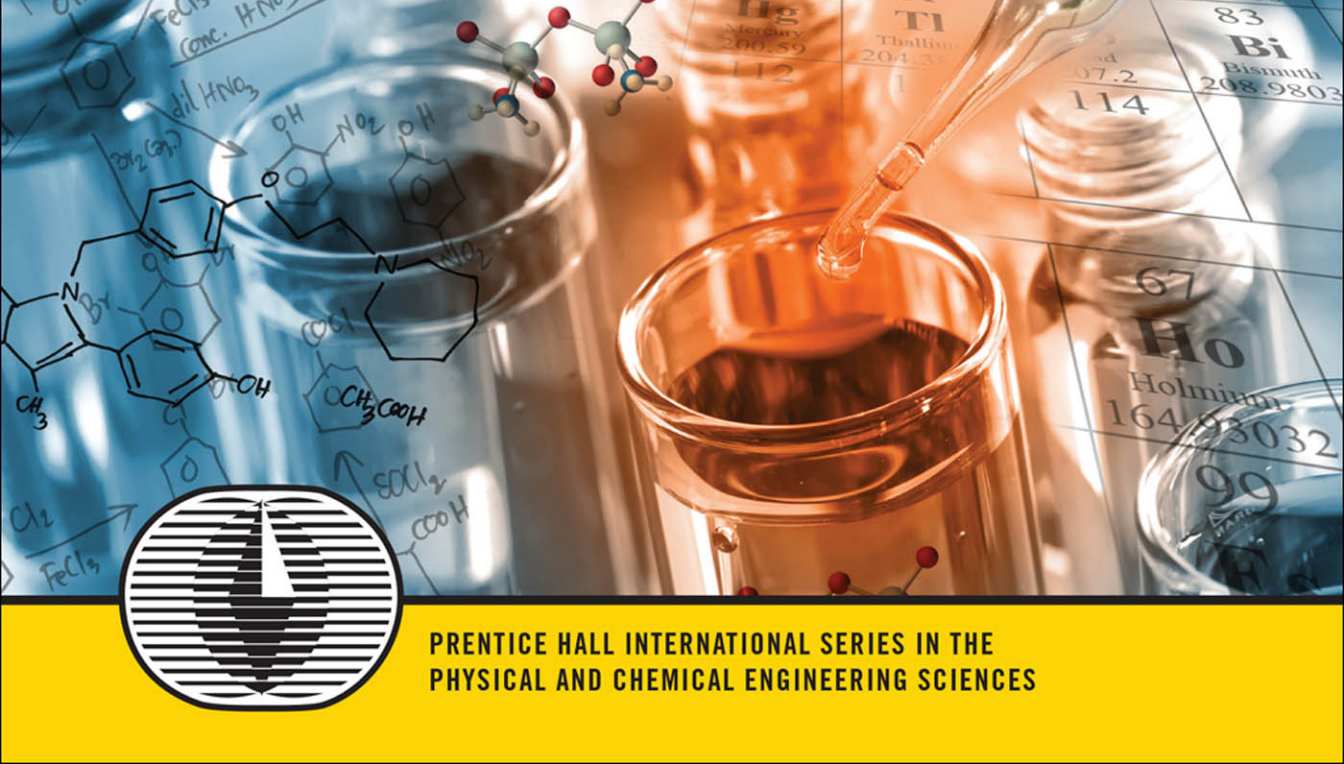
PRENTICE HALL

Bioprocess Engineering

Basic Concepts

Third Edition

Michael L. Shuler • Fikret Kargi • Matthew DeLisa



PRENTICE HALL INTERNATIONAL SERIES IN THE
PHYSICAL AND CHEMICAL ENGINEERING SCIENCES

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Basic Concepts

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Retired professor of Dokuz Eylul University

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1 17

For Andy, Kristin, Eric, and Kathy, and for Karen

—Michael L. Shuler

To my family and my parents

—Fikret Kargi

To my entire family, especially Kerry, Sam, and Charlie,
for their unwavering love, support, and encouragement

—Matthew P. DeLisa

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Contents

PREFACE ***xvii***

ABOUT THE AUTHORS ***xxi***

Part 1 The Basics of Biology: An Engineer's Perspective **1**

1 WHAT IS A BIOPROCESS ENGINEER? ***1***

1.1. Biotechnology and Bioprocess Engineering 2
1.2. Differing Approaches to Research for Biologists and Engineers 3
1.3. The Story of Penicillin: How Biologists and Engineers Work Together 4
1.4. Bioprocesses: Regulatory Constraints 9
Suggestions for Further Reading 11
Questions 11

2 AN OVERVIEW OF BIOLOGICAL BASICS ***13***

2.1. Microbial Diversity 13
2.1.1. Naming Cells, 14
2.1.2. Viruses, 16
2.1.3. Procaryotes, 18
2.1.4. Eucaryotes, 22

2.2. Cell Construction 28
2.2.1. Amino Acids and Proteins, 29
2.2.2. Carbohydrates: Mono- and Polysaccharides, 37
2.2.3. Lipids, Fats, and Steroids, 42
2.2.4. Nucleic Acids, RNA, and DNA, 45
2.3. Cell Nutrients 51
2.3.1. Macronutrients, 52
2.3.2. Micronutrients, 54
2.3.3. Growth Media, 55
2.4. Summary 56
Suggestions for Further Reading 58
Questions 58

3 ENZYMES 61

3.1. How Enzymes Work 62
3.2. Enzyme Kinetics 63
3.2.1. Mechanistic Models for Simple Enzyme Kinetics, 65
3.2.2. Determining Rate Parameters for Michaelis–Menten Kinetics, 67
3.2.3. Models for More Complex Enzyme Kinetics, 71
3.2.4. Effects of pH and Temperature, 80
3.2.5. Insoluble Substrates, 83
3.2.6. Multiphase Enzymatic Reactions, 84
3.3. Immobilized Enzyme Systems 86
3.3.1. Methods of Immobilization, 86
3.3.2. Diffusional Limitations in Immobilized Enzyme Systems, 91
3.3.3. Electrostatic and Steric Effects in Immobilized Enzyme Systems, 98
3.4. Large-Scale Production of Enzymes 98
3.5. Medical and Industrial Utilization of Enzymes 100
3.6. Summary 103
Suggestions for Further Reading 104
Problems 104

4 HOW CELLS WORK 113

4.1. The Central Dogma 114
4.2. DNA Replication: Preserving and Propagating the Message 117
4.3. Transcription: Sending the Message 119
4.4. Translation: Going from Message to Product 123
4.4.1. Genetic Code: Universal Message, 123
4.4.2. Translation: How the Machinery Works, 124
4.4.3. Posttranslational Processing: Making the Product Useful, 125

4.5. Metabolic Regulation 130
4.5.1. Genetic-Level Control: Which Proteins Are Synthesized?, 130
4.5.2. Metabolic Pathway Control, 133
4.6. How the Cell Senses its Extracellular Environment 135
4.6.1. Transporting Small Molecules across Cellular Membranes, 135
4.6.2. Role of Cell Receptors in Metabolism and Cellular Differentiation, 137
4.7. Summary 139
4.8. Appendix: Example Regulation of Complex Pathways 140
Suggestions for Further Reading 142
Problems 143

5 MAJOR METABOLIC PATHWAYS 145

5.1. Bioenergetics 146
5.2. Glucose Metabolism: Glycolysis and the TCA Cycle 149
5.3. Respiration 152
5.4. Control Sites in Aerobic Glucose Metabolism 154
5.5. Metabolism of Nitrogenous Compounds 155
5.6. Nitrogen Fixation 156
5.7. Metabolism of Hydrocarbons 156
5.8. Biodegradation of Xenobiotics 157
5.9. Overview of Biosynthesis 158
5.10. Overview of Anaerobic Metabolism 161
5.11. Overview of Autotrophic Metabolism 163
5.12. Summary 165
Suggestions for Further Reading 166
Questions 168

6 HOW CELLS GROW 169

6.1. Batch Growth 170
6.1.1. Quantifying Cell Concentration, 170
6.1.2. Growth Patterns and Kinetics in Batch Culture, 175
6.1.3. How Environmental Conditions Affect Growth Kinetics, 184
6.1.4. Heat Generation by Microbial Growth, 190
6.2. Quantifying Growth Kinetics 191
6.2.1. Unstructured Nonsegregated Models, 192
6.2.2. Models for Transient Behavior, 201
6.2.3. Cybernetic Models, 207
6.3. Cell Growth in Continuous Culture 208
6.3.1. Specific Devices for Continuous Culture, 208
6.3.2. The Ideal Chemostat, 210

6.3.3. The Chemostat as a Tool, *217*
6.3.4. Deviations from Ideality, *218*
6.4. Summary 219
Suggestions for Further Reading 219
Problems 220

7 STOICHIOMETRY OF MICROBIAL GROWTH AND PRODUCT FORMATION **227**

7.1. Coefficients for ATP Consumption and Oxygen 227
7.2. Stoichiometric Calculations 229
7.2.1. Elemental Balances, *230*
7.2.2. Degree of Reduction, *230*
7.3. Theoretical Predictions of Yield Coefficients 235
7.4. Estimation of Elemental Cell Composition 236
7.5. Stoichiometry by Oxidation-Reduction Half-Reactions 237
7.6. Thermodynamics of Biological Reactions 240
7.7. Summary 242
Suggestions for Further Reading 242
Problems 243

8 HOW CELLULAR INFORMATION IS ALTERED **247**

8.1. Evolving Desirable Biochemical Activities Through Mutation and Selection 247
8.1.1. How Mutations Occur, *248*
8.1.2. Selecting for Desirable Mutants, *250*
8.2. Natural Mechanisms for Gene Transfer and Rearrangement 252
8.2.1. Genetic Recombination, *252*
8.2.2. Transformation, *254*
8.2.3. Transduction, *254*
8.2.4. Episomes and Conjugation, *256*
8.2.5. Transposons: Internal Gene Transfer, *257*
8.3. Genetically Engineering Cells 257
8.3.1. Basic Elements of Genetic Engineering, *258*
8.3.2. Genetic Engineering of Higher Organisms, *265*
8.3.3. Genome Engineering, *266*
8.4. Genomics 267
8.4.1. Experimental Techniques, *268*
8.4.2. Computational Techniques, *271*
8.5. Summary 272
Suggestions for Further Reading 272
Problems 273

Part 2 Engineering Principles for Bioprocesses 275

9 OPERATING CONSIDERATIONS FOR BIOREACTORS FOR SUSPENSION AND IMMOBILIZED CULTURES 275

9.1. Choosing the Cultivation Method 276
9.2. Modifying Batch and Continuous Reactors 278
9.2.1. Chemostat with Recycle, 278
9.2.2. Multistage Chemostat Systems, 281
9.2.3. Fed-Batch Operation, 288
9.2.4. Perfusion Systems, 293
9.2.5. Membrane Bioreactors, 294
9.3. Immobilized Cell Systems 298
9.3.1. Active Immobilization of Cells, 298
9.3.2. Passive Immobilization: Biological Films, 303
9.3.3. Diffusional Limitations in Immobilized Cell Systems, 304
9.3.4. Bioreactor Considerations in Immobilized Cell Systems, 309
9.4. Hybrid Bioreactors: Attached and Suspended Cells 311
9.5. Solid-State Fermentations 313
9.6. Summary 316
Suggestions for Further Reading 317
Problems 318

10 SELECTION, SCALE-UP, OPERATION, AND CONTROL OF BIOREACTORS 323

10.1. Scale-Up and its Difficulties 323
10.1.1. Overview of Traditional Reactor Types, 324
10.1.2. Reactors with Internal Mechanical Agitation, 324
10.1.3. Bubble Column and Loop Reactor, 329
10.1.4. Single-Use Bioreactors, 330
10.1.5. Considerations in Aeration, Agitation, and Heat Transfer, 331
10.1.6. Approaches to Scale-Up, 337
10.1.7. Scale-Down and Microbioreactors, 342
10.2. Bioreactor Instrumentation and Control 349
10.2.1. Instrumentation for Measurements of Active Fermentation, 349
10.2.2. Using the Information Obtained, 352
10.3. Sterilization of Process Fluids 356
10.3.1. The Kinetics of Death, 356
10.3.2. Sterilization of Liquids, 357
10.3.3. Sterilization of Gases, 362

10.4. Summary 364
Suggestions for Further Reading 365
Problems 366

11 RECOVERY AND PURIFICATION OF PRODUCTS 371

11.1. Strategies to Recover and Purify Products 371
11.2. Separation of Insoluble Products 374
11.2.1. Filtration, 374
11.2.2. Centrifugation, 378
11.2.3. Coagulation and Flocculation, 382
11.3. Cell Disruption 382
11.3.1. Mechanical Methods, 383
11.3.2. Nonmechanical Methods, 384
11.4. Separation of Soluble Products 385
11.4.1. Liquid–Liquid Extraction, 385
11.4.2. Aqueous Two-Phase Extraction, 389
11.4.3. Precipitation, 390
11.4.4. Dialysis, 392
11.4.5. Reverse Osmosis, 393
11.4.6. Ultrafiltration and Microfiltration, 395
11.4.7. Cross-Flow Ultrafiltration and Microfiltration, 398
11.4.8. Adsorption, 403
11.4.9. Chromatography, 409
11.4.10. Electrophoresis, 420
11.4.11. Electrodialysis, 421
11.5. Finishing Steps for Purification 422
11.5.1. Crystallization, 422
11.5.2. Drying, 423
11.6. Integration of Reaction and Separation 424
11.7. Summary 426
Suggestions for Further Reading 426
Problems 427

12 BIOPROCESS CONSIDERATIONS IN USING ANIMAL CELL CULTURES 431

12.1. Structure and Biochemistry of Animal Cells 431
12.2. Methods Used for the Cultivation of Animal Cells 434
12.2.1. Basic Techniques for Animal Cell Culture, 434
12.2.2. Growth Media, 437
12.2.3. Growth Dynamics for Animal Cells, 441
12.3. Bioreactor Considerations for Animal Cell Culture 443
12.4. Bioreactor Systems for Animal Cell Culture 444
12.4.1. Nonstirred Reactor Systems, 444
12.4.2. Systems for Entrapped Cells in Stirred Reactors, 445

12.4.3. Suspended Cultures, *447*
12.5. Products of Animal Cell Cultures 447
12.6. Summary 448
Suggestions for Further Reading 449
Problems 450

13 BIOPROCESS CONSIDERATIONS IN USING PLANT CELL CULTURES **451**

13.1. Why Plant Cell Cultures? 451
13.2. Plant Cells in Culture Compared to Microbes 457
13.3. Bioreactor Considerations 461
13.3.1. Bioreactors for Suspension Cultures, *462*
13.3.2. Reactors Using Cell Immobilization, *463*
13.3.3. Bioreactors for Organized Tissues, *466*
13.4. Economics of Plant Cell Tissue Cultures 467
13.5. Summary 468
Suggestions for Further Reading 468
Problems 469

14 UTILIZING GENETICALLY ENGINEERED ORGANISMS **471**

14.1. How the Product Influences Process Decisions 471
14.2. Guidelines for Choosing Host–Vector Systems 474
14.2.1. Escherichia Coli, *475*
14.2.2. Gram-Positive Bacteria, *477*
14.2.3. Lower Eucaryotic Cells, *477*
14.2.4. Mammalian Cells, *479*
14.2.5. Insect Cell–Baculovirus System, *480*
14.2.6. Transgenic Animals, *482*
14.2.7. Transgenic Plants and Plant Cell Culture, *483*
14.2.8. Cell-Free Protein Synthesis, *484*
14.2.9. Comparison of Strategies, *484*
14.3. Process Constraints: Genetic Instability 485
14.3.1. Segregational Loss, *486*
14.3.2. Plasmid Structural Instability, *488*
14.3.3. Host Cell Mutations, *488*
14.3.4. Growth-Rate-Dominated Instability, *489*
14.4. Avoiding Process Problems in Plasmid Design 490
14.5. Predicting Host–Vector Interactions and Genetic Instability 493
14.6. Regulatory Constraints on Genetic Processes 503
14.7. Metabolic Engineering 506
14.8. Synthetic and Systems Biology 509
14.9. Protein Engineering 511
14.10. Summary 513

Suggestions for Further Reading 514
Problems 516

15 MEDICAL APPLICATIONS OF BIOPROCESS ENGINEERING 519

15.1. Tissue Engineering 519
15.1.1. What Is Tissue Engineering?, 520
15.1.2. Tissue-Engineered Skin Replacements, 521
15.1.3. Chondrocyte Culture for Cartilage Replacement, 522
15.2. Gene Therapy Using Viral Vectors 523
15.2.1. Models of Viral Infection, 524
15.2.2. Mass Production of Retrovirus, 527
15.3. Bioreactors 528
15.3.1. Stem Cells and Hematopoiesis, 529
15.3.2. Extracorporeal Artificial Liver, 530
15.3.3. Body-on-a-Chip Systems, 530
15.4. Summary 531
Suggestions for Further Reading 532
Problems 532

16 BIOPROCESSES UTILIZING MIXED CULTURES 535

16.1. Major Classes of Interactions in Mixed Cultures 536
16.2. Simple Models Describing Mixed-Culture Interactions 539
16.3. Mixed Cultures in Nature 545
16.4. Industrial Utilization of Mixed Cultures 546
16.5. Biological Waste Treatment 549
16.5.1. Biological Waste-Treatment Processes, 551
16.5.2. Advanced Wastewater Treatment Systems, 566
16.5.3. Conversion of Wastewater to Useful Products, 571
16.6. Summary 572
Suggestions for Further Reading 572
Problems 573

APPENDIX TRADITIONAL INDUSTRIAL BIOPROCESSES 577

A.1. Anaerobic Bioprocesses 577
A.1.1. Ethanol Production, 578
A.1.2. Lactic Acid Production, 581
A.1.3. Acetone–Butanol Production, 584
A.2. Aerobic Processes 586
A.2.1. Citric Acid Production, 586
A.2.2. Production of Baker's Yeast, 588
A.2.3. Production of Penicillins, 590
A.2.4. Production of High-Fructose Corn Syrup, 593

A.3. Bioprocess Technologies: Biofuel and Bioenergy Production from Biomass 596
A.3.1. Production of Liquid Fuels, *596*
A.3.2. Production of Gaseous Fuels from Biomass, *597*
A.3.3. Bioelectricity Generation from Wastes Using Microbial Fuel Cells, *598*
Suggestions for Further Reading 600

INDEX ***601***

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Preface

This third edition of *Bioprocess Engineering: Basic Concepts* updates the prior two editions. Although the principles of bioprocess engineering stated in the first two editions remains sound, the field has made considerable advances in improving the productivity, consistency, and safety of bioprocesses.

Some of the major changes in the book come from biology itself with the development of tools that have greatly increased our ability to both understand and manipulate cell biology more effectively and cheaply. On the bioprocess side, tremendous advances have been made in production of biologicals with greater emphasis on processes incorporating animal cells. In particular, productivity in making many heterologous proteins has increased by several orders of magnitude over the last 16 years. The commercial use of animal cell culture has increased significantly over this period of time, and plant cell culture is starting to see commercial applications. Processes with microbes based on recombinant DNA and related technology have matured greatly. For any cell type, consistent authentic posttranslational processing of proteins remains challenging. The development of the concepts in systems and synthetic biology is now having an impact on the thinking of bioprocess engineers.

In this edition, we either introduce or expand discussion of the following issues:

- The role of small RNAs as regulators
- Cell-free processes

- Biofuels and energy
- Synthetic biology
- Role of genomics
- Development of single-use technology in bioprocesses
- Stem cell technology and utilization
- Use of techniques of microfabrication and nanobiotechnology as well as 3D printing
- Animal and plant cell biotechnology
- Development in environmental biotechnology

Teaching a subject as broad as bioprocess engineering in one semester is a difficult challenge, considering the range of backgrounds students may have. Although many engineering students have some formal exposure to specific areas of biology, they often have not had an opportunity to complete courses from several areas of biology. The biological concepts necessary for an engineer are described in Part 1, "The Basics of Biology: An Engineer's Perspective." Although this condensed treatment cannot replace an in-depth knowledge of biochemistry, genetics, microbiology, and cell biology, it serves as a useful starting point for a bioprocess professional. On the process side, an instructor might wish to emphasize Part 2, "Engineering Principles for Bioprocesses," dealing with biopharmaceuticals, bioenergy, or waste treatment, as they decide which topics would be effective for their students.

We hope this book imparts the excitement of bioprocess engineering as well as the basic concepts and knowledge to become a leader in this field.

Register your copy of *Bioprocess Engineering, Third Edition,* at informit.com for convenient access to downloads, updates, and corrections as they become available. To start the registration process, go to informit.com/register and log in or create an account. Enter the product ISBN (9780137062706) and click Submit. Once the process is complete, you will find any available bonus content under "Registered Products."

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PART 1
The Basics of Biology: An Engineer's Perspective

1
What Is a Bioprocess Engineer?

We can now manipulate life at its most basic level—the genetic. For thousands of years, people have practiced genetic engineering at the level of selection and breeding. But now it can be done in a purposeful, predetermined manner with the molecular-level manipulation of DNA. We now have tools to probe the mysteries of life in a way unimaginable even a few decades ago. We are at the doorstep of being able to chemically synthesize whole genomes and transfer them into a host bacterium to create semisynthetic life. We now have a whole host of new techniques to rationally design novel organisms.

With this intellectual revolution emerged new visions and new hopes: new medicines, semisynthetic organs grown in large vessels, abundant and nutritious foods, computers based on biological molecules rather than silicon chips, superorganisms to degrade pollutants, a wide array of consumer products, and potentially new sources of energy.

These dreams will remain dreams without hard work. Engineers will play an essential role in converting these visions into reality. Biological systems are very complex and beautifully constructed, but they obey the rules of chemistry and physics and are susceptible to engineering analysis. Living cells are predictable, and the processes to use them can be rationally constructed on commercial scales. Doing this is the job of the bioprocess engineer.

Probably the reason you are reading this book is your desire to participate in this intellectual revolution and to make an important contribution to society. You *can* do it, but it is not easy to combine the skills of the engineer with those of the biologist. This book's intent is to help you begin to develop these skills. This book and a one-term course are not enough to make you a complete bioprocess engineer, but it will help you form the necessary foundation.

In this chapter, we discuss the origins of bioprocess engineering, how bioprocess engineering evolved, and the complementary roles of biologists and engineers. We then consider the regulatory constraints on bioprocesses and how they impact the engineer.

1.1. BIOTECHNOLOGY AND BIOPROCESS ENGINEERING

When new fields emerge from new ideas, old terminology is usually not adequate to describe these fields. Biotechnology and what constitutes engineering in this field are best described with examples rather than single words or short phrases. However, it is critical to understand the cell's inner workings to harness the cellular machinery and engineer the cell to perform a designated task.

Biotechnology usually implies the use or development of methods of direct genetic manipulation for a socially desirable goal. Such goals might be the production of a particular chemical, but they may also involve the production of better plants or seeds, or gene therapy, or the use of specially designed organisms to degrade wastes. The key element for many professionals is the use of sophisticated techniques outside the cell for genetic manipulation. Others interpret biotechnology in a much broader sense and equate it with the technical use of biology for production of pharmaceuticals, chemicals, food, and diagnostics; they may include engineering as a subcomponent of biotechnology.

Many terms are used to describe engineers working with biotechnology. *Bioengineering* is a broad title and includes work on medical, bioprocess, agricultural, and environmental systems; its practitioners include agricultural, electrical, mechanical, industrial, environmental, and chemical engineers as well as others. *Biological engineering* is similar but emphasizes applications to plants and animals. *Metabolic engineering* is the design of cells with genetically altered pathways to make small molecules that are often novel for that cell. This term stresses human design of cells for manufacture of specialty chemicals. *Biochemical engineering* has usually meant the extension of chemical engineering principles to systems using a biological catalyst to bring about desired chemical transformations. It is often subdivided into bioreaction engineering and bioseparations, particularly for production of biologics, chemicals, and fuels. *Biomedical engineering* has been considered to be separate from biochemical engineering, although the boundary between the two is increasingly vague, particularly in the areas of cell-surface receptors and animal cell culture. It focuses on the human body with an emphasis on application of engineering principles from a variety of disciplines to design medical devices, synthetic organs, and novel methods for drug delivery and to develop diagnostics and instrumentation. Another relevant term is *biomolecular engineering*, which has been defined by the National Institutes of Health as "research at the interface of biology and chemical engineering and is focused at the molecular level."

There is a difference between bioprocess engineering and biochemical engineering. In addition to chemical engineering, *bioprocess engineering* includes the work of mechanical, electrical, environmental, and industrial engineers to apply the principles of their disciplines to processes based on using living cells or subcomponents of such cells. The problems of detailed equipment design, sensor development, control algorithms, and manufacturing strategies can utilize principles from these disciplines. Biochemical engineering is more limited in the sense that it draws primarily from chemical engineering principles and broader in the sense that it is not restricted to well-defined, artificially constructed processes but can be applied to natural systems.

This book focuses primarily on the application of chemical engineering principles to systems containing biological catalysts, but with an emphasis on those systems making use of biotechnology. The rapidly increasing ability to determine the complete sequence of genes in an organism offers new opportunities for bioprocess engineers in the design and monitoring of bioprocesses. The cell itself is now a designable component of the overall process.

1.2. DIFFERING APPROACHES TO RESEARCH FOR BIOLOGISTS AND ENGINEERS

The fundamental trainings of biologists and engineers are distinctly different. In the development of knowledge in the life sciences, unlike chemistry and physics, mathematical theories and quantitative methods (except statistics) have played a secondary role. Most progress has been due to improvements in experimental tools. Results are qualitative, and descriptive models are formulated and tested. Consequently, biologists often have incomplete backgrounds in mathematics but are very strong with respect to laboratory tools and, more importantly, with respect to the interpretation of laboratory data from complex systems.

Engineers usually possess a solid background in the physical and mathematical sciences. Often, a theory leads to mathematical formulations, and the validity of the theory is tested by comparing predicted responses to those in experiments. Quantitative models and approaches, even to complex systems, are strengths. Biologists are usually adept at the formation of testable hypotheses, experimental design, and data interpretation from complex systems. Engineers quite often are unfamiliar with the experimental techniques and strategies used by life scientists.

The skills of the engineer and life scientist are complementary. To convert the promises of molecular biology into new processes to make new products requires the integration of these skills. To function at this level, the engineer needs a solid understanding of biology and its experimental tools. This book provides sufficient biological background for you to understand how to apply engineering principles to biosystems. However, if you are serious about becoming a bioprocess engineer, you will need to take further courses in microbiology, biochemistry, and cell biology, as well as more advanced work in bioengineering. If you already have these courses, this book can be used for review and to provide a coherent framework for applications to bioprocessing.

1.3. THE STORY OF PENICILLIN: HOW BIOLOGISTS AND ENGINEERS WORK TOGETHER

In September 1928, Alexander Fleming at St. Mary's Hospital in London was trying to isolate the bacterium *Staphylococcus aureus*, which causes boils. The technique in use was to grow the bacterium on the surface of a nutrient solution. One of the dishes had been contaminated inadvertently with a foreign particle. Normally, such a contaminated plate would be tossed out. However, Fleming noticed that no bacteria grew near the invading substance (see Figure 1.1).

Fleming's genius was to realize that this observation was meaningful and not a "failed" experiment. Fleming recognized that the cell killing must be due to an antibacterial agent. He recovered the foreign particle and found that it was a common mold of the *Penicillium* genus (later identified as *Penicillium notatum*). Fleming nurtured the mold to grow and, using the crude extraction methods then available, managed to obtain a tiny quantity of secreted material. He then demonstrated that this material had powerful antimicrobial properties and named the product penicillin. Fleming carefully preserved the culture, but the discovery lay essentially dormant for over a decade.

World War II provided the impetus to resurrect the discovery. Sulfa drugs have a rather restricted range of activity, and an antibiotic with minimal side effects and broader applicability was desperately needed. Howard Florey and Ernst Chain of Oxford decided to build on Fleming's observations. Norman Heatley played the key role in producing sufficient material for Chain and Florey to test the effectiveness of penicillin. Heatley, trained as a biochemist, performed as a bioprocess engineer.

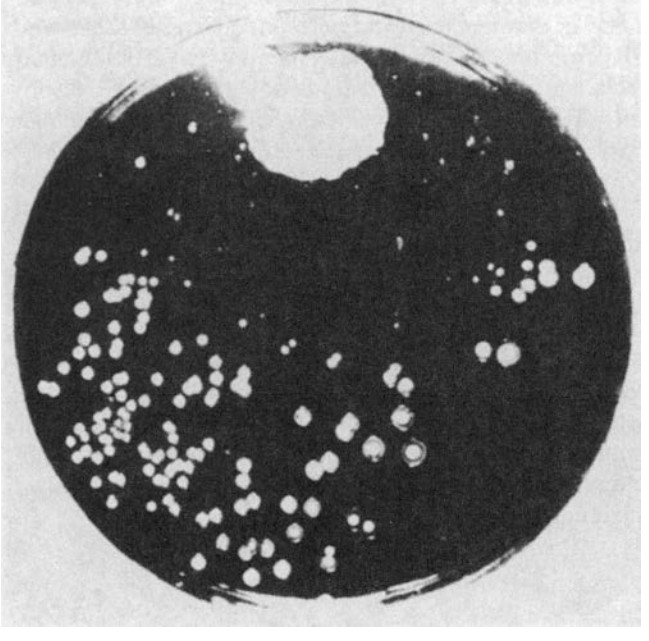


Figure 1.1. Alexander Fleming's original plate showing the growth of the mold *Penicillium notatum* and its inhibitory action on bacterial growth. (With permission, from Corbis Corporation.)

He developed an assay to monitor the amount of penicillin made so as to determine the kinetics of the fermentation, developed a culture technique that could be implemented easily, and devised a novel back-extraction process to recover the very delicate product. After months of immense effort, they produced enough penicillin to treat some laboratory animals.

Eighteen months after starting on the project, they began to treat a London bobby for a blood infection. The penicillin worked wonders initially and brought the patient to the point of recovery. Most unfortunately, the supply of penicillin was exhausted, and the man relapsed and died. Nonetheless, Florey and Chain had demonstrated the great potential for penicillin, if it could be made in sufficient amount. To make large amounts of penicillin would require a process, and for such a process development, engineers, in addition to microbial physiologists and other life scientists, would be needed.

The war further complicated the situation. Great Britain's industrial facilities were already totally devoted to the war. Florey and his associates approached pharmaceutical firms in the United States to persuade them to develop the capacity to produce penicillin, since the United States was not at war at that time.

Many companies and government laboratories, assisted by many universities, took up the challenge. Particularly prominent were Merck, Pfizer, Squibb, and the USDA Northern Regional Research Laboratory in Peoria, Illinois.

The first efforts with fermentation were modest. A large effort went into attempts to chemically synthesize penicillin. This effort involved hundreds of chemists. Consequently, many companies were at first reluctant to commit to the fermentation process beyond the pilot-plant stage. It was thought that the pilot-plant fermentation system could produce sufficient penicillin to meet the needs of clinical testing, but large-scale production would soon be done by chemical synthesis. At that time, U.S. companies had achieved a great deal of success with chemical synthesis of other drugs, which gave the companies significant control over the drug's production. The chemical synthesis of penicillin proved to be exceedingly difficult. (It was accomplished in the 1950s, and the synthesis route is still not competitive with fermentation.) However, in 1940 fermentation for the production of a pharmaceutical was an unproved approach, and most companies were betting on chemical synthesis to ultimately dominate.

The early clinical successes were so dramatic that in 1943 the War Production Board appointed A. L. Elder to coordinate the activities of producers to greatly increase the supply of penicillin. The fermentation route was chosen. As Elder recalls, "I was ridiculed by some of my closest scientific friends for allowing myself to become associated with what obviously was to be a flop—namely, the commercial production of penicillin by a fermentation process" (from Elder, 1970). The problems facing the fermentation process were indeed very formidable.

The problem was typical of most new fermentation processes: a valuable product made at very low levels. The low rate of production per unit volume would necessitate very large and inefficient reactors, and the low concentration (titer) made product recovery and purification very difficult. In 1939 the final concentration in a typical penicillin fermentation broth was one part per million (ca. 0.001 g/l). Furthermore, penicillin is a fragile and unstable product, which places significant constraints on the approaches used for recovery and purification.

Life scientists at the Northern Regional Research Laboratory made many major contributions to the penicillin program. One was the development of a corn steep liquor–lactose-based medium. This medium increased productivity about tenfold. A worldwide search by the laboratory for better producer strains of *Penicillium* led to the isolation of a *Penicillium chrysogenum* strain. This strain, isolated from a moldy cantaloupe at a Peoria fruit market, proved superior to hundreds of other isolates tested. Its progeny have been used in almost all commercial penicillin fermentations.

The other hurdle was to decide on a manufacturing process. One method involved the growth of the mold on the surface of moist bran. This bran method was discarded because of difficulties in temperature control, sterilization, and equipment size. The surface method involved growth of the mold on top of a quiescent liquid medium. The surface method used a variety of containers, including milk bottles, and the term "bottle plant" indicated such a manufacturing technique. The surface method gave relatively high yields but had a long growing cycle and was very labor intensive. The first manufacturing plants were bottle plants because the method worked and could be implemented quickly.

However, it was clear that the surface method would not meet the full need for penicillin. If the goal of the War Production Board was met by bottle plants, it was estimated that the necessary bottles would fill a row stretching from New York City to San Francisco. Engineers generally favored a submerged tank process. The submerged process presented challenges in terms of both mold physiology and tank design and operation. Large volumes of absolutely clean, oil- and dirt-free sterile air were required. What were then very large agitators were required, and the mechanical seal for the agitator shaft had to be designed to prevent the entry of organisms. Even today, problems of oxygen supply and heat removal are important constraints on antibiotic fermenter design. Contamination by foreign organisms could degrade the product as fast as it was formed, consume nutrients before they were converted to penicillin, or produce toxins.

In addition to these challenges in reactor design, there were similar hurdles in product recovery and purification. The very fragile nature of penicillin required the development of special techniques. A combination of pH shifts and rapid liquid–liquid extraction proved useful.

Soon, processes using tanks of about 10,000 gal were built. Pfizer completed in less than 6 months the first plant for commercial production of penicillin by submerged fermentation (Hobby, 1985). The plant had 14 tanks, each of 7000-gal capacity. Large-scale fermenters (typically 50,000 to 100,000 l) now used in antibiotic fermentations are shown in Figures 1.2 and 1.3.

By a combination of good luck and hard work, the United States had the capacity by the end of World War II to produce enough penicillin for almost 100,000 patients per year. The impact of penicillin was immediate, and the advent of antibiotics saved many lives. Although the first impact was in war zones, the overall effect was critical globally to fighting many diseases (see Figure 1.4).

This accomplishment required a high level of multidisciplinary work. For example, Merck realized that men who understood both engineering and biology were not available. Merck assigned a chemical engineer and microbiologist together

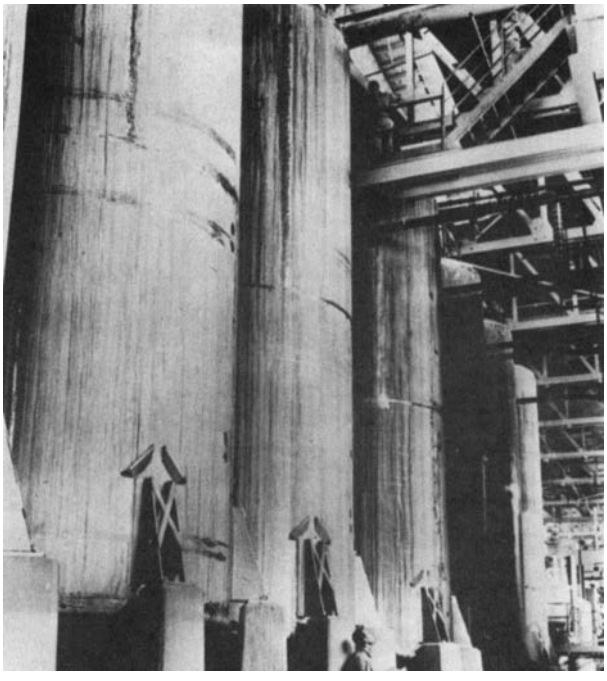


Figure 1.2. Series of large-scale antibiotic fermenters. (Courtesy of Pfizer, Inc.)

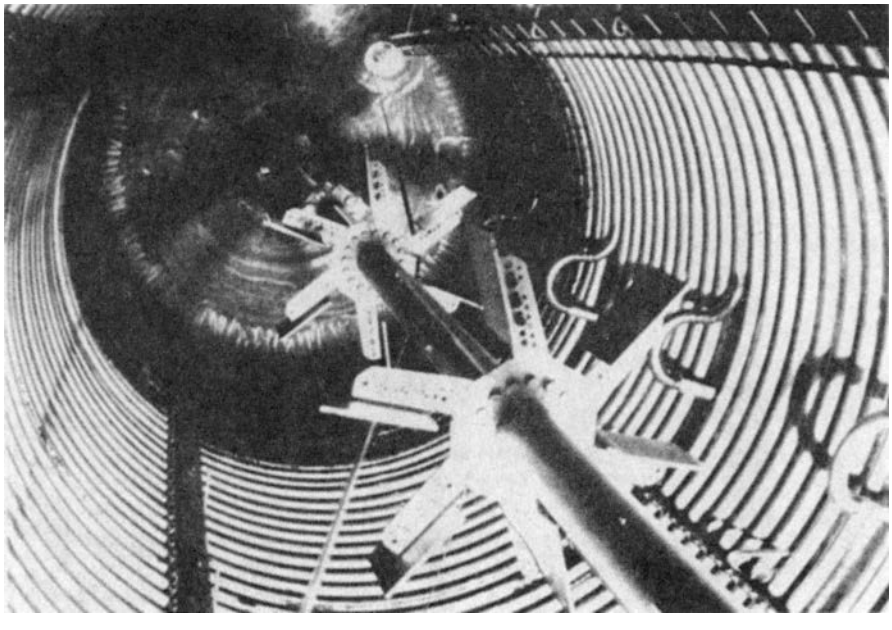


Figure 1.3. Inside view of a large antibiotic fermenter. (From *Trends in Biotechnology 3*(6), 1985. Used with permission of Elsevier Science Publishers.)



Figure 1.4. First impact of penicillin in war zones. (WW II Museum, New Orleans, LA.)

to each aspect of the problem. They planned, executed, and analyzed the experimental program jointly, "almost as if they were one man" (Silcox in Elder, 1970).

Progress with penicillin fermentation has continued, as has the need for the interaction of biologists and engineers. From 1939 to now, the yield of penicillin has gone from 0.001 g/l to over 50 g/l of fermentation broth. Progress has involved better understanding of mold physiology, metabolic pathways, penicillin structure, methods of mutation and selection of mold genetics, process control, and reactor design (see Figure 1.5).

Before the penicillin process, almost no chemical engineers sought specialized training in the life sciences. With the advent of modern antibiotics, the concept of a bioprocess engineer was born. The penicillin process also established a paradigm for bioprocess development and biochemical engineering. This paradigm still guides much of our profession's thinking. The mindset of bioprocess engineers was cast with the penicillin experience. It is for this reason that we have focused on the penicillin story rather than on an example for production of a protein from a genetically engineered organism. Although many parallels can be made between the penicillin process and our efforts to use recombinant DNA, no similar paradigm has yet emerged from our experience with genetically engineered cells. We must continually reexamine the prejudices the field has inherited from the penicillin experience.

It is you, the student, who will best be able to challenge these prejudices.

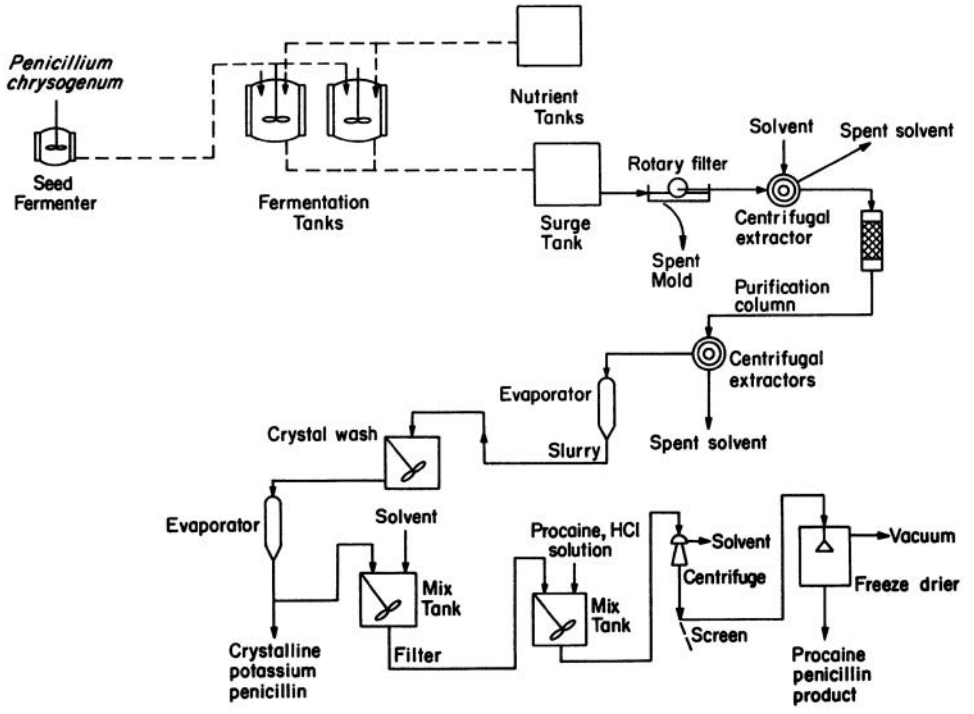


Figure 1.5. Schematic of penicillin production process.

1.4. BIOPROCESSES: REGULATORY CONSTRAINTS

To understand the mindset of a bioprocess engineer, you must understand the regulatory climate in which many bioprocess engineers work. The U.S. FDA (Food and Drug Administration) and its equivalents in other countries must insure the safety and efficacy of medicines. For bioprocess engineers working in the pharmaceutical or biotechnology industry, the primary concern is not reduction of manufacturing cost (although that is still a very desirable goal), but the production of a product of consistently high quality in amounts to satisfy the medical needs of the population.

Consider briefly the process by which a drug obtains FDA approval. A typical drug undergoes 6.5 years of development from the discovery stage through preclinical testing in animals. Human clinical trials are conducted in three phases. Phase 1 clinical trials (about 1 year) are used to test safety; typically 20 to 80 volunteers are used. Phase II clinical trials (about 2 years) use 100 to 300 patients, and the emphasis is on efficacy (i.e., whether it helps the patient) as well as further determining which side effects exist. Compounds that are still promising enter phase III clinical trials (about 3 years) with 1000 to 3000 patients. Since individuals vary in body chemistry, it is important to test the range of responses in terms of both side effects and efficacy by using a representative cross section of the population. Data from clinical trials is presented to the FDA for review (about 18 months). If the clinical trials are

well designed and demonstrate statistically significant improvements in health with acceptable side effects, the drug is likely to be approved. Even after this point, there is continued monitoring of the drug for adverse effects. The whole drug discovery-through-approval process takes 12 to 15 years on the average and costs up to about $5 billion (in 2013). Only one in ten drugs that enter human clinical trials receives approval. Recent FDA reforms have decreased the time to obtain approval for life-saving drugs in treatment of diseases such as cancer and AIDS, but the overall process is still lengthy.

This process greatly affects a bioprocess engineer. FDA approval is for the product *and* the process together. There are tragic examples in which a small process change has allowed a toxic trace compound to form or become incorporated in the final product, resulting in severe side effects, including death. For example, heparin contaminated with oversulfated chondroitin sulfate caused significant problems. Thus, process changes may require new clinical trials to test the safety of the resulting product. Since clinical trials are very expensive, process improvements are made under a limited set of circumstances. Even during clinical trials it is difficult to make major process changes.

Drugs sold on the market or used in clinical trials must come from facilities that are certified as *good manufacturing practice* (GMP). GMP concerns the actual manufacturing facility design and layout, the equipment and procedures, training of production personnel, control of process inputs (e.g., raw materials and cultures), and handling of product. The plant layout and design must prevent contamination of the product and dictates the flow of material, personnel, and air. Equipment and procedures must be *validated*. Procedures include not only operation of a piece of equipment but also cleaning and sterilization. Computer software used to monitor and control the process must be validated. Off-line assays done in laboratories must satisfy *good laboratory practices* (GLP). Procedures are documented by *standard operating procedures* (SOP).

The GMP guidelines stress the need for documented procedures to validate performance: "Process validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality characteristics" and "There shall be written procedures for production and process-control to assure that products have the identity, strength, quality, and purity they purport or are represented to possess."[1]

The actual process of doing validation is often complex, particularly when a whole facility design is considered. The FDA provides extensive information and guidelines, which are updated regularly. Bioprocess engineers involved in biomanufacturing for pharmaceuticals need to consult these sources. For example, the recent introduction of disposable bioreactors requires considerations of how FDA regulations can be applied. However, certain key concepts do not change. These concepts are written documentation, consistency of procedures, consistency of product, and demonstrable measures of product quality, particularly purity

1. *Guidelines on General Principles of Process Validation*, Federal Register of May 11, 1987 (52 FR 17638).

and safety. These tasks are demanding and require careful attention to detail. Bioprocess engineers often find that much of their effort is to satisfy these regulatory requirements.

The key point is that process changes cannot be made without considering their significant regulatory impact.

SUGGESTIONS FOR FURTHER READING

Synthetic Biology

Gibson, D. G., et al., Creation of a Bacterial Cell Controlled by a Chemically Synthesized Genome, *Science* 329(5987): 52–56, 2010.

History of Penicillin

Elder, A. L., ed., *The History of Penicillin Production*, Chem. Eng. Prog. Symp. Ser. 66 (#100), American Institute of Chemical Engineers, New York, 1970.

Hobby, G. L., *Penicillin. Meeting the Challenge*, Yale University Press, New Haven, CT, 1985.

Lax, E., *The Mold in Dr. Florey's Coat: The Story of the Penicillin Miracle,* Henry Holt & Company, New York, New York, 2004.

Moberg, C. L., Penicillin's Forgotten Man: Norman Heatley, *Science* 253(5021): 734–735, 1991.

Regulatory Issues

Agalloco, J. P., and Carleton, F. J., *Validation of Pharmaceutical Processes*, 3d ed., CRC Press, Boca Raton, FL, 2007.

Kishimoto, T. K., et al., Contaminated Heparin Associated with Adverse Clinical Events and Activation of the Contact System, *N. Engl. J. Med.* 358(23): 2457–2567, 2008.

Mullard, A., New Drugs Cost US $2.6 Billion to Develop, *Nat. Rev. Drug Discov.* 13: 877, 2014.

Naglak, T. J., Keith, M. G., and Omstead, D. R., Validation of Fermentation Processes, *Biopharm.* (July/Aug.): 28–36, 1994.

Rao, G., Moreira, A., and Bronson, K., Disposable Bioprocessing: The Future Has Arrived, *Biotechnol. Bioeng.* 102: 348–356, 2009.

Education

Lee-Parsons, C. W. T., Biochemical Engineering Taught in the Context of Drug Discovery to Manufacturing. *Chem. Eng. Edu.* 39(Pt 3): 208–221, 2005.

QUESTIONS

1.1. What is GMP, and how does it relate to the regulatory process for pharmaceuticals?

1.2. When the FDA approves a process, it requires *validation* of the process. Explain what validation means in the FDA context.

1.3. Why does the FDA approve the process and product together?

1.4. Carefully specify the differences among biotechnologist, bioprocess engineer, and bioengineer.

1.5. What is achieved by submerged fermentation in penicillin production versus surface fermentation?

2

An Overview of Biological Basics

A basic understanding of biology is essential to the biochemical engineer. Here we present selected aspects of basic biology at a level that will allow the student to function as a biochemical engineer. Although specific courses in biochemistry, genetics, microbiology, and cell biology provide much more depth, most engineering students do not have an opportunity to take three or four courses in the biological sciences, and some have no opportunity to take any of these courses. Thus, we have selected the parts of microbiology, biochemistry, genetics, and cell biology most directly relevant to bioprocesses to emphasize throughout chapters 2 to 8 and present this material with examples of application to bioprocesses.

2.1. MICROBIAL DIVERSITY

Life is very tenacious and can exist in extreme environments. Living cells can be found almost anywhere that water is in the liquid state. The right temperature, pH, and moisture levels vary from one organism to another.

Organisms from extreme environments *(extremophiles)* often provide the human race with important tools for processes to make useful chemicals and medicinals. They are also key to the maintenance of natural cycles and can be used in the

recovery of metals from low-grade ores or in the desulfurization of coal or other fuels. The fact that organisms can develop the capacity to exist and multiply in almost any environment on earth is extremely useful.

Some cells can grow at −20°C (in a brine to prevent freezing), while others can grow at 120°C (where water is under high enough pressure to prevent boiling). Cells that grow best at low temperatures (below 20°C) are usually called *psychrophiles*, and those with temperature optima in the range of 20° to 50°C are *mesophiles*. Organisms that grow best at temperatures greater than 50°C are *thermophiles*.

Most organisms grow around neutral pH (6 to 8) and are called *neutrophiles*. There are some, however, that have pH optima far from neutrality, preferring pH values down to 1 or 2 *(acidophiles)* or well above pH 9 *(alkaliphiles)*. Some organisms can grow at both low pH values and high temperatures. Although most organisms can grow only where water activity is high, others can grow on barely moist solid surfaces or in solutions with high salt concentrations *(halophiles)*.

Some cells require oxygen for growth and metabolism. Such organisms can be termed *aerobic*. Other organisms are inhibited by the presence of oxygen and grow only *anaerobically*. Some organisms can switch the metabolic pathways to allow them to grow under either circumstance. Such organisms are *facultative*.

Often, organisms can grow in environments with almost no obvious source of nutrients. Some *cyanobacteria* (formerly called blue-green algae) can grow in an environment with only a little moisture and a few dissolved minerals. These bacteria are photosynthetic and can convert CO_2 from the atmosphere into the organic compounds necessary for life. They can also convert N_2 into NH_3 for use in making the essential building blocks of life. Such organisms are important in colonizing nutrient-deficient environments.

Not only do organisms occupy a wide variety of habitats, but they also come in a wide range of sizes and shapes. Spherical, cylindrical, ellipsoidal, spiral, and pleomorphic cells exist. Special names are used to describe the shape of bacteria. A cell with a spherical or elliptical shape is often called a *coccus* (plural, *cocci*); a cylindrical cell is a rod or *bacillus* (plural, *bacilli*); a spiral-shaped cell is a *spirillum* (plural, *spirilla*). Some cells may change shape in response to changes in their local environment.

Thus, organisms can be found in the most extreme environments and have a wondrous array of different shapes, sizes, and metabolic capabilities. This great diversity provides the engineer with an immense variety of potential tools, many of which have been exploited. Take, for example, the heat-resistant enzyme *Taq* DNA polymerase, an enzyme which is derived from a thermophilic bacterium named *Thermus aquaticus*. *Taq* has become one of the most important enzymes in molecular biology because of its use in a DNA amplification technique known as the *polymerase chain reaction* (PCR; discussed in Chapter 8, "How Cellular Information Is Altered").

2.1.1. Naming Cells

How cells are named is complicated by the bewildering variety of organisms present. A systematic approach to classifying these organisms is an essential aid to their intelligent use. *Taxonomy* is the development of approaches to organize and

summarize our knowledge about the variety of organisms that exist. Although knowledge of taxonomy may seem remote from the needs of the engineer, it is necessary for efficient communication among engineers and scientists working with living cells. Taxonomy can also play a critical role in patent litigation involving bioprocesses.

While taxonomy is concerned with approaches to classification, *nomenclature* refers to the actual naming of organisms. For microorganisms, we use a dual name (binary nomenclature). The names are given in Latin, or are Latinized. A genus is a group of related species, and a species includes organisms that are substantially alike. A common gut organism that has been well studied is *Escherichia coli. Escherichia* is the genus and *coli* the species. When writing a report or paper, it is common practice to give the full name when the organism is first mentioned but in subsequent discussion to abbreviate the *genus* to the first letter. In this case, we would use *E. coli*. Although organisms that belong to the same species all share the same major characteristics, there are subtle and often technologically important variations within species. An *E. coli* used in one laboratory may differ from that used in another. Thus, various strains and substrains are designated by the addition of letters and numbers. For example, *E. coli* B/r A will differ in growth and physiological properties from *E. coli* K12.

Now that we know how to name organisms, we could consider broader classification up to the level of kingdoms. There is no universal agreement on how to classify microorganisms at this level. Such classification is rather arbitrary and need not concern us. However, we must be aware that there are two primary cell types: *eucaryotic* and *procaryotic*. The primary difference between them is the presence or absence of a membrane around the cell's genetic information.

Procaryotes have a simple structure with a single chromosome. Procaryotic cells have no nuclear membrane and no organelles, such as the mitochondria and endoplasmic reticulum. Eucaryotes have a more complex internal structure, with more than one chromosome (DNA molecule) in the nucleus. Eucaryotic cells have a true nuclear membrane and contain mitochondria, endoplasmic reticulum, Golgi apparatus, and a variety of specialized organelles. We describe each of these components in Section 2.1.4. A detailed comparison of procaryotes and eucaryotes is presented in Table 2.1. Structural differences between procaryotes and eucaryotes are discussed later.

There is further distinction of subgroups within procaryotes. Evidence suggests that a common or universal ancestor gave rise to three distinctive branches of life: eucaryotes, eubacteria (or "true" bacteria), and archaebacteria. Table 2.2 summarizes some of the distinctive features of these groups. All of the genes of whole organisms, known as the *genome*, can now be readily sequenced and this information is having a great impact on our understanding of how these families evolved and are related.

Viruses cannot be classified under any of these categories, as they are not free-living organisms. Let's consider first some of the characteristics of these rather simple "organisms."

TABLE 2.1. A Comparison of Procaryotes with Eucaryotes

Characteristic	Procaryotes	Eucaryotes
Genome		
Number of DNA molecules	One	More than one
DNA in organelles	No	Yes
DNA observed as chromosomes	No	Yes
Nuclear membrane	No	Yes
Mitotic and meiotic division of the nucleus	No	Yes
Formation of partial diploid	Yes	No
Organelles		
Mitochondria	No	Yes
Endoplasmic reticulum	No	Yes
Golgi apparatus	No	Yes
Photosynthetic apparatus	Chlorosomes	Chloroplasts
Carboxysomes	Yes	No
Flagella	Single protein, simple structure	Complex structure, with microtubules
Spores	Endospores	Endo- and exospores
Heat resistance	High	Low

With permission, from N. F. Millis in *Comprehensive Biotechnology*, M. Moo-Young, ed., Vol. 1, Elsevier Science, 1985.

TABLE 2.2. Primary Subdivisions of Cellular Organisms That Are Now Recognized

Group	Cell Structure	Properties	Constituent Groups
Eucaryotes	Eucaryotic	Multicellular; extensive differentiation of cells and tissues	Plants (seed plants, ferns, mosses)
			Animals (vertebrates, invertebrates)
		Unicellular, coenocytic or mycelial; little or no tissue differentiation	Protists (algae, fungi, protozoa)
Eubacteria	Procaryotic	Cell chemistry similar to eucaryotes	Most bacteria
Archaebacteria	Procaryotic	Distinctive cell chemistry	Methanogens, halophiles, thermoacidophiles

R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, and P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.

2.1.2. Viruses

Viruses are very small and are obligate parasites of other cells, such as bacterial, yeast, plant, and animal cells. Viruses cannot capture or store free energy and are not functionally active except when inside their host cells. The sizes of viruses vary from 30 to 200 nanometers (nm). Viruses contain either DNA (DNA viruses) or RNA (RNA viruses) as genetic material. DNA stands for *deoxyribonucleic acid*, and RNA is *ribonucleic acid*; we will soon discuss these molecules in more detail. In free-living cells, all genetic information is contained in the DNA, whereas viruses can use either RNA or DNA to encode such information. The viral genome is surrounded by

a protein coat called a *capsid*. Some viruses have an outer envelope of a lipoprotein and some do not.

Almost all cell types are susceptible to viral infections. Viruses infecting bacteria are called *bacteriophages*. Some bacteriophages have a hexagonal head, tail, and tail fibers. Bacteriophages attach to the cell wall of a host cell with tail fibers, alter the cell wall of the host cell, and inject the viral genetic material into the host cell. Figure 2.1 describes the attachment of a virus onto a host cell. Bacteriophage nucleic acids reproduce inside the host cells to produce more phages. At a certain stage of viral reproduction, host cells lyse, or break apart, and phage particles are released, which can infect new host cells. This mode of reproduction of viruses is called the *lytic cycle*. In some cases, phage DNA may be incorporated into the host DNA, and the host may continue to multiply in this state, which is called the *lysogenic cycle*.

Viruses are the cause of many diseases, and antiviral agents are important targets for drug discovery. Additionally, viruses are directly important to bioprocess technology. For example, a phage attack on an *E. coli* fermentation to make a recombinant protein product can be extremely destructive, causing the loss of the entire culture in vessels of many thousands of liters. However, phages can be used as agents

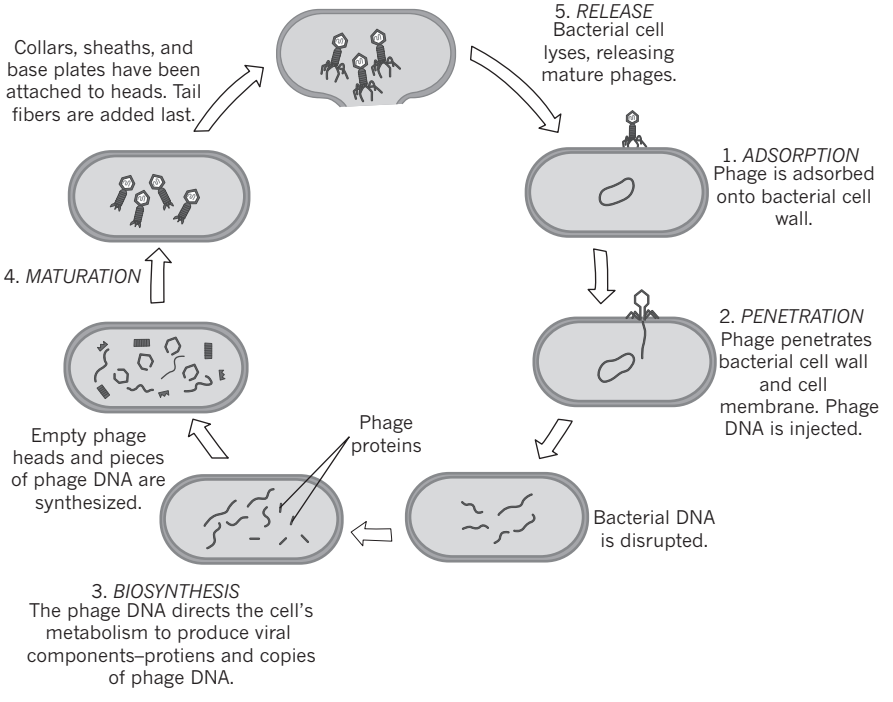


Figure 2.1. Replication of a virulent bacteriophage. A virulent phage undergoes a lytic cycle to produce new phage particles within a bacterial cell. Cell lysis releases new phage particles that can infect more bacteria. (J. G. Black, *Microbiology: Principles and Applications,* 3d ed., © 1996.)

to move desired genetic material into *E. coli*. Modified animal viruses can be used as vectors in genetically engineering animal cells to produce proteins from recombinant DNA technology. In some cases, a killed virus preparation is used as a vaccine. In other cases, genetic engineering allows production of viruslike particles (VLPs) that are empty shells; the shell is the capsid, and all nucleic acid is removed. Such particles can be used as vaccines without fear of viral replication because all of the genetic material has been removed. For gene therapy, one approach is to use a virus in which viral genetic material has been modified or replaced with the desired gene to be inserted into the patient. The viral capsid can act as a Trojan horse to protect the desired gene in a hostile environment and then deliver it selectively to a particular cell type. Thus, although viruses can do great harm, they are also important biotechnological tools.

2.1.3. Procaryotes

The sizes of most procaryotes vary from 0.5 to 3 micrometers (μm) in equivalent radius. Different species have different shapes, such as spherical or coccus (e.g., *Staphylococci*), cylindrical or bacillus (e.g., *E. coli*), or spiral or spirillum (e.g., *Rhodospirillum*). Procaryotic cells grow rapidly, with typical doubling times of less than 30 minutes to several hours. Also, procaryotes can utilize a variety of nutrients as carbon source, including carbohydrates, hydrocarbons, proteins, and CO_2.

2.1.3.1. Eubacteria. Eubacteria can be divided into several different groups. One distinction is based on the Gram stain (developed by Hans Christian Gram in 1884). The staining procedure first requires fixing the cells by heating. The basic dye, crystal violet, is added; all bacteria will stain purple. Next, iodine is added, followed by the addition of ethanol. *Gram-positive* cells remain purple, whereas *gram-negative* cells become colorless. Finally, counterstaining with safranin leaves gram-positive cells purple, whereas gram-negative cells turn red. The ability to react with the Gram stain reveals intrinsic differences in the structure of the cell envelope.

A typical gram-negative cell is *E. coli* (see Figure 2.2). It has an *outer membrane* supported by a thin peptidoglycan layer. *Peptidoglycan* is a complex polysaccharide with amino acids and forms a structure somewhat analogous to a chain-link fence. A second membrane (the inner or *cytoplasmic* membrane) exists and is separated from the outer membrane by the *periplasmic space*. The cytoplasmic membrane contains by weight about 50% protein, 30% lipids, and 20% carbohydrates. The cell envelope serves to retain important cellular compounds and to preferentially exclude undesirable compounds in the environment. *Lipopolysaccharide* (LPS), also known as *lipoglycans* and *endotoxins*, are macromolecules consisting of a lipid covalently linked to a polysaccharide composed of O-antigen, outer core, and inner core; they are found in the outer membrane of gram-negative bacteria and elicit strong immune responses in animals. Consequently, LPS removal is a key consideration when using gram-negative bacteria such as *E. coli* for producing therapeutic molecules. Conversely, LPS and its subcomponents (e.g., O-antigen) are the focus of numerous vaccine development efforts. Loss of membrane integrity leads to *cell lysis* (cells breaking open) and cell death. The cell envelope is crucial to the transport of selected material in and out of the cell.

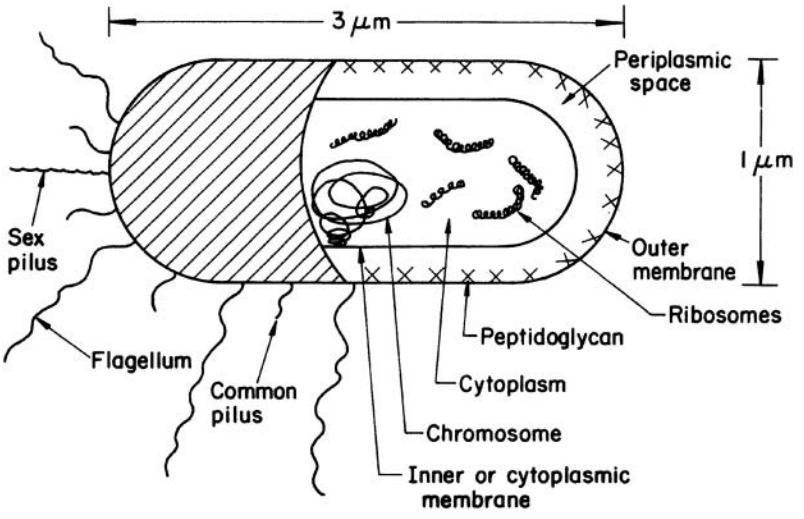


Figure 2.2. Schematic of a typical gram-negative bacterium *(E. coli)*. A gram-positive cell would be similar, except that it would have no outer membrane, its peptidoglycan layer would be thicker, and the chemical composition of the cell wall would differ significantly from the outer envelope of the gram-negative cell.

A typical gram-positive cell is *Bacillus subtilis*. Gram-positive cells do not have an outer membrane. Rather, they have a very thick, rigid cell wall with multiple layers of peptidoglycan. Gram-positive cells also contain *teichoic acids* covalently bonded to the peptidoglycan. Because gram-positive bacteria have only a cytoplasmic membrane, they are often much better suited to excretion of proteins into the culture media. Such excretion can be technologically advantageous when the protein is a desired product.

Some bacteria are not gram-positive or gram-negative. For example, the *Mycoplasma* genus of bacteria have no cell walls. These bacteria are important not only clinically (e.g., primary atypical pneumonia) but also because they commonly contaminate media used industrially for animal cell culture.

Actinomycetes are bacteria, but, morphologically, they resemble molds with their long and highly branched hyphae. However, the lack of a nuclear membrane and the composition of the cell wall require classification as bacteria. Actinomycetes are important sources of antibiotics. Some possess amylolytic and cellulolytic enzymes and are effective in enzymatic hydrolysis of starch and cellulose. *Actinomyces*, *Thermomonospora*, and *Streptomyces* are examples of genera belonging to this group.

Other distinctions within the eubacteria can be made on the basis of cellular nutrition and energy metabolism. One important example is photosynthesis. The cyanobacteria (formerly called blue-green algae) have chlorophyll and fix CO_2 into sugars. Anoxygenic photosynthetic bacteria (the purple and green bacteria) have light-gathering pigments called *bacteriochlorophyll*. Unlike true photosynthesis, the purple and green bacteria do not obtain reducing power from the splitting of water and do not form oxygen.

When stained properly, the area occupied by the procaryotic cell's DNA can be easily seen. Procaryotes may also have other visible structures when viewed under the microscope, such as *ribosomes*, *storage granules*, *bacterial microcompartments* (BMCs), *spores*, and *volutins*. Ribosomes are the site of protein synthesis. A typical bacterial cell contains approximately 10,000 ribosomes per cell, although this number can vary greatly with growth rate. A ribosome typically is 10 to 20 nm in size and consists of approximately 63% RNA and 37% protein.

Storage granules (which are not present in every bacterium) can be used as a source of key metabolites and often contain polysaccharides, lipids, phosphate, and sulfur granules. The sizes of storage granules vary between 0.5 and 1 μm. Along similar lines, BMCs are organelles that enclose enzymes and other proteins. BMCs are typically about 40 to 200 nm in diameter and are made entirely of proteins that self-assemble into icosahedral or quasi-icosahedral structures. The protein shell of BMCs is selectively permeable, functioning like a membrane. The majority are involved in either carbon fixation (carboxysomes) or aldehyde oxidation (metabolosomes), and efforts to engineer them to contain customized cargo are currently being pursued.

Some bacteria make intracellular spores (often called endospores in bacteria). Bacterial spores are produced as a resistance to adverse conditions such as high temperature, radiation, and toxic chemicals. The usual concentration is 1 spore per cell, with a spore size of about 1 μm. Spores can germinate under favorable growth conditions to yield actively growing bacteria.

Volutin is another granular intracellular structure, made of inorganic polymetaphosphates, that is present in some species. Some photosynthetic bacteria, such as *Rhodospirillum*, have chromatophores that are large inclusion bodies (50 to 100 nm) utilized in photosynthesis for the absorption of light.

Extracellular products can adhere to or be incorporated within the surface of the cell. Certain bacteria have a coating or outside cell wall called *capsule*, which is usually a polysaccharide or sometimes a polypeptide. Extracellular polymers are important to biofilm formation and response to environmental challenges (e.g., viruses). Similar to LPS, capsular polysaccharides of pathogenic bacteria have also become an important target for vaccine development. Table 2.3 summarizes the architecture of most bacteria.

2.1.3.2. Archaebacteria. The archaebacteria appear under the microscope to be nearly identical to many of the eubacteria. However, these cells differ greatly at the molecular level. In many ways, the archaebacteria are as similar to the eucaryotes as they are to the eubacteria. Some examples of differences between archaebacteria and eubacteria are as follows:

- Archaebacteria have no peptidoglycan.
- The nucleotide sequences in the ribosomal RNA are similar within the archaebacteria but distinctly different from eubacteria.
- The lipid composition of the cytoplasmic membrane is very different for the two groups.

TABLE 2.3. Characteristics of Various Components of Bacteria

Part	Size	Composition and Comments
Slime layer		
Microcapsule	5–10 nm	Protein–polysaccharide–lipid complex responsible for the specific antigens of enteric bacteria and other species.
Capsule	0.5–2.0 μm	Mainly polysaccharides (e.g., *Streptococcus*); sometimes polypeptides (e.g., *Bacillus anthracis*).
Slime	Indefinite	Mainly polysaccharides (e.g., *Leuconostoc*); sometimes polypeptides (e.g., *B. subtilis*).
Cell wall		
Gram-positive species	10–20 nm	Confers shape and rigidity upon the cell; 20% dry weight of the cell. Consists mainly of macromolecules of a mixed polymer of *N*-acetyl muramic peptide, teichoic acids, and polysaccharides.
Gram-negative species	10–20 nm	Consists mostly of a protein–polysaccharide–lipid complex with a small amount of the muramic polymer.
Cell membrane	5–10 nm	Semipermeable barrier to nutrients; 5–10% dry weight of the cell, consisting of 50% protein, 28% lipid, and 15–20% carbohydrate in a double-layered membrane.
Flagellum	10–20 nm by 4–12 μm	Protein of the myosin–keratin–fibrinogen class, molecular weight (MW) of 40,000. Arises from the cell membrane and is responsible for motility.
Pilus (fimbria)	5–10 nm by 0.5–2.0 μm	Rigid protein projections from the cell. Especially long ones are formed by *E. coli*.
Inclusions		
Spore	1.0–1.5 μm by 1.6–2.0 μm	One spore is formed per cell intracellularly. Spores show great resistance to heat, dryness, and antibacterial agents.
Storage granule	0.5–2.0 μm	Glycogen, sulfur, or lipid granules may be found in some species.
Chromatophore	50–100 nm	Organelles in photosynthetic species. *Rhodospirillum rubrum* contains about 6,000 per cell.
Ribosome	10–30 nm	Organelles for synthesis of protein. About 10,000 ribosomes per cell. They contain 63% RNA and 37% protein.
Volutin	0.5–1.0 μm	Inorganic polymetaphosphates that stain metachromatically.
Microcompartments	40–2,000 nm	Organelles consisting of a protein shell that enclose enzymes and other proteins.
Nuclear material		Composed of DNA that functions genetically as if the genes were arranged linearly on a single endless chromosome, but that appears by light microscopy as irregular patches with no nuclear membrane or distinguishable chromosomes. Autoradiography confirms the linear arrangement of DNA and suggests a MW of at least 1000×10^6.

With permission, from S. Aiba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, 2d ed., University of Tokyo Press, Tokyo, 1973.

The archaebacteria usually live in extreme environments and possess unusual metabolism. Methanogens, which are methane-producing bacteria, as well as the thermoacidophiles and halobacteria, belong to this group. The thermoacidophiles can grow at high temperatures and low pH values, whereas the halobacteria can live only in very strong salt solutions. Together, these organisms are important sources for catalytically active proteins (enzymes) with novel properties.

2.1.4. Eucaryotes

Fungi (yeasts and molds), algae, protozoa, and animal and plant cells constitute the eucaryotes. Eucaryotes are five to ten times larger than procaryotes in diameter (e.g., yeast about 5 μm, animal cells about 10 μm, and plants about 20 μm). Eucaryotes have a true nucleus and a number of cellular organelles inside the cytoplasm. Figure 2.3 is a schematic of two typical eucaryotic cells.

In cell-wall and cell-membrane structure, eucaryotes are similar to procaryotes. The plasma membrane is made of proteins and phospholipids that form a bilayer structure. Major proteins of the membrane are hydrophobic and are embedded in the phospholipid matrix. One major difference is the presence of sterols in the cytoplasmic membrane of eucaryotes. Sterols strengthen the structure and make the membrane less flexible. The cell wall of eucaryotic cells shows considerable variations. Some eucaryotes have a peptidoglycan layer in their cell wall; some have polysaccharides and cellulose (e.g., algae). The plant cell wall is composed of cellulose fibers embedded in pectin aggregates, which impart strength to the cell wall. Animal cells do not have a cell wall but only a cytoplasmic membrane. For this reason, animal cells are very shear-sensitive and fragile. This factor significantly complicates the design of large-scale bioreactors for animal cells.

The *nucleus* of eucaryotic cells contains *chromosomes* as nuclear material (DNA molecules with some closely associated small proteins), surrounded by a membrane. The nuclear membrane consists of a pair of concentric and porous membranes. The nucleolus is an area in the nucleus that stains differently and is the site of ribosome synthesis. However, many chromosomes contain small amounts of RNA and basic proteins called *histones* attached to the DNA. Each chromosome contains a single linear DNA molecule on which the histones are attached.

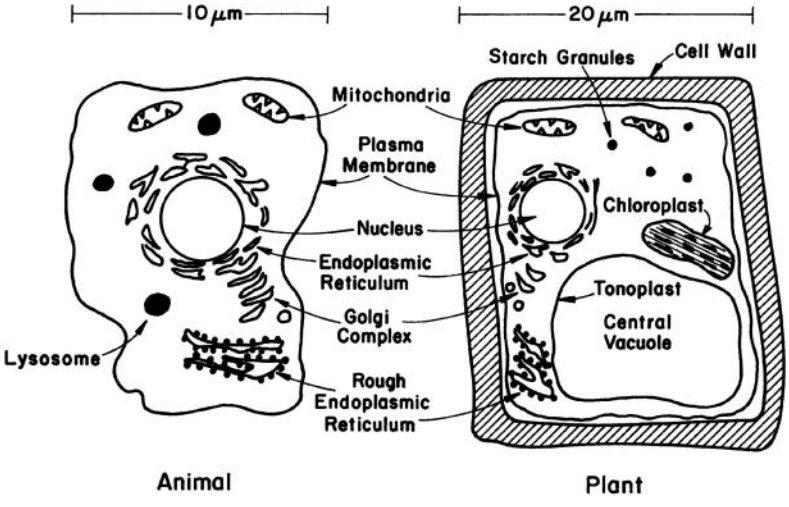


Figure 2.3. The two primary types of higher eucaryotic cells. Neither sketch is complete, but each summarizes the principal differences and similarities of such cells.

Cell division (asexual) in eucaryotes involves several major steps, such as DNA synthesis, nuclear division, cell division, and cell separation. Sexual reproduction in eucaryotic cells involves the conjugation of two cells called *gametes* (egg and sperm cells). The single cell formed from the conjugation of gametes is called a *zygote*. The zygote has twice as many chromosomes as does the gamete. Gametes are *haploid* cells, while zygotes are *diploid*. For humans, a haploid cell contains 23 chromosomes, and diploid cells have 46. The cell-division cycle (asexual reproduction) in a eucaryotic cell is depicted in Figure 2.4.

The cell-division cycle is divided into four phases. The M phase consists of *mitosis* whereby the nucleus divides and *cytokinesis* whereby the cell splits into separate daughter cells. All of the phases between one M phase and the next are known collectively as the *interphase*. The interphase is divided into three phases: G_1, S, and G_2. The cell increases in size during the interphase period. In the S phase, the cell replicates its nuclear DNA. There are key checkpoints in the cycle when the cell machinery must commit to entry into the next phase. Checkpoints exist for entry into the S and M phases and exit from M phase. Cells may also be in a G_0 state, which is a resting state in which no growth occurs.

The *mitochondria* are the powerhouses of a eucaryotic cell, where respiration and oxidative phosphorylation take place. Mitochondria have a nearly cylindrical shape 1 μm in diameter and 2 to 3 μm in length. The typical structure of a mitochondrion is shown in Figure 2.5. The external membrane is made of a phospholipid bilayer with proteins embedded in the lipid matrix. The mitochondria contain a complex system of inner membranes called *cristae*. A gel-like matrix containing large amounts of protein fills the space inside the cristae. Some enzymes of oxidative respiration are bound to the cristae. A mitochondrion has its own DNA and protein-synthesizing machinery and reproduces independently.

The *endoplasmic reticulum* is a complex, convoluted membrane system leading from the cell membrane into the cell. The rough endoplasmic reticulum contains ribosomes on the inner surfaces and is the site of protein synthesis and modifications

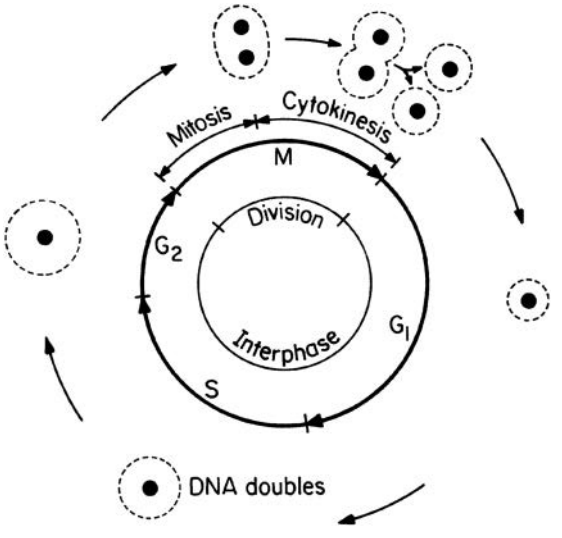


Figure 2.4. Schematic of cell-division cycle in a eucaryote. (See text for details.)

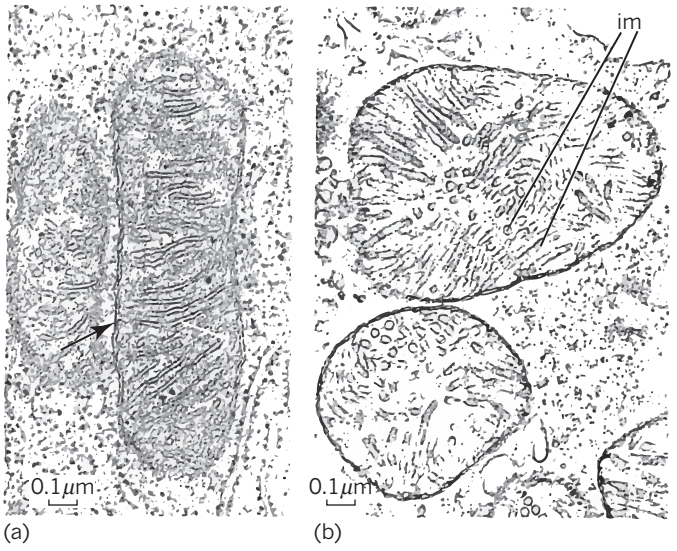


Figure 2.5. The structure of mitochondria as seen in electron micrographs of thin sections of eucaryotic cells. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, and P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

of protein structure after synthesis. The smooth endoplasmic reticulum is more involved with lipid synthesis.

Lysosomes are very small, membrane-bound particles that contain and release digestive enzymes. Lysosomes contribute to the digestion of nutrients and invading substances.

Peroxisomes are similar to lysosomes in their structure but not in function. Peroxisomes carry out oxidative reactions that produce hydrogen peroxide.

Glyoxysomes are also very small, membrane-bound particles that contain the enzymes of the glyoxylate cycle.

Golgi bodies are very small particles composed of membrane aggregates and are responsible for the secretion of certain proteins. Golgi bodies are sites where proteins are modified by the addition of various complex sugars known as *glycans* in a process called *glycosylation*. Such modifications are important to protein function in the body.

Vacuoles are membrane-bound organelles of low density and are responsible for food digestion, osmotic regulation, and waste-product storage. Vacuoles may occupy a large fraction of cell volume (up to 90% in plant cells).

Chloroplasts are relatively large, chlorophyll-containing, green organelles that are responsible for photosynthesis in photosynthetic eucaryotes, such as algae and plant cells. Every chloroplast contains an outer membrane and a large number of inner membranes called *thylakoids*. Chlorophyll molecules are associated with thylakoids, which have a regular membrane structure with lipid bilayers. Chloroplasts are autonomous units containing their own DNA and protein-synthesizing machinery.

Certain procaryotic and eucaryotic organisms contain *flagella*—long, filamentous structures that are attached to one end of the cell and are responsible for

the motion of the cell. Eucaryotic flagella contain 2 central fibers surrounded by 18 peripheral fibers, which exist in doublets. Fibers are in a tube structure called a *microtubule* and are composed of proteins called *tubulin*. The whole fiber assembly is embedded in an organic matrix and is surrounded by a membrane.

The *cytoskeleton* (in eucaryotic cells) consists of filaments that provide an internal framework to organize the cell's internal activities and control its shape. These filaments are critical in cell movement, transduction of mechanical forces into biological responses, and separation of chromosomes into the two daughter cells during cell division. Three types of fibers are present: *actin filaments*, *intermediate filaments*, and *microtubules*.

Cilia are flagella-like structures but are numerous and shorter. Only one group of protozoa, called *ciliates*, contains cilia. *Paramecium* species contain nearly 10^4 cilia per cell. Ciliated organisms move much faster than flagellated ones.

This completes our summary of eucaryotic cell structure. Now let us turn our attention to the microscopic eucaryotes.

Fungi are heterotrophs that are widespread in nature. Fungal cells are larger than bacterial cells, and their typical internal structures, such as nucleus and vacuoles, can be seen easily with a light microscope. Two major groups of fungi are yeasts and molds.

Yeasts are single small cells of 5 to 10 μm in size. Yeast cells are usually spherical, cylindrical, or oval. Yeasts can reproduce by asexual or sexual means. Asexual reproduction is by either budding or fission. In budding, a small bud cell forms on the cell and gradually enlarges and separates from the mother cell. Asexual reproduction by *fission* is similar to that of bacteria. Only a few species of yeast can reproduce by fission. In fission, cells grow to a certain size and divide into two equal cells. Sexual reproduction of yeasts involves the formation of a zygote (a diploid cell) from fusion of two haploid cells, each having a single set of chromosomes. The nucleus of the diploid cells divides several times to form *ascospores*. Each ascospore eventually becomes a new haploid cell and may reproduce by budding and fission. The life cycle of a typical yeast cell is presented in Figure 2.6.

The classification of yeasts is based on reproductive modes (e.g., *budding* or *fission*) and the nutritional requirements of cells. The most widely used yeast, *Saccharomyces cerevisiae*, is used in alcohol formation under anaerobic conditions (e.g., in wine, beer, and whiskey making) and also for baker's yeast production under aerobic conditions.

Molds are filamentous fungi and have a mycelial structure. The *mycelium* is a highly branched system of tubes that contains a mobile mass of cytoplasm with many nuclei. Long, thin filaments on the mycelium are called *hyphae*. Certain branches of mycelium may grow in the air, and asexual spores called *conidia* are formed on these aerial branches. Conidia are nearly spherical in structure and are often pigmented. Some molds reproduce by sexual means and form sexual spores. These spores provide resistance against heat, freezing, drying, and some chemical agents. Both sexual and asexual spores of molds can germinate and form new hyphae. Figure 2.7 describes the structure and asexual reproduction of molds.

Molds usually form long, highly branched cells and easily grow on moist, solid nutrient surfaces. The typical size of a filamentous form of mold is 5 to 20 μm. When

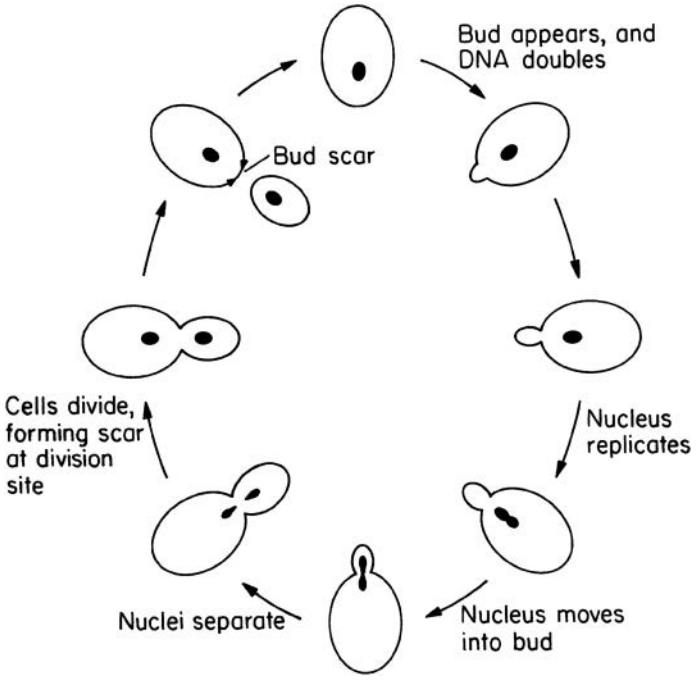


Figure 2.6. Cell-division cycle of a typical yeast, *Saccharomyces cerevisiae*. (With permission, from T. D. Brock, D. W. Smith, and M. T. Madigan, *Biology of Microorganisms*, 4th ed., Prentice Hall, Englewood Cliffs, NJ, 1984, p. 80.)

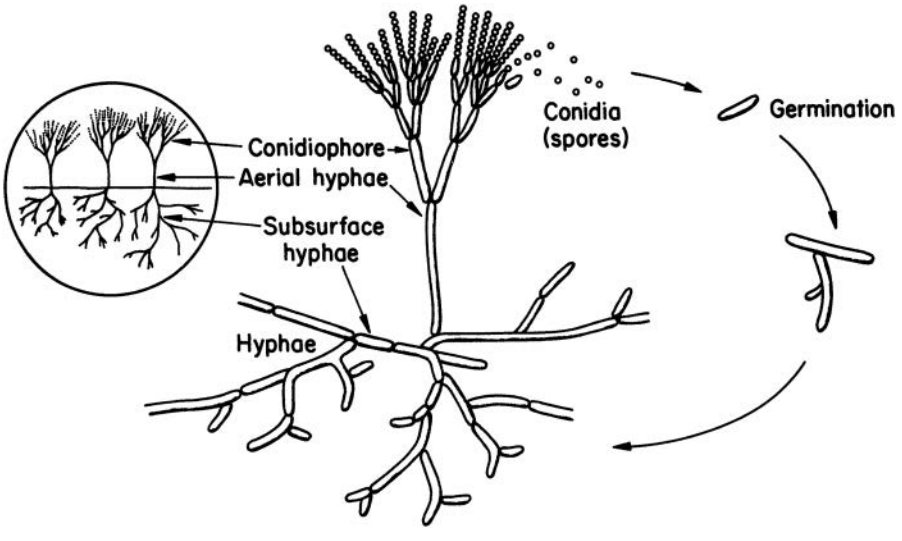


Figure 2.7. Structure and asexual reproduction of molds. (Thomas D. Brock, Katherine M. Brock, and David M. Ward, *Basic Microbiology with Applications*, 3d ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

grown in submerged culture, molds often form cell aggregates and pellets. The typical size of a mold pellet varies between 50 μm and 1 mm, depending on the type of mold and growth conditions. Pellet formation can cause some nutrient-transfer (mainly oxygen) problems inside the pellet. However, pellet formation reduces broth viscosity, which can improve bulk oxygen transfer.

On the basis of their mode of sexual reproduction, fungi are grouped in four classes:

- The phycomycetes are algae-like fungi; however, they do not possess chlorophyll and cannot photosynthesize. Aquatic and terrestrial molds belong to this category.
- The ascomycetes form sexual spores called ascospores, which are contained within a sac (a capsule structure). Some molds of the genera *Neurospora* and *Aspergillus* and yeasts belong to this category.
- The basidiomycetes reproduce by basidiospores, which are extended from the stalks of specialized cells called the basidia. Mushrooms are basidiomycetes.
- The deuteromycetes *(Fungi imperfecti)* cannot reproduce by sexual means. Only asexually reproducing molds belong to this category. Some pathogenic fungi, such as *Trichophyton*, which causes athlete's foot, belong to the deuteromycetes.

Molds are used for the production of citric acid *(Aspergillus niger)* and many antibiotics, such as penicillin *(Penicillium chrysogenum)*. Mold fermentations make up a large fraction of the fermentation industry.

Algae are usually unicellular organisms. However, some plantlike multicellular structures are present in both marine and fresh waters. All algae are photosynthetic and contain chloroplasts, which normally impart a green color to the organisms. The chloroplasts are the sites of chlorophyll pigments and are responsible for photosynthesis. The size of a typical unicellular alga is 10 to 30 μm. Multicellular algae sometimes form a branched or unbranched filamentous structure. Some algae contain silica or calcium carbonate in their cell wall. Diatoms containing silica in their cell wall are used as filter aids in industry. Some algae, such as *Chlorella*, *Scenedesmus*, *Spirulina*, and *Dunaliella*, are used for wastewater treatment with simultaneous single-cell protein production. Certain gelling agents, such as agar and alginic acid, are obtained from marine algae and seaweeds. Some algae are brown or red because of the presence of other pigments. Certain microalgae species accumulate high levels of oil (>30%) and are of commercial interest because they may be used to provide a renewable platform for production of biodiesel.

Protozoa are unicellular, motile, relatively large (5 to 50 μm) eucaryotic cells that lack cell walls. Protozoa usually obtain food by ingesting other small organisms, such as bacteria, or other food particles. Protozoa are usually uninucleate and reproduce by sexual or asexual means. They are classified on the basis of their motion. The *amoebae* move by ameboid motion, whereby the cytoplasm of the cell flows forward to form a pseudopodium (false foot), and the rest of the cell flows toward this lobe. The *flagellates* move using their flagella. *Trypanosomes* move by flagella and cause a number of diseases in humans. The *ciliates* move by motion of a large number of small appendages on the cell surface called *cilia*. The *sporozoans* are nonmotile and contain members that are human and animal parasites. These protozoa do not engulf food particles but absorb

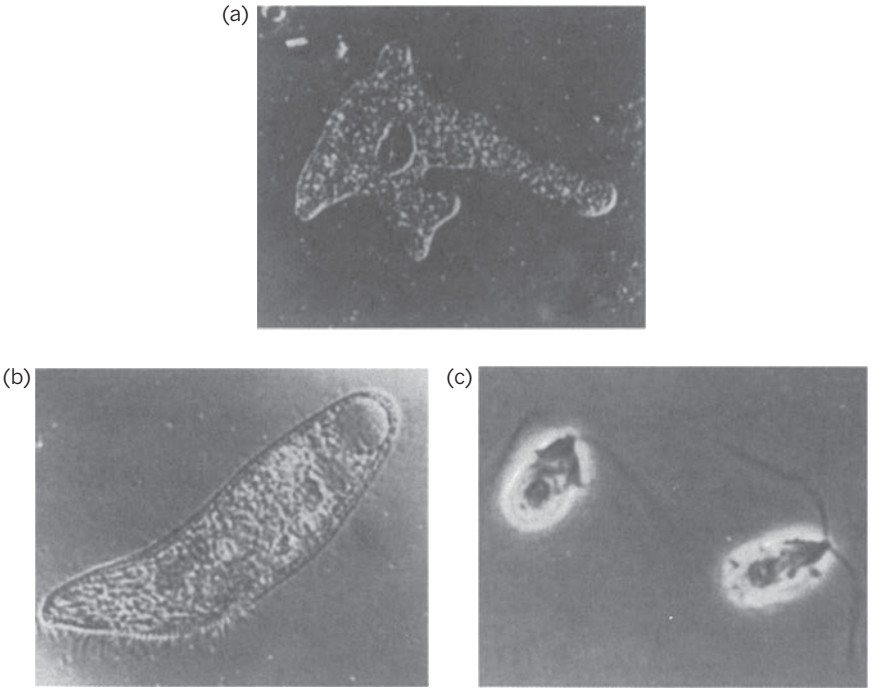


Figure 2.8. Microscopic images of protozoa. (a) An amoeba, *Amoeba proteus*. Magnification, 125X. (b) A ciliate, *Blepharisma*. Magnification, 120X. (c) A flagellate, *Dunaliella*. Magnification, 1900X. (Thomas D. Brock, Katherine M. Brock, and David M. Ward, *Basic Microbiology with Applications*, 3d ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

dissolved food components through their membranes. Protozoa cause some diseases, such as malaria and dysentery. Protozoa may have a beneficial role in removing bacteria from wastewater in biological wastewater treatment processes and helping to obtain clean effluents. Microscopic pictures of some protozoa are presented in Figure 2.8.

2.2. CELL CONSTRUCTION

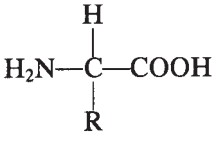
Living cells are composed of high-molecular-weight polymeric compounds such as proteins, nucleic acids, polysaccharides, lipids, and other storage materials (fats, polyhydroxybutyrate, glycogen). These biopolymers constitute the major structural elements of living cells. For example, a typical bacterial cell wall contains polysaccharides, proteins, and lipids; cell cytoplasm contains proteins mostly in the form of enzymes; in eucaryotes, the cell nucleus contains nucleic acids mostly in the form of DNA. In addition to these biopolymers, cells contain metabolites in the form of inorganic salts (e.g., NH_4^+, PO_4^{3-}, K^+, Ca^{2+}, Na^+, SO_4^{2-}), metabolic intermediates (e.g., pyruvate, acetate), and vitamins. The elemental composition of a typical bacterial cell is 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% phosphorus, and 1% sulfur, with small amounts of K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, and vitamins.

The cellular macromolecules are functional only when in the proper three-dimensional configuration. The interaction among them is very complicated. Each macromolecule is part of an intracellular organelle and functions in its unique micro-environment. Information transfer from one organelle to another (e.g., from nucleus to ribosomes) is mediated by special molecules (e.g., messenger RNA). Most of the enzymes and metabolic intermediates are present in cytoplasm. However, other organelles, such as mitochondria, contain enzymes and other metabolites. A living cell can be visualized as a very complex reactor in which more than 2000 reactions take place. These reactions (metabolic pathways) are interrelated and are controlled in a complicated fashion.

Despite all their complexity, an understanding of biological systems can be simplified by analyzing the system at several different levels: molecular (molecular biology, biochemistry); cellular (cell biology, microbiology); population (microbiology, ecology); and production (bioprocess engineering). An emergent interdisciplinary field known as *systems biology* seeks to understand this complexity using a holistic approach (holism instead of the more traditional reductionism) to study the interactions within biological systems. This section is devoted mainly to the structure and function of biological molecules.

2.2.1. Amino Acids and Proteins

Proteins are the most abundant organic molecules in living cells, constituting 40% to 70% of their dry weight. Proteins are polymers built from amino acid monomers. Proteins typically have molecular weights (MWs) of 6000 to several hundred thousand. The α-amino acids are the building blocks of proteins and contain at least one carboxyl group and one α-amino group, but they differ from each other in the structure of their R groups or side chains:



L-amino acid

Although the sequence of amino acids determines a protein's *primary* structure, the *secondary* and *tertiary* structures are determined by the weak interactions among the various side groups. The ultimate three-dimensional structure is critical to the biological activity of the protein. Two major types of protein conformation are fibrous proteins and globular proteins. Figure 2.9 depicts examples of fibrous and globular proteins. Proteins have diverse biological functions, which can be classified in five major categories:

- Structural (e.g., collagen, elastin, keratin)
- Catalytic (e.g., amylase, catalase, lysozyme, pepsin)
- Transport (e.g., aquaporin, dopamine transporter, hemoglobin, serum albumin)

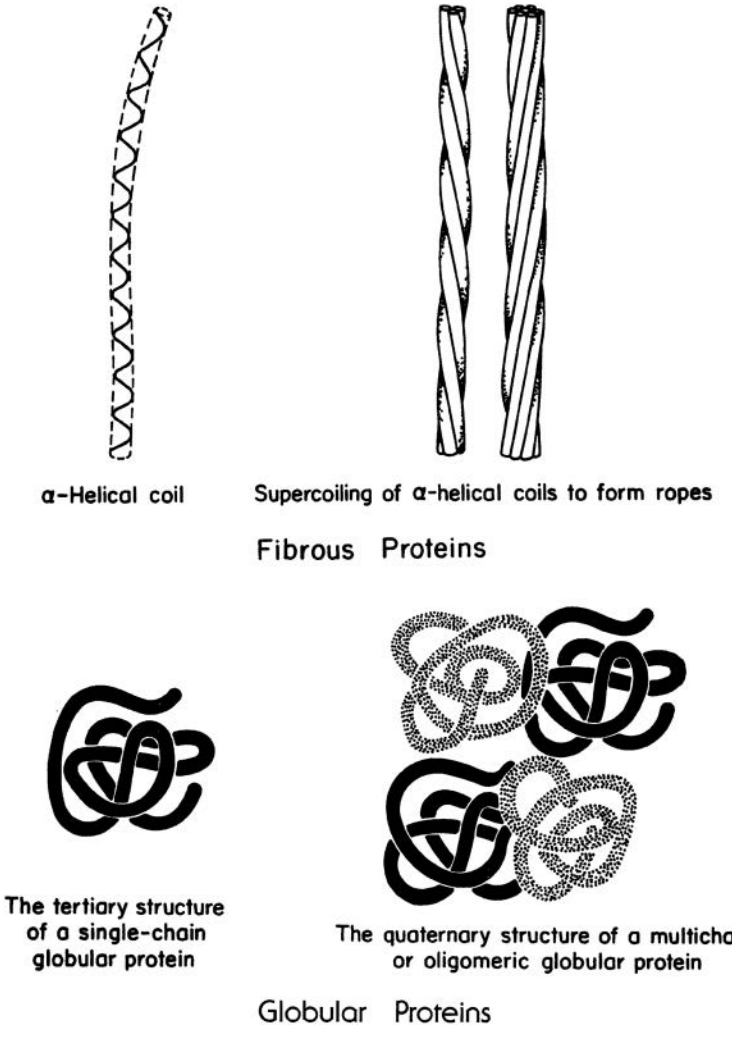


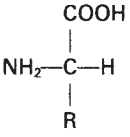
Figure 2.9. Fibrous and globular proteins. (From *Lehninger Principles of Biochemistry*, 6th ed., by David L. Nelson et al., Copyright 2013. All rights reserved. Reprinted by permission of W. H. Freeman.)

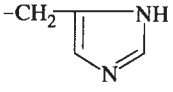
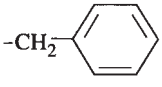
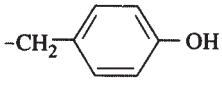
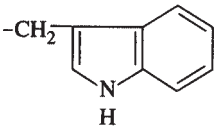
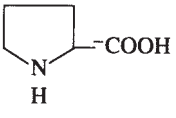
- Regulatory (e.g., human growth hormone, insulin sigma factor, transcription factor)
- Protective (e.g., antibodies, thrombin)

Enzymes represent the largest class of proteins. Over 2000 different kinds of enzymes are known. Enzymes are highly specific in their function and have extraordinary catalytic power. Enzyme molecules contain an *active site* to which specific substrates are bound during catalysis. Some enzymes regulate cellular reactions and are thus called *regulatory* enzymes. Although most enzymes are *globular* proteins, some reside in cellular membranes and are known as *integral membrane proteins*.

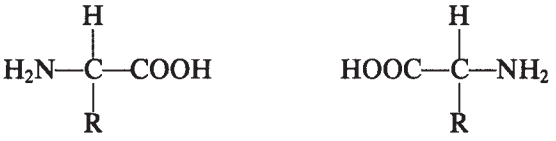
The building blocks of proteins are α-amino acids. There are 22 amino acids that can be naturally incorporated into polypeptides and are called *proteinogenic* or *natural* amino acids. Twenty of these are encoded by the universal genetic code, and the remaining two, *selenocysteine* and *pyrrolysine* (which are derivatives of cysteine and lysine), are incorporated into proteins by unique synthetic mechanisms. A list of the 20 common amino acids that are found in proteins is given in Table 2.4. Amino

TABLE 2.4. Chemical Structure of 20 Amino Acids of the General Structure



R Group	Name	Abbreviation	Symbol	Class
—H	Glycine	GLY	G	Aliphatic
—CH_3	Alanine	ALA	A	
—$CH(CH_3)_2$	Valine	VAL	V	
—$CH_2CH(CH_3)_2$	Leucine	LEU	L	
—$CHCH_3CH_2CH_3$	Isoleucine	ILU	I	
—CH_2OH	Serine	SER	S	Hydroxyl or sulfur containing
—$CHOHCH_3$	Threonine	THR	T	
—CH_2SH	Cysteine	CYS	C	
—$(CH_2)_2SCH_3$	Methionine	MET	M	
—CH_2COOH	Aspartic acid	ASP	D	Acids and corresponding amides
—CH_2CONH_2	Asparagine	ASN	N	
—$(CH_2)_2COOH$	Glutamic acid	GLU	E	
—$(CH_2)_2CONH_2$	Glutamine	GLN	Q	
—$(CH_2)_3CH_2NH_2$	Lysine	LYS	K	Basic
—$(CH_2)_3NHCNHNH_2$	Arginine	ARG	R	
	Histidine	HIS	H	
	Phenylalanine	PHE	F	Aromatic
	Tyrosine	TYR	Y	
	Tryptophan	TRP	W	
	Proline	PRO	P	Amino acid

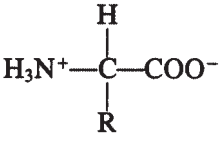
acids are named on the basis of the side (R) group attached to the α-carbon. Amino acids are optically active and occur in two isomeric forms:



L-amino acid D-amino acid

Only L-amino acids are found in proteins. D-amino acids are rare in nature; they are found in the cell walls of some microorganisms and in some antibiotics.

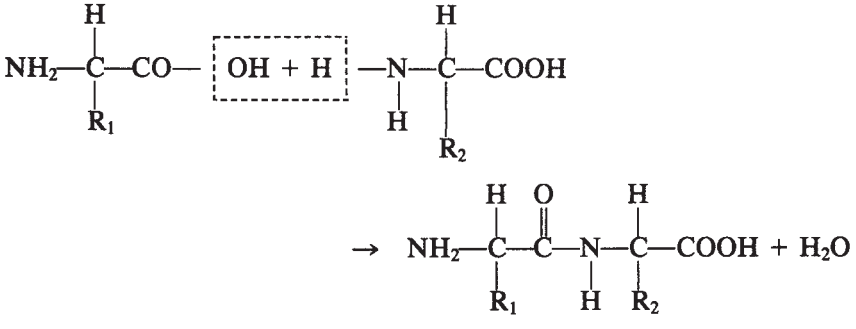
Amino acids have acidic (—COOH) and basic ($—NH_2$) groups. The acidic group is neutral at low pH (—COOH) and negatively charged at high pH ($—COO^-$). At intermediate pH values, an amino acid has positively and negatively charged groups, a dipolar molecule called a *zwitterion*:



zwitterion

The pH value at which amino acids have no net charge is called the *isoelectric point*, which varies depending on the R group of amino acids. At its isoelectric point, an amino acid does not migrate under the influence of an electric field. Knowledge of the isoelectric point can be used in developing processes for protein purification.

Proteins are linear chains of covalently linked amino acids. The condensation reaction between two amino acids results in the formation of a *peptide bond*:



(2.1)

The peptide bond is planar. Peptides contain two or more amino acids linked by peptide bonds. Polypeptides usually contain fewer than 50 amino acids. Larger amino acid chains are called *proteins*. Many proteins contain organic and/or inorganic components other than amino acids. These components are called *prosthetic groups*, and

the proteins containing prosthetic groups are named *conjugated proteins*. Hemoglobin is a conjugated protein and has four heme groups, which are iron-containing organometallic complexes.

The three-dimensional structure of proteins can be described at four levels:

- *Primary structure:* The primary structure of a protein is its linear sequence of amino acids. Each protein has not only a definite amino acid composition but also a unique sequence. The one-dimensional structure of proteins (the amino acid sequence) has a profound effect on the resulting three-dimensional structure and, therefore, on the function of proteins.
- *Secondary structure*: This structure is the means by which the polypeptide chain is extended and is a result of hydrogen bonding between residues not widely separated. Two major types of secondary structure are helixes and sheets. Helical structures can be either α-helical or triple helix. In an α-helical structure, hydrogen bonding can occur between the α-carboxyl group of one residue and the —NH group of its neighbor four units down the chain, as shown in Figure 2.10. The triple-helix structure present in collagen consists of three α-helixes intertwined in a superhelix. The triple-helix structure is rigid and stretch resistant. The α-helical structure can be easily disturbed, since H bonds are not highly stable. However, the sheet structure (β-pleated sheet) is more stable. The hydrogen bonds between parallel chains stabilize the sheet structure and provide resistance to stretching (Figure 2.11).

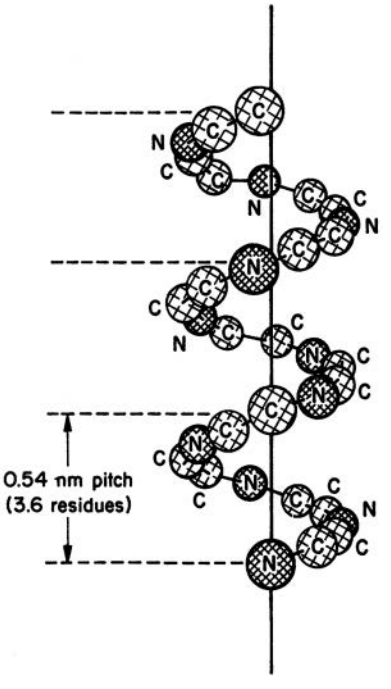


Figure 2.10. The α-helical structure of fibrous proteins.

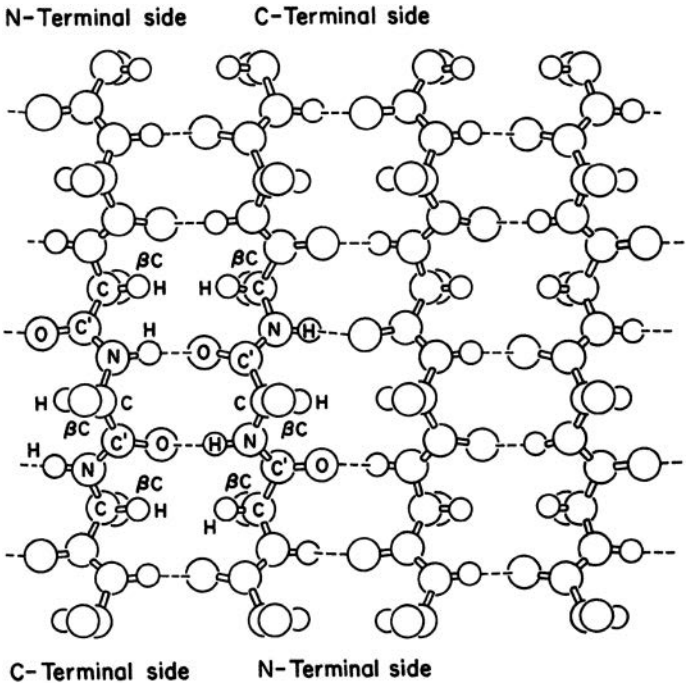


Figure 2.11. Representation of an antiparallel β-pleated sheet. Dashed lines indicate hydrogen bonds between strands.

- *Tertiary structure:* This structure is a result of interactions between R groups widely separated along the chain. The folding or bending of an amino acid chain, induced by interaction between R groups, determines the tertiary structure of proteins. R groups may interact by covalent, disulfide, or hydrogen bonds. Hydrophobic and hydrophilic interactions may also be present among R groups. A disulfide bond can cross-link two polypeptide chains (e.g., insulin). Disulfide bonds are also critical in proper chain folding, as shown in Figure 2.12. The tertiary structure of a protein has a profound effect on its function.
- *Quaternary structure:* Only proteins with more than one polypeptide chain have quaternary structure. Interactions among polypeptide chains determine the quaternary structure (Figure 2.9). Hemoglobin has four subunits (oligomeric), and interaction among these subunits results in a quaternary structure. The forces between polypeptide chains can be disulfide bonds or other weak interactions. The subunit structure of enzymes has an important role in the control of their catalytic activity.

Antibodies or *immunoglobulins* are proteins that bind to particular molecules or portions of large molecules with a high degree of specificity. Antibody (Ab)

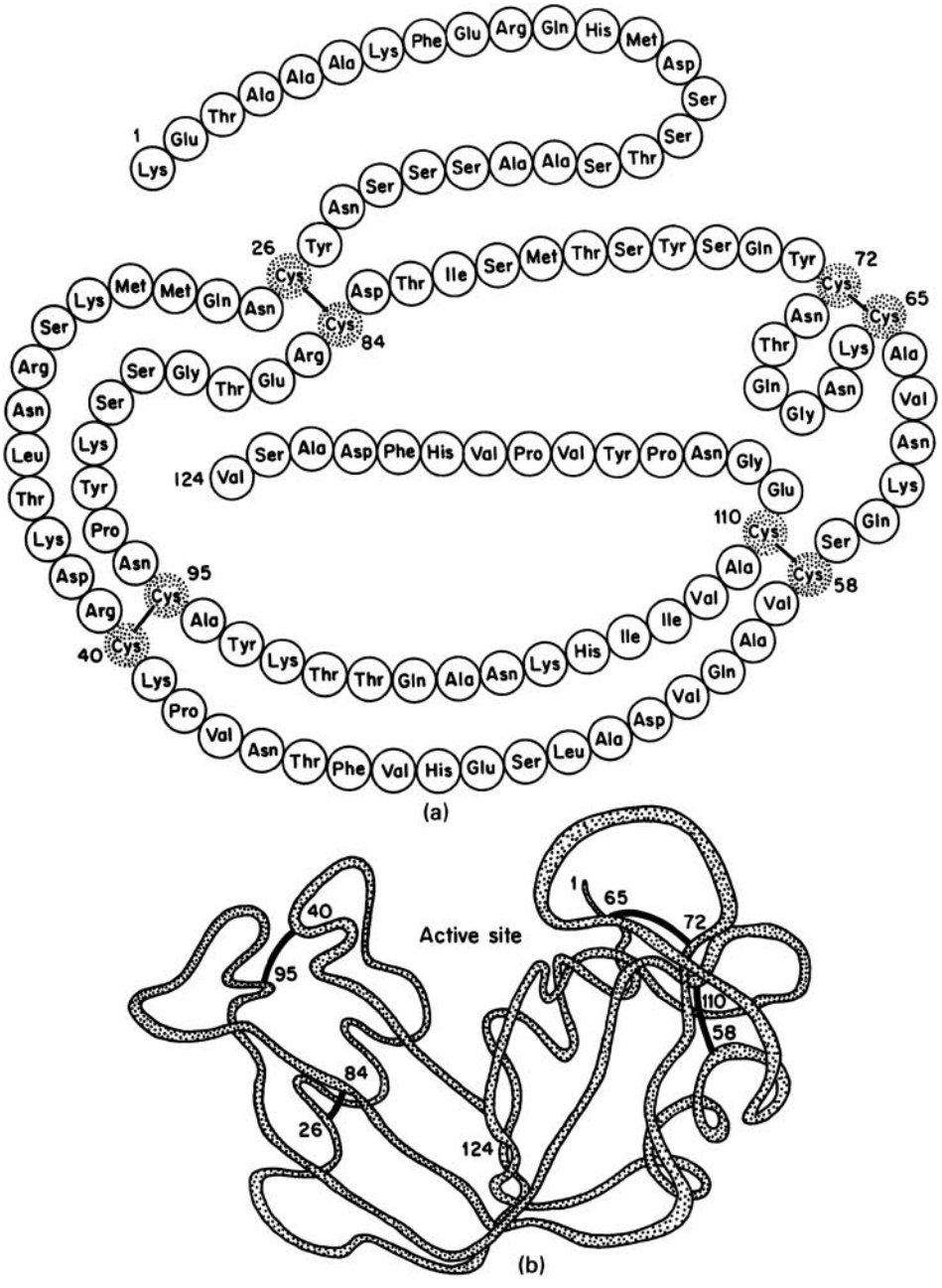


Figure 2.12. Structure of the enzyme ribonuclease. (a) Primary amino acid sequence, showing how sulfur–sulfur bonds between cysteine residues cause folding of the chain. (b) The three-dimensional structure of ribonuclease, showing how the macromolecule folds so that a site of enzymatic activity is formed, the active site. (Thomas D. Brock, Katherine M. Brock, and David M. Ward, *Basic Microbiology with Applications*, 3d ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

molecules appear in the blood serum and in certain cells of a vertebrate in response to foreign macromolecules. The foreign macromolecule, called an *antigen* (Ag), is typically a pathogen, such as a bacterium or virus, or their constituents (protein, lipid, polysaccharide). A specific antibody molecule can combine with the antigen to form an *antibody–antigen complex*. Complex formation between antibodies and antigens is a key facet of the *humoral immune response* to a foreign invader. This interaction can be exploited for medical and industrial applications. For example, natural and engineered antibodies have emerged as a powerful modality for treating various human diseases in a strategy known as *immunotherapy*. One type of engineered antibody, known as an *antibody-drug conjugate* (ADC), has become a key element in the delivery of some anticancer drugs. In addition to their clinical significance, antibodies are important industrial products for use in diagnostic kits and protein separation schemes. Taken together, antibodies have emerged as one of the most important products of biotechnology.

Antibody and antigen molecules interact by spatial complementarity (lock and key). That is, antibodies have binding sites that are specific for and complementary to the structural features of the antigen's *epitopes*, surface features arranged discontinuously along the antigen that are recognized by the antibody. Antibody molecules usually have two binding sites and can form a three-dimensional lattice of alternating antigen and antibody molecules. This complex precipitates from the serum and is called *precipitin*. Antibodies are highly specific for the foreign macromolecules that induce their formation.

The five major classes of immunoglobulins in human blood plasma are IgG, IgA, IgD, IgM, and IgE, of which the IgG globulins are the most abundant and the best understood. Immunoglobulin MWs are about 150 kilodaltons (kD); however, IgM forms a pentamer that has a MW of 900 kD, while the secreted form of IgA (sIgA) is composed of two to four IgA monomers linked by two additional chains and has a MW of 385 kD. A *dalton* is a unit of mass equivalent to a hydrogen atom. Immunoglobulins have four polypeptide chains: two heavy (H) chains (about 430 amino acids) and two light (L) chains (about 214 amino acids). These chains are linked together by disulfide bonds into a Y-shaped, flexible structure (Figure 2.13). The heavy chains contain a covalently bound oligosaccharide component known as a *glycan*. Each chain has a region of constant amino acid sequence and a variable-sequence region. The antibody molecule has two binding sites for the antigen; the variable portions of the L and H chains contribute to these binding sites. The variable sections have *hypervariable regions* or *complementarity determining regions* (CDRs) in which the frequency of amino acid replacement is high.

How cells develop and produce antibodies is a heavily studied research topic worldwide. Recent developments have also led to insights on how to impart novel activities to antibodies, including cell penetration, multiantigen targeting (e.g., bispecific antibodies), and catalytic functions (e.g., abzymes). Coupling new developments in protein engineering to antibodies promises the development of extremely specific catalytic agents that can be delivered to specific cells or tissues by design.

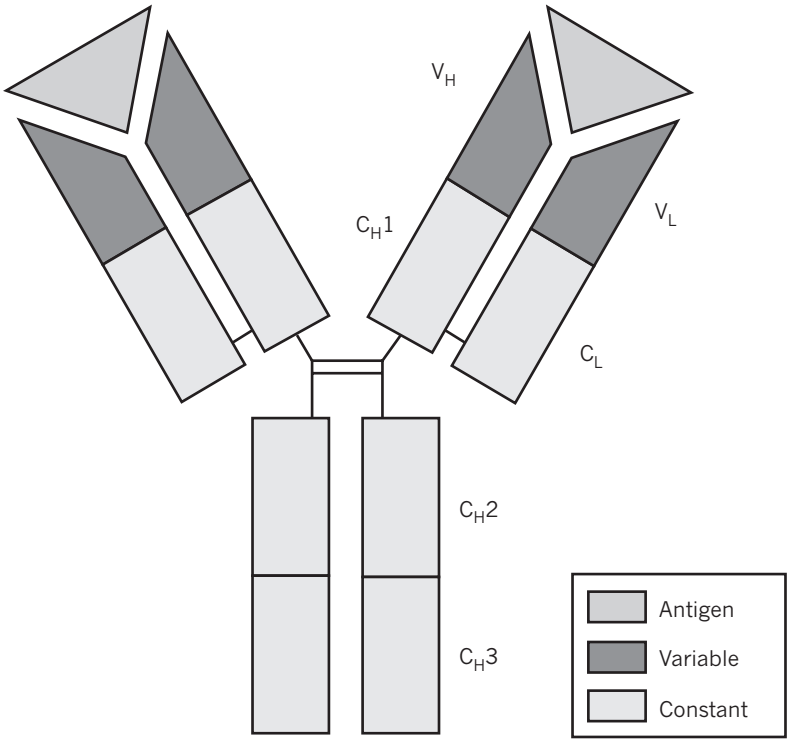
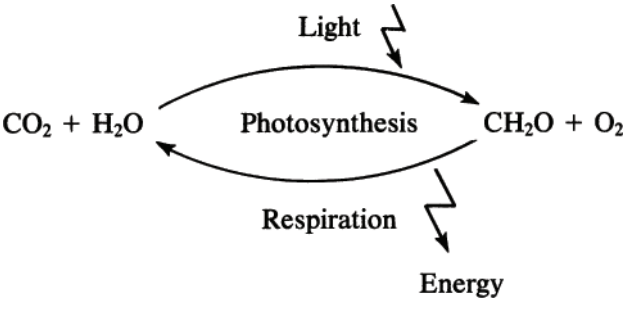


Figure 2.13. Immunoglobulin G structure. (Thomas D. Brock, Katherine M. Brock, and David M. Ward, *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

2.2.2. Carbohydrates: Mono- and Polysaccharides

Carbohydrates play key roles as structural and storage compounds in cells. They also appear to play critical roles in modulating some aspects of chemical signaling in animals and plants. Carbohydrates are represented by the general formula $(CH_2O)_n$, where $n \geq 3$, and are synthesized through photosynthesis.

The gases CO_2 and H_2O are converted through photosynthesis into sugars in the presence of sunlight and are then polymerized to yield polysaccharides, such as cellulose or starch.

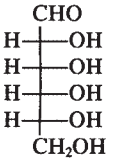


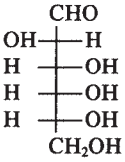
Monosaccharides are the smallest carbohydrates and contain three to nine carbon atoms. Common monosaccharides are presented in Table 2.5. Common monosaccharides are either aldehydes or ketones. For example, glucose is an aldohexose. Glucose may be present in the form of a linear or ring structure. In solution,

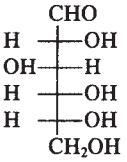
TABLE 2.5. Common Monosaccharides

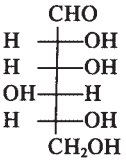
I. Aldoses

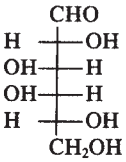
a. D-Hexoses

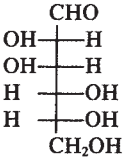

D-Allose

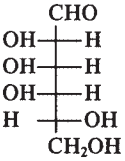

D-Altrose

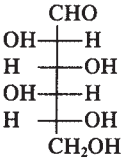

D-Glucose


D-Gulose

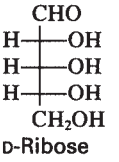

D-Galactose

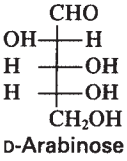

D-Mannose

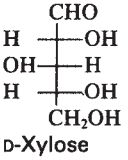

D-Talose

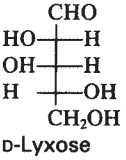

D-Idose

b. D-Pentoses

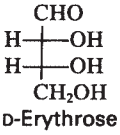

D-Ribose

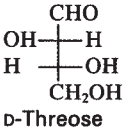

D-Arabinose


D-Xylose

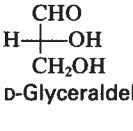

D-Lyxose

c. D-Tetroses


D-Erythrose

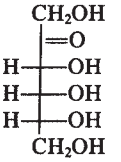

D-Threose

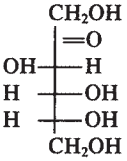
d. D-Trioses

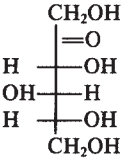

D-Glyceraldehyde

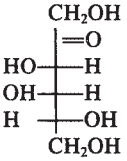
II. Ketoses

a. Ketohexoses


D-Psicose


D-Fructose

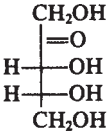

D-Sorbose


D-Togatose

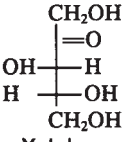
(*Continued*)

TABLE 2.5. (*Continued*)

b. Ketopentoses

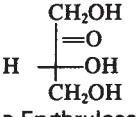


D-Ribulose



D-Xylulose

c. Ketotetroses



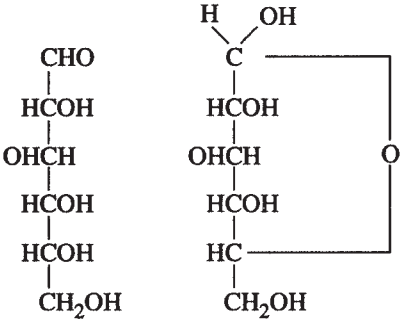
D-Erythrulose

d. Ketotriose



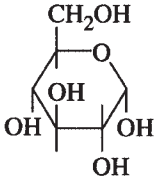
Dihydroxyacetone

D-glucose is in the form of a ring (pyranose) structure. The L-form plays a minor role in biological systems.

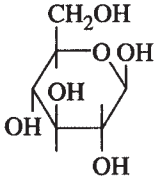


D-Glucose, linear structure

D-Glucose, ring (pyranose) structure

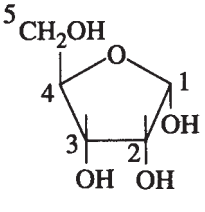


α-D-Glucose

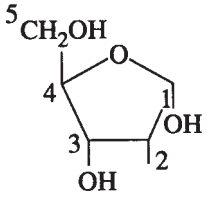


β-D-Glucose

A particularly important group of monosaccharides are D-ribose and deoxyribose. These are five carbon ring-structured sugar molecules and are essential components of DNA and RNA.

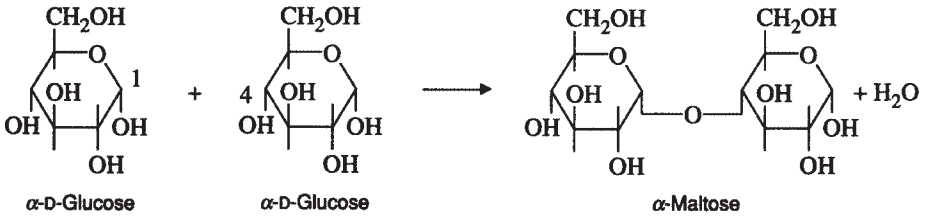


D-Ribose

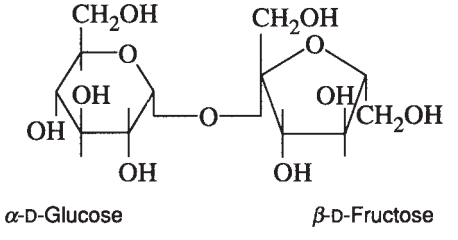


Deoxyribose

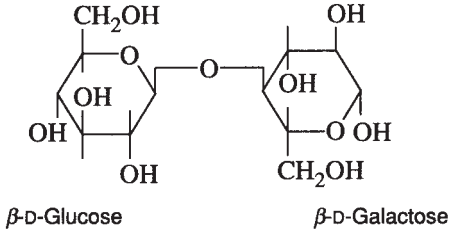
Disaccharides are formed by the condensation of two monosaccharides. For example, maltose is formed by the condensation of two glucose molecules via an α-1,4 glycosidic linkage.



Sucrose is a disaccharide of α-D-glucose and β-D-fructose. Lactose is a disaccharide of β-D-glucose and β-D-galactose.



Sucrose

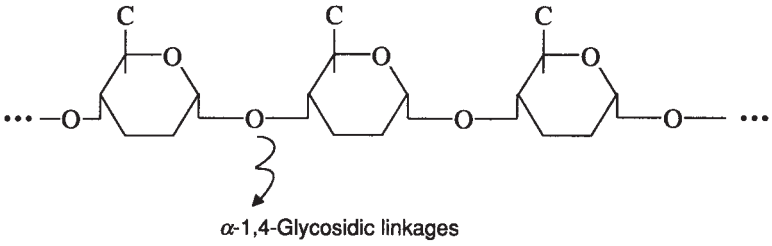


Lactose

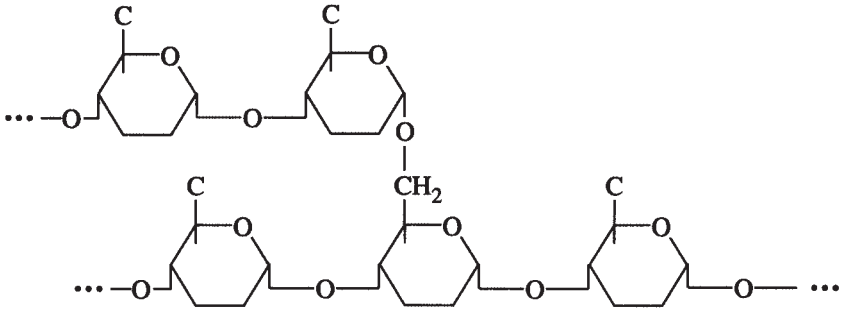
Lactose is found in milk and whey, and sucrose is the major sugar in photosynthetic plants. Whey utilization remains an important biotechnological challenge, and sucrose is often a major component in artificial growth media.

Polysaccharides are formed by the condensation of more than two monosaccharides by glycosidic bonds and can be assembled in linear and branched configurations. The polysaccharide processing industry makes extensive use of enzymatic processing and biochemical engineering.

Amylose is a straight chain of glucose molecules linked by α-1,4 glycosidic linkages. The MW of amylose is between several thousand and one-half million daltons. Amylose is water insoluble and constitutes about 20% of starch.



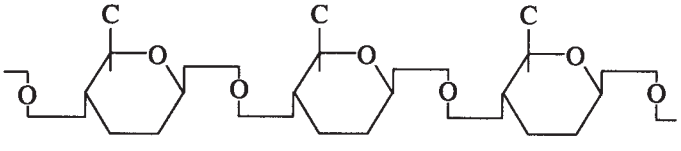
Amylopectin is a branched chain of D-glucose molecules. Branching occurs between the glycosidic —OH of one chain and the —6 carbon of another glucose, which is called an α-1,6 glycosidic linkage.



Amylopectin molecules are much larger than those of amylose, with a MW of 1 to 2 million daltons. Amylopectin is water soluble. Partial hydrolysis of starch (acidic or enzymatic) yields glucose, maltose, and dextrins, which are branched sections of amylopectin. Dextrins are used as thickeners.

Glycogen is a branched chain of glucose molecules that resembles amylopectin. Glycogen is highly branched and contains about 12 glucose units in straight-chain segments. The MW of a typical glycogen molecule is less than 5×10^6 daltons.

Cellulose is a long, unbranched chain of D-glucose with a MW between 50,000 and 1 million daltons. The linkage between glucose monomers in cellulose is a β-1,4 glycosidic linkage.



The β-1,4 glycosidic bond is resistant to enzymatic hydrolysis. Only a few microorganisms can hydrolyze β-1,4 glycosidic bonds of cellulose. α-1,4 glycosidic bonds in starch or glycogen are relatively easy to break by enzymatic or acid hydrolysis.

Efficient cellulose hydrolysis remains one of the most challenging problems in attempts to convert cellulosic materials into valuable fuels or chemicals.

Polysaccharides can exist in a free form or as attachments to other biomolecules. As conjugates, polysaccharides or glycans add an additional layer of information to biomolecules and have diverse biological roles such as regulating protein structure and function and altering cell surface properties. The process whereby a glycan is enzymatically attached to a hydroxyl or other functional group of another biomolecule (e.g., protein, lipid) is referred to as *glycosylation*. When attached to proteins, glycans can influence folding, stability, molecular interactions, and quality control. Over half of all eucaryotic proteins are predicted to be glycosylated, although yeasts have fewer total glycoproteins than multicellular eucaryotes. Not surprisingly, approximately 70% of therapeutic proteins either approved or in clinical and preclinical development are glycoproteins.

2.2.3. Lipids, Fats, and Steroids

Lipids are hydrophobic biological compounds that are insoluble in water but soluble in nonpolar solvents such as benzene, chloroform, and ether. They are usually present in the nonaqueous biological phases, such as plasma membranes. Fats are lipids that can serve as biological fuel-storage molecules. Lipoproteins and lipopolysaccharides are other types of lipids, which appear in the biological membranes of cells. Cells can alter the mix of lipids in their membranes to compensate (at least partially) for changes in temperature or to increase their tolerance to the presence of chemical agents such as ethanol.

The major component in most lipids is *fatty acids*, which consist of a straight chain of hydrocarbon (hydrophobic) groups, with a carboxyl group (hydrophilic) at the end. A typical fatty acid can be represented as follows:

$$CH_3 — (CH_2)_n — COOH$$

The value of n is typically between 12 and 20. Unsaturated fatty acids contain double —C = C— bonds, such as oleic acid:

$$\text{Oleic aid:} \quad CH_3 — (CH_2)_7 — HC = CH — (CH_2)_7 — COOH$$

A list of common fatty acids is presented in Table 2.6. The hydrocarbon chain of a fatty acid is hydrophobic (water insoluble), but the carboxyl group is hydrophilic (water soluble).

Fats are esters of fatty acids with glycerol. The formation of a fat molecule can be represented by the following reaction:

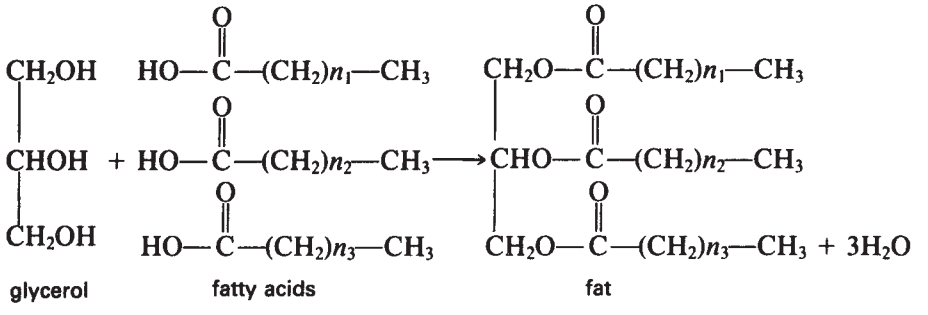
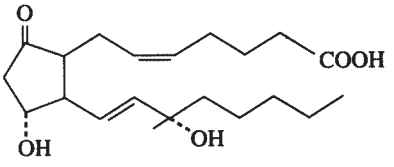


TABLE 2.6. Examples of Common Fatty Acids

Acid	Structure
Saturated fatty acids	
Acetic acid	CH_3COOH
Propionic acid	CH_3CH_2COOH
Butyric acid	$CH_3(CH_2)_2COOH$
Caproic acid	$CH_3(CH_2)_4COOH$
Decanoic acid	$CH_3(CH_2)_8COOH$
Lauric acid	$CH_3(CH_2)_{10}COOH$
Myristic acid	$CH_3(CH_2)_{12}COOH$
Palmitic acid	$CH_3(CH_2)_{14}COOH$
Stearic acid	$CH_3(CH_2)_{16}COOH$
Arachidic acid	$CH_3(CH_2)_{18}COOH$
Behenic acid	$CH_3(CH_2)_{20}COOH$
Lignoceric acid	$CH_3(CH_2)_{22}COOH$
Monoenoic fatty acids	
Oleic acid	$CH_3(CH_2)_7CH \overset{cis}{=} CH(CH_2)_7COOH$
Dienoic fatty acid	
Linoleic acid	$CH_3(CH_2)_4(CH \overset{cis}{=} CHCH_2)_2(CH_2)_6COOH$
Trienoic fatty acids	
α-Linolenic acid	$CH_3CH_2(CH \overset{cis}{=} CHCH_2)_3(CH_2)_6COOH$
γ-Linolenic acid	$CH_3(CH_2)_4(CH \overset{cis}{=} CHCH_2)_3(CH_2)_3COOH$
Tetraenoic fatty acid	
Arachidonic acid	$CH_3(CH_2)_4(CH \overset{cis}{=} CHCH_2)_4(CH_2)_2COOH$
Unusual fatty acids	
Tariric acid	$CH_3(CH_2)_{10}C{\equiv}C(CH_2)_4COOH$
Lactobacillic acid	$CH_3(CH_2)_5CH(CH_2)CH(CH_2)_9COOH$
Prostaglandin (PGE_2)	

Phosphoglycerides have similar structures to fats, the only difference being that phosphoric acid replaces a fatty acid and is esterified at one end to glycerol.

Membranes with selective permeability are key to life. Cells must control the entry and exit of molecules. *Phospholipids* are key components, but membranes contain large amounts of proteins. Biological membranes are based on a lipid bilayer. The hydrophobic tails of the phospholipids associate with each other in the core of the membrane. The hydrophilic heads form the outsides of the membrane and associate with the aqueous cytosol or the aqueous extracellular fluid. Some proteins span across the membrane *(integral membrane proteins)*, whereas others are attached to one of the surfaces *(peripheral membrane proteins)*. Membranes are dynamic structures, and lipids and proteins can diffuse rapidly. Typical membrane phospholipids

include phosphatidylcholine, phosphatidylserine, phosphatidyl glycerol, and phosphatidyl inositol.

Another class of lipids of increasing technological importance is the polyhydroxyalkanoates (PHA). In particular, *polyhydroxybutyrate* (PHB) is a good example. It can be used to form a clear, biodegradable polymeric sheet. Polymers with a variety of PHAs are being commercially developed. In some cells, PHB is formed as a storage product.

Steroids can also be classified as lipids. Naturally occurring steroids are hormones that are important regulators of animal development and metabolism at very low concentrations (e.g., 10^{-8} *M*). A well-known steroid, cholesterol, is present in membranes of animal tissues. Figure 2.14 depicts the structures of some important steroids. Cortisone is an anti-inflammatory used to treat rheumatoid arthritis and some skin diseases. Derivatives of estrogens and progesterone are used as contraceptives. The commercial production of steroids is very important and depends on microbial conversions. Because of the large number of asymmetric centers, the total synthesis of steroids is difficult. Plants provide a source of abundant lipid precursors for these steroids, but the highly specific hydroxylation of these substrates at positions 11 (and 16) or dehydrogenations at position 1 are necessary to convert the precursors

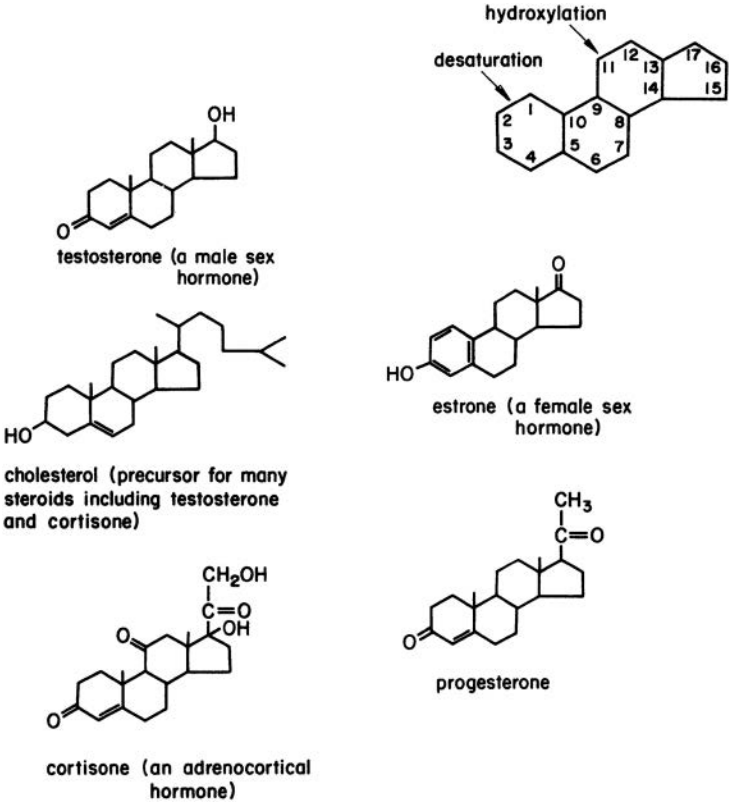


Figure 2.14. Examples of important steroids. The basic numbering of the carbon atoms in these molecules is also shown.

into compounds similar to those made in the adrenal gland. Such conversions cannot be done easily with chemical means and is done commercially using microbes that contain enzymes mediating specific hydroxylations or dehydrogenations.

2.2.4. Nucleic Acids, RNA, and DNA

Nucleic acids play a central role in reproduction of living cells. *Deoxyribonucleic acid* (DNA) stores and preserves genetic information. *Ribonucleic acid* (RNA) plays a central role in protein synthesis. Both DNA and RNA are large polymers made of their corresponding nucleotides.

Nucleotides are the building blocks of DNA and RNA and also serve as molecules to store energy and reducing power. The three major components in all nucleotides are phosphoric acid, pentose (ribose or deoxyribose), and a base (purine or pyrimidine). Figure 2.15 depicts the structure of nucleotides and purine-pyrimidine bases. Two major purines present in nucleotides are adenine (A) and guanine (G), and three major pyrimidines are thymine (T), cytosine (C), and uracil (U). DNA contains A, T, G, and C, and RNA contains A, U, G, and C as bases. It is the base sequence in DNA that carries genetic information for protein synthesis. In Chapter 4, "How Cells Work," and Chapter 8, "How Cellular Information Is Altered," we discuss how this information is expressed and passed on from one generation to another.

In Chapter 5, "Major Metabolic Pathways," we discuss further the role of nucleotides in cellular energetics. The triphosphates of adenosine and to a lesser extent guanosine are the primary energy currency of the cell. The phosphate bonds in *ATP (adenosine triphosphate)* and *GTP (guanosine triphosphate)* are high-energy bonds. The formation of these phosphate bonds or their hydrolysis is the primary means by which cellular energy is stored or used. For example, the synthesis of a compound that is thermodynamically unfavorable can be coupled to hydrolysis of ATP to ADP (adenosine diphosphate) or AMP (adenosine monophosphate). The coupled reaction can proceed to a much greater extent, since the free-energy change becomes much more negative. In reactions that release energy (e.g., oxidation of a sugar), the energy is "captured" and stored by the formation of a phosphate bond in a coupled reaction where ADP is converted into ATP.

In addition to using ATP to store energy, the cell stores and releases hydrogen atoms from biological oxidation-reduction reactions by using nucleotide derivatives. The two most common carriers of reducing power are *nicotinamide adenine dinucleotide* (NAD) and *nicotinamide adenine dinucleotide phosphate* (NADP).

In addition to this important role in cellular energetics, the nucleotides are important monomers. The polynucleotides (DNA and RNA) are formed by the condensation of nucleotides. The nucleotides are linked together between the 3′ and 5′ carbons' successive sugar rings by phosphodiester bonds. The structures of DNA and RNA are illustrated in Figure 2.16.

DNA is a very large, threadlike macromolecule (MW, 2×10^9 D in *E. coli*) and has a double-helical three-dimensional structure. The sequence of bases (purines and pyrimidines) in DNA carries genetic information, whereas sugar and phosphate groups perform a structural role. The base sequence of DNA is written in the $5' \rightarrow 3'$ direction, such as pAGCT. The double-helical structure of DNA is depicted in Figure 2.17. In this

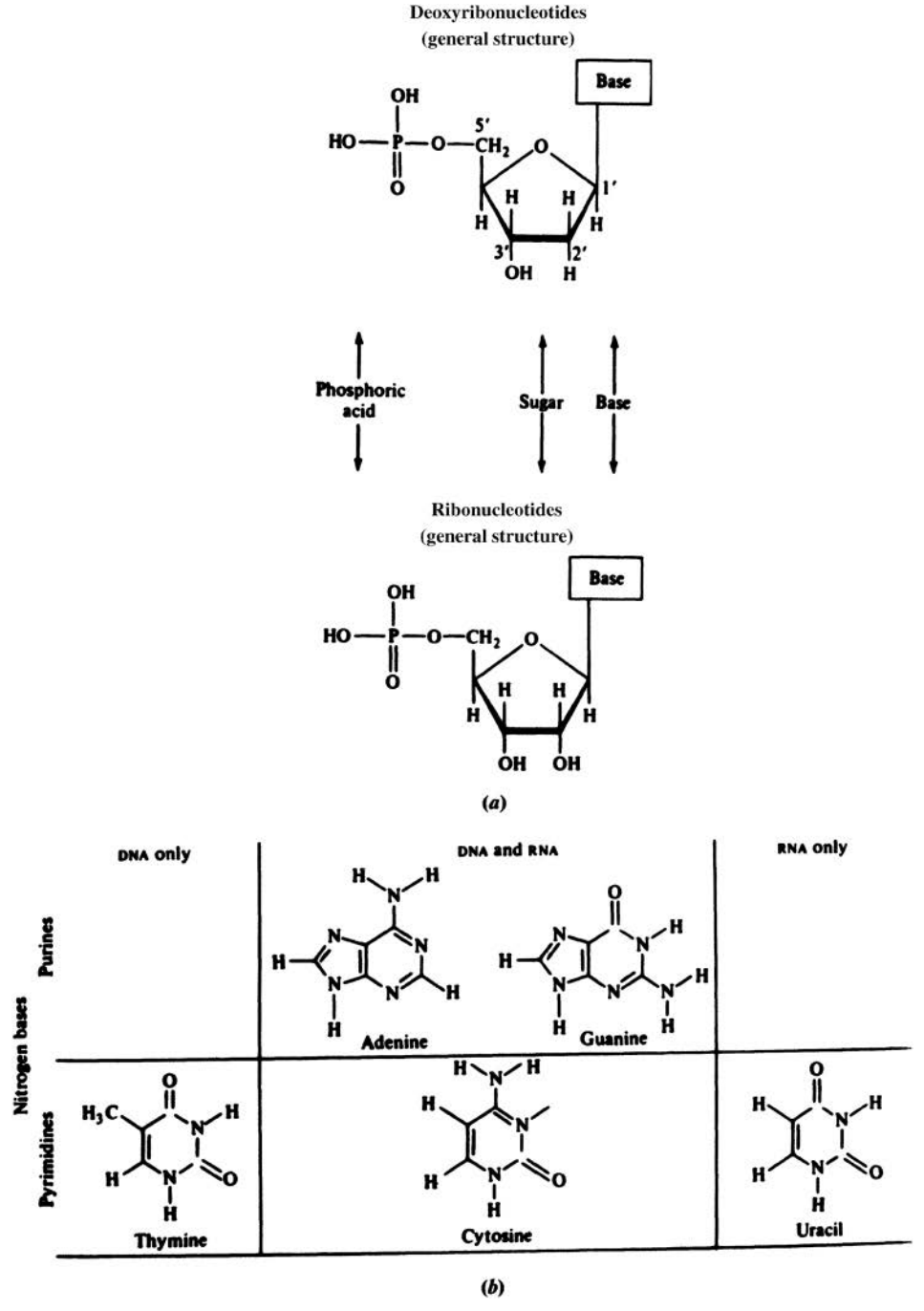


Figure 2.15. (a) General structure of ribonucleotides and deoxyribonucleotides. (b) Five nitrogenous bases found in DNA and RNA. (With permission, from J. E. Bailey and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill, New York, 1986, p. 43.)

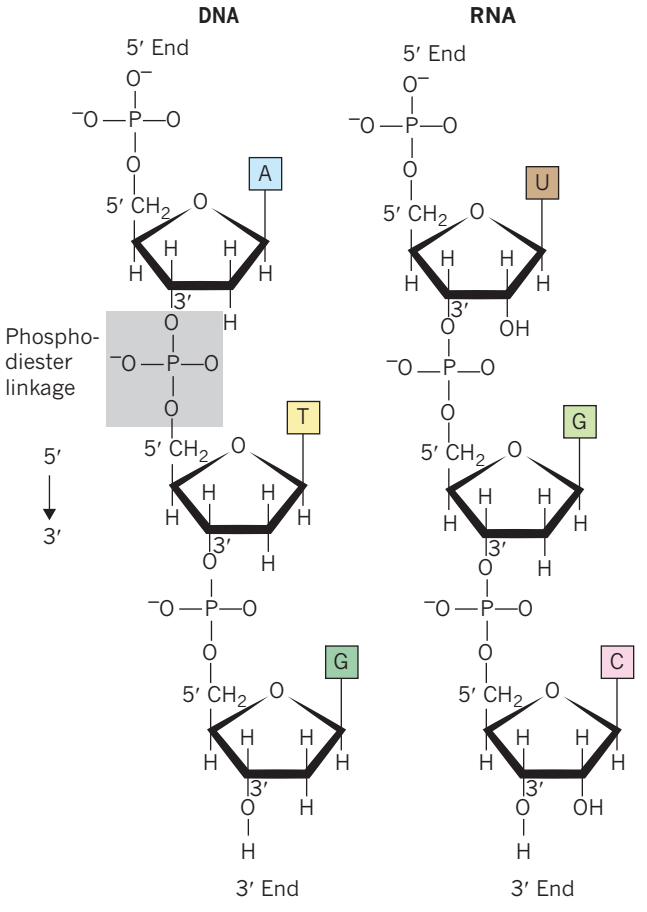


Figure 2.16. Structure of DNA and RNA molecules. Phosphodiester bonds are formed between 3′ and 5′ carbon atoms. (From *Lehninger Principles of Biochemistry*, 6th ed., by David L. Nelson et al., Copyright 2013. All rights reserved. Reprinted by permission of W. H. Freeman.)

structure, two helical polynucleotide chains are coiled around a common axis to form a double-helical DNA, and the chains run in opposite directions, 5′ → 3′ and 3′ → 5′. The main features of double-helical DNA structures are as follows:

- The phosphate and deoxyribose units are on the outer surface, but the bases point toward the chain center. The planes of the bases are perpendicular to the helix axis.
- The diameter of the helix is 2 nm. The helical structure repeats after 10 residues on each chain, at an interval of 3.4 nm.
- The two chains are held together by hydrogen bonding between pairs of bases. *Adenine is always paired with thymine* (two H bonds); *guanine is always paired with cytosine* (three H bonds). *This feature is essential to the genetic role of DNA.*
- The sequence of bases along a polynucleotide is not restricted in any way, although each strand must be complementary to the other. The precise sequence of bases carries the genetic information.

The large number of H bonds formed between base pairs provides molecular stabilization. Regeneration of DNA from original DNA segments is known as *DNA replication*.

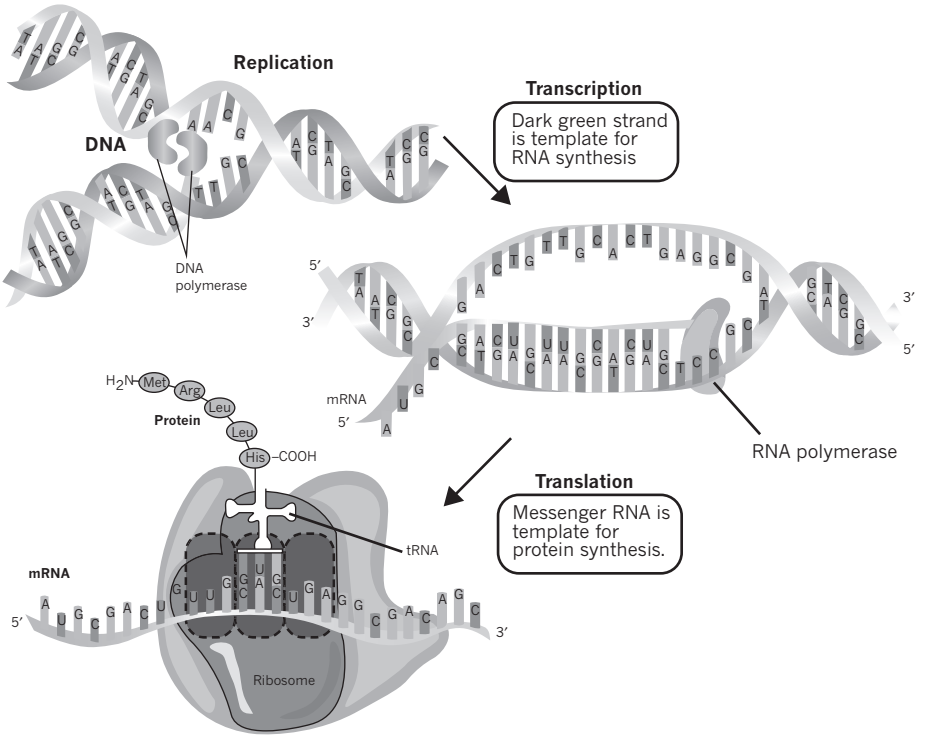


Figure 2.17. Double-helical structure of DNA, showing overall process of replication by complementary base pairing. (Thomas D. Brock, Katherine M. Brock, and David M. Ward, *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

When DNA segments are replicated, one strand of the new DNA segment comes directly from the parent DNA, and the other strand is newly synthesized using the parent DNA segment as a template. Therefore, DNA replication is semiconservative, as depicted in Figure 2.18. The replication of DNA is discussed in more detail in Chapter 4.

Some cells contain circular DNA segments in the cytoplasm that are called *plasmids.* (Linear rather than circular plasmids can be found in some yeasts and other organisms.) Plasmids are nonchromosomal, autonomous, self-replicating DNA segments. Plasmids are easily moved in and out of the cells and are often used for genetic engineering, which is discussed in more detail in Chapter 8. Naturally occurring plasmids can encode factors that protect cells from antibiotics or harmful chemicals.

The major function of DNA is to carry genetic information in its base sequence. The genetic information in DNA is transcribed by RNA molecules and translated in protein synthesis. The templates for RNA synthesis are DNA molecules, and RNA molecules are the templates for protein synthesis. The formation of RNA molecules from DNA is known as *DNA transcription*, and the formation of peptides and proteins from RNA is called *translation*.

Certain RNA molecules function as the genetic information-carrying intermediates in protein synthesis *(messenger, mRNA)*, whereas other RNA molecules

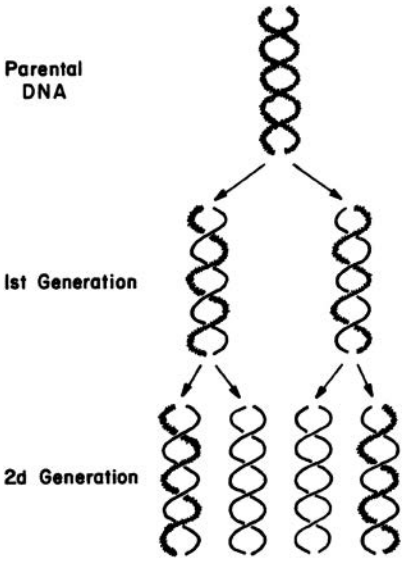


Figure 2.18. Semiconservative replication of DNA.

(*transfer [tRNA]* and *ribosomal [rRNA]*) are part of the machinery of protein synthesis. The rRNA is located in ribosomes, which are small particles made of protein and RNA. *Ribosomes* are cytoplasmic organelles (usually attached on the inner surfaces of endoplasmic reticulum in eucaryotes) and are the sites of protein synthesis.

RNA is a long, unbranched macromolecule consisting of nucleotides joined by $3' \rightarrow 5'$ phosphodiester bonds. An RNA molecule may contain from 70 to several thousand nucleotides. RNA molecules are usually single stranded, except some viral RNA. However, certain RNA molecules contain regions of double-helical structure, like hairpin loops. Figure 2.19 describes the cloverleaf structure of tRNA. In double-helical regions of tRNA, A pairs with U and G pairs with C. The RNA content of cells is usually two to six times higher than the DNA content.

It is important to note that an organism's DNA includes sequences that code for proteins *(coding sequences)* as well as sequences that do not (*noncoding DNA*; originally termed "junk" DNA). Some of the noncoding DNA is transcribed into the noncoding RNA (ncRNA) molecules discussed earlier (e.g., tRNAs, rRNAs) as well as into regulatory RNAs. Two types of important small regulatory RNAs are microRNAs (miRNAs) and small interfering RNAs (siRNAs), which can bind to other specific mRNA molecules and either increase or decrease their activity, for example, by preventing an mRNA from producing a protein. Likewise, long ncRNAs (lncRNAs) play a functional role in regulating gene transcription as well as posttranscriptional mRNA processing.

Other functions of noncoding DNA include mediating structural organization of the chromatin within the nucleus and regulating the transcription and translation of protein-coding sequences by defining the location where transcription factors can attach and control transcription of the genetic code from DNA to mRNA.

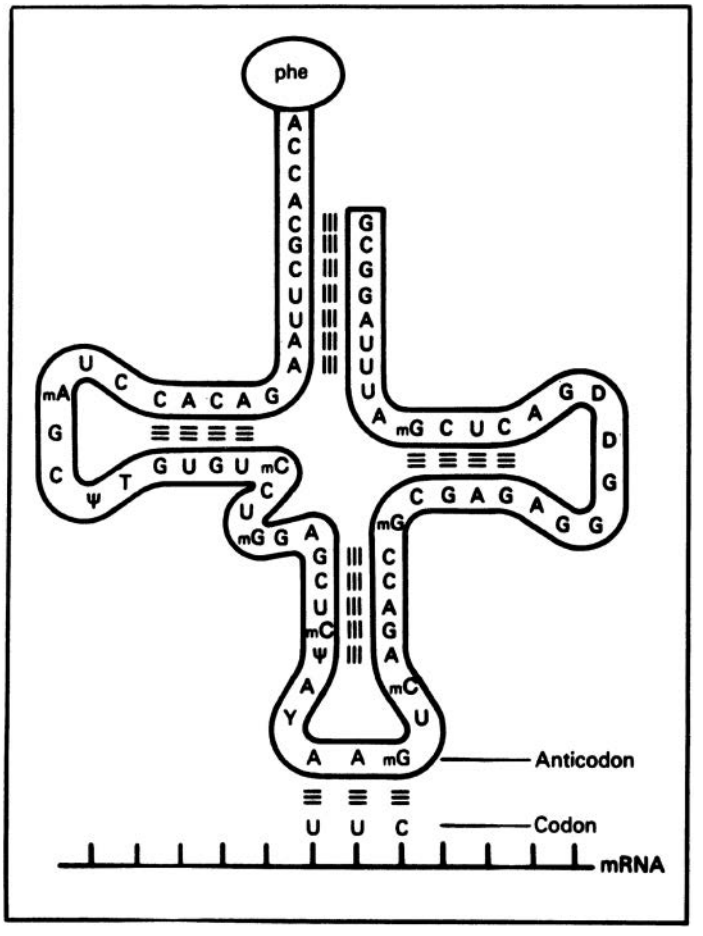


Figure 2.19. The structure of a transfer RNA (tRNA) molecule and the manner in which the anticodon of tRNA associates with the codon on messenger RNA (mRNA) by complementary base pairing. The amino acid corresponding to this codon (UUC) is phenylalanine, which is bound to the opposite end of the tRNA molecule. Many tRNA molecules contain unusual bases, such as methyl cytosine (mC) and pseudouridine (ψ). (Thomas D. Brock, Katherine M. Brock, and David M. Ward, *Basic Microbiology with Applications*, 3d ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

Let us summarize the roles of each class of RNA species:

- *Messenger RNA* (mRNA) is synthesized on the chromosome and carries genetic information from the chromosome for synthesis of a particular protein to the ribosomes. The mRNA molecule is a large one with a short half-life.
- *Transfer RNA* (tRNA) is a relatively small and stable molecule that carries a specific amino acid from the cytoplasm to the site of protein synthesis on ribosomes. tRNAs contain 70 to 90 nucleotides and have a MW range of 23 to 28 kD. Each one of 20 amino acids has at least one corresponding tRNA.

- *Ribosomal RNA* (rRNA) is the major component of ribosomes, constituting nearly 65%. The remainder is various ribosomal proteins. Three distinct types of rRNAs present in the *E. coli* ribosome are specified as 23S, 16S, and 5S, respectively, on the basis of their sedimentation coefficients (determined in a centrifuge). The symbol S denotes a Svedberg unit. The MWs are 35 kD for 5S, 550 kD for 16S, and 1100 kD for 23S. These three rRNAs differ in their base sequences and ratios. Eucaryotic cells have larger ribosomes and four different types of rRNAs: 5S, 7S, 18S, and 28S. rRNAs make up a large fraction of total RNA. In *E. coli*, about 85% of the total RNA is rRNA, while tRNA is about 12% and mRNA is 2% to 3%.
- *Noncoding RNA* (ncRNA) is any RNA molecule that is not translated into a protein. This class includes the highly abundant and functionally important tRNA and rRNA molecules. It also includes small regulatory RNAs such as miRNAs, siRNAs, and small nucleolar RNAs (snoRNAs) among others, as well as long ncRNAs (lncRNAs). The number of nucleotides for these RNAs is approximately 22 for miRNAs, 20 to 25 for siRNAs, 20 to 200 for snoRNAs, and longer than 200 for lncRNAs.

2.3. CELL NUTRIENTS

A cell's composition differs greatly from its environment. A cell must selectively remove desirable compounds from its extracellular environment and retain other compounds intracellularly. A semipermeable membrane is the key to this selectivity. Since the cell differs so greatly in composition from its environment, it must expend energy to maintain itself away from thermodynamic equilibrium. Thermodynamic equilibrium and death are equivalent for a cell.

All organisms except viruses contain large amounts of water (about 80%). About 50% of cellular dry weight is protein, and the proteins are largely enzymes (proteins that act as catalysts). The nucleic acid content (which contains the genetic code and machinery to make proteins) of cells varies from 10% to 20% of dry weight. However, viruses may contain nucleic acids up to 50% of their dry weight. Typically, the lipid content of most cells varies between 5% and 15% of dry weight. However, some cells accumulate PHB up to 90% of the total mass under certain culture conditions. In general, the intracellular composition of cells varies depending on the type and age of the cells and the composition of the nutrient media. Typical compositions for major groups of organisms are summarized in Table 2.7.

Most of the products formed by organisms are produced as a result of their response to environmental conditions, such as nutrients, growth hormones, and ions. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation. Nutrients required by cells can be classified in two categories:

- *Macronutrients* are needed in concentrations larger than 10^{-4} *M*. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+} are major macronutrients.
- *Micronutrients* are needed in concentrations of less than 10^{-4} *M*. Trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, vitamins, growth hormones, and metabolic precursors are micronutrients.

TABLE 2.7. Chemical Analyses, Dry Weights, and the Populations of Different Microorganisms Obtained in Culture

Organism	Composition (% dry weight)			Typical Population in Culture (numbers/ml)	Typical Dry Weight of This Culture (g/100 ml)
	Protein	Nucleic Acid	Lipid		
Viruses	50–90	5–50	<1	10^8–10^9	0.0005[a]
Bacteria	40–70	13–34	10–15	2×10^8–2×10^{11}	0.02–2.9
Filamentous fungi	10–25	1–3	2–7		3–5
Yeast	40–50	4–10	1–6	1–4×10^8	1–5
Small unicellular algae	10–60(50)	1–5(3)	4–80(10)	4–8×10^7	0.4–0.9

2.3.1. Macronutrients

Carbon compounds are major sources of cellular carbon and energy. Microorganisms are classified in two categories on the basis of their carbon source: heterotrophs and autotrophs. *Heterotrophs* use organic compounds such as carbohydrates, lipids, and hydrocarbons as a carbon and energy source. *Autotrophs* use carbon dioxide as a carbon source. Mixotrophs concomitantly grow under both autotrophic and heterotrophic conditions; however, autotrophic growth is stimulated by certain organic compounds. Facultative autotrophs normally grow under autotrophic conditions; however, they can grow under heterotrophic conditions in the absence of CO_2 and inorganic energy sources. *Chemoautotrophs* utilize CO_2 as a carbon source and obtain energy from the oxidation of inorganic compounds. *Photoautotrophs* use CO_2 as a carbon source and utilize light as an energy source.

The most common carbon sources in industrial fermentations are molasses (sucrose), starch (glucose, dextrin), corn syrup, and waste sulfite liquor (glucose). In laboratory fermentations, glucose, sucrose, and fructose are the most common carbon sources. Methanol, ethanol, and methane also constitute cheap carbon sources for some fermentations. In aerobic fermentations, about 50% of substrate carbon is incorporated into cells and about 50% of it is used as an energy source. In anaerobic fermentations, a large fraction of substrate carbon is converted to products and a smaller fraction is converted to cell mass (less than 20%).

Nitrogen constitutes about 10% to 14% of cell dry weight. The most widely used nitrogen sources are ammonia or the ammonium salts (NH_4Cl, $[NH_4]_2SO_4$, NH_4NO_3), proteins, peptides, and amino acids. Nitrogen is incorporated into cell mass in the form of proteins and nucleic acids. Some organisms such as *Azotobacter* species and many species of cyanobacteria fix nitrogen from the atmosphere to form ammonium. Urea may also be used as a nitrogen source by some organisms. Organic nitrogen sources such as yeast extract and peptone are expensive compared to ammonium salts. Some carbon and nitrogen sources utilized by the fermentation industry are summarized in Table 2.8.

TABLE 2.8. Some Carbon and Nitrogen Sources Used by the Fermentation Industry

Carbon Sources	Nitrogen Sources
Starch waste (maize and potato)	Soya meal
Molasses (cane and beet)	Yeast extract
Whey	Distillers solubles
n-Alkanes	Cottonseed extract
Gas oil	Dried blood
Sulfite waste liquor	Corn steep liquor
Domestic sewage	Fish solubles and meal
Cellulose waste	Groundnut meal
Carbon bean	

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Oxygen is present in all organic cell components and cellular water and constitutes about 15% to 20% of the dry weight of cells. Molecular oxygen is required as a terminal electron acceptor in the aerobic metabolism of carbon compounds. Gaseous oxygen is introduced into growth media by sparging air or by surface aeration.

Hydrogen constitutes about 8% of cell dry weight and is derived primarily from carbon compounds, such as carbohydrates. Some bacteria, such as methanogens, can utilize hydrogen as an energy source.

Magnesium is a cofactor for some enzymes and is present in cell walls and membranes. Ribosomes specifically require Mg^{2+} ions. ATP molecules in the cytoplasm are usually bound to Mg^{2+}. Magnesium is usually supplied as $MgSO_4 \cdot 7H_2O$ or $MgCl_2$.

Phosphorus constitutes about 2% to 3% of cell dry weight and is present in nucleic acids and in the cell wall of some gram-positive bacteria such as teichoic acids. Some bacteria deposit polyphosphate granules. Inorganic phosphate salts, such as KH_2PO_4 and K_2HPO_4, are the most common phosphate salts. Glycerophosphates can also be used as organic phosphate sources. Phosphorus is a key element in the regulation of cell metabolism. The phosphate level in the media should be less than 1 mM for the formation of many secondary metabolites such as antibiotics.

Potassium is a cofactor for some enzymes and is required in carbohydrate metabolism. Cells tend to actively take up K^+ and Mg^{2+} and exclude Na^+ and Ca^{2+}. The most commonly used potassium salts are K_2HPO_4, KH_2PO_4, and K_3PO_4.

Sulfur constitutes nearly 0.5% to 1% of cell dry weight and is present in proteins and some coenzymes. Sulfate salts such as $(NH_4)_2SO_4$ are the most common sulfur source. Sulfur-containing amino acids can also be used as a sulfur source. Certain autotrophs utilize S^{2+} and S^0 as energy sources.

Table 2.9 lists the eight major macronutrients and their physiological role.

TABLE 2.9. Macronutrient Elements and Physiological Functions and Growth Requirements

Element	Physiological Function	Required Concentration (mol/l)
Carbon	Constituent of organic cellular material. Often the energy source	$>10^{-2}$
Nitrogen	Constituent of proteins, nucleic acids, and coenzymes	10^{-3}
Hydrogen	Organic cellular material and water	—
Oxygen	Organic cellular material and water. Required for aerobic respiration	—
Magnesium	Cofactor for many enzymes and chlorophylls (photosynthetic microbes) and present in cell walls and membranes	10^{-4} to 10^{-3}
Phosphorus	Constituent of nucleic acids, phospholipids, nucleotides, and certain coenzymes	10^{-4} to 10^{-3}
Potassium	Principal inorganic cation in the cell and cofactor for some enzymes	10^{-4} to 10^{-3}
Sulfur	Constituent of proteins and certain coenzymes	10^{-4}

With permission, from G. M. Dunn in *Comprehensive Biotechnology,* M. Moo-Young, ed., Vol. I, Elsevier Science, 1985.

2.3.2. Micronutrients

Trace elements are essential to microbial nutrition. Lack of essential trace elements increases the lag phase (the time from inoculation to active cell replication in batch culture) and may decrease the specific growth rate and the yield. Following are the three major categories of micronutrients:

- *Commonly added:* The most widely needed trace elements are Fe, Zn, and Mn. Iron (Fe) is present in ferredoxin and cytochrome and is an important cofactor. Iron also plays a regulatory role in some fermentation processes (e.g., iron deficiency is required for the excretion of riboflavin by *Ashbya gossypii*, and iron concentration regulates penicillin production by *P. chrysogenum*). Zinc (Zn) is a cofactor for some enzymes and also regulates some fermentations such as penicillin fermentation. Manganese (Mn) is also an enzyme cofactor and plays a role in the regulation of secondary metabolism and excretion of primary metabolites.
- *Sometimes added:* Trace elements needed under specific growth conditions are Cu, Co, Mo, Ca, Na, Cl, Ni, and Se. Copper (Cu) is present in certain respiratory-chain components and enzymes. Copper deficiency stimulates penicillin and citric acid production. Cobalt (Co) is present in corrinoid compounds such as vitamin B_{12}. Propionic bacteria and certain methanogens require cobalt. Molybdenum (Mo) is a cofactor of nitrate reductase and nitrogenase and is required for growth on NO_3 and N_2 as the sole source of nitrogen. Calcium (Ca) is a cofactor for amylases and some proteases and is also present in some bacterial spores and in the cell walls of some cells, such as plant cells. Sodium (Na) is needed in trace amounts by some bacteria, especially by

methanogens for ion balance. Sodium is important in the transport of charged species in eucaryotic cells. Chloride (Cl^-) is needed by some halobacteria and marine microbes, which require Na^+, too. Nickel (Ni) is required by some methanogens as a cofactor, and Selenium (Se) is required in formate metabolism of some organisms.

- *Rarely added:* Trace elements that are rarely required are B, Al, Si, Cr, V, Sn, Be, F, Ti, Ga, Ge, Br, Zr, W, Li, and I. These elements are required in concentrations of less than 10^{-6} *M* and are toxic at high concentrations, such as 10^{-4} *M*.

Some ions such as Mg^{2+}, Fe^{3+}, and PO_4^{3-} may precipitate in nutrient medium and become unavailable to the cells. *Chelating agents* are used to form soluble compounds with the precipitating ions. Chelating agents have certain groups termed *ligands* that bind to metal ions to form soluble complexes. Major ligands are carboxyl (—COOH), amine ($—NH_2$), and mercapto (—SH) groups. Citric acid, EDTA (ethylenediaminetetraacetic acid), polyphosphates, histidine, tyrosine, and cysteine are the most commonly used chelating agents. Na_2-EDTA is the most common chelating agent. EDTA may remove some metal ion components of the cell wall, such as Ca^{2+}, Mg^{2+}, and Zn^{2+}, and may cause cell-wall disintegration. Citric acid is metabolizable by some bacteria. Chelating agents are included in media in low concentrations (e.g., 1 m*M*).

Growth factors stimulate the growth and synthesis of some metabolites. Vitamins, hormones, and amino acids are major growth factors. Vitamins usually function as coenzymes. Some commonly required vitamins are thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), biotin, cyanocobalamin (B_{12}), folic acid, lipoic acid, *p*-amino benzoic acid, and vitamin K. Vitamins are required at a concentration range of 10^{-6} to 10^{-12} *M*. Depending on the organism, some or all of the amino acids may need to be supplied externally in concentrations from 10^{-6} to 10^{-13} *M*. Some fatty acids, such as oleic acid and sterols, are also needed in small quantities by some organisms. Higher forms of life, such as animal and plant cells, require hormones to regulate their metabolism. Insulin is a common hormone for animal cells, and auxin and cytokinins are plant-growth hormones.

2.3.3. Growth Media

Two major types of growth media are defined and complex media. *Defined media* contain specific amounts of pure chemical compounds with known chemical compositions. A medium containing glucose, $(NH_4)_2SO_4$, KH_2PO_4, and $MgCl_2$ is a defined medium. *Complex media* contain natural compounds whose chemical composition is not exactly known. A medium containing yeast extracts, peptone, molasses, or CSL is a complex medium. A complex medium usually can provide the necessary growth factors, vitamins, hormones, and trace elements, often resulting in higher cell yields compared to the defined medium. Often, complex media are less expensive than defined media. The primary advantage of defined media is that the results are more reproducible and the operator has better control of the fermentation. Further, recovery and purification of a product is often easier and cheaper in defined media. Table 2.10 summarizes typical defined and complex media.

TABLE 2.10. Compositions of Typical Defined and Complex Media

Defined Medium		
Constituent	Purpose	Concn (g/l)
Group A		
Glucose	C, energy	30
KH_2PO_4	K, P	1.5
$MgSO_4 \cdot 7H_2O$	Mg, S	0.6
$CaCl_2$	Ca	0.05
$Fe_2(SO_4)_3$	Fe	15×10^{-4}
$ZnSO_4 \cdot 7H_2O$	Zn	6×10^{-4}
$CuSO_4 \cdot 5H_2O$	Cu	6×10^{-4}
$MnSO_4 \cdot H_2O$	Mn	6×10^{-4}
Group B		
$(NH_4)_2HPO_4$	N	6
$(NH_4)H_2PO_4$	N	5
Group C		
$C_6H_5Na_3O_7 \cdot 2H_2O$	Chelator	4
Group D		
Na_2HPO_4	Buffer	20
KH_2PO_4	Buffer	10
Complex Medium Used in a Penicillin Fermentation		
Glucose or molasses (by continuous feed)		10% of total
Corn steep liquor		1–5% of total
Phenylacetic acid (by continuous feed)		0.5–0.8% of total
Lard oil (or vegetable oil) antifoam by continuous addition		0.5% of total
pH to 6.5 to 7.5 by acid or alkali addition		

2.4. SUMMARY

Microbes can grow over an immense range of conditions: temperatures above boiling and below freezing, high salt concentrations, high pressures (>1000 atm), and at low and high pH values (about 1 to 10). Cells that must use oxygen are known as *aerobic*. Cells that find oxygen toxic are *anaerobic*. Cells that can adapt to growth either with or without oxygen are *facultative*.

The two major groups of cells are *procaryotic* and *eucaryotic*. Eucaryotic cells are more complex. The major differences between procaryotic and eucaryotic cells are the absence (procaryotes) or presence (eucaryotes) of a membrane around the chromosomal or genetic material and cellular organelles.

The procaryotes can be divided into two major groups: the *eubacteria* and *archaebacteria*. The archaebacteria are a group of ancient organisms; subdivisions include methanogens (methane producing), halobacteria (live in high-salt environments), and thermoacidophiles (grow best under conditions of high temperature and high acidity). Most eubacteria can be separated into *gram-positive* and *gram-negative* cells. Gram-positive cells have an inner membrane and strong cell wall. Gram-negative cells have an inner membrane

and an outer membrane. The outer membrane is supported by peptidoglycan and the cell envelope is less rigid than in gram-positive cells. The *cyanobacteria* (blue-green algae) are photosynthetic procaryotes classified as a subdivision of the eubacteria.

The eucaryotes contain both single-celled organisms and multicellular systems. The fungi and yeasts, the algae, and the protozoa are all examples of single-celled eucaryotes. Plants and animals are multicellular eucaryotes.

Viruses are replicating particles that are obligate parasites. Some viruses use DNA to store genetic information, while others use RNA. Viruses that infect bacteria are called *bacteriophages* or *phages*.

All cells contain the following macromolecules: *protein*, *RNA*, and *DNA*. Other essential components of the cell are lipids and carbohydrates. Proteins are polymers of amino acids; typically, 20 different amino acids are used. Each amino acid has a distinctive side group. The sequence of amino acids determines the *primary structure* of the protein. Interactions among the side groups of the amino acids (hydrogen bonding, disulfide bonds, regions of hydrophobicity or hydrophilicity) determine the *secondary* and *tertiary structure* of the molecule. If separate polypeptide chains associate to form the final structure, then we speak of *quaternary structure*. The three-dimensional shape of a protein is critical to its function.

DNA and RNA are polymers of nucleotides. DNA contains the cell's genetic information that encodes the cell's proteins *(coding DNA)*. RNA is involved in transcribing and translating that information into real proteins *(coding RNA)*. Messenger RNA transcribes the code; transfer RNA is an adapter molecule that transports a specific amino acid to the reaction site for protein synthesis; and ribosomal RNAs are essential components of ribosomes, which are the structures responsible for protein synthesis. Both DNA and RNA also have noncoding functions. *Non-coding DNA* is transcribed into functional *noncoding RNA* molecules (e.g., transfer RNAs, ribosomal RNAs, and regulatory RNAs) and also functions in various aspects of transcriptional and translational regulation. Noncoding RNA functions to regulate gene expression at the transcriptional and posttranscriptional level.

In addition to their role as monomers for DNA and RNA synthesis, nucleotides play important roles in cellular energetics. The *high-energy phosphate bonds* in *ATP* can store energy. The hydrolysis of ATP, when coupled to otherwise energetically unfavorable reactions, can drive the reaction toward completion. *NAD* and *NAPH* are important carriers of *reducing power*.

Carbohydrates consist of sugars, and the polymerized products of sugars are called *polysaccharides*. Sugars represent convenient molecules for the rapid oxidation and release of energy. Polysaccharides play an important structural role (as in cellulose) or can be used as a cellular reserve of carbon and energy (as in starch). Complex sugars or *glycans* are frequently conjugated to other biomolecules (lipids, proteins) in a process called *glycosylation.* As attachments to other biomolecules, glycans play important structural, functional, and regulatory roles.

Lipids and related compounds are critical in the construction of cellular membranes. Some fats also form reserve sources. A number of growth factors or hormones involve lipid materials. *Phospholipids* are the primary components of biological membranes.

The maintenance of cellular integrity requires the selective uptake of nutrients. One class of nutrients is the *macronutrients*, and these are used in large amounts. The *micronutrients* and trace nutrients are used in low concentrations; some of these compounds become toxic if present at too high a level.

In a *defined medium*, all components added to the medium are identifiable chemical species. In a *complex medium*, one or more components are not chemically defined (e.g., yeast extract).

SUGGESTIONS FOR FURTHER READING

Alberts, B., D. Bray, K. Hopkin, A. Johnson, J. Lewis, M. Ruff, K. Roberts, and P. Walter, *Essential Cell Biology*, 4th ed., Garland Science, New York, 2013.

Hardin, J., G. Bertoni, and L. J. Kleinsmith, *Becker's World of the Cell*, 9th ed., Pearson, Boston, 2015.

Madigan, M. T., J. M. Martinko, K. S. Bender, D. H. Buckley, and D. A. Stahl, *Brock Biology of Microorganisms*, 14th ed., Pearson, Boston, 2014.

Mathews, C. K., K. E. Van Holde, D. R. Appling, and S. J. Anthony-Cahill, *Biochemistry*, 4th ed., Pearson, Toronto, Canada, 2013.

Pace, N. R., Microbial Ecology and Diversity, *Am. Soc. Microbiol. News* 65: 328–333, 1999.

QUESTIONS

2.1. Briefly compare procaryotes with eucaryotes in terms of internal structure and functions.

2.2. Briefly explain the following terms:
- **a.** Heterotrophs
- **b.** Autotrophs
- **c.** Thermophiles
- **d.** Acidophiles

2.3. What are the major differences between eubacteria and archaebacteria?

2.4. Give characteristic dimensions for each of these organisms:
- **a.** *E. coli*
- **b.** Yeast (*S. cerevisiae*)
- **c.** Liver cell (hepatocyte)
- **d.** Plant cell

2.5. What are the differences in cell envelope structure between gram-negative and gram-positive bacteria? How might these differences become important if you wish to genetically engineer bacteria to excrete proteins into the extracellular fluid?

2.6. Briefly explain the functions of the following organelles present in eucaryotes:
- **a.** Mitochondria
- **b.** Endoplasmic reticulum
- **c.** Golgi bodies
- **d.** Lysosomes
- **e.** Chloroplasts

2.7. What are the major classes of fungi? Cite the differences among these classes briefly.

2.8. Briefly describe distinct features of actinomycetes and their important products.

2.9. Briefly compare protozoa with algae in terms of their cellular structures and functions.

2.10. Name some industrially important microalgae with their industrial use.

2.11. Cite five major biological functions of proteins.

2.12. Briefly describe the primary, secondary, tertiary, and quaternary structure of proteins. What could happen if tyrosine is substituted for cysteine in the active site? What might happen if the substitution occurred elsewhere?

2.13. Briefly explain the major groups of antibodies and their functions.

2.14. Briefly describe the structure of the following carbohydrates:

- **a.** Sucrose
- **b.** Maltose
- **c.** Lactose
- **d.** Amylose
- **e.** Amylopectin

2.15. What are differences between lipids, fats, and steroids? Why are steroids important to cells?

2.16. Compare structure and functions of DNA and RNA. Cite at least four differences.

2.17. Briefly explain the three major types of RNA and their functions.

2.18. Briefly explain the following terms:

- **a.** Replication
- **b.** Transcription
- **c.** Translation
- **d.** Plasmids

2.19. What are the macronutrients, micronutrients, and trace elements? Provide some examples.

2.20. What are the typical major sources of carbon, nitrogen, and phosphorus in industrial fermentations for antibiotics?

2.21. Explain the functions of the following trace elements in microbial metabolism: Fe, Zn, Cu, Co, Ni, Mn, vitamins.

2.22. What are chelating agents? Explain their function with an example.

2.23. Compare the advantages and disadvantages of chemically defined and complex media.

2.24. You are asked to develop a medium for production of an antibiotic. The antibiotic is to be made in large amounts (ten 100,000 l fermenters) and is relatively inexpensive. The host cell is a soil isolate of a fungal species, and the nutritional requirements for rapid growth are uncertain. Will you try to develop a defined or complex medium? Why?

2.25. You wish to produce a high-value protein using recombinant DNA technology. Would you try to develop a chemically defined medium or a complex medium? Why?

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3

Enzymes

Enzymes are usually proteins of high molecular weight (15,000 < MW < several million daltons) that act as catalysts. Recently, it has been shown that some RNA molecules are also catalytic, but the vast majority of cellular reactions are mediated by protein catalysts. RNA molecules that have catalytic properties are called *ribozymes*. Both ribozymes and enzymes are specific, versatile, and very effective biological catalysts, resulting in much higher reaction rates as compared to chemically catalyzed reactions under ambient conditions.

More than 2000 enzymes are known. Enzymes are named by adding the suffix *-ase* to the end of the substrate, such as *urease*, or to the end of the reaction catalyzed, such as *alcohol dehydrogenase*. Some enzymes have a simple structure, such as a folded polypeptide chain (typical of most hydrolytic enzymes). Many enzymes have more than one subunit. Some protein enzymes require a nonprotein group for their activity. This group is either a cofactor, such as metal ions, Mg, Zn, Mn, Fe, or a coenzyme, such as a complex organic molecule, nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), coenzyme A (CoA), or some vitamins. An enzyme containing a nonprotein group is called a *holoenzyme*. The protein part of this enzyme is the *apoenzyme* (holoenzyme = apoenzyme + cofactor). Enzymes that occur in several different molecular forms, but catalyze the same reaction, are called *isozymes*. Some enzymes are grouped to form enzyme complexes. Enzymes are

substrate specific and are classified according to the reaction they catalyze. Major classes of enzymes and their functions are listed in Table 3.1.

3.1. HOW ENZYMES WORK

Enzymes lower the activation energy of the reaction catalyzed by binding the substrate and forming an enzyme–substrate complex. Enzymes do not affect the free-energy change or the equilibrium constant. Figure 3.1 illustrates the action of an enzyme from the activation-energy point of view. For example, the activation energy for the decomposition of hydrogen peroxide varies depending on the type of catalysis. The activation energy of the uncatalyzed reaction at 20°C is 18 kilocalories per mole (kcal/mol), whereas the ΔE values for chemically catalyzed (by colloidal platinum) and enzymatically catalyzed (catalase) decomposition are 13 and 7 kcal/mol, respectively. That is, catalase accelerates the rate of reaction by a factor of about 10^8. This large change in rate for a relatively small change in activation energy is due to the exponential dependence of rate on activation energy.

TABLE 3.1. International Classification of Enzymes

Class No.	Class Name	Type of Reaction Catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Cleavage of C—C, C—O, C—N, or other bonds by elimination, leaving double bonds or rings, or addition of groups to double bonds
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C—C, C—S, C—O, and C—N bonds by condensation reactions coupled to cleavage of ATP or similar cofactor

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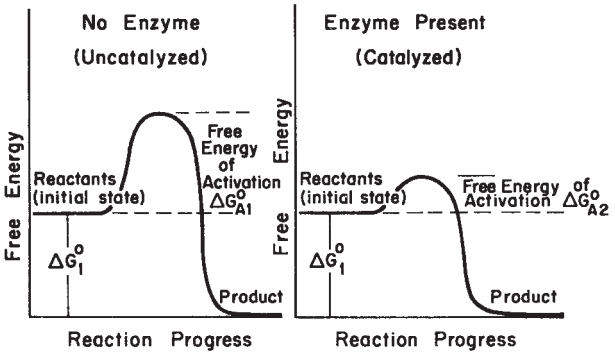


Figure 3.1. Activation energies of enzymatically catalyzed and uncatalyzed reactions. Note that $|\Delta G^\circ_{A_2}| < |\Delta G^\circ_{A_1}|$.

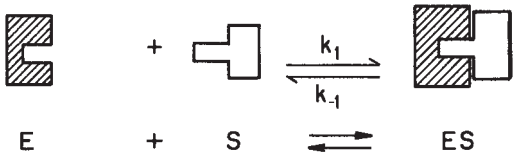


Figure 3.2. Schematic of the lock-and-key model of enzyme catalysis.

The molecular aspects of enzyme–substrate interaction are not yet fully understood. This interaction varies from one enzyme–substrate complex to another. Various studies using x-ray and Raman spectroscopy have revealed the presence of the enzyme–substrate complex. The interaction between the enzyme and its substrate is usually by weak forces. In most cases, van der Waals forces and hydrogen bonding are responsible for the formation of enzyme–substrate complexes. The substrate binds to a specific site on the enzyme known as the *active site*. The substrate is a relatively small molecule and fits into a certain region on the enzyme molecule, which is a much larger molecule. The simplest model describing this interaction is the lock-and-key model, in which the enzyme represents the lock and the substrate represents the key, as described in Figure 3.2. The induced fit model is a more widely accepted mechanism for enzyme–substrate interaction where the active site of the enzyme goes through some conformational changes to bind the substrate. Enzymatic reaction takes place as a result of those conformational changes.

In multisubstrate enzyme–catalyzed reactions, enzymes can hold substrates such that reactive regions of substrates are close to each other and to the enzyme's active site, which is known as the *proximity effect*. Also, enzymes may hold the substrates at certain positions and angles to improve the reaction rate, which is known as the *orientation effect*. In some enzymes, the formation of an enzyme–substrate complex causes slight changes in the three-dimensional shape of the enzyme. This induced fit of the substrate to the enzyme molecule may contribute to the catalytic activity of the enzyme, too. The enzymes lysozyme and carboxypeptidase A have been observed to change their three-dimensional structure upon complexing with the substrate. Enzyme catalysis is affected not only by the primary structure of enzymes but also by the secondary, tertiary, and quaternary structures. The properties of the active site of enzymes and the folding characteristics have a profound effect on the catalytic activity of enzymes. Certain enzymes require coenzymes and cofactors for proper functioning. Table 3.2 lists some enzymes and their cofactors and coenzymes.

3.2. ENZYME KINETICS

A mathematical model of the kinetics of single-substrate-enzyme-catalyzed reactions was first developed by V. C. R. Henri in 1902 and by L. Michaelis and M. L. Menten in 1913. Kinetics of simple enzyme–catalyzed reactions are often referred to as Michaelis–Menten kinetics or *saturation* kinetics. The qualitative features of enzyme kinetics are similar to Langmuir–Hinshelwood kinetics (see Figure 3.3). These models are based on data from batch reactors with constant liquid volume in which the

TABLE 3.2. Cofactors (Metal Ions) and Coenzymes of Some Enzymes

Some Inorganic Ions That Serve as Cofactors for Enzymes	
Ions	Enzymes
Cu^{2+}	Cytochrome oxidase
Fe^{2+} or Fe^{3+}	Cytochrome oxidase, catalase, peroxidase
K^{+}	Pyruvate kinase
Mg^{2+}	Hexokinase, glucose 6-phosphatase, pyruvate kinase
Mn^{2+}	Arginase, ribonucleotide reductase
Mo	Dinitrogenase
Ni^{2+}	Urease
Zn^{2+}	Carbonic anhydrase, alcohol dehydrogenase, carboxypeptidase A and B

Some Coenzymes That Serve as Transient Carriers of Specific Atoms or Functional Groups		
Coenzyme	Examples of Chemical Groups Transferred	Dietary Precursor in Mammals
Biocytin	CO_2	Biotin
Coenzyme A	Acyl groups	Pantothenic acid and other compounds
5′-Deoxyadenosylcobalamin (coenzyme B_{12})	H atoms and alkyl groups	Vitamin B_{12}
Flavin adenine dinucleotide	Electrons	Riboflavin (vitamin B_2)
Lipoate	Electrons and acyl groups	Not required in diet
Nicotinamide adenine dinucleotide	Hydride ion ($:H^-$)	Nicotinic acid (niacin)
Pyridoxal phosphate	Amino groups	Pyridoxine (vitamin B_6)
Tetrahydrofolate	One-carbon groups	Folate
Thiamine pyrophosphate	Aldehydes	Thiamine (vitamin B_1)

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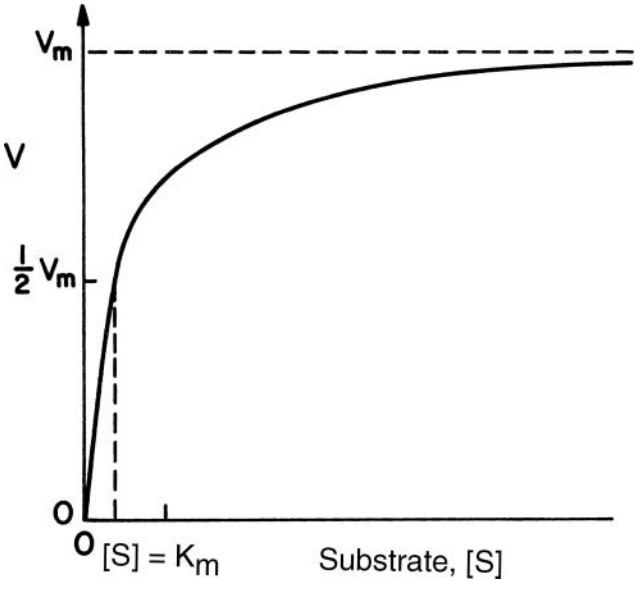


Figure 3.3. Effect of substrate concentration on the rate of an enzyme–catalyzed reaction.

initial substrate, $[S_0]$, and enzyme, $[E_0]$, concentrations are known. More complicated enzyme–substrate interactions such as multisubstrate–multienzyme reactions can take place in biological systems.

An enzyme solution has a fixed number of active sites to which substrates can bind. At high substrate concentrations, all these sites may be occupied by substrates, or the enzyme is *saturated*. Saturation kinetics can be obtained from a simple reaction scheme that involves a reversible step for enzyme–substrate complex formation and a dissociation step of the enzyme–substrate complex:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \tag{3.1}$$

It is assumed that the enzyme–substrate complex is established rather rapidly and the rate of the reverse reaction of the second step is negligible. The assumption of an irreversible second reaction often holds only when product accumulation is negligible at the beginning of the reaction. Two major approaches used in developing a rate expression for the enzyme–catalyzed reactions are rapid-equilibrium approach and quasi-steady-state approach.

3.2.1. Mechanistic Models for Simple Enzyme Kinetics

Both the quasi-steady-state approximation and the assumption of rapid equilibrium share the same few initial steps in deriving a rate expression for the mechanism in equation 3.1, where the rate of product formation is

$$v = \frac{d[P]}{dt} = k_2[\mathrm{ES}] \tag{3.2}$$

where v is the rate of product formation or substrate consumption in mol/l-s.

The rate constant k_2 is often denoted as k_{cat} in the biological literature. The rate of variation of the enzyme–substrate complex is

$$\frac{d[\mathrm{ES}]}{dt} = k_1[\mathrm{E}][\mathrm{S}] - k_{-1}[\mathrm{ES}] - k_2[\mathrm{ES}] \tag{3.3}$$

Since the enzyme is not consumed, the conservation equation on the enzyme yields

$$[\mathrm{E}] = [\mathrm{E}_0] - [\mathrm{ES}] \tag{3.4}$$

At this point, an assumption is required to achieve an analytical solution.

3.2.1.1. The rapid-equilibrium assumption. Henri, Michaelis, and Menten used essentially this approach. Assuming a rapid equilibrium between the enzyme and substrate to form an [ES] complex, we can use the equilibrium coefficient to express [ES] in terms of [S].

The equilibrium constant is

$$K_m' = \frac{k_{-1}}{k_1} = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]} \tag{3.5}$$

Since $[E] = [E_0] - [ES]$, if enzyme is conserved, then

$$[ES] = \frac{[E_0][S]}{(k_{-1}/k_1) + [S]} \tag{3.6}$$

$$[ES] = \frac{[E_0][S]}{K_m' + [S]} \tag{3.7}$$

where $K_m' = k_{-1}/k_1$, which is the dissociation constant of the enzyme–substrate complex. Substituting equation 3.7 into equation 3.2 yields

$$v = \frac{d[P]}{dt} = k_2 \frac{[E_0][S]}{K_m' + [S]} = \frac{V_m[S]}{K_m' + [S]} \tag{3.8}$$

where $V_m = k_2[E_0]$.

In this case, the maximum forward velocity of the reaction is V_m. V_m changes if more enzyme is added, but the addition of more substrate has no influence on V_m. K_m' is often called the Michaelis–Menten constant, and the prime reminds us that it was derived by assuming rapid equilibrium in the first step. A low value of K_m' suggests that the enzyme has a high affinity for the substrate. Also, K_m' corresponds to the substrate concentration, giving the half-maximal reaction velocity.

An equation of exactly the same form as equation 3.8 can be derived with a different, more general assumption applied to the reaction scheme in equation 3.1.

3.2.1.2. The quasi-steady-state assumption. In many cases, the assumption of rapid equilibrium following mass-action kinetics is not valid, although the enzyme–substrate reaction still shows saturation-type kinetics.

G. E. Briggs and J. B. S. Haldane first proposed using the quasi-steady-state assumption. In most experimental systems, a closed system (batch reactor) is used in which the initial substrate concentration greatly exceeds the initial enzyme concentration. They suggest that since $[E_0]$ was small, $d[ES]/dt \approx 0$. (This logic is flawed. Do you see why?) Computer simulations of the actual time course represented by equations 3.2, 3.3, and 3.4 have shown that *in a closed system, the quasi-steady-state hypothesis holds* after a brief transient *if* $[S_0] >> [E_0]$ (e.g., 100×). Figure 3.4 displays one such time course.

By applying the quasi-steady-state assumption to equation 3.3, we find

$$[ES] = \frac{k_1[E][S]}{k_{-1} + k_2} \tag{3.9}$$

Substituting the enzyme conservation equation 3.4 in equation 3.9 yields

$$[ES] = \frac{k_1([E_0] - [ES])[S]}{k_{-1} + k_2} \tag{3.10}$$

Solving equation 3.10 for [ES],

$$[ES] = \frac{[E_0][S]}{\frac{k_{-1} + k_2}{k_1} + [S]} \tag{3.11}$$

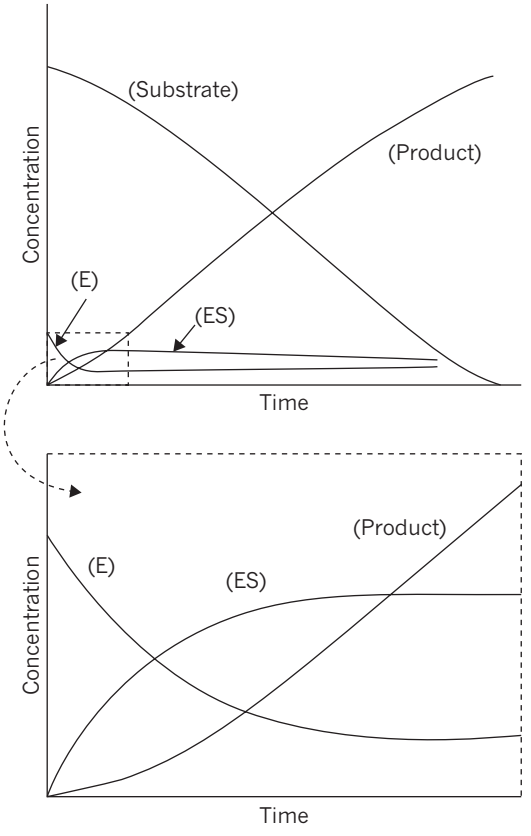


Figure 3.4. Time course of the formation of an enzyme–substrate complex and initiation of the steady state, as derived from computer solutions of data obtained in an actual experiment on a typical enzyme. The portion in the dashed box in the top graph is shown in magnified form on the lower graph. (From Lehninger, *Principles of Biochemistry*, 6th ed., by David L. Nelson et al., Copyright 2013. All rights reserved. Reprinted by permission of W. H. Freeman.)

Substituting equation 3.11 into equation 3.2 yields

$$v = \frac{d[\mathrm{P}]}{dt} = \frac{k_2[\mathrm{E}_0][\mathrm{S}]}{\dfrac{k_{-1} + k_2}{k_1} + [\mathrm{S}]} \tag{3.12a}$$

$$v = \frac{V_m[\mathrm{S}]}{K_m + [\mathrm{S}]} \tag{3.12b}$$

where K_m is $(k_{-1} + k_2)/k_1$ and V_m is $k_2[\mathrm{E}_0]$. Under most circumstances (simple experiments), it is impossible to determine whether K_m or K_m' is more suitable. Since K_m results from the more general derivation, we use it in the rest of our discussions.

3.2.2. Determining Rate Parameters for Michaelis–Menten Kinetics

The determination of values for K_m and V_m with high precision may be difficult. Typically, experimental data are obtained from *initial-rate experiments*. A batch reactor is charged with a known amount of substrate $[\mathrm{S}_0]$ and enzyme $[\mathrm{E}_0]$. The product (or substrate concentration) is plotted against time. The initial slope of this curve is

estimated (i.e., $v = d[P]/dt|_{t=0} = d[S]/dt|_{t=0}$). This value of v then depends on the values of $[E_0]$ and $[S_0]$ in the charge to the reactor. Many such experiments at different initial substrate concentrations can be performed to generate many pairs of v_0 and $[S]_0$ data. The initial rate data at different $[S]_0$ levels could be plotted as in Figure 3.3; however, the accurate determination of K_m from such a plot is very difficult. Consequently, other methods of analyzing such data have been suggested.

3.2.2.1. Double-reciprocal plot (Lineweaver–Burk plot). Equation 3.12b can be linearized in double-reciprocal form:

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]} \tag{3.13}$$

A plot of $1/v$ versus $1/[S]$ yields a linear line with a slope of K_m/V_m and y-axis intercept of $1/V_m$, as depicted in Figure 3.5. A double-reciprocal plot gives good estimates on V_m but not necessarily on K_m. Because the error about the reciprocal of a data point is not symmetric, be cautious in applying regression analysis (least squares) to such plots. Data points at low substrate concentrations influence the slope and intercept more than those at high substrate concentrations.

3.2.2.2. Eadie–Hofstee plot. Equation 3.12b can be rearranged as

$$v = V_m - K_m\frac{v}{[S]} \tag{3.14}$$

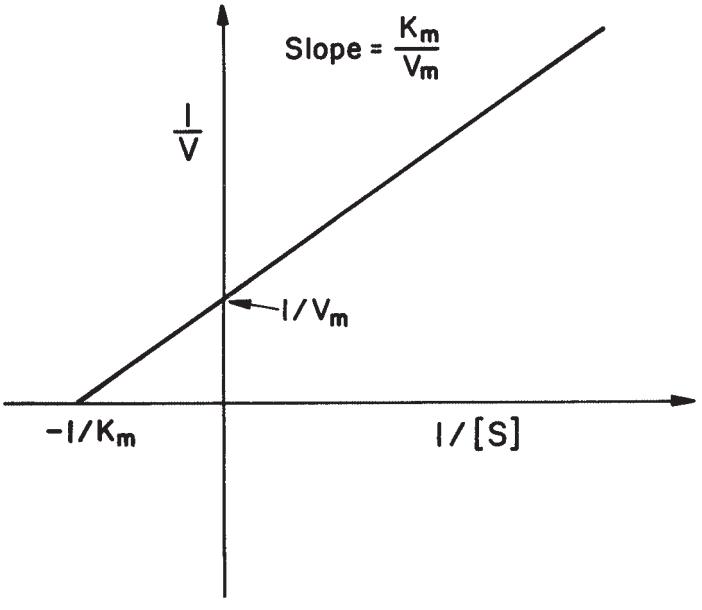


Figure 3.5. Double-reciprocal (Lineweaver–Burk) plot.

A plot of v versus v/[S] results in a line of slope $-K_m$ and y-axis intercept of V_m, as depicted in Figure 3.6. Eadie–Hofstee plots can be subject to large errors because both coordinates contain υ, but there is less bias on points at low [S]. This is a good method for estimation of K_m but not V_m.

3.2.2.3. Hanes–Woolf plot. Rearrangement of equation 3.12b yields

$$\frac{[S]}{v} = \frac{K_m}{V_m} + \frac{1}{V_m}[S] \tag{3.15}$$

A plot of [S]/v versus [S] results in a line of slope $1/V_m$ and y-axis intercept of K_m/V_m, as depicted in Figure 3.7. This plot is used to determine V_m more accurately.

It is preferable to determine kinetic constants from any of these approaches based on the slope rather than the intercept. The intercept often designates hypothetical cases where S or V approach zero or infinity. Multivariable regression analyses are also commonly used for determination of the kinetic constants using the experimental data.

3.2.2.4. Batch kinetics. The time course of variation of [S] in a batch enzymatic reaction can be determined from

$$v = -\frac{d[S]}{dt} = \frac{V_m[S]}{K_m + [S]} \tag{3.12b}$$

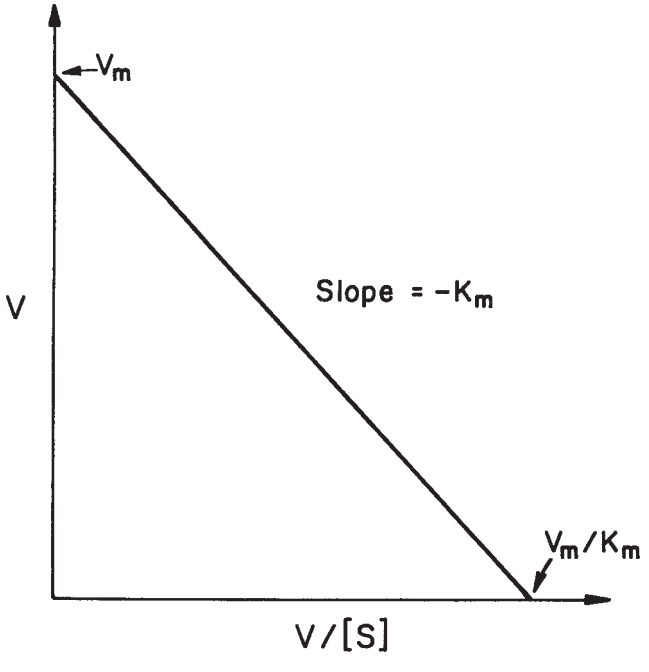


Figure 3.6. Eadie–Hofstee plot.

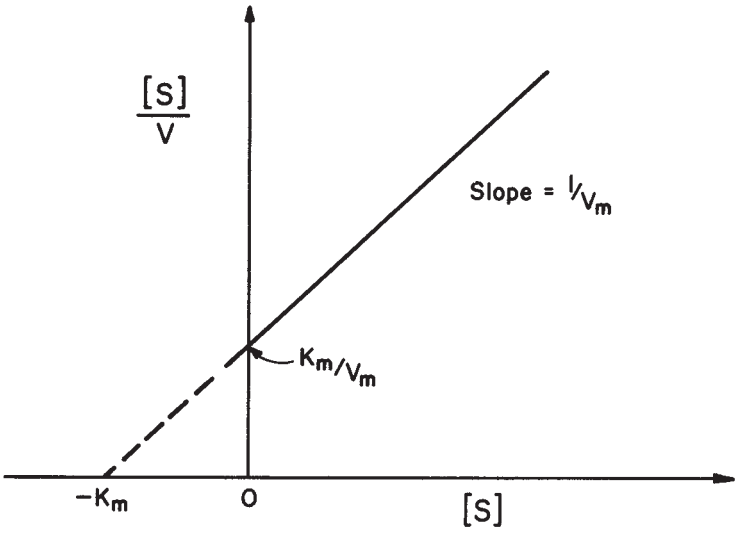


Figure 3.7. Hanes–Woolf plot.

Equation 3.12b can be integrated to yield

$$V_m t = [S_0] - [S] + K_m \ln \frac{[S_0]}{[S]} \tag{3.16}$$

Equation 3.16 can be rearranged to give

$$V_m - \frac{[S_0] - [S]}{t} = \frac{K_m}{t} \ln \frac{[S_0]}{[S]} \tag{3.17}$$

A plot of $1/t$ Ln$[S_0]/[S]$ versus $\{[S_0] - [S]\}/t$ results in a line of slope $-1K_m$ and intercept of V_m/K_m. However, V_m may be better determined from a plot of Ln $[S_0]/[S]$ versus time (t), where the slope is V_m/K_m and the intercept is $\{[S_0] - [S]\}/K_m$.

3.2.2.5. Interpretation of K_m and V_m. While K_m (or K_m') is an intrinsic parameter, V_m is not. K_m is solely a function of rate parameters and is expected to change with temperature or pH. However, V_m is a function of the rate parameter k_2 and the initial enzyme level, $[E_0]$. As $[E_0]$ changes, so does V_m. Of course, k_2 can be readily calculated if $[E_0]$ is known. For highly purified enzyme preparations, it may be possible to express $[E_0]$ in terms of mol/l or g/l.

When the enzyme is part of a crude preparation, its concentration is in terms of "units." A *unit* is the amount of enzyme that gives a predetermined amount of catalytic activity under specific conditions. For example, one unit would be the formation of one μmol product per minute at a specified pH and temperature with a substrate concentration much greater than the value of K_m. The *specific activity* is the number of units of activity per amount of total protein. For example, a crude cell lysate might have a specific activity of 0.2 units/mg protein, which upon purification may increase to 10 units/mg protein. Only enzyme that remains catalytically active will be

measured. The enzyme may be *denatured* if it unfolds or has its three-dimensional shape altered by extreme pH or temperature changes during purification. The denatured enzyme will have no activity.

Example 3.1 Determining Specific Activity

To measure the amount of glucoamylase in a crude enzyme preparation, 1 ml of the crude enzyme preparation containing 8 mg protein is added to 9 ml of a 4.44% starch solution. One unit of activity of glucoamylase is defined as the amount of enzyme which produces 1 μmol of glucose per minute in a 4% solution of Lintner starch at pH 4.5 and at 60°C. Initial rate experiments show that the reaction produces 0.6 μmol of glucose/ml-min. What is the specific activity of the crude enzyme preparation?

Solution The total amount of glucose made is 10 ml × 0.6 μmol glucose/ml-min or 6 μmol glucose per minute. The specific activity is then

$$\begin{aligned}\text{specific activity} &= \frac{6 \text{ units}}{1 \text{ ml protein solution} \cdot 8 \text{ mg/ml}} \\ &= 6 \text{ units/8 mg protein} \\ &= 0.75 \text{ units/mg protein}\end{aligned}$$

V_m must have units such as μmol product/ml-min. Since $V_m = k_2 E_0$, the dimensions of k_2 must reflect the definition of units in E_0. In Example 3.1, we had a concentration of enzyme of 8 mg protein/10 ml solution · 0.75 units/mg protein, or 0.6 units/ml. If, for example, V_m = 1 μmol/ml-min, then we get k_2 = 1 μmol/ml-min ÷ 0.6 units/ml, or k_2 = 1.67 μmol/unit-min.

3.2.3. Models for More Complex Enzyme Kinetics

The kinetics of enzyme reactors can become quite complex. Here we discuss some examples of common kinetic expressions that involve multiple binding sites. These expressions show inhibition or activation by small molecules. These complex kinetic forms are often required within a cell to describe enzymes that regulate cellular function. For example, if too much of a product is formed, the cell may need to slow its production rate. Conversely, it may be necessary to activate an enzyme to a higher rate of conversion to respond to changes in other intracellular chemical concentrations. Some of these examples of cell regulation are given in Chapter 4, "How Cells Work." Here we focus on the kinetics of individual isolated enzymes, often for industrial use outside of the cell. We must be aware of these kinetics to design properly processes using enzymes as industrial catalysts.

3.2.3.1. Allosteric enzymes. Some enzymes have more than one substrate-binding site. The binding of one substrate to the enzyme facilitates binding of other substrate molecules. This behavior is known as *allostery* or *cooperative binding*, and regulatory enzymes show this behavior. The rate expression in this case is

$$v = -\frac{d[\mathrm{S}]}{dt} = \frac{V_m[\mathrm{S}]^n}{K_m'' + [\mathrm{S}]^n} \tag{3.18}$$

where n = cooperativity coefficient and $n > 1$ indicates positive cooperativity. Figure 3.8 compares Michaelis–Menten kinetics with allosteric enzyme kinetics, indicating a sigmoidal shape of υ–[S] plot for allosteric enzymes.

The cooperativity coefficient can be determined by rearranging equation 3.18 as follows and by plotting Ln $v/(V_m - v)$ versus Ln[S], as depicted in Figure 3.9:

$$\text{Ln}\frac{v}{V_m - v} = n\,\text{Ln}[\text{S}] - \text{Ln}\,K_m'' \tag{3.19}$$

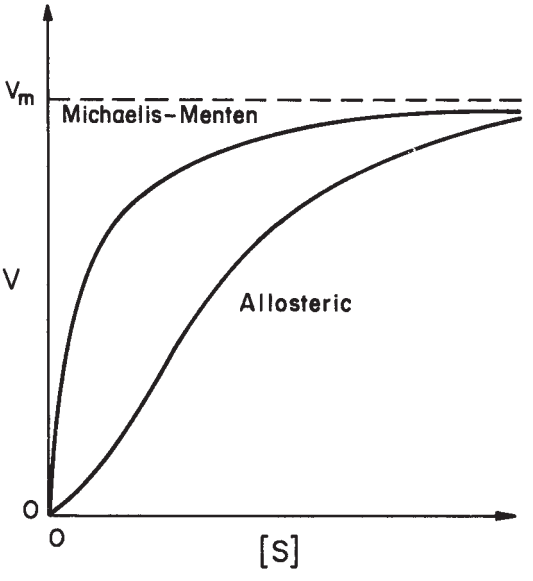


Figure 3.8. Comparison of Michaelis–Menten and allosteric enzyme kinetics.

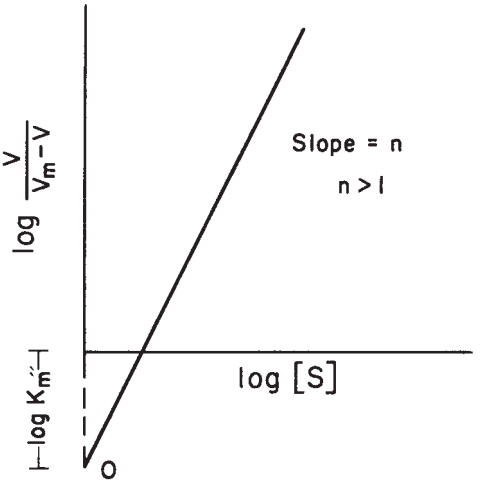


Figure 3.9. Determination of cooperativity coefficient.

Depending on the value of n, substrate binding may be cooperative when $n > 1$, or noncooperative (prohibitive) when $n < 1$. Substrate binding is independent or not affected from the other bound substrates when $n = 1$, which is a major assumption for Michaelis–Menten kinetics.

3.2.3.2. Inhibited enzyme kinetics. Certain compounds may bind to enzymes and reduce their activity. These compounds are known to be *enzyme inhibitors*. Enzyme inhibitions may be irreversible or reversible. Irreversible inhibitors such as heavy metals (lead, cadmium, mercury, and others) form a stable complex with enzyme and reduce enzyme activity. Such enzyme inhibition may be reversed only by using chelating agents such as EDTA (ethylenediaminetetraacetic acid) and citrate. Reversible inhibitors may dissociate more easily from the enzyme after binding. The three major classes of reversible enzyme inhibitions are *competitive*, *noncompetitive*, and *uncompetitive inhibitions*. The substrate may act as an inhibitor in some cases.

Competitive inhibitors are usually substrate analogs and compete with substrate for the active site of the enzyme. The competitive enzyme inhibition scheme can be described as

$$\begin{array}{ccccc} \mathrm{E} + \mathrm{S} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \mathrm{ES} & \xrightarrow{k_2} & \mathrm{E} + \mathrm{P} \\ + & & & & \\ \mathrm{I} & & & & \\ \Vert\downarrow K_1 & & & & \\ \mathrm{EI} & & & & \end{array} \tag{3.20}$$

Let us assume rapid equilibrium and a definition of

$$\begin{aligned} K_m' &= \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]}, \quad K_I = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} \\ [\mathrm{E}_0] &= [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{EI}] \quad \text{and} \quad v = k_2[\mathrm{ES}] \end{aligned} \tag{3.21}$$

We can then develop the following equation for the rate of enzymatic conversion:

$$v = \frac{V_m[\mathrm{S}]}{K_m'\left[1 + \dfrac{[\mathrm{I}]}{K_I}\right] + [\mathrm{S}]} \tag{3.22}$$

or

$$v = \frac{V_m[\mathrm{S}]}{K_{m,\,\mathrm{app}}' + [\mathrm{S}]} \tag{3.23}$$

where

$$K_{m,\mathrm{app}}' = K_m'\left(1 + \frac{[\mathrm{I}]}{K_1}\right).$$

The net effect of competitive inhibition is an increased value of $K'_{m,\text{app}}$ and, therefore, reduced reaction rate. Competitive inhibition can be overcome by high concentrations of substrate. Figure 3.10 describes competitive enzyme inhibition in the form of a double-reciprocal plot.

Noncompetitive inhibitors are not substrate analogs. Inhibitors bind on sites other than the active site and reduce enzyme affinity to the substrate. Noncompetitive enzyme inhibition can be described as follows:

$$\begin{array}{ccc} \mathrm{E}+\mathrm{S} & \underset{K'_m}{\rightleftharpoons} & \mathrm{ES} \xrightarrow[k_2]{} \mathrm{E}+\mathrm{P} \\ + & & + \\ \mathrm{I} & & \mathrm{I} \\ {\scriptstyle K_I}\downarrow\uparrow & & \downarrow\uparrow \\ \mathrm{EI}+\mathrm{S} & \underset{K'_m}{\rightleftharpoons} & \mathrm{ESI} \end{array} \tag{3.24}$$

With the definitions of

$$K'_m = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]} = \frac{[\mathrm{EI}][\mathrm{S}]}{[\mathrm{ESI}]}, \quad K_I = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} = \frac{[\mathrm{ES}][\mathrm{I}]}{[\mathrm{ESI}]}$$
$$[\mathrm{E}_0] = [\mathrm{E}]+[\mathrm{ES}]+[\mathrm{EI}]+[\mathrm{ESI}] \text{ and } v = k_2[\mathrm{ES}] \tag{3.25}$$

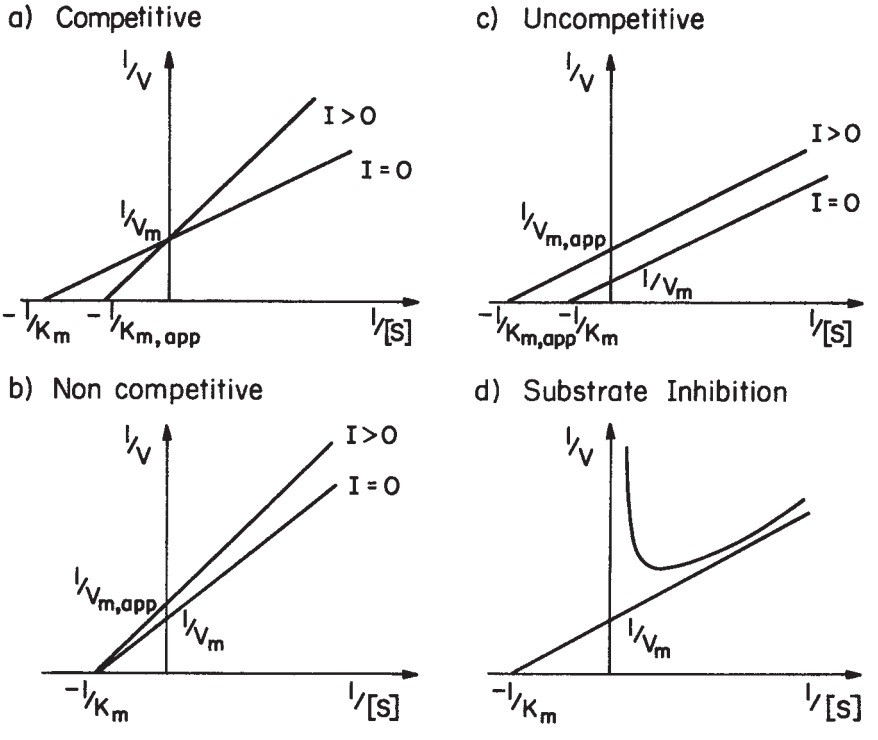


Figure 3.10. Different forms of inhibited enzyme kinetics.

we can develop the following rate equation:

$$v = \frac{V_m}{\left(1 + \frac{[\mathrm{I}]}{K_1}\right)\left(1 + \frac{K_m'}{[\mathrm{S}]}\right)} \tag{3.26}$$

or

$$v = \frac{V_{m,\mathrm{app}}}{\left(1 + \frac{K_m'}{[\mathrm{S}]}\right)} \tag{3.27}$$

where

$$V_{m,\mathrm{app}} = \frac{V_m}{\left(1 + \frac{[\mathrm{I}]}{K_1}\right)}$$

The net effect of noncompetitive inhibition is a reduction in V_m. High substrate concentrations would not overcome noncompetitive inhibition. Other reagents need to be added to block binding of the inhibitor to the enzyme. In some forms of noncompetitive inhibition V_m is reduced and K_m' is increased. This occurs if the complex ESI can form product.

Uncompetitive inhibitors bind to the ES complex only and have no affinity for the enzyme itself. The scheme for uncompetitive inhibition follows:

$$\begin{array}{ccc} \mathrm{E} + \mathrm{S} \overset{K_m'}{\rightleftharpoons} \mathrm{ES} & \xrightarrow{k_2} & \mathrm{E} + \mathrm{P} \\ & + & \\ & \mathrm{I} & \\ & \Big\uparrow\Big\downarrow K_1 & \\ & \mathrm{ESI} & \end{array} \tag{3.28}$$

With the definitions of

$$K_m' = \frac{[\mathrm{E}][\mathrm{S}]}{[\mathrm{ES}]},\ K_1 = \frac{[\mathrm{ES}][\mathrm{I}]}{[\mathrm{ESI}]}$$
$$[\mathrm{E}_0] = [\mathrm{E}] + [\mathrm{ES}] + [\mathrm{ESI}] \text{ and } v = k_2[\mathrm{ES}] \tag{3.29}$$

we can develop the following equation for the rate of reaction:

$$v = \frac{\frac{V_m}{\left(1 + \frac{[\mathrm{I}]}{K_1}\right)}[\mathrm{S}]}{\frac{K_m'}{\left(1 + \frac{[\mathrm{I}]}{K_1}\right)} + [\mathrm{S}]} \tag{3.30}$$

or

$$v = \frac{V_{m,\text{app}}[\text{S}]}{K'_{m,\text{app}} + [\text{S}]} \tag{3.31}$$

The net effect of uncompetitive inhibition is a reduction in both V_m and K'_m values. Reduction in V_m has a more pronounced effect than reduction in K'_m, and the net result is a reduction in reaction rate. Uncompetitive inhibition is described in Figure 3.10 in the form of a double-reciprocal plot.

Mixed inhibition mechanism is somewhat similar to the noncompetitive enzyme inhibition with the only difference that both the maximum reaction rate (V_m) decreases and the K'_m increases as follows:

$$V_{m,\text{app}} = V_m / (1 + I / K_I), \quad K'_{m,\text{app}} = K'_m (1 + I / K'_I) \tag{3.32a}$$

and

$$V = V_{m,\text{app}}(S) / (K'_{m,\text{app}} + (S)) \tag{3.32b}$$

In this case, the inhibitors do not bind to the active site allowing the substrate to bind the EI complex to form ESI which has some catalytic activity to produce product.

High substrate concentrations may cause inhibition in some enzymatic reactions, known as *substrate inhibition*. Substrate inhibition is described in Figure 3.11.

The reaction scheme for uncompetitive substrate inhibition is

$$\begin{array}{ccccc} \text{E} + \text{S} & \overset{K'_m}{\rightleftharpoons} & \text{ES} & \xrightarrow{k_2} & \text{E} + \text{P} \\ & & + & & \\ & & \text{S} & & \\ & & \Vert\, K_{S_1} & & \\ & & \text{ES}_2 & & \end{array} \tag{3.33a}$$

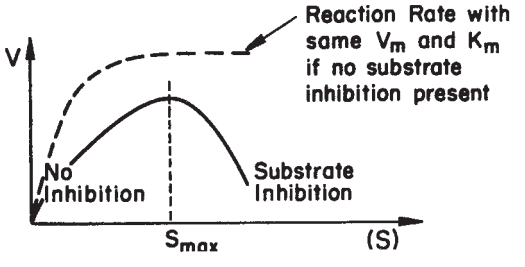


Figure 3.11. Comparison of substrate-inhibited and uninhibited enzymatic reactions.

Consider the following definitions:

$$K_{s_1} = \frac{[S][ES]}{[ES_2]}, \quad K_m' = \frac{[S][E]}{[ES]} \tag{3.33b}$$

The assumption of rapid equilibrium yields

$$v = \frac{V_m[S]}{K_m' + [S] + \dfrac{[S]^2}{K_{s_1}}} \tag{3.34}$$

A double-reciprocal plot describing substrate inhibition is given in Figure 3.10.

At low substrate concentrations, $[S]^2/K_{s_1} \ll 1$, and inhibition effect is not observed. The rate is

$$v = \frac{V_m}{\left[1 + \dfrac{K_m'}{[S]}\right]} \tag{3.35}$$

or

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m'}{V_m}\frac{1}{[S]} \tag{3.36}$$

A plot of $1/v$ versus $1/[S]$ results in a line of slope K_m'/V_m and intercept of $1/V_m$.

At high substrate concentrations, $K_m'/[S] \ll 1$, and inhibition is dominant. The rate is

$$v = \frac{V_m}{\left(1 + \dfrac{[S]}{K_{s_1}}\right)} \tag{3.37}$$

or

$$\frac{1}{v} = \frac{1}{V_m} + \frac{[S]}{K_{s_1}V_m} \tag{3.38}$$

A plot of $1/v$ versus $[S]$ results in a line of slope $1/K_{s_1} \cdot V_m$ and intercept of $1/V_m$.

The substrate concentration resulting in the maximum reaction rate can be determined by setting $dv/d[S] = 0$. The $[S]_{max}$ is given by

$$[S]_{max} = \sqrt{K_m' K_{s_1}} \tag{3.39}$$

Example 3.2 Effect of Initial Substrate Concentration

The following data have been obtained for two different initial enzyme concentrations for an enzyme–catalyzed reaction.

$v([E_0] = 0.015$ g/l) (g/l-min)	[S] (g/l)	$v([E_0] = 0.00875$ g/l) (g/l-min)
1.14	20.0	0.67
0.87	10.0	0.51
0.70	6.7	0.41
0.59	5.0	0.34
0.50	4.0	0.29
0.44	3.3	
0.39	2.9	
0.35	2.5	

a. Find K_m.
b. Find V_m for $[E_0] = 0.015$ g/l.
c. Find V_m for $[E_0] = 0.00875$ g/l.
d. Find k_2.

Solution A Hanes–Woolf plot (Figure 3.12) can be used to determine V_m and K_m:

$$\frac{[S]}{v} = \frac{K_m}{V_m} + \frac{1}{V_m}[S]$$

which results in the following table:

$[S]/v$ ($E_0 = 0.015$) (min)	$[S]/v$ ($E_0 = 0.00875$) (min)	[S] (g/l)
17.5	30	20.0
11.5	20	10.0
9.6	16	6.7
8.5	15	5.0
8.0	14	4.0
7.6		3.3
7.3		2.9
7.1		2.5

From a plot of $[S]/v$ versus [S] for $E_0 = 0.015$ g/l, the slope is found to be 0.6 min/g/l and $V_m = 1/0.6 = 1.7$ g/l-min. The y-axis intercept is $K_m/V_m = 5.5$ min and $K_m = 9.2$ g [S]/l.

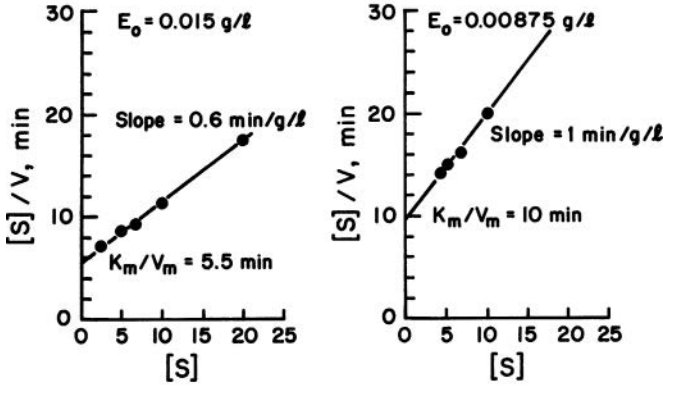


Figure 3.12. Hanes–Woolf plots for $E_0 = 0.015$ g/l and $E_0 = 0.00875$ g/l (Example 3.2).

Also, $V_m = k_2 E_0$ and $k_2 = 1.7/0.015 = 110$ g/g enzyme-min. The Hanes–Woolf plot for $E_0 = 0.00875$ g/l gives a slope of 1.0 min/g/l and $V_m = 1.0$ g/l-min; $k_2 = V_m/E_0 = 1.0/0.00875 = 114$ g/g enzyme-min.

Example 3.3

The hydrolysis of urea by urease is an only partially understood reaction and shows inhibition. Data for the hydrolysis of the reaction are given next.

	0.2 *M*		0.2 *M*	
Substrate Concentration:	1/*v*	I	1/*v*	I
	0.22	0	0.68	0
	0.33	0.0012	1.02	0.0012
	0.51	0.0027	1.50	0.0022
	0.76	0.0044	1.83	0.0032
	0.88	0.0061	2.04	0.0037
	1.10	0.0080	2.72	0.0044
	1.15	0.0093	3.46	0.0059

Here, v = mol/l-min and I is inhibitor molar concentration.

a. Determine the Michaelis–Menten constant (K_m') for this reaction.
b. What type of inhibition reaction is this? Substantiate the answer.
c. Based on the answer to part (b), what is the value of K_i?

Solution A double-reciprocal plot of 1/v versus 1/[S] for inhibitor concentrations I = 0, 0.0012, 0.0044, and 0.006 indicates that the inhibition is noncompetitive (Figure 3.13).

From the x-axis intercept of the plot, $-1/K_m' = -13$ and $K_m' \approx 7.77 \times 10^{-2}$ *M*. For [S] = 0.2 *M* and I = 0 from the intercept of $1/V$ versus 1/S, $1/v_m = 0.2$ and $V_m \cong 5$ mol/l-min. For I = 0.0012 *M* and [S] = 0.2 *M*, $v = 3$ mol/l-min. Substituting these values in

$$v = \frac{V_m}{\left(1 + \dfrac{[\mathrm{I}]}{K_I}\right)\left(1 + \dfrac{K_m'}{[\mathrm{S}]}\right)}$$

gives us $K_I = 6 \times 10^{-3}$ *M*.

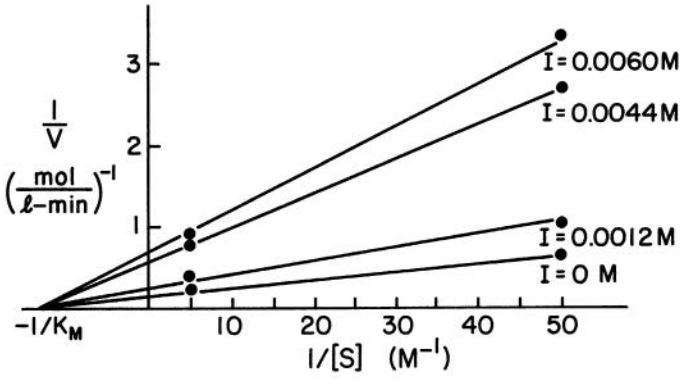


Figure 3.13. Double-reciprocal plot for different inhibitor concentrations (Example 3.3).

3.2.3.3. Enzyme activation. Just as enzyme inhibitors lower enzymatic activity, some molecules may bind to the enzymes to increase the enzymatic activity. These compounds are known as the *enzyme activators*, which are usually involved in allosteric enzymes for regulation of the metabolism.

A good example of an enzyme activator is fructose 2,6 biphosphate, which activates phosphofructokinase and increases the rate of metabolism in response to the hormone glucagon.

The activation energy of the enzymatic reaction is reduced upon binding the activator to the enzyme molecule increasing the reaction rate.

An activator molecule can increase the V_m or decrease the $K_m^{'}$ or can do both with a net effect of increasing the rate of enzymatic reaction. In the presence of an activator the enzyme kinetics may take the following form:

$$V_{m,\text{app}} = V_m\left(1+(A)/K_A\right), \quad K_{m,\text{app}}^{'} = K_m^{'}/(1+(A)/K_A) \tag{3.40a}$$

and

$$V = V_{m,\text{app}}(S)/(K_{m,\text{app}}^{'} + (S)) \tag{3.40b}$$

Here (A) is the activator concentration and K_A is the dissociation constant for the enzyme–activator complex. The term $(1 + (A)/K_A)$ is known as the activation factor.

In the presence of an inhibitor 1, the activator may bind to the inhibitor molecule preventing the inhibitor to bind the enzyme and hence improving the reaction rate. In this case only the $K_m^{'}$ value decreases yielding higher reaction rates.

$$\begin{aligned} &E + S \rightleftharpoons ES \rightarrow E + P \\ &+ \\ &I + A \rightleftharpoons IA \end{aligned} \tag{3.40c}$$

3.2.4. Effects of pH and Temperature

Enzyme activity responds directly to changes in the physical environment, such as pH and temperature. It is important to incorporate these effects into kinetic expressions for enzyme activity.

3.2.4.1. pH effects. Certain enzymes have ionic groups on their active sites, and these ionic groups must be in a suitable form (acid or base) to function. Variations in the pH of the medium result in changes in the ionic form of the active site and changes in the activity of the enzyme and hence the reaction rate. Changes in pH may also alter the three-dimensional shape of the enzyme. For these reasons, enzymes are active only over a certain pH range. The pH of the medium may affect the maximum reaction rate, K_m, and the stability of the enzyme. In some cases, the substrate may contain ionic groups, and the pH of the medium affects the affinity of the substrate to the enzyme.

The following scheme may be used to describe pH dependence of the enzymatic reaction rate for ionizing enzymes:

$$\begin{array}{ccccc} & & E^- + H^+ & & \\ & K_2 \uparrow\downarrow & & & \\ EH + S & \overset{K'_m}{\rightleftharpoons} & EHS & \xrightarrow{k_2} & EH + P \\ & + & & & \\ & H^+ & & & \\ & K_1 \uparrow\downarrow & & & \\ & EH_2^+ & & & \end{array} \tag{3.41}$$

For example, from the definition of

$$\begin{aligned} K'_m &= \frac{[EH][S]}{[EHS]} \\ K_1 &= \frac{[EH][H^+]}{[EH_2^+]} \\ K_2 &= \frac{[E^-][H^+]}{[EH]} \\ [E_0] &= [E^-] + [EH] + [EH_2^+] + [EHS], \quad v = k_2[EHS] \end{aligned} \tag{3.42}$$

we can derive the following rate expression:

$$v = \frac{V_m[S]}{K'_m\left[1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}\right] + [S]} \tag{3.43}$$

or

$$v = \frac{V_m[S]}{K'_{m,app} + [S]} \tag{3.44}$$

where

$$K'_{m,app} = K'_m\left[1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}\right]$$

As a result of this behavior, the pH optimum of the enzyme is between pK_1 and pK_2.

For ionizing substrate, the following scheme and rate expression can be developed:

$$\begin{array}{ccccc} SH^+ + E & \overset{K'_m}{\rightleftharpoons} & ESH^+ & \xrightarrow{k_2} & E + HP^+ \\ \downarrow\uparrow_{K_1} & & & & \\ S + H^+ & & & & \end{array} \tag{3.45}$$

$$v = \frac{V_m[\mathrm{S}]}{K_m'\left(1+\dfrac{K_1}{[\mathrm{H}^+]}\right)+[\mathrm{S}]} \tag{3.46}$$

Theoretical prediction of the pH optimum of enzymes requires knowledge of the active site characteristics of enzymes, which is very difficult to obtain. The pH optimum for an enzyme is usually determined experimentally. Figure 3.14 depicts variation of enzymatic activity with pH for the enzymes (A) trypsin and (B) cholinesterase.

3.2.4.2. Temperature effects. The rate of enzyme–catalyzed reactions increases with temperature up to a certain limit. Above a certain temperature, enzyme activity decreases with temperature because of enzyme denaturation. Figure 3.15 depicts the variation of reaction rate with temperature and the presence of an optimal temperature.

The ascending part of Figure 3.15 is known as *temperature activation.* The rate varies according to the Arrhenius equation in this region:

$$v = k_2[E] \tag{3.47a}$$

$$k_2 = Ae^{-E_a/RT} \tag{3.47b}$$

Here E_a is the activation energy (kcal/mol) and [E] is the active enzyme concentration. A plot of Ln υ versus $1/T$ results in a line of slope $-E_a/R$.

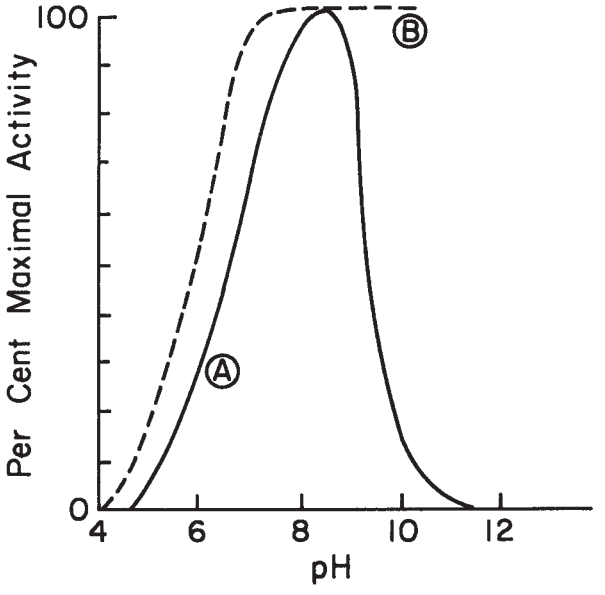


Figure 3.14. The pH-activity profiles of two enzymes. (A) approximate activity for trypsin; (B) approximate activity for cholinesterase.

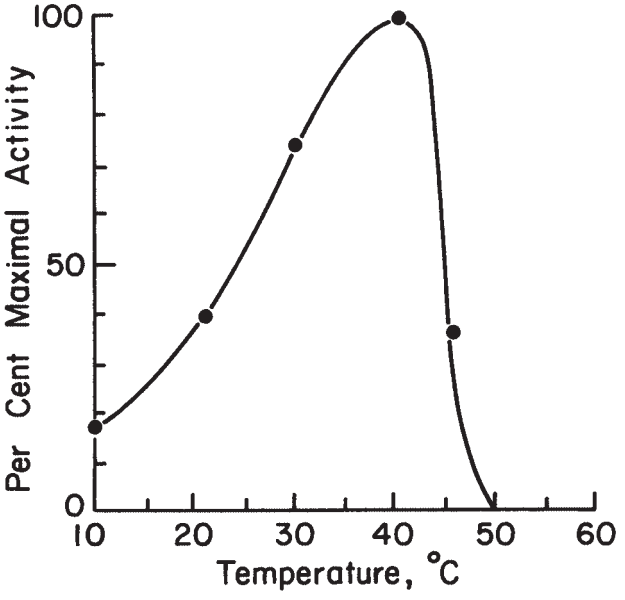


Figure 3.15. Effect of temperature on the activity of an enzyme. Here we have assumed a value of $E_a = 11$ kcal/g-mol and $E_d = 70$ kcal/g-mol. The descending portion of the curve is due to thermal denaturation and is calculated assuming a 10-min exposure to the temperature. Note that the nature of the plot will depend on the length of time the reaction mixture is exposed to the test temperature.

The descending part of Figure 3.15 is known as *temperature inactivation* or *thermal denaturation*. The kinetics of thermal denaturation can be expressed as follows:

$$-\frac{d[\mathrm{E}]}{dt} = k_d[\mathrm{E}] \tag{3.48}$$

or

$$[\mathrm{E}] = [\mathrm{E}_0]e^{-k_d t} \tag{3.49}$$

Here $[\mathrm{E}_0]$ is the initial enzyme concentration, and k_d is the denaturation constant. k_d also varies with temperature according to the Arrhenius equation:

$$k_d = A_d e^{-E_a/RT} \tag{3.50}$$

Here E_d is the deactivation energy (kcal/mol). Consequently, we get the following:

$$v = Ae^{-E_a/RT}\mathrm{E}_0 e^{-k_d t} \tag{3.51}$$

The activation energies of enzyme–catalyzed reactions are within the 4 to 20 kcal/g-mol range (mostly about 11 kcal/g-mol). Deactivation energies E_d vary between 40 and 130 kcal/g-mol (mostly about 70 kcal/g-mol). That is, enzyme denaturation by temperature is much faster than enzyme activation. A rise in temperature from 30° to 40°C results in a 1.8-fold increase in enzyme activity but a 41-fold increase in enzyme denaturation. Variations in temperature may affect both V_m and K_m values of enzymes.

3.2.5. Insoluble Substrates

Enzymes are often used to attack large, insoluble substrates such as wood chips (in biopulping for paper manufacture) or cellulosic residues from agriculture (e.g., cornstalks).

In these cases, access to the reaction site on these biopolymers by enzymes is often limited by enzyme diffusion. The number of potential reactive sites exceeds the number of enzyme molecules. This situation is opposite that of the typical situation with soluble substrates, where access to the enzyme's active site limits reaction. If we consider initial reaction rates and if the reaction is first order with respect to the concentration of enzyme bound to substrate (i.e., [ES]), then we can derive a rate expression:

$$v = \frac{V_{\max,S}[E]}{K_{eq} + [E]} \tag{3.52a}$$

where

$$V_{\max,S} = k_2[S_0] \tag{3.52b}$$

and

$$K'_{eq} = k_{des}/k_{ads} \tag{3.52c}$$

The previous equation assumes slow binding of enzyme (i.e., $[E] \approx [E_0]$), S_0 is the number of substrate bonds available initially for breakage, and k_{des} and k_{ads} refer to rates of enzyme desorption and adsorption onto the insoluble matrix, respectively.

3.2.6. Multiphase Enzymatic Reactions

Enzymes function in aqueous media, since most of the cells contain nearly 80% water yielding high water activity in the cytoplasm. When the substrate is effectively not soluble in aqueous media, but primarily soluble in an organic phase, then substrate containing organic phase and enzyme containing aqueous phase would have to come in contact for effective enzymatic catalysis (see Figure 3.16). Organic phase is assumed to be insoluble in water; and the solubility of the substrate between the organic and the aqueous phase differ greatly.

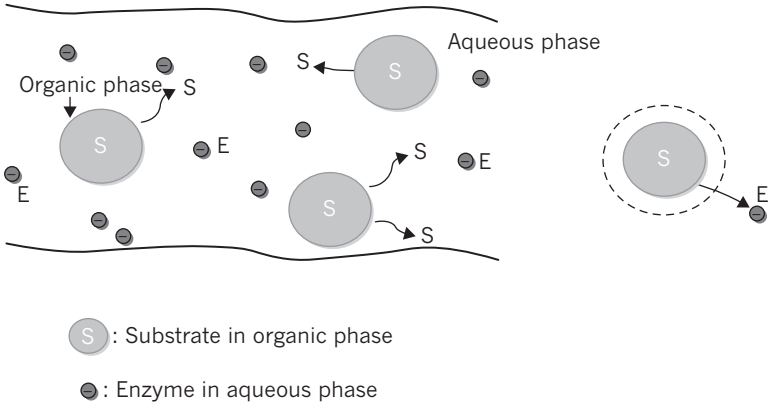


Figure 3.16. A schematic of the two-phase enzymatic reactions.

The substrate dissolved in the organic phase diffuses to the aqueous phase, and equilibrium is established between the two phases in the absence of reaction. The ratio of the solubilities of the substrate in organic and aqueous phase is known as the *partition coefficient* (p).

$$p = S_{o,e}/S_{w,e} \tag{3.53}$$

In this equation, $S_{o,e}$ and $S_{w,e}$ are the substrate concentrations in the organic and aqueous phases at equilibrium, respectively.

The rate of enzymatic reaction in the aqueous phase is limited typically by the rate of substrate transfer from the organic phase because the solubility of the substrate in aqueous phase is very low.

The rate of substrate transfer (RST) from the organic to the aqueous phase is as follows:

$$RST = K_L a\left(S_{w,e} - S_w\right) = K_L a(S_o/p - S_w) \tag{3.54}$$

Here K_L is the mass-transfer coefficient for the substrate transfer from organic to the aqueous phase; a is the interfacial area of the organic and aqueous phases per reaction volume ($a = A_i/V_w$); and S_o and S_w are the substrate concentrations in the organic and aqueous phases, respectively. No substrate transfer takes place when $S_w = S_{w,e}$.

The rate of enzymatic reaction in the aqueous phase is as follows:

$$V = \frac{V_m\ S_w}{K_S + S_w} \tag{3.55}$$

At steady state, the substrate transfer and conversion rates are equal:

$$K_L a\ \left(S_o/p\ -\ S_w\right)\ = \frac{V_m S_w}{K_S + S_w} \tag{3.56}$$

When the rate is severely mass-transfer limited, then S_w is negligible. In this case the rate of enzymatic reaction can be approximated as equal to the substrate transfer rate as follows:

$$V = K_L a\ S_o/p \tag{3.57}$$

In case of considerable substrate concentration in the aqueous phase, S_w is calculated from equation 3.56 in terms of the other parameters, and equation 3.54 or equation 3.55 can be used for calculation of the enzymatic reaction rate.

The substrate concentration in the aqueous phase can also be determined graphically. When the rate of substrate transfer and the enzymatic reaction rates were plotted with respect to S_w, the intersection point would yield the S_w on the x-axis and the rate of substrate transfer on the y-axis.

3.3. IMMOBILIZED ENZYME SYSTEMS

The restriction of enzyme mobility in a fixed space is known as *enzyme immobilization*. Immobilization of enzymes provides important advantages, such as enzyme reutilization and elimination of enzyme recovery and purification processes and may provide a better environment for enzyme activity. Because enzymes are expensive, catalyst reuse is critical for many processes. Since some of the intracellular enzymes are membrane bound, immobilized enzymes provide a model system to mimic and understand the action of some membrane-bound intracellular enzymes. Product purity is usually improved, and effluent-handling problems are minimized by immobilization.

3.3.1. Methods of Immobilization

Major methods of immobilization are summarized in Figure 3.17. The two major categories are entrapment and surface immobilization.

3.3.1.1. Entrapment. Entrapment is the physical enclosure of enzymes in a small space. Matrix entrapment and membrane entrapment, including microencapsulation, are the two major methods of entrapment.

Matrices used for enzyme immobilization are usually polymeric materials such as calcium-alginate, agar, κ-carrageenin, polyacrylamide, and collagen. However, some solid matrices, such as activated carbon, porous ceramic, and diatomaceous earth, can also be used for this purpose. The matrix can be a particle, a membrane, or a fiber. When immobilizing in a polymer matrix, enzyme solution is mixed with polymer solution before polymerization takes place. Polymerized gel-containing enzyme can be extruded, or else a template is used to shape the particles from a liquid polymer–enzyme mixture. Entrapment and surface attachment may be used in combination in some cases.

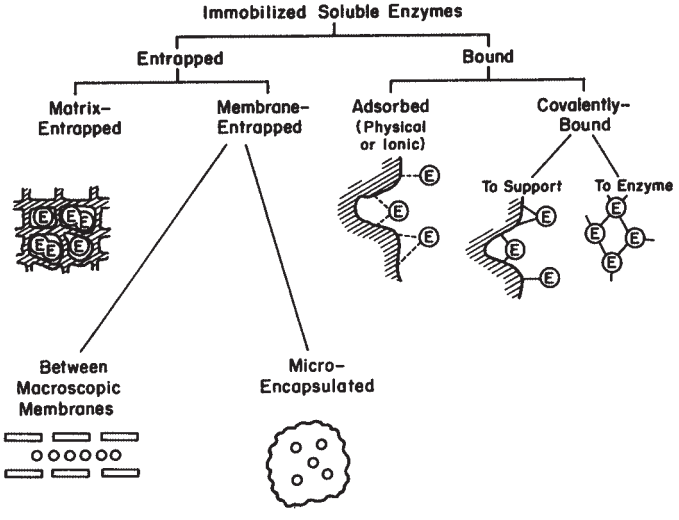


Figure 3.17. Major immobilization methods.

Membrane entrapment of enzymes is possible; for example, hollow fiber units have been used to entrap an enzyme solution between thin, semipermeable membranes. Membranes of nylon, cellulose, polysulfone, and polyacrylate are commonly used. Configurations, other than hollow fibers, are possible, but in all cases a semipermeable membrane is used to retain high-molecular-weight compounds (enzyme) while allowing small-molecular-weight compounds (substrate or products) access to the enzyme.

A special form of membrane entrapment is *microencapsulation*. In this technique, microscopic hollow spheres are formed. The spheres contain the enzyme solution, and the spheres are enclosed within a porous membrane. The membrane can be polymeric or an enriched interfacial phase formed around a microdrop.

Despite the aforementioned advantages, enzyme entrapment may have its inherent problems, such as enzyme leakage into solution, significant diffusional limitations, reduced enzyme activity and stability, and lack of control of microenvironmental conditions. Enzyme leakage can be overcome by reducing the molecular weight cutoff of membranes or the pore size of solid matrices. Diffusion limitations can be eliminated by reducing the particle size of matrices and/or capsules. Reduced enzyme activity and stability are due to unfavorable microenvironmental conditions, which are difficult to control. However, by using different matrices and chemical ingredients, by changing processing conditions, and by reducing particle or capsule size, more favorable microenvironmental conditions can be obtained. Diffusion barrier is usually less significant in microcapsules as compared to gel beads.

3.3.1.2. Surface immobilization. The two major types of immobilization of enzymes on the surfaces of support materials are adsorption and covalent binding.

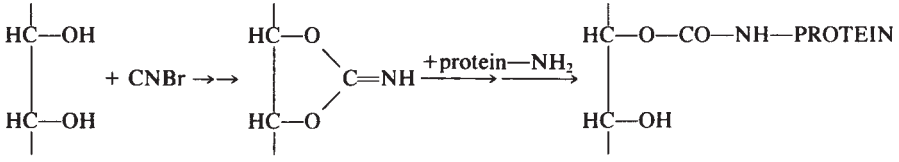
Adsorption is the attachment of enzymes on the surfaces of support particles by weak physical forces, such as van der Waals or dispersion forces. The active site of the adsorbed enzyme is usually unaffected, and nearly full activity is retained upon adsorption. However, desorption of enzymes is a common problem, especially in the presence of strong hydrodynamic forces, because binding forces are weak. Adsorption of enzymes may be stabilized by cross-linking with glutaraldehyde. Glutaraldehyde treatment can denature some proteins. Support materials used for enzyme adsorption can be inorganic materials, such as alumina, silica, porous glass, ceramics, diatomaceous earth, clay, and bentonite, or organic materials, such as cellulose (CMC, DEAE-cellulose), starch, activated carbon, and ion-exchange resins, such as Amberlite, Sephadex, and Dowex. The surfaces of the support materials may need to be pretreated (chemically or physically) for effective immobilization.

Covalent binding is the retention of enzymes on support surfaces by covalent bond formation. Enzyme molecules bind to support material via certain functional groups, such as amino, carboxyl, hydroxyl, and sulfhydryl groups. These functional groups must not be in the active site. One common trick is to block the active site by flooding the enzyme solution with a competitive inhibitor prior to covalent binding. Functional groups on support material are usually activated by using chemical reagents, such as cyanogen bromide, carbodiimide, and glutaraldehyde. Support materials with various functional groups and the chemical reagents used for the covalent binding of proteins are listed in Table 3.3. Binding groups on the protein

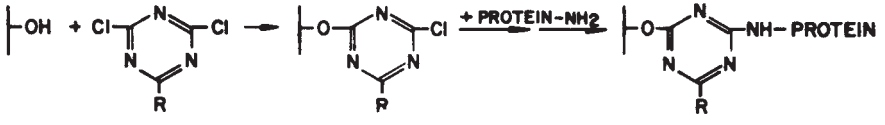
TABLE 3.3. Methods of Covalent Binding of Enzymes to Supports

Supports with —OH

(a) Using cyanogen bromide

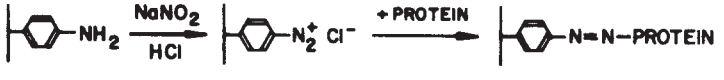


(b) Using S-triazine derivatives

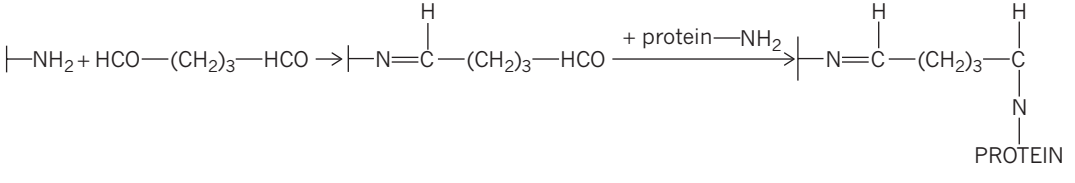


Supports with —NH_2

(a) By diazotization



(b) Using glutaraldehyde

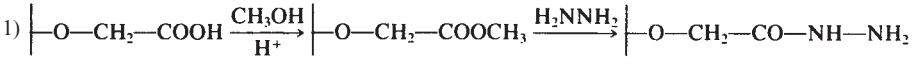


Supports with —COOH

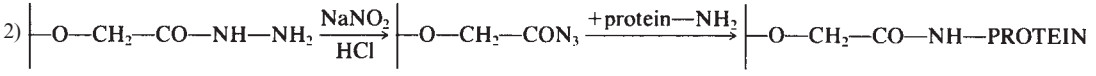
(a) Via azide derivative



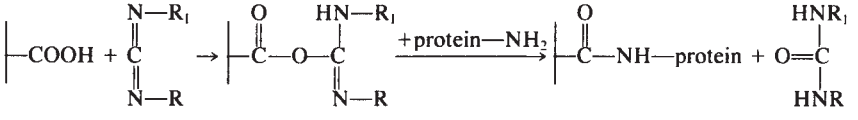
1)



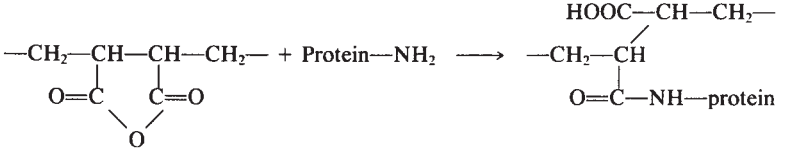
2)



(b) Using a carbodiimide



Supports Containing Anhydrides



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molecule are usually side groups (R) or the amino or carboxyl groups of the polypeptide chain.

The cross-linking of enzyme molecules using agents such as glutaraldehyde, *bis*-diazobenzidine, and 2,2-disulfonic acid is another method of enzyme immobilization. Cross-linking can be achieved in several ways: enzymes can be cross-linked with glutaraldehyde to form an insoluble aggregate, adsorbed enzymes may be cross-linked, or cross-linking may take place following the impregnation of porous support material with enzyme solution. Cross-linking may cause significant changes in the active site of enzymes, and severe diffusion limitations may result.

The most suitable support material and immobilization method vary depending on the enzyme and particular application. Two major criteria used in the selection of support material are the binding capacity of the support material, which is a function of charge density, functional groups, porosity, and hydrophobicity of the support surface; and stability and retention of enzymatic activity, which is a function of functional groups on support material and microenvironmental conditions. If immobilization causes some conformational changes on the enzyme, or if reactive groups on the active site of the enzyme are involved in binding, a loss in enzyme activity can take place upon immobilization. Usually, immobilization results in a loss in enzyme activity and stability. However, in some cases, immobilization may cause an increase in enzyme activity and stability due to more favorable microenvironmental conditions. Because enzymes often have more than one functional site that can bind the surface, an immobilized enzyme preparation may be very heterogeneous. Even when binding does not alter enzyme structure, some enzyme can be bound with the active site oriented away from the substrate solution and toward the support surface, decreasing the access of the substrate to the enzyme. Retention of activity varies with the method used. Table 3.4 summarizes the retention of activity of aminoacylase immobilized by different methods.

TABLE 3.4. Immobilization Effects on Enzymatic Activity of Aminoacylase

Support	Method	Observed Activity (units)	Enzyme Activity Immobilized (%)
Polyacrylamide	Entrapment	526	52.6
Nylon	Encapsulation	360	36.0
DEAE-cellulose	Ionic binding	668	55.2
DEAE-Sephadex A-5O	Ionic binding	680	56.2
CM-Sephadex C-5O	Ionic binding	0	0
Iodoacetyl cellulose	Covalent binding	472	39.0
CNBr-activated Sephadex	Covalent binding	12	1.0
AE-cellulose	Cross-linked with glutaraldehyde	8	0.6

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3.3.2. Diffusional Limitations in Immobilized Enzyme Systems

Diffusional resistances may be observed at different levels in immobilized enzymes. These resistances vary depending on the nature of the support material (porous, nonporous), hydrodynamical conditions surrounding the support material, and distribution of the enzyme inside or on the surface of the support material. Whether diffusion resistance has a significant effect on the rate of enzymatic reaction rate depends on the relative rate of the reaction rate and diffusion rate, which is characterized by the Damköhler number (Da):

$$\text{Da} = \frac{\text{maximum rate of reaction}}{\text{maximum rate of diffusion}} = \frac{V_{m'}}{k_L[S_b]} \tag{3.58}$$

where $[S_b]$ is substrate concentration in bulk liquid, and k_L is the mass-transfer coefficient.

The rate of enzymatic conversion may be limited by diffusion of the substrate or reaction, depending on the value of the Damköhler number. If Da >> 1, then the diffusion rate is limiting. For Da << 1, the reaction rate is limiting, and for Da ≈ 1, the diffusion and reaction resistances are comparable. Diffusion and enzymatic reactions may be simultaneous, with enzymes entrapped in a solid matrix, or may be two consecutive phenomena for adsorbed enzymes.

3.3.2.1. Diffusion effects on surface-bound enzymes. Assume a situation where enzymes are bound and evenly distributed on the surface of a nonporous support material, all enzyme molecules are equally active, and substrate diffuses through a thin liquid film surrounding the support surface to reach the reactive surfaces, as depicted in Figure 3.18. Assume further that the process of immobilization has not altered the protein structure, and the intrinsic kinetic parameters (V_m, K_m) are unaltered.

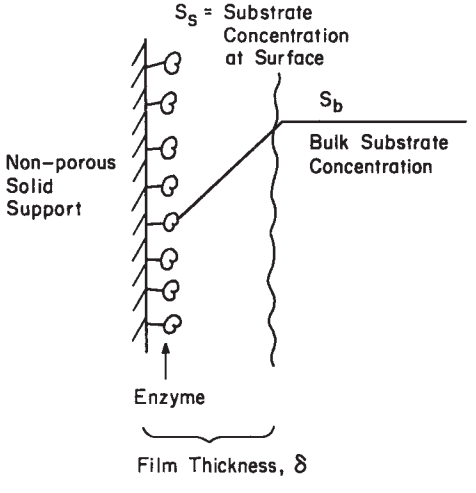


Figure 3.18. Substrate concentration profile in a liquid film around adsorbed enzymes.

At steady state, the reaction rate is equal to the mass-transfer rate:

$$J_s = k_L([S_b] - [S_s]) = \frac{V_m'[S_s]}{K_m + [S_s]} \tag{3.59}$$

where V_m' is the maximum reaction rate per unit of external surface area, and k_L is the liquid mass-transfer coefficient. This equation is quadratic in $[S_s]$, the substrate concentration at the surface. It can be solved analytically, but the solution is cumbersome. Furthermore, the value of $[S_s]$ is not amenable to direct experimental observation.

Equation 3.59 can be solved graphically, as depicted in Figure 3.19. Such a plot also makes it easy to visualize the effects of parameter changes such as in stirring rate, bulk substrate concentration, or enzyme loading.

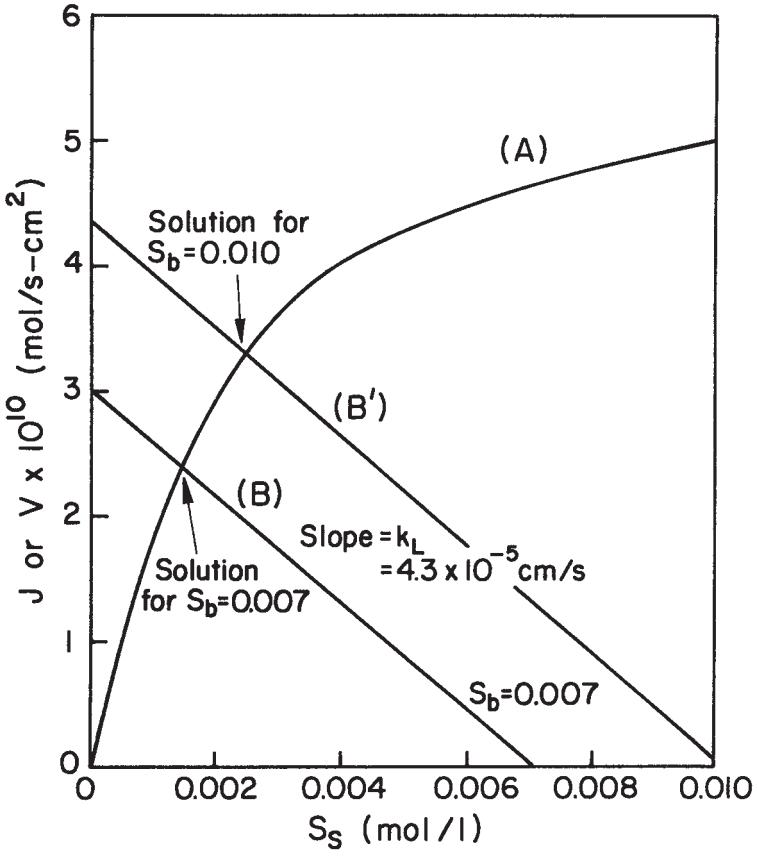


Figure 3.19. Graphical solution for amount of reaction per unit surface area for enzyme immobilized on a nonporous catalyst. Curve A results from the intrinsic solution-based kinetic parameters and the surface loading of enzyme. Line B is the mass-transfer equation. The intersection of the two lines is the reaction rate that can be sustained in the system. The responses for two different bulk substrate concentrations are shown.

When the system is strongly mass-transfer limited, $[S_s] \approx 0$, since the reaction is rapid compared to mass transfer. As a result, the system behaves as a pseudo-first-order reaction:

$$v \approx k_L[S_b], \quad (\text{for Da} >> 1) \tag{3.60}$$

When the system is reaction limited (Da << 1), the reaction rate is often expressed as

$$v = \frac{V_m'[S_b]}{K_{m,\text{app}} + [S_b]} \tag{3.61a}$$

where, with appropriate assumptions,

$$K_{m,\text{app}} = K_m \left\{ 1 + \frac{V_m'}{k_L([S_b] + K_m)} \right\} \tag{3.61b}$$

Under these circumstances, the apparent Michaelis–Menten constant is a function of stirring speed. Usually, $K_{m,\text{app}}$ is estimated experimentally as the value of $[S_b]$, giving one-half of the maximal reaction rate.

Example 3.4 Effect of Diffusion on Reaction Rate

Consider a system where a flat sheet of polymer coated with enzyme is placed in a stirred beaker. The intrinsic maximum reaction rate (V_m) of the enzyme is 6×10^{-6} mol/s-mg enzyme. The amount of enzyme bound to the surface has been determined to be 1×10^{-4} mg enzyme/cm^2 of support. In solution, the value of K_m has been determined to be 2×10^{-3} mol/l. The mass-transfer coefficient can be estimated from standard correlations for stirred vessels. We assume in this case a very poorly mixed system where $k_L = 4.3 \times 10^{-5}$ cm/s. What is the reaction rate when (a) the bulk concentration of the substrate is 7×10^{-3} mol/l? (b) $S_b = 1 \times 10^{-2}$ mol/l?

Solution The solution is given in Figure 3.19. The key is to note that the mass-transfer rate equals the reaction rate at steady state, and consequently, the right side of equation 3.59 must be equal to the left side. In case (a), this occurs at a substrate surface concentration of about 0.0015 mol/l with a reaction rate of 2.3×10^{-10} mol/s-cm^2. By increasing the bulk substrate concentration to 0.01 mol/l, the value of $[S_s]$ increases to 0.0024 mol/l with a reaction rate of about 3.3×10^{-10} mol/s-cm^2.

3.3.2.2. Diffusion effects in enzymes immobilized in a porous matrix. When enzymes are immobilized on internal pore surfaces of a porous matrix, substrate diffuses through the tortuous pathway among pores and reacts with enzyme immobilized on pore surfaces. Diffusion and reaction are simultaneous in this case, as depicted in Figure 3.20.

Assume that the enzyme is uniformly distributed in a spherical support particle; the reaction kinetics are expressed by Michaelis–Menten kinetics, and there is no partitioning of the substrate between the exterior and interior of the support. Then we write the following equation, stating that diffusion rate is equal to reaction rate at steady state:

$$D_e\left(\frac{d^2[S]}{dr^2} + \frac{2}{r}\frac{d[S]}{dr} \right) = \frac{V_m''[S]}{K_m + [S]} \tag{3.62}$$

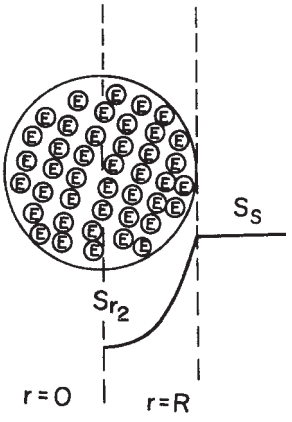


Figure 3.20. Substrate concentration profile in a porous support particle containing immobilized enzymes. Here it is assumed that no external substrate limitation exists so that the bulk and surface concentrations are the same.

Boundary conditions are [S] = [S_s] at $r = R$ and d[S]/dr = 0 at $r = 0$, where V_m' is the maximum reaction rate per unit volume of support, and D_e is the effective diffusivity of substrate within the porous matrix.

Equation 3.62 can be written in dimensionless form by defining the following dimensionless variables:

$$\overline{S} = \frac{[\mathrm{S}]}{[S_s]}, \quad \overline{r} = \frac{r}{R}, \quad \beta = \frac{K_m}{[\mathrm{S}_s]}$$
$$\frac{d^2\overline{S}}{d\overline{r}^2} + \frac{2}{\overline{r}}\frac{d\overline{S}}{d\overline{r}} = \frac{R^2 V_m''}{S_s D_e}\left(\frac{\overline{S}}{\overline{S}+\beta}\right) \tag{3.63a}$$

or

$$\frac{d^2\overline{S}}{d\overline{r}^2} + \frac{2}{\overline{r}}\frac{d\overline{S}}{d\overline{r}} = \phi^2 \frac{\overline{S}}{1+\overline{S}/\beta} \tag{3.63b}$$

where

$$\phi = R\sqrt{\frac{V_m''/K_m}{D_e}} = \text{Thiele modulus} \tag{3.63c}$$

With boundary conditions of $\overline{S} = 1$ at $\overline{r} = 1$ and $d\overline{S}/d\overline{r} = 0$ at $\overline{r} = 0$, equation 3.63b can be numerically solved to determine the substrate profile inside the matrix. The rate of substrate consumption is equal to the rate of substrate transfer through the external surface of the support particle at steady state into the sphere:

$$r_s = N_s = 4\pi R^2 D_e \left.\frac{d[\mathrm{S}]}{dr}\right|_{r=R} \tag{3.64}$$

Under diffusion limitations, the rate per unit volume is usually expressed in terms of the effectiveness factor:

$$r_s = \eta \frac{V_m'' [S_s]}{K_m + [S_s]} \tag{3.65}$$

The *effectiveness factor* is defined as the ratio of the reaction rate with diffusion limitation (or diffusion rate) to the reaction rate with no diffusion limitation. The value of the effectiveness factor is a measure of the extent of diffusion limitation. For $\eta < 1$, the conversion is diffusion limited, whereas for $\eta \approx 1$ values, conversion is limited by the reaction rate and diffusion limitations are negligible. The factor is a function of ϕ and β, as depicted in Figure 3.21.

For a zero-order reaction rate ($\beta \to 0$), and consequently $\eta \approx 1$ for a large range of Thiele modulus values such as $1 < \phi < 100$. For a first-order reaction rate ($\beta \to \infty$), $\eta = (\phi,\beta)$ and η is approximated to the following equation for high values of ϕ:

$$\eta = \frac{3}{\phi}\left[\frac{1}{\tanh\phi} - \frac{1}{\phi}\right] \tag{3.66}$$

When internal diffusion limits the enzymatic reaction rate, the rate-constant $V_{m,\text{app}}$ and $K_{m,\text{app}}$ values are not true intrinsic rate constants but apparent values. To obtain true intrinsic rate constants in immobilized enzymes, diffusion resistances should be eliminated by using small particle sizes, a high degree of turbulence around the particles, and high substrate concentrations.

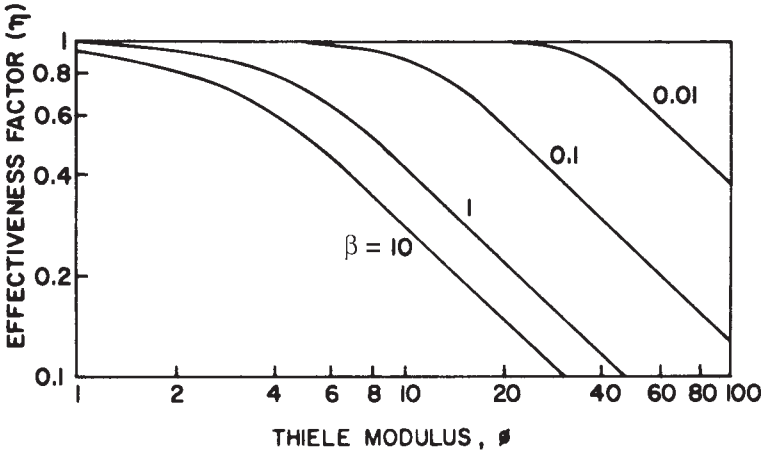


Figure 3.21. Theoretical relationship between the effectiveness factor and first-order Thiele modulus. (D. I. C. Wang et al., *Fermentation and Enzyme Technology*, John Wiley & Sons, New York, 1979. Reproduced with permission of John Wiley & Sons, Incorporated, in the format Book via Copyright Clearance Center.)

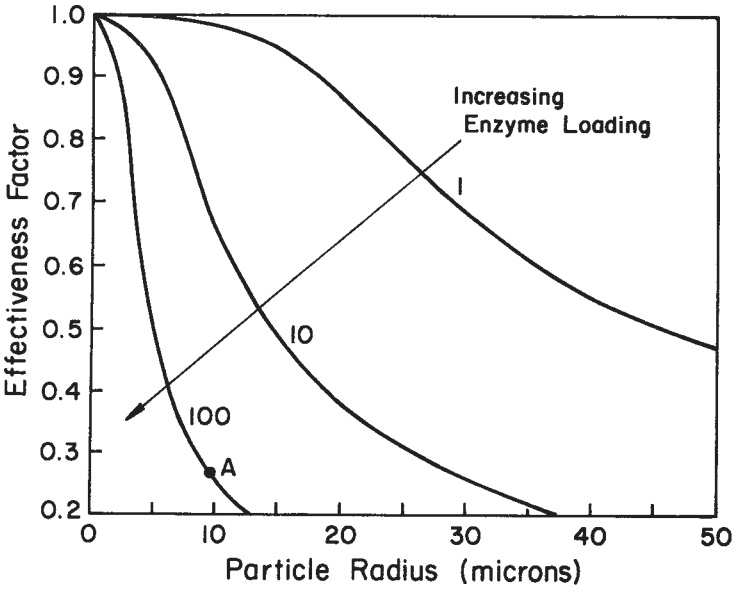


Figure 3.22. The effectiveness factor decreases with increases in enzyme loading or with increases in particle diameter. Point A represents the value of the effectiveness factor for a particle radius of 10 μm with an enzyme loading of 100 mg/cm^3, an enzyme activity of 100 μmol/min per mg enzyme, a substrate diffusivity of 5×10^{-6} cm^2/s, and a bulk substrate concentration tenfold higher than K_m.

When designing immobilized enzyme systems using a particular support, the main variables are V_m and R, since the substrate concentration, K_m, and D_e are fixed. The particle size (R) should be as small as possible within the constraints of particle integrity, resistance to compression, and the nature of the particle recovery systems. The maximum reaction rate is determined by enzyme activity and concentration in the support. High enzyme content will result in high enzyme activity per unit of reaction volume but low effectiveness factor. Low enzyme content will result in lower enzyme activity per unit volume but a high effectiveness factor. For maximum conversion rates, particle size should be small ($D_p \leq 10$ μm) and enzyme loading should be optimized. As depicted in the example in Figure 3.22, $D_p \leq 10$ μm and enzyme loadings of less than 10 mg/cm^3 are required for high values of the effectiveness factor ($\eta \geq 0.8$).

Example 3.5 Determining Whether Diffusional Limitations Exist

D. Thornton and coworkers studied the hydrolysis of sucrose at pH = 4.5 and 25°C using crude invertase obtained from baker's yeast in free and immobilized form. The following initial velocity data were obtained with 408 units of crude enzyme (1 unit = quantity of enzyme hydrolyzing 1 μmol of sucrose/min when incubated with 0.29 *M* sucrose in a buffer at pH 4.5 and 25°C).

V_0 (mmol hydrolyzed/l-min)		
Free Enzyme	Immobilized Enzyme	S_0 (mol/l)
0.083	0.056	0.010
0.143	0.098	0.020
0.188	0.127	0.030
0.222	0.149	0.040
0.250	0.168	0.050
0.330	0.227	0.100
0.408	0.290	0.290

a. Determine the K_m and V_m for this reaction using both free and immobilized enzyme.
b. Do the data indicate any diffusion limitations in the immobilized enzyme preparation?

Solution From a double-reciprocal plot of $1/v$ versus $1/S$ for free enzyme (Figure 3.23), $-1/K_m = -20$ and $K_m = 0.05$ M. $1/V_m = 2$ and $V_m = 0.5$ mmol/l-min. From a double-reciprocal plot of $1/v$ versus $1/S$ for the immobilized enzyme, $-1/K_m = -20$ and $K_m = 0.05$ M. $1/V_m = 3$ and $V_m = 0.33$ mmol/l-min. Since the K_m values for free and immobilized enzymes are the same, there is no diffusion limitation.

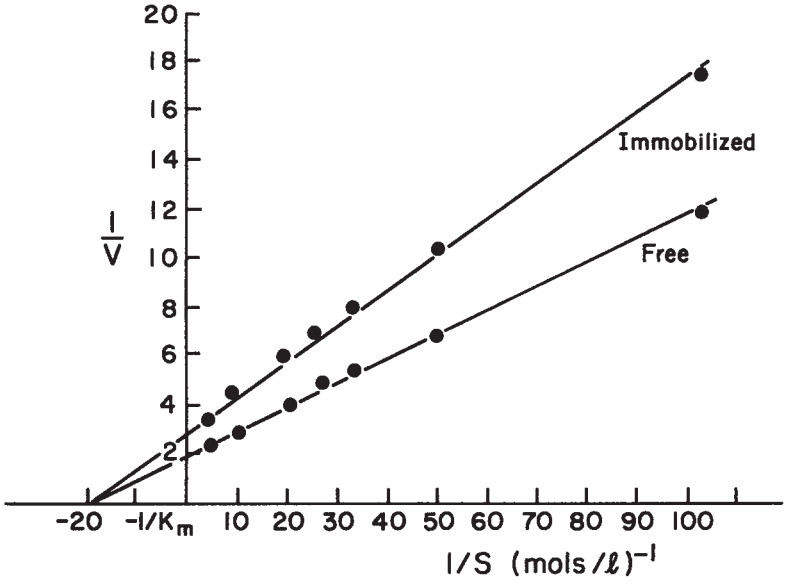


Figure 3.23. Double-reciprocal plots for free and immobilized enzymes (Example 3.5).

3.3.3. Electrostatic and Steric Effects in Immobilized Enzyme Systems

When enzymes are immobilized in a charged matrix as a result of a change in the microenvironment of the enzyme, the apparent bulk pH optimum of the immobilized enzyme will shift from that of soluble enzyme. The charged matrix will repel or attract substrates, product, cofactors, and H^+ depending on the type and quantity of surface charge. For an enzyme immobilized onto a charged support, the shift in the pH-activity profile is given by

$$\Delta\text{pH} = \text{pH}_i - \text{pH}_e = 0.43\frac{zF\psi}{RT} \tag{3.67}$$

where pH_i and pH_e are internal and external pH values, respectively; z is the charge (valence) on the substrate; F is the Faraday constant (96,500 coulomb/mol); ψ is the electrostatic potential; and R is the gas constant. Expressions similar to equation 3.67 apply to other nonreactive charged medium components. The intrinsic activity of the enzyme is altered by the local changes in pH and ionic constituents. Further alterations in the apparent kinetics are due to the repulsion or attraction of substrates or inhibitors.

The activity of an enzyme toward a high-molecular-weight substrate is usually reduced upon immobilization to a much greater extent than for a low-molecular-weight substrate, mainly because of steric hindrance by the support. Certain substrates, such as starch, have molecular weights comparable to those of enzymes and may therefore not be able to penetrate to the active sites of immobilized enzymes.

Immobilization also affects the thermal stability of enzymes. Thermal stability often increases upon immobilization due to the presence of thermal diffusion barriers and the constraints on protein unfolding. However, decreases in thermal stability have been noted in a few cases. The pH stability of enzymes may also increase upon immobilization.

3.4. LARGE-SCALE PRODUCTION OF ENZYMES

Among various enzymes produced at large scale are proteases (subtilisin, rennet), hydrolases (pectinase, lipase, lactase), isomerases (glucose isomerase), and oxidases (glucose oxidase). These enzymes are produced using overproducing strains of certain organisms. Separation and purification of an enzyme from an organism require disruption of cells, removal of cell debris and nucleic acids, precipitation of proteins, ultrafiltration of the desired enzyme, chromatographic separations (optional), crystallization, and drying. The process scheme varies depending on whether the enzyme is intracellular or extracellular. In some cases, it may be more advantageous to use inactive (dead or resting) cells with the desired enzyme activity in immobilized form. This approach eliminates costly enzyme separation and purification steps and is therefore economically more feasible. Details of protein separations are covered in Chapter 11, "Recovery and Purification of Products."

The first step in the large-scale production of enzymes is to cultivate the organisms producing the desired enzyme. Enzyme production can be regulated and fermentation conditions can be optimized for overproduction of the enzyme. Proteases are produced by using overproducing strains of *Bacillus*, *Aspergillus*, *Rhizopus*, and *Mucor*; pectinases are produced by *Aspergillus niger*; lactases are produced by yeast and *Aspergillus*; lipases are produced by certain strains of yeasts and fungi; glucose isomerase is produced by *Flavobacterium arborescens* or *Bacillus coagulans*. After the cultivation step, cells are separated from the media usually by filtration or sometimes by centrifugation. Depending on the intracellular or extracellular nature of the enzyme, either the cells or the fermentation broth is further processed to separate and purify the enzyme. The recovery of intracellular enzymes is more complicated and involves the disruption of cells and removal of cell debris and nucleic acids. Figure 3.24 depicts a schematic of an enzyme plant producing intracellular enzymes.

In some cases, an enzyme may be both intracellular and extracellular, which requires processing of both broth and cells. Intracellular enzymes may be released by increasing the permeability of the cell membrane. Certain salts, such as $CaCl_2$, and other chemicals, such as dimethylsulfoxide (DMSO), and pH shift may be used for this purpose. If enzyme release is not complete, then cell disruption may be essential.

The processes used to produce these industrial enzymes have much in common with the processes used to make proteins from recombinant DNA, discussed later.

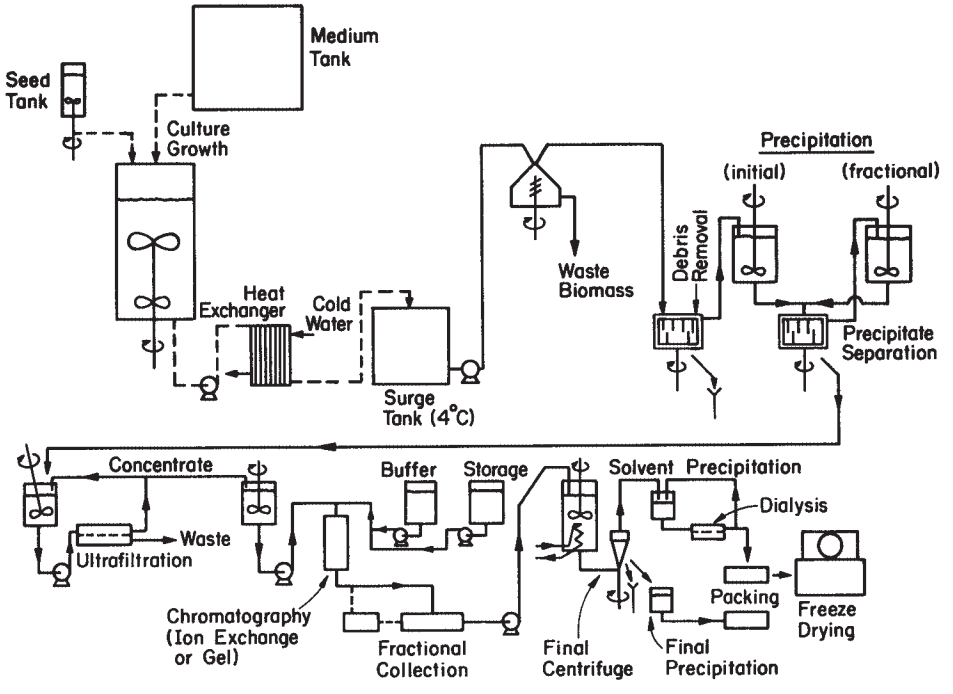


Figure 3.24. A flowsheet for the production of an extracellular enzyme.

3.5. MEDICAL AND INDUSTRIAL UTILIZATION OF ENZYMES

Enzymes have been significant industrial products for more than a hundred years. However, the range of potential applications is increasing rapidly. With the advent of recombinant DNA technology, it has become possible to make formerly rare enzymes in large quantities and hence to reduce cost. Also, in pharmaceutical manufacturing, the desire to make chirally pure compounds is leading to new opportunities. Chirality is important in a product; in a racemic mixture, one enantiomer is often therapeutically useful while the other may cause side effects and add no therapeutic value. The ability of enzymes to recognize chiral isomers and react with only one of them can be a key component in pharmaceutical synthesis. Processes that depend on a mixture of chemical and enzymatic synthesis are being developed for a new generation of pharmaceuticals.

Technological advances have facilitated the use of enzymes over an increasingly broad range of process conditions. Enzymes from organisms that grow in unusual environments (e.g., deep ocean, salt lakes, and hot springs) are increasingly available for study and potential use. New enzymes and better control of reaction conditions allow the use of enzymes in the presence of high concentrations of organics, in high-salt aqueous environments, or at extreme temperatures, pH, or pressures. As we couple new insights into the relationship of enzyme structure to biological function with recombinant DNA technology, we are able to produce enzymes that are human designed or manipulated (see Chapter 14, "Utilizing Genetically Engineered Organisms," Section 14.9, on protein engineering). We no longer need to depend solely on natural sources for enzymes.

Another emerging technology is the use of cell-free systems. This approach uses cell lysates; such crude mixtures have been used historically for industrial purposes for nonenergy-requiring single-step reactions and are relatively inexpensive. Recent advances allow the use of cell-free production for complex, multistep synthesis of high-value products. Such approaches potentially allow intensification of bioprocesses for complex reaction networks and show potential for industrial applications for products that would normally be intracellular. Such systems have been scaled up to over 100 l.

In 2014, U.S. sales of industrial enzymes were about $4.5 billion, and sales are projected to grow to $6.5 billion by 2020. The products made by enzymatic processes are worth many more billions of dollars. Food and beverage account for about one-third of the market. The detergent market is next in size with animal feed, biofuels, paper and pulp, and textiles being significant. Table 3.5 lists some industrially important enzymes.

Proteases hydrolyze proteins into smaller peptide units and constitute a large and industrially important group of enzymes. Proteases constitute about 60% of the total enzyme market. Industrial proteases are obtained from bacteria (*Bacillus*), molds (*Aspergillus, Rhizopus,* and *Mucor*), animal pancreas, and plants. Most of the industrial proteases are endoproteases. Proteases are used in food processing, such as cheese making (rennet), baking, meat tenderization (papain, trypsin), and brewing (trypsin, pepsin); in detergents for the hydrolysis of protein stains (subtilisin); and in tanning and the medical treatment of wounds.

Pectinases are produced mainly by *A. niger*. The major components in pectinases are pectin esterase, polygalacturonase, and polymethylgalacturonatelyase. Pectinases are used in fruit juice processing and wine making to increase juice yield, reduce viscosity, and clear the juice.

TABLE 3.5. Some Industrially Important Enzymes

Name	Example of Source	Application
Amylase	*Bacillus subtilis, Aspergillus niger*	Starch hydrolysis, glucose production
Glucoamylase	*A. niger, Rhizopus niveus, Endomycopsis*	Saccharification of starch, glucose production
Trypsin	Animal pancreas	Meat tenderizer, beer haze removal
Papain	Papaya	Digestive aid, meat tenderizer, medical applications
Pepsin	Animal stomach	Digestive aid, meat tenderizer
Rennet	Calf stomach/recombinant *E. coli*	Cheese manufacturing
Glucose isomerase	*Flavobacterium arborescens, B. coagulans, Lactobacillus brevis*	Isomerization of glucose to fructose
Penicillinase	*B. subtilis*	Degradation of penicillin
Glucose oxidase	*A. niger*	Glucose → gluconic acid, dried-egg manufacture
Lignases	Fungal	Biopulping of wood for paper manufacture
Lipases	*Rhizopus,* pancreas	Hydrolysis of lipids, flavoring and digestive aid
Invertase	*S. cerevisiae*	Hydrolysis of sucrose for further fermentation
Pectinase	*A. oryzae, A. niger, A. flavus*	Clarification of fruit juices, hydrolysis of pectin
Cellulase	*Trichoderma viride*	Cellulose hydrolysis

Lipases hydrolyze lipids into fatty acids and glycerol and are produced from animal pancreas, some molds, and yeasts. Lipases may be used to hydrolyze oils for soap manufacture and to hydrolyze the lipid-fat compounds present in wastewater streams. Interesterification of oils and fats may be catalyzed by lipases. Lipases may also be used in the cheese and butter industry to impart flavor as a result of the hydrolysis of fats. Lipase-containing detergents are an important application of lipases.

Amylases are used for the hydrolysis of starch and are produced by many different organisms, including *A. niger* and *B. subtilis*. Three major types of amylases are α-amylase, β-amylase, and glucoamylase. α-amylase breaks α-1,4 glycosidic bonds randomly on the amylose chain and solubilizes amylose. For this reason, α-amylase is known as the starch-liquefying enzyme. β-amylase hydrolyzes α-1,4 glycosidic bonds on the nonreducing ends of amylose and produces maltose residues. β-amylase is known as a saccharifying enzyme. α-1,6 glycosidic linkages in the amylopectin fraction of starch are hydrolyzed by glucoamylase, which is also known as a saccharifying enzyme. In the United States, on the average, nearly 1.3×10^9 lb/yr of glucose is produced by the enzymatic hydrolysis of starch. The enzyme pullulanase also hydrolyzes α-1,6 glycosidic linkages in starch selectively.

Cellulases are used in the hydrolysis of cellulose and are produced by some *Trichoderma* species, such as *Trichoderma viride* or *T. reesei*; by some molds, such as *A. niger* and *Thermomonospora*; and by some *Clostridium* species. Cellulase is an enzyme complex, and its formation is induced by cellulose. *Trichoderma* cellulase hydrolyzes crystalline cellulose, but *Aspergillus* cellulase does not. Cellulose is first hydrolyzed to cellobiose by cellulase, and cellobiose is further hydrolyzed to glucose by β-glucosidase. Both of these enzymes are inhibited by their end products,

cellobiose and glucose. Cellulases are used in cereal processing, alcohol fermentation from biomass, brewing, and waste treatment.

Hemicellulases hydrolyze hemicellulose to five-carbon sugar units and are produced by some molds, such as white rot fungi and *A. niger*. Hemicellulases are used in combination with other enzymes in baking doughs, brewing mashes, alcohol fermentation from biomass, and waste treatment.

Lactases are used to hydrolyze lactose in whey to glucose and galactose and are produced by yeast and some *Aspergillus* species. Lactases are used in the fermentation of cheese whey to ethanol.

Other microbial β-1,4 glucanases produced by *B. amyloliquefaciens*, *A. niger*, and *Penicillium emersonii*, are used in brewing mashes containing barley or malt. These enzymes improve wort filtration and extract yield.

Penicillin acylase is used by the antibiotic industry to convert penicillin G to 6-aminopenicillanic acid (6-APA), which is a precursor for semisynthetic penicillin derivatives.

Among other important industrial application of enzymes is the conversion of fumarate to L-aspartate by aspartase. In industry, this conversion is realized in a packed column of immobilized dead *E. coli* cells with active aspartase enzyme. Fumarate solution is passed through the column, and aspartate is obtained in the effluent stream. Aspartate is further coupled with L-phenylalanine to produce aspartame, which is a low-calorie sweetener known as NutraSweet.

The conversion of glucose to fructose by immobilized glucose isomerase is an important industrial process. Fructose is nearly 1.7 times sweeter than glucose and is used as a sweetener in soft drinks. Glucose isomerase is an intracellular enzyme and is produced by different organisms, such as *Flavobacterium arborescens*, *B. licheniformis*, and some *Streptomyces* and *Arthrobacter* species. Immobilized inactive whole cells with glucose isomerase activity are used in a packed column for fructose formation from glucose. Cobalt (Co^{2+}) and magnesium (Mg^{2+}) ions (4×10^{-4} *M*) enhance enzyme activity. Different immobilization methods are used by different companies. One uses flocculated whole cells of *F. arborescens* treated with glutaraldehyde in the form of dry spherical particles. Entrapment of whole cells in gelatin treated with glutaraldehyde, the use of glutaraldehyde-treated lysed cells in the form of dry particles, and immobilization of the enzyme on inorganic support particles such as silica and alumina are methods used by other companies.

DL-Acylamino acids are converted to a mixture of L- and D-amino acids by immobilized aminoacylase. L-Amino acids are separated from D-acylamino acid, which is recycled back to the column. L-Amino acids have important applications in food technology and medicine.

Enzymes are commonly used in medicine for diagnosis, therapy, and treatment purposes. Trypsin can be used as an anti-inflammatory agent; lysozyme, which hydrolyzes the cell wall of gram-positive bacteria, is used as an antibacterial agent; streptokinase is used as an anti-inflammatory agent; urokinase is used in dissolving and preventing blood clots. Asparaginase, which catalyzes the conversion of L-asparagine to L-aspartate, is used as an anticancer agent. Cancer cells require L-asparagine and are inhibited by asparaginase. Asparaginase is produced by *E. coli*. Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide, which can

easily be detected. Glucose oxidase is used for the determination of glucose levels in blood and urine. Penicillinases hydrolyze penicillin and are used to treat allergic reactions against penicillin. Tissue plasminogen activator and streptokinase are used in the dissolution of blood clots (particularly following a heart attack or stroke).

The development of biosensors using enzymes as integral components is proceeding rapidly. Two examples of immobilized enzyme electrodes are those used in the determination of glucose and urea by using glucose oxidase and urease immobilized on the electrode membrane. Scarce enzymes (e.g., tissue plasminogen activator) are finding increasing uses, as the techniques of genetic engineering now make it possible to produce usable quantities of such enzymes.

3.6. SUMMARY

Enzymes are protein or glycoprotein molecules that catalyze biologically important reactions. Some RNA molecules may have catalytic activity too. Enzymes are very effective, specific, and versatile biocatalysts. Enzymes bind substrate molecules and reduce the activation energy of the reaction catalyzed, resulting in significant increases in reaction rate. Some protein enzymes require a nonprotein group for their activity as a cofactor.

Simple single-enzyme–catalyzed reaction kinetics can be described by Michaelis–Menten kinetics, which has a hyperbolic form in terms of substrate concentration. The activity of some enzymes can be altered by inhibitory compounds, which bind the enzyme molecule and reduce its activity. Enzyme inhibition may be competitive, noncompetitive, and uncompetitive. High substrate and product concentrations may be inhibitory, too. Enzyme activators increase enzymatic reaction rate upon binding the enzyme molecules.

Enzymes require optimal conditions (pH, temperature, ionic strength) for their maximum activity. Enzymes with an ionizing group on their active site show a distinct optimal pH that corresponds to the natural active form of the enzyme. The activation energy of enzyme–catalyzed reactions is within 4 to 20 kcal/g-mol. Above the optimal temperature, enzymes lose their activity, and the inactivation energy is on the order of 40 to 130 kcal/g-mol.

Enzymes can be used in suspension or in immobilized form. Enzymes can be immobilized by entrapment in a porous matrix, by encapsulation in a semipermeable membrane capsule or between membranes, such as in a hollow-fiber unit, or by adsorption onto a solid support surface. Enzyme immobilization provides enzyme reutilization, eliminates costly enzyme recovery and purification processes, and may result in increased activity by providing a more suitable microenvironment for the enzyme. Enzyme immobilization may result in diffusion limitations within the matrix. Immobilization may also cause enzyme instability, loss of activity, and a shift in optimal conditions (pH, ionic strength). To obtain maximum reaction rates, the particle size of the support material and enzyme loading need to be optimized, and a support material with the correct surface characteristics must be selected.

Enzymes are widely used in industry and have significant medical applications. Among the most widely used enzymes are proteases (papain, trypsin, subtilisin), amylases (starch hydrolysis), rennet (cheese manufacturing), glucose isomerase (glucose-to-fructose conversion), glucose oxidase (glucose-to-gluconic acid conversion),

lipases (lipid hydrolysis), and pectinases (pectin hydrolysis). Enzyme production and utilization are a multibillion-dollar business with a great potential for expansion.

SUGGESTIONS FOR FURTHER READING

Bailey, J. E., and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill, New York, 1986.

Blanch, H. W., and D. S. Clark, *Biochemical Engineering*, Marcel Dekker, New York, 1996.

Datta, S., L. R. Chistena, and Y. R. S. Rajaram, Enzyme Immobilization: An Overview on Techniques and Support Materials, *3 Biotech* 3(1): 1–9, 2013.

Fedor, M. J., and J. R. Williamson, The Catalytic Diversity of RNAs, *Nat. Rev. Mol. Cell Biol.* 6(5): 399–412, 2005.

Liszka, M. J., M. E. Clark, E. Schneider, and D. S. Clark, Nature versus Nurture: Developing Enzymes That Function under Extreme Conditions, *Ann. Rev. Chem. Biomol. Eng.* 3(1): 77–102, 2012.

Schmid, A., J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, and B. Witholt, Industrial Biocatalysis Today and Tomorrow, *Nature* 409(6817): 258–268, 2001.

Sellek, G. A., and J. B. Chaudhuri, Biocatalysis in Organic Media Using Enzymes from Extremophiles, *Enzyme Microb. Technol.* 25(6): 471–482, 1999.

Swartz, J. R., Transforming Biochemical Engineering with Cell-Free Biology, *AIChE J.* 58(1): 5–13, 2012.

PROBLEMS

3.1. Consider the following reaction sequence:

$$\mathrm{S} + \mathrm{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (\mathrm{ES})_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (\mathrm{ES})_2 \xrightarrow{k_5} \mathrm{P} + \mathrm{E}$$

Develop a suitable rate expression for production formation [$v = k_5(\mathrm{ES})_2$] by using (a) the equilibrium approach, and (b) the quasi-steady-state approach.

3.2. Consider the reversible product-formation reaction in an enzyme–catalyzed bioreaction:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\mathrm{ES}) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}$$

Develop a rate expression for product-formation using the quasi-steady-state approximation and show that

$$v = \frac{d[\mathrm{P}]}{dt} = \frac{(v_s/K_m)[\mathrm{S}] - (v_p/K_p)[\mathrm{P}]}{1 + \dfrac{[\mathrm{S}]}{K_m} + \dfrac{[\mathrm{P}]}{K_p}}$$

where $K_m = \dfrac{k_{-1} + k_2}{k_1}$ and $K_p = \dfrac{k_{-1} + k_2}{k_{-2}}$ and $V_s = k_2[\mathrm{E}_0], V_p = k_{-1}[\mathrm{E}_0]$.

3.3. The enzyme fumarase has the following kinetic constants:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$

where $k_1 = 10^9\ M^{-1}\ s^{-1}$

$k_{-1} = 4.4 \times 10^4\ s^{-1}$

$k_2 = 10^3\ s^{-1}$

a. What is the value of the Michaelis constant for this enzyme?

b. At an enzyme concentration of $10^{-6}\ M$, what will be the initial rate of product formation at a substrate concentration of $10^{-3}\ M$?

[Courtesy of D. J. Kirwan from "Collected Coursework Problems in Biochemical Engineering" compiled by H. W. Blanch for 1977 American Society for Engineering Education Summer School.]

3.4. Data on enzymatic degradation of dichlorophenol at different concentrations are given in the following table:

So (mg/l)	30	60	90	120	150	200	250	300
Rate (mg P/l-h)	2.1	3.8	5.7	7.4	8.5	7.1	4.5	2.5

Assuming the substrate-inhibited kinetics is given by the following equation, determine the constants V_m, K_m, and K_{SI}:

$$V = \frac{V_m}{\left(1 + K_m/S\right)\left(1 + S/K_{SI}\right)}$$

3.5. Biological oxidation of phenylacetic acid is inhibited by propionic acid present in wastewater. The following data were obtained for enzymatic oxidation of phenylacetic acid at different concentrations of propionic acid:

S (mM)	0.25	0.40	0.50	0.60	0.75	1.00
V (mM/l-h), I = 0	1.0	1.7	1.9	2.1	2.4	2.5
V (mM/l-h), I = 1 mM	0.65	1.1	1.3	1.4	1.8	2.2
V (mM/l-h), I = 2 mM	0.55	0.9	1.0	1.3	1.4	1.8

a. What kind of inhibition is this?

b. Determine V_m, K_m, and K_I.

3.6. Hydrogenase enzyme responsible for hydrogen gas synthesis in photoheterotrophic bacteria is activated by the addition of sodium molybdate into the medium. Hydrogen formation rate varies with the Mo concentration as follows:

Rate (mlH2/h)	0.1	0.2	0.3	0.5	0.7	0.9	1.2
Mo (µg/l)	0	10	30	50	80	100	125

a. Assuming that the activator only affects the maximum reaction rate (V_m), determine the activation constant (K_A).

b. What would be the reaction rate at Mo = 60 µg/l?

3.7. Enzymes are immobilized on flat surfaces of plates in the form of monolayer (or very thin film) and placed in an agitated reactor for enzymatic reaction. The reactor is operated continuously. The bulk substrate concentration is S_0 = 200 mg/l at steady state. When the reactor was operated at different agitation speeds, the following data were obtained:

RPM	25	50	100	200	300	400
K_L (m/h)	0.18	0.24	0.33	0.42	0.88	2.25
V (mgS/l-h)	30	36	40	41.5	44	45

a. Determine surface concentration of the substrate for each RPM.
b. Determine kinetic constants of V_m and K_m for the enzymatic reaction.

3.8. The hydration of CO_2 is catalyzed by carbonic anhydrase as follows:

$$H_2O + CO_2 \overset{E}{\rightleftharpoons} HCO_3^- + H^+$$

The following data were obtained for the forward and reverse reaction rates at pH 7.1 and an enzyme concentration of 2.8×10^{-9} M:

Hydration		Dehydration	
$1/v$, M^{-1} $(s \times 10^{-3})$	$[CO_2]$ $(M \times 10^{-3})$	$1/v$, M^{-1} $(s \times 10^{-3})$	$[HCO_3^-]$ $(M \times 10^{3})$
36	1.25	95	2
20	2.5	45	5
12	5	29	10
6	20	25	15

v is the *initial* reaction rate at the given substrate concentration. Calculate the forward and reverse catalytic and Michaelis constants.

(Courtesy of D. J. Kirwan from "Collected Coursework Problems in Biochemical Engineering" compiled by H. W. Blanch for 1977 American Society for Engineering Education Summer School. © American Society for Engineering Education.)

3.9. An inhibitor (I) is added to the enzymatic reaction at a level of 1.0 g/l. The following data were obtained for K_m = 9.2 g S/l:

v	S
0.909	20
0.658	10
0.493	6.67
0.40	5
0.333	4
0.289	3.33
0.227	2.5

a. Is the inhibitor competitive or noncompetitive?
b. Find K_I.

3.10. During a test of kinetics of an enzyme–catalyzed reaction, the following data were recorded:

E_0 (g/l)	T (°C)	I (mmol/ml)	S (mmol/ml)	V (mmol/ml-min)
1.6	30	0	0.1	2.63
1.6	30	0	0.033	1.92
1.6	30	0	0.02	1.47
1.6	30	0	0.01	0.96
1.6	30	0	0.005	0.56
1.6	49.6	0	0.1	5.13
1.6	49.6	0	0.033	3.70
1.6	49.6	0	0.01	1.89
1.6	49.6	0	0.0067	1.43
1.6	49.6	0	0.005	1.11
0.92	30	0	0.1	1.64
0.92	30	0	0.02	0.90
0.92	30	0	0.01	0.58
0.92	30	0.6	0.1	1.33
0.92	30	0.6	0.033	0.80
0.92	30	0.6	0.02	0.57

a. Determine the Michaelis–Menten constant for the reaction with no inhibitor present at 30°C and at 49.6°C.

b. Determine the maximum velocity of the uninhibited reaction at 30°C and an enzyme concentration of 1.6 g/l.

c. Determine the K_I for the inhibitor at 30°C and decide what type of inhibitor is being used.

3.11. An enzyme ATPase has a molecular weight of 5×10^4 daltons, a K_M value of $10^{-4}\,M$, and a k_2 value of $k_2 = 10^4$ molecules ATP/min molecule enzyme at 37°C. The reaction catalyzed is the following:

$$\mathrm{ATP} \xrightarrow{\text{ATPase}} \mathrm{ADP} + \mathrm{P}_i$$

This can also be represented as

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}$$

Here S is ATP. The enzyme at this temperature is unstable. The enzyme inactivation kinetics are first order:

$$\mathrm{E} = \mathrm{E}_0 e^{-k_d t}$$

Here E_0 is the initial enzyme concentration and $k_d = 0.1\,\mathrm{min}^{-1}$. In an experiment with a partially pure enzyme preparation, $10\,\mu\mathrm{g}$ of total crude protein (containing enzyme) is added to a 1 ml reaction mixture containing 0.02 *M* ATP and incubated at 37°C. After 12 hours, the reaction ends (i.e., $t \to \infty$) and the inorganic phosphate (P_i) concentration is found to be 0.002 *M*, which was initially zero. What fraction of the crude protein preparation was the enzyme? *Hint*: Since $[\mathrm{S}] >> K_m$, the reaction rate can be represented by

$$\frac{d(\mathrm{P})}{dt} = k_2[\mathrm{E}]$$

3.12. Assume that for an enzyme immobilized on the surface of a nonporous support material, the external mass-transfer resistance for substrate is not negligible as compared to the reaction rate. The enzyme is subject to substrate inhibition (equation 3.34).

a. Are multiple states possible? Why or why not?

b. Could the effectiveness factor be greater than one?

3.13. The following data were obtained for an enzyme–catalyzed reaction. Determine V_{max} and K_m by inspection. Plot the data using the Eadie–Hofstee method and determine these constants graphically. Explain the discrepancy in your two determinations. The initial rate data for the enzyme–catalyzed reaction are as follows:

[S] mol/l	v μmol/min
5.0×10^{-4}	125
2.0×10^{-4}	125
6.0×10^{-5}	121
4.0×10^{-5}	111
3.0×10^{-5}	96.5
2.0×10^{-5}	62.5
1.6×10^{-5}	42.7
1.0×10^{-5}	13.9
8.0×10^{-6}	7.50

Do these data fit into Michaelis–Menten kinetics? If not, what kind of rate expression would you suggest? Use graphical methods.

3.14. H. H. Weetall and N. B. Havewala report the following data for the production of dextrose from corn starch using both soluble and immobilized (azo-glass beads) glucoamylase in a fully agitated continuous stirred-tank reactor system:

a. Soluble data: $T = 60°C, [S_0] = 168$ mg starch/ml, $[E_0] = 11,600$ units, volume = 1000 ml

b. Immobilized data: $T = 60°C, [S_0] = 336$ mg starch/ml, $[E_0] = 46,400$ units initially, immobilized, volume = 1000 ml

	Product concentration (mg dextrose/ml)	
Time (min)	Soluble	Immobilized
0	12.0	18.4
15	40.0	135
30	76.5	200
45	94.3	236
60	120.0	260
75	135.5	258
90	151.2	262
105	150.4	266
120	155.7	278
135	160.1	300
150	164.9	310
165	170.0	306
225	—	316
415	—	320

Determine the maximum reaction velocity, V_m (mg/ml-min · unit of enzyme) and the saturation constant, K_M (mg/ml).

The same authors studied the effect of temperature on the maximum rate of the hydrolysis of corn starch by glucoamylase. The results are tabulated next:

	V_{max} (m mol/min 10^6)	
T, °C	Soluble	Azo-immobilized
25	0.62	0.80
35	1.42	1.40
45	3.60	3.00
55	8.0	6.2
65	16.0	11.0

Determine the activation energy (ΔE kcal/g-mol) for the soluble and immobilized enzyme reaction.

Using these results, determine if immobilized enzyme is diffusion limited.

(Courtesy of A. E. Humphrey from "Collected Coursework Problems in Biochemical Engineering" compiled by H. W. Blanch for 1977 American Society for Engineering Education Summer School. © American Society for Engineering Education.)

3.15. Michaelis–Menten kinetics are used to describe intracellular reactions. Yet $[E_0] \approx [S_0]$. In in vitro batch reactors, the quasi-steady-state hypothesis does not hold for $[E_0] \approx [S_0]$. The rapid equilibrium assumption also will not hold. Explain why Michaelis–Menten kinetics and the quasi-steady-state approximation are still reasonable descriptions of intracellular enzyme reactions.

3.16. You are working for company A and you join a research group working on immobilized enzymes. Harry, the head of the lab, claims that immobilization improves the stability of the enzyme. His proof is that the enzyme has a half-life of 10 days in free solution, but under identical conditions of temperature, pH, and medium composition, the measured half-life of a packed column is 30 days. The enzyme is immobilized in a porous sphere 5 mm in diameter. Is Harry's reasoning right? Do you agree with him? Why or why not?

3.17. The following data were obtained from enzymatic oxidation of phenol by phenol oxidase at different phenol concentrations:

S (mg/l)	10	20	30	50	60	80	90	110	130	140	150
v (mg/l-h)	5	7.5	10	12.5	13.7	15	15	12.5	9.5	7.5	5.7

a. What type of inhibition is this?
b. Determine the constants V_m, K_m, and K_{si}.
c. Determine the oxidation rate at $[S] = 70$ mg/l.

3.18. Uric acid is degraded by uricase enzyme immobilized in porous Ca-alginate beads. Experiments conducted with different bead sizes result in the following rate data:

Bead diameter, Dp (cm)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Rate, v (mg/l.h)	200	198	180	140	100	70	50	30

a. Determine the effectiveness factor for particle sizes $Dp = 0.5$ cm and $Dp = 0.7$ cm.
b. The following data were obtained for $Dp = 0.5$ cm at different bulk uric acid concentrations. Assuming negligible liquid film resistance, calculate V_m and K_s for the enzyme. Assume no substrate or product inhibition.

S_0 (mg UA/l)	10	25	50	100	200	250
v (mg UA/l-h)	10	20	30	40	45	46

3.19. The enzyme urease is immobilized in Ca-alginate beads 2 mm in diameter. When the urea concentration in the bulk liquid is 0.5 mM, the rate of urea hydrolysis is $v = 10$ mmol/l-h. Diffusivity of urea in Ca-alginate beads is $D_e = 1.5 \times 10^{-5}$ cm^2/sec, and the Michaelis constant for the enzyme is $K_m' = 0.2$ mM. By neglecting the liquid film resistance on the beads (i.e., $[S_o] = [S_s]$) determine the following:

a. Maximum rate of hydrolysis V_m, Thiele modulus (ϕ), and effectiveness factor (η).

b. The V_m, ϕ, and η values for a particle size of Dp = 4 mm.
Hint: Assume $\eta = 3/\phi$ for large values of ϕ ($\phi > 2$).

3.20. Decarboxylation of glyoxalate (S) by mitochondria is inhibited by malonate (I). The following data was obtained in batch experiments:

	Rate of CO_2 Evolution, v (mmoles/l-h)		
Glyox,S (mM)	I = 0	I = 1.26 mM	I = 1.95 mM
0.25	1.02	0.73	0.56
0.33	1.39	0.87	0.75
0.40	1.67	1.09	0.85
0.50	1.89	1.30	1.00
0.60	2.08	1.41	1.28
0.75	2.44	1.82	1.39
1.00	2.50	2.17	1.82

a. What type of inhibition is this?

b. Determine the constants V_m, K_m', and K_I.

3.21. Urea dissolved in aqueous solution is degraded to ammonia and CO_2 by the enzyme urease immobilized on surfaces of nonporous polymeric beads. Conversion rate is controlled by transfer of urea to the surface of the beads through liquid film, and the conversion takes place on the surfaces of the beads. The following parameters are given for the system:

$$k_L = 0.2 \text{ cm/s}; \; K_m = 200 \text{ mg/l}$$

$$V_m' = 0.1 \text{ mg urea/cm}^2 \text{ support surface -s.}$$

$$S_b = 1000 \text{ mg urea/l}$$

a. Determine the surface concentration of urea.

b. Determine the rate of urea degradation under mass-transfer-controlled conditions.

3.22. Two enzymes are immobilized on the same flat, nonporous surface. For enzyme A, the substrate is S_1. For enzyme B, the substrate is S_2. The product of the first reaction is S_2:

$$S_1 \xrightarrow[E_A]{} S_2 \xrightarrow[E_B]{} P$$

a. Figure 3.P1 depicts the rate of the first reaction on the surface as a function of local concentrations of S_1. If the bulk concentration of S_1 is 100 mg/l and the mass-transfer coefficient is 4×10^{-5} cm/s, what is the rate of consumption of S_1 for a 1 cm^2 surface? What is the surface concentration of S_1?

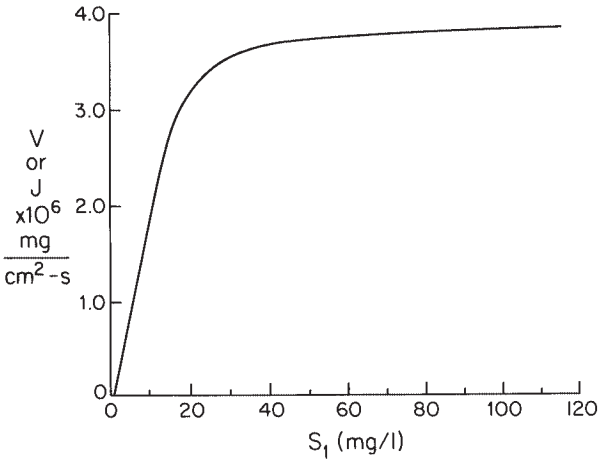


Figure 3P.1. Reaction rate data for problem 3.22. Reaction rate dependence on substrate 1 for reaction catalyzed by E_A.

b. The rate of the second reaction follows:

$$-d[S_2]/dt = d[P]/dt = \frac{V_m'' S_{2surface}}{K_m + S_{2surface}}$$

Here $K_m = 5$ mg/l (or 5×10^{-3} mg/cm^3) and $V_m'' = 4.0 \times 10^{-6}$ mg/cm^2 = s. The bulk concentration of S_2 [$S_{2\text{bulk}}$] is maintained as 5 mg/l, and the mass-transfer coefficient is the same for S_1 and S_2. Calculate [$S_{2\text{surface}}$] and the rate of formation of P (assuming all stoichiometric coefficients are one).

3.23. Consider the case of two enzymes immobilized on the same nonporous, planar surface. S is a substrate used by both enzymes in the following reactions:

$$S + E_1 \longrightarrow E_1 + P_1 \quad (1)$$

$$S + E_2 \longrightarrow E_2 + P_2 \quad (2)$$

The final product P_3 is formed by the spontaneous reaction of P_1 and P_2:

$$P_1 + P_2 \longrightarrow P_3 \quad (3)$$

Reactions 1 and 2 occur only at the surface, and reaction 3 is a homogeneous reaction occurring throughout the bulk liquid phase.

Figure 3.P2 gives the predicted reaction-rate dependence of reaction 1 (bottom curve) alone and reaction 2 (top curve) alone based on the measured amount of each enzyme immobilized and assuming the intrinsic reaction kinetics are not altered in the process of immobilization.

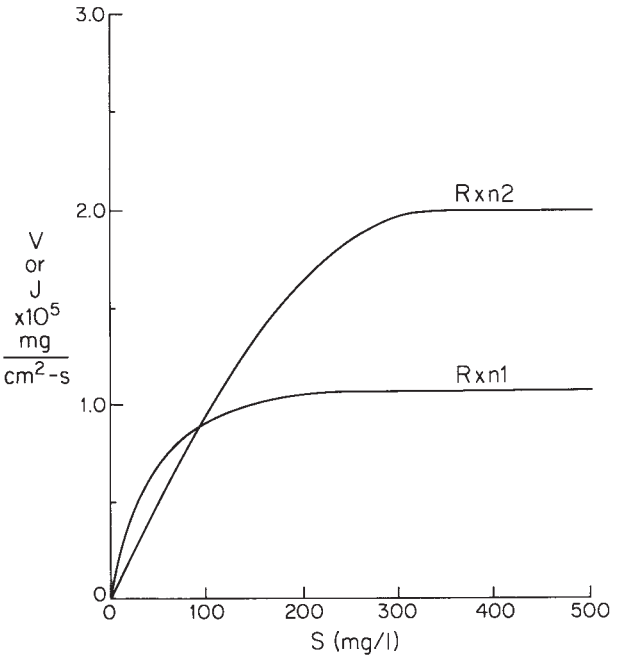


Figure 3P.2. Reaction rate data for problem 3.23. The reaction rate data for two different enzymes (E_1 and E_2) are shown.

a. If $k_L = 6 \times 10^{-5}$ cm/s and the bulk concentration of substrate is 500 mg/l, what is the *total* rate of substrate disappearance?
b. What is the overall effectiveness factor under the conditions of part a?
c. What will be the ratio of P_2 to P_1 under the conditions of part a?
d. If you want to produce equimolar amounts of P_1 and P_2 and if $k_L = 6 \times 10^{-5}$ cm/s, what value of bulk substrate concentration must you pick?

4

How Cells Work

So far you have learned something about how cells are constructed and how enzymes function. But a cell is much more than a bag filled with lipids, amino acids, sugars, enzymes, and nucleic acids. The cell must control how these components are made and how they interact with each other. Controlling and predicting the behavior of a cell is made more challenging by the phenomenon of macromolecular crowding, a condition that occurs routinely in living cells that may be essential for the efficient operation of metabolism. For example, the cytosol of the bacterium *Escherichia coli* contains about 300 to 400 mg/mL of macromolecules. Crowding from these high concentrations of macromolecules reduces the volume of solvent available for other molecules in the solution, which has the result of increasing their effective concentrations. The result of this crowding effect is that molecules in cells may behave in radically different ways than in test-tube assays. Therefore, measurements of the properties of individual enzymes or metabolic processes that are made in dilute solutions in the laboratory (in vitro) may differ by many orders of magnitude from the values seen in living cells (in vivo). In this chapter, we explore some examples of metabolic regulation. It is this ability to coordinate a wide variety of chemical reactions in the face of macromolecular crowding that makes a cell a cell.

The key to metabolic regulation is the flow and control of information. The advent of computers has led many to speak of the "Information Age." There are

many analogies between human society's need to use and exchange information and a cell's need to use and exchange information between subcellular components. Human society depends primarily on electronic signals for information storage, processing, and transmission; cells use chemical signals for the same purposes. Molecular biology is primarily the study of information flow and control.

4.1. THE CENTRAL DOGMA

Fortunately, almost all living systems have the same core approach to the storage, expression, and utilization of information. Information is stored in the DNA molecule and, like information on a computer tape or disk, can be replicated. It can also be played back or transcribed to produce a message. The message must translate into some action, such as a series of calculations with the ultimate display of the results, for the message to be useful.

Cells operate with an analogous system. Figure 4.1 displays the *central dogma* of biology. Information is stored in the DNA molecule. That information can be *replicated* directly to form a second identical DNA molecule. Further segments of information on the molecule can be *transcribed* to yield RNAs. Using a variety of RNAs, this information is *translated* into proteins. The proteins then perform a structural or enzymatic role, mediating almost all the metabolic functions in the cell.

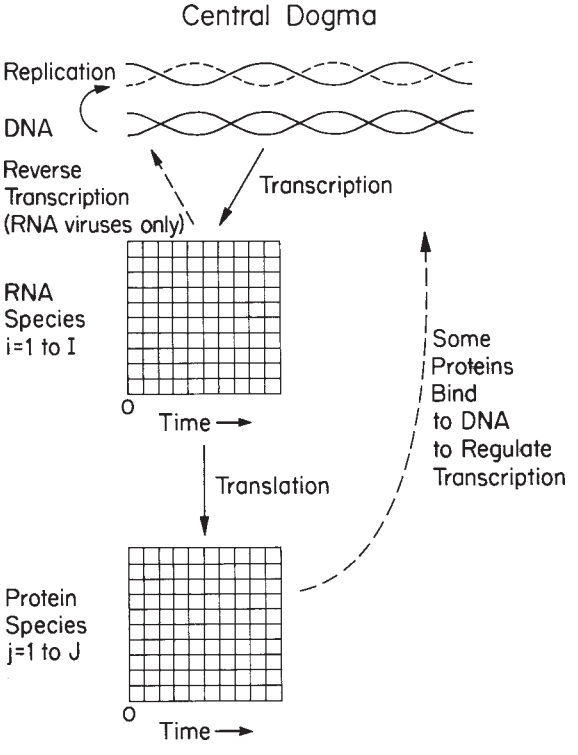


Figure 4.1. The primary tenet of molecular biology is the central dogma, which applies to all organisms.

The information content of the DNA molecule is static; changes occur slowly through infrequent mutations or rearrangements. Which species of RNA are present and in what amount varies with time and with changes in culture conditions. Likewise, the proteins that are present will change with time but on a different time scale than for RNA species. Some of the proteins produced in the cell bind to DNA and regulate the transcriptional process to form RNAs.

The key feature of the central dogma is its universality from the simplest to most complex organisms. The process depicted in Figure 4.1 is essentially applicable to any cell of commercial significance. Of note is that several important but unusual transfers of biological sequential information are known to exist (i.e., occur only under specific conditions in the case of some viruses or in a laboratory). One such special transfer is known as *reverse transcription* (the reverse of normal transcription) and is the process by which genetic information from RNA is transcribed into new DNA. This process is known to occur in *retroviruses*, such as the human immunodeficiency virus (HIV), as well as in eucaryotes in the case of *retrotransposons* and *telomere synthesis*. In HIV, the virus encodes information on an RNA molecule. In the host cell, a viral enzyme called *reverse transcriptase* produces a viral DNA molecule using the viral RNA as a template. Such viruses are clinically important, and one approach to their treatment involves selective inhibition of reverse transcriptase. In addition to serving as an antiviral target, the reverse transcriptase enzyme is an important tool in genetic engineering. Another special transfer is *RNA replication*, which is the copying of one RNA to another and is used by many viruses to replicate. The enzymes that copy RNA to create new RNA are called *RNA-dependent RNA polymerases*. These enzymes are also found in many eucaryotes where they participate in a mechanism known as *RNA silencing*. Finally, another type of transfer is the direct translation from DNA to protein, a process that bypasses transcription but has only been demonstrated in a cell-free system (i.e., in a test tube using extracts from *E. coli* that contain ribosomes but not intact cells). At present, it is unclear whether this mechanism of translation corresponds specifically to the genetic code and whether it occurs naturally in a living cell.

Other key transfers of biological information that are not explicitly covered in the central dogma theory include posttranslational protein modification, inteins, and DNA methylation. *Posttranslational modification* occurs after a protein amino acid sequence has been translated from nucleic acid chains, and are catalyzed by dedicated enzymes. These modifications can occur *cotranslationally* in concert with the translation process (i.e., while the protein chain is still emerging from the ribosome) or *posttranslationally* after the protein has been fully synthesized and released from the ribosomal machinery. Important examples of such processing include the following:

- *Glycosylation:* The addition of a sugar group to the side chain of a specific amino acid (e.g., asparagine, serine, threonine) to form a glycoprotein; discussed in detail shortly
- *Disulfide bond formation:* The coupling of two thiol (—SH) groups present in cysteine residues
- *Acylation:* The addition of an acetyl group either at the N-terminus or at lysine residues

- *Alkylation:* The addition of an alkyl group (e.g., methyl, ethyl) typically at lysine or arginine residues
- *Hydroxylation:* The introduction of a hydroxyl group (—OH) typically to proline residues
- *Lipidation:* The addition of a lipid to a protein chain; specific types of lipidation include *N*-myristoylation, palmitoylation, and GPI-anchor addition
- *Phosphorylation:* The addition of a phosphate group (PO_4^{3-}) usually to serine or threonine
- *Sulfation:* The addition of a sulfate group to a tyrosine residue
- *Ubiquitination:* The addition of a small protein tag called *ubiquitin* to a lysine residue

Proteins can also change their own primary sequence from the sequence originally encoded by the DNA in a process called *protein splicing*. This process involves an *intein*, which is a "parasitic" segment of a protein that is able to excise itself from the chain of amino acids as they emerge from the ribosome and rejoin the remaining portions *(exteins)* with a peptide bond in such a manner that the main protein "backbone" does not fall apart. Inteins are often referred to as "protein introns" owing to their mechanistic resemblance to *introns*, which mediate *mRNA splicing* in eucaryotes (discussed shortly).

Variation in methylation states of DNA can alter gene expression levels significantly. Methylation variation of DNA is a process that usually involves the action of enzymes known as *DNA methylases*. Importantly, the effective information content is changed by methylation on DNA, but the primary DNA sequence is not altered. Functionally relevant changes to an organism's genome, such as methylation or histone modification, that do not involve a change in the nucleotide sequence are known as *epigenetic* modifications. Unlike genetics whereby changes occur to the DNA sequence, the changes in gene expression or cellular phenotype of epigenetics have other causes, thus use of the prefix *epi-* from Greek meaning "over, outside of, or around."

For information storage and exchange to take place, there must be a language. We can conceive of all life as using a four-letter alphabet made up of the nucleotides discussed in Chapter 2, "An Overview of Biological Basics" (that is, A, T, G, and C in DNA). All words are three letters long; such words are called *codons*. With four letters and only three-letter words, we have a maximum of 64 words. These words, when expressed, represent a particular amino acid or "stop" protein synthesis.

When these words are put into a sequence, they can make a "sentence" (i.e., a *gene*), which when properly transcribed and translated is a protein. Other combinations of words regulate when the gene is expressed. Carrying the analogy to the extreme, we may look at the complete set of information in an organism's DNA (i.e., the *genome*) as a book. (For the human genome, it would be more than 1000 books the size of this one.)

This simple language of 64 words is all that is necessary to summarize your total physical makeup at birth and all your natural capabilities. It is essentially universal,

the same for *E. coli* and *Homo sapiens* (humans). This universality has helped us to make great strides in understanding life and is a practical tool in genetic engineering and commercial biotechnology. (Recently, it has been demonstrated that this process can be manipulated to allow the incorporation of unnatural amino acids.)

Each of the steps in information storage and transfer (Figure 4.1) requires a macromolecular template. Let us examine how these templates are made and how this genetic-level language is preserved and expressed.

4.2. DNA REPLICATION: PRESERVING AND PROPAGATING THE MESSAGE

The double-helix structure of DNA discussed in Chapter 2 is extremely well suited to its role of preserving genetic information. Information resides simply in the linear arrangement of the four nucleotide letters (A, T, G, and C). Because G can hydrogen-bind only to C and A only to T, the strands must be complementary if an undistorted double helix is to result. Replication is *semiconservative* (see Figure 2.18); each daughter chromosome contains one parental strand and one newly generated strand.

To illustrate the replication process, let us briefly consider DNA replication in *E. coli*. The enzyme responsible for covalently linking the monomers is DNA polymerase. *E. coli* has three DNA polymerases (named Pol I, Pol II, and Pol III). A DNA polymerase is an enzyme that links deoxynucleotides together to form a DNA polymer. Pol III enzymatically mediates the addition of nucleotides to an RNA primer. Pol I can hydrolyze an RNA primer and duplicate single-stranded regions of DNA; it is also active in the repair of DNA molecules. The exact role of Pol II is still unclear.

In addition to the enzyme, the enzymatic reaction requires activated monomer and the template. The activated monomers are the nucleoside triphosphates. The formation of the 5′–3′ phosphodiester bond to link a nucleotide with the growing DNA molecule results in the release of a pyrophosphate, which provides the energy for such a biosynthetic reaction. The resulting nucleoside monophosphates are the constituent monomers of the DNA molecule.

Replication of the chromosome normally begins at a predetermined site, the *origin of replication*, which in *E. coli* is attached to the plasma membrane at the start of replication. Initiator proteins bind to DNA at the origin of replication, break hydrogen bonds in the local region of the origin, and force the two DNA strands apart. When DNA replication begins, the two strands separate to form a Y-shaped structure called a *replication fork*. Movement of the fork must be facilitated by the energy-dependent action of *DNA gyrase* and *unwinding enzymes*. In *E. coli*, the chromosome is circular. In *E. coli* (but not all organisms), the synthesis of DNA is *bidirectional*. Two forks start at the origin and move in opposite directions until they meet again, approximately 180° from the origin.

To initiate DNA synthesis, an *RNA primer* is required; RNA polymerase requires no primer to initiate the chain-building process, whereas DNA

polymerase does. (We can speculate on why this is so. In DNA replication, it is critical that no mistakes be made in the addition of each nucleotide. The DNA polymerase Pol III can *proofread*, in part because of the enzyme's 3′-to-5′ exonuclease activity, which can remove mismatches by moving backward. In contrast, a mistake in RNA synthesis is not nearly so critical, so RNA polymerase lacks this proofreading capacity.) Once a short stretch of RNA complementary to one of the DNA strands is made, DNA synthesis begins with Pol III. Next, the RNA portion is degraded by Pol I, and DNA is synthesized in its place. This process is summarized in Figure 4.2.

DNA polymerase works only in the 5′-to-3′ direction, which means that the next nucleotide is always added to the exposed 3′-OH group of the chain. Thus, one strand (the *leading strand*) can be formed continuously if it is synthesized in the same direction as the replication fork is moving. The other strand (the *lagging strand*) must be synthesized discontinuously. Short pieces of DNA attached to RNA are formed on the lagging strand. These fragments are called *Okazaki fragments*. The whole process is summarized in Figure 4.3. The enzyme *DNA ligase*, which joins the two short pieces of DNA on the continuous strand, is important in our discussions of genetic engineering.

This brief discussion summarizes the essentials of how one DNA molecule is made from another and thus preserves and propagates the genetic information in the original molecule. Now we turn our attention to how this genetic information can be transferred.

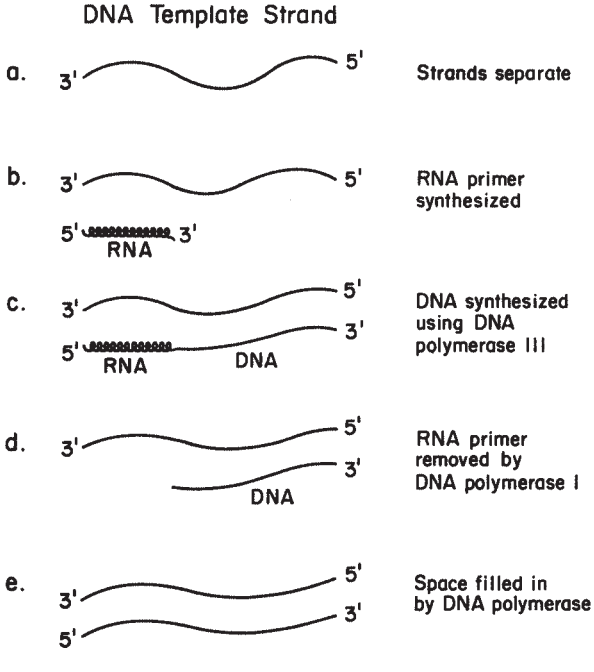


Figure 4.2. Initiation of DNA synthesis requires the formation of an RNA primer.

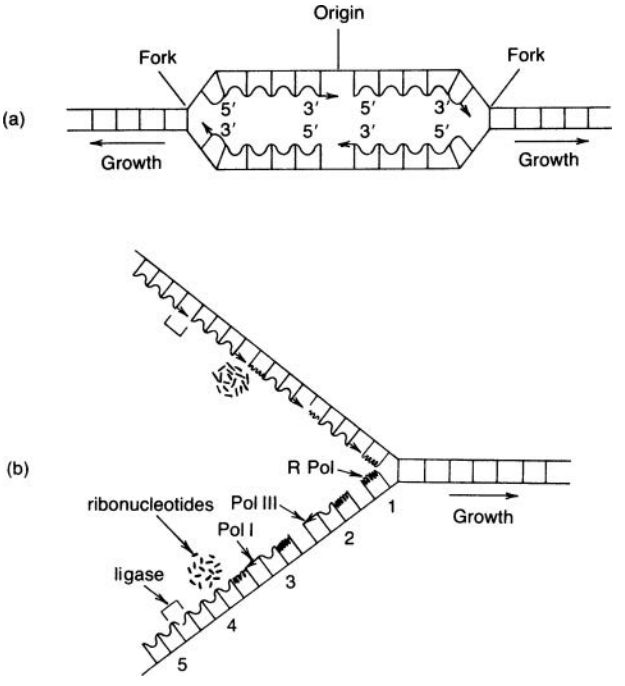


Figure 4.3. Schematic representation of the steps of replication of the bacterial chromosome. The newly polymerized strands of DNA (wavy lines) are synthesized in the 5′-to-3′ direction (indicated by the arrows), using the preexisting DNA strands (solid lines) as a template. The process creates two replication forks, which travel in opposite directions until they meet on the opposite side of the circular chromosome, completing the replication process. Part (b) represents a more detailed view of one of the replicating forks and shows the process by which short lengths of DNA are synthesized and eventually joined to produce a continuous new strand of DNA. For purposes of illustration, four short segments of nucleic acid are illustrated at various stages. In (1), primer RNA (thickened area) is being synthesized by an RNA polymerase (R Pol). Then, successively, in (2) DNA is being polymerized to it by DNA polymerase III (Pol III); in (3) a preceding primer RNA is being hydrolyzed, while DNA is being polymerized in its place by the exonuclease and polymerase activities of DNA polymerase I (Pol I); finally, the completed short segment of DNA (4) is joined to the continuous strand (5) by the action of DNA ligase. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

4.3. TRANSCRIPTION: SENDING THE MESSAGE

The primary products of transcription are the three major types of RNA we introduced in Chapter 2: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Their rates of synthesis determine the cell's capacity to make proteins. RNA synthesis from DNA is mediated by the enzyme *RNA polymerase*. To be functional, RNA polymerase must have two major subcomponents: the *core* enzyme and the *sigma factor*. The core enzyme contains the catalytic site, whereas the sigma factor is a protein essential to locating the appropriate beginning for the message. The core enzyme plus the sigma factor constitutes the *holoenzyme*.

One may wonder which of the two strands of DNA is actually transcribed. It turns out that either strand can be read. RNA polymerase always reads in the 3′-to-5′ direction, so the direction of reading will be opposite on each strand. On one part of the chromosome, one strand of DNA may serve as the template, or *sense strand*, and on another portion of the chromosome, the other strand may serve as a template.

The processes of initiation, elongation, and termination are summarized in Figure 4.4. The sigma factor is involved only in initiation. The sigma factor

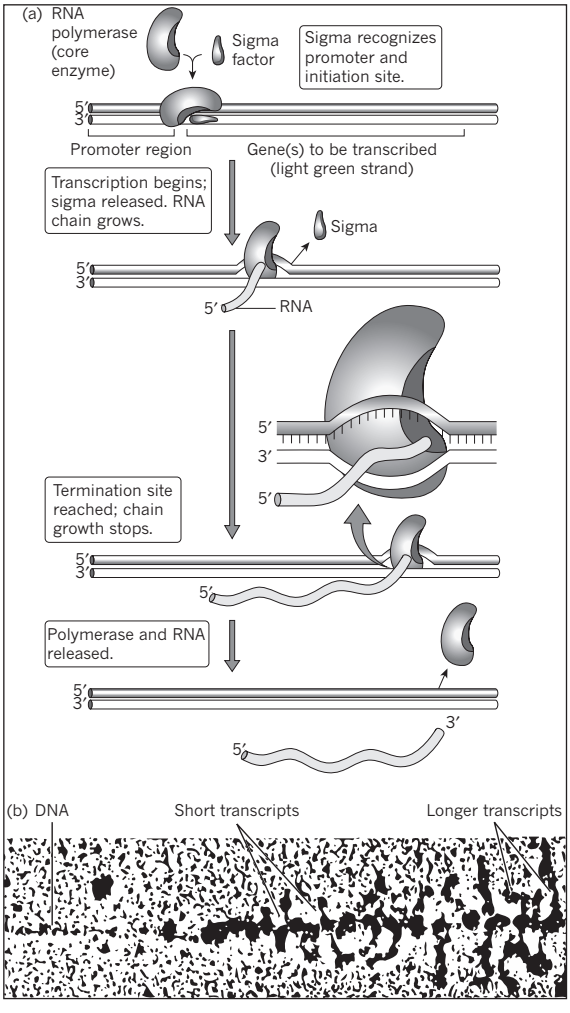


Figure 4.4. Transcription. Steps in messenger RNA synthesis. The start and stop sites are specific nucleotide sequences on the DNA. RNA polymerase moves down the DNA chain, causing temporary opening of the double helix and transcription of one of the DNA strands. Rho binds to the termination site and stops chain growth; termination can also occur at some sites without rho. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

recognizes a specific sequence of nucleotides on a DNA strand. This sequence is the *promoter region*. Promoters can vary somewhat, and variations alter the affinity of the sigma factor (and consequently the holoenzyme) for a particular promoter. A *strong promoter* is one with a high affinity for the holoenzyme. The rate of formation of transcripts is determined primarily by the frequency of initiation of transcription, which is directly related to promoter strength. This information is important in our discussions of genetic engineering. Cells usually have one dominant sigma factor that is required to recognize the vast majority of promoters in the cell. However, other sigma factors can play important roles under different growth conditions (particularly stress) and are used to initiate transcription from promoters that encode proteins important to the cell for coping with unusual growth conditions or stress.

After the initiation site is recognized, elongation of the transcript begins. As soon as elongation is established, the sigma factor is released so it can be reused. The synthesis of the growing RNA molecule requires energy, so activated triphosphate monomers of the ribonucleotides are required.

The transcript is made until the RNA polymerase encounters a stop signal, or *transcription terminator*. At this point, the RNA polymerase disassociates from the DNA template, and the RNA transcript is released. In some cases, an additional protein, the *rho* protein, is required for termination. Terminators can be strong or weak. If a weak terminator is coupled with a strong promoter, some of the RNA polymerase will *read through* the terminator, creating an artificially long transcript and possibly disrupting subsequent control regions on that DNA strand. We must consider terminator regions and their strength when constructing recombinant DNA systems.

The transcripts that are formed may be roughly lumped as either stable or unstable RNA species. The stable RNA species are rRNA and tRNA. mRNA is highly unstable (about a 1-min half-life for a typical *E. coli* mRNA, although mRNA may be considerably more stable in cells from higher plants and animals). Consider for a moment why mRNA is relatively unstable. The answer should become apparent as we discuss translation and regulation.

Although the general features of transcription are universal, there are some significant differences in transcription between procaryotic and eucaryotic cells. One example is that in procaryotes, related proteins are often encoded in a row without interspacing terminators. Thus, transcription from a single promoter may result in a *polygenic* message. *Polygenic* indicates many genes; *each single gene encodes a separate protein*. Thus, the regulation of transcription from a single promoter can provide efficient regulation of functionally related proteins; such a strategy is particularly important for relatively small and simple cells. By contrast, eucaryotic cells do not normally produce polygenic messages, although cells have been engineered to do so.

In procaryotic cells, there is no physical separation of the chromosome from the cytoplasma and ribosomes. Often, an mRNA will bind to a ribosome and begin translation immediately, even while part of it is still being transcribed! However, in eucaryotes, where the nuclear membrane separates chromosomes and ribosomes, the mRNA is often subject to processing before translation (see Figure 4.5). The DNA can encode for a transcript with an intervening sequence (called an *intron*) in the middle of the transcript. This intron is then cut out of the transcript at two specific sites.

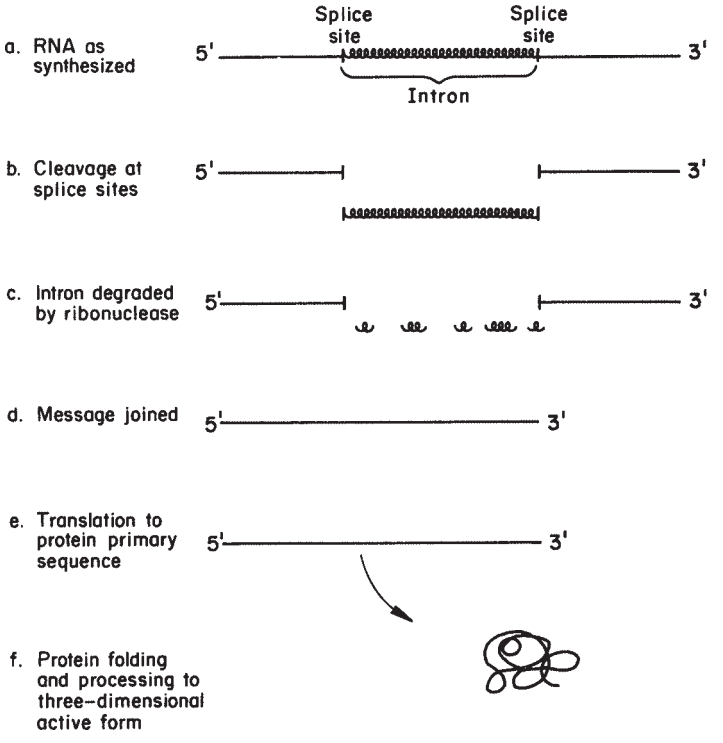


Figure 4.5. In eucaryotes, RNA splicing is important. The presence of introns is a complication in cloning genes from eucaryotes to procaryotes.

The ends of the remaining fragments are joined by a process called *mRNA splicing*. The spliced message can then be translated into an actual protein. The part of the transcript forming the intron is degraded and the monomers recycled. When mRNA is recovered from the cytosol, it will be in the mature form, while mRNA within the nucleus has introns. Many eucaryotic genes contain "nonsense DNA," which encodes for the intronic part of the transcript. The word *nonsense* denotes that that particular sequence of DNA does not encode for amino acids. The role of introns is not well understood, but they likely play a role in either evolution or cellular regulation or both. The presence of introns complicates the transfer of eucaryotic genes to protein production systems in procaryotes such as *E. coli*.

Two other mRNA processing steps occur in eucaryotic cells that do not typically occur in procaryotes. One is *RNA capping*, in which the 5′ end is modified by the addition of a guanine nucleotide with a methyl group attached. The other is *polyadenylation*, in which a string of adenine nucleotides are added to the 3′ end. This tail of adenines is often several hundred nucleotides long. These two modifications are thought to increase mRNA stability and facilitate transport across the nuclear membrane.

Once we understand transcription, we can tackle translation. The translation of information on mRNA into proteins occupies a very large fraction of the cells' resources. Like a large automobile plant, the generation of blueprints and the construction of the manufacturing machinery is worthless until product is made.

4.4. TRANSLATION: GOING FROM MESSAGE TO PRODUCT

Translation is the critical process of converting the information from RNA into peptides and proteins. After the proteins are made, they may be further modified to make the protein useful to the cell through posttranslational processing.

4.4.1. Genetic Code: Universal Message

The blueprint for any living cell is the genetic code. The code, as discussed previously, is made up of three-letter words (codons) with an alphabet of four letters. Sixty-four words are possible, but many of these words are redundant. Although such a "language" may appear to be ridiculously simple, it is sufficient to serve as a complete blueprint for the "construction" of the reader.

The dictionary for this language is given in Table 4.1, and an illustration of the relationship of nucleotides in the chromosome and mRNA to the final protein product is given in Figure 4.6. The code is *degenerate* in that more than one codon can specify a particular amino acid (e.g., UCU, UCC, UCA, and UCG all specify serine). Three codings, UAA, UAG, and UGA, are called *nonsense codons* because they do not encode normally for amino acids. These codons act as stop points in translation and are encoded at the end of each gene. It should also be pointed out that scientists have been able to artificially modify the genetic code. Such efforts have effectively

TABLE 4.1. The Genetic Code: Correspondence between Codons and Amino Acids

	Second Bases							
First Base	U		C		A		G	
U	UUU	phe[a]	UCU	ser	UAU	tyr	UGU	cys
	UUC	phe	UCC	ser	UAC	tyr	UGC	cys
	UUA	leu	UCA	ser	UAA	(none)[b]	UGA	(none)[b]
	UUG	leu	UCG	ser	UAG	(none)[b]	UGG	try
C	CUU	leu	CCU	pro	CAU	his	CGU	arg
	CUC	leu	CCC	pro	CAC	his	CGC	arg
	CUA	leu	CCA	pro	CAA	glu-N	CGA	arg
	CUG	leu	CCG	pro	CAG	glu-N	CGG	arg
A	AUU	ileu	ACU	thr	AAU	asp-N	AGU	ser
	AUC	ileu	ACC	thr	AAC	asp-N	AGC	ser
	AUA	ileu	ACA	thr	AAA	lys	AGA	arg
	AUG	met	ACG	thr	AAG	lys	AGG	arg
G	GUU	val	GCU	ala	GAU	asp	GGU	gly
	GUC	val	GCC	ala	GAC	asp	GGC	gly
	GUA	val	GCA	ala	GAA	glu	GGA	gly
	GUG	val	GCG	ala	GAG	glu	GGG	gly

[a]Amino acids are abbreviated as the first three letters in each case, except for glutamine (glu-N), asparagine (asp-N), and isoleucine (ileu).
[b]The codons UAA, UAG, and UGA are nonsense codons; UAA and UAG are called the ochre codon and the amber codon, respectively.
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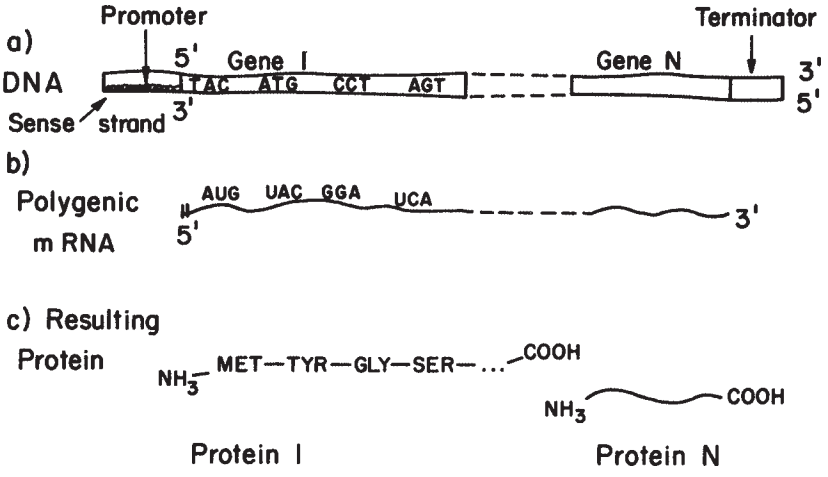


Figure 4.6. Overview of the transfer of information from codons on the DNA template to proteins. In procaryotes, messages are often polygenic, whereas in eucaryotes, polygenic messages are not made.

expanded the genetic code by reallocating one or more specific codons, typically the nonsense codons or a four-base codon, to encode an amino acid that is not among the 20 encoded proteinogenic amino acids (e.g., *O*-methyltyrosine, naphthylalanine, and the photo-cross-linking benzoylphenylalanine). The ability to insert nonstandard amino acids into specific sites within proteins enriches the repertoire of useful tools available to scientists and engineers and holds great promise for a variety of biotechnological applications.

The genetic code is essentially universal, although some exceptions exist (particularly in the mitochondria and inclusion of rare amino acids such as selenocysteine [Sec] and pyrrolysine [Pyl], which are the 21st and 22nd amino acids in the genetic code). This essential universality greatly facilitates genetic engineering. The language used to make a human protein is understood in *E. coli* and yeast, and these simple cells will faithfully produce the same amino acid sequence as a human cell.

Knowing the genetic language, we may ask more about the mechanism by which proteins are actually made.

4.4.2. Translation: How the Machinery Works

The process of translation consists of three primary steps: *initiation*, *elongation*, and *termination*.

For initiation, mRNA must bind to the ribosome. All protein synthesis begins with an AUG codon (or GUG) on the mRNA. This AUG encodes for a modified methionine, *N*-formylmethionine. In the middle of a protein, AUG encodes for methionine, so the question is how the cell knows that a particular AUG is an initiation codon for *N*-formylmethionine. The answer lies about 10 nucleotides upstream of the AUG, where the ribosome binding site (Shine-Delgarno box) is located. Ribosome

binding sites can vary in strength and are an important consideration in genetic engineering. The initiation of polymerization in procaryotes requires an *initiation complex* composed of a 30S ribosomal unit with an *N*-formylmethionine bound to its initiation region, a 50S ribosomal unit, three proteins called *initiation factors* (IF1, IF2, and IF3), and the phosphate bond energy from guanosine triphosphate (GTP).

The elongation of the amino acid chain uses tRNAs as decoders. One end of the tRNA contains the *anticodon*, which is complementary to the codon on the mRNA. The other end of the tRNA binds a specific amino acid. The tRNA is called *charged* when it is carrying an amino acid. The binding of an amino acid to the tRNA molecule requires the energy from two phosphate bonds and enzymes known as *aminoacyl-tRNA synthetases*. Figure 2.19 depicts a tRNA molecule.

The actual formation of the peptide bond between the two amino acids occurs on adjacent sites on the ribosome: the P or *peptidyl* site and the A or *aminoacyl* site (see Figure 4.7). The growing protein occupies the P site, and the next amino acid to be added occupies the A site. As the peptide bond is formed, the tRNA associated with the P site is released, and a rachet mechanism moves the mRNA down one codon so as to cause the tRNA that was in the A site to be in the P site. Then a charged tRNA with the correct anticodon can be recognized and inserted into the A site. The whole process is then repeated. The cell expends a total of four phosphate bonds to add one amino acid to each growing polypeptide (two to charge the tRNA and two in the process of elongation), and this accounts for most of the cellular energy expenditure in bacteria.

When a nonsense or stop codon is reached, the protein is released from the ribosome with the aid of a protein *release factor* (RF). The 70S ribosome then dissociates into 30S and 50S subunits. An mRNA typically is being read by many (e.g., 10 to 20) ribosomes at once; as soon as one ribosome has moved sufficiently far along the message that the ribosome binding site is not physically blocked, another ribosome can bind and initiate synthesis of a new polypeptide chain.

4.4.3. Posttranslational Processing: Making the Product Useful

Often, the polypeptide formed from the ribosome must undergo further processing before it can become truly useful. First, the newly formed chain must fold into the proper structure; in some cases, several different chains must associate to form a particular enzyme or structural protein. Additionally, *chaperones* are an important class of proteins that assist in the proper folding of peptides. There are distinct pathways to assist in folding polypeptides. The level of chaperones in a cell increases in response to environmental stresses such as high temperature. Misfolded proteins are subject to degradation if they remain soluble. Often, misfolded proteins aggregate and form insoluble particles (i.e., inclusion bodies). High levels of expression of foreign proteins through recombinant DNA technology in *E. coli* often overwhelm the processing machinery, resulting in inclusion bodies. The formation of proteins in inclusion bodies greatly complicates any bioprocess, since in vitro methods to unfold and refold the protein product must be employed. Even when a cell properly folds a protein, additional cellular processing steps must occur to make a useful product.

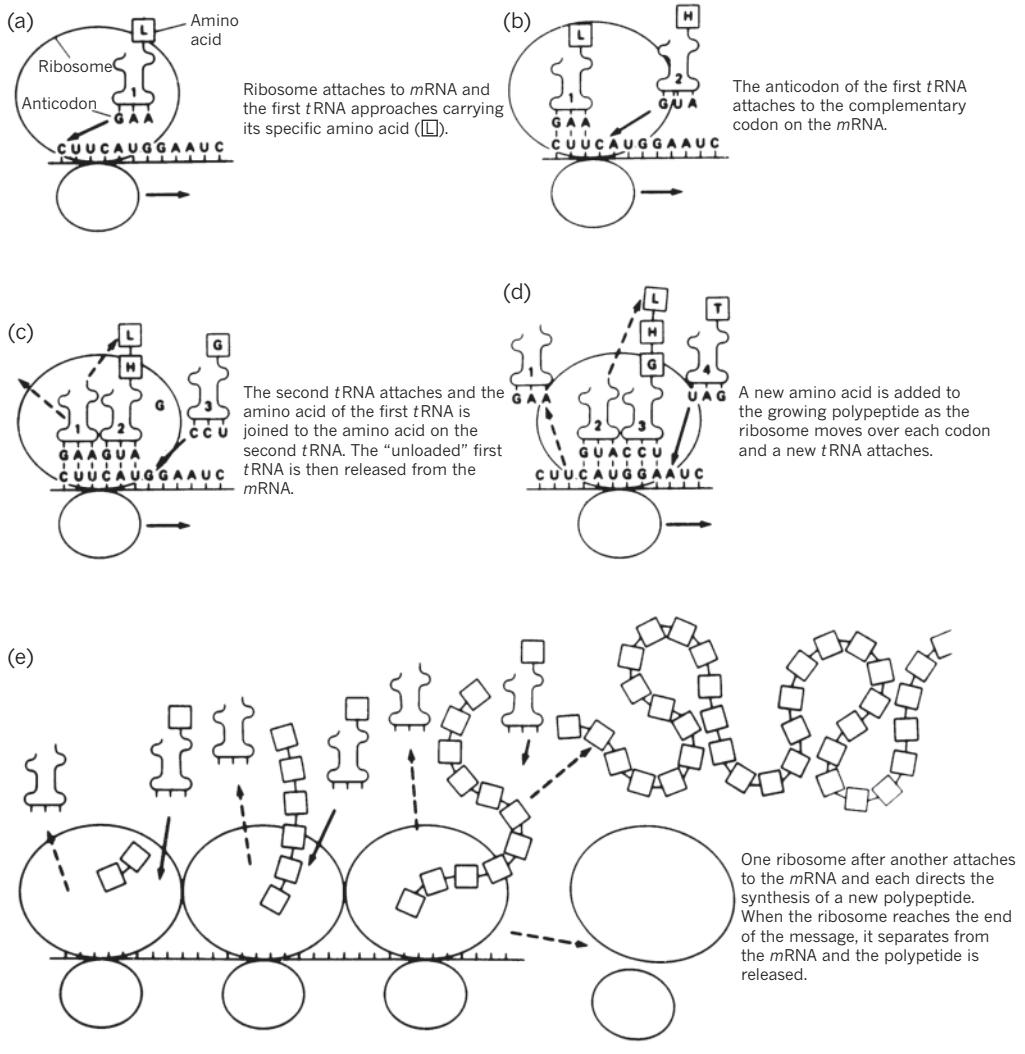


Figure 4.7. Translation of genetic information from a nucleotide sequence to an amino acid sequence. (M. W. Jensen, D. N. Wright, *Introduction to Medical Microbiology*, 1st ed., ©1985. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

Many proteins are secreted through a membrane. In many cases, the translocation of the protein across the membrane is done *cotranslationally* (during translation), whereas in some cases, *posttranslation* movement across the membrane occurs. When proteins move across a membrane, they have a *signal peptide* (about 20 to 25 amino acids). This signal peptide is clipped off during secretion. Such proteins exist in a preform and mature form. The preform is what is made from the mRNA, but the actual active form is the mature form. The preform is the signal sequence plus the mature form.

In procaryotes, secretion of proteins occurs through the cytoplasmic membrane. In *E. coli* and most gram-negative bacteria, the outer membrane blocks release of the secreted protein into the extracellular compartment. In gram-positive cells, secreted proteins readily pass the cell wall into the extracellular compartment. Whether a protein product is retained in a cell or released has a significant impact on bioprocess design.

In eucaryotic cells, proteins are released by two pathways. Both involve *exocytosis*, where *transport vesicles* fuse with the plasma membrane and release their contents. Transport vesicles mediate the transport of proteins and other chemicals from the endoplasmic reticulum (ER) to the Golgi apparatus and from the Golgi apparatus to other membrane-enclosed compartments. Such vesicles bud from a membrane and enclose an aqueous solution with specific proteins, lipids, or other compounds. In the secretory pathway, vesicles carrying proteins bud from the ER, enter the *cis* face of the Golgi apparatus, exit the Golgi *trans* face, and then fuse with the plasma membrane. Only proteins with a signal sequence are processed in the ER to enter the secretory pathway. Two forms of the secretary pathway exist. One is the *constitutive exocytosis pathway*, which operates at all times and delivers lipids and proteins to the plasma membrane. The second is the *regulated exocytosis pathway*, which typically is in specialized secretory cells. These cells secrete proteins or other chemicals only in response to specific chemical signals. Other modifications to proteins, known as *post-translational modifications* (PTMs), can take place, as discussed earlier. These modifications involve the addition (or subtraction in the case of inteins) of components such as sugars, lipids, chemical groups, and proteinaceous tags and are typically vital to protein folding, regulation, and/or function. A bioprocess engineer must be aware that many proteins are subject to extensive processing after the initial polypeptide chain is made. These modifications can be quite complex and are important considerations in the choice of host organisms for the production of proteins.

One of the most abundant PTMs in cellular proteins is *glycosylation*, which is the addition of complex sugars known as *glycans*. The two main varieties of glycosylation are *N*-linked glycosylation (the glycans are attached to the nitrogen of an asparagine amino acid) and *O*-linked glycosylation (the glycans are attached to the hydroxyl oxygen of serine or threonine residue). The glycosylation pattern can serve to target the protein to a particular compartment or to control its degradation and removal from the organism. For therapeutic proteins injected into the human body, these issues are critical. A protein product may be ineffective if the *N*- or *O*-linked glycosylation pattern is not humanlike, as the protein may not reach the target tissue or may be cleared (i.e., removed) from the body before it exerts the desired action. Further, undesirable immunogenic responses can occur if a protein has a nonhuman glycosylation pattern. Thus, the glycan pattern *(glycoform)* of a protein product is a key issue in bioprocesses to make therapeutic proteins (see Chapter 14, "Utilizing Genetically Engineered Organisms").

Because therapeutic glycoproteins must harbor human or humanlike glycans, production of therapeutic glycoproteins has traditionally been limited to mammalian cell lines that can replicate human or humanlike *N*-glycosylation reactions. However, not all eucaryotic cells produce proteins with humanlike *N*-linked glycosylation. For example, yeasts, lower fungi, and insect cells often produce partially processed products. Even mammalian cells (including human cells) will show altered patterns of glycosylation when cultured in bioreactors, and these patterns can shift upon scale-up in bioreactor

size. To address the nonhuman glycan patterns produced by lower eucaryotes, scientists have engineered the *N*-linked glycosylation process in yeast for the production of glycoproteins bearing homogeneous, human *N*-glycan structures. The industrial workhorse *E. coli* remains the preferred host for producing therapeutic proteins such as insulin and human growth hormone that do not require glycosylation; however, the lack of glycosylation machinery in *E. coli* has so far restricted its use to non-glycosylated products.

The process of *N*-linked glycosylation in eucaryotes is depicted in Figure 4.8. The glycoform shown is "typical," and many variants are possible. The natural

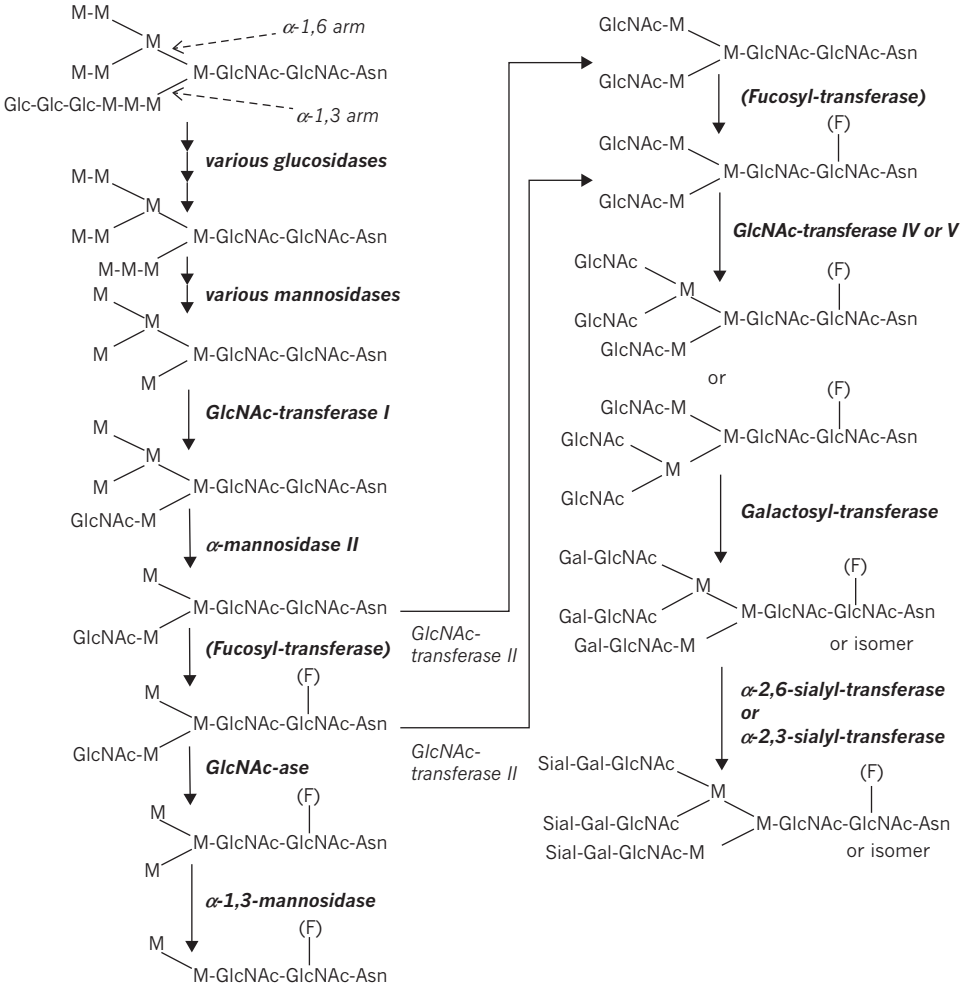


Figure 4.8. Example of an *N*-linked glycosylation pathway (Glc = glucose, M = mannose, GlcNAc = *N*-acetylglucosamine, F = fucose, Gal = galactose, Sial = sialic acid). The oligosaccharide side chain is bound to an asparagine (Asn) of the protein. The upper arm represents the α-1,6 arm and the lower one the α-1,3 arm. The parentheses refer to an optional fucosylation. The GlcNAc-ase step is important in insect cells, but not mammalian cells. (Courtesy of C. Joosten.)

proteins in the human body usually display a range of glycoforms; a single form is not observed. Eucaryotic *N*-linked protein glycosylation pathways span the cytosol, ER, and Golgi apparatus. Protein glycosylation can be divided into two distinct processes: the assembly of lipid-linked oligosaccharides (LLOs) at the ER membrane and the transfer of the oligosaccharide from the lipid anchor dolichyl pyrophosphate (Dol-PP) to selected asparagine residues of nascent polypeptides. The characteristics of *N*-linked protein glycosylation—namely, the use of Dol-PP as carrier for oligosaccharide assembly, the transfer of the completely assembled *N*-glycan, and the recognition of asparagine residues characterized by the sequence Asn-Xaa-Ser/Thr where X can be any amino acid except proline—are highly conserved in eucaryotes. The *oligosaccharyltransferase* (OST) catalyzes the transfer of a preformed branched glycan comprising 14 monosaccharides from the lipid donor Dol-PP to the acceptor protein. In yeast, eight different membrane proteins have been identified that constitute the OST complex in vivo. STT3 represents the catalytic subunit and is the most conserved subunit in the OST complex.

Following transfer to the protein, the 14-residue glycan is first "trimmed" by a set of specific glycosidases. In yeast, oligosaccharide processing often stops in the ER, leading to *simple glycoforms* (or high mannose or oligomannose forms). The initial trimming takes place in the ER, followed by transfer to the Golgi apparatus where final trimming occurs, followed by addition of various sugars or aminosugars. These units are added through the action of various glycosyltransferases using nucleotide-activated sugar cosubstrates as sugar donors. In insect cells, high levels of *N*-acetylglucosaminidase activity results typically in dead-end structures with a mannose cap. *Complex glycoforms* have sugar residues (*N*-acetylglucosamine, galactose, and/or sialic acid) added to all branches of the oligosaccharide structure. *Hybrid glycoforms* have at least one branch modified with one of these sugar residues and one or more with mannose as the terminal residue.

Until recently, *N*-linked protein glycosylation was thought to be the exclusive domain of eucaryotes. However, *bona fide N*-linked glycosylation pathways are now known to exist in all domains of life. In the case of procaryotes, *N*-linked glycosylation was first discovered in the pathogenic bacterium *Campylobacter jejuni* and subsequently transferred to laboratory strains of *E. coli*. This procaryotic glycosylation system shares many functional similarities with the eucaryotic process, such as assembly of glycans on a lipid carrier in the bacterial cytoplasmic membrane (the equivalent of the eucaryotic ER membrane) and transfer of the glycans to asparagines in secretory or membrane proteins by an integral membrane OST. There are also several major differences between the systems. Most notably, the structures of bacterial *N*-linked glycans are significantly divergent from those produced in eucaryotes. Hence, glycoproteins derived from natural procaryotic glycosylation systems are likely to be immunogenic in humans and thus of limited therapeutic value. However, bottom-up engineering of a synthetic pathway for performing eucaryotic *N*-linked glycosylation reactions in *E. coli* has been described recently. This development paves the way for using laboratory strains of *E. coli* for production of therapeutically relevant glycoproteins.

4.5. METABOLIC REGULATION

Metabolic regulation is the heart of any living cell. Regulation takes place principally at the genetic level and at the cellular level (principally, control of enzyme activity and through cell surface receptors). Let us first consider genetic-level changes, as these fit most closely with our discussion of transcription and translation.

4.5.1. Genetic-Level Control: Which Proteins Are Synthesized?

Recall that the formation of a protein requires transcription of a gene. Transcriptional control of protein synthesis is the most common control strategy used in bacteria. Control of protein synthesis in eucaryotes can be more complex, but the same basic concepts hold. In the simplest terms, the cell senses that it has too much or too little of a particular protein and responds by increasing or decreasing the rate of transcription of that gene. One form of regulation is *feedback repression*; in this case, the end product of enzymatic activity accumulates and blocks transcription. Another form of regulation is *induction*; a metabolite (often a substrate for a pathway) accumulates and acts as an *inducer* of transcription. These concepts are summarized in Figures 4.9 and 4.10. In both cases, a repressor protein is required. The repressor can bind to the *operator* region and hinder RNA polymerase binding. For repression, a corepressor (typically the end product of the pathway) is required, and the repressor can block transcription only when bound to the corepressor. For induction, the inducer (typically a substrate for a reaction) will combine with the repressor, and the complex is inactive as a repressor.

Note in Figures 4.9 and 4.10 that several genes are under the control of a single promoter. A set of contiguous genes, encoding proteins with related functions, under the control of a single promoter-operator, is called an *operon*. The operon concept is central to understanding microbial regulation.

Control can be even more complex than indicated in Figures 4.9 and 4.10. The lactose (or lac) operon controls the synthesis of three proteins involved in lactose utilization as a carbon and energy source in *E. coli*. These genes are *lacZ*, *lacY*, and *lacA*. The *lacZ* gene encodes the LacZ protein, known as β-galactosidase (or lactase),

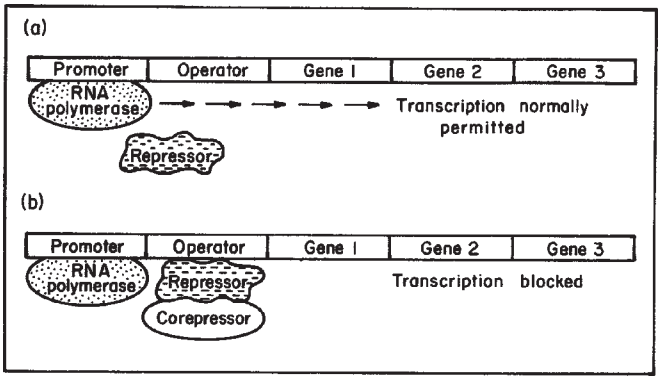


Figure 4.9. Process of enzyme repression. (a) Transcription of the operon occurs because the repressor is unable to bind to the operator. (b) After a corepressor (small molecule) binds to the repressor, the repressor now binds to the operator and blocks transcription. mRNA and the proteins it codes for are not made. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

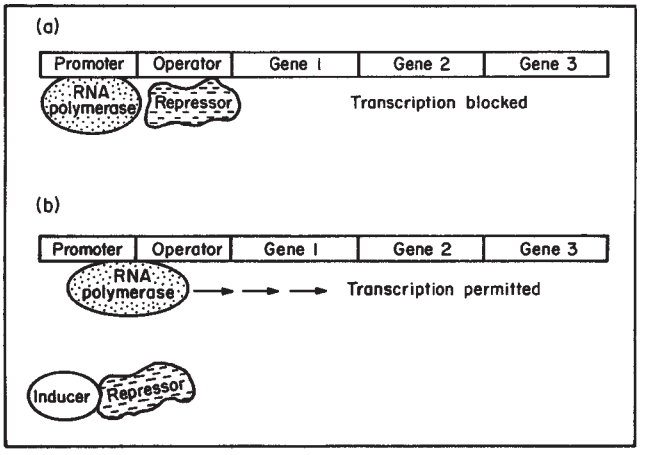


Figure 4.10. Process of enzyme induction. (a) A repressor protein binds to the operator region and blocks the action of RNA polymerase. (b) Inducer molecule binds to the repressor and inactivates it. Transcription by RNA polymerase occurs, and an mRNA for that operon is formed. In the lac operon, gene 1 is *lacZ*, gene 2 is *lacY*, and gene 3 is *lacA*. The repressor is made on a separate gene called lac i. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

which is an enzyme that cleaves lactose to glucose and galactose. The *lacY* gene encodes the LacY enzyme, a *permease* that acts to increase the rate of uptake of lactose into the cell. Lactose is modified in the cell to allolactose, which acts as the inducer. The conversion of lactose to allolactose is through a secondary activity of the enzyme β-galactosidase. Repression of transcription in uninduced cells is incomplete, and a low level *(basal level)* of proteins from the operon is made. Allolactose acts as indicated in Figure 4.10, but induction by allolactose is necessary but not sufficient for maximum transcription. Further regulation is exerted through *catabolite repression* (also called the *glucose effect*).

When *E. coli* senses the presence of a carbon–energy source preferred to lactose, it will not use the lactose until the preferred substrate (e.g., glucose) is fully consumed. This control mechanism is exercised through a protein called *CAP* (cyclic-AMP-activating protein). Cyclic AMP (cAMP) levels increase as the amount of energy available to the cell decreases. Thus, if glucose or a preferred substrate is depleted, the level of cAMP will increase. Under these conditions, cAMP will readily bind to CAP to form a complex that binds near the lac promoter. This complex greatly enhances RNA polymerase binding to the lac promoter. *Enhancer* regions exist in both procaryotes and eucaryotes.

The reader should now see how the cellular control strategy emerges. If the cell has an energetically favorable carbon–energy source available, it will not expend significant energy to create a pathway for utilization of a less favorable carbon–energy source. If, however, energy levels are low, then it seeks an alternative carbon–energy source. If and only if lactose is present will it activate the pathway necessary to utilize it.

Catabolite repression is a global response that affects more than lactose utilization. Furthermore, even for lactose, the glucose effect can work at levels other than genetic. The presence of glucose inhibits the uptake of lactose, even when an active uptake system exists. This is called *inducer exclusion*.

The role of global regulatory systems is still emerging. One concept is that of a *regulon*. Many noncontiguous gene products under the control of separate promoters can be coordinately expressed in a regulon. The best studied regulon is the *heat shock regulon*. The cell has a specific response to a sudden increase in temperature (or other

stresses that result in abnormal protein formation or membrane disruption), which results in the elevated synthesis of specific proteins. Evidence now exists that this regulon works by employing the induction of an alternative sigma factor, which leads to high levels of transcription from promoters that do not readily recognize the normal *E. coli* sigma factor. Examples of other regulons involve nitrogen and phosphate starvation, as well as a switch from aerobic to anaerobic conditions.

Although many genes are regulated, others are not. Unregulated genes are termed *constitutive*, which means that their gene products are made at a relatively constant rate irrespective of changes in growth conditions. Constitutive gene products are those that a cell expects to utilize under almost any condition; the enzymes involved in glycolysis are an example.

Example 4.1.

Diauxic growth is the sequential use of two different carbon–energy sources for cellular growth. Industrially, diauxic growth is observed when fermenting a mixture of sugars, such as might result from the hydrolysis of biomass. The classic example of diauxic growth is growth of *E. coli* on a glucose-lactose mixture. Observations on this system led to formulation of the operon hypothesis and the basis for a Nobel prize (for J. Monod and F. Jacob). Consider the plot in Figure 4.11, where the utilization of glucose and lactose and the growth of a culture are depicted for a batch culture (batch reactor). As we will discuss in more detail in Chapter 6, "How Cells Grow," the amount of biomass in a culture, X, accumulates exponentially. Note that at 2 hours after inoculation, cells are growing rapidly, glucose is being consumed, and lactose is not being utilized. At 7 hours, cell mass accumulation is zero. All the glucose has been consumed. At 10 hours, the culture is growing and lactose is being consumed, but the rate of growth (cell mass accumulation) is less than at 2 hours.

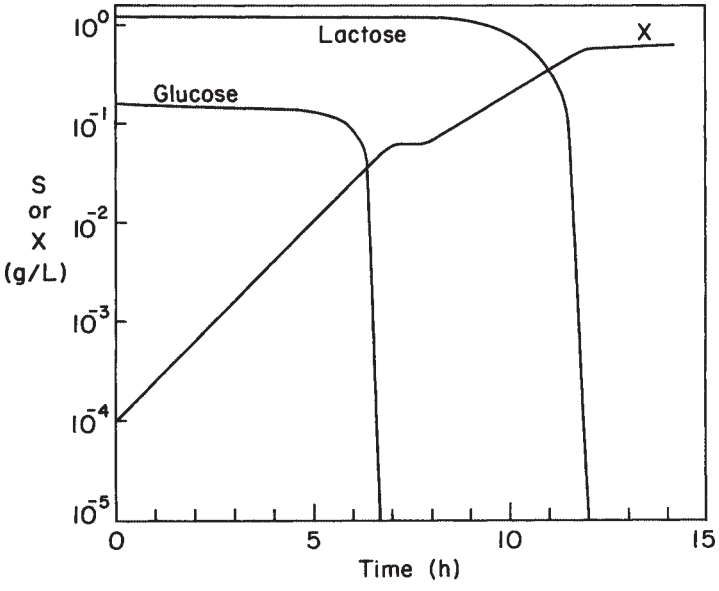


Figure 4.11. Diauxic growth curve for *Escherichia coli* on glucose and lactose.

Explain what is happening with intracellular control to account for the observations at 2, 7, and 10 hours. What rate of β-galactosidase formation would you expect to find in the culture at these times in comparison to the basal rate (which is <1% of the maximum rate)?

Solution

At 2 hours, the lac operon is fully induced, since lactose converted to allolactose combines with the *LacI* repressor protein, inactivating it. With the repressor protein deactivated, RNA polymerase is free to bind to the promoter but does so inefficiently. Glucose levels are still high, which results in higher levels of ATP and low levels of AMP and cAMP. Consequently, little cAMP–CAP complex is formed, and the interaction of the lac promoter with RNA polymerase is weak in the absence of cAMP–CAP. The rate of β-galactosidase formation would be slightly increased from the basal level—perhaps 5% of the maximal rate.

At 7 hours, the glucose has been fully consumed. The cell cannot generate energy, and the level of ATP decreases and cAMP increases. The cAMP–CAP complex level is high, which increases the efficiency of binding RNA polymerase to the lac promoter. This increased binding leads to increased transcription and translation. The rate of β-galactosidase formation is maximal and much higher than the basal rate or the 2-hour rate. However, the cells have not yet accumulated sufficient intracellular concentrations of β-galactosidase and LacY permease to allow efficient use of lactose and rapid growth.

At 10 hours, the intracellular content of proteins made from the lac operon is sufficiently high to allow maximal growth on lactose. However, this growth rate on lactose is slower than on glucose, since lactose utilization generates energy less efficiently. Consequently, the cAMP level remains higher than when the cell was growing on glucose. The level of production of β-galactosidase is thus higher than the basal or 2-hour level.

Irrespective of whether an enzyme is made from a regulated or constitutive gene, its activity in the cell is regulated. Let us now consider control at the enzymatic level.

4.5.2. Metabolic Pathway Control

In Chapter 3, "Enzymes," we learned how enzyme activity could be modulated by inhibitors or activators. Here we discuss how the activities of a group of enzymes (a pathway) can be controlled. The cell will attempt to make the most efficient use of its resources; the fermentation specialist tries to disrupt the cell's control strategy so as to cause the cell to overproduce the product of commercial interest. An understanding of how cells control their pathways is vital to the development of many bioprocesses.

First, consider the very simple case of a linear pathway making a product, P_1. Most often the first reaction in the pathway is inhibited by accumulation of the product (*feedback inhibition* or *end-product inhibition*). The enzyme for the entry of substrate into the pathway would be an allosteric enzyme (as described in Chapter 3), where the binding of the end product in a secondary site distorts the enzyme so as to render the primary active site ineffective. Thus, if the cell has a sufficient supply of P_1 (perhaps through an addition to the growth medium), it will deactivate the pathway so that the substrates normally used to make P_1 can be utilized elsewhere.

This simple concept can be extended to more complicated pathways with many branch points (see Figure 4.12). Assuming that P_1 and P_2 are both essential

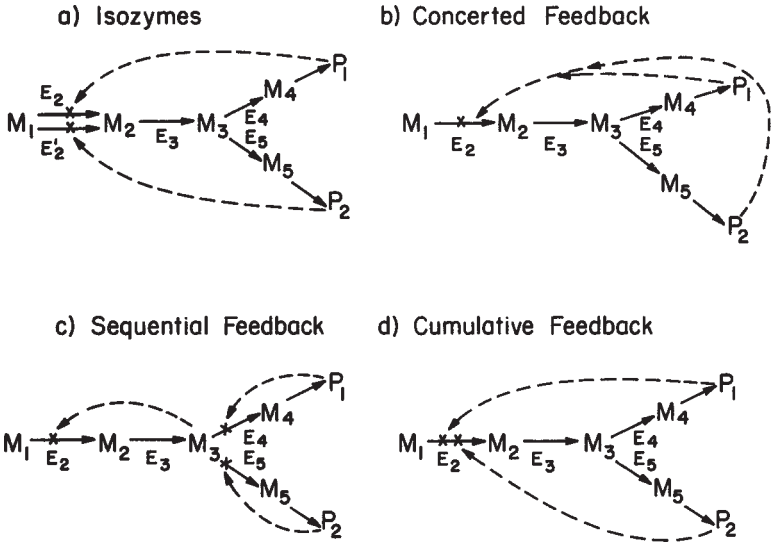


Figure 4.12. Examples of feedback control of branched pathways. P_1 and P_2 are the desired end products. M_1, M_2, . . . , M_j are intermediates, and E_j is the enzyme involved in converting metabolite M_{j-1} to M_j. Possible paths of inhibition are shown by dashed lines.

metabolites, the cell may use one of several strategies to ensure adequate levels of P_1 and P_2 with efficient utilization of substrates.

One strategy is the use of isofunctional enzymes *(isozymes)*. Two separate enzymes are made to carry out the same conversion, while each is sensitive to inhibition by a different end product. Thus, if P_1 is added in excess in the growth medium, it inhibits one of the isozymes (E_2), while the other enzyme (E'_2) is fully active. Sufficient activity remains through isozyme E'_2 to ensure adequate synthesis of P_2.

An alternative approach is *concerted feedback inhibition*. Here a single enzyme with two allosteric binding sites (for P_1 and P_2) controls entry into the pathway. A high level of either P_1 or P_2 is not sufficient by itself to inhibit enzyme E_2, while a high level of both P_1 and P_2 will result in full inhibition.

A third possibility is *sequential feedback inhibition*, by which an intermediate at the branch point can accumulate and act as the inhibitor of metabolic flux into the pathway. High levels of P_1 and P_2 inhibit enzymes E_4 and E_5, respectively. If either E_4 or E_5 is blocked, M_3 will accumulate, but not as rapidly as when both E_4 or E_5 are blocked. Thus, intermediate flux levels are allowed if either P_1 or P_2 is high, but the pathway is inactivated if both P_1 and P_2 are high.

Other effects are possible in more complex pathways. A single allosteric enzyme may have effector sites for several end products of a pathway; each effector causes only partial inhibition. Full inhibition is a cumulative effect, and such control is called *cumulative feedback inhibition* or *cooperative feedback inhibition*. In other cases, effectors from related pathways may also act as activators. Typically, this situation occurs when the product of one pathway was the substrate for another pathway. An example of control of a complex pathway (for aspartate) is described in Section 4.8.

Let us pause to consider the differences between feedback inhibition and repression. Inhibition occurs at the enzyme level and is rapid; repression occurs at the genetic level and is slower and more difficult to reverse. In bacteria where growth rates are high, unwanted enzymes are diluted out by growth. Would such a strategy work for higher cells in differentiated structures? Clearly not, since growth rates would be nearly zero. In higher cells (animals and plants), the control of enzyme levels is done primarily through the control of protein degradation rather than at the level of synthesis. Most of our discussion has centered on procaryotes; the extension of these concepts to higher organisms must be done carefully.

Another caution is that the control strategy that one organism adopts for a particular pathway may differ greatly from that adopted by even a closely related organism with an identical pathway. Even if an industrial organism is closely related to a well-studied organism, it is prudent to check whether the same regulatory strategy has been adopted by both organisms. Knowing the cellular regulatory strategy facilitates choosing optimal fermenter operating strategy, as well as guiding strain improvement programs.

4.6. HOW THE CELL SENSES ITS EXTRACELLULAR ENVIRONMENT

We have touched on some aspects of cellular metabolic regulation. A related form of regulation that we are just now beginning to appreciate has to do with the cell-surface receptors and how they sense the extracellular environment and cause the cell to respond to extracellular changes.

4.6.1. Transporting Small Molecules across Cellular Membranes

A cell must take nutrients from its extracellular environment if it is to grow or retain metabolic activity. As we discussed in Example 4.1, which nutrients enter the cell and at what rate can be important in regulating metabolic activity.

Molecules enter the cell through either energy-independent or energy-dependent mechanisms. The two primary examples of energy-independent uptake are *passive diffusion* and *facilitated diffusion*. Energy-dependent uptake mechanisms include *active transport* and *group translocation*.

In passive diffusion, molecules move down a concentration gradient (from high to low concentration) that is thermodynamically favorable, as follows:

$$J_A = K_p(C_{AE} - C_{AI}) \tag{4.1}$$

where J_A is the flux of species A across the membrane (mol/cm^2-s), K_p is the permeability (cm/s), C_{AE} is the extracellular concentration of species A (mol/cm^3), and C_{AI} is the intracellular concentration. The cytoplasmic membrane consists of a lipid core with perhaps very small pores. For charged or large molecules, the value K_p is very low and the flow of material across the membrane is negligible. The cellular uptake of water and oxygen appears to be due to passive diffusion. Furthermore, lipids or

other highly hydrophobic compounds have relatively high diffusivities (10^{-8} cm^2/s) in cellular membranes, and passive diffusion can be a mechanism of quantitative importance in their transport.

With facilitated transport, a carrier molecule (protein) can combine specifically and reversibly with the molecule of interest. The carrier protein is considered embedded in the membrane. By mechanisms that are not yet understood, the carrier protein, after binding the target molecule, undergoes conformational changes, which result in release of the molecule on the intracellular side of the membrane. The carrier can bind to the target molecule on the intracellular side of the membrane, resulting in the efflux or exit of the molecule from the cell. Thus, the net flux of a molecule depends on its concentration gradient.

The carrier protein effectively increases the solubility of the target molecule in the membrane. Because the binding of the molecule to the carrier protein is saturable (just as the active sites in the enzyme solution can be saturated), the flux rate of the target molecule into the cell depends on concentration differently than indicated in equation 4.1. Following is a simple equation to represent uptake by facilitated transport:

$$J_A = J_{A\ \mathrm{MAX}}\left[\frac{C_{AE}}{K_{MT}+C_{AE}} - \frac{C_{AI}}{K_{MT}+C_{AI}}\right] \tag{4.2}$$

where K_{MT} is related to the binding affinity of the substrate (mol/cm^3) and $J_{A\ \mathrm{MAX}}$ is the maximum flux rate of A (mol/cm^2-s). When $C_{AE} > C_{AI}$, the net flux will be into the cell. If $C_{AI} > C_{AE}$, there will be a net efflux of A from the cell. The transport is down a concentration gradient and is thermodynamically favorable. Facilitated transport of sugars and other low-molecular-weight organic compounds is common in eucaryotic cells but infrequent in procaryotes. However, the uptake of glycerol in enteric bacteria (such as *E. coli*) is a good example of facilitated transport.

Active transport is similar to facilitated transport in that proteins embedded in the cellular membrane are necessary components. The primary difference is that active transport occurs *against a concentration gradient*. The intracellular concentration of a molecule may be a hundred-fold (or more) greater than the extracellular concentration. The movement of a molecule up a concentration gradient is thermodynamically unfavorable and will not occur spontaneously; energy must be supplied. In active transport, several energy sources are possible: the electrostatic or pH gradients of the proton-motive force, and secondary gradients (e.g., of Na^+ or other ions) derived from the proton-motive force by other active transport systems and by the hydrolysis of ATP.

The *proton-motive force* results from the extrusion of hydrogen as protons. The respiratory system of cells (see Chapter 5, "Major Metabolic Pathways," section 5.3) is configured to ensure the formation of such gradients. Hydrogen atoms, removed from hydrogen carriers (most commonly nicotinamide adenine dinucleotide plus hydrogen [NADH]) on the inside of the membrane, are carried to the outside of the membrane, while the electrons removed from these hydrogen atoms return to the cytoplasmic side of the membrane. These electrons are passed to a final electron acceptor, such as O_2. When O_2 is reduced, it combines with H^+ from the cytoplasm, causing the net formation of OH^- on the inside. Because the flow of H^+ and

OH^- across the cellular membrane by passive diffusion is negligible, the concentration of chemical species cannot equilibrate. This process generates a pH gradient and an electrical potential across the cell. The inside of the cell is alkaline compared to the extracellular compartment. The cytoplasmic side of the membrane is electrically negative, and the outside is electrically positive. The proton-motive force is essential to the transport of many species across the membrane, and any defect in the cellular membrane that allows free movement of H^+ and OH^- across the cell boundary can collapse the proton-motive force and lead to cell death.

Some molecules are actively transported into the cell without coupling to the ion gradients generated by the proton-motive force. By a mechanism that is not fully understood, the hydrolysis of ATP to release phosphate bond energy is utilized directly in transport (e.g., the transport of maltose in *E. coli*).

For these mechanisms of active transport, irrespective of energy source, we can write an equation analogous to Michaelis–Menten kinetics to describe uptake:

$$J_A = J_{A\,\mathrm{MAX}} \frac{C_{AE}}{K_{MT} + C_{AE}} \tag{4.3}$$

The use of equation 4.3 is meaningful only when the cell is in an energy-sufficient state.

Another energy-dependent approach to the uptake of nutrients is group translocation. The key factor here is the chemical modification of the substrate during the process of transport. The best-studied system of this type is the *phosphotransferase system*. This system is important in the uptake of many sugars in bacteria. The biological system itself is complex, consisting of four separate phosphate-carrying proteins. The source of energy is phosphoenolpyruvate (PEP).

Effectively, the process can be represented as follows:

$$\mathrm{sugar}_{(\mathrm{extracellular})} + \mathrm{PEP}_{(\mathrm{intracellular})} \rightarrow \mathrm{sugar\text{-}P}_{(\mathrm{intracellular})} + \mathrm{pyruvate}_{(\mathrm{intracellular})} \tag{4.4}$$

By converting the sugar to the phosphorylated form, the sugar is trapped inside the cell. The asymmetric nature of the cellular membrane and this process make the process essentially irreversible. Because the phosphorylation of sugars is a key step in their metabolism, nutrient uptake of these compounds by group translocation is energetically preferable to active transport. In active transport, energy would be expended to move the unmodified substrate into the cell, and then further energy would be expended to phosphorylate it.

Certainly, the control of nutrient uptake is a critical cellular interface with its extracellular environment. In some cases, however, cells can sense their external environment without the direct uptake of nutrients.

4.6.2. Role of Cell Receptors in Metabolism and Cellular Differentiation

Almost all cells have receptors on their surfaces. These receptors can bind a chemical in the extracellular space. Such receptors are important in providing a cell with information about its environment. Receptors are particularly important in animals

in facilitating cell-to-cell communication. Animal cell-surface receptors are important in transducing signals for growth or cellular differentiation. These receptors are also prime targets for the development of therapeutic drugs. Many viruses mimic certain chemicals (e.g., a growth factor) and use cell-surface receptors as a means to entering a cell.

Simpler examples exist with bacteria. Some motile bacteria have been observed to move up concentration gradients for nutrients or down gradients of toxic compounds. This response is called *chemotaxis*. Some microbes also respond to gradients in oxygen *(aerotaxis)* or light *(phototaxis)*. Such tactic phenomena are only partially understood. However, the mechanism involves receptors binding to specific compounds, and this binding reaction results in changes in the direction of movement of the flagella. Motile cells move in a random-walk fashion; the binding of an attractant extends the length of time the cell moves on a "run" toward the attractant. Similarly, repellents decrease the length of runs up the concentration gradient. Chemotaxis is described in Figure 4.13. These drawings are two-dimensional projections of the three-dimensional movement.

Microbial communities can be highly structured (e.g., biofilms), and cell-to-cell communication is important in the physical structure of the biofilm. Cell-to-cell communication is also important in microbial phenomena such as bioluminescence, exoenzyme synthesis, and virulence factor production. Basically, these phenomena depend on local cell concentration. How do bacteria count? They produce a chemical known as *quorum sensing molecule*, whose accumulation is related to cell concentration. When the quorum sensing molecule reaches a critical concentration, it activates

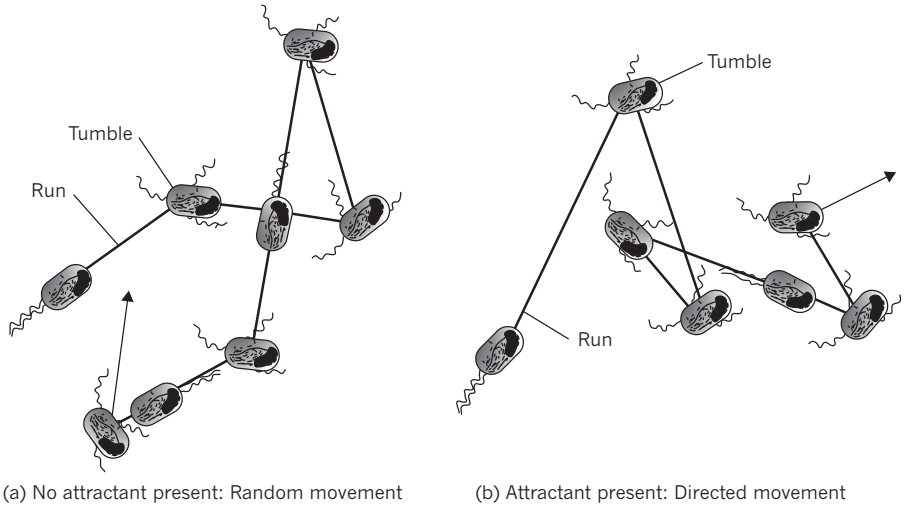


Figure 4.13. Chemotaxis in a peritrichously flagellated bacterium such as *Escherichia coli*. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

a response in all of the cells present. A typical quorum sensing molecule is an acylated homoserine lactone. The mechanism of quorum sensing depends on an intracellular receptor protein, while chemotaxis depends on surface receptor proteins.

With higher cells, the timing of events in cellular differentiation and development is associated with surface receptors. With higher organisms, these receptors are highly evolved. Some receptors respond to steroids *(steroid hormone receptors)*. Steroids do not act by themselves in cells, but rather the hormone-receptor complex interacts with specific gene loci to activate the transcription of a target gene.

A host of other animal receptors respond to a variety of small proteins that act as *hormones* or *growth factors*. These growth factors are normally required for the cell to initiate DNA synthesis and replication. Such factors are a critical component in the large-scale use of animal tissue cultures. Other cell-surface receptors are important in the attachment of cells to surfaces. Cell adhesion can lead to changes in cell morphology, which are often critical to animal cell growth and normal physiological function. The exact mechanism by which receptors work is only now starting to emerge. One possibility for growth factors that stimulate cell division is that binding of the growth factor to the receptor causes an alteration in the structure of the receptor. This altered structure possesses catalytic activity (e.g., tyrosine kinase activity), which begins a cascade of reactions leading to cellular division. Surface receptors are continuously internalized, complexes degraded, and receptors recycled to supplement newly formed receptors. Thus, the ability of cells to respond to changes in environmental signals is continuously renewed. Such receptors will be important in our later discussions on animal cell culture in Chapter 12, "Bioprocess Considerations in Using Animal Cell Cultures."

4.7. SUMMARY

In this chapter, you learned some of the elementary concepts of how cells control their composition in response to an ever-changing environment. The essence of an organism resides in the chromosome as a linear sequence of nucleotides that form a language *(genetic code)* to describe the production of cellular components. The cell controls the storage and transmission of such information, using macromolecular templates. DNA is responsible for its own *replication* and is also a template for *transcription* of information into RNA species that serve both as machinery and template to *translate* genetic information into proteins. Proteins often must undergo posttranslational processing to perform their intended functions.

The cell controls both the amount and activity of proteins it produces. Many proteins are made on *regulated genes* (e.g., *repressible* or *inducible*), although other genes are *constitutive*. With regulated genes, small effector molecules alter the binding of regulatory proteins to specific sequences of nucleotides in the *operator* or *promoter* regions. Such regulatory proteins can block transcription or in other cases enhance it. A group of contiguous genes under the control of a single promoter-operator is called an *operon*. More global control through *regulons* is also evident. Some gene products are not regulated, and their synthesis is constitutive.

Once a protein is formed, its activity may be continuously modulated through *feedback inhibition*. A number of alternative strategies are employed by the cell to control the flux of material through a pathway. Another form of regulation occurs through the interaction of extracellular compounds with cell surface protein receptors.

4.8. APPENDIX: EXAMPLE REGULATION OF COMPLEX PATHWAYS[†]

In *E. coli*, the conversion of aspartic acid to aspartyl phosphate is mediated by three isofunctional enzymes, of which two (designated as a and c in Figure 4A.1) also mediate the conversion of aspartic acid semialdehyde to homoserine. Enzyme **a**, possessing both these functions, is feedback inhibited, and its synthesis is repressed by threonine. Enzyme **c**, which similarly possesses both functions, is inhibited and is repressed by lysine. The third, aspartokinase (enzyme **b**), is not subject to end-product inhibition, but its synthesis is repressed by methionine (Table 4A.1).

The enzymes of the L-lysine branch (**m–q**) and the L-methionine branch (**r–v**) catalyze reactions leading in each case to a single end product and are subject to specific repression by that end product (L-lysine and L-methionine, respectively).

The third branch of the aspartate pathway is subject to much more complex regulation, for two reasons. First, L-threonine, formed through this branch, is both a component of proteins and an intermediate in the synthesis of another amino acid, L-isoleucine. Second, four of the five enzymes (**e–h**) that catalyze L-isoleucine synthesis from L-threonine also catalyze analogous steps in the completely separate biosynthetic pathway by which L-valine is synthesized from pyruvic acid. The intermediate of this latter pathway, α-ketoisovaleric acid, is also a precursor of the amino acid L-leucine. These interrelationships are shown in Figure 4A.1(b).

L-isoleucine is an end-product inhibitor of the enzyme **d**, catalyzing the first step in its synthesis from L-threonine; this enzyme has no other biosynthetic role. L-valine is an end-product inhibitor of an enzyme (**e**) that has a dual metabolic role, since it catalyzes steps in both isoleucine and valine biosynthesis. In certain strains of *E. coli*, this enzyme is extremely sensitive to valine inhibition, with the result that exogenous valine prevents growth, an effect that can be reversed by the simultaneous provision of exogenous isoleucine. The L-leucine branch of the valine pathway is regulated by L-leucine, which is an end-product inhibitor of the first enzyme, **i**, specific to this branch. These interrelationships are shown in Figure 4A.1(b).

As shown in Table 4A.2, many of the enzymes that catalyze steps in the synthesis of L-isoleucine, L-valine, and L-leucine are subject to repression only by a mixture of the three end products, a phenomenon known as *multivalent repression*. However, the five enzymes specific to L-leucine synthesis are specifically repressed by this amino acid alone.

[†]With permission, from R. Y. Stanier and others, *The Microbial World*, 5th ed., Prentice Hall, Englewood Cliffs, NJ, 1986.

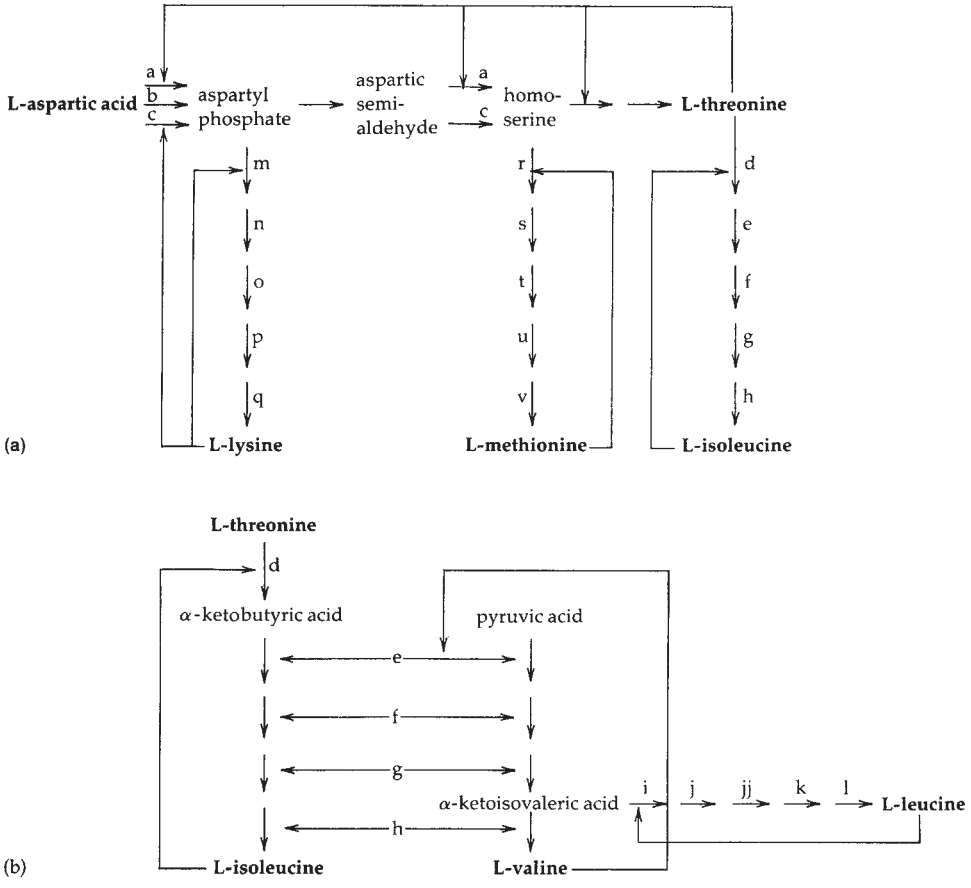


Figure 4A.1. A simplified diagram of the aspartate pathway in *E. coli*. Each solid arrow designates a reaction catalyzed by one enzyme. The biosynthetic products of the pathway (in boldface) are all allosteric inhibitors of one or more reactions. Careful study of this diagram reveals that with a single exception (the inhibition exerted by valine) the inhibition imposed by one amino acid does not cause starvation for a different amino acid. (a) The regulatory interrelationships of the L-lysine, L-methionine, and L-isoleucine branches of the pathway. (b) The regulatory interrelationships of the L-isoleucine, L-valine, and L-leucine branches.

TABLE 4A.1. Control of the First Step of the Aspartate Pathway

Enzyme	Corepressor	Allosteric Inhibitor
Aspartokinase I	Threonine and isoleucine	Threonine
Aspartokinase II	Methionine	No allosteric control
Aspartokinase III	Lysine	Lysine

TABLE 4A.2. Repressive Control of the Enzymes (See Figure 4A.1)

Enzymes	Corepressor
d, e, f, g, h	Isoleucine + valine + leucine
i, j, jj, k, l	Leucine

SUGGESTIONS FOR FURTHER READING

Alberts, B., D. Bray, K. Hopkin, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *Essential Cell Biology*, 4th ed., Garland Science, New York, 2013.

Black, J. G., *Microbiology: Principles and Applications*, 3d ed., Prentice Hall, Upper Saddle River, NJ, 1996.

Cain, J. A., N. Solis, and S. J. Cordwell, "Beyond Gene Expression: The Impact of Protein Post-translational Modifications in Bacteria," *J. Proteomics* 97: 268–286, 2014.

Chubukov, V., L. Gerosa, K. Kochanowski, and U. Sauer, "Coordination of Microbial Metabolism," *Nature Rev. Microbiol.* 12(5): 327–340, 2014.

Cook, P. R., "The Organization of Replication and Transcription," *Science* 284(5421): 1790–1795, 1999.

Elgard, L., M. Molinari, and A. Helenius, "Setting the Standards: Quality Control in the Secretory Pathway," *Science* 286(5446): 1882–1888, 1999.

Hardin, J., G. Bertoni, and L. J. Kleinsmith, *Becker's World of the Cell*, 9th ed., Pearson, New York, 2015.

Kelly, M. T., and T. R. Hoover, "Bacterial Enhancers Function at a Distance," *ASM News* 65: 484–489, 1999.

Kolter, R., and R. Losick, "One for All and All for One," *Science* 280(5361): 226–227, 1998. (This article is an overview related to a more detailed article on quorum sensing in biofilms; see Davies, D. G., and others, "The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Film," *Science* 280: 295–298, 1998).

Madigan, M. T., J. M. Martinko, K. S. Bender, D. H. Buckley, and D. A. Stahl, *Brock Biology of Microorganisms,* 14th ed., Pearson, Boston, 2015.

Moran, L. A., K. G. Scrimgeour, H. R. Horton, R. S. Ochs, and J. D. Rown, *Biochemistry,* 2d ed., Prentice Hall, Upper Saddle River, NJ, 1994.

Nothaft, H., and C. M. Szymanski, "Protein Glycosylation in Bacteria: Sweeter Than Ever," *Nature Rev. Microbiol.* 8(11): 765–778, 2010.

Stanier, R. Y., and others, *The Microbial World*, 5th ed., Prentice Hall, Englewood Cliffs, NJ, 1986.

von Hippel, P. H., "An Integrated Model of the Transcription Complex in Elongation, Termination, and Editing," *Science* 281(5377): 660–665, 1998.

Wadhams, G. H., and J. P. Armitage, "Making Sense of It All: Bacterial Chemotaxis," *Nature Rev. Mol. Cell Biol.* 5(12): 1024–1037, 2004.

Weerapana, E., and B. Imperiali, "Asparagine-Linked Protein Glycosylation: From Eukaryotic to Prokaryotic Systems," *Glycobiology* 16(6): 91R–101R, 2006.

PROBLEMS

4.1. Consider the aspartic acid pathway shown in Figure 4A.1. Assume you have been asked to develop a high-lysine-producing mutant. What strategy would you pursue? (That is, which steps would you modify by removing feedback inhibition, and what changes in medium composition would you make over a simple mineral salts–glucose base medium?)

4.2. Why is mRNA so unstable in most bacteria (half-life of about 1 min)? In many higher organisms, mRNA half-lives are much longer (>1 h). Why?

4.3. What would be the consequence of one base deletion at the beginning of the message for a protein?

4.4. How many ribosomes are actively synthesizing proteins at any instant in an *E. coli* cell growing with a 45-min doubling time? The birth size of *E. coli* is 1-μm diameter and 2-μm length. The water content is 75%. About 60% of the dry material is protein, and the rate of amino acid addition per ribosome is 20 amino acids per second. The average molecular weight of free amino acids in *E. coli* is 126.

4.5. Describe simple experiments to determine if the uptake of a nutrient is by passive diffusion, facilitated diffusion, active transport, or group translocation.

4.6. Consider the following mRNA nucleotide code:

CCG UAU CGA CUU GUA ACA ACG CGC

a. Deduce the corresponding sequence of amino acids.

b. What is the corresponding nucleotide sequence on the chromosome? This sequence codes for a part of insulin.

4.7. Consider the pathway in Figure 4A.1 for production of lysine, methionine, isoleucine, and threonine. You need to produce lysine. Describe a strategy for making large amounts of lysine. Your strategy can consist of adding various amino acids to the medium and choosing the mutant cells altered in regulation. Say you can identify up to two points of mutation (e.g., removal of feedback inhibition).

4.8. Suggest an experiment to determine if the uptake of a compound is by either facilitated or active transport.

4.9. What is catabolite repression, and how does it affect the level of protein expression from the lac operon?

4.10. Explain the difference between feedback inhibition and feedback repression.

4.11. You are asked to produce a human protein in *E. coli*. Because you have learned some of the differences in the way that procaryotes and eucaryotes make proteins, you worry about at least two factors that could complicate production of an authentic protein for human use.

a. What complication might you worry about if the human DNA encoding the protein were placed directly in *E. coli*?

b. Assume that the correct primary sequence of amino acids has been produced. What posttranslational steps do you worry about, and why?

4.12. Consider the process of *N*-linked glycosylation.

a. What organelles are required?

b. What is the residual sugar on a glycoprotein that has simple glycosylation?

c. If glycosylation is complete, what will be the final sugar on the glycoform?

d. Why may *N*-linked glycosylation be important?

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5

Major Metabolic Pathways

A major challenge in bioprocess development is to select an organism that can efficiently make a desired product. Before about 1980 only naturally occurring organisms were available. With the advent of genetic engineering, it is possible to remove and add genes to an organism to alter its metabolic functions in a predetermined manner *(metabolic engineering)*. Further, with mixed cultures of multiple species for food fermentations and waste treatment, metabolic cross-feeding may be critical to the behavior of such populations, particularly biodegradation of xenobiotics. In any case, the bioprocess developer must understand the metabolic capabilities of natural organisms either to use them directly or to know how to metabolically engineer them to make a desired, perhaps novel, product. Consequently, we turn our focus toward learning about some essential metabolic pathways.

Differences in microbial metabolism can be attributed partly to genetic differences and/or to differences in their responses to changes in their environment. Even the same species may produce different products when grown under different nutritional and environmental conditions. The control of metabolic pathways by nutritional and environmental regulation has become an important consideration in bioprocess engineering. For example, *Saccharomyces cerevisiae* (baker's yeast) produces ethanol when grown under anaerobic conditions. However, the major product is yeast cells (baker's yeast) when growth conditions are aerobic. Moreover, even under aerobic conditions, at high glucose concentrations some ethanol formation is observed, which

indicates metabolic regulation not only by oxygen but also by glucose. This effect is known as the *Crabtree effect*. Ethanol formation during baker's yeast fermentation may be reduced or eliminated with intermittent addition of glucose. The major metabolic pathways and products of various microorganisms are briefly covered in this chapter. Metabolic pathways are subgrouped as aerobic and anaerobic metabolism.

There are two key concepts in our discussion. *Catabolism* is the intracellular process of degrading a compound into smaller and simpler products (e.g., glucose to CO_2 and H_2O). Catabolism produces energy for the cell. *Anabolism* is involved in the synthesis of more complex compounds (e.g., glucose to glycogen) and requires energy.

5.1. BIOENERGETICS

Living cells require energy for biosynthesis, transport of nutrients, motility, and maintenance. This energy is obtained from the catabolism of carbon compounds, mainly carbohydrates. Carbohydrates are synthesized from CO_2 and H_2O in the presence of light by photosynthesis. The sun is the ultimate energy source for the life processes on earth.[†]

Metabolic reactions and resulting networks are fairly complicated and vary from one organism to another. However, these reactions can be classified in three major categories. A schematic diagram of these reactions is presented in Figure 5.1. The major categories are (I) degradation of nutrients, (II) biosynthesis of small molecules (amino acids, nucleotides), and (III) biosynthesis of large molecules. These reactions take place in the cell simultaneously. As a result of metabolic reactions, end products are formed and released from the cells. These end products (organic acids, amino acids, antibiotics) are often valuable products for human and animal consumption.

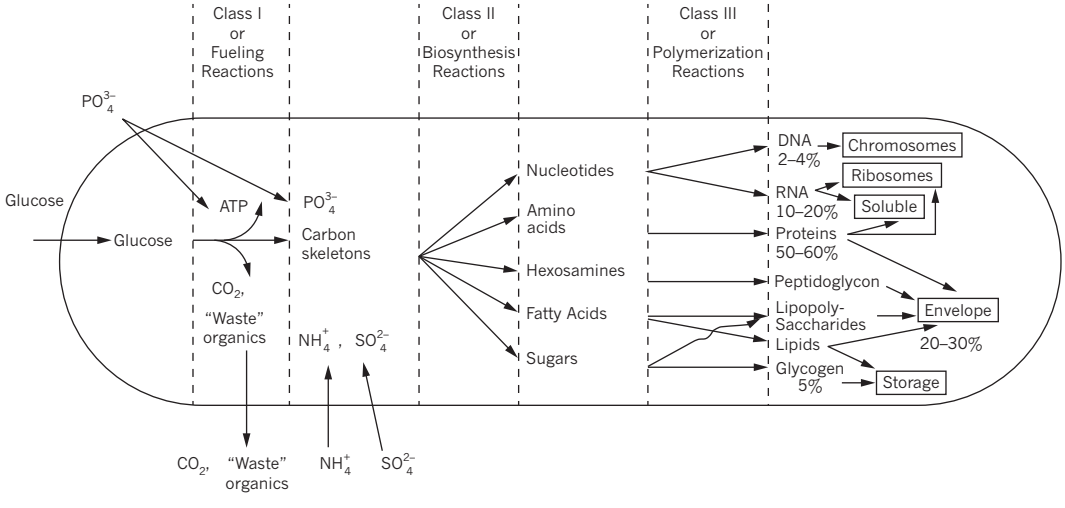


Figure 5.1. Schematic diagram of reactions in a bacterial cell.

[†]An exception is near some thermal vents at the bottom of the ocean, where nonphotosynthetic ecosystems exist independently of sunlight.

Energy in biological systems is primarily stored and transferred via adenosine triphosphate (ATP), which contains high-energy phosphate bonds.

The active form of ATP is complexed with Mg^{2+}. The standard free-energy change for the hydrolysis of ATP is 7.3 kcal/mol. The actual free-energy release in the cell may be substantially higher because the concentration of ATP is often much greater than that for adenosine diphosphate (ADP):

$$ATP + H_2O \rightleftarrows ADP + P_i, \quad \Delta G° = -7.3 \text{ kcal/mol} \tag{5.1}$$

Biological energy is stored in ATP by reversing this reaction to form ATP from ADP and Pi (inorganic phosphate). Similarly, ADP dissociates to release energy:

$$ADP + H_2O \rightleftarrows AMP + P_i, \quad \Delta G° = -7.3 \text{ kcal/mol} \tag{5.2}$$

Analog compounds of ATP, such as guanosine triphosphate (GTP), uridine triphosphate (UTP), and cytidine triphosphate (CTP), also store and transfer high-energy phosphate bonds but not to the extent of ATP. High-energy phosphate compounds produced during metabolism, such as phosphoenol pyruvate and 1,3-diphosphoglycerate, transfer their ~P group into ATP. Energy stored in ATP is later transferred to lower-energy phosphate compounds such as glucose-6-phosphate and glycerol-3-phosphate, as depicted in Figure 5.2.

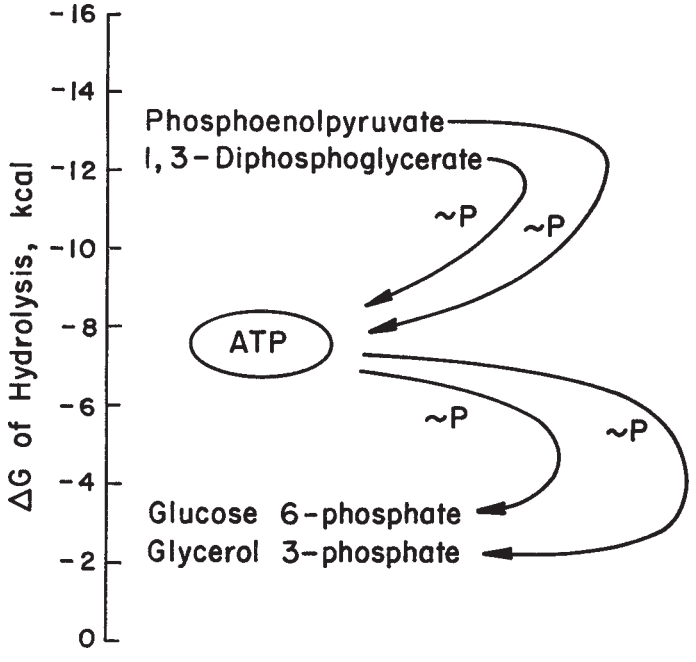


Figure 5.2. Transfer of biological energy from high-energy to low-energy compounds via adenosine triphosphate (ATP). Phosphoenolpyruvate and 1,3-diphosphoglycerate are high-energy phosphate compounds and act as phosphate donors. Low-energy compounds such as glucose 6-phosphate and glycerol 3-phosphate are phosphate acceptors.

Hydrogen atoms released in biological oxidation–reduction reactions are carried by nucleotide derivatives, especially by nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) (see Figure 5.3). The oxidation–reduction reaction described is readily reversible. Reduced form of NAD (NADH) can donate electrons to certain compounds and accept from others, depending on the oxidation–reduction potential of the compounds. NADH has two major functions in biological systems:

- *Reducing power:* NADH and reduced form of NADP (NADPH) supply hydrogen in biosynthetic reactions, such as CO_2 fixation by autotrophic organisms:

$$CO_2 + 4H \longrightarrow CH_2O + H_2O \tag{5.3}$$

- *ATP formation in respiratory metabolism:* The electrons (or H atoms) carried by NADH are transferred to oxygen via a series of intermediate compounds (respiratory chain). The energy released from this electron transport results in the formation of up to three ATP molecules.[†] ATP can be formed from the reducing power in NADH in the absence of oxygen if an alternative electron acceptor is available (e.g., NO_3^-).

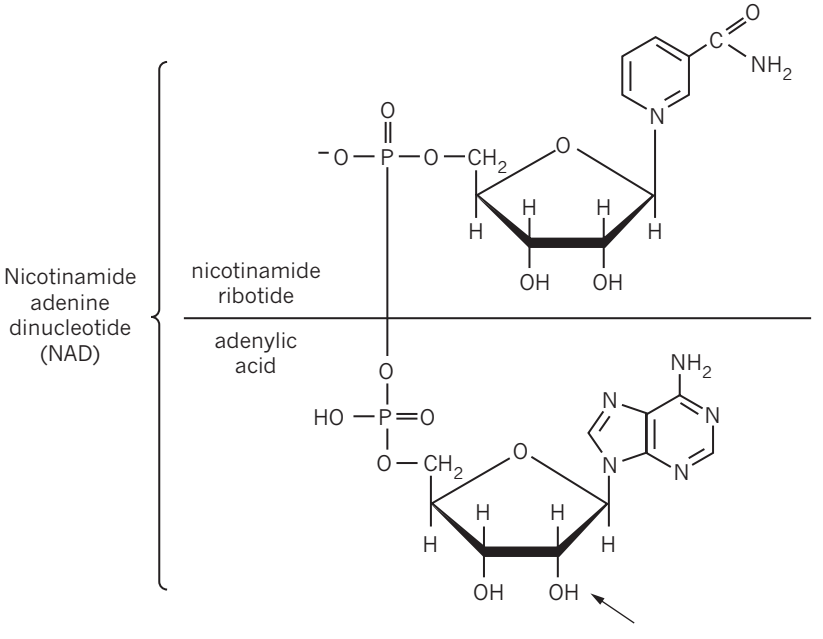


Figure 5.3. Structure of NAD (nicotinamide adenine dinucleotide). (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

[†]Some research suggests that the theoretical limit may be 2.5 ATP, rather than 3 ATP, for each NADH.

5.2. GLUCOSE METABOLISM: GLYCOLYSIS AND THE TCA CYCLE

Glucose is a major carbon and energy source for many organisms. Several different metabolic pathways are used by different organisms for the catabolism of glucose. The catabolism of glucose by *glycolysis*, or the *Embden–Meyerhof–Parnas* (EMP) pathway, is the primary pathway in many organisms. Other pathways, such as the *hexose monophosphate* (HMP) and *Entner–Doudoroff* (ED) pathways are covered later in this chapter.

Aerobic catabolism of organic compounds such as glucose may be considered in three phases:

- EMP pathway for fermentation of glucose to pyruvate.
- *Krebs*, *tricarboxylic acid (TCA)*, or *citric acid cycle* for conversion of pyruvate to CO_2 and NADH.
- Respiratory or *electron transport chain* for formation of ATP by transferring electrons from NADH to an electron acceptor.

The final phase, respiration, changes reducing power into a biologically useful energy form (ATP). *Respiration* may be aerobic or anaerobic, depending on the final electron acceptor. If oxygen is used as the final electron acceptor, the respiration is called *aerobic respiration*. When other electron acceptors, such as NO_3^-, SO_4^{3-}, Fe^{3+}, Cu^{2+}, and S^0, are used, the respiration is termed *anaerobic respiration*.

Glycolysis, or the EMP pathway, results in the breakdown of glucose to two pyruvate molecules. The enzymatic reaction sequence involved in glycolysis is presented in Figure 5.4. The first step in glycolysis is phosphorylation of glucose to glucose-6-phosphate (G-6P) by hexokinase. Phosphorylated glucose can be kept inside the cell. Glucose-6-phosphate is converted to fructose-6-phosphate (F-6P) by phosphoglucose isomerase, which is converted to fructose 1,6-diphosphate by phosphofructokinase. The first and the third reactions are the only two ATP-consuming reactions in glycolysis. They are irreversible. The breakdown of fructose-1,6-diphosphate into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA-3P) by aldolase is one of the key steps in glycolysis (e.g., C_6 to 2 C_3). DHAP and GA-3P are in equilibrium. As GA-3P is utilized in glycolysis, DHAP is continuously converted to GA-3P. GA-3P is first oxidized with the addition of inorganic phosphate (Pi) to 1,3-diphosphoglycerate (1,3-dP-GA) by GA-3P dehydrogenase. 1,3-dP-GA releases one phosphate group to form ATP from ADP and is converted to 3-phosphoglycerate (3P-GA) by 3-phosphoglycerate kinase. 3P-GA is further converted to 2-phosphoglycerate (2P-GA) by phosphoglyceromutase. Dehydration of 2P-GA to phosphoenolpyruvate (PEP) by enolase is the next step. PEP is further dephosphorylated to pyruvate (Pyr) by pyruvate kinase, with the formation of an ATP. Reactions after DHAP and GA-3P formation repeat twice during glycolysis.

The end-product pyruvate is a key metabolite in metabolism. Under anaerobic conditions, pyruvate may be converted to lactic acid, ethanol, or other products, such as acetone, butanol, and acetic acid. Anaerobic conversion of glucose to the aforementioned compounds used to be known as *fermentation*. However, that term today

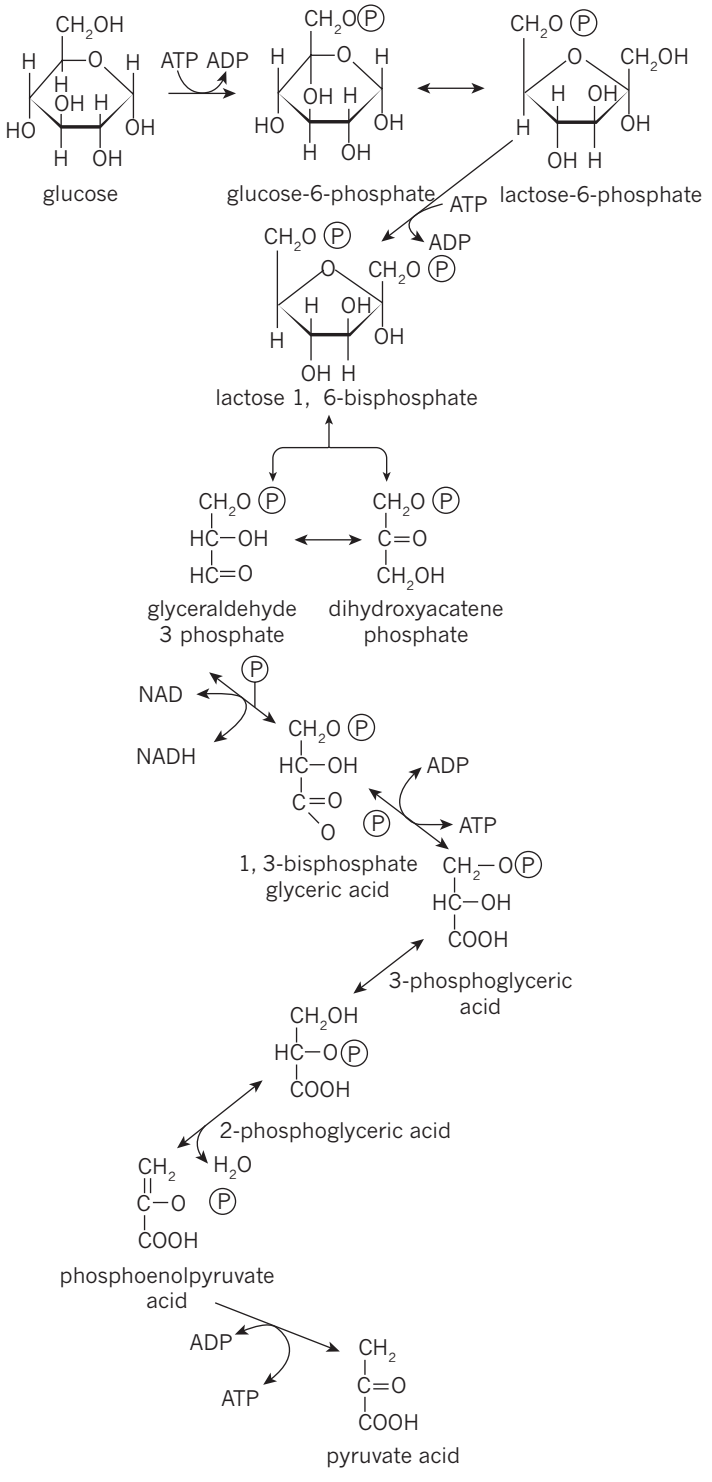


Figure 5.4. The Embden–Meyerhol (glycolytic) pathway of conversion of glucose to pyruvic acid. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

covers a whole range of enzymatic and microbial conversions. Under aerobic conditions, pyruvate is converted to CO_2 and NADH through the TCA cycle.

The overall reaction in glycolysis follows:

$$\text{glucose} + 2\,\text{ADP} + 2\,\text{NAD}^+ + 2\,\text{Pi} \longrightarrow 2\,\text{pyruvate} + 2\,\text{ATP} + 2(\text{NADH} + \text{H}^+) \quad (5.4)$$

The net ATP yield in glycolysis is 2 mol ATP/glucose under anaerobic conditions.

Pyruvate produced in the EMP pathway transfers its reducing power to NAD^+ via the Krebs cycle. Glycolysis takes place in cytoplasm, whereas the site for the Krebs cycle is the matrix of mitochondria in eucaryotes. In procaryotes, these reactions are associated with membrane-bound enzymes. Entry into the Krebs cycle is provided by the acylation of coenzyme-A by pyruvate:

$$\text{pyruvate} + \text{NAD}^+ + \text{CoA-SH} \xrightarrow{\text{pyruvate dehydrogenase}} \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (5.5)$$

Acetyl coenzyme A (CoA) is transferred through mitochondrial membrane at the expense of the conversion of the two NADHs produced in glycolysis to two FADH (flavin adenine dinucleotide). Acetyl-CoA is a key intermediate in the metabolism of amino acids and fatty acids.

The reactions involved in the TCA cycle are presented in Figure 5.5. Condensation of acetyl-CoA with oxaloacetic acid results in citric acid, which is further converted to isocitric acid and then to α-ketoglutaric acid (α-KGA) with a release of CO_2. α-KGA is decarboxylated and oxidized to succinic acid (SA), which is further oxidized to fumaric acid (FA). Hydration of fumaric acid to malic acid (MA) and oxidation of malic acid to oxaloacetic acid (OAA) are the two last steps of the TCA cycle. For each pyruvate molecule entering the cycle, three CO_2, four NADH + H^+, and one $FADH_2$ are produced. The succinate and α-ketoglutarate produced during the TCA cycle are used as precursors for the synthesis of certain amino acids. The reducing power (NADH + H^+ and $FADH_2$) produced is used either for biosynthetic pathways or for ATP generation through the electron transport chain.

The overall reaction of the TCA cycle follows:

$$\begin{aligned}\text{acetyl-CoA} + 3\,\text{NAD}^+ + \text{FAD} + \text{GDP} + \text{Pi} + 2\text{H}_2\text{O} \longrightarrow \\ \text{CoA} + 3(\text{NADH} + \text{H}^+) + \text{FADH}_2 + \text{GTP} + 2\,\text{CO}_2\end{aligned} \quad (5.6)$$

Note from Figure 5.5 that GTP can be converted easily into ATP; some descriptions of the TCA cycle show directly the conversion of ADP plus inorganic phosphate into ATP, as succinyl-CoA is converted to succinate.

The major roles of the TCA cycle are to provide electrons (NADH) for the electron transport chain and biosynthesis, to supply C skeletons for amino acid synthesis, and to generate energy.

Many of the intermediates in the TCA cycle are used extensively in biosynthesis. Removal of these intermediates "short-circuits" the TCA cycle. To maintain a functioning TCA cycle, the cell can fix CO_2 (heterotrophic CO_2 fixation). In some microbes, PEP can be combined with CO_2 to yield oxaloacetate. Three enzymes that can catalyze such a conversion have been found (PEP carboxylase, PEP carboxykinase,

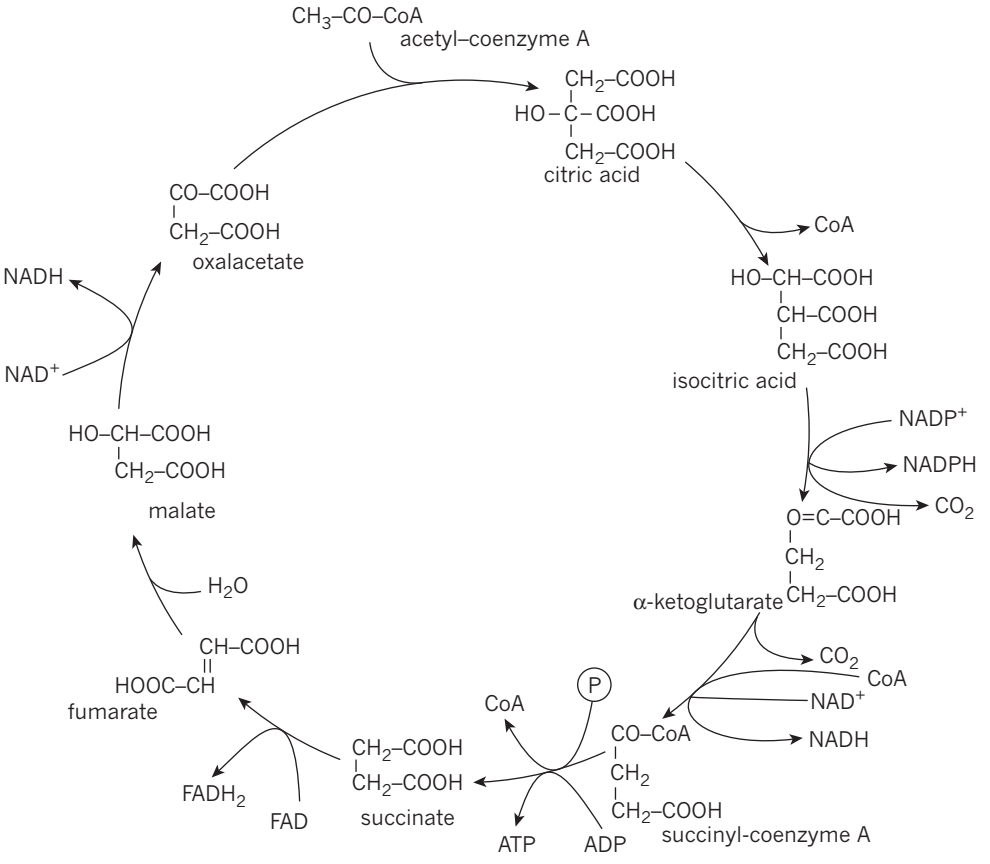


Figure 5.5. The tricarboxylic acid (TCA) cycle by which acetyl-CoA is oxidized. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

and PEP carboxytransphosphorylase). Pyruvate can be combined with CO_2 to yield oxaloacetate, with the expenditure of one ATP, using the enzyme pyruvate carboxylase. Malate dehydrogenase promotes the reversible formation of malate from pyruvate and CO_2, using the reducing power of one molecule of $NADH + H^+$.

Heterotrophic CO_2 fixation can be an important factor in culturing microbes. When a culture is initiated at low density (i.e., few cells per unit volume) with little accumulation of intracellular CO_2, or when a gas sparge rate into a fermentation tank is high, then growth may be limited by the rate of CO_2 fixation to maintain the TCA cycle.

5.3. RESPIRATION

The respiration reaction sequence is also known as the *electron transport chain*. The process of forming ATP from the electron transport chain is known as *oxidative phosphorylation*. Electrons carried by $NADH + H^+$ and $FADH_2$ are transferred to oxygen

via a series of electron carriers, and ATPs are formed. Three ATPs are formed from each NADH + H^+ and two ATPs for each $FADH_2$ in eucaryotes. The details of the respiratory (cytochrome) chain are depicted in Figure 5.6. The major role of the electron transport chain is to regenerate NADs for glycolysis and ATPs for biosynthesis. The term *P/O ratio* is used to indicate the number of phosphate bonds made (ADP + Pi → ATP) for each oxygen atom used as an electron acceptor (e.g., $\frac{1}{2}O_2$ + NADH + $H^+ \rightarrow H_2O$ + NAD^+).

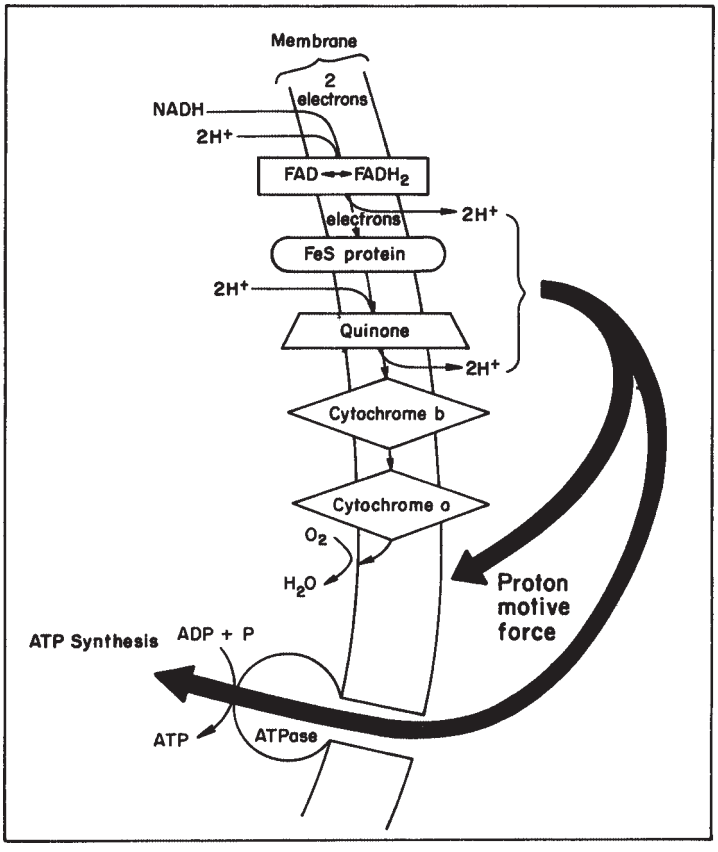


Figure 5.6. Electron transport and electron transport phosphorylation. *Top:* Oxidation of NADH and the flow of electrons through the electron transport system, leading to the transfer of protons (H^+) from the inside to the outside of the membrane. The tendency of protons to return to the inside is called *proton-motive force*. *Bottom:* ATP synthesis occurs as protons reenter the cell. An ATPase enzyme uses the proton-motive force for the synthesis of ATP. The proton-motive force is discussed in Section 4.6. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 3rd Ed., ©1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

TABLE 5.1. Summary of NADH, $FADH_2$, and ATP Formation during Aerobic Catabolism of Glucose

	NADH	$FADH_2$	ATP	Total ATP[a]
Glycolysis	2	–	2	6[b]
Oxidative decarboxylation of pyruvate	2	–	–	6
TCA cycle	6	2	2	24
Total	10	2	4	36 mol ATP

[a]Assumes a P/O ratio of 3 (i.e., three ATP phosphates made for each proton transported by the electron transport chain) for NADH and a P/O ratio of 2 for each $FADH_2$.

[b]The yield is 6 ATP rather than 8 because two NADH are converted to two $FADH_2$ for the transfer of acetyl-CoA into mitochondria.

Formation of $NADH + H^+$, $FADH_2$, and ATP at different stages of the aerobic catabolism of glucose is summarized in Table 5.1. The overall reaction (assuming 3 ATP/NADH) of aerobic glucose catabolism in eucaryotes is as follows:[†]

$$\text{glucose} + 36\ \text{Pi} + 36\ \text{ADP} + 6\ \text{O}_2 \longrightarrow 6\ \text{CO}_2 + 6\ \text{H}_2\text{O} + 36\ \text{ATP} \qquad (5.7)$$

The energy deposited in 36 mol of ATP is 263 kcal/mol glucose. The free-energy change in the direct oxidation of glucose is 686 kcal/mol glucose. Therefore, the energy efficiency of glycolysis is 38% under standard conditions. With the correction for nonstandard conditions, this efficiency is estimated to be greater than 60%, which is significantly higher than the efficiency of man-made machines. The remaining energy stored in glucose is dissipated as heat. However, in procaryotes the conversion of the reducing power to ATP is less efficient. The number of ATP generated from $NADH + H^+$ is usually ≤2, and only one ATP may be generated from $FADH_2$. Thus, in procaryotes a single glucose molecule will yield less than 24 ATP, and the P/O ratio is generally between 1 and 2.

5.4. CONTROL SITES IN AEROBIC GLUCOSE METABOLISM

Several enzymes involved in glycolysis are regulated by feedback inhibition. The major control site in glycolysis is the phosphorylation of fructose-6-phosphate by phosphofructokinase:

$$\text{fructose-6-phosphate} + \text{ATP} \longrightarrow \text{fructose-1,6-diphosphate} + \text{ADP} \qquad (5.8)$$

The enzyme phosphofructokinase is an allosteric enzyme activated by ADP and Pi but inactivated by ATP:

$$\text{phosphofructokinase (active)} \underset{\text{ADP}}{\overset{\text{ATP}}{\rightleftharpoons}} \text{phosphofructokinase (inactive)} \qquad (5.9)$$

[†]The yield is about 30 ATP if we assume 2.5 ATP/NADH, which may be more representative of actual metabolism.

At high ATP/ADP ratios, this enzyme is inactivated, resulting in a reduced rate of glycolysis and reduced ATP synthesis.

The concentration of dissolved oxygen or oxygen partial pressure has a regulatory effect on rate of glycolysis, known as the *Pasteur effect*. The rate of glycolysis under anaerobic conditions is higher than that under aerobic conditions. In the presence of oxygen, ATP yield is high, since the TCA cycle and electron transport chain are operating. As a result of high levels of ATP, ADP and Pi become limiting, and phosphofructokinase becomes inhibited. Also, some enzymes of glycolysis with — SH groups are inhibited by high levels of oxygen. A high $NADH/NAD^+$ ratio also reduces the rate of glycolysis.

Certain enzymes of the Krebs cycle are also regulated by feedback inhibition. Pyruvate dehydrogenase is inhibited by ATP, NADH, and acetyl-CoA and activated by ADP, AMP, and NAD^+. Similarly, citrate synthase is inactivated by ATP and activated by ADP and AMP; succinyl CoA synthetase is inhibited by NAD^+. In general, high ATP/ADP and $NADH/NAD^+$ ratios reduce the processing rate of the TCA cycle.

Several steps in the electron transport chain are inhibited by cyanide, azide, carbon monoxide, and certain antibiotics, such as amytal. Such inhibitions are important because of their potential to alter cellular metabolism.

5.5. METABOLISM OF NITROGENOUS COMPOUNDS

Most of the organic nitrogen compounds have an oxidation level between carbohydrates and lipids. Consequently, organic nitrogenous compounds can be used as a nitrogen, carbon, and energy source. Proteins are hydrolyzed to peptides and further to amino acids by proteases. Amino acids are first converted to organic acids by deamination (removal of amino group). Deamination reaction may be oxidative, reductive, or dehydrative, depending on the enzyme systems involved. A typical oxidative deamination reaction can be represented as follows:

$$R-\underset{\displaystyle NH_2}{\underset{|}{CH}}-COOH + H_2O + NAD^+ \longrightarrow R-\overset{\displaystyle O}{\overset{\|}{C}}-COOH + NH_3 + NADH + H^+ \qquad (5.10)$$

Ammonia released from deamination is utilized in protein and nucleic acid synthesis as a nitrogen source, and organic acids can be further oxidized for energy production (ATP).

Transamination is another mechanism for conversion of amino acids to organic acids and other amino acids. The amino group is exchanged for the keto group of α-KGA. Following is a typical transamination reaction:

$$\text{glutamic acid} + \text{oxaloacetic acid} \longrightarrow \alpha\text{-ketoglutaric acid} + \text{aspartic acid} \qquad (5.11)$$

Nucleic acids can also be utilized by many organisms as a carbon, nitrogen, and energy source. The first step in nucleic acid utilization is enzymatic hydrolysis by

specific nucleases hydrolyzing RNA and DNA. Nucleases with different specificities hydrolyze different bonds in nucleic acid structure, producing ribose/deoxyribose, phosphoric acid, and purines/pyrimidines. Sugar molecules are metabolized by glycolysis and the TCA cycle, producing CO_2 and H_2O under aerobic conditions. Phosphoric acids are used in ATP, phospholipid, and nucleic acid synthesis.

Purines/pyrimidines are degraded into urea and acetic acid and then to ammonia and CO_2. For example, the hydrolysis of adenine and uracil can be represented as follows:

$$\text{adenine} \longrightarrow CO_2 + NH_3 + \text{acetic acid} + \text{urea} \longrightarrow 5\,NH_3 + 5\,CO_2 \qquad (5.12)$$

$$\text{uracil} \longrightarrow \text{alanine} + NH_3 + CO_2 \longrightarrow 2\,NH_3 + 4\,CO_2 \qquad (5.13)$$

5.6. NITROGEN FIXATION

Certain microorganisms fix atmospheric nitrogen to form ammonia under reductive or microaerophilic conditions. Organisms capable of fixing nitrogen under aerobic conditions include *Azotobacter*, *Azotomonas*, *Azotococcus*, and *Beijerinckia*. Nitrogen fixation is catalyzed by the enzyme *nitrogenase*, which is inhibited by oxygen. Typically, these aerobic organisms sequester nitrogenase in compartments that are protected from oxygen. Equation 5.14 summarizes the nitrogen fixation process:

$$N_2 + 6\,H^+ + 6\,e \longrightarrow 2\,NH_3 \qquad (5.14)$$

Azotobacter species present in soil provide ammonium for plants by fixing atmospheric nitrogen, and some form associations with plant roots. Some facultative anaerobes, such as *Bacillus*, *Klebsiella*, *Rhodopseudomonas*, and *Rhodospirillum*, fix nitrogen under strict anaerobic conditions, and strict anaerobes such as *Clostridia* can also fix nitrogen under anaerobic conditions. Certain cyanobacteria, such as *Anabaena* sp., fix nitrogen under aerobic conditions. The lichens are *associations* of cyanobacteria and fungi. *Cyanobacteria* provide nitrogen to fungi by fixing atmospheric nitrogen. *Rhizobium* sp. are heterotrophic organisms growing in the roots of leguminous plants. *Rhizobium* fix atmospheric nitrogen under low oxygen pressure and provide ammonium to plants. *Rhizobium* and *Azospirillum* are widely used for agricultural purposes and are bioprocess products.

5.7. METABOLISM OF HYDROCARBONS

The metabolism of aliphatic hydrocarbons is important in some bioprocesses and often critical in applications such as bioremediation. Such metabolism requires oxygen, and only few organisms (e.g., *Pseudomonas*, *Mycobacteria*, certain yeasts and molds) can metabolize hydrocarbons. The low solubility of hydrocarbons in water is a barrier to rapid metabolism.

The first step in metabolism of aliphatic hydrocarbons is oxygenation by oxygenases. Hydrocarbon molecules are converted to an alcohol by incorporation of oxygen into the end of the carbon skeleton. The alcohol molecule is further oxidized to aldehyde and then to an organic acid, which is further converted to acetyl-CoA, which is metabolized by the TCA cycle.

Oxidation of aromatic hydrocarbons takes place by the action of oxygenases and proceeds more slowly than those of aliphatic hydrocarbons. Catechol is the key intermediate in this oxidation sequence and can be further broken down ultimately to acetyl-CoA or TCA cycle intermediates. Following is a depiction of aerobic metabolism of benzene:

$$\text{benzene} \longrightarrow \text{catechol} \longrightarrow \textit{cis-cis}\text{-muconate} \longrightarrow \beta\text{-ketoadipate} \downarrow \text{acetyl-CoA} + \text{succinate} \tag{5.15}$$

Anaerobic metabolism of hydrocarbons is more difficult. Only a few organisms can metabolize hydrocarbons under anaerobic conditions. The C–C bonds are cleaved and saturated with hydrogen to yield methane.

5.8. BIODEGRADATION OF XENOBIOTICS

Xenobiotics encompass toxic aromatic compounds, including nitroaromatic compounds (NACs), halogenated aromatic compounds (HACs), and polyaromatic hydrocarbons (PAH). Herbicides; pesticides; insecticides used for agricultural activities; and synthetic chemicals such as plasticizers, dyes, pigments, solvents, and pharmaceuticals, which are toxic, refractory compounds, all are considered xenobiotics. Biodegradation of xenobiotics is especially important for soil bioremediation. Biodegradation mechanisms of xenobiotics vary depending on the type of bacteria and environmental conditions. Aerobic and anaerobic bacteria use different pathways for biodegradation of xenobiotics. Aerobic mechanisms are usually oxidative, leading to a TCA cycle. Anaerobic metabolism is reductive, leading to formation of amino or carboxylic groups followed by ring cleavage and methane formation. *Pseudomonas*, *Rhodococcus*, *Nocardia*, *Alcaligenes*, *Mycobacterium*, and *Arthrobacter* spp. are some of the aerobic bacteria capable of degrading xenobiotic compounds.

In aerobic degradation of nitroaromatics, the first step is removal of the attached nitro groups. Formation of hydroxyl groups by oxygenation, ring cleavage, and formation of carboxyl groups are the next steps in which the carboxylated organic compounds enter the TCA cycle for further degradation. As an example, the major steps involved in oxidative biodegradation of p-nitrophenol are as follows:

$$\begin{aligned} &\text{p}-\text{nitrophenol} \rightarrow \text{p}-\text{benzoquinone} \rightarrow \text{hydroquinone} \rightarrow \\ &\text{hydroxy}-\text{muconic acid} \rightarrow \beta-\text{ketoadipate} \rightarrow\rightarrow\rightarrow \text{TCA cycle} \end{aligned} \tag{5.16}$$

The first step is nitrite removal, followed by ketone, alcohol, and organic acid formation along with the ring cleavage.

Biodegradation of PAHs such as naphthalene takes place by carboxylation and ring cleavage. Carboxylated organics enter the TCA cycle for further degradation. Biodegradability of PAHs decreases with the increasing number of rings and the attached groups. Two major mechanisms are identified for biodegradation of halogenated aromatics:

- Halogen groups are removed as the first step in biodegradation by oxidative, reductive, or hydrolytic mechanisms.
- Cleavage of the aromatic ring takes place first, followed by dehalogenation from an aliphatic intermediate.

Cleavage of the halogen–carbon bond is the critical step in biodegradation of halogenated aromatics. The rate of biodegradation varies depending on the number, type, and the position of halogen groups on the aromatic ring. Biodegradation may take place under oxic (O_2 as the electron acceptor), anoxic (nitrate or sulfate as the electron acceptor), or anaerobic (no O_2) conditions. Monochlorinated and dichlorinated phenols are degraded by aerobic bacteria through chlorinated catechol. Usually, chlorophenols are oxidized to chlorocatechols by phenol monooxygenase and then degraded by a ring cleavage. Dechlorination takes place after the cleavage of the aromatic ring.

Biodegradation of xenobiotics may result in formation of even more toxic intermediates. Complete biodegradation (mineralization) of xenobiotics to CO_2, H_2O, and HCl (or HNO_3) may be accomplished by using a consortium of bacteria with different metabolic capabilities.

5.9. OVERVIEW OF BIOSYNTHESIS

The TCA cycle and glycolysis are critical catabolic pathways and also provide important precursors for the biosynthesis of amino acids, nucleic acids, lipids, and polysaccharides. Although many additional pathways exist, we describe only two more, one in the context of biosynthesis and the other under anaerobic metabolism.

The first is the *pentose–phosphate pathway* or *hexose–monophosphate (HMP) pathway* (see Figure 5.7). Although this pathway produces significant reducing power, which could be used, in principle, to supply energy to the cell, its primary role is to provide carbon skeletons for biosynthetic reactions and the reducing power necessary to support anabolism. Normally, NADPH is used in biosynthesis, whereas NADH is used in energy production. The HMP pathway provides an array of small, organic compounds with three, four, five, and seven carbon atoms. These compounds are particularly important for the synthesis of ribose, purines, coenzymes, and the aromatic amino acids. The GA-3P formed can be oxidized to yield energy through conversion to pyruvate and further oxidation of pyruvate in the TCA cycle.

A vital component of biosynthesis, which consumes a large amount of cellular building material, is the production of amino acids. Many amino acids are also important commercial products, and the alteration of pathways to induce overproduction (see Chapter 4, "How Cells Work") is critical to commercial success. The 20 amino

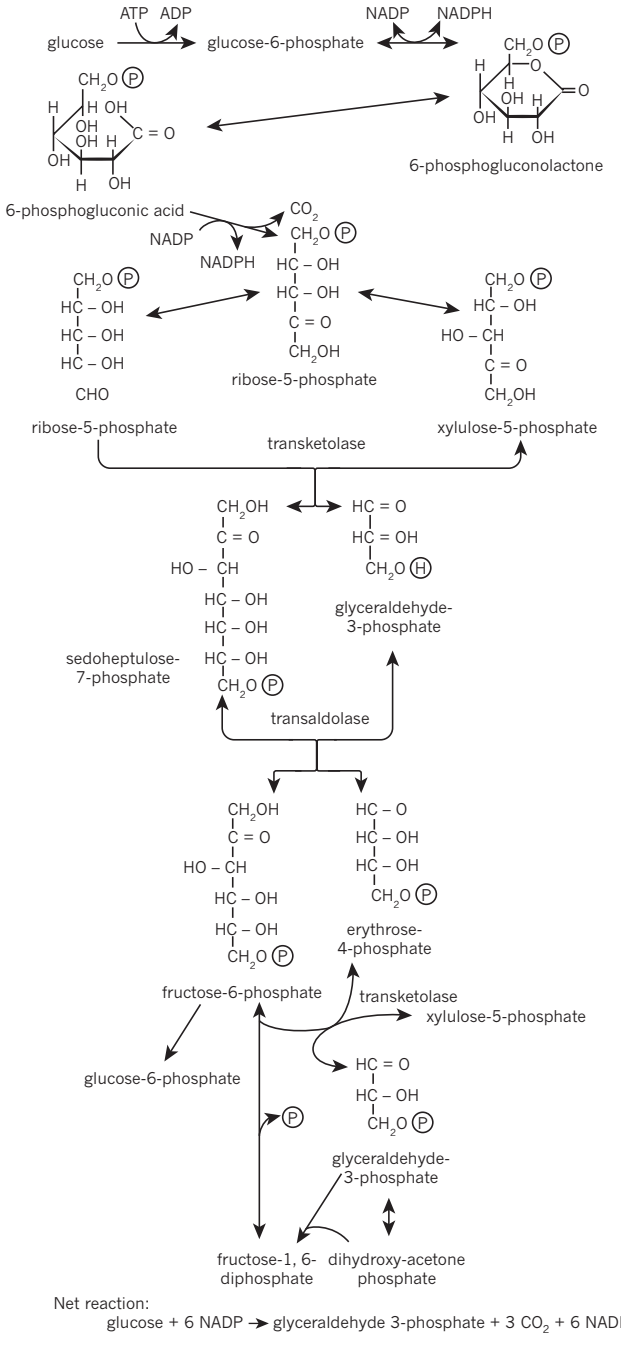


Figure 5.7. The pentose–phosphate pathway of glucose oxidation. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

acids can be grouped into various families. Figure 5.8 summarizes these families and the compounds from which they are derived. The amino acid histidine is not included in Figure 5.8. Its biosynthesis is fairly complicated and cannot be easily grouped with the others. However, ribose-5-phosphate from HMP is a key precursor in its synthesis.

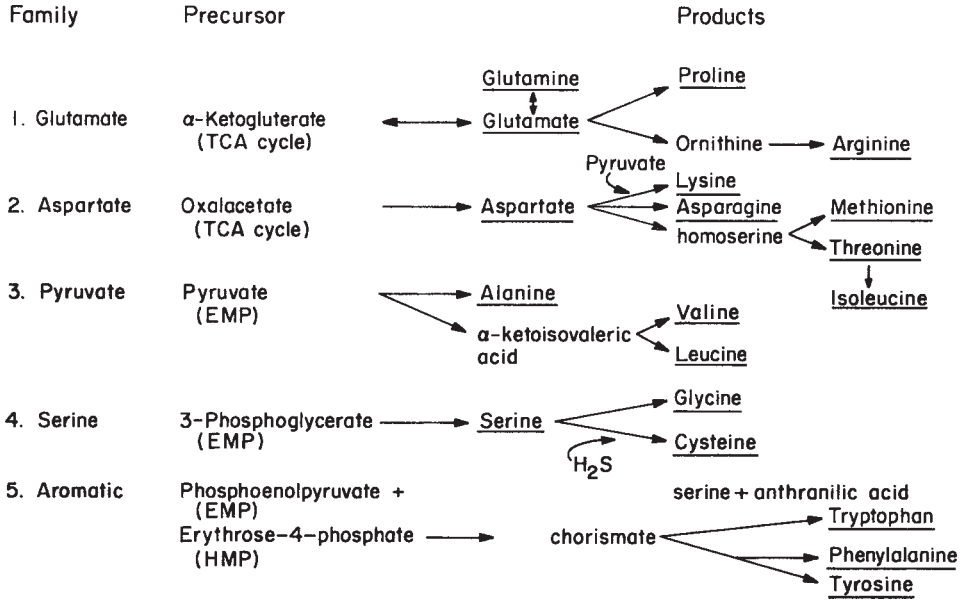


Figure 5.8. Summary of the amino acid families and their synthesis from intermediates in the Embden–Meyerhof–Parnas, tricarboxylic acid, and hexose monophosphate pathways. The amino acids are underlined.

In addition to synthesizing amino acids and nucleic acids, the cell must be able to synthesize lipids and polysaccharides. The key precursor is acetyl-CoA (see Figure 5.5 for the TCA cycle). Fatty acid synthesis consists of the stepwise buildup of acetyl-CoA. Also, CO_2 is an essential component in fatty acid biosynthesis. Acetyl-CoA and CO_2 produce malonyl-CoA, which is a three-carbon-containing intermediate in fatty acid synthesis. This requirement for CO_2 can lengthen the start-up phase (or lag phase; see Chapter 6, "How Cells Grow") for commercial fermentations if the system is not operated carefully. The requirement for CO_2 can be eliminated if the medium is formulated to supply key lipids, such as oleic acid.

The synthesis of most of the polysaccharides from glucose or other hexoses is readily accomplished in most organisms. However, if the carbon energy source has less than six carbons, special reactions need to be used. Essentially, the EMP pathway needs to be operated in reverse to produce glucose. The production of glucose is called *gluconeogenesis*. Since several of the key steps in the EMP pathway are irreversible, the cell must circumvent these irreversible reactions with energy-consuming reactions. Since pyruvate can be synthesized from a wide variety of pathways, it is the starting point. However, in glycolysis the final step to convert PEP into pyruvate is irreversible. In gluconeogenesis, PEP is produced from pyruvate as follows:

$$\text{pyruvate} + CO_2 + \text{ATP} + H_2O \longrightarrow \text{oxaloacetate} + \text{ADP} + \text{Pi} + 2H^+ \quad (5.17)$$

and

$$\text{oxaloacetate} + \text{ATP} \longrightarrow \text{PEP} + \text{ADP} + \text{CO}_2 \tag{5.18}$$

These two equations can be summarized as follows:

$$\text{pyruvate} + 2\,\text{ATP} + \text{H}_2\text{O} \longrightarrow \text{PEP} + 2\,\text{ADP} + 2\,\text{H}^+ \tag{5.19}$$

The reactions in glycolysis are reversible (under appropriate conditions) up to the formation of fructose-1,6-diphosphate. To complete glucogenesis, two enzymes (fructose-1,6-diphosphatase and glucose-6-phosphatase) not in the EMP pathway are required. Thus, an organism with these two enzymes and the ability to complete the reaction shown in equation 5.19 should be able to grow a wide variety of nonhexose carbon–energy sources.

So far, we have concentrated on aerobic metabolism. Many of the reactions we have described would be operable under anaerobic conditions. The primary feature of anaerobic metabolism is energy production in the absence of oxygen and in most cases the absence of other external electron acceptors. The cell must also balance its generation and consumption of reducing power. In the next section, we show how the pathways we have discussed can be adapted to the constraints of anaerobic metabolism.

5.10. OVERVIEW OF ANAEROBIC METABOLISM

The production of energy in the absence of oxygen can be accomplished by *anaerobic respiration* (see Section 5.1). The same pathways as employed in aerobic metabolism can be used; the primary difference is the use of an alternative electron acceptor. One excellent example is the use of nitrate NO_3^-, which can act as an electron acceptor. Its product, nitrous oxide (N_2O^-), is also an acceptor leading to the formation of dinitrogen (N_2). This process, known as *denitrification*, is important environmentally. Many advanced biological waste-treatment systems are operated to promote denitrification.

Many organisms grow without using the electron transport chain. The generation of energy without the electron transport chain is called *fermentation*. This definition is the exact and original meaning of the term fermentation, although currently it is often used in a broader context. Since no electron transport is used, the organic substrate must undergo a balanced series of oxidative and reductive reactions. This constraint requires that the rates of conversion of NAD^+ and $NADP^+$ to NADH and NADPH must equal the rates of conversion of NADH and NADPH to NAD^+ and $NADP^+$. For example, with the EMP pathway, the 2 mol of NAD reduced in this pathway in the production of pyruvate are reoxidized by oxidation of pyruvate to other products. Two prime examples are lactic acid and ethanol production (see Figure 5.9). Both lactic acid and ethanol are important commercial products from

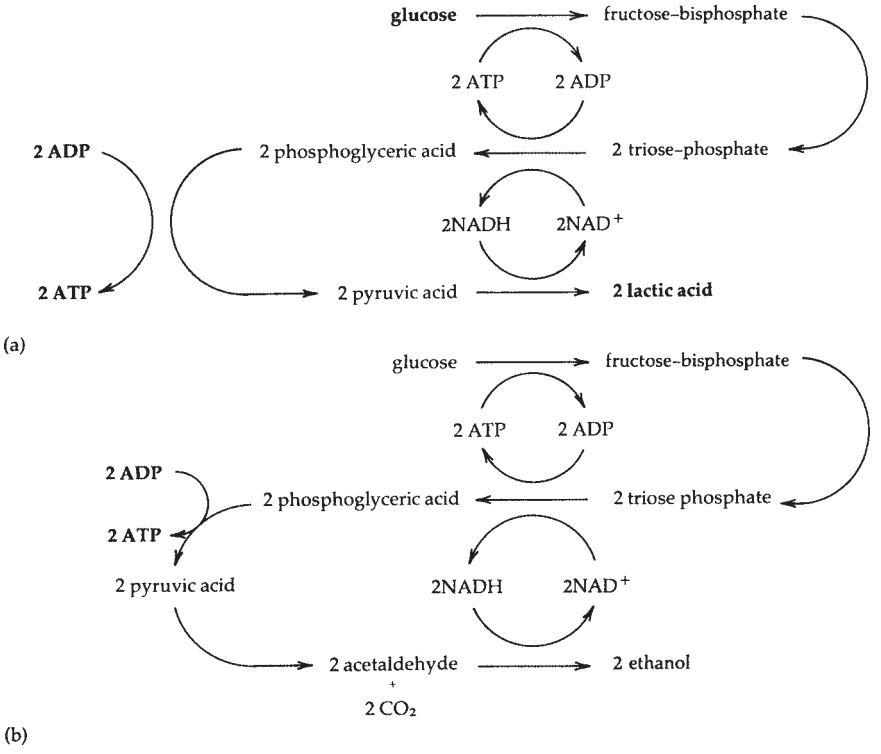


Figure 5.9. Comparison between (a) lactic acid and (b) alcoholic fermentations. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

bioprocesses. Some other commercially important products of fermentation are acetone–butanol, propionic acid, acetic acid (for vinegar), 2,3-butanediol, isopropanol, and glycerol.

Figure 5.10 summarizes common routes to some of these fermentation end products. Pyruvate is a key metabolite in these pathways. In most cases, pyruvate is formed through glycolysis. However, alternative pathways to form pyruvate exist. The most common of these is the *Entner–Doudoroff pathway* (see Figure 5.11). This pathway is important in the fermentation of glucose by the bacterium *Zymomonas*. The use of *Zymomonas* to convert glucose into ethanol is of potential commercial interest, because the use of the Entner–Doudoroff pathway produces only 1 mol of ATP per mole of glucose. This low energy yield forces more glucose into ethanol and less into cell mass, yielding a higher specific rate of ethanol formation as compared to ethanol fermentations by yeasts.

No one organism alone makes all the products indicated in Figure 5.10. Different organisms contain different combinations of pathways. Thus, it is important to screen a wide variety of organisms to select one that maximizes the yield of a desired product while minimizing the formation of by-products.

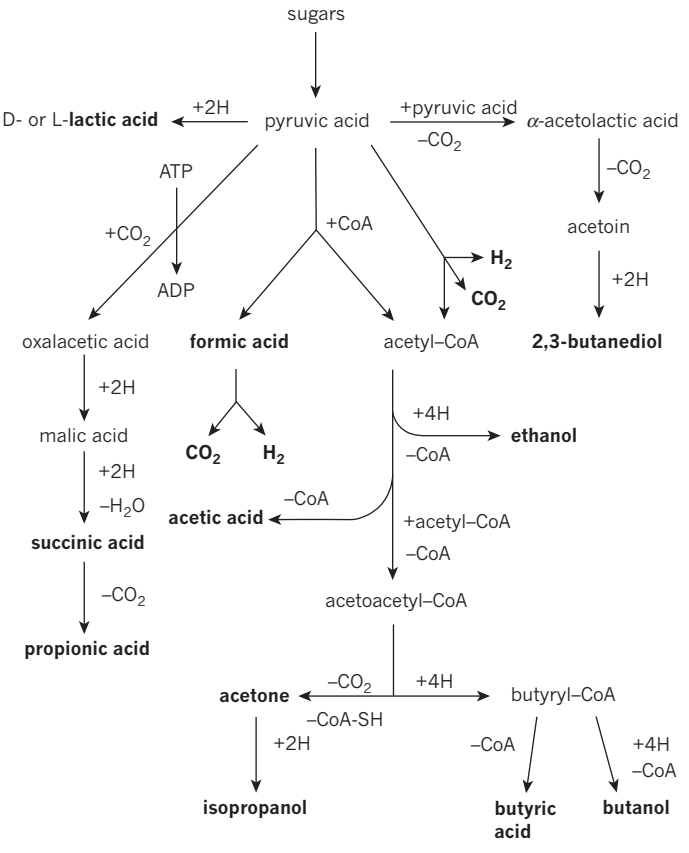


Figure 5.10. Derivations of some major end products of the bacterial fermentations of sugars from pyruvic acid. The end products are shown in boldface type. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

5.11. OVERVIEW OF AUTOTROPHIC METABOLISM

So far we have been concerned primarily with *heterotrophic growth* (e.g., organic molecules serve as carbon–energy sources). However, *autotrophs* obtain nearly all their carbon from CO_2. Most autotrophs (either photoautotrophs or chemoautotrophs) fix or capture CO_2 by a reaction catalyzed by the enzyme ribulose bisphosphate carboxylase, which converts ribulose-1,5-diphosphate plus CO_2 and H_2O into two molecules of glyceric acid-3-phosphate. This is the key step in the *Calvin cycle* (or *Calvin–Benson cycle*). This cycle is summarized in Figure 5.12 and provides the building blocks for autotrophic growth.

Energy for autotrophic growth can be supplied by light (photoautotroph) or chemicals (chemoautotroph). Here we consider the special case of photoautotrophic growth.

Photosynthesis takes place in two phases. The overall reaction follows:

$$6\,CO_2 + 6\,H_2O \xrightarrow{\text{light}} C_6H_{12}O_6 + 6O_2 \qquad (5.20)$$

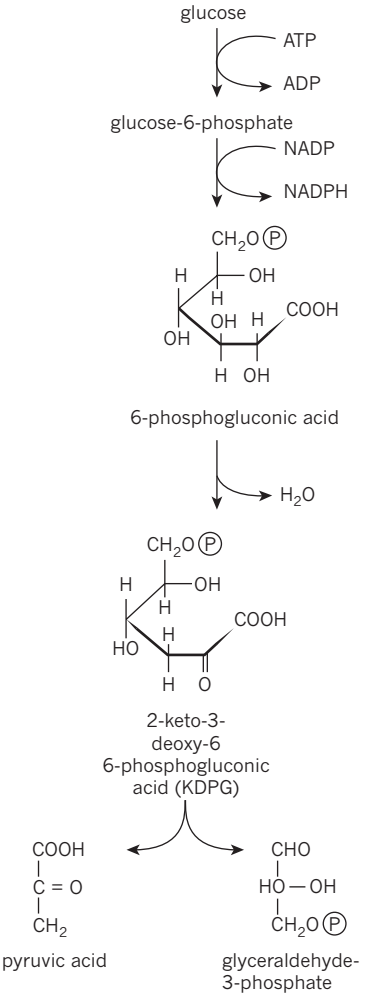


Figure 5.11. Entner–Doudoroff pathway. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

The first phase of photosynthesis is known as the *light phase*. Light energy is captured and converted into biochemical energy in the form of ATP and reducing agents, such as NADPH. In this process, hydrogen atoms are removed from water molecules and are used to reduce NADP, leaving behind molecular oxygen. Simultaneously, ADP is phosphorylated to ATP. Following is the light-phase reaction of photosynthesis:

$$H_2O + NADH^+ + P_i + ADP \xrightarrow{\text{light}} \text{oxygen} + NADPH + H^+ + ATP \qquad (5.21)$$

In the second phase, the energy-rich products of the first phase, NADPH and ATP, are used as the sources of energy to reduce the CO_2 to yield glucose (see Figure 5.12). Simultaneously, NADPH is reoxidized to $NADP^+$, and the ATP is converted into ADP and phosphate. This *dark phase* is described by the following reaction:

$$CO_2 + NADPH + H^+ + ATP \longrightarrow \tfrac{1}{6}\,\text{glucose} + NADP^+ + ADP + P_i \qquad (5.22)$$

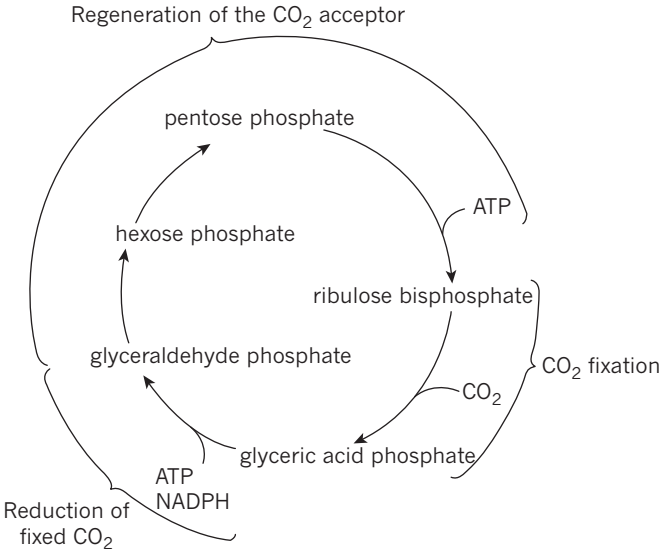


Figure 5.12. Schematic representation of the Calvin–Benson cycle, illustrating its three phases: CO_2 fixation, reduction of fixed CO_2, and regeneration of the CO_2 acceptor. (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

Both procaryotic and eucaryotic cells can fix CO_2 by photosynthesis. In procaryotes (e.g., cyanobacteria), photosynthesis takes place in stacked membranes, whereas in eucaryotes an organelle called the *chloroplast* conducts photosynthesis. Both systems contain chlorophyll to absorb light. Light absorption by chlorophyll molecules results in an electronic excitation. The excited chlorophyll molecule returns to the normal state by emitting light quanta in a process known as *fluorescence*. The excited chlorophyll donates an electron to a sequence of enzymes, and ATP is produced as the electrons travel through the chain. This ATP-generation process is called *photophosphorylation*. Electron carriers in this process are ferredoxin and several cytochromes.

The light phase of photosynthesis consists of two photosystems. Photosystem I (PS I) can be excited by light of wavelength shorter than 700 nm and generates NADPH. Photosystem II (PS II) requires light of wavelength shorter than 680 nm and splits H_2O into $\frac{1}{2}O_2 + 2H^+$. ATPs are formed as electrons flow from PS II to PS I.

5.12. SUMMARY

Cellular metabolism is concerned with two primary functions: *catabolism* and *anabolism*. Catabolism involves the degradation of a substrate to more highly oxidized end products for the purpose of generating energy and reducing power. Anabolism is the biosynthesis of more complex compounds from simpler compounds, usually with the consumption of energy and reducing power. The key compound to store and release energy is *adenosine triphosphate*, or *ATP*. Reducing power is stored by *nicotinamide adenine dinucleotide* (NADH) or *nicotinamide adenine dinucleotide phosphate* (NADPH).

Three of the most important pathways in the cell are the *Embden–Meyerhof–Parnas* (EMP) pathway, or *glycolysis*, which converts glucose into pyruvate; the *tricarboxylic acid* (TCA) *cycle*, which can oxidize pyruvate through acetyl-CoA into CO_2 and H_2O; and the *pentose–phosphate* or *hexose–monophosphate* (HMP) pathway, which converts glucose-6-phosphate into a variety of carbon skeletons (C_3, C_4, C_5, C_6, and C_7), with glyceraldehyde-3-phosphate as the end product. Although all three pathways can have catabolic and anabolic roles, the EMP pathway and TCA cycle are the primary means for energy generation, and HMP plays a key role in supplying carbon skeletons and reducing power for direct use in biosynthesis. In this chapter, we briefly considered the relationship of these pathways to amino acid, fatty acid, and polysaccharide biosynthesis. The conversion of pyruvate to glucose, necessary for polysaccharide biosynthesis when the carbon source does not have six carbons, is called *glucogenesis*.

Reducing power can be used to generate ATP through the *electron transport chain*. If oxygen is the final electron acceptor for this reducing power, the process is called *aerobic respiration*. If another electron acceptor is used in conjunction with the electron transport chain, then the process is called *anaerobic respiration*. Cells that obtain energy without using the electron transport chain use *fermentation*. *Substrate-level phosphorylation* supplies ATP. The end products of fermentative metabolism (e.g., ethanol, acetone–butanol, and lactic acid) are important commercially and are formed in response to the cell's need to balance consumption and the production of reducing power.

Autotrophic organisms use CO_2 as their carbon source and rely on the *Calvin* (or *Calvin–Benson*) cycle to incorporate (or fix) carbon from CO_2 into cellular material. Energy is obtained either through light *(photoautotroph)* or through oxidation of inorganic chemicals *(chemoautotroph)*. Figure 5.13 summarizes the major metabolic pathways and their interrelationship. Metabolic pathways can be altered with the use of modern biochemical tools such as genetic engineering. We discuss later in the book metabolic engineering, which combines a knowledge of the existing metabolic pathways with the modern tolls of genetic and epigenetic manipulations.

SUGGESTIONS FOR FURTHER READING

General Information on Metabolic Pathways

Alberts, B. D., D. Bray, K. Hopkin, A. D. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *Essential Cell Biology*, 4th ed., Garland Science, New York, 2013.

Black, J. G., and L. J. Black, *Microbiology: Principles and Applications*, 9th ed., Wiley, Hoboken, NJ, 2014.

Jain, R. K., M. Kapur, S. Labana, B. Lal, P. M. Sarma, D. Bhattacharya, and I. S. Thakur, Microbial Diversity: Application of Microorganisms for Biodegradation of Xenobiotics, *Curr. Sci.* 89(1): 101–112, 2005.

Madigan, M. T., J. M. Martinko, K. S. Binder, D. H. Buckley, and D. A. Stahl, *Brock Biology of Microorganisms*, 14th ed., Pearson Education, New York, 2015.

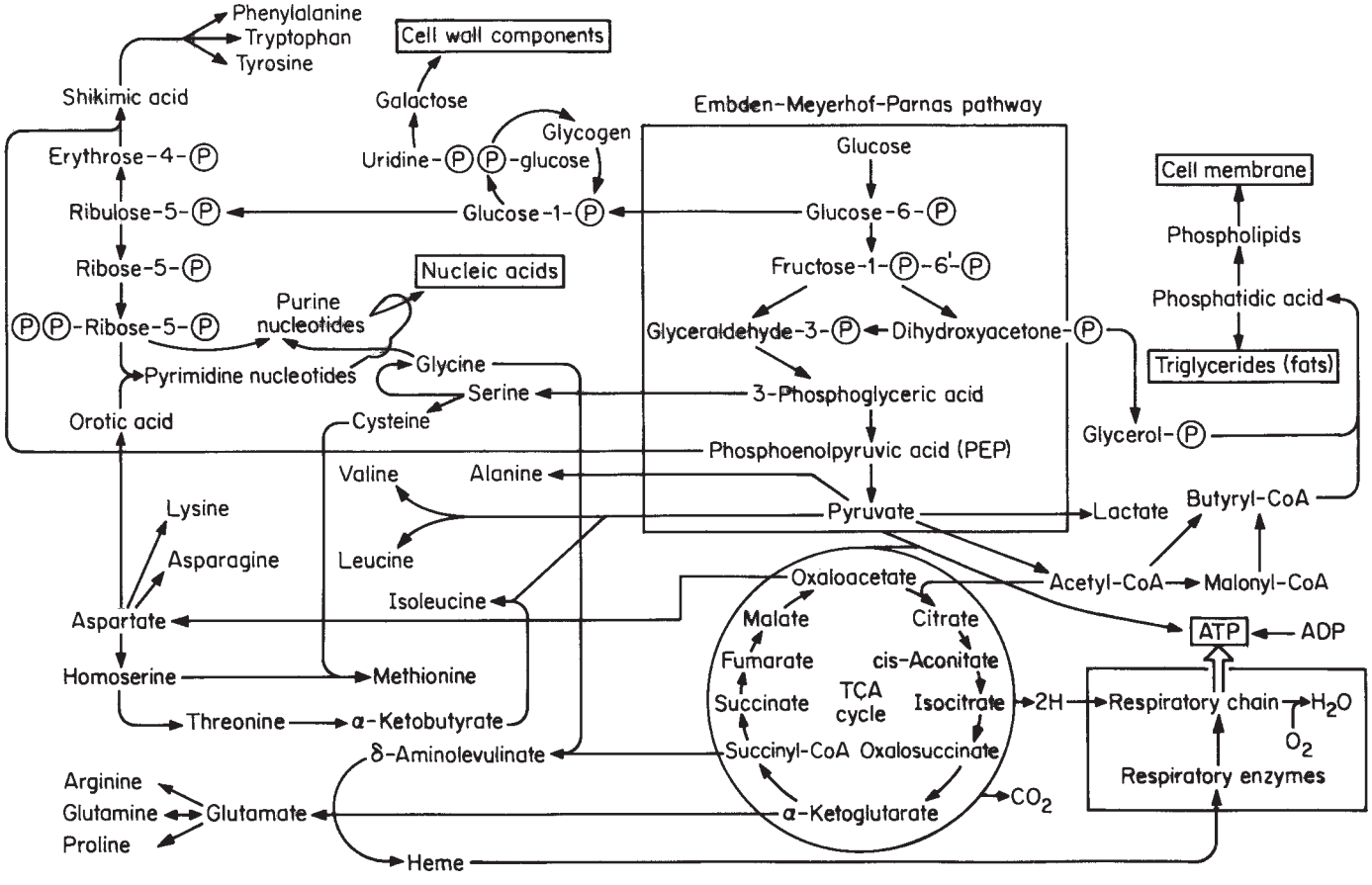


Figure 5.13. Interrelationship of major metabolic pathways in *Escherichia coli*. (Adapted from J. D. Watson, *Molecular Biology of the Gene*, 2d ed., W. A. Benjamin, Inc., New York, 1970.)

Specific Information on Interaction of Metabolism and Product Formation

CRUEGER, W., AND A. CRUEGER, *Biotechnology. A Textbook of Industrial Microbiology* (T. D. Brock, ed., English edition), 2d ed., Sinauer Associates, Sunderland, MA, 1990.

WAITES, M. J., N. L. MORGAN, J. S. ROCKEY, AND G. HIGTON, *Industrial Microbiology: An Introduction*, Blackwell Science, Malden, MA, 2001.

QUESTIONS

5.1. Cite the ATP-consuming and ATP-generating steps in glycolysis.

5.2. Briefly specify major functions of the TCA cycle.

5.3. What are the major control sites in glycolysis?

5.4. What is the Pasteur effect? Explain in terms of regulation of metabolic flow into a pathway.

5.5. How is glucose synthesized from pyruvate?

5.6. Explain the major functions of the dark and light reaction phases in photosynthesis.

5.7. What are the major differences between microbial and plant photosynthesis?

5.8. What is transamination? Provide an example.

5.9. Briefly explain the Crabtree effect.

5.10. What are the major steps in aerobic metabolism of hydrocarbons? What are the end products?

5.11. What is nitrogen fixation? Compare the aerobic and anaerobic nitrogen fixation mechanisms.

5.12. What are the major mechanisms for aerobic biodegradation of halogenated aromatics?

5.13. What are the major steps involved in degradation of nitroaromatics?

6

How Cells Grow

For microbes, growth is their most essential response to their physiochemical environment. Growth is a result of both replication and change in cell size. Microorganisms can grow under a variety of physical, chemical, and nutritional conditions. In a suitable nutrient medium, organisms extract nutrients from the medium and convert them into biological compounds. Part of these nutrients are used for energy production and part are used for biosynthesis and product formation. As a result of nutrient utilization, microbial mass increases with time and can be described simply by

$$\begin{aligned} \text{substrates} + \text{cells} &\longrightarrow \text{extracellular products} + \text{more cells} \\ \Sigma S + X &\longrightarrow \Sigma P + nX \end{aligned} \tag{6.1}$$

Microbial growth is a good example of an autocatalytic reaction. The rate of growth is directly related to cell concentration, and cellular reproduction is the normal outcome of this reaction.

The rate of microbial growth is characterized by the net *specific growth rate*, defined as

$$\mu_{net} \equiv \frac{1}{X}\frac{dX}{dt} \tag{6.2a}$$

$$\mu_{net} = \mu_g - k_d \tag{6.2b}$$

where X is cell mass concentration (g/l), t is time (h), and μ_{net} is net specific growth rate (h^{-1}). The net specific growth is the difference between a gross specific growth rate, $\mu_g(h^{-1})$, and the rate of loss of cell mass due to cell death or endogenous metabolism, $k_d(h^{-1})$.

Microbial growth can also be described in terms of cell number concentration, N, as well as X:

$$\mu_R \equiv \frac{1}{N}\frac{dN}{dt} \tag{6.3}$$

where μ_R is the net specific replication rate (h^{-1}). If we ignore cell death, k_d, then we use the symbol μ'_R, and in cases where cell death is unimportant, μ_R will equal μ'_R.

Similar to specific rate of growth, specific rates of substrate consumption (q_s) and product formation (q_p) can be defined as

$$q_s = -\frac{1}{X}\frac{dS}{dt} = -\frac{1}{X}\frac{\Delta S}{\Delta t} \tag{6.4}$$

$$q_p = \frac{1}{X}\frac{dP}{dt} = \frac{1}{X}\frac{\Delta P}{\Delta t} \tag{6.5}$$

where q_s and q_p are in units of g substrate/g biomass-h and g product/g biomass-h, respectively.

In this chapter, we discuss how the specific growth, substrate consumption, and product formation rates change with their environment. First, we consider growth in batch culture, where growth conditions are constantly changing.

6.1. BATCH GROWTH

Batch growth refers to culturing cells in a vessel with an initial charge of medium that is not altered by further nutrient addition or removal. This form of cultivation is simple and widely used both in the laboratory and industrially.

6.1.1. Quantifying Cell Concentration

The quantification of cell concentration in a culture medium is essential for the determination of the kinetics and stoichiometry of microbial growth. The methods used in the quantification of cell concentration can be classified in two categories: direct and indirect. In many cases, the direct methods are not feasible because of the presence of suspended solids or other interfering compounds in the medium. Either cell number or cell mass can be quantified depending on the type of information needed and the properties of the system. Cell mass concentration is often preferred to the measurement of cell number density when only one is measured, but the combination of the two measurements is often desirable.

6.1.1.1. Determining cell number density. A Petroff–Hausser counting chamber, or *hemocytometer*, is often used for direct cell counting. In this method, a calibrated grid is placed over the culture chamber, and the number of cells per grid square is counted using a microscope. To be statistically reliable, at least 20 grid squares must be counted and averaged. The culture medium should be clear and free of particles that could hide cells or be confused with cells. Stains can be used to distinguish between dead and live cells. This method is suitable for nonaggregated cultures. It is difficult to count molds under the microscope because of their mycelial nature.

Plates containing appropriate growth medium gelled with agar (Petri dishes) are used for counting viable cells. (The word *viable* used in this context means capable of reproduction.) Culture samples are diluted and spread on the agar surface and the plates are incubated. Colonies are counted on the agar surface following the incubation period. The results are expressed in terms of colony-forming units (CFU). If cells form aggregates, then a single colony may not be formed from a single cell. This method *(plate counts)* is more suitable for bacteria and yeasts and much less suitable for molds. A large number of colonies must be counted to yield a statistically reliable number. Growth media have to be selected carefully, since some media support growth better than others. The *viable count* may vary, depending on the composition of the growth medium. From a single cell, it may require 25 generations to form an easily observable colony. Unless the correct medium and culture conditions are chosen, some cells that are metabolically active may not form colonies.

In an alternative method, an agar–gel medium is placed in a small ring mounted on a microscope slide, and cells are spread on this miniature culture dish. After an incubation period of a few doubling times, the slide is examined with a microscope to count cells. This method has many of the same limitations as plate counts, but it is faster, and cells capable of only limited reproduction will be counted.

Another method is based on the relatively high electrical resistance of cells (Figure 6.1). Commercial *particle counters* employ two electrodes and an electrolyte solution. One electrode is placed in a tube containing an orifice. A vacuum is applied to the inner tube, which causes an electrolyte solution containing the cells to be sucked through the orifice. An electrical potential is applied across the electrodes. As cells pass through the orifice, the electrical resistance increases and causes pulses in electrical voltage. The number of pulses is a measure of the number of particles; particle concentration is known, since the counter is activated for a predetermined sample volume. The height of the pulse is a measure of cell size. Probes with various orifice sizes are used for different cell sizes. This method is suitable for discrete cells in a particulate-free medium and cannot be used for mycelial organisms.

The number of particles in solution can be determined from the measurement of scattered light intensity with the aid of a phototube (nephelometry). Light passes through the culture sample, and a phototube measures the light scattered by cells in the sample. The intensity of the scattered light is proportional to cell concentration. This method gives best results for dilute cell and particle suspensions.

6.1.1.2. Determining cell mass concentration. Determination of cellular *dry weight* is the most commonly used *direct method* for determining cell mass concentration and is applicable only for cells grown in solids-free medium. If noncellular

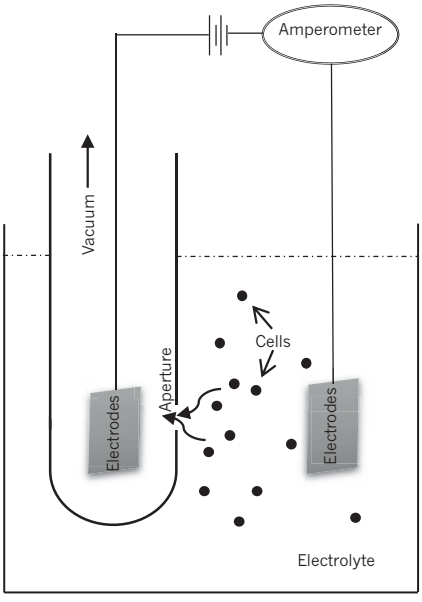


Figure 6.1. Schematic of a particle counter using the electrical resistance method to measure the cell number and cell size distribution.

solids, such as molasses solids, cellulose, or corn steep liquor, are present, the dry weight measurement will be inaccurate. Typically, samples of culture broth are centrifuged or filtered and washed with a buffer solution or water. The washed wet cell mass is then dried at 80°C for 24 hours; then dry cell weight is measured.

Packed cell volume is used to rapidly but roughly estimate the cell concentration in a fermentation broth (e.g., industrial antibiotic fermentations). Fermentation broth is centrifuged in a tapered graduated tube under standard conditions (rpm and time), and the volume of cells is measured.

Another rapid method is based on the absorption of light by suspended cells in sample culture media. The intensity of the transmitted light is measured using a spectrometer. Turbidity or *optical density* measurement of the culture medium provides a fast, inexpensive, and simple method of estimating cell density in the absence of other solids or light-absorbing compounds. The extent of light transmission in a sample chamber is a function of cell density and the thickness of the chamber. Light transmission is modulated by both absorption and scattering. Pigmented cells give different results than unpigmented ones. Background absorption by components in the medium must be considered. The medium should be essentially particle free. Proper procedure entails using a wavelength that minimizes absorption by medium components (600 to 700 nm wavelengths are often used), "blanking" against medium, and using a calibration curve. The calibration curve relates optical density (OD) to dry-weight measurements. Such calibration curves can become nonlinear at high OD values (>0.3) and depend to some extent on the physiological state of the cells.

In many fermentation processes, such as mold fermentations, direct methods cannot be used. In such cases, *indirect methods* are used, which are based mainly on

the measurement of substrate consumption and/or product formation during the course of growth.

Intracellular components of cells such as RNA, DNA, and protein can be measured as indirect measures of cell growth. During a batch growth cycle, the concentrations of these intracellular components change with time. Figure 6.2 depicts the variation of certain intracellular components with time during a batch growth cycle. Concentration of RNA (RNA/cell weight) varies significantly during a batch growth cycle; however, DNA and protein concentrations remain fairly constant. Therefore, in a complex medium, DNA concentration can be used as a measure of microbial growth. Cellular protein measurements can be achieved using different methods. Total amino acids, Biuret, Lowry (Folin reagent), and Kjeldahl nitrogen measurements can be used for this purpose. Total amino acids and the Lowry method are the most reliable. Recently, protein determination kits from several vendors have been developed for simple and rapid protein measurements. However, many media contain proteins as substrates, which limits the usefulness of this approach.

The intracellular adenosine triphosphate (ATP) concentration (mg ATP/mg cells) is approximately constant for a given organism. Thus, the ATP concentration in a fermentation broth can be used as a measure of biomass concentration. The method is based on luciferase activity, which catalyzes oxidation of luciferin at the expense of oxygen and ATP with the emission of light:

$$\text{luciferin} + O_2 + \text{ATP} \xrightarrow{\text{luciferase}} \text{light} \tag{6.6}$$

When oxygen and luciferin are in excess, total light emission is proportional to total ATP present in the sample. Photometers can be used to detect emitted light. Small concentrations of biomass can be measured by this method, since very low concentrations of ATP (10^{-12} g ATP/l) can be measured by photometers or scintillation counters. The ATP content of a typical bacterial cell is 1 mg ATP/g dry-weight cell, approximately.

Sometimes, nutrients used for cellular mass production can be measured to follow microbial growth. Nutrients used for product formation are not suitable for this purpose. Nitrate, phosphate, or sulfate measurements can be used. The utilization of a carbon source or oxygen uptake rate can be measured to monitor cellular growth when cell mass is the major product.

The products of cell metabolism can be used to monitor and quantify cellular growth. Certain products produced under anaerobic conditions, such as ethanol and lactic acid, can be related nearly stoichiometrically to microbial growth. Products must be either growth associated (ethanol) or mixed growth associated (lactic acid) to be correlated with microbial growth. For aerobic fermentations, CO_2 is a common product and can be related to microbial growth. In some cases, changes in the pH or acid–base addition to control pH can be used to monitor nutrient uptake and microbial growth. For example, the utilization of ammonium results in the release of hydrogen ions (H^+) and therefore a drop in pH. The amount of base added to neutralize the H^+ released is proportional to ammonium uptake and

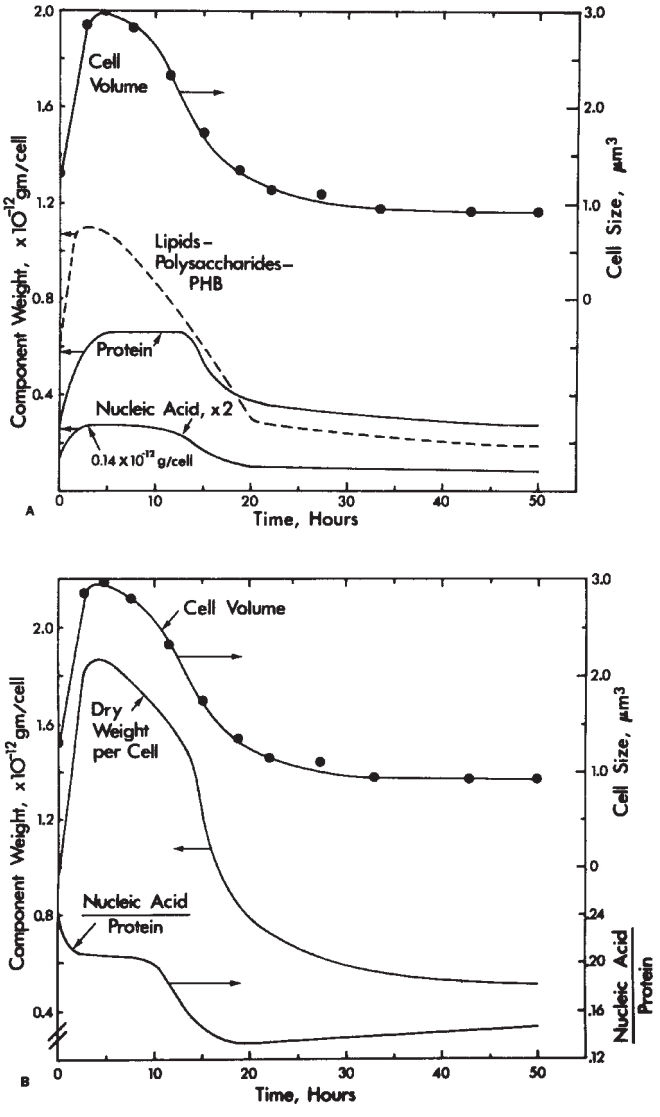


Figure 6.2. Time-dependent changes in cell composition and cell size for *Azotobacter vinelandii* in batch culture. (With permission, from M. L. Shuler and H. M. Tsuchiya, "Cell Size as an Indicator of Changes in Intracellular Composition of *Azotobacter vinelandii*," *Canadian Journal of Microbiology*, 1975, 21(6): 927–935, National Research Council of Canada, Ottawa. © Canadian Science Publishing or its licensors.)

growth. Similarly, when nitrate is used as the nitrogen source, hydrogen ions are removed from the medium, resulting in an increase in pH. In this case, the amount of acid added is proportional to nitrate uptake and therefore to microbial growth.

In some fermentation processes, as a result of mycelial growth or extracellular polysaccharide formation, the viscosity of the fermentation broth increases during

the course of fermentation. If the substrate is a biodegradable polymer, such as starch or cellulose, then the viscosity of the broth decreases with time as biohydrolysis continues. Changes in the viscosity of the fermentation broth can be correlated with the extent of microbial growth. Although polymeric broths are usually non-Newtonian, the apparent viscosity measured at a fixed rate can be used to estimate cell or product concentration.

6.1.2. Growth Patterns and Kinetics in Batch Culture

When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass. A typical batch growth curve includes the following phases: lag phase, logarithmic or exponential growth phase, deceleration phase, stationary phase, and death phase. Figure 6.3 describes a batch growth cycle.

The *lag phase* occurs immediately after inoculation and is a period of adaptation of cells to a new environment. Microorganisms reorganize their molecular constituents when they are transferred to a new medium. Depending on the composition of nutrients, new enzymes are synthesized, the synthesis of some other enzymes is repressed, and the internal machinery of cells is adapted to the new environmental

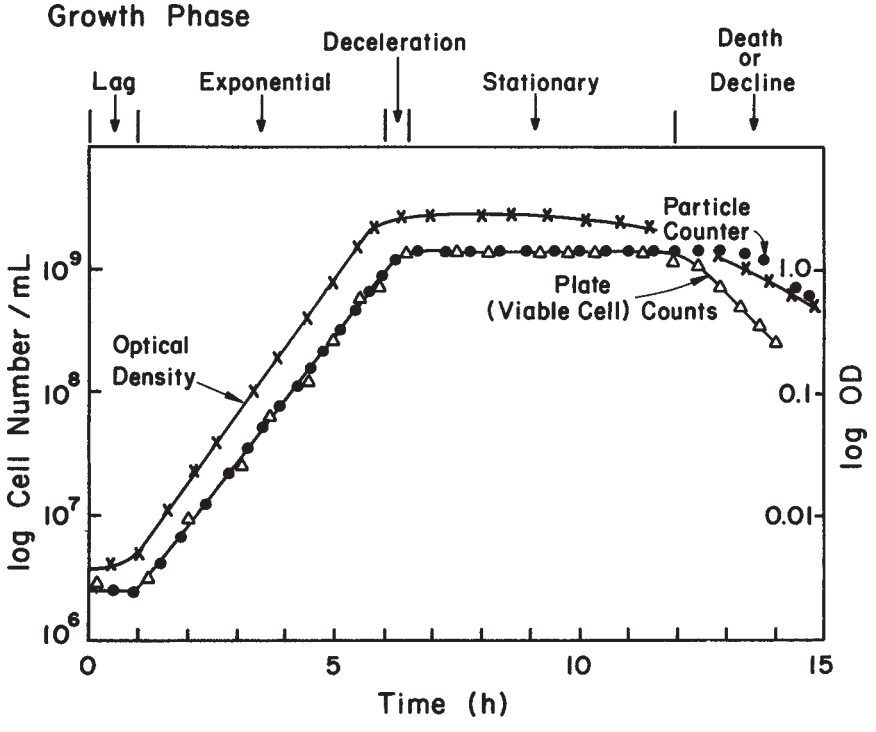


Figure 6.3. Typical growth curve for a bacterial population. Note that the phase of growth (shown here for cell number) depends on the parameter used to monitor growth.

conditions. These changes reflect the intracellular mechanisms for the regulation of the metabolic processes discussed in Chapter 4, "How Cells Work." During this phase, cell mass may increase a little, without an increase in cell number density. When the inoculum is small and has a low fraction of cells that are viable, there may be a pseudolag phase, which is a result not of adaptation but of small inoculum size or poor condition of the inoculum.

Low concentration of some nutrients and growth factors may also cause a long lag phase. For example, the lag phase of *Enterobacter aerogenes* (formerly *Aerobacter aerogenes*) grown in glucose and phosphate buffer medium increases as the concentration of Mg^{2+}, which is an activator of the enzyme phosphatase, is decreased. As another example, even heterotrophic cells require CO_2 fixation (to supplement intermediates removed from key energy-producing metabolic cycles during rapid biosynthesis), and excessive sparging can remove metabolically generated CO_2 too rapidly for cellular restructuring to be accomplished efficiently, particularly with a small inoculum.

The age of the inoculum culture has a strong effect on the length of lag phase. The age refers to how long a culture has been maintained in a batch culture. Usually, the lag period increases with the age of the inoculum. In some cases, there is an optimal inoculum age resulting in minimum lag period. To minimize the duration of the lag phase, cells should be adapted to the growth medium and conditions before inoculation, and cells should be young (or exponential phase cells) and active, and the inoculum size should be large (5% to 10% by volume). The nutrient medium may need to be optimized and certain growth factors included to minimize the lag phase. Figure 6.4 shows an example of variation of the lag phase with $MgSO_4$ concentration. Many commercial fermentation plants rely on batch culture; to obtain high productivity from a fixed plant size, the lag phase must be as short as possible.

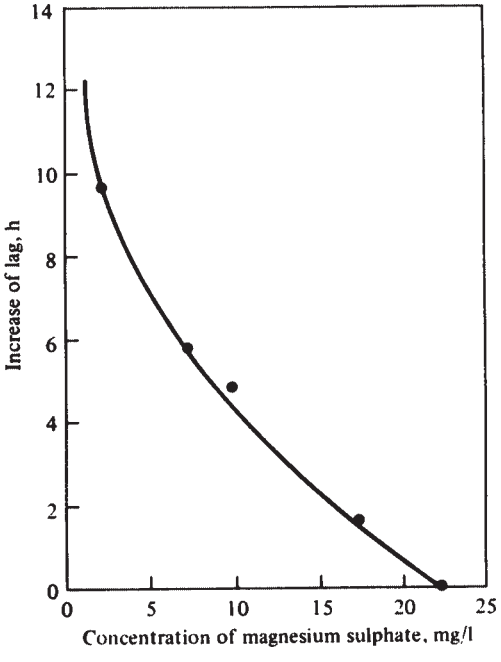


Figure 6.4. Influence of Mg^{2+} concentration on the lag phase in *E. aerogenes* culture. (With permission, from A. C. R. Dean, C. Hinshelwood, *Growth, Function, and Regulation in Bacterial Cells*. Oxford Press, London, 1966, p. 55.)

Multiple lag phases may be observed when the medium contains more than one carbon source. This phenomenon, known as *diauxic growth*, is caused by a shift in metabolic pathways in the middle of a growth cycle (see Example 4.1). After one carbon source is exhausted, the cells adapt their metabolic activities to utilize the second carbon source. The first carbon source is more readily utilizable than the second, and the presence of more readily available carbon source represses the synthesis of the enzymes required for the metabolism of the second substrate.

The *exponential growth phase* is also known as the *logarithmic growth phase*. In this phase, the cells have adjusted to their new environment. After this adaptation period, cells can multiply rapidly, and cell mass and cell number density increase exponentially with time. This is a period of *balanced growth* in which all components of a cell grow at the same rate. That is, the average composition of a single cell remains approximately constant during this phase of growth. During balanced growth, the net specific growth rate determined from either cell number or cell mass would be the same. Since the nutrient concentrations are large in this phase, the growth rate is independent of nutrient concentration. The exponential growth rate is first order:

$$\frac{dX}{dt} = \mu_{\text{net}} X, \quad X = X_0 \quad \text{at} \quad t = 0 \tag{6.7}$$

Integration of equation 6.7 yields

$$\ln \frac{X}{X_0} = \mu_{\text{net}}\, t, \quad \text{or} \quad X = X_0 e^{\mu_{\text{net}} t} \tag{6.8}$$

where X and X_0 are cell concentrations at time t and $t = 0$.

The time required to double the microbial mass is given by equation 6.9. The exponential growth is characterized by a straight line on a semilogarithm plot of Ln X versus time, where τ_d is the doubling time of cell mass:

$$\tau_d = \frac{\ln 2}{\mu_{\text{net}}} = \frac{0.693}{\mu_{\text{net}}} \tag{6.9}$$

Similarly, we can calculate a doubling time based on cell numbers and the net specific rate of replication:

$$\tau'_d = \frac{\ln 2}{\mu_R} \tag{6.10}$$

where τ'_d is the doubling time based on the replication rate. During balanced growth, τ_d will equal τ'_d, since the average cell composition and size will not change with time.

The *deceleration growth phase* follows the exponential phase. In this phase, growth decelerates due to either depletion of one or more essential nutrients or the accumulation of toxic by-products of growth. For a typical bacterial culture, these changes occur over a very short period of time. The rapidly changing environment results in *unbalanced growth*. During unbalanced growth, cell composition and size will change and τ_d and τ'_d will *not* be equal. In the exponential phase, the cellular metabolic control system is set to achieve maximum rates of reproduction. In the deceleration phase, the stresses induced by nutrient depletion or waste accumulation cause a restructuring of the cell to increase the prospects of cellular survival in a hostile environment. These observable changes are the result of the molecular mechanisms of repression and induction that we discussed in Chapter 4. Because of the rapidity of these changes, cell physiology under conditions of nutrient limitation is more easily studied in continuous culture, as discussed later in this chapter.

The *stationary phase* starts at the end of the deceleration phase, when the net growth rate is zero (no cell division) or when the growth rate is equal to the death rate. Even though the net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. *Primary metabolites* are growth-related products and *secondary metabolites* are nongrowth-related. In fact, the production of certain metabolites (e.g., antibiotics, some hormones) is enhanced during the stationary phase due to metabolite deregulation. During the course of the stationary phase, one or more of the following phenomena may take place:

- Total cell mass concentration may stay constant, but the number of viable cells may decrease.
- Cell lysis may occur and viable cell mass may drop. A second growth phase may occur and cells may grow on lysis products of lysed cells (cryptic growth).
- Cells may not be growing but may have active metabolism to produce secondary metabolites. Cellular regulation changes when concentrations of certain metabolites (carbon, nitrogen, phosphate) are low. Secondary metabolites are produced as a result of metabolite deregulation.

During the stationary phase, the cell catabolizes cellular reserves for new building blocks and for energy-producing monomers. This is called *endogenous metabolism*. The cell must always expend energy to maintain an energized membrane (i.e., proton-motive force) and transport of nutrients and for essential metabolic functions such as motility and repair of damage to cellular structures. This energy expenditure is called *maintenance energy*. The appropriate equation describing catabolism of cellular mass for maintenance energy or the loss of cell mass due to cell lysis during the stationary phase is

$$\left(\frac{dX}{dt}\right)_{end} = -k_d X \qquad \text{or} \qquad X = X_{so} e^{-k_d t} \tag{6.11}$$

where k_d is a first-order rate constant for endogenous metabolism, and X_{so} is the cell mass concentration at the beginning of the stationary phase. Because S is zero, μ_g is zero in the stationary phase.

The reason for termination of growth may be either exhaustion of an essential nutrient or accumulation of toxic products. If an inhibitory product is produced and accumulates in the medium, the growth rate will slow down, depending on inhibitor production, and at a certain level of inhibitor concentration, growth will stop. Ethanol production by yeast is an example of a fermentation in which the product is inhibitory to growth. Dilution of toxified medium, addition of an unmetabolizable chemical compound complexing with the toxin, or simultaneous removal of the toxin would alleviate the adverse effects of the toxin and yield further growth.

The *death phase* (or decline phase) follows the stationary phase. However, some cell death may start during the stationary phase, and a clear demarcation between these two phases is not always possible. Often, dead cells lyse, and intracellular nutrients released into the medium are used by the living organisms during the stationary phase. At the end of the stationary phase, because of either nutrient depletion or toxic product accumulation, the death phase begins.

The rate of death usually follows first-order kinetics:

$$\frac{dN}{dt} = -k'_d N \qquad \text{or} \qquad N = N_s e^{-k'_d t} \tag{6.12}$$

where N_s is the concentration of cells at the end of the stationary phase and k'_d is the first-order death-rate constant. A plot of Ln N versus t yields a line of slope $-k'_d$. During the death phase, cells may or may not lyse, and the reestablishment of the culture may be possible in the early death phase if cells are transferred into a nutrient-rich medium. In both the death and stationary phases, it is important to recognize that there is a distribution of properties among individuals in a population. With a narrow distribution, cell death will occur nearly simultaneously; with a broad distribution, a subfraction of the population may survive for an extended period. It is this subfraction that would dominate the reestablishment of a culture from inoculum derived from stationary or death-phase cultures. Thus, using an old inoculum may select for variants of the original strain having altered metabolic capabilities.

To better describe growth kinetics, we define some stoichiometrically related parameters. Yield coefficients are defined on the basis of the amount of consumption of another material. For example, the growth yield coefficient in fermentation is

$$Y_{X/S} \equiv -\frac{\Delta X}{\Delta S} \tag{6.13}$$

At the end of the batch growth period, we have an *apparent growth yield* (or observed growth yield). Because culture conditions can alter patterns of substrate utilization, the apparent growth yield is not a true constant.

The consumption of a substrate for chemoheterotrophic growth is used to supply both energy and carbon as a building block for the synthesis of cellular components. There is also the concept of *maintenance*. Maintenance was first described as energy consumed for functions other than production of new cell material, but these "nongrowth" uses of substrate are complex and context dependent (see van Bodegom[†] for a more complete description). We will adapt a fairly standard approach to the description of these components, but understand that there are many subtitles to these concepts.

If we take as an example the consumption of a sugar (such as glucose) in a simple chemically defined medium, we can summarize the use of the glucose as follows:

$$\Delta S = \Delta S_{\substack{\text{assimilated}\\ \text{into}\\ \text{biomass}}} + \Delta S_{\substack{\text{assimilated}\\ \text{into an}\\ \text{extracellular}\\ \text{product}}} + \Delta S_{\substack{\text{growth}\\ \text{energy}}} + \Delta S_{\substack{\text{maintenance}\\ \text{energy}}} \tag{6.14}$$

Equation 6.14 summarizes the major ways glucose is utilized. The first is to use glucose to make cellular components such as DNA, RNA, proteins, polysaccharides, and other small carbon-containing molecules. In addition to these intracellular components, glucose supplies the carbon for extracellular products, such as proteins made from recombinant DNA techniques. The term $\Delta S_{\text{growth energy}}$ denotes the glucose consumed to generate energy, typically in the form of ATP or GTP (guanosine triphosphate), which is then used in biosynthesis of macromolecules and transport of substrates in or product out of the cell and other growth-related functions. $\Delta S_{\text{maintenance energy}}$ refers to substrate consumed to produce ATP or GTP to sustain the cell for nongrowth-related functions such as repair of damaged cellular components, osmotic pressure balancing, motility, defense against O_2 stress, and energy-spilling reactions. The breakdown of glucose to supply energy may result in the formation of CO_2 due to oxygen consumption (aerobic) or due to formation of a partially oxidized compound such as ethanol and CO_2 (anaerobic). Such compounds (e.g., ethanol and CO_2) are also "products" from the breakdown of glucose, and indeed, as shown in Chapter 5, "Major Metabolic Pathways," many are important commercial products.

In Section 6.3, we differentiate between the true (or maximum) growth yield, which is constant, and the apparent yield. Yield coefficients based on other substrates or product formation may be defined as

$$Y_{X/O_2} = -\frac{\Delta X}{\Delta O_2} \tag{6.15}$$

$$Y_{P/S} = -\frac{\Delta P}{\Delta S} \tag{6.16}$$

For organisms growing aerobically on glucose, $Y_{X/S}$ is typically 0.4 to 0.6 g/g for most yeast and bacteria, while Y_{X/O_2} is 0.9 to 1.4 g/g. Anaerobic growth is less efficient, and the yield coefficient is reduced substantially (see Figure 6.5). With

[†]P. van Bodegom, "Microbial Maintenance: A Critical Review on Its Quantification," *Microbial Ecology* 53(4): 513–523, 2007.

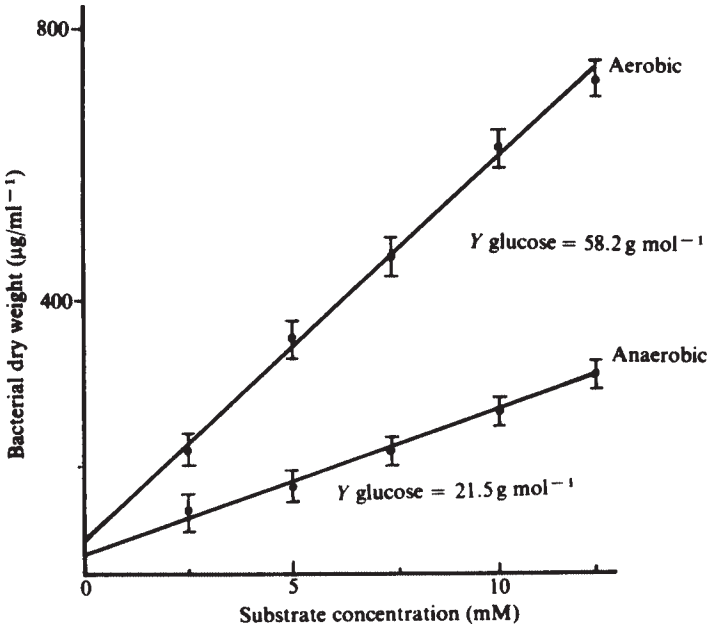


Figure 6.5. Aerobic and anaerobic growth yields of *Streptococcus faecalis* with glucose as substrate. (B. Atkinson and F. Mavituna, *Biochemical Engineering and Biotechnology Handbook*, Macmillan, Inc., New York, 1983. Reproduced with permission of Palgrave Macmillan.)

substrates that are more or less reduced than glucose, the value of the apparent yield coefficient will change. For methane, $Y_{X/S}$ would assume values of 0.6 to 1.0 g/g, with the corresponding Y_{X/O_2} decreasing to about 0.2 g/g. In most cases, the yield of biomass on a carbon-energy source is 1.0 ± 0.4 g biomass per g of carbon consumed. Table 6.1 lists some examples of $Y_{X/S}$ and Y_{X/O_2} for a variety of substrates and organisms.

Two closely linked parameters are often used to describe nongrowth-related functions. There are difficulties in employing these parameters and disagreement among authors. These nongrowth functions are most easily observed and discussed in terms of continuous culture, which is described in Section 6.3. The maintenance coefficient, m, is often defined as

$$m \equiv -\frac{(dS/dt)_m}{X} \tag{6.17}$$

where $(dS/dt)_m$ represents the consumption of substrate for maintenance functions. Given the complexity of what is included in maintenance and the conceptual difficulty of applying the concept to a simple model in which there is no explicit accounting of the internal structure of the cell, the term $(dS/dt)_m$ should be viewed as a bookkeeping term.

TABLE 6.1. Yield Factors for Aerobic Growth

Organism	Substrate	$Y_{X/S}$ g/g	$Y_{X/S}$ g/mol	$Y_{X/S}$ g/g-C	Y_{X/O_2}[a] g/g
Enterobacter aerogenes	Maltose	0.46	149.2	1.03	1.50
	Mannitol	0.52	95.2	1.32	1.18
	Fructose	0.42	76.1	1.05	1.46
	Glucose	0.40	72.7	1.01	1.11
Candida utilis	Glucose	0.51	91.8	1.28	1.32
Penicillium chrysogenum	Glucose	0.43	77.4	1.08	1.35
Pseudomonas fluorescens	Glucose	0.38	68.4	0.95	0.85
Rhodopseudomonas spheroides	Glucose	0.45	81.0	1.12	1.46
Saccharomyces cerevisiae	Glucose	0.50	90.0	1.25	0.97
Enterobacter aerogenes	Ribose	0.35	53.2	0.88	0.98
	Succinate	0.25	29.7	0.62	0.62
	Glycerol	0.45	41.8	1.16	0.97
	Lactate	0.18	16.6	0.46	0.37
	Pyruvate	0.20	17.9	0.49	0.48
	Acetate	0.18	10.5	0.43	0.31
Candida utilis	Acetate	0.36	21.0	0.90	0.70
Pseudomonas fluorescens	Acetate	0.28	16.8	0.70	0.46
Candida utilis	Ethanol	0.68	31.2	1.30	0.61
Pseudomonas fluorescens	Ethanol	0.49	22.5	0.93	0.42
Klebsiella sp.	Methanol	0.38	12.2	1.01	0.56
Methylomonas sp.	Methanol	0.48	15.4	1.28	0.53
Pseudomonas sp.	Methanol	0.41	13.1	1.09	0.44
Methylococcus sp.	Methane	1.01	16.2	1.34	0.29
Pseudomonas sp.	Methane	0.80	12.8	1.06	0.20
Pseudomonas sp.	Methane	0.60	9.6	0.80	0.19
Pseudomonas methanica	Methane	0.56	9.0	0.75	0.17

[a] Y_{X/O_2} is the yield factor relating grams of cells formed per gram of O_2 consumed.

With permission, from S. Nagai in *Advances in Biochemical Engineering*, vol. 11, T. K. Ghose, A. Fiechter, and N. Blakebrough, eds., Springer-Verlag, New York, p. 53, 1979. With permission of Springer.

Depending on the culture conditions, maintenance may be largely due to endogenous metabolism (although some nongrowth functions are not included in endogenous metabolism but are included in maintenance). When substrate and energy are present in large amounts, maintenance and endogenous metabolism are quantitatively small terms, whereas in stationary growth, when external substrate is present at a low level, endogenous metabolism dominates. Endogenous metabolism consists of the breakdown of cellular compounds such as polysaccharides, but also proteins and RNA, for monomers to make new macromolecules and for energy for cellular function. The term k_d in equation 6.11 is the rate constant for endogenous metabolism.

Microbial growth, product formation, and substrate utilization rates are usually expressed in the form of specific rates (e.g., normalized with respect to X), since bioreactions are autocatalytic. The specific rates are used to compare the effectiveness of various fermentation schemes and biocatalysts.

Microbial products can be classified in three major categories (see Figure 6.6):

- *Growth-associated products* are produced simultaneously with microbial growth. The specific rate of product formation is proportional to the specific rate of growth, μ_g. Note that μ_g differs from μ_{net}, the net specific growth rate, when endogenous metabolism is nonzero:

$$q_P = \frac{1}{X}\frac{dP}{dt} = Y_{P/X}\mu_g \tag{6.18}$$

  The production of a constitutive enzyme is an example of a growth-associated product.
- *Nongrowth-associated product formation* takes place during the stationary phase when the growth rate is zero. The specific rate of product formation is constant:

$$q_P = \beta = \text{constant} \tag{6.19}$$

  Many secondary metabolites, such as antibiotics (e.g., penicillin), are non-growth-associated products.
- *Mixed-growth-associated product formation* takes place during the slow growth and stationary phases. In this case, the specific rate of product formation is given by the following equation:

$$q_P = \alpha\mu_g + \beta \tag{6.20}$$

  Lactic acid fermentation, xanthan gum, and some secondary metabolites from cell culture are examples of mixed-growth-associated products. Equation 6.20 is a *Luedeking–Piret* equation. If $\alpha = 0$, the product is only nongrowth associated, and if $\beta = 0$, the product would be only growth associated, and consequently α would be equal to $Y_{P/X}$.

Some of the concepts concerning growth rate and yield are illustrated in Example 6.1.

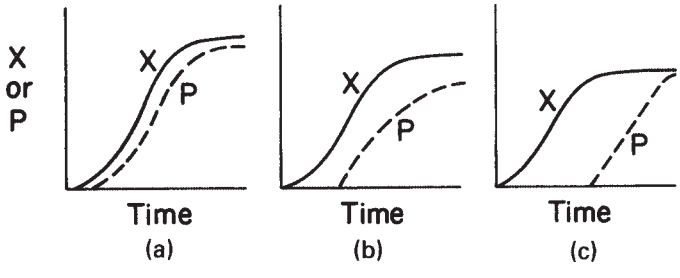


Figure 6.6. Kinetic patterns of growth and product formation in batch fermentations: (a) growth-associated product formation, (b) mixed-growth-associated product formation, and (c) nongrowth-associated product formation.

Example 6.1 Calculation of Growth Parameters

A strain of mold was grown in a batch culture on glucose, and the following data were obtained:

Time	Cell Concentration (g/l)	Glucose Concentration (g/l)
0	1.25	100
9	2.45	97
16	5.1	90.4
23	10.5	76.9
30	22	48.1
34	33	20.6
36	37.5	9.38
40	41	0.63

a. Calculate the maximum net specific growth rate.
b. Calculate the apparent growth yield.
c. What maximum cell concentration could one expect if 150 g of glucose were used with the same size inoculum?

Solution
A plot of Ln X versus t yields a slope of 0.1 h.

a. $\mu_{net} = \dfrac{\ln X_2 - \ln X_1}{t_2 - t_1} = \dfrac{\ln 37.5 - \ln 5.1}{36 - 16} \cong 0.1\,\text{h}^{-1}$

b. $Y = -\dfrac{\Delta X}{\Delta S} = -\dfrac{41 - 1.25}{0.625 - 100} \cong 0.4\,\text{g cells/g substrate}$

c. $X_{max} = X_0 + YS_0 = 1.25 + 0.4(150) = 60.25\,\text{g cells/l}$

6.1.3. How Environmental Conditions Affect Growth Kinetics

The patterns of microbial growth and product formation we have just discussed are influenced by environmental conditions such as temperature, pH, and dissolved-oxygen concentration.

Temperature is an important factor affecting the performance of cells. According to their temperature optima, organisms can be classified in three groups: psychrophiles (T_{opt} < 20°C), mesophiles (T_{opt} = from 20° to 50°C), and thermophiles (T_{opt} > 50°C). As the temperature is increased toward optimal growth temperature, the growth rate approximately doubles for every 10°C increase in temperature. Above the optimal temperature range, the growth rate decreases and thermal death may occur. The net specific replication rate for temperature above optimal level can be expressed by

$$\frac{dN}{dt} = (\mu_R' - k_d')N \tag{6.21}$$

At high temperatures, the thermal death rate exceeds the growth rate, which causes a net decrease in the concentration of viable cells.

Both μ'_R and k'_d vary with temperature according to the Arrhenius equation:

$$\mu'_R = Ae^{-E_a/RT}, \quad k'_d = A'e^{-E_d/RT} \tag{6.22}$$

where E_a and E_d are activation energies for growth and thermal death. The activation energy for growth is typically 10 to 20 kcal/mol, and for thermal death, 60 to 80 kcal/mol. That is, thermal death is more sensitive to temperature changes than microbial growth.

Temperature also affects product formation. However, the temperature optimum for growth and product formation may be different. The yield coefficient is also affected by temperature. In some cases, such as single-cell protein production, temperature optimization to maximize the yield coefficient ($Y_{X/S}$) is critical. When temperature is increased above the optimum temperature, the maintenance requirements of cells increase. That is, the maintenance coefficient (see equation 6.17) increases with increasing temperature with an activation energy of 15 to 20 kcal/mol, resulting in a decrease in the yield coefficient.

Temperature also may affect the rate-limiting step in a fermentation process. At high temperatures, the rate of bioreaction might become higher than the diffusion rate, and diffusion would then become the rate-limiting step (e.g., in an immobilized cell system). The activation energy of molecular diffusion is about 6 kcal/mol. The activation energy for most bioreactions is more than 10 kcal/mol, so diffusional limitations must be carefully considered at high temperatures. Figure 6.7 depicts a typical variation of growth rate with temperature.

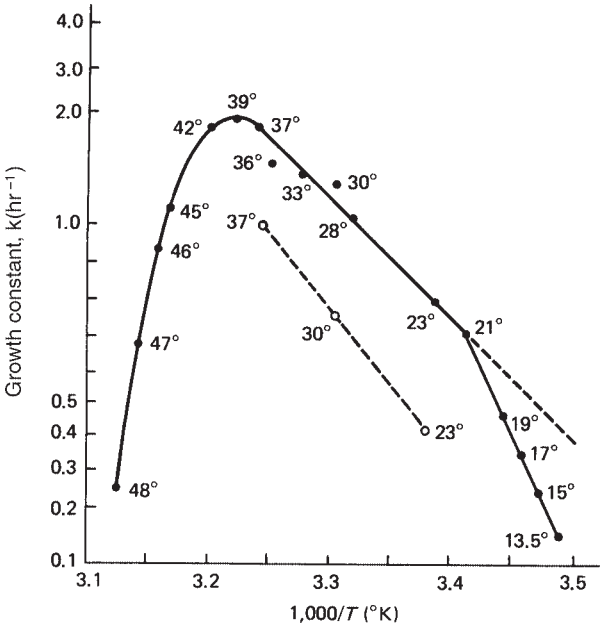


Figure 6.7. Arrhenius plot of growth rate of *Escherichia coli* B/r. Individual data points are marked with corresponding degrees Celsius. *E. coli* B/r was grown in a rich complex medium (●) and a glucose-mineral salts medium (○). (After S. L. Herendeen, R. A. VanBogelen, F. C. Neidhardt, "Levels of Major Protein of Escherichia coli during Growth at Different Temperatures," *J. Bacteriol*. 139:195, 1979, as drawn in R. Y. Stanier and others, *The Microbial World*, 5th ed., Pearson Education, Upper Saddle River, NJ, 1986, p. 207.)

Hydrogen-ion concentration (pH) affects the activity of enzymes and therefore the microbial growth rate. The optimal pH for growth may be different from that for product formation. Generally, the acceptable pH range varies about the optimum by ±1 to 2 pH units. Different organisms have different pH optima: the pH optimum for many bacteria ranges from pH = 3 to 8; for yeast, pH = 3 to 6; for molds, pH = 3 to 7; for plant cells, pH = 5 to 6; and for animal cells, pH = 6.5 to 7.5. Many organisms have mechanisms to maintain intracellular pH at a relatively constant level in the presence of fluctuations in environmental pH. When pH differs from the optimal value, the maintenance-energy requirements increase. One consequence of different pH optima is that the pH of the medium can be used to select one organism over another.

In most fermentations, pH can vary substantially. Often, the nature of the nitrogen source can be important. If ammonium is the sole nitrogen source, hydrogen ions are released into the medium as a result of the microbial utilization of ammonia, resulting in a decrease in pH. If nitrate is the sole nitrogen source, hydrogen ions are removed from the medium to reduce nitrate to ammonia, resulting in an increase in pH. Also, pH can change because of the production of organic acids, the utilization of acids (particularly amino acids), or the production of bases. The evolution or supply of CO_2 can alter pH greatly in some systems (e.g., seawater or animal cell culture). Thus, pH control by means of a buffer or an active pH control system is important. Variation of specific growth rate with pH is depicted in Figure 6.8, indicating a pH optimum.

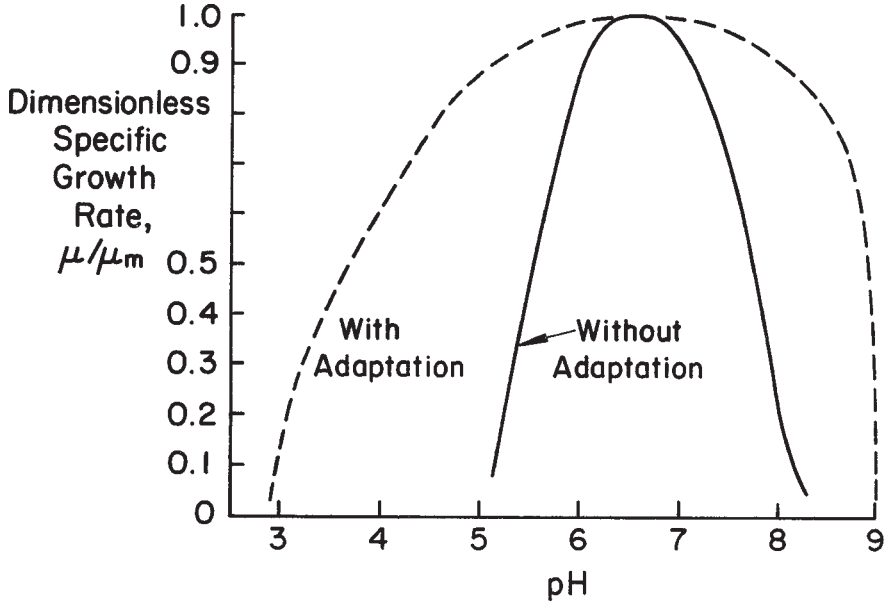


Figure 6.8. Typical variation of specific growth rate with pH. The units are arbitrary. With some microbial cultures, it is possible to adapt cultures to a wider range of pH values if pH changes are made in small increments from culture transfer to transfer.

Dissolved oxygen (DO) is an important substrate in aerobic fermentations and may be a limiting substrate, since oxygen gas is sparingly soluble in water. At high cell concentrations, the rate of oxygen consumption may exceed the rate of oxygen supply, leading to oxygen limitations. When oxygen is the rate-limiting factor, specific growth rate varies with DO concentration according to saturation kinetics; below a critical concentration, growth or respiration approaches a first-order rate dependence on the DO concentration.

Above a *critical oxygen concentration*, the growth rate becomes independent of the DO concentration. Figure 6.9 depicts the variation of specific growth rate with DO concentration. Oxygen is a growth-rate-limiting factor when the DO level is below the critical DO concentration. In this case, another medium component (e.g., glucose, ammonium) becomes growth-extent limiting. For example, with *Azotobacter vinelandii* at a DO = 0.05 mg/l, the growth rate is about 50% of maximum even if a large amount of glucose is present. However, the maximum amount of cells formed is not determined by the DO, as oxygen is continually resupplied. If glucose were totally consumed, growth would cease even if DO = 0.05 mg/l. Thus, the extent of growth (mass of cells formed) would depend on

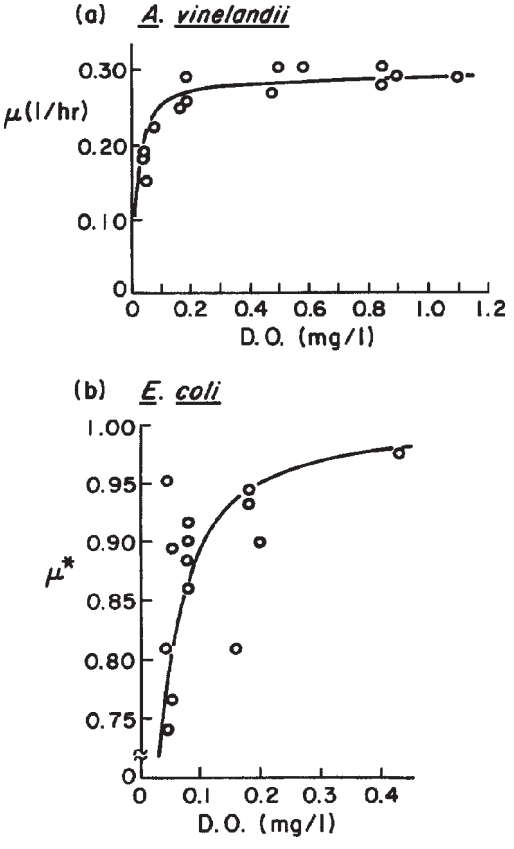


Figure 6.9. Growth-rate dependence on dissolved oxygen for (a) *Azotobacter vinelandii*, a strictly aerobic organism, and (b) *Escherichia coli*, which is facultative. *E. coli* grows anaerobically at a rate of about 70% of its aerobic growth in minimal medium. Note that μ* is defined as follows: $\mu^* = \frac{\mu - \mu_m^{\text{anaerobic}}}{\mu_m^{\text{aerobic}} - \mu_m^{\text{anaerobic}}}$

glucose, while the growth rate for most of the culture period would depend on the value of DO.

The critical oxygen concentration is about 5% to 10% of the saturated DO concentration for bacteria and yeast and about 10% to 50% of the saturated DO concentration for mold cultures, depending on the pellet size of molds. Saturated DO concentration in water at 25°C and 1 atm pressure is about 7 ppm. The presence of dissolved salts and organics can alter the saturation value, whereas increasingly high temperatures decrease the saturation value.

Oxygen is usually introduced to fermentation broth by sparging air through the broth. Oxygen transfer from gas bubbles to cells is usually limited by oxygen transfer through the liquid film surrounding the gas bubbles. The rate of oxygen transfer from the gas to liquid phase is given by

$$N_{O_2} = k_L a(C^* - C_L) = \text{OTR} \tag{6.23}$$

where k_L is the oxygen transfer coefficient (cm/h), a is the gas–liquid interfacial area (cm^2/cm^3), k_La is the volumetric oxygen transfer coefficient (h^{-1}), C^* is saturated DO concentration (mg/l), C_L is the actual DO concentration in the broth (mg/l), and N_{O_2} is the rate of oxygen transfer (mg O_2/l-h). Also, the term *oxygen transfer rate (OTR)* is used.

The rate of oxygen uptake is denoted as *OUR (oxygen uptake rate)* and when maintenance can be neglected is given by equation 6.24:

$$\text{OUR} = q_{O2}X = \frac{\mu_g X}{Y_{X/O_2}} \tag{6.24}$$

where q_{O_2} is the specific rate of oxygen consumption (mg O_2/g dw cells-h), Y_{X/O_2} is the yield coefficient on oxygen (g dw cells/g O_2), and X is cell concentration (g dw cells/l).

When oxygen transfer is the rate-limiting step, the rate of oxygen consumption is equal to the rate of oxygen transfer. If the maintenance requirement of O_2 is negligible compared to growth, then we get

$$\frac{\mu_g X}{Y_{X/O_2}} = k_L a(C^* - C_L) \tag{6.25}$$

or

$$\frac{dX}{dt} = Y_{X/O_2} k_L a(C^* - C_L) \tag{6.26}$$

Growth rate varies nearly linearly with the OTR under oxygen-transfer limitations. Among the various methods used to overcome DO limitations are the use of oxygen-enriched air or pure oxygen and operation under high atmospheric

pressure (2 to 3 atm). Oxygen transfer has a big impact on reactor design (see Chapter 10, "Selection, Scale-Up, Operation, and Control of Bioreactors").

The *redox potential* is an important parameter that affects the rate and extent of many oxidative–reductive reactions. In a fermentation medium, the redox potential is a complex function of DO, pH, and other ion concentrations, such as reducing and oxidizing agents. The electrochemical potential of a fermentation medium can be expressed by

$$E_h = E_0' + \frac{2.3RT}{4F}\log P_{O_2} + 2.3\frac{RT}{F}\log(H^+) \tag{6.27}$$

The electrochemical potential is measured in millivolts by a pH/voltmeter, and P_{O_2} is in atmospheres.

The redox potential of a fermentation medium can be reduced by passing nitrogen gas or by the addition of reducing agents such as cysteine HCl or Na_2S. Oxygen gas can be passed or some oxidizing agents can be added to the fermentation medium to increase the redox potential.

Dissolved carbon dioxide (DCO_2) concentration may have a profound effect on performance of organisms. Very high DCO_2 concentrations may be toxic to some cells. However, cells require a certain DCO_2 level for proper metabolic functions. The dissolved carbon dioxide concentration can be controlled by changing the CO_2 content of the air supply and the agitation speed.

The ionic strength of the fermentation medium affects the transport of certain nutrients into and out of cells, the metabolic functions of cells, and the solubility of certain nutrients, such as DO. The ionic strength is given by

$$I = \frac{1}{2}\Sigma C_i Z_i^2 \tag{6.28}$$

where C is the concentration of an ion, Z_i is its charge, and I is the ionic strength of the medium.

High substrate concentrations that are significantly above stoichiometric requirements are inhibitory to cellular functions. Inhibitory levels of substrates vary depending on the type of cells and substrate. Glucose may be inhibitory at concentrations above 200 g/l (e.g., ethanol fermentation by yeast), probably due to a reduction in water activity. Certain salts, such as NaCl, may be inhibitory at concentrations above 40 g/l due to high osmotic pressure. Some refractory compounds, such as phenol, toluene, and methanol, are inhibitory at much lower concentrations (e.g., 1 g/l). Typical maximum noninhibitory concentrations of some nutrients are glucose, 100 g/l; ethanol, 50 g/l for yeast, much less for most organisms; ammonium, 5 g/l; phosphate, 10 g/l; and nitrate, 5 g/l. Substrate inhibition can be overcome by intermittent addition of the substrate to the medium.

6.1.4. Heat Generation by Microbial Growth

About 40% to 50% of the energy stored in a carbon and energy source is converted to biological energy (ATP) during aerobic metabolism, and the rest of the energy is released as heat. For actively growing cells, the maintenance requirement is low, and heat evolution is directly related to growth.

The heat generated during microbial growth can be calculated using the heat of combustion of the substrate and of cellular material. A schematic of an enthalpy balance for microbial utilization of substrate is presented in Figure 6.10. The heat of combustion of the substrate is equal to the sum of the metabolic heat and the heat of combustion of the cellular material:

$$\frac{\Delta H_s}{Y_{X/S}} = \Delta H_c + \frac{1}{Y_H} \tag{6.29a}$$

where ΔH_s is the heat of combustion of the substrate (kJ/g substrate), $Y_{X/S}$ is the substrate yield coefficient (g cell/g substrate), ΔH_c is the heat of combustion of cells (kJ/g cells), and $1/Y_H$ is the metabolic heat evolved per gram of cell mass produced (kJ/g cells).

Equation 6.29a can be rearranged to yield

$$Y_H = \frac{Y_{X/S}}{\Delta H_s - Y_{X/S}\Delta H_c} \tag{6.29b}$$

ΔH_s and ΔH_c can be determined from the combustion of substrate and cells. Typical ΔH_c values for bacterial cells are 20 to 25 kJ/g cells. Typical values of Y_H are glucose, 0.42 g/kcal; malate, 0.30 g/kcal; acetate, 0.21 g/kcal; ethanol, 0.18 g/kcal; methanol, 0.12 g/kcal; and methane, 0.061 g/kcal. Clearly, the degree of oxidation of the substrate has a strong effect on the amount of heat released. The total rate of heat evolution in a batch fermentation is

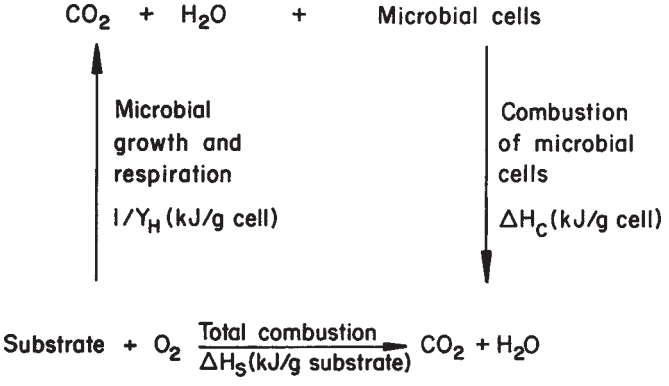


Figure 6.10. Heat balance on microbial utilization of substrate.

$$Q_{GR} = V_L \mu_{\text{net}} X \frac{1}{Y_H} \tag{6.30}$$

where V_L is the liquid volume (l) and X is the cell concentration (g/l).

In aerobic fermentations, the rate of metabolic heat evolution can roughly be correlated to the rate of oxygen uptake, since oxygen is the final electron acceptor:

$$Q_{GR} \cong 0.12 Q_{O_2} \tag{6.31}$$

where Q_{GR} is in units of kcal/h, and Q_{O_2} is in millimoles of O_2/h.

Metabolic heat released during fermentation can be removed by circulating cooling water through a cooling coil or cooling jacket in the fermenter. Often, temperature control (adequate heat removal) is an important limitation on reactor design (see Chapter 10). The ability to estimate heat-removal requirements is essential to proper reactor design.

6.2. QUANTIFYING GROWTH KINETICS

In the previous section, we described some key concepts in the growth of cultures. Clearly, we can think of the growth dynamics in terms of kinetic descriptions. It is essential to recall that cellular composition and biosynthetic capabilities change in response to new growth conditions *(unbalanced growth)*, although a constant cellular composition and balanced growth can predominate in the exponential growth phase. If the decelerating growth phase is due to substrate depletion rather than inhibition by toxins, the growth rate decreases in relation to decreasing substrate concentrations. In the stationary and death phases, the distribution of properties among individuals is important (e.g., cryptic death). Although these kinetic ideas are evident in batch culture, they are equally evident and important in other modes of culture (e.g., continuous culture).

The complete description of the growth kinetics of a culture would involve recognition of the *structured* nature of each cell and the *segregation* of the culture into individual units (cells) that may differ from each other. Models can have these same attributes. A chemically structured model divides the cell mass into components. If the ratio of these components can change in response to perturbations in the extracellular environment, then the model is behaving analogously to a cell changing its composition in response to environmental changes. Consider in Chapter 4, "How Cells Work," our discussion of cellular regulation, particularly the induction of whole pathways. Any of these metabolic responses results in changes in intracellular structure. Furthermore, if a model of a culture is constructed from discrete units, it begins to mimic the segregation observed in real cultures. Models may be structured and segregated, structured and nonsegregated, unstructured and segregated, or unstructured and nonsegregated. Models containing both structure and segregation are the most realistic, but they are also computationally complex.

The degree of realism and complexity required in a model depends on what is being described; the modeler should always choose the simplest model that can adequately describe the desired system. An unstructured model assumes fixed cell composition, which is equivalent to assuming *balanced growth*. The balanced-growth assumption is valid primarily in single-stage, steady-state continuous culture and the exponential phase of batch culture; it fails during any transient condition. How fast the cell responds to perturbations in its environment and how fast these perturbations occur determine whether pseudobalanced growth can be assumed. If cell response is fast compared to external changes and if the magnitude of these changes is not too large (e.g., a 10% or 20% variation from initial conditions), then the use of unstructured models can be justified, since the deviation from balanced growth may be small. Culture response to large or rapid perturbations cannot be described satisfactorily by unstructured models.

For many systems, segregation is not a critical component of culture response, so nonsegregated models will be satisfactory under many circumstances. An important exception is the prediction of the growth responses of plasmid-containing cultures (see Chapter 14, "Utilizing Genetically Engineered Organisms").

Because of the introductory nature of this book, we will concentrate our discussion on unstructured and nonsegregated models. Be aware of the limitations on these models. Nonetheless, such models are simple and applicable to some situations of practical interest.

6.2.1. Unstructured Nonsegregated Models

The simplest models are unstructured, nonsegregated models, but they can be powerful. Primarily, they are effective at predicting specific growth as a function of substrate concentrations. They also can be extended to predict response to a variety of inhibitors or activators. They can be applied not only to bacteria but also to filamentous organisms. These models are useful in the design of bioreactors.

6.2.1.1. Substrate-limited growth. As shown in Figure 6.11, the relationship of specific growth rate to substrate concentration often assumes the form of saturation kinetics. Here we assume that a single chemical species, S, is growth-rate limiting (i.e., an increase in S influences growth rate, while changes in other nutrient concentrations have no effect). These kinetics are similar to the Langmuir–Hinshelwood (or Hougen–Watson) kinetics in traditional chemical kinetics or Michaelis–Menten kinetics for enzyme reactions. When applied to cellular systems, these kinetics can be described by the *Monod equation:*

$$\mu_g = \frac{\mu_m S}{K_S + S} \tag{6.32}$$

where μ_m is the maximum specific growth rate when $S \gg K_s$. If endogenous metabolism is unimportant, then $\mu_{net} = \mu_g$. The constant K_s is known as the *saturation constant* or *half-velocity constant* and is equal to the concentration of the rate-limiting substrate

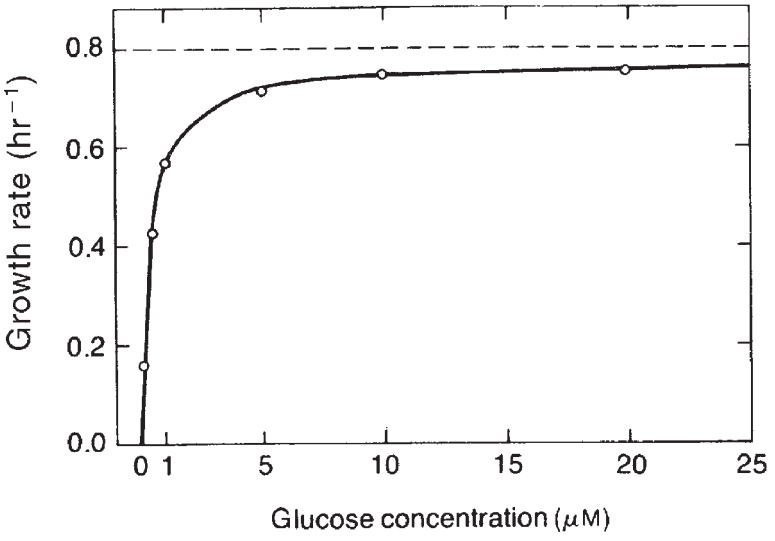


Figure 6.11. Effect of nutrient concentration on the specific growth rate of *Escherichia coli.* (R. Y. Stainer, J. L. Ingraham, M. L. Wheelis, P. R. Painter, *The Microbial World*, 5th ed., © 1986. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

when the specific rate of growth is equal to one-half of the maximum. That is, $K_s = S$ when $\mu_g = \frac{1}{2}\mu_{max}$ In general, $\mu_g = \mu_m$ for $S \gg K_s$, and $\mu_g = (\mu_m/K_s)S$ for $S \ll K_s$. The Monod equation is semiempirical; it derives from the premise that a single-enzyme system with Michaelis–Menten kinetics is responsible for uptake of S, and the amount of that enzyme or its catalytic activity is sufficiently low to be growth-rate limiting.

This simple premise is rarely, if ever, true; however, the Monod equation empirically fits a wide range of data satisfactorily and is the most commonly applied unstructured, nonsegregated model of microbial growth.

The Monod equation describes substrate-limited growth only when growth is slow and population density is low. Under these circumstances, environmental conditions can be related simply to S. If the consumption of a carbon–energy substrate is rapid, then the release of toxic waste products is more likely (due to energy-spilling reactions). At high population levels, the buildup of toxic metabolic by-products becomes more important. The following rate expressions have been proposed for rapidly growing dense cultures:

$$\mu_g = \frac{\mu_m S}{K_{S0} S_0 + S} \tag{6.33}$$

or

$$\mu_g = \frac{\mu_m S}{K_{S1} + K_{S0} S_0 + S} \tag{6.34}$$

where S_0 is the initial concentration of the substrate and K_{S0} is dimensionless.

Other equations have been proposed to describe the substrate-limited growth phase. Depending on the shape of the μ–S curve, one of these equations may be more plausible than the others. The following equations are alternatives to the Monod equation:

$$\text{Blackman equation: } \mu_g = \mu_m, \qquad \text{iff } S \geq 2K_S$$

$$\mu_g = \frac{\mu_m}{2K_S} S, \quad \text{iff } S < 2K_S \tag{6.35}$$

$$\text{Tessier equation: } \mu_g = \mu_m(1 - e^{-KS}) \tag{6.36}$$

$$\text{Moser equation: } \mu_g = \frac{\mu_m S^n}{K_S + S^n} = \mu_m(1 + K_S S^{-n})^{-1} \tag{6.37}$$

$$\text{Contois equation: } \mu_g = \frac{\mu_m S}{K_{sx} X + S} \tag{6.38}$$

Although the Blackman equation often fits the data better than the Monod equation, the discontinuity in the Blackman equation is troublesome in many applications. The Tessier equation has two constants (μ_m, K), and the Moser equation has three constants (μ_m, K_S, n). The Moser equation is the most general form of these equations, and it is equivalent to the Monod equation when $n = 1$. The Contois equation has a saturation constant proportional to cell concentration that describes substrate-limited growth at high cell densities. According to this equation, the specific growth rate decreases with decreasing substrate concentrations and eventually becomes inversely proportional to the cell concentration in the medium (i.e., $\mu_g \propto X^{-1}$).

These equations can be described by a single differential equation:

$$\frac{d\upsilon}{dS} = K\upsilon^a(1 - \upsilon)^b \tag{6.39}$$

where $\upsilon = \mu_g/\mu_m$, S is the rate-limiting substrate concentration, and K, a, and b are constants. The values of these constants are different for each equation and are listed in Table 6.2.

The correct rate form to use when more than one substrate is potentially growth-rate limiting is an unresolved question. However, under most circumstances, the noninteractive approach works best:

$$\mu_g = \mu_g(S_1) \quad \text{or} \quad \mu_g(S_2) \quad \text{or} \quad \ldots \mu_g(S_n) \tag{6.40}$$

The lowest value of $\mu_g(S_i)$ is used.

TABLE 6.2. Constants of the Generalized Differential Specific Growth Rate Equation 6.39 for Different Models

	a	b	K
Monod	0	2	$1/K_s$
Tessier	0	1	$1/K$
Moser	$1-1/n$	$1+1/n$	$n/K_s^{1/n}$
Contois	0	2	$1/K_{SX}$

6.2.1.2. Models with growth inhibitors. At high concentrations of substrate or product and in the presence of inhibitory substances in the medium, growth becomes inhibited, and growth rate depends on inhibitor concentration. The inhibition pattern of microbial growth is analogous to enzyme inhibition. If a single-substrate enzyme-catalyzed reaction is the rate-limiting step in microbial growth, then kinetic constants in the rate expression are biologically meaningful. Often, the underlying mechanism is complicated, and kinetic constants do not have biological meanings and are obtained from experimental data by curve fitting.

At high substrate concentrations, microbial growth rate is inhibited by the substrate. As in enzyme kinetics, *substrate inhibition* of growth may be competitive or noncompetitive. If a single-substrate enzyme-catalyzed reaction is the rate-limiting step in microbial growth, then inhibition of enzyme activity results in inhibition of microbial growth by the same pattern.

The major substrate-inhibition patterns and expressions are as follows:

For noncompetitive substrate inhibition,

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_s}{S}\right)\left(1+\frac{S}{K_I}\right)} \tag{6.41}$$

Or, if $K_I \gg K_S$, then

$$\mu_g = \frac{\mu_m S}{K_s + S + S^2 / K_I} \tag{6.42}$$

For competitive substrate inhibition,

$$\mu_g = \frac{\mu_m S}{K_s\left(1+\frac{S}{K_I}\right)+S} \tag{6.43}$$

Note that equation 6.43 is different from equations 6.41 and 6.42, and K_I in equations 6.42 and 6.43 is different. Substrate inhibition may be alleviated by slow, intermittent addition of the substrate to the growth medium.

High concentrations of product can be inhibitory for microbial growth. *Product inhibition* may be competitive or noncompetitive, and in some cases when the underlying mechanism is not known, the inhibited growth rate is approximated to exponential or linear decay expressions.

Equations 6.44 and 6.45 present important examples of the product inhibition rate expression.

For competitive product inhibition,

$$\mu_g = \frac{\mu_m S}{K_s\left(1+\frac{P}{K_p}\right)+S} \tag{6.44}$$

For noncompetitive product inhibition,

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_s}{S}\right)\left(1+\frac{P}{K_p}\right)} \tag{6.45}$$

Ethanol fermentation from glucose by yeasts is a good example of noncompetitive product inhibition, and ethanol is the inhibitor at concentrations above about 5%. Other rate expressions used for ethanol inhibition are as follows:

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_s}{S}\right)}\left(1-\frac{P}{P_m}\right)^n \tag{6.46}$$

where P_m is the product concentration at which growth stops.

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_s}{S}\right)} e^{-P/K_p} \tag{6.47}$$

where K_p is the product inhibition constant.

Analogous to enzyme inhibition, the following rate expressions are used for competitive (eq. 6.48), noncompetitive (eq. 6.49), and uncompetitive (eq. 6.50) inhibition by an inhibitor other than substrate or product:

$$\mu_g = \frac{\mu_m S}{K_s\left(1+\frac{\mathrm{I}}{K_\mathrm{I}}\right)+S} \tag{6.48}$$

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_s}{S}\right)\left(1+\frac{\mathrm{I}}{K_\mathrm{I}}\right)} \tag{6.49}$$

$$\mu_g = \frac{\mu_m S}{\left(\frac{K_s}{\left(1+\frac{I}{K_I}\right)} + S\right)\left(1+\frac{I}{K_I}\right)} \tag{6.50}$$

In some cases, the presence of toxic compounds in the medium results in the inactivation of cells or death. The net specific rate expression in the presence of death has the form

$$\mu_g = \frac{\mu_m S}{K_s + S} - k'_d \tag{6.51}$$

where k'_d is the death-rate constant (h^{-1}).

6.2.1.3. The logistic equation. When plotted on arithmetic paper, the batch growth curve assumes a sigmoidal shape (see Figure 6.3). This shape can be predicted by combining the Monod equation (6.32) with the growth equation (6.2) and an equation for the yield of cell mass based on substrate consumption. Combining equations 6.32 and 6.2a and assuming no endogenous metabolism yields the following:

$$\frac{dX}{dt} = \frac{\mu_m S}{K_s + S} X \tag{6.52}$$

The relationship between microbial growth yield and substrate consumption is

$$X - X_0 = Y_{X/S}(S_0 - S) \tag{6.53}$$

where X_0 and S_0 are initial values, and $Y_{X/S}$ is the cell mass yield based on the limiting nutrient. Substituting for S in equation 6.53 yields the following rate expression:

$$\frac{dX}{dt} = \frac{\mu_m (Y_{X/S} S_0 + X_0 - X)}{(K_S Y_{X/S} + Y_{X/S} S_0 + X_0 - X)} X \tag{6.54}$$

The integrated form of the rate expression in this phase is

$$\frac{(K_S Y_{X/S} + S_0 Y_{X/S} + X_0)}{(Y_{X/S} S_0 + X_0)} \ln\left(\frac{X}{X_0}\right) - \frac{K_S Y_{X/S}}{(Y_{X/S} S_0 + X_0)} \ln\{(Y_{X/S} S_0 + X_0 - X)/Y_{X/S} S_0\} = \mu_m t \tag{6.55}$$

This equation describes the sigmoidal-shaped batch growth curve, and the value of X asymptotically reaches to the value of $Y_{X/S} S_0 + X_0$.

Equation 6.54 requires a predetermined knowledge of the maximum cell mass in a particular environment. We denote this maximum cell mass as X_∞; it is identical to the ecological concept of *carrying capacity*. Equation 6.55 is implicit in its dependence on S.

Logistic equations are a set of equations that characterize growth in terms of carrying capacity. The usual approach is based on a formulation in which the specific growth rate is related to the amount of unused carrying capacity:

$$\mu_g = k\left(1 - \frac{X}{X_\infty}\right) \tag{6.56}$$

Thus, we have the following:

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{X_\infty}\right) \tag{6.57}$$

The integration of equation 6.57 with the boundary condition $X(0) = X_0$ yields the logistic curve:

$$X = \frac{X_0 e^{kt}}{1 - \frac{X_0}{X_\infty}(1 - e^{kt})} \tag{6.58}$$

Equation 6.58 is represented by the growth curve in Figure 6.12.

Equations of the form of 6.58 can also be generated by assuming that a toxin generated as a by-product limits the growth at X_∞ (which is the carrying capacity allowed due to build-up of a toxin). Example 6.2 illustrates the use of the logistic approach.

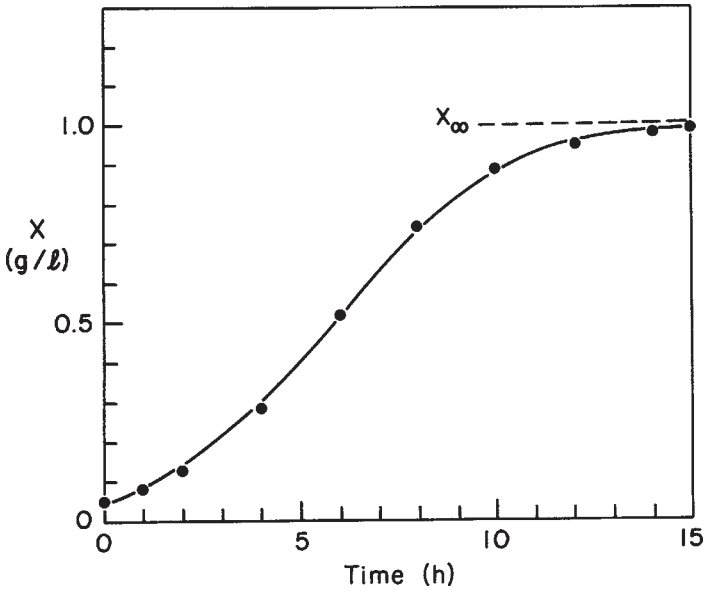


Figure 6.12. Logistic growth curve.

Example 6.2 Logistic Equation

Ethanol formation from glucose is accomplished in a batch culture of *Saccharomyces cerevisiae,* and the following data were obtained:

Time (h)	Glucose (S), g/l	Biomass (X), g/l	Ethanol (P), g/l
0	100	0.5	0.0
2	95	1.0	2.5
5	85	2.1	7.5
10	58	4.8	20.0
15	30	7.7	34.0
20	12	9.6	43.0
25	5	10.4	47.5
30	2	10.7	49.0

a. By fitting the biomass data to the logistic equation, determine the carrying-capacity coefficient k.

b. Determine yield coefficients $Y_{P/S}$ and $Y_{X/S}$.

Solution

a. Equation 6.57 can be rewritten as follows:

$$\frac{1}{X}\frac{dX}{dt} = k\left(1 - \frac{\bar{X}}{X_\infty}\right)$$

or

$$k = \frac{1}{\bar{X}}\frac{\Delta X}{\Delta t} \div \left(1 - \frac{\bar{X}}{X_\infty}\right)$$

Here $\bar{X}$ is average biomass concentration during Δt, and X_∞ is about 10.8 g/l, since growth is almost complete at 30 hours. Thus we get the following data:

Δt (h)	$\bar{X}$ (g/L)	$\frac{1}{\bar{X}}\Delta X/\Delta t\ (\mathrm{h}^{-1})$	$\left(1 - \frac{\bar{X}}{X_\infty}\right)$	$k\ (\mathrm{h}^{-1})$
2	0.75	0.333	0.931	0.36
3	1.55	0.236	0.856	0.28
5	3.45	0.156	0.681	0.23
5	6.25	0.093	0.416	0.22
5	8.65	0.044	0.200	0.22
5	10.00	0.016	0.074	0.22
5	10.55	0.0057	0.023	0.25

A value of $k = 0.24\ \mathrm{h}^{-1}$ would describe most of the data, although it would slightly underestimate the initial growth rate. Another approach would be to take the log of the preceding equation:

$$\log\frac{1}{X}\frac{dX}{dt} = \log k + \log\left(1 - \frac{\bar{X}_0}{X_\infty}\right)$$

We then fit the data to this equation and estimate k from the intercept. In this case, k would be about 0.25 h^{-1}.

b. The yields are estimated directly from the data as follows:

$$Y_{P/S} = \frac{-\Delta P}{\Delta S} = \frac{-(49-0)}{(2-100)} = 0.5 \frac{gP}{gS}$$

$$Y_{X/S} = \frac{-\Delta X}{\Delta S} = \frac{-(10.7-0.5)}{(2-100)} = 0.104 \frac{gX}{gS}$$

The estimate of $Y_{X/S}$ is only approximate, as maintenance effects and endogenous metabolism have been neglected.

6.2.1.4. The Gompertz equation for batch growth and product formation. Neither the logistic (equation 6.58) nor the integrated Monod (equation 6.55) equations consider the lag phase in batch microbial growth and product formation. The Gompertz equation considers the maximum growth potential, maximum growth rate, and duration of the lag phase in batch microbial growth and was developed originally to model the growth of tumors.

The Gompertz equation for batch microbial growth has the following form:

$$X = X_m exp\left[-exp\left[\left(R_{xm}e / X_m\right)(\lambda - \mathbf{t}) + 1\right]\right] \tag{6.59}$$

where X is the concentration of biomass at any time during batch growth, X_m is the maximum biomass concentration, R_{xm} is the maximum rate of growth, λ is duration of the lag phase, e is 2.718, and t is time.

A similar equation can be written for product formation:

$$P = P_m exp\left[-exp\left[\left(R_{pm}e / P_m\right)(\lambda - \mathbf{t}) + 1\right]\right] \tag{6.60}$$

where P is the concentration of product at any time during batch growth, P_m is the maximum potential product concentration, R_{pm} is the maximum rate of product formation, λ is duration of the lag phase, e is approximately 2.718, and t is time. It is assumed that initial biomass (X_o) and product (P_o) concentrations are negligible in equations 6.59 and 6.60.

Variation of substrate concentration with time can be estimated by using the following equation:

$$S = S_o - \frac{X - X_o}{Y_{x/s}} \tag{6.61}$$

With a negligible X_o, equation 6.61 takes the following form:

$$S = S_o - X / Y_{x/s} = S_o - \frac{1}{Y_{x/s}}\left[X_m exp\left[-exp\left[\left(R_{xm}e / X_m\right)(\lambda - \mathbf{t}) + 1\right]\right]\right] \tag{6.62}$$

Due to inclusion of the lag time, Gompertz equations may correlate with the batch experimental data better than the logistic and the integrated Monod equations do.

6.2.1.5. Growth models for filamentous organisms. Filamentous organisms such as molds often form microbial pellets at high cell densities in suspension culture. Cells growing inside pellets may be subject to diffusion limitations. The growth models of molds should include the simultaneous diffusion and consumption of nutrients within the pellet at large pellet sizes. This problem is the same one we face in modeling the behavior of bacteria or yeasts entrapped in spherical gel particles (see Chapter 9, "Operating Considerations for Bioreactors for Suspension and Immobilized Cultures").

Alternatively, filamentous cells can grow on the surface of a moist solid. Such growth is usually a complicated process, involving not only growth kinetics but the diffusion of nutrients and toxic metabolic by-products. However, for an isolated colony growing on a rich medium, we can ignore some of these complications.

In the absence of mass-transfer limitations, it has been observed that the radius of a microbial pellet in a submerged culture or of a mold colony growing on an agar surface increases linearly with time:

$$\frac{dR}{dt} = k_P = \text{constant} \tag{6.63}$$

In terms of growth rate of a mold colony, equation 6.63 can be expressed as

$$\frac{dM}{dt} = \rho 4\pi R^2 \frac{dR}{dt} = k_P 4\pi R^2 \rho \tag{6.64a}$$

or

$$\frac{dM}{dt} = \gamma M^{2/3} \tag{6.64b}$$

where $\gamma = k_P(36\pi\rho)^{1/3}$. Integration of equation 6.64b yields the following:

$$M = \left(M_0^{1/3} + \frac{\gamma t}{3} \right)^3 \approx \left(\frac{\gamma t}{3} \right)^3 \tag{6.65}$$

The initial biomass, M_0, is usually very small compared to M, and therefore M varies with t^3. This behavior has been supported by experimental data.

6.2.2. Models for Transient Behavior

In most practical applications of microbial cultures, the environmental or culture conditions can shift, dramatically leading to changes in cellular composition and biosynthetic capabilities. These cellular changes are not instantaneous but occur over an

observable period of time. In this section, we examine models that can describe or predict such time-dependent (or transient) changes.

6.2.2.1. Models with time delays. The unstructured growth models we have described so far are limited to balanced or pseudobalanced growth conditions. These unstructured models can be improved for use in dynamic situations through the addition of time delays. The use of time delays incorporates structure implicitly. It is built on the premise that the dynamic response of a cell is dominated by an internal process with a time delay on the order of the response time under observation. Other internal processes are assumed to be too fast (essentially always at a pseudoequilibrium) or too slow to influence greatly the observed response. By using black-box techniques equivalent to the traditional approach to the control of chemical processes, it is possible to generate transfer functions that can represent the dynamic response of a culture. An example of the results from such an approach is given in Figure 6.13. However, it should be recognized that such approaches are limited to cultures with similar growth histories and subjected to qualitatively similar perturbations.

6.2.2.2. Chemically structured models. A much more general approach with much greater a priori predictive power is a model capturing the important kinetic interactions among cellular subcomponents. Initially, chemically structured models were based on two components, but at least three components appear

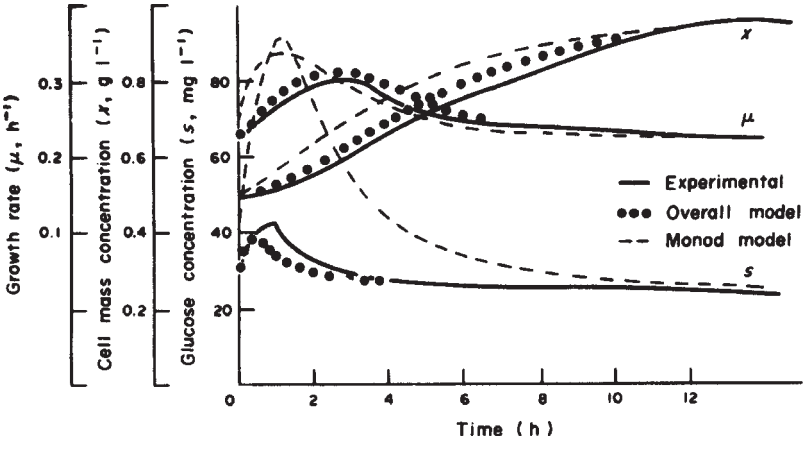


Figure 6.13. Comparison of predictions from a model derived from a system-analysis perspective, predictions from a Monod model, and experiment. The experimental system was a chemostat for a glucose-limited culture of *Saccharomyces cerevisiae* operating at a dilution rate of 0.20 h^{-1}. In this particular experiment, the system was perturbed with a stepwise increase in feed glucose concentration from 1.0 and 2.0 g l^{-1}. X is biomass concentration, μ is growth rate, and S is substrate concentration. (With permission, from T. B. Young III and H. R. Bungay, "Dynamic Analysis of a Microbial Process: A Systems Engineering Approach," *Biotechnol. Bioeng*. 15:377, 1973, and John Wiley & Sons, Inc., New York.)

necessary to give good results. More sophisticated models with 20 to 40 components are being used in many laboratories. A schematic of one such model is given in Figure 6.14.

Writing such models requires that the modeler understand the physical system at a level of greater detail than that at which the model is written, so that the appropriate assumptions can be made. A detailed discussion of such models is appropriate for more advanced texts. However, two important guidelines in writing such models should be understood by even the beginning student. The first is that all reactions should be expressed in terms of intrinsic concentrations. An *intrinsic* concentration is the amount of a compound per unit cell mass or cell volume. *Extrinsic* concentrations, the amount of a compound per unit reactor volume, cannot be used in kinetic expressions. Although this may seem self-evident, all the early structured models were flawed by the use of extrinsic concentrations. A second consideration, closely related to the first, is that the dilution of intrinsic concentration by growth must be considered.

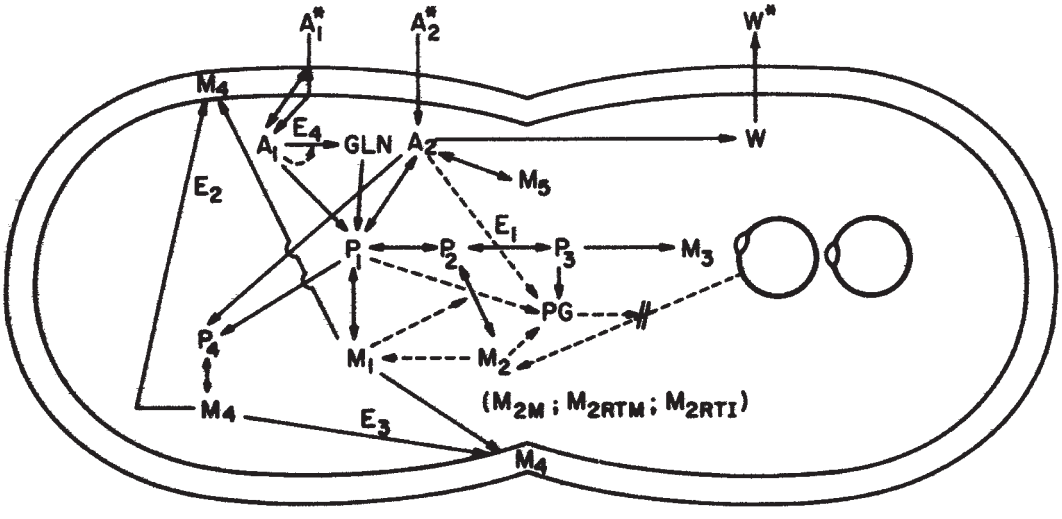


Figure 6.14. An idealized sketch of the model *Escherichia coli* B/rA growing in a glucose–ammonium salts medium with glucose or ammonia as the limiting nutrient. At the time shown, the cell has just completed a round of DNA replication and initiated cross-wall formation and a new round of DNA replication. Solid lines indicate the flow of material, and dashed lines indicate flow of information. The symbols are A_1, ammonium ion; A_2, glucose (and associated compounds in the cell); W, waste products (CO_2, H_2O, and acetate) formed from energy metabolism during aerobic growth; P_1, amino acids; P_2, ribonucleotides; P_3, deoxyribonucleotides; P_4, cell envelope precursors; M_1, protein (both cytoplasmic and envelope); M_{2RTI}, immature "stable" RNA; M_{2RTM}, mature "stable" RNA (*r*RNA and *t*RNA—assume 85% *r*RNA throughout); M_{2M}, messenger RNA; M_3, DNA; M_4, nonprotein part of cell envelope (assume 16.7% peptidoglycan, 47.6% lipid, and 35.7% polysaccharide); M_5, glycogen; PG, ppGpp; E_1, enzymes in the conversion of P_2 to P_3; E_2, E_3, molecules involved in directing cross-wall formation and cell envelope synthesis; GLN, glutamine; E_4, glutamine synthetase; * indicates that the material is present in the external environment. (With permission, from M. L. Shuler and M. M. Domach, in *Foundations of Biochemical Engineering*, H. W. Blanch, E. T. Papoutsakis, and G. Stephanopoulos, ed., ACS Symposium Series 207, American Chemical Society, Washington, DC, 1983, p. 93.)

The appropriate equation to use in a nonflow reactor is

$$\underbrace{\frac{d[V_R C_i]}{dt}}_{\substack{\text{rate of change}\\ \text{in amount of } i\\ \text{in the reactor}}} = \underbrace{V_R X}_{\substack{\text{total}\\ \text{biomass}\\ \text{in reactor}}} \times \underbrace{r_{fi}}_{\substack{\text{rate of}\\ \text{formation}\\ \text{of } i \text{ per}\\ \text{unit biomass}\\ \text{based on intrinsic}\\ \text{concentrations}}} \tag{6.66}$$

where V_R is the total volume in the reactor, X is the extrinsic biomass concentration, and C_i is the extrinsic concentration of component i.

Equation 6.66 can also be rewritten in terms of intrinsic concentrations. For simplicity, we use mass fractions (e.g., C_i/X). Note the following:

$$\frac{d(C_i/X)}{dt} = \left(\frac{1}{X}\frac{dC_i}{dt}\right) - \left(C_i/X\,\frac{dX/dt}{X}\right) \tag{6.67}$$

Recall equation 6.2a:

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{6.2a}$$

We now have

$$\frac{d(C_i/X)}{dt} = \left(\frac{1}{X}\frac{dC_i}{dt}\right) - \mu C_i/X \tag{6.68}$$

Substituting equation 6.66 for $(1/X)(dC_i/dt)$ after dividing the equation by $V_R X$ and assuming V_R is a constant, we get the following:

$$\frac{d(C_i/X)}{dt} = r_{fi} - \frac{\mu_{\text{net}} C_i}{X} \tag{6.69}$$

In equation 6.69, the r_{fi} term must be in terms of intrinsic concentrations and the term $\mu_{\text{net}} C_i/X$ represents dilution by growth. These concepts are illustrated in Example 6.3.

The model indicated in Figure 6.14 is that for a single cell. The single-cell model response can be directly related to culture response if all cells are assumed to behave identically. In this case, each cell has the same division cycle. A population will have, at steady state, twice as many cells at "birth" as at division. The average concentrations in the culture will be at the geometric mean (a time equal to $\sqrt{2}$ multiplied by the division time) for each cell component. Used in this way, the model is a structured, nonsegregated model. If, however, a cellular population is divided into subpopulations, with each subpopulation represented by a separate single-cell model, then a population model containing a high level of structure, as well as aspects of segregation, can be built.

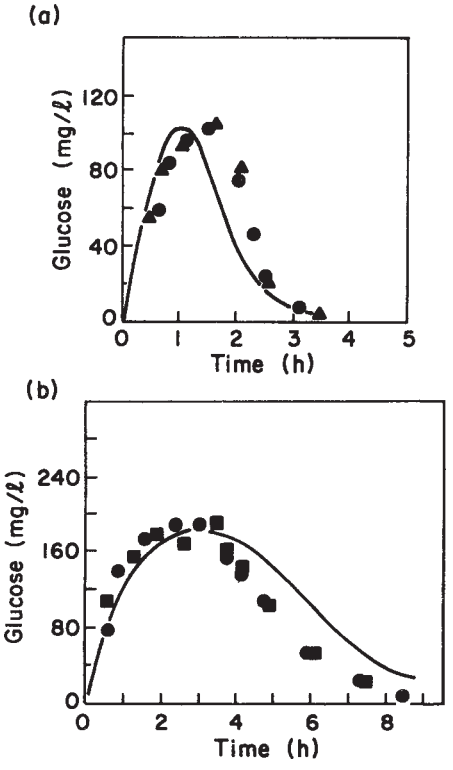


Figure 6.15. Prediction of transient response in a continuous-flow system to perturbations in limiting substrate concentration or flow rate. Note that the predictions are not fitted to the data; predictions are completely a priori. (a) Transient glucose concentration in response to step change in glucose feed concentration from 1.0 to 1.88 g/l in anaerobic continuous culture of *Escherichia coli* B/r at dilution rate of $0.38\ h^{-1}$; ● and ▲, results of two duplicate experimental runs; solid line, computer prediction. (b) Transient glucose concentration in response to step change in dilution rate from $D = 0.16$ to $D = 0.55\ h^{-1}$; ● and ■, results of two duplicate experimental runs; solid line, computer prediction. (With permission, from M. M. Ataai and M. L. Shuler, Simulation of CFSTR through development of a mathematical model for anaerobic growth of *Escherichia coli* cell population. *Biotechnol. Bioeng*. 27:7, p. 1051, 1985, and John Wiley & Sons, Inc., New York.)

Such a finite-representation technique has been used and is capable of making good a priori predictions of dynamic response in cultures (see Figure 6.15). When used in this context, at least one random element in the cell cycle must be included to lead to realistic prediction of distributions. Some examples of such randomness include placement of the cell cross wall, timing of the initiation of chromosome synthesis or cross-wall synthesis, and distribution of plasmids at division. Models that incorporate genomic detail have been developed. Such models reveal the explicit relationship of genetic modifications to physiological response. While these models are beyond the scope of an introductory text, be aware that many advances have been made in this regard.

With this section, you should have a good overview of the basic concepts in modeling and some simple tools to describe microbial growth. We need these tools to adequately discuss the cultivation of cells in continuous culture. Example 6.3 illustrates how a structured model might be developed.

Example 6.3 Building a Simple Single-Cell Model

Write the equations describing the following system. An organism consists of active biomass and a storage component. The storage component is made when the internal concentration of the carbon–energy sources is high and is degraded when it is low. Use the symbols A = active biomass, P = polymeric storage compound, S^* = external

concentration of S, and S = internal concentration of S. Assume that S is the growth-rate-limiting nutrient.

Solution When measuring the concentration of A, P, and S^*, the natural units are grams per liter of reactor volume. These are extrinsic concentrations. The following will be the total biomass concentration, since the internal concentration of S will be low [$<0.001(A + P)$]:

$$X = A + P + S \approx A + P \tag{a}$$

By considering S^* and S separately, transients in S can be captured. However, these transients will be fast in comparison to changes in A and P. The Monod and other unstructured models assume an instantaneous equilibrium between S^* and S.

A number of kinetic descriptions would be defensible. However, let us assume the following pseudochemical equations:

$$A + \alpha_1 S + \cdots \rightarrow 2A + \cdots \tag{b}$$

Here α_1 g of S is consumed to make 1 g of A plus by-products; the reaction requires both A as a "catalyst" and S for building blocks. Similarly, for P, we assume the following:

$$A + \alpha_2 S + \cdots \rightleftharpoons P + A + \cdots \tag{c}$$

where P is formed reversibly in this parallel reaction requiring A as the catalyst, and α_2 is a stoichiometric coefficient between the monomer S and the polymer P (g · S consumed/g · P formed).

The actual reaction mechanisms implied in these reactions are likely very complex. Most reactions in a cell are saturable and subject to feedback control. For these reactions, the intracellular level of S is the main control variable. Thus, a reasonable set of equations based on equation 6.66 for constant V_R follows:

$$\frac{dS^*}{dt} = X\frac{k_1 S^*}{K_{xs^*} + S^*} \tag{d}$$

where k_1 is the rate of uptake per unit cell mass $\left(\text{g } S \text{ transported/g } X\text{-h}\right)$ and K_{XS^*} is the saturation parameter for uptake. Since S^* is not a cellular component, extrinsic concentrations can be used in the rate expression. For S we write

$$\frac{dS}{dt} = X\left\{\underbrace{\frac{k_1 S^*}{K_{XS^*} + S}}_{\substack{\text{rate of} \\ \text{uptake}}} - \alpha_1 \underbrace{\frac{k_2 S/X}{K_{AS} + S/X}(A/X)}_{\substack{\text{rate of } S \text{ used} \\ \text{to form } A}} - \alpha_2\left[\frac{k_3 S/X}{K_{PSf} + S/X}(A/X) - \frac{k_4 K_{PSd}}{K_{PSd} + S/X}(P/X)\right]\right\} \tag{e}$$

net rate of S consumption to form P

where k_2 is g A formed/g$\cdot$ A present-h, k_3 is g P formed/g$\cdot$ A present-h, and k_4 is a first-order degradation rate for $P(h^{-1})$. The parameters α_1 and α_2 are defined in equations (b) and (c), while K_{AS}, K_{PSf}, and K_{PSd} are saturation parameters in units of mass fraction. Similarly, we write the following:

$$\frac{dA}{dt} = X\left[\frac{k_2 S/X}{K_{AS}+S/X}(A/X) - k_5(A/X)\right] \tag{f}$$

rate of formation of A — decomposition of A to provide maintenance energy

where k_5 is a first-order coefficient (h^{-1}) and

$$\frac{dP}{dt} = X\left[\frac{k_3 S/X}{K_{PSf}+S/X}(A/X) - \frac{k_4 K_{PSd}}{K_{PSd}+S/X}(P/X)\right] \tag{g}$$

Equations (f) and (g) describe the rate of formation of A and P.

The value of k_1 could be determined by the uptake of a nonmetabolizable analog of the substrate, use of vesicles, or material balance information on exponentially growing cells. Values of k_2 and k_3 can be estimated from measurements on total biomass and polymer under conditions where S/X should be large (e.g., exponential growth), while k_4 and k_5 can be estimated from time-dependent measurements of cell composition in the stationary phase, where S/X should be low. If experimental values of S/X are available at various times, then more refined estimates of k_2, k_3, k_4, and k_5 can be made, as well as estimates of the saturation parameters. The rate form chosen in equation (g) requires that $K_{PSd} << K_{PSf}$, so that P is made only when S/X is in excess and degraded only when S/X is low. The values for α_1 and α_2 are essentially the reciprocal of the yield coefficients and can be readily estimated from growth experiments and compositional data on P.

The rate forms used here are by no means the only correct solution. A more extensive base of experimental observations would be needed to eliminate other possible formulations.

6.2.3. Cybernetic Models

Another modeling approach has been developed primarily to predict growth under conditions when several substrates are available. These substrates may be complementary (e.g., carbon or nitrogen) or substitutable. For example, glucose and lactose would be substitutable, as these compounds both supply carbon and energy. We discussed the diauxic phenomenon for sequential use of glucose and lactose in Chapter 4, "How Cells Work." That experimental observation led us to an understanding of regulation of the *lac* operon and catabolite repression. This metabolic regulation was necessary for the transition from one primary pathway to another. We might infer that the culture had as its objective function the maximization of its growth rate.

One approach to modeling growth on multiple substrates is a *cybernetic* approach. Cybernetic means that a process is goal seeking (e.g., maximization of growth rate). While this approach was initially motivated by a desire to predict the response of a microbial culture to growth on a set of substitutable carbon

sources, it has been expanded to provide an alternative method of identifying the regulatory structure of a complex biochemical reaction network (such as cellular metabolism) in a simple manner. Typically, a single objective, such as maximum growth rate, is chosen and an objective-oriented mathematical analysis is employed. This analysis is similar to many economic analyses for resource distribution. For many practical situations, this approach describes satisfactorily growth of a culture on a complex medium. However, the potential power of this approach is now being realized in metabolic engineering and in relating information on DNA sequences to physiological functions of organisms (see Chapter 8, "How Cellular Information Is Altered").

This approach has limitations, as the objective function for any organism is maximizing its long-term survival as a species. Maximization of growth rate or of growth yield are really subobjectives that can dominate under some environmental conditions; these conditions are often of great interest to the bioprocess engineer. Consequently, the cybernetic approach is often a valuable tool. It is too complex for us to describe in detail in this book; consult the references at the end of this chapter for more information.

6.3. CELL GROWTH IN CONTINUOUS CULTURE

The culture environment changes continually in a batch culture. Growth, product formation, and substrate utilization terminate after a certain time interval, whereas in continuous culture, fresh nutrient medium is continually supplied to a well-stirred culture, and products and cells are simultaneously withdrawn. Growth and product formation can be maintained for prolonged periods in continuous culture. After a certain period of time, the system usually reaches a steady state where cell, product, and substrate concentrations remain constant. Continuous culture provides constant environmental conditions for growth and product formation and supplies uniform-quality product. Continuous culture is an important tool to determine the response of microorganisms to their environment and to produce the desired products under optimal environmental conditions. In Chapter 9, we will compare batch and continuous culture in terms of their suitability for large-scale operation.

6.3.1. Specific Devices for Continuous Culture

The primary types of continuous cultivation devices are the *chemostat* and *turbidostat*, although plug flow reactors (PFRs) are used. In some cases, these units are modified by the recycling of cells.

Figure 6.16 is a schematic of a continuous culture device (chemostat). Cellular growth is usually limited by one essential nutrient, and other nutrients are in excess. As we will show, when a chemostat is at steady state, the nutrient, product, and cell concentrations are constant. For this reason, the name *chemostat* refers to constant chemical environment.

Figure 6.17 is a schematic of a turbidostat in which the cell concentration in the culture vessel is maintained constant by monitoring the optical density of the culture and controlling the feed flow rate. When the turbidity of the medium exceeds the set point, a pump is activated and fresh medium is added. The culture volume is

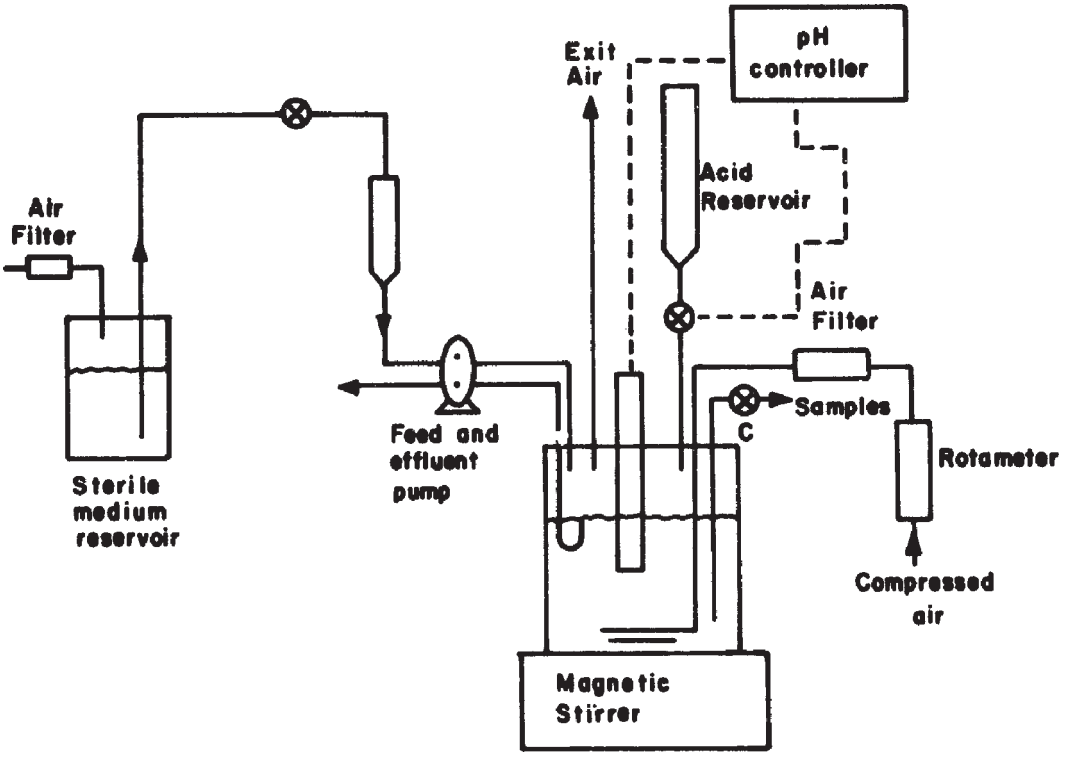


Figure 6.16. A continuous-culture laboratory setup (chemostat). (With permission, from D. I. C. Wang and others, *Fermentation and Enzyme Technology*, John Wiley & Sons, Inc., New York, 1979, p. 99. Permission conveyed through Copyright Clearance Center, Inc.)

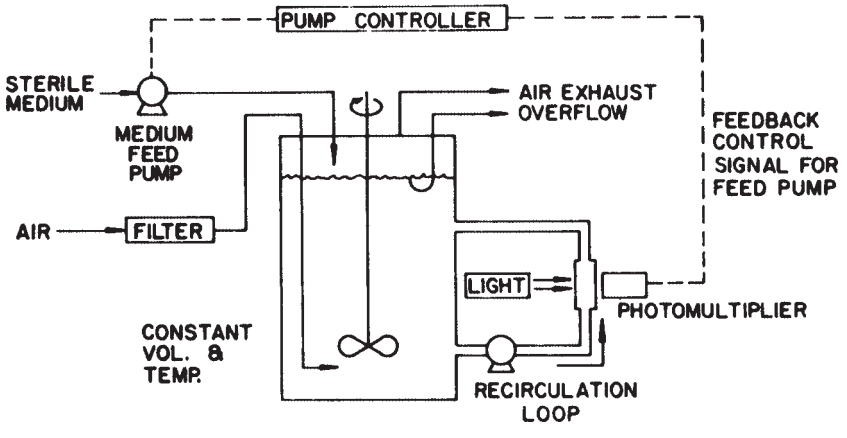


Figure 6.17. Typical laboratory setup for a turbidostat. (With permission, from D. I. C. Wang and others, *Fermentation and Enzyme Technology*, John Wiley & Sons, Inc., New York, 1979, p. 100. Permission conveyed through Copyright Clearance Center, Inc.)

kept constant by removing an equal amount of culture fluid. The turbidostat is less used than the chemostat, since it is more elaborate than a chemostat and because the environment is dynamic. Turbidostats can be very useful in selecting subpopulations able to withstand a desired environmental stress (e.g., high ethanol concentrations), because the cell concentration is maintained constant. The selection of variants or mutants with desirable properties is very important (see Chapter 8).

A PFR can also be used for continuous cultivation purposes. Since there is no backmixing in an ideal PFR, fluid elements containing active cells cannot inoculate other fluid elements at different axial positions. Liquid recycle is required for continuous inoculation of nutrient media. In a PFR, substrate and cell concentrations vary with axial position in the vessel. An ideal PFR resembles a batch reactor in which distance along the fermenter replaces incubation time in a batch reactor. In waste treatment, some units approach PFR behavior, and multistage chemostats tend to approach PFR dynamics if the number of stages is large (five or more).

6.3.2. The Ideal Chemostat

An ideal chemostat is the same as a perfectly mixed continuous-flow, stirred-tank reactor (CFSTR). Most chemostats require some control elements, such as pH and DO control units, to be useful. Fresh sterile medium is fed to the completely mixed and aerated (if required) reactor, and cell suspension is removed at the same rate. Liquid volume in the reactor is kept constant. A critical feature of a chemostat is that a steady state arises spontaneously where the growth rate is determined by the feed flow rate to per unit of reactor volume (or dilution rate as defined in equation 6.71).

Figure 6.18 is a schematic of a simplified chemostat. A material balance on the cell concentration around the chemostat yields

$$FX_0 - FX + V_R \mu_g X - V_R k_d X = V_R \frac{dX}{dt} \tag{6.70}$$

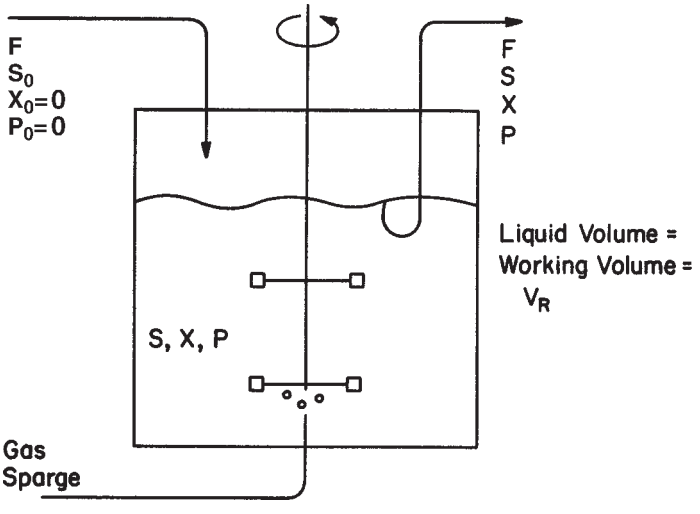


Figure 6.18. Simplified schematic of a chemostat.

where F is the flow rate of nutrient solution (l/h), V_R is the culture volume (l) (assumed constant), X is the cell concentration (g/l), and μ_g and k_d are growth and endogenous (or death) rate constants, respectively (h^{-1}). Note that if cell mass is the primary parameter, it is difficult to differentiate cell death from endogenous metabolism. When we use k_d, we imply that endogenous metabolism is the primary mechanism for cell mass decrease. With k'_d, we imply that cell death and lysis are the primary mechanisms of decrease in mass. Also note that if equation 6.70 had been written in terms of cell number, k_d could only be a cell death rate. When balances are written in terms of cell number, the influence of endogenous metabolism can appear only in the substrate balance equation. Since most experiments are done by measuring total cell mass rather than number, we write our examples based on X. However, be aware of the ambiguity introduced when equations are written in terms of X.

Equation 6.70 can be rearranged as follows:

$$\frac{dX}{dt} = DX_0 + (\mu_g - k_d - D)X \tag{6.71}$$

where D is *dilution rate* and $D = F/V_R$. D is the reciprocal of hydraulic residence time (V/F).

Usually, the feed media are sterile, $X_0 = 0$, and if the endogenous metabolism or death rate is negligible compared to the growth rate ($k_d \ll \mu_g$) and if the system is at steady state $(dX/dt = 0)$, then we get the following:

$$\mu_g = D \quad (\text{if } k_d = 0) \tag{6.72}$$

In a chemostat, cells are removed at a rate equal to their growth rate, and the growth rate of cells is equal to the dilution rate. This property allows the investigator to manipulate growth rate as an independent parameter and makes the chemostat a powerful experimental tool.

Since growth rate is limited by at least one substrate in a chemostat, a simple description of chemostat performance can be made by substituting the Monod equation (equation 6.32) for μ_g in equation 6.72:

$$\mu_g = D = \frac{\mu_m S}{K_s + S} \tag{6.73}$$

Here S is the steady-state limiting substrate concentration (g/l). If D is set at a value greater than μ_m, the culture cannot reproduce quickly enough to maintain itself and is *washed out* (i.e., no substrate is used [$S = S_0$] and no biomass is formed [$X = 0$]). Equation 6.73 is identical to that for Michaelis–Menten kinetics, and as we discussed in Chapter 3, "Enzymes," a plot of $1/\mu_g$ versus $1/S$ can be used to estimate values for μ_m and K_s.

Using equation 6.73, we can relate effluent substrate concentration to dilution rate for $D < \mu_m$:

$$S = \frac{K_s D}{\mu_m - D} \tag{6.74}$$

A material balance on the limiting substrate yields the following:

$$FS_0 - FS - V_R u_g X\left(1/Y^M_{x/s}\right) - V_R q_p X\left(1/Y_{p/s}\right) - m_s X V_R = V_R\left(dS/dt\right) \qquad (6.75)$$

Here S_0 and S are feed and effluent substrate concentrations (g/l), q_P is the specific rate of extracellular product formation (g P/g X-h), $Y^M_{X/S}$, and $Y_{P/S}$ are yield coefficients (g X/g S and gP/gS), and m_s is the maintenance coefficient based on substrate (g S/g X-h). The use of the superscript M on $Y_{X/S}$ denotes a maximum value of the yield coefficient; such a superscript will be important in discussing the effects of maintenance energy. Equation 6.75 can be written as follows:

$$\frac{F(S_0 - S)}{V_R X} = q_s = \frac{\mu_g}{Y^M_{X/S}} + \frac{q_p}{Y_{P/S}} + m_s \qquad (6.76)$$

Equation 6.76 is known as the *linear equation of substrate consumption*, which is an explicit expression of the substrate balance given by equation 6.14.

When the extracellular product formation and the maintenance are negligible (q_p and m_s are negligible) and the system is at steady state (dS/dt = 0), equation 6.76 takes the following form:

$$D(S_0 - S) = \frac{\mu_g X}{Y^M_{Y/S}} \qquad (6.77)$$

Since $\mu_g = D$ at steady state, if $k_d = 0$, then we have the following:

$$X = Y^M_{X/S}(S_0 - S) \qquad (6.78)$$

Using equation 6.74, the steady-state cell concentration can be expressed as follows:

$$X = Y^M_{Y/S}\left(S_0 - \frac{K_s D}{\mu_m - D}\right) \qquad (6.79)$$

In equations 6.77 and 6.78, the yield coefficient $\left(Y_{X/S}\right)$ is assumed to be constant and equal to $Y^M_{X/S}$, which is an approximation, since endogenous metabolism has been neglected. Usually, $Y_{X/S}$ varies with the limiting nutrient and growth rate. When endogenous metabolism or death rate are not negligible, equation 6.72 takes the following form:

$$D = \mu_g - k_d = \mu_{net} \qquad (6.80a)$$

or

$$\mu_g = D + k_d \qquad (6.80b)$$

By substituting equation 6.80b into the steady-state substrate balance, assuming no extracellular product formation (equation 6.77), we find the following:

$$D(S_0 - S) - 1/Y^M_{X/S}(D + k_d)X = 0 \tag{6.80c}$$

Here $Y^M_{X/S}$ denotes the maximum yield coefficient (no endogenous metabolism or maintenance energy). $Y^M_{X/S}$ is constant and independent of growth rate. Since this approach is based on an unstructured model, it does not allow the direct conversion of extracellular substrates into maintenance energy. Rather, S must be first incorporated into the cell mass, where it is degraded by endogenous metabolism. This viewpoint is the proper one when working with an unstructured model. With a structured model, which explicitly recognizes intracellular S as a subcomponent of the biomass, the direct consumption of S for maintenance functions could be modeled. However, with unstructured models, substrate that is no longer extracellular becomes part of the biomass, X.

Equation 6.80c can be rearranged as follows:

$$D\left(\frac{S_0 - S}{X}\right) - 1/Y^M_{X/S}(D + k_d) = 0 \tag{6.81}$$

or

$$D\left(\frac{1}{Y^{AP}_{X/S}}\right) - \frac{D}{Y^M_{X/S}} - \frac{k_d}{Y^M_{X/S}} = 0 \tag{6.82a}$$

$$\frac{1}{Y^M_{X/S}} + \frac{k_d}{Y^M_{X/S} \cdot D} = \frac{1}{Y^{AP}_{X/S}} \tag{6.82b}$$

or

$$\frac{1}{Y^{AP}_{X/S}} = \frac{1}{Y^M_{X/S}} + \frac{m_s}{D} \tag{6.83}$$

where

$$m_s = \frac{k_d}{Y^M_{X/S}} \tag{6.84}$$

where m_s is the *maintenance coefficient* based on substrate S. $Y^{AP}_{X/S}$ is the apparent yield. When $Y_{X/S}$ is written, it should be interpreted as $Y^{AP}_{X/S}$. While $Y^M_{X/S}$ is a constant, $Y^{AP}_{X/S}$ varies with growth conditions if $k_d > 0$.

Values of $Y^M_{X/S}$ and m_s can be obtained from chemostat experiments by plotting $1/Y^{AP}_{X/S}$ against $1/D$. The slope is m_s and the intercept is $1/Y^M_{X/S}$ (see Figure 6.19).

In the presence of endogenous metabolism [$\mu_{net} = \mu_m S/(K_s + S) - k_d$], we can also show the following:

$$S = \frac{K_s(D + k_d)}{\mu_m - D - k_d} \tag{6.85}$$

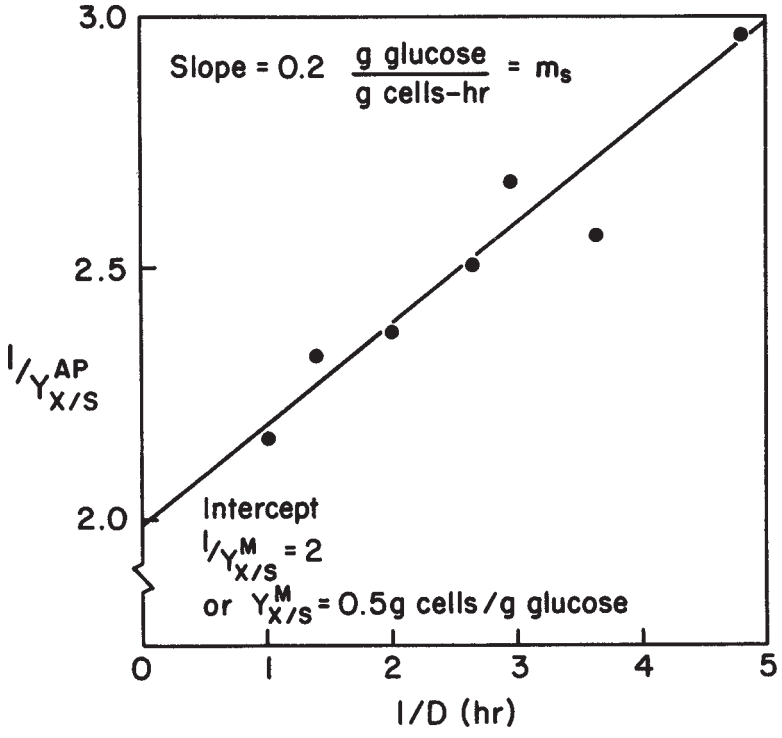


Figure 6.19. Graphical approach to estimating $Y^M_{X/S}$ and m_S for chemostat data for *Escherichia coli* growing on glucose as the limiting nutrient.

and

$$X = Y^M_{X/S}[S_0 - S] \cdot \frac{D}{D + k_d} \tag{6.86}$$

In addition to the effects of maintenance, it is often appropriate to consider the conversion of extracellular substrate into extracellular product. With an unstructured model, we must view this as instantaneous conversion. Otherwise, a perceptible period of substrate uptake, prior to conversion to product, would cause a change in the amount of X. This is unlike maintenance, since the period between substrate uptake and use for maintenance functions may be long, so we allow S to become incorporated into X, and X is then degraded when needed; the following equations assume instantaneous conversion of substrate to extracellular product, with the cell acting as a catalyst. The balance on product formation is

$$DP = q_P X \tag{6.87}$$

where q_P is described by equation 6.19 or 6.20. For nongrowth-associated product formation, q_P is constant (β), and for growth-associated products, it is a function

of μ_g. In the presence of product formation and with negligible maintenance, equation 6.76 becomes

$$D(S_0 - S) = \frac{1}{Y^M_{X/S}}(D + k_d)X + \frac{1}{Y_{P/S}} q_P X \tag{6.88}$$

The biomass balance is unchanged from the case with endogenous metabolism (given in eq. 6.85) and yields

$$S = \frac{K_s(D + k_d)}{\mu_m - D - k_d}$$

Equation 6.87 can be solved for X:

$$X = Y^M_{X/S}(S_0 - S)\left(\frac{D}{D + k_d + q_P \dfrac{Y^M_{X/S}}{Y_{P/S}}}\right) \tag{6.89}$$

The productivity of a chemostat for product and biomass (Pr_x) can be found from DP and DX, respectively. The dilution rate that maximizes productivity is found by differentiating DP or DX with respect to D and setting the derivative equal to zero. The optimal value of D (D_{opt}) will depend on whether endogenous metabolism and/or product formation are considered. When $k_d = 0$ and $q_P = 0$, equations 6.78 and 6.74 apply. Then D_{opt} for biomass production (DX) becomes

$$D_{\text{opt}} = \mu_m\left(1 - \sqrt{\frac{K_s}{K_s + S_0}}\right) \tag{6.90}$$

Since S_0 is usually much greater than K_s, D_{opt} will approach $D = \mu_m$, or the washout point. Stable chemostat operation with $D \simeq \mu_m$ is very difficult unless the flow rate and liquid volume can be maintained exactly constant. Consequently, a value of D slightly less than D_{opt} may be a good compromise between stability and biomass productivity. It should also be apparent that D_{opt} for biomass formation will not necessarily be optimal for product formation.

Examples 6.4 and 6.5 illustrate the use of these equations to characterize the performance of chemostats.

Example 6.4. Calculating Basic Chemostat Parameters

A new strain of yeast is being considered for biomass production. The following data were obtained using a chemostat. An influent substrate concentration of 800 mg/l and an excess of oxygen were used at a pH of 5.5 and T = 35°C. Using the following data, calculate μ_m, K_s, $Y^M_{X/S}$, k_d, and m_s, assuming $\mu_{\text{net}} = \mu_m S/(K_s + S) - k_d$.

Dilution Rate (h^{-1})	Carbon Substrate Concentration (mg/l)	Cell Concentration (mg/l)
0.1	16.7	366
0.2	33.5	407
0.3	59.4	408
0.4	101	404
0.5	169	371
0.6	298	299
0.7	702	59

Solution The first step is to plot $1/Y_{X/S}^{AP}$ versus $1/D$. $Y_{X/S}^{AP}$ is calculated from $X/(S_0 - S)$. The intercept is $1/Y_{X/S}^{M} = 1.58$ or $Y_{X/S}^{M} = 0.633\,\text{g}\,X/\text{g}\,S$. The slope is 0.06 g S/g X-h, which is the value for m_s. Recall $m_s = k_d / Y_{X/S}^{M}$; then $k_d = m_s Y_{X/S}^{M} = (0.06\ \text{g}S/\text{g}\ X\text{-h})(0.633\ \text{g}X/\text{g}\ S) = 0.038\ \text{h}^{-1}$. For the second step, recall the following:

$$D = \mu_g - k_d = \frac{\mu_m S}{K_s + S} - k_d \tag{a}$$

Then recall the following:

$$\frac{1}{D + k_d} = \frac{1}{\mu_m} + \frac{K_s}{\mu_m}\frac{1}{S} \tag{b}$$

We now plot $1/(D + k_d)$ versus $1/S$. The intercept is 1.25 h or $\mu_m = 0.8\ \text{h}^{-1}$. The slope is 100 h/(mg/l). Thus, $K_s/m_m = 100$ or $K_s = 80$ mg/l.

Example 6.5. Effect of Inhibitors on Chemostat Growth

The specific growth rate for inhibited growth in a chemostat is given by the following equation:

$$\mu_g = \frac{\mu_m S}{K_s + S + IK_s / K_I} \tag{a}$$

Assume that the following parameters have been evaluated for this culture:

$$S_0 = 10\,\text{g/l} \qquad K_s = 1\,\text{g/l} \qquad I = 0.05\,\text{g/l} \qquad Y_{X/S}^{M} = 0.1\,\frac{\text{g cells}}{\text{g subs}}$$

$$X_0 = 0 \qquad K_I = 0.01\,\text{g/l} \qquad \mu_m = 0.5\,\text{h}^{-1} \qquad k_d = 0$$

a. Determine X and S as a function of D when $I = 0$.
b. With inhibitor added to a chemostat, determine the effluent substrate concentration and X as a function of D.
c. Determine the cell productivity, DX, as a function of dilution rate.

Solution

a. The following equations apply when there is no inhibitor:

$$S = \frac{K_s D}{\mu_m - D} = \frac{D}{0.5 - D}$$

$$X = Y_{X/S}^{M}(S_0 - S) = 0.1\left(10 - \frac{D}{0.5 - D}\right)$$

b. In the presence of inhibitor, we use the following:

$$\mu_g = \frac{\mu_m S}{K_s\left(1 + \frac{\mathrm{I}}{K_1}\right) + S} = D$$

$$S = \frac{K_s\left(1 + \frac{\mathrm{I}}{K_1}\right)D}{\mu_m - D} = \frac{I\left(1 + \frac{0.05}{0.01}\right)D}{0.5 - D}$$

$$S = \frac{6D}{0.5 - D}$$

$$X = Y_{X/S}^{M}(S_0 - S) = 0.1\left(10 - \frac{6D}{0.5 - D}\right)$$

c. The cell productivity can then be calculated from the following:

$$Pr_x = DX = D\,Y_{X/S}(S_0 - S) = 0.1D\left(10 - \frac{6D}{0.5 - D}\right)$$

6.3.3. The Chemostat as a Tool

The chemostat can be used as a tool to study the mutation and selection of cultures and also to study the effect of changes in the environment on cell physiology. The molecular aspects of mutation and selection are discussed in Chapter 8.

Natural or induced *mutations* can take place in a chemostat culture. Errors in DNA replication take place with an average frequency of about 10^{-6} to 10^{-8} genes per generation. With a cell concentration of 10^9 cells/ml in culture, the probability is high in a chemostat that a wide variety of mutant cells will be formed. The vast majority of natural mutations in a chemostat are of little significance unless the mutation alters the function of a protein involved in growth in the chemostat environment. If the specific growth rate of the mutant is larger than that of the wild type, then the mutant outgrows the wild type in a chemostat. This selection for a variant cell type can be accomplished by creating a more favorable environment for growth of the mutant organism.

A chemostat culture can be used for the selection of special organisms. Selection or enrichment nutrient media need to be used for this purpose. For example, if it is desired to select an organism growing on ethanol, a nutrient medium containing

ethanol and mineral salts is used as a feed to a chemostat culture. An organism capable of oxidizing some toxic refractory compounds can be selected from a mixed culture by slowly feeding this compound to a chemostat. A thermophilic organism can be selected from a natural population by operating a chemostat at an elevated temperature (e.g., 50° to 60°C). Selection in chemostats also presents significant problems in the culture of cells containing recombinant DNA. The most productive cells often grow more slowly and are displaced by less productive cells. We discuss this problem in more detail in Chapter 14, "Utilizing Genetically Engineered Organisms."

6.3.4. Deviations from Ideality

In fermentations such as the utilization or production of polysaccharides or mycelial fermentations, the fermentation broth may be highly viscous and it may be difficult to maintain complete mixing. Also, certain cells tend to grow in the form of a film on solid surfaces in fermenters, such as on fermenter walls or probe surfaces. Incomplete mixing is the rule in industrial-scale fermenters. The presence of incompletely mixed regions in a "chemostat" culture is common. The term *chemostat* is actually applicable only to perfectly mixed vessels. Here we denote nonideality by putting "chemostat" in quotation marks. A segregated reactor model can be used to analyze an incompletely mixed continuous-flow fermenter.

A simple, two-compartmental model of a "chemostat" culture is depicted in Figure 6.20, where the reactor is divided into two regions: a well-mixed region and a stagnant region. Feed and effluent streams pass through the well-mixed region 1, and mass exchange takes place between the two regions.

In an imperfectly mixed chemostat with a stagnant biotic phase, the dilution rate can exceed μ_m without washout. The washout dilution rate is obtained by setting $S_0 = S_1 = S_2$:

$$D_{wo} = \frac{\mu_m S_0}{K_m + S_0}\left[\frac{(1-\alpha)^2 \mu_m S_0}{K_s + S_0 - (1-\alpha)D'\mu_m S_0} + 1\right] \tag{6.91}$$

where D_{wo} is the washout dilution rate.

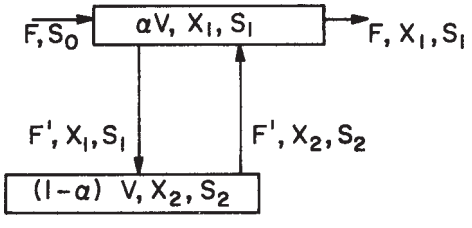


Figure 6.20. Simple two-compartmental model for an incompletely mixed "chemostat."

6.4. SUMMARY

During batch cultivation, a population of cells typically exhibits several different growth phases. During the lag phase, the cell builds the biosynthetic pathways necessary for maximal growth rates in the fresh medium. During *exponential growth*, cell replication is maximal, and the chemical composition of the cell population is nearly constant (e.g., balanced growth). When substrate is nearly exhausted or when toxic metabolic by-products have built to a critical level, the growth rate begins to drop rapidly, causing significant changes in biosynthetic pathways. In the *stationary phase*, there is no net growth; cells now reorient their metabolic machinery to increase the probability of long-term survival. At some point, some cells can no longer obtain enough energy from their reserves or enough of another critical resource, and the culture enters the *death phase*. Dead cells do not have an energized membrane and often lyse (or break apart). Nutrients released by lysed cells can be utilized by survivors, allowing cryptic growth.

Products formed by cells can be related to this batch-culture growth cycle. Primary products are growth associated. Secondary products are nongrowth associated and are made in the stationary phase. Some products have both growth-associated and nongrowth-associated components.

Cell-growth kinetics can be modeled. Models that are *structured* break the population into distinct subcomponents. *Unstructured* models quantify cell mass as a single component. Unstructured models cannot describe transient behavior very well. Some models are *segregated*; they recognize that not all members of the population are identical, and the distribution of properties among individual cells is important. *Nonsegregated models* ignore these distributions and assume that population-average parameters are adequate.

These models apply not only to batch culture but also to continuous culture. The primary form of continuous culture is a steady-state CFSTR, or *chemostat*. A chemostat ensures a time-invariant growth environment. The net growth rate is equal to the dilution rate, which is determined by the flow rate to the chemostat. Thus, the growth rate can be manipulated by the investigator. A *turbidostat* adjusts flow rate to maintain a constant cell density. A turbidostat operates well at high flow rates (near the washout point) and is useful in selecting cellular subpopulations that have adapted to a particular stress.

SUGGESTIONS FOR FURTHER READING

Atlas, J. P., P. Foley, and M. L. Shuler, "Modeling a Minimal Cell," *Meth. Mol. Biol.* 881: 573–610, 2012.

Bailey, J. E., and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill, New York, 1986.

Blanch, H. W., and D. S. Clark, *Biochemical Engineering*, Marcel Dekker, New York, 1996.

Doran, P. M., *Bioprocess Engineering Properties*, 2d ed., Academic Press, San Diego, CA, 2013.

Frederickson, A. G., R. D. Megee III, and H. M. Tsuchiya, "Mathematical Models for Fermentation Processes," *Adv. Appl. Microbiol.* 23(10): 419, 1970.

Ramkrishna, D., and H. Song, "Dynamic Models of Metabolism: Review of the Cybernetic Approach," *AIChE Journal* 58: 986–992, 2012.

Villadsen, J., J. Nielsen, and G. Liden, *Bioreaction Engineering Principles*, 2d ed., Springer, New York, 2011.

PROBLEMS

6.1. By using the following information, determine the cell number concentration in a fermenter containing 1 g dry weight/l biomass:
Cell dimensions: 1 × 2 μm cylindrical cells
Cell density: 1.05 g/cm^3
Water content of cells: 80%

6.2. Two different bacterial species with doubling times of 2 h and 4 h are growing in a batch nutrient-rich medium with their maximum growth rate. The initial concentrations are the same.
a. What would be the ratio of X_1/X_2 after 10 h of batch growth?
b. How long should the batch growth of the mixed cultures last in order to obtain $X_1/X_2 = 10$? Assume exponential growth.

6.3. A simple, batch fermentation of an aerobic bacterium growing on methanol gave the results shown in the following table. Calculate the following:
a. Maximum growth rate (μ_{max})
b. Yield on substrate ($Y_{X/S}$)
c. Mass doubling time (t_d)
d. Saturation constant (K_s)
e. Specific growth rate (μ_{net}) at t = 10 h

Time (h)	X (g/l)	S (g/l)
0	0.2	9.23
2	0.211	9.21
4	0.305	9.07
8	0.98	8.03
10	1.77	6.8
12	3.2	4.6
14	5.6	0.92
16	6.15	0.077
18	6.2	0

6.4. The growth of a microbial population is a function of pH and is given by the following equation:

$$\mu_g = \frac{1}{X}\frac{dX}{dt} = \frac{\mu_m S}{K_s\left(1+\dfrac{H^+}{k_1}\right)+S}$$

a. With a given set of experimental data (X and S versus t), describe how you would determine the constants μ_m, K_s, and k_1.

b. How would the double-reciprocal plot $1/\mu_g$ versus $1/S$ change with pH (or H^+) concentration?

6.5. The following data were obtained for the effect of temperature on the fermentative production of lactic acid by a strain of *Lactobacillus delbrueckii*. From these data, calculate the value of the activation energy for this process. Is the value of the activation energy typical of this sort of biological conversion? (See Chapter 3.)

Temperature (°C)	Rate Constant (mol/l-h)
40.4	0.0140
36.8	0.0112
33.1	0.0074
30.0	0.0051
25.1	0.0036

(Courtesy of A. E. Humphrey from "Collected Coursework Problems in Biochemical Engineering," compiled by H. W. Blanch for 1977 Am. Soc. Eng. Educ. Summer School. © American Society for Engineering Education.)

6.6. The logistic equation for batch microbial growth is given by the following equation:

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{X_m}\right)$$

a. Use the following approximations:

$$X_m = Y_{x/s}S_0,\ X = Y_{x/s}(S_0 - S)$$

Prove that the logistic equation can be expressed as follows:

$$\frac{dX}{dt} = kX\frac{S}{S_0}$$

b. By using the following relationship, develop an expression describing the variation of substrate concentration with time according to the logistic equation:

$$\frac{dX}{dt} = -Y_{x/s}\frac{dS}{dt}$$

c. By comparing the form of logistic equation derived in part (a) with the Monod equation at low substrate concentrations (first-order kinetics), determine the logistic equation constant (k) in terms of Monod kinetic constants.

6.7. It is desired to model the growth of an *individual* bacterium. The cell transports S_1 into the cell enzymatically, and the permease is subject to product inhibition. S_1 is converted into precursors, P, that are converted finally into the macromolecular portion of the cell, M. The catalyst of all reactions is M.

a. $S_1^* \xrightarrow[M]{} S_1$ (per unit surface area), where S^* = outside concentration of S

b. $S_1 \xrightarrow[M]{} P$

c1. $\left.\begin{array}{l}\text{Energy} + P \xrightarrow[M]{} M \\ S_1 \xrightarrow[M]{} \text{energy}\end{array}\right\}$ coupled reaction

c2. Or, $S_1 + P \xrightarrow[M]{} M$

d. The dry weight of the cell is T and is equal to $T = S_1 + P + M_1 = \rho V$, where ρ = cell density and V = cell volume

Write the mass balance equations and define all symbols necessary to describe the changes in S_1, P, M, and T within the cell. Remember that the cell volume is always changing.

6.8. A biochemical engineer has determined in her lab that the optimal productivity of a valuable antibiotic is achieved when the carbon nutrient, in this case molasses, is metered into the fermenter at a rate proportional to the growth rate. However, she cannot implement her discovery in the antibiotic plant, since there is no reliable way to measure the growth rate (dX/dt) or biomass concentration (X) during the course of the fermentation. It is suggested that an oxygen analyzer be installed on the plant fermenters so that the OUR (oxygen uptake rate, g/l-h) may be measured.

a. Derive expressions that may be used to estimate X and dX/dt from OUR and time data, assuming that a simple yield and maintenance model may be used to describe the rate of oxygen consumption by the culture.

b. Calculate values for the yield (Y_{X/O_2}) and maintenance (m_{O_2}) parameters from the following data:

Time	OUR (g/el-h)	X (g/l)
0	0.011	0.60
1	0.008	0.63
2	0.084	0.63
3	0.153	0.76
4	0.198	1.06
5	0.273	1.56
6	0.393	2.23
7	0.493	2.85
8	0.642	4.15
9	0.915	5.37
10	1.031	7.59
11	1.12	9.40
12	1.37	11.40
13	1.58	12.22
14	1.26	13.00
15	1.58	13.37
16	1.26	14.47
17	1.12	15.37
18	1.20	16.12
19	0.99	16.18
20	0.86	16.67
21	0.90	17.01

(Courtesy of D. Zabriskie from "Collected Coursework Problems in Biochemical Engineering," compiled by H. W. Blanch for 1977 American Society Engineering Education Summer School.)

6.9. *Pseudomonas* sp. has a mass doubling time of 2.4 h when grown on acetate. The saturation constant using this substrate is 1.3 g/l (which is unusually high), and cell yield on acetate is 0.46 g cell/g acetate. If we operate a chemostat on a feed stream containing 38 g/l acetate, find the following:

a. Cell concentration when the dilution rate is one-half of the maximum

b. Substrate concentration when the dilution rate is 0.8 D_{max}

c. Maximum dilution rate

d. Cell productivity at 0.8 D_{max}

(Courtesy of E. Dunlop from "Collected Coursework Problems in Biochemical Engineering," compiled by H. W. Blanch for 1977 Am. Soc. Eng. Educ. Summer School. © American Society for Engineering Education.)

6.10. The following data were obtained in a chemostat for the growth of *E. aerogenes* on a glycerol-limited growth medium:

D, h^{-1} Dilution Rate	$1/D$	S, mg/ml, Glycerol	$1/S$	X, mg/ml Cell Conc.	ΔS	$\Delta S/X$	$\Delta S/X \cdot D$
0.05	20	0.012	83.3	3.2	9.988	3.12	0.156
0.10	10	0.028	35.7	3.7	9.972	2.7	0.270
0.20	5.0	0.05	20	4.0	9.95	2.49	0.498
0.40	2.5	0.10	10	4.4	9.90	2.25	0.90
0.60	1.67	0.15	6.67	4.75	9.85	2.075	1.245
0.70	1.43	0.176	5.68	4.9	9.824	2.005	1.405
0.80	1.25	0.80	1.25	4.5	9.20	2.045	1.635
0.84	1.19	9.00	0.11	0.5	—	—	—

Note: S_0 = 10 mg/ml

For this system, estimate the following values:

a. K_s, mg glycerol/ml

b. μ_m, h^{-1}

c. $Y_{X/S}$, mg cells/mg glycerol

d. m_s, mg glycerol/mg cell-h

(Courtesy of A. E. Humphrey from "Collected Coursework Problems in Biochemical Engineering," compiled by H. W. Blanch for 1977 American Society for Engineering Summer School. © American Society for Engineering Education.)

6.11. The kinetics of microbial growth, substrate consumption, and mixed-growth-associated product formation for a chemostat culture are given by the following equations:

$$\frac{dX}{dt} = \frac{\mu_m S}{K_s + S} X$$

$$\frac{dS}{dt} = \frac{\mu_m S}{(K_s + S) Y_{X/S}} X$$

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X = (\alpha \mu_g + \beta) X$$

The kinetic parameter values are $\mu_m = 0.7\ h^{-1}$, $K_s = 20$ mg/l, $Y_{X/S} = 0.5$ g dw/g substrate, $Y_{P/X} = 0.15$ gP/g·dw, $\alpha = 0.1$, and $S_0 = 1$ g/l.

a. Determine the optimal dilution rate maximizing the productivity of product formation (PD) when $\beta = 0.02\ h^{-1}$ and $0.01\ h^{-1}$.

b. Determine the optimal dilution rate maximizing the productivity of cell (biomass) formation for the same value of β as in part (a) (DX).

(Problem adapted from one suggested by L. Erickson and modified by C. Lee.)

6.12. Ethanol is to be used as a substrate for single-cell protein production in a chemostat. The available equipment can achieve an oxygen transfer rate of 10 g O_2/l of liquid per hour. Assume the kinetics of cell growth on ethanol is of the Monod type, with $\mu_m = 0.5\ h^{-1}$, $K_s = 30$ mg/l, $Y_{X/S} = 0.5$ cells/g ethanol, and $Y_{O_2/S} = 2$ g O_2/g EtOH. We wish to operate the chemostat with an ethanol concentration in the feed of 22 g/l. We also wish to maximize the biomass productivity and minimize the loss of unused ethanol in the effluent. Determine the required dilution rate and whether sufficient oxygen can be provided.

6.13. Plot the response of a culture to diauxic growth on glucose and lactose based on the following: $\mu_{glucose} = 1.0\ h^{-1}$, $\mu_{lactose} = 0.6\ h^{-1}$, $Y_{glucose} = Y_{lactose} = 0.5$; enzyme induction requires 30 min to complete. Plot cell mass, glucose, and lactose concentrations, assuming initial values of 2 g/l glucose, 3 g/l lactose, and 0.10 g/l cells.

6.14. The following data are obtained in oxidation of pesticides present in wastewater by a mixed culture of microorganisms in a continuously operating aeration tank:

$D\ (h^{-1})$	S (Pesticides), mg/l	X (mg/l)
0.05	15	162
0.11	25	210
0.24	50	250
0.39	100	235
0.52	140	220
0.7	180	205
0.82	240	170

Assuming the pesticide concentration in the feed wastewater stream as $S_0 = 500$ mg/l, determine $Y^M_{X/S}, k_d, \mu_m$, and K_s.

6.15. Wastewater is treated in a completely mixed aeration tank operated in continuous mode. The rate of chemical oxygen demand (COD) removal is given by the following equation:

$$R_s = \frac{Q(S_0 - S)}{V} = D(S_0 - S) = D\left(S_0 - \frac{K_s D}{\mu_m - D}\right)$$

Here μ_m is the maximum specific growth rate constant (h^{-1}), D is the dilution rate (h^{-1}), K_s is saturation constant (mg/L), and S is the effluent COD concentration (mg/l).

a. Determine the optimum dilution rate or hydraulic residence time (HRT) maximizing COD removal rate.

b. Determine the optimal HRT, the maximum R_s and the biomass concentration for aerobic treatment where $\mu_m = 0.1\ h^{-1}$, $K_s = 0.1$ g/l, $Y = 0.4$ gX/g S, and $S_0 = 1$ g/l.

c. Determine the optimal HRT, the maximum R_s and biomass concentration for anaerobic treatment where $\mu_m = 0.01\ h^{-1}$, $K_s = 0.25$ g/l, $Y = 0.06$ gX/g S, and $S_0 = 1$ g/l.

6.16. *Pseudomonas putida* is used for fermentation of lactose present in cheese whey in a continuously operating aeration tank at a dilution rate of $D = 0.28\ h^{-1}$. The lactose concentration in the feed and the effluent are $S_0 = 2$ g/L and $S_e = 0.1$ g/l. The growth rate and lactose removal is limited by oxygen transfer. The following information is available: $Y_{x/s}^{M} = 0.45$ gX/g S, $Y_{x/O2} = 0.25$ gX/g O_2 and $C^* = 8$ mg/l (saturation DO).

a. Determine the steady-state biomass concentration (X) and the specific rate of oxygen consumption (q_{O2}).

b. What should be the oxygen transfer coefficient ($K_L a$) to overcome oxygen transfer limitations if the desired DO concentration in the fermentation medium is 2 mg/l?

6.17. In a chemostat, you know that if a culture obeys the Monod equation, the residual substrate is independent of the feed substrate concentration. You observe that in your chemostat, an increase in S_0 causes an increase in the residual substrate concentration. Your friend suggests that you consider whether the Contois equation may describe the situation better. The Contois equation (equation 6.38) follows:

$$\mu = \frac{\mu_m S}{K_{sx} X + S}$$

a. Derive an expression for S in terms of D, μ_m, K_{sx}, and X for a steady-state CFSTR (chemostat).

b. Derive an equation for S as a function of S_0, D, K_{sx}, $Y_{X/S}^{M}$, and μ_m.

c. If S_0 increases twofold, by how much will S increase?

6.18. The maximum growth yield coefficient for *Bacillus subtilis* growing on methanol is 0.4 g X/g S. The heat of combustion of cells is 21 kJ/g cells, and for substrate it is 7.3 kcal/g. Determine the metabolic heat generated by the cells per unit mass of methanol consumption.

6.19. Calculate the productivity (i.e., DP) of a chemostat under the following conditions:

a. Assume Monod kinetics applies. Assume that negligible amounts of biomass (<1%) must be converted to product.

b. Assume the Luedeking–Piret equation for product formation (equation 6.20) applies.

c. Assume steady state:

$$D = 0.8\mu_m \qquad Y_{X/S}^{M} = 0.5\ \text{g}X/\text{g}\ S$$
$$\mu_m = 1.0\ h^{-1} \qquad S_0 = 1000\ \text{mg/l}$$
$$K_s = 10\ \text{mg/l} \qquad \beta = 0.5\ h^{-1}\ \text{mg}\ P/\text{g}\ X$$
$$\alpha = 0.4\ \text{mg}\ P/\text{g}\ X$$

6.20. Consider a chemostat. You wish to know the *number* of cells in the reactor and the *fraction* of the cells that are viable (i.e., alive as determined by ability to divide).

a. Write an equation for viable cell number (n_v). Assume the following:

$$\mu_{\text{net,rep}} = \frac{\mu_{m,\text{rep}} S}{K_{s,\text{rep}} + S} - k_d'$$

Here $\mu_{net,rep}$ = net specific replication rate, $\mu_{m,rep}$ = maximum specific replication rate, and k'_d = death rate. $K_{s,rep}$ is the saturation parameter.

b. Derive an expression for the value of S at steady state.

c. Write the number balance in the chemostat on dead cells (n_d).

d. Derive an expression for the fraction of the total population which are dead cells.

6.21. *E. coli* is cultivated in continuous culture under aerobic conditions with a glucose limitation. When the system is operated at $D = 0.2\ h^{-1}$, determine the effluent glucose and biomass concentrations by using the following equations ($S_0 = 5$ g/l):

a. Monod equation: $\mu_m = 0.25\ h^{-1}, K_s = 100$ mg/l

b. Tessier equation: $\mu_m = 0.25\ h^{-1}, K = 0.005\ (mg/l)^{-1}$

c. Moser equation: $\mu_m = 0.25\ h^{-1}, K_s = 100$ mg/l, $n = 1.5$

d. Contois equation: $\mu_m = 0.25\ h^{-1}, K_{sx} = 0.04, Y^M_{X/S} = 0.4$ g X/g S

$$S_0 = 5 \text{ g/l}$$

Compare and comment on the results.

6.22. Consider steady-state operation of a chemostat. Assume that growth is substrate inhibited and that endogenous metabolism can be ignored such that we get the following:

$$\mu_{net} = \frac{\mu_m S}{K_S + S + S^2/K_I}$$

a. Derive an expression for the residual substrate concentration (i.e., S) as a function of dilution rate and the kinetic parameters (μ_m, K_S, K_I).

b. What are the implications for operation of a chemostat when the organism is subjected to substrate inhibition?

6.23. Formation of lactic acid from glucose is realized in a continuous culture by *Streptococcus lactis*. The following information was obtained from experimental studies:

$$S_0 = 5 \text{ g/l}, \mu_m = 0.2\ h^{-1}, K_S = 200 \text{ mg/l}, k_d = 0.002\ h^{-1}, Y^M_{X/S} = 0.4 \text{ g } X/\text{g } S,$$
$$Y_{P/S} = 0.2 \text{ g } P/\text{g } S, q_P = 0.1 \text{ g } P/\text{g } X\text{-h}.$$

a. Plot the variations of *S, X, P,* DX, and DP with dilution rate.

b. Determine (graphically) the optimum dilution rate maximizing the productivities of biomass (DX) and the product (DP).

7

Stoichiometry of Microbial Growth and Product Formation

Cell growth and product formation are complex processes reflecting the overall kinetics and stoichiometry of the thousands of intracellular reactions that can be observed within a cell. For many process calculations, we wish to compare potential substrates in terms of cell mass yield, or product yield, or evolution of heat. Also, we may need to know how close the system is operating to its thermodynamic limits. (That is, is product yield constrained by kinetic or thermodynamic considerations?) If a system is close to its thermodynamic limit, it would be unwise to try to improve production through mutation or genetic engineering.

Although the cell is complex, the stoichiometry of conversion of substrates into products and cellular materials is often represented by a simple pseudochemical equation. In this chapter, we discuss how these equations can be written and how useful estimates of key yield coefficients can be made.

7.1. COEFFICIENTS FOR ATP CONSUMPTION AND OXYGEN

In Chapter 6, "How Cells Grow," we discussed the definitions of yield and maintenance coefficients, and we learned how to estimate their values using chemostat culture. In particular, we discussed the overall growth yield coefficient $Y^{M}_{X/S}$, which is the

maximum yield of cell mass per unit mass of substrate consumed when no maintenance is considered.

Two other yield and maintenance coefficients of importance are related to adenosine triphosphate (ATP) consumption and oxygen. The ATP yield coefficient, $Y_{X/\mathrm{ATP}}$, represents the amount of biomass synthesized per mole of ATP generated. Surprisingly, it has been observed that for many substrates and organisms, $Y^M_{X/\mathrm{ATP}}$ is nearly constant at 10 to 11 g dry weight/mol ATP for heterotrophic growth under anaerobic conditions. The ATP yield for many autotrophic organisms (recall that autotrophic organisms fix CO_2) is approximately $Y^M_{X/\mathrm{ATP}} \approx 6.5$ g/mol ATP. Under aerobic conditions, the values for $Y^M_{X/\mathrm{ATP}}$ are usually greater than 10.5 (see Table 7.1).

Table 7.2 shows calculated ATP yields (maximum theoretical values) for a variety of media. A maintenance coefficient can also be estimated using an equation analogous to the one we developed for substrate yield coefficient in a chemostat:

$$\frac{1}{Y^{AP}_{X/\mathrm{ATP}}} = \frac{1}{Y^{M}_{X/\mathrm{ATP}}} + \frac{m_{\mathrm{ATP}}}{D} \tag{7.1}$$

TABLE 7.1. Growth Parameters of Organisms Growing Anaerobically in a Chemostat

Organism	Growth-Limiting Factor	m_{ATP} (mol ATP/g-cells-h)	$Y^M_{X/\mathrm{ATP}}$ (g cells/mol ATP)
Lactobacillus casei	Glucose	1.5	24.3
Enterobacter aerogenes	Glucose[a]	6.8	14.0
	Glucose[b]	2.3	17.6
	Tryptophan	38.7	25.4
	Citrate	2.2	9.0
Escherichia coli	Glucose	18.9	10.3
		6.9	8.5
Saccharomyces cerevisiae	Glucose	0.5	11.0
		0.25	13.0
Saccharomyces cerevisiae (petite)	Glucose	0.7	11.3
Candida parapsilosis	Glucose	0.2	12.5
Clostridium acetobutylicum	Glucose	—	23.8
Streptococcus cremoris	Lactose[c]	2.3	12.6

[a] Minimal medium.
[b] Complex medium.
[c] In the presence of a high extracellular lactate concentration.

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TABLE 7.2. ATP Yields in Various Growth Media

Growth Medium	$Y_{X/\mathrm{ATP}}$ (g cells/mol ATP)
Glucose + amino acids + nucleic acids	31.9
Glucose + inorganic salts	28.8
Pyruvate + amino acids + nucleic acids	21
Pyruvate + inorganic salts	13.5
CO_2 + inorganic salts (autotrophic growth)	6.5

Here $Y_{X/\mathrm{ATP}}^{AP}$ is the "apparent" yield of biomass and m_{ATP} is the rate of ATP consumption for maintenance energy.

Similarly, yields based on oxygen consumption can be defined and calculated as follows:

$$\frac{1}{Y_{X/\mathrm{O_2}}^{AP}} = \frac{1}{Y_{X/\mathrm{O_2}}^{M}} + \frac{m_{\mathrm{O_2}}}{D} \tag{7.2}$$

As indicated in Chapter 6, Table 6.1, values of $Y_{X/\mathrm{O_2}}$ can vary from 0.17 to 1.5 g biomass/g O_2, depending on substrate and organism.

Information from some measurements can be usefully combined. A particularly important derived parameter is the respiratory quotient (RQ), which is defined as the moles of CO_2 produced per mole of oxygen consumed. The RQ value provides an indication of metabolic state (e.g., aerobic growth versus ethanol fermentation in baker's yeast) and can be used in process control.

We discussed in Chapter 5, "Major Metabolic Pathways," the P/O ratio, which is the ratio of phosphate bonds formed per unit of oxygen consumed (gmol P/g atom O). The P/O ratio indicates the efficiency of conversion of reducing power into high-energy phosphate bonds in the respiratory chain. For eucaryotes, the P/O ratio approaches 3 when glucose is the substrate, whereas it is significantly less than 3 in procaryotes. A closely related parameter is the proton/oxygen ratio (H/O). This ratio is the number of H^+ ions released per mole of oxygen consumed. Electron generation is directly related to proton release. Usually, 4 moles of electrons are generated per mole of oxygen consumed. The generation of electrons results in the expulsion of H^+ that can be used directly to drive the transport of some substrates or to generate ATP.

The complexity of mass and energy balances for cellular growth can be decreased greatly through the recognition that some parameters are nearly the same irrespective of the species or substrate involved. These parameters can be referred to as *regularities*. For example, Table 7.1 shows that $Y_{X/\mathrm{ATP}}^{M} \geq 10.5$ g dry wt/mol ATP under aerobic conditions. Three important regularities (identified first by I. G. Minkevich and V. K. Eroshin) are 26.95 kcal/g equivalent of available electrons transferred to oxygen (coefficient of variation of 4%), 4.291 g equivalent of available electrons per quantity of biomass containing 1 g atom carbon, and 0.462 g carbon in biomass per gram of dry biomass. It has also been observed that $Y_{X/e^-} = 3.14 \pm 0.11$ g dry wt/g equivalent of electrons. These observed average values of cell composition and yields facilitate estimation of other growth-related parameters.

7.2. STOICHIOMETRIC CALCULATIONS

Conservation laws are absolute and powerful aids in understanding biological reactions and systems. Here we discuss the use of elemental balances and analysis of the energetics of reactions as both constraints on biological systems and as tools to understand what such systems are allowed to do.

7.2.1. Elemental Balances

A material balance on biological reactions can easily be written when the compositions of substrates, products, and cellular material are known. Usually, electron–proton balances are required in addition to elemental balances to determine the stoichiometric coefficients in bioreactions. Accurate determination of the composition of cellular material is a major challenge. Variations in cellular composition with different types of organisms are shown in Table 7.3. A typical cellular composition can be represented as $CH_{1.8}O_{0.5}N_{0.2}$. One mole of biological material is defined as the amount containing 1 gram atom of carbon, such as $CH_\alpha O_\beta N_\delta$.

Consider the following simplified biological conversion in which no extracellular products other than H_2O and CO_2 are produced:

$$CH_mO_n + a\,O_2 + b\,NH_3 \rightarrow c\,CH_\alpha O_\beta N_\delta + d\,H_2O + e\,CO_2 \tag{7.3}$$

where CH_mO_n represents 1 mole of carbohydrate, and $CH_\alpha O_\beta N_\delta$ stands for 1 mole of cellular material. Simple elemental balances on C, H, O, and N yield the following equations:

$$\begin{aligned} &\text{C:} \quad 1 = c + e \\ &\text{H:} \quad m + 3b = c\alpha + 2d \\ &\text{O:} \quad n + 2a = c\beta + d + 2e \\ &\text{N:} \quad b = c\delta \end{aligned} \tag{7.4}$$

The RQ is

$$RQ = \frac{e}{a} \tag{7.5}$$

Equations 7.4 and 7.5 constitute five equations for five unknowns: a, b, c, d, and e. With a measured value of RQ, these equations can be solved to determine the stoichiometric coefficients.

7.2.2. Degree of Reduction

In more complex reactions, as in the formation of extracellular products, an additional stoichiometric coefficient is added, requiring more information. Also, elemental balances provide no insight into the energetics of a reaction. Consequently, the concept of *degree of reduction* has been developed and used for proton–electron balances in bioreactions. The degree of reduction, γ, for organic compounds may be defined as the number of equivalents of available electrons per gram atom C. The available electrons are those that would be transferred to oxygen upon oxidation of a compound to CO_2, H_2O, and NH_3. The degrees of reduction for some key elements are C = 4, H = 1, N = −3, O = −2, P = 5, and S = 6. The degree of reduction of any element in a compound is equal to the valence of this element. For example, 4 is the valence of carbon in CO_2, and −3 is the valence of N in NH_3. Degrees of reduction

TABLE 7.3. Elemental Composition of Microorganisms

Microorganism	Limiting Nutrient	μ (h^{-i})	Composition (% by wt)							Empirical Chemical Formula	Formula "Molecular" Weight
			C	H	N	O	P	S	Ash		
Bacteria			53.0	7.3	12.0	19.0			8	$CH_{1.666}N_{0.20}O_{0.27}$	20.7
Bacteria			47.1	7.8	13.7	31.3				$CH_2N_{0.25}O_{0.5}$	25.5
Aerobacter aerogenes			48.7	7.3	13.9	21.1			8.9	$CH_{1.78}N_{0.24}O_{0.33}$	22.5
Klebsiella aerogenes	Glycerol	0.1	50.6	7.3	13.0	29.0				$CH_{1.74}N_{0.22}O_{0.43}$	23.7
K aerogenes	Glycerol	0.85	50.1	7.3	14.0	28.7				$CH_{1.73}N_{0.24}O_{0.43}$	24.0
Yeast			47.0	6.5	7.5	31.0			8	$CH_{1.66}N_{0.13}O_{0.40}$	23.5
Yeast			50.3	7.4	8.8	33.5				$CH_{1.75}N_{0.15}O_{0.5}$	23.9
Yeast			44.7	6.2	8.5	31.2	1.08	0.6		$CH_{1.64}N_{0.16}O_{0.52}P_{0.01}S_{0.005}$	26.9
Candida utilis	Glucose	0.08	50.0	7.6	11.1	31.3				$CH_{1.82}N_{0.19}O_{0.47}$	24.0
C. utilis	Glucose	0.45	46.9	7.2	10.9	35.0				$CH_{1.84}N_{0.2}O_{0.56}$	25.6
C. utilis	Ethanol	0.06	50.3	7.7	11.0	30.8				$CH_{1.82}N_{0.19}O_{0.46}$	23.9
C. utilis	Ethanol	0.43	47.2	7.3	11.0	34.6				$CH_{1.84}N_{0.2}O_{0.55}$	25.5

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for various organic compounds are given in Table 7.4. The following are examples of how to calculate the degree of reduction for substrates:

Methane (CH_4): $1(4) + 4(1) = 8, \quad \gamma = 8/1 = 8$

Glucose ($C_6H_{12}O_6$): $6(4) + 12(1) + 6(-2) = 24, \quad \gamma = 24/6 = 4$

Ethanol (C_2H_5OH): $2(4) + 6(1) + 1(-2) = 12, \quad \gamma = 12/2 = 6$

A high degree of reduction indicates a low degree of oxidation. That is, $\gamma_{CH_4} > \gamma_{EtOH} > \gamma_{glucose}$.

Consider the aerobic production of a single extracellular product:

$$\underset{\text{substrate}}{CH_mO_n} + aO_2 + bNH_3 \rightarrow c\underset{\text{biomass}}{CH_\alpha O_\beta N_\delta} + d\underset{\text{product}}{CH_xO_yN_z} + eH_2O + fCO_2 \tag{7.6}$$

Following are the degrees of reduction of substrate, biomass, and product:

$$\gamma_s = 4 + m - 2n \tag{7.7}$$

$$\gamma_b = 4 + \alpha - 2\beta - 3\delta \tag{7.8}$$

$$\gamma_p = 4 + x - 2y - 3z \tag{7.9}$$

Note that for CO_2, H_2O, and NH_3 the degree of reduction is zero.

Equation 7.6 can lead to elemental balances on C, H, O, and N; an available electron balance; an energy balance; and a total mass balance. Of the equations, only

TABLE 7.4. Degree of Reduction and Weight of One Carbon Equivalent of One Mole of Some Substrates and Biomass

Compound	Molecular Formula	Degree of Reduction, γ	Weight, m (per carbon mole)
Biomass	$CH_{1.64}N_{0.16}O_{0.52}$	4.17 (NH_3)	24.5
	$P_{0.0054}S_{0.005}$[a]	4.65 (N_2) 5.45 (HNO_3)	
Methane	CH_4	8	16.0
n-Alkane	$C_{15}H_{32}$	6.13	14.1
Methanol	CH_4O	6.0	32.0
Ethanol	C_2H_6O	6.0	23.0
Glycerol	$C_3H_8O_3$	4.67	30.7
Mannitol	$C_6H_{14}O_6$	4.33	30.3
Acetic acid	$C_2H_4O_2$	4.0	30.0
Lactic acid	$C_3H_6O_3$	4.0	30.0
Glucose	$C_6H_{12}O_6$	4.0	30.0
Formaldehyde	CH_2O	4.0	30.0
Gluconic acid	$C_6H_{12}O_7$	3.67	32.7
Succinic acid	$C_4H_6O_4$	3.50	29.5
Citric acid	$C_6H_8O_7$	3.0	33.5
Formic acid	CH_2O_2	2.0	46.0
Oxalic acid	$C_2H_2O_4$	1.0	45.0

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five will be independent. If all the equations are written, then the extra equations can be used to check the consistency of an experimental data set. Because the amount of water formed or used in such reactions is difficult to determine and water is present in great excess, the hydrogen and oxygen balances are difficult to use. For such a data set, we would typically choose a carbon, a nitrogen, and an available-electron balance:

$$c + d + f = 1 \tag{7.10}$$

$$c\delta + dz = b \tag{7.11}$$

$$c\gamma_b + d\gamma_p = \gamma_s - 4a \tag{7.12}$$

With partial experimental data, it is possible to solve this set of equations. Measurements of RQ and a yield coefficient would, for example, allow the calculation of the remaining coefficients. It should be noted that the coefficient, c, is $Y_{X/S}$ (on a molar basis) and d is $Y_{P/S}$ (also on a molar basis).

An energy balance for aerobic growth follows:

$$Q_0 c\gamma_b + Q_0 d\gamma_p = Q_0\gamma_s - Q_0 4a \tag{7.13}$$

If Q_0, the heat evolved per equivalent of available electrons transferred to oxygen, is constant, equation 7.13 is *not* independent of equation 7.12. Recall that an observed regularity is 26.95 kcal/g equivalent of available electrons transferred to oxygen, which allows the prediction of heat evolution based on estimates of oxygen consumption.

Equations 7.12 and 7.13 also allow estimates of the fractional allocation of available electrons or energy for an organic substrate. Equation 7.12 can be rewritten as

$$1 = \frac{c\gamma_b}{\gamma_s} + \frac{d\gamma_p}{\gamma_s} + \frac{4a}{\gamma_s} \tag{7.14a}$$

$$1 = \xi_b + \xi_p + \varepsilon \tag{7.14b}$$

where ε is the fraction of available electrons in the organic substrate that is transferred to oxygen, ξ_b is the fraction of available electrons that is incorporated into biomass, and ξ_p is the fraction of available electrons that is incorporated into extracellular products.

Example 7.1.

Assume that experimental measurements for a certain organism have shown that cells can convert two-thirds (wt/wt) of the substrate carbon (alkane or glucose) to biomass.

a. Calculate the stoichiometric coefficients for the following biological reactions:

$$\text{Hexadecane: } C_{16}H_{34} + a\,O_2 + b\,NH_3 \rightarrow c(C_{4.4}H_{7.3}N_{0.86}O_{1.2}) + d\,H_2O + e\,CO_2$$

$$\text{Glucose: } C_6H_{12}O_6 + a\,O_2 + b\,NH_3 \rightarrow c(C_{4.4}H_{7.3}N_{0.86}O_{1.2}) + d\,H_2O + e\,CO_2$$

b. Calculate the yield coefficients $Y_{X/S}$ (g dw cell/g substrate), Y_{X/O_2} (g dw cell/g O_2) for both reactions. Comment on the differences.

Solution

a. For hexadecane:

$$\text{Amount of carbon in 1 mole of substrate} = 16(12) = 192\,\text{g}$$

$$\text{Amount of carbon converted to biomass} = 192(2/3) = 128\,\text{g}$$

Then, $128 = c(4.4)(12); c = 2.42$.

$$\text{Amount of carbon converted to } CO_2 = 192 - 128 = 64\,\text{g}$$

$$64 = e(12), \quad e = 5.33$$

The nitrogen balance yields

$$14b = c(0.86)(14)$$
$$b = (2.42)(0.86) = 2.085$$

The hydrogen balance is

$$34(1) + 3b = 7.3c + 2d$$
$$d = 12.43$$

The oxygen balance yields

$$2a(16) = 1.2c(16) + 2e(16) + d(16)$$
$$a = 12.427$$

For glucose:

$$\text{Amount of carbon in 1 mole of substrate is } 72\,\text{g}$$

$$\text{Amount of carbon converted to biomass is } 72(2/3) = 48\,\text{g}$$

Then, $48 = 4.4c(12)$, and thus $c = 0.909$.

$$\text{Amount of carbon converted to } CO_2 \text{ is } 72 - 48 = 24\,\text{g}.$$

$$24 = 12e; \text{ and thus } e = 2$$

The nitrogen balance yields

$$14b = 0.86c(14)$$

Consequently, $b = 0.782$.

The hydrogen balance is

$$12 + 3b = 7.3c + 2d$$

resulting in $d = 3.854$.

The oxygen balance yields

$$6(16) + 2(16)a = 1.2(16)c + 2(16)e + 16d$$
$$a = 1.473$$

which allows calculation of a.

b. For hexadecane:

$$Y_{X/S} = \frac{2.42(\text{MW})_{\text{biomass}}}{(\text{MW})_{\text{substrate}}}$$

$$Y_{X/S} = \frac{2.42(91.34)}{226} = 0.98 \text{ gdw cells/g substrate}$$

$$Y_{X/O_2} = \frac{2.42(\text{MW})_{\text{biomass}}}{12.43(\text{MW})_{O_2}}$$

$$Y_{X/O_2} = \frac{2.42(91.34)}{(12.43)(32)} = 0.557 \text{ gdw cells/g } O_2$$

For glucose:

$$Y_{X/S} = \frac{(0.909)(91.34)}{180} = 0.461 \text{ gdw cells/g substrate}$$

$$Y_{X/O_2} = \frac{(0.909)(91.34)}{(1.473)(32)} = 1.76 \text{ gdw cells/g } O_2$$

The growth yield on more reduced substrate (hexadecane) is higher than that on partially oxidized substrate (glucose), assuming that two-thirds of all the entering carbon is incorporated in cellular structures. However, the oxygen yield on glucose is higher than that on the hexadecane because glucose is partially oxidized.

7.3. THEORETICAL PREDICTIONS OF YIELD COEFFICIENTS

In aerobic fermentations, the growth yield per available electron in oxygen molecules is approximately 3.14 ± 0.11 gdw cells/electron, when ammonia is used as the nitrogen source. The number of available electrons per oxygen molecule (O_2) is four. When the number of oxygen molecules per mole of substrate consumed is known, the growth yield coefficient, $Y_{X/S}$, can easily be calculated. Consider the aerobic catabolism of glucose:

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$$

The total number of available electrons in 1 mole of glucose is 24. The cellular yield per available electron is $Y_{X/S} = 24(3.14) = 76$ gdw cells/mol glucose.

The predicted growth yield coefficient is $Y_{X/S} = 76/180 = 0.4$ gdw cells/g glucose. Most measured values of $Y_{X/S}$ for aerobic growth on glucose are 0.38 to 0.51 g/g (see Table 6.1).

The ATP yield ($Y_{X/\text{ATP}}$) in anaerobic fermentations is approximately 10.5 ± 2 gdw cells/mol ATP. In aerobic fermentations, this yield varies between 6 and 29. When the energy yield of a metabolic pathway is known (N moles of ATP produced per gram of substrate consumed), the growth yield $Y_{X/S}$ can be calculated using the following equation:

$$Y_{X/S} = Y_{X/\text{ATP}} N$$

Example 7.2.

Estimate the theoretical growth and product yield coefficients for ethanol fermentation by *S. cerevisiae*, as described by the following overall reaction:

$$C_6H_{12}O_6 \rightarrow 2\,C_2H_5OH + 2\,CO_2$$

Solution

Since $Y_{X/ATP} \approx 10.5$ gdw/mol ATP and glucose fermentation to ethanol yields 2 ATP/mol of glucose in yeast, we have the following:

$$Y_{X/S} \approx 10.5 \text{ gdw/mol ATP} \cdot 2 \frac{\text{moles ATP}}{180 \text{ g glucose}}$$

or

$$Y_{X/S} \approx 0.117 \text{ gdw/g glucose}$$

For complete conversion of glucose to ethanol by the yeast pathway, the following would be the maximal yield:

$$Y_{P/S} = \frac{2(46)}{180} = 0.51 \text{ g ethanol / g glucose}$$

For CO_2, the maximum yield is

$$Y_{CO_2/S} = 2(44)/180 = 0.49 \text{ g } CO_2\text{/g glucose}$$

In practice, these maximum yields are not obtained. The product yields are about 90% to 95% of the maximum values because the glucose is converted into biomass and other metabolic by-products (e.g., glycerol or acetate).

7.4. ESTIMATION OF ELEMENTAL CELL COMPOSITION

Cells are made of C, N, O, H, P, S, and minerals (Ca, Mg, Fe, Zn, and others). Elemental composition of the cells varies depending on the type of cells (bacteria, yeasts, molds, animal and plant cells), type of substrate, and growth conditions (T, pH, ORP, DO). A typical bacterial cell contains nearly 45% to 50% C, 10% to 14% N, 20% to 25% O, 6% to 8% H, 2% to 3% P, 0.5% to 1% S, and 5% to 6% ash and is usually symbolized by $C_5H_7NO_2$ with a molar weight of 113 g/mol bacteria. However, this composition varies depending on the composition of growth medium (C, N, P, O sources) and environmental conditions.

Elemental composition of the cells needs to be determined before balancing biological reactions. This determination is done by filtering and drying the cells at 105°C until constant weight. Elemental analyses are carried out on dry cell mass to determine the percentages of C, N, O, H, P, and S content. Dried cells are further burned in a furnace at 550°C to remove organic fractions (volatile suspended solids) and to determine the ash content. The mass fraction of every element in dry cell mass is then converted to mole fractions to determine the empirical formula for one mole of cell.

The mole number of the *i*th element is

$$N_i = m_i / M_i \tag{7.15}$$

where N_i is the mole number, m_i is the weight, and M_i is the molar weight of the *i*th elements. The total number of moles is

$$N_T = \Sigma N_i = N_c + N_N + N_o + N_H + N_P + N_\Sigma \tag{7.16}$$

The mole fraction for the *i*th element is

$$X_i = N_i / N_T \tag{7.17}$$

Once the mole fractions are determined, the empirical formula is established as follows:

$$C_{Xc} H_{XH} N_{XN} O_{XO} P_{XP} S_{XS}$$

Example 7.3.

One gram of dry cell mass is analyzed for major elements, and the following results are obtained: $m_C = 0.48$g, $m_H = 0.07$ g, $m_O = 0.24$ g, $m_N = 0.12$ g, and $m_{ash} = 0.09$ g. Ash includes P, S, and minerals. Determine the empirical formula of the cell.

Solution Mole numbers of each element are as follows:

$N_C = 0.48/12 = 0.04$, $N_H = 0.07$ mole, $N_O = 0.24/16 = 0.015$, $N_N = 0.12/14 = 0.0086$ mole

Total number of moles (mole number of cell) $N_T = 0.1336$ mole.

Mole fractions of the elements are as follows:

$X_C = 0.04/0.1336 = 0.30$, $X_H = 0.524$, $X_O = 0.112$, $X_N = 0.064$

Then the following is the empirical formula of the cell:

$$C_{0.3}\, H_{0.524}\, O_{0.112}\, N_{0.064} \text{ or } C_{4.68}\, H_{8.2}\, O_{1.75}\, N$$

7.5. STOICHIOMETRY BY OXIDATION-REDUCTION HALF-REACTIONS

In biological conversions, a substrate such as glucose is both the source of energy and the material used for biosynthesis of cells and the products. A fraction of the total electrons present in the substrate is transferred to the electron acceptor by metabolic reactions to generate energy in the form of ATPs (e_e), and the remaining fraction ends up in the newly synthesized cellular mass (e_s). The sum of e_e and e_s is 1.0. When electrons are transferred from the substrate to the electron acceptor, the net reaction can be represented by two half-reactions in the form of oxidation and reduction. The electron donor (substrate) is oxidized, and the electron acceptor is reduced. In aerobic oxidation of glucose to CO_2 and H_2O, glucose-carbon is the electron donor and oxygen is

the electron acceptor. The electron donor (C in glucose) is oxidized to CO_2 by donating electrons to the electron acceptor (O_2), yielding water. This bioconversion can be represented by the following half-reactions based on one mole electron transfer:

$$\textit{Oxidation}: \quad 1/24\ C_6H_{12}O_6 + \tfrac{1}{4}\ H_2O \rightarrow \tfrac{1}{4}\ CO_2 + H^+ + e^- \tag{7.18}$$

$$\textit{Reduction}: \quad 1/4\ O_2 + H^+ + e^- \rightarrow \tfrac{1}{2}\ H_2O \tag{7.19}$$

$$\textit{Overall}: \quad 1/24\ C_6H_{12}O_6 + \tfrac{1}{4}\ O_2 \rightarrow \tfrac{1}{4}\ H_2O + \tfrac{1}{4}\ CO_2 \tag{7.20}$$

By multiplying both sides of the overall reaction by 24, the overall reaction is obtained:

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O \tag{7.21}$$

A list of half-reactions for common electron donors is presented in Table 7.5. Electron acceptor is oxygen in aerobic respiration. However, nitrate, sulfate, or Fe (III) may be the electron acceptor in anaerobic respiration. Carbon dioxide is the electron acceptor in anaerobic metabolism of carbonaceous compounds yielding methane as the end product. Table 7.6 summarizes half-reactions for common electron acceptors.

Table 7.5. Major Electron-Donor Half-Reactions

Compound	Half-Reaction	ΔG^o (kJ/eq e^-)
Glucose	$1/24\ C_6H_{12}O_6 + \tfrac{1}{4}\ H_2O \rightarrow \tfrac{1}{4}\ CO_2 + H^+ + e^-$	−41.35
Glycerol	$1/14\ C_3H_5(OH)_3 + 3/14\ H_2O \rightarrow 3/14\ CO_2 + H^+ + e^-$	−38.88
Ethanol	$1/12\ C_2H_5OH + \tfrac{1}{4}\ H_2O \rightarrow 1/6\ CO_2 + H^+ + e^-$	−31.18
Methanol	$1/6\ CH_3OH + 1/6\ H_2O \rightarrow 1/6\ CO_2 + H^+ + e^-$	−36.84
Methane	$1/8\ CH_4 + \tfrac{1}{4}\ H_2O \rightarrow 1/8\ CO_2 + H^+ + e^-$	−23.53
Acetate	$1/8\ CH_3COO^- + 3/8\ H_2O \rightarrow 1/8\ CO_2 + 1/8\ HCO_3^- + H^+ + e^-$	−27.40
Benzoate	$1/30\ C_6H_5COO^- + 13/30\ H_2O \rightarrow 1/5\ CO_2 + 1/30\ HCO_3^- + H^+ + e^-$	−27.34
Lactate	$1/12\ CH_3CHOHCOO^- + 1/3\ H_2O \rightarrow 1/6\ CO_2 + 1/12\ HCO_3^- + H^+ + e^-$	−32.29
Propionate	$1/14\ CH_3CH_2COO^- + 5/14\ H_2O \rightarrow 1/7\ CO_2 + 1/14\ HCO_3^- + H^+ + e^-$	−27.63
Pyruvate	$1/10\ CH_3COCOO^- + 2/5\ H_2O \rightarrow 1/5\ CO_2 + 1/10\ HCO_3^- + H^+ + e^-$	−35.09
Cell Synthesis	$1/20\ C_5H_7NO_2 + 9/20\ H_2O \rightarrow 1/5\ CO_2 + 1/20\ NH_4^+ + 1/20\ HCO_3^- + H^+ + e^-$	

Adapted from Rittmann & McCarty, *Environmental Biotechnology*, McGraw Hill, 2001.

Table 7.6. Major Electron-Acceptor Half-Reactions

Compound	Half-Reaction	ΔG^o (kJ/eq e^-)
Oxygen	$\tfrac{1}{4}\ O_2 + H^+ + e^- \rightarrow \tfrac{1}{2}\ H_2O$	−78.72
Nitrate	$1/5\ NO_3^- + 6/5\ H^+ + e^- \rightarrow 1/10\ N_2 + 3/5\ H_2O$	−72.20
Sulfate	$1/8\ SO_4^{-2} + 19/16\ H^+ + e^- \rightarrow 1/16\ H_2S + 1/16\ HS^- + 1/2\ H_2O$	−20.85
Fe(III)	$Fe(III) + e^- \rightarrow Fe(II)$	−74.27
CO_2	$1/8\ CO_2 + H^+ + e^- \rightarrow 1/8\ CH_4 + \tfrac{1}{4}\ H_2O$	23.53

Adapted from Rittmann & McCarty, *Environmental Biotechnology*, McGraw Hill, 2001.

As mentioned before, a fraction of electrons present in the substrate ends up in the newly synthesized biomass. The empirical formula for bacterial cell is usually assumed to be $C_5H_7NO_2$ which varies depending on the type of cell (yeasts, molds, animal and plant cells) and medium used. As shown in Section 7.4 it is possible to determine a more exact formula for a particular culture. Considering inorganic carbon (CO_2 and HCO_3^-) as carbon and NH_4^+ as nitrogen source, the half-reaction for cell synthesis can be written as follows for one mole electron transfer:

$$\frac{1}{5}CO_2 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7NO_2 + \frac{9}{20}H_2O \qquad (7.22)$$

This is a reduction half-reaction where inorganic carbon is the electron acceptor. When nitrate is used as the N-source, the half-reaction for cell synthesis can be written as follows:

$$\frac{1}{28}NO_3^- + \frac{5}{28}CO_2 + \frac{29}{28}H^+ + e^- \rightarrow \frac{1}{28}C_5H_7NO_2 + \frac{11}{28}H_2O \qquad (7.23)$$

Cell synthesis half-reactions are the electron-acceptor reactions that must accompany an electron-donor reaction such as the reaction shown in equation 7.18.

The fraction of electrons used for energy generation (e_e) and for cell synthesis (e_s) need to be known in balancing microbial reactions. Net energy generation and net biosynthesis reactions are

$$R_e = R_a + R_d \text{ and } R_s = R_c + R_d \qquad (7.24)$$

where R_e and R_s are the net energy generation and cell synthesis reactions and R_a, R_d, and R_c are the electron acceptor, electron donor, and cell synthesis (electron acceptor) half-reactions, respectively.

The net reaction based on fractional electron utilization is

$$R = e_e R_e + e_s R_s = e_e (R_a + R_d) + e_s (R_c + R_d) \qquad (7.25)$$

Since $e_e + e_s = 1$, equation 7.25 can be written as

$$R = e_e R_a + e_s R_c + R_d \qquad (7.26)$$

Equation 7.25 or 7.26 can be used in balancing microbial reactions.

Example 7.4.

Aerobic oxidation of glucose is accompanied by microbial growth. NH_4^+ is used as the nitrogen source and the end products are CO_2 and H_2O. The formula for bacterial cell is $C_5H_7NO_2$. Determine the coefficients for this microbial conversion. Assume that 40% of total electrons are used for biosynthesis and 60% are used for energy generation.

Solution

Following are half-reactions for microbial oxidation of glucose:

Electron-donor reaction (R_d): 1/24 $C_6H_{12}O_6$ + ¼ $H_2O \rightarrow$ ¼ $CO_2 + H^+ + e^-$

Electron-acceptor reaction (R_a): 1/4 $O_2 + H^+ + e^- \rightarrow$ ½ H_2O

The half-reaction for cell synthesis (R_c) is

$$\frac{1}{5}CO_2 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7NO_2 + \frac{9}{20}H_2O$$

The net energy generating reaction is

$$R_e = R_a + R_d = \frac{1}{24}C_6H_{12}O_6 + ¼\ O_2 \rightarrow ¼\ H_2O + ¼\ CO_2$$

The net cell synthesis reaction is

$$R_s = R_c + R_d = 1/24\ C_6H_{12}O_6 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- \rightarrow \frac{1}{20}C_5H_7NO_2 + \frac{1}{20}CO_2 + \frac{1}{5}H_2O$$

The overall reaction is

$$R = e_e R_e + e_s R_s = 0.60R_e + 0.40\ R_s = 0.0417C_6H_{12}O_6 + 0.02\ NH_4^+ + 0.15\ O_2 + 0.02HCO_3^- \rightarrow 0.02C_5H_7NO_2 + 0.17\ CO_2 + 0.23\ H_2O$$

or

$$C_6H_{12}O_6 + 0.48NH_4^+ + 3.6O_2 + 0.48HCO_3^- \rightarrow 0.48C_5H_7NO_2 + 4.08CO_2 + 5.52H_2O$$

The growth yield coefficient and the respiratory quotient for this reaction are $Y_{x/s} = 0.48$ (113)/180 = 0.30 g cell/g glucose, and RQ = 4.08/3.6 = 1.33 mol CO_2/mol O_2.

7.6. THERMODYNAMICS OF BIOLOGICAL REACTIONS

Like chemical reactions, biological reactions obey thermodynamic laws too. Energy is conserved in all biological reactions. Spontaneous bioreactions result in negative free-energy changes. Therefore, by looking at the free-energy changes in bioreactions, it is possible to determine which reactions would take place spontaneously and which require energy input. Free-energy changes in bioreactions are calculated by using the formation free energies of products and reactants. In general, free-energy change in a reaction is calculated by using the following equation:

$$\Delta G = \Sigma\ a_i\ G_i - \Sigma\ b_j G_j \tag{7.27}$$

where $\Sigma\ a_i\ G_i$ is the sum of free energies of the products, $\Sigma\ b_j G_j$ is the sum of free energies of reactants, and ΔG is the free-energy change of the reaction.

Free energy of any compound is defined as

$$G_i = G_i^o + RT\ LnC_i \tag{7.28}$$

where G_i^o is the standard free energy (25°C, 1 atm), R is the gas constant (1.99 cal/mol-K), T is the absolute temperature (K), and C_i is the molar concentration of the ith compound in aqueous solution. Therefore, free-energy change for the following bioreaction is

$$aA + bB \rightarrow cC + dD \tag{7.29}$$

$$\Delta G = \Delta G^{o} + RT\ LnQ = \Delta G^{o} + RT\ Ln\frac{(C)^{c}(D)^{d}}{(A)^{a}(B)^{b}} \tag{7.30}$$

Usually, standard free energies are calculated from the half-reactions of the electron donors and acceptors to determine the energy requirement of a reaction.

For aerobic microbial oxidation of glucose, the electron-donor half-reaction is

$$1/24\ C_6H_{12}O_6 + ¼\ H_2O \rightarrow ¼\ CO_2 + H^+ + e^-$$

The standard free-energy change for this reaction is

$$\Delta G_d^o = ¼\ G_{CO2}^o + G_{H+}^o - 1/24\ G_{Glu}^o - ¼\ G_{H2O}^o$$

$$= ¼(-394.36) + (-39.87) - 1/24(-917.22) - 1/4(-237.18) = -40.956\ \text{kJ/eq}\ e^-$$

The electron-acceptor half-reaction for the same bioconversion is

$$1/4\ O_2 + H^+ + e^- \rightarrow ½\ H_2O$$

The standard free-energy change for this reaction is

$$\Delta G_a^o = 1/2G_{H2O}^o - G_{H+}^o - 1/4G_{O2}^o = ½(-237.18) - (-39.87) - ¼(0) = -78.72\ \text{kJ/eq}\ e^-$$

Therefore, the free-energy change for the net reaction is

$$1/24\ C_6H_{12}O_6 + ¼\ H_2O \rightarrow ¼\ CO_2 + H^+ + e^- \qquad \Delta G^o{}_d = -40.96\ \text{kJ/ eq}\ e^-$$

$$1/4\ O_2 + H^+ + e^- \rightarrow ½\ H_2O \qquad \Delta G^o{}_a = -78.72\ \text{kJ/ eq}\ e^-$$

$$1/24\ C_6H_{12}O_6 + ¼\ O_2 \rightarrow ¼\ CO_2 + ¼\ H_2O \qquad \Delta G^o = -119.68\ \text{kJ/ eq}\ e^-$$

Therefore, this bioreaction is spontaneous and energetically favorable. Free-energy changes for different electron donor and acceptors have been calculated. Figure 7.1 depicts variation of free-energy changes for different electron donors and acceptors. When glucose is used as the electron donor and oxygen as the electron acceptor in a bioreaction, the free-energy change is around −120 kJ/eq e^-, whereas with the nitrate as the electron acceptor the free-energy change becomes nearly −110 kJ/eq e^-. The free-energy change is much lower when sulfate is used as the electron acceptor (about −20kJ/eq e^-). Under anaerobic conditions, where glucose is the electron donor and CO_2 is the acceptor, the free-energy change is −18 kJ/eq e^-. The difference between the free-energy changes of the same reaction under aerobic and anaerobic conditions is the reason for differences in growth yield coefficients. Large free-energy changes in aerobic metabolism of glucose result in high aerobic-growth-yield coefficients as compared to that of anaerobic growth.

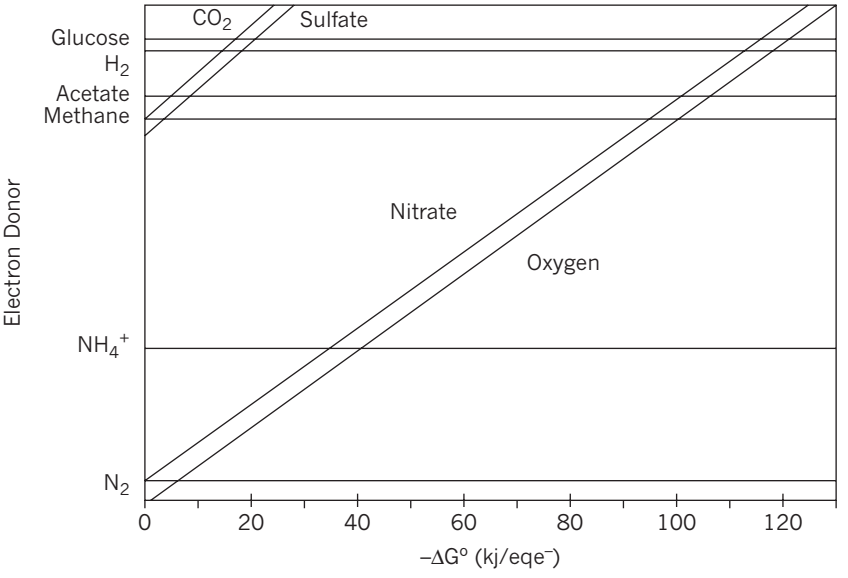


FIGURE 7.1. Variation of reaction free energies for different electron donors and acceptors. The lines represent electron acceptors. (Adapted from Rittmann & McCarty, *Environmental Biotechnology*, McGraw Hill, 2001.)

7.7. SUMMARY

This chapter reviewed simple methods to determine the reaction stoichiometry for bioreactions. These methods lead to the possibility of predicting yield coefficients for various fermentations using a variety of substrates. By coupling these equations to experimentally measurable parameters, such as the respiratory quotient, we can infer a great deal about the progress of fermentation. Such calculations can also assist in initial process design equations by allowing the prediction of the amount of oxygen required (and consequently heat generated) for a certain conversion of a particular substrate. The prediction of yield coefficients is usually not exact, because unknown or unaccounted metabolic pathways and products may be present. Nonetheless, such calculations provide useful first estimates of such parameters.

SUGGESTIONS FOR FURTHER READING

Bailey, J. E., and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill, New York, 1986.

Doran, P. M., *Bioprocess Engineering Principles*, 2d ed., Academic Press, Waltham, MA, 2013.

Erickson, L. E., I. G. Minkevich, and V. K. Eroshin, "Application of Mass and Energy Balance Regularities in Fermentation," *Biotechnology and Bioengineering 20*: 1595, 1978.

MINKEVICH, I. G., "Mass and Energy Balance for Microbial Product Synthesis: Biochemical and Cultural Aspects," *Biotechnology and Bioengineering 25*: 1267, 1983.

RITTMANN, B. E., AND P. L. MCCARTY, *Environmental Biotechnology: Principles and Applications*, McGraw-Hill, New York, 2001.

ROELS, J. A., *Energetics and Kinetics in Biotechnology*, Elsevier Science, New York, 1983.

VILLADSEN, J., J. NIELSON, AND G. LIDEN, *Bioreaction Engineering Principles*, 3d ed., Springer US, New York, 2011.

VAN GULIK, W. M., AND J. J. HEIJNEN, "A Metabolic Network Stoichiometry Analysis of Microbial Growth and Product Formation," *Biotechnology and Bioengineering 48*: 181, 1995.

PROBLEMS

7.1. Determine the amount of $(NH_4)_2SO_4$ to be supplied in a fermentation medium where the final cell concentration is 30 g/l in a 10^3 l culture volume. Assume that the cells are 12% nitrogen by weight and $(NH_4)_2SO_4$ is the only nitrogen source.

7.2. The growth of baker's yeast (*S. cerevisiae*) on glucose may be simply described by the following equation:

$$C_6H_{12}O_6 + 3\ O_2 + 0.48\ NH_3 \rightarrow 0.48\ \underset{\text{yeast}}{C_6H_{10}NO_3} + 4.32\ H_2O + 3.12\ CO_2$$

In a batch reactor of volume 10^5 l, the final desired yeast concentration is 50 gdw/l.

- **a.** Determine the concentration and total amount of glucose and $(NH_4)_2SO_4$ in the nutrient medium.
- **b.** Determine the yield coefficients $Y_{X/S}$ (biomass/glucose) and $Y_{X/O}$ (biomass/oxygen).
- **c.** Determine the total amount of oxygen required.
- **d.** If the rate of growth at exponential phase is $r_x = 0.7$ gdw/l-h, determine the rate of oxygen consumption $(g\ O_2/l\text{-}h)$.
- **e.** Calculate the heat-removal requirements for the reactor (recall equation 6.26).

7.3. The growth of *S. cerevisiae* on glucose under anaerobic conditions can be described by the following overall reaction:

$$C_6H_{12}O_6 + \beta\ NH_3 \rightarrow 0.59\ CH_{1.74}N_{0.2}O_{0.45}\ (\text{biomass}) + 0.43\ C_3H_8O_3 + 1.54\ CO_2 + 1.3\ C_2H_5OH + 0.036\ H_2O$$

- **a.** Determine the biomass yield coefficient $Y_{X/S}$.
- **b.** Determine the product yield coefficients $Y_{EtOH/S}, Y_{CO_2/S}, Y_{C_3H_2O/S}$.
- **c.** Determine the coefficient β.

7.4. Aerobic growth of *S. cerevisiae* on ethanol is simply described by the following overall reaction:

$$C_2H_5OH + a\ O_2 + b\ NH_3 \rightarrow c\ CH_{1.704}N_{0.149}O_{0.408} + d\ CO_2 + e\ H_2O$$

- **a.** Determine the coefficients *a*, *b*, *c*, and *d*, where RQ = 0.66.
- **b.** Determine the biomass yield coefficient, $Y_{X/S}$, and oxygen yield coefficient, $Y_{X/O_2}\ (gdw/g\ O_2)$.

7.5. Aerobic degradation of benzoic acid by a mixed culture of microorganisms can be represented by the following reaction:

$$\underset{\text{(substrate)}}{C_6H_5COOH} + a\,O_2 + b\,NH_3 \rightarrow \underset{\text{(bacteria)}}{c\,C_5H_7NO_2} + d\,H_2O + e\,CO_2$$

a. Determine a, b, c, d, and e if $RQ = 0.9$.
b. Determine the yield coefficients, $Y_{X/S}$ and Y_{X/O_2}.
c. Determine degree of reduction for the substrate and bacteria.

7.6. Aerobic degradation of an organic compound by a mixed culture of organisms in waste water can be represented by the following reaction:

$$C_3H_6O_3 + a\,O_2 + b\,NH_3 \rightarrow c\,C_5H_7NO_2 + d\,H_2O + e\,CO_2$$

a. Determine a, b, c, d, and e if $Y_{X/S} = 0.4$ g X/g S.
b. Determine the yield coefficients Y_{X/O_2} and Y_{X/NH_3}.
c. Determine the degree of reductions for the substrate, bacteria, and RQ for the organisms.

7.7. Biological denitrification of nitrate-containing waste waters can be described by the following overall reaction:

$$NO_3^{-1} + a\,CH_3OH + H^+ \rightarrow b\,C_5H_7NO_2 + c\,N_2 + d\,CO_2 + e\,H_2O$$

a. Determine a, b, c, d, and e if $Y_{X/S} = 0.5$ g X/g N.
b. Determine the degree of reduction of bacteria and methanol.

7.8. The following biological oxidation reaction takes place in biological treatment of an industrial wastewater:

$$C_6H_4CH_3OH + a\,O_2 + b\,NH_3 \rightarrow c\,C_5H_7NO_2 + d\,CO_2 + e\,H_2O$$

a. Determine the coefficients of the equation (a, b, c, d, and e) if RQ = 0.5.
b. Determine $Y_{x/s}$, and $Y_{X/O2}$.
c. Determine γ_s and γ_b.

7.9. *Clostridium* sp. were used for anaerobic conversion of glucose to acetic acid and H_2 as described by the following reaction:

$$C_6H_{12}O_6 + a\,NH_3 + b\,H_3PO_4 \rightarrow c\,C_5H_7NO_2P_{0.2} + d\,CH_3COOH + e\,CO_2 + f\,H_2$$

a. Determine the coefficients by writing elemental balances if $Y_{x/s} = 0.1$ g/g.
b. Determine γ_s, γ_b, γ_{Ac}, and γ_{H_2} and write down degree of reduction balance to see if it is satisfied.
c. Determine $Y_{p/s}$ for acetic acid and hydrogen.

7.10. *Rhodobacter* sp. ferments acetic acid to hydrogen gas and CO_2 under photoheterotrophic conditions (anoxygenic photosynthesis) as depicted in the following reaction:

$$CH_3COOH + a\,NH_3 + b\,H_3PO_4 \xrightarrow{\text{Light}} c\,C_5H_7NO_2P_{0.2} + d\,CO_2 + e\,H_2$$

a. Determine the coefficients by using elemental balances.
b. Determine the growth yield coefficient $Y_{x/s}$ and $Y_{p/s}$.
c. Write down degree of reduction balance for the conversion.

7.11. Elemental analysis of dry cell mass indicates that the cell is composed of 45% C, 25% O, 5% H, 12% N, 2.5% P, 0.5% S, and 10% minerals.

a. By considering 1 g of cell dry weight, determine mole fractions of every element and the cell formula.

b. If the cell formula is $C_4\ H_8\ O_{2.2}\ N_{1.2}\ P_{0.08}\ S_{0.02}$, determine the percent elemental composition (i.e., mass fractions) of dry cell mass.

7.12. In ethanol fermentation from glucose, carbon dioxide is the electron acceptor, and glucose is the electron donor.

a. Write down the electron donor and acceptor half-reactions for ethanol fermentation for one mole electron transfer along with free-energy changes.

b. Determine the overall fermentation reaction for one mole of glucose and the free-energy change without cell-synthesis reaction.

c. Assuming 20% of the electrons in glucose are used for cell synthesis and 80% for energy generation ($e_e = 0.80$ and $e_s = 0.20$), write down the reactions for Ra, Rd, and Rc, and determine the overall reaction stoichiometry for one mole of NH_4-N.

7.13. In biological nitrification reaction NH_4-N is converted to NO_3-N under autotrophic conditions by using CO_2 as the carbon source in the presence of oxygen. NH_4-N is the electron donor, and oxygen is the electron acceptor in this bioconversion, which is realized in the absence of organic carbon source. Ammonium is also used as N-source for cell synthesis. If 85% of electrons are transferred to the product for energy generation and only 15% ends up in cell biomass, determine the following:

a. Write down the electron donor and acceptor half-reactions (R_d and R_a).

b. Write down the half-reaction for cell synthesis (R_c).

c. Write down the overall reaction by considering the electron fractionation.

7.14. Methane formation from acetate is realized under anaerobic conditions where NH_4 is the nitrogen source for cell synthesis. Acetate is the electron donor and CO_2 is the electron acceptor in this bioconversion. Assuming the cell formula is $C_5H_7NO_2$ and only 10% of the electrons in the substrate are transferred to the cell biomass, determine the following:

a. Write down the electron donor and the acceptor half-reactions (R_d and R_a).

b. Write down the cell synthesis half-reaction (R_c).

c. Write down the overall reaction by considering that $e_e = 0.90$ and $e_s = 0.10$.

7.15. Determine the free-energy changes for methane formation from the electron donors of glucose, acetate, H_2 and ethanol where CO_2 is the electron acceptor by using Figure 7.1. Comment on the results in terms of cell yield from different substrates.

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8

How Cellular Information Is Altered

We have discussed some aspects of how cells inherit information and how a chemostat (covered in Chapter 6, "How Cells Grow") can be used as a tool to select for individual cells with different or augmented metabolism. The mechanism for DNA replication in procaryotes was summarized in Chapter 4, "How Cells Work." This process is a good example of the exchange of genetic information from one generation to another. However, some individual cells can receive additional genetic information through natural or artificial means. The initial genetic information within a cell may also undergo rearrangements or alterations. In this chapter, we discuss some mechanisms causing alterations in a cell's genetic information and ways that we can manipulate those mechanisms to improve bioprocesses.

8.1. EVOLVING DESIRABLE BIOCHEMICAL ACTIVITIES THROUGH MUTATION AND SELECTION

Although the cell has a well-developed system to prevent errors in DNA replication and an active repair system to correct damage to a DNA molecule, mistakes can still occur. These mistakes are called *mutations*. Before we discuss mutations, we need to establish the working vocabulary of microbial genetics.

The sum of the genetic construction of an organism constitutes its *genotype*. The characteristics expressed by a cell constitute its *phenotype*. The phenotypic response of a culture may change reversibly with alterations in environmental conditions, whereas the genotype is constant irrespective of the environment. A *mutation* is a genotypic change and is irreversible. A whole culture undergoes a phenotypic response, whereas only a rare individual will undergo a genotypic change. For example, if a culture changes in color from white to green when oxygen levels fall and then changes from green to white upon an increase in dissolved oxygen, the change would be phenotypic. Now consider an alternative experiment where white cells were removed from a culture, and placed on a plate (a small circular dish filled with nutrients solidified with agar) and allowed to grow into separate colonies. If one colony, but not the others, turned green and if cells obtained from the green colony remain green when cultured under the original conditions, it would be evidence for a genotypic change. In this case, the white cells would be the *wild type* and the green cells the *mutants*.

Let us consider what mechanisms may lead to genotypic change.

8.1.1. How Mutations Occur

Most mutations occur because of mistakes in DNA synthesis. Some examples are shown in Figure 8.1. One common form is a *point mutation*. A point mutation results from the change of a single base (e.g., cytosine instead of thymine). Some point mutations are *silent mutations* because the altered codon still codes for the same amino acid (e.g., UCU and UCA both code for serine). Even if the point mutation causes the substitution of a different amino acid, it may or may not alter protein activity substantially. A change of amino acid near the active site might alter protein activity greatly, whereas the same substitution at another site might have little to no effect.

One type of point mutation that usually has a profound effect is a *nonsense* mutation, whereby a stop codon is introduced into a DNA sequence (e.g., the CAA codon for glutamine changed to the UAA codon for "stop" in the mRNA derived from the altered DNA). A nonsense mutation results in premature termination of translation and a truncated, incomplete, and usually nonfunctional protein product.

Generally, *deletion mutations* have profound effects on cellular metabolism. By deleting or adding one or more bases, we can alter the whole composition of a protein, not just a single amino acid. A deletion can shift the *reading frame* when translating the resulting mRNA. This effect is illustrated in Figure 8.2.

Additions often take place through *insertion-sequence (IS) elements*. These elements are about 700 to 1400 base pairs in length; there are 37 different IS elements present in the genome of *Escherichia coli* strain MG1655. These elements can move on the chromosome from essentially any site to another. Often, they will insert in the middle of a gene, totally destroying its function.

Back mutations or *reversions* are possible. Revertants are cells for which the original wild-type phenotype has been restored. Restoration of a function can occur due to a

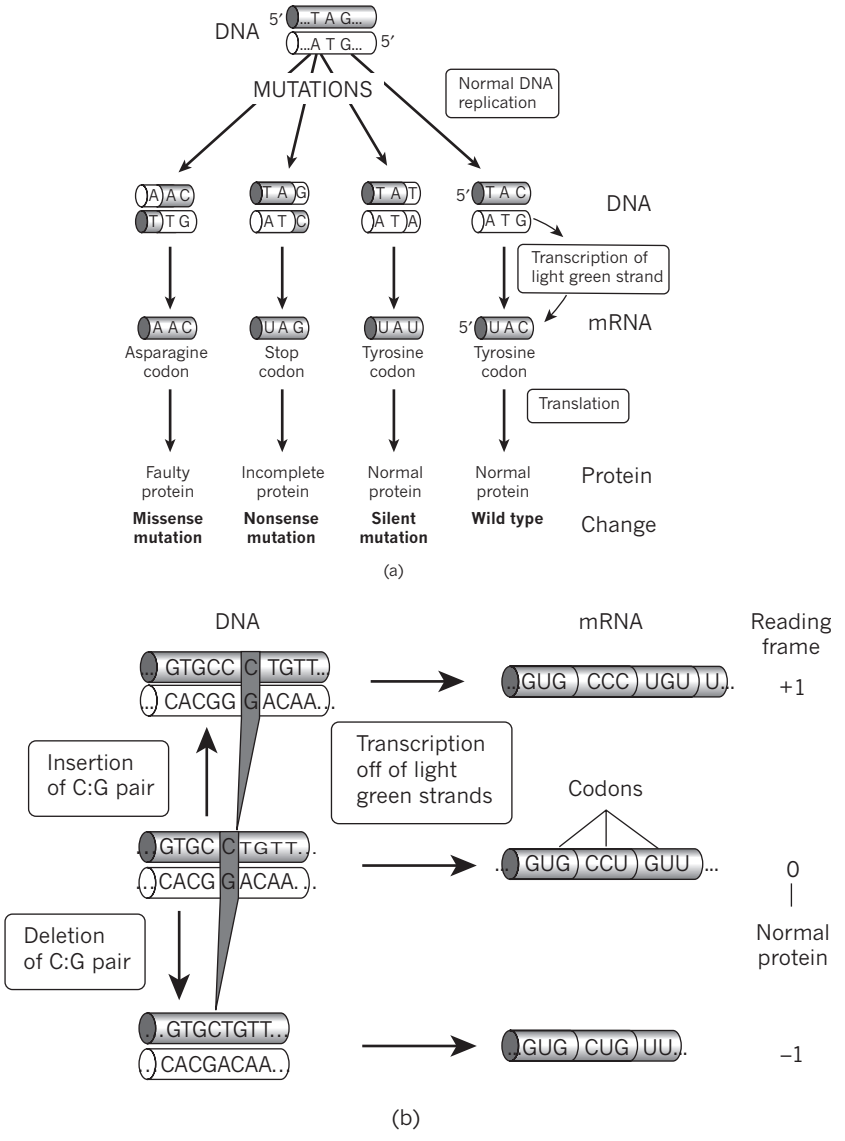


Figure 8.1. (a) Possible effects of base-pair substitution in a gene encoding a protein. (b) Shifts in the reading frame of mRNA caused by insertions and deletions. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

direct change at the original mutation (e.g., if the original mutation was CAA to UAA, then a second mutation for UAA to CAA restores the original genotype and phenotype). Second-site revertants can occur that restore phenotype *(suppressor mutations)* but not genotype (e.g., a second deletion mutation that restores the gene to the normal reading frame or a mutation in another gene that restores the wild-type phenotype).

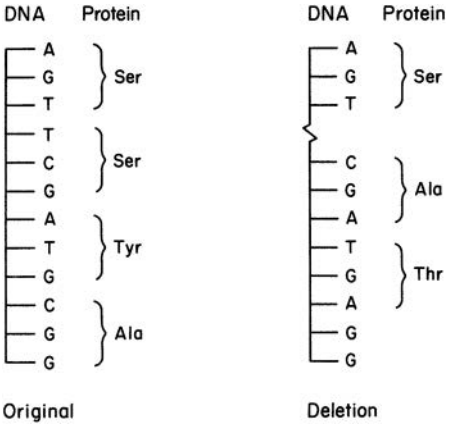


Figure 8.2. Effect of deletion of a base on the reading frame and the protein encoded.

8.1.2. Selecting for Desirable Mutants

Mutants can serve as powerful tools to better understand cell physiology; they are also valuable as industrial organisms because mutation can be used to alter metabolic regulation and cause overproduction of a desired compound. Methods to induce mutations and then select for mutants are important tools for catalyst development in bioprocessing.

Natural *(spontaneous)* rates of mutation vary greatly from gene to gene (10^{-3} to 10^{-9} per cell division), with 10^{-6} mutations in a gene per cell division being typical. Chemical agents *(mutagens)* and/or radiation are often used in the laboratory to increase mutation rates. Mutagens are nonspecific and may affect any gene.

The selection of a mutant with desirable properties is no easy task. Mutations are classified as *selectable* and *unselectable*. A selectable mutation confers upon the mutant an advantage for growth or survival under a specific set of environmental conditions; thus, the mutant can grow and the wild type will die. An unselectable mutant requires a cell-by-cell examination to find a mutant with the desired characteristics (e.g., green pigment). Even with mutagens, the frequency of mutation is sufficiently low to make prohibitive a brute-force screening effort for most unselectable mutants.

Selection can be direct or indirect. An example of direct selection would be to find a mutant resistant to an antibiotic or toxic compound. A culture fluid containing 10^8 to 10^{10} cells/ml is subjected to a mutagenic agent. A few drops of culture fluid are spread evenly on a plate, with the antibiotic incorporated into the solidified medium. Only antibiotic-resistant cells can grow, so any colonies that form must arise from antibiotic-resistant mutants. If one in a million cells has this particular mutation, we would expect to find about 10 to 100 colonies per plate if 0.1 ml of culture fluid was tested.

Indirect selection is used for isolating mutants that are deficient in their capacity to produce a necessary growth factor (e.g., an amino acid or a vitamin). Wild-type *E. coli* grow on glucose and mineral salts. *Auxotrophic* mutants would not grow on such a simple medium unless it were supplemented with the growth factor that the cell could no longer make (e.g., a lysine auxotroph has lost the capacity to make lysine, so lysine must be added to the glucose and salts to enable the cell to grow). The wild-type cell that needs no supplements to a minimal medium is called a *prototroph*.

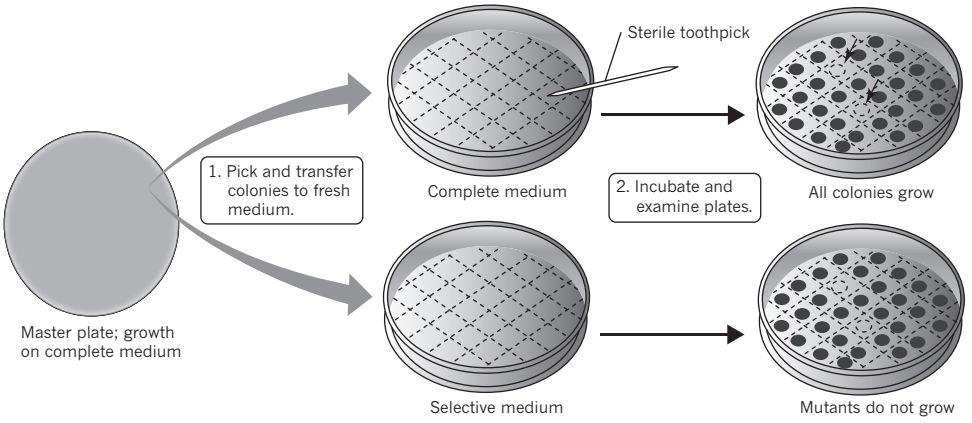


Figure 8.3. Screening for nutritional auxotrophs. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

Consider the selection of a rare mutant cell that is auxotrophic for lysine from a population of wild-type cells. This selection cannot be done directly because both cell types would grow in the minimal medium supplemented with lysine.

A method that facilitates selection greatly is called *replica plating* (see Figure 8.3). A master plate using lysine-supplemented medium will grow both the auxotroph and wild-type cells. Once colonies are well formed on the master plate, an imprint is made on sterile velveteen. The bristles on the velveteen capture some of the cells from each colony. The orientation of the master plate is carefully noted. Then a test plate with minimal medium is pressed against the velveteen; some cells, at the point of each previous colony, then serve to inoculate the test plate at positions identical to those on the master plate. After incubation (approximately 24 hours for *E. coli*), the test plate is compared to the master plate. Colonies that appear at the same positions on both plates arise from wild-type cells, whereas colonies that exist only on the master plate must arise from the auxotrophic mutants.

Another class of mutants is called *conditional* mutants. Mutations that would normally be lethal to the cell could not be detected by methods we have described so far. However, mutated proteins are often more temperature sensitive than normal proteins. Thus, temperature sensitivity can often be used to select for conditionally lethal mutations. For example, the mutant may be unable to grow at the normal growth temperature (e.g., 37°C for *E. coli*) but will grow satisfactorily at a lower temperature (e.g., 25°C). Table 8.1 summarizes a variety of mutants and how they may be detected.

Mutation and selection have been used to tremendous advantage to probe the basic features of cell physiology and regulation. They also have been the mainstay of industrial programs for the improvement of production strains. For example, mutation and selection strategies have been primarily responsible for increasing the yield of penicillin from 0.001 g/l in 1939 to current values of about 50 g/l of fermentation broth.

TABLE 8.1. Kinds of Mutants

Description	Nature of Change	Detection of Mutant
Nonmotile	Loss of flagella; nonfunctional flagella	Compact colonies instead of flat, spreading colonies
Noncapsulated	Loss or modification of surface capsule	Small, rough colonies instead of larger, smooth colonies
Rough colony	Loss or change in lipopolysaccharide outer layer	Granular, irregular colonies instead of smooth, glistening colonies
Nutritional	Loss of enzyme in biosynthetic pathway	Inability to grow on medium lacking the nutrient
Sugar fermentation	Loss of enzyme in degradative pathway	Do not produce color change on agar containing sugar and a pH indicator
Drug resistant	Impermeability to drug or drug target is altered or drug is detoxified	Growth on medium containing a growth-inhibitory concentration of the drug
Virus resistant	Loss of virus receptor	Growth in the presence of large amounts of virus
Temperature sensitive	Alteration of any essential protein so that it is more heat sensitive	Inability to grow at a temperature normally supporting growth (e.g., 37°C) but still growing at a lower temperature (e.g., 25°C)
Cold sensitive	Alteration in an essential protein so that it is inactivated at low temperature	Inability to grow at a low temperature (e.g., 20°C) that normally supports growth

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8.2. NATURAL MECHANISMS FOR GENE TRANSFER AND REARRANGEMENT

Bacteria can gain and express wholly different biochemical capabilities (e.g., the ability to degrade an antibiotic or detoxify a hazardous chemical in their environment) literally overnight. These alterations cannot be explained through inheritance and small evolutionary changes in the chromosome. Rather, they arise from gene transfer from one organism to another and/or large rearrangements in chromosomal DNA. In this section, we discuss genetic recombination, gene transfer, and genetic rearrangements—all mechanisms that can be exploited to genetically engineer cells (see Table 8.1).

8.2.1. Genetic Recombination

Genetic recombination is a process that brings genetic elements from two different genomes into one unit, resulting in new genotypes in the absence of mutations. Genetic recombination in procaryotes is a rare event but sufficiently frequent to be important ecologically as well as industrially. The three main mechanisms for gene transfer are *transformation*, *transduction*, and *conjugation*. Transformation is a process in which free DNA is taken up by a cell. Transduction is a process in which DNA is transferred by a bacteriophage, and conjugation is DNA transfer between intact cells that are in direct contact with one another.

Once *donor DNA* is inside a cell, the mechanism for recombination is essentially independent of how the donor DNA was inserted. Figure 8.4 summarizes the molecular-level events in general recombination. The donor DNA must be homologous, or nearly so, to a segment of DNA on the recipient DNA.† Under the right conditions, cellular enzymes cut out the homologous section of recipient DNA, allow insertion of the donor DNA, and then ligate or join the ends of the donor DNA to the recipient DNA. Note that pieces of donor DNA that a cell recognizes as foreign are usually degraded by enzymes called *restriction endonucleases* (these enzymes are essential in genetic engineering) and

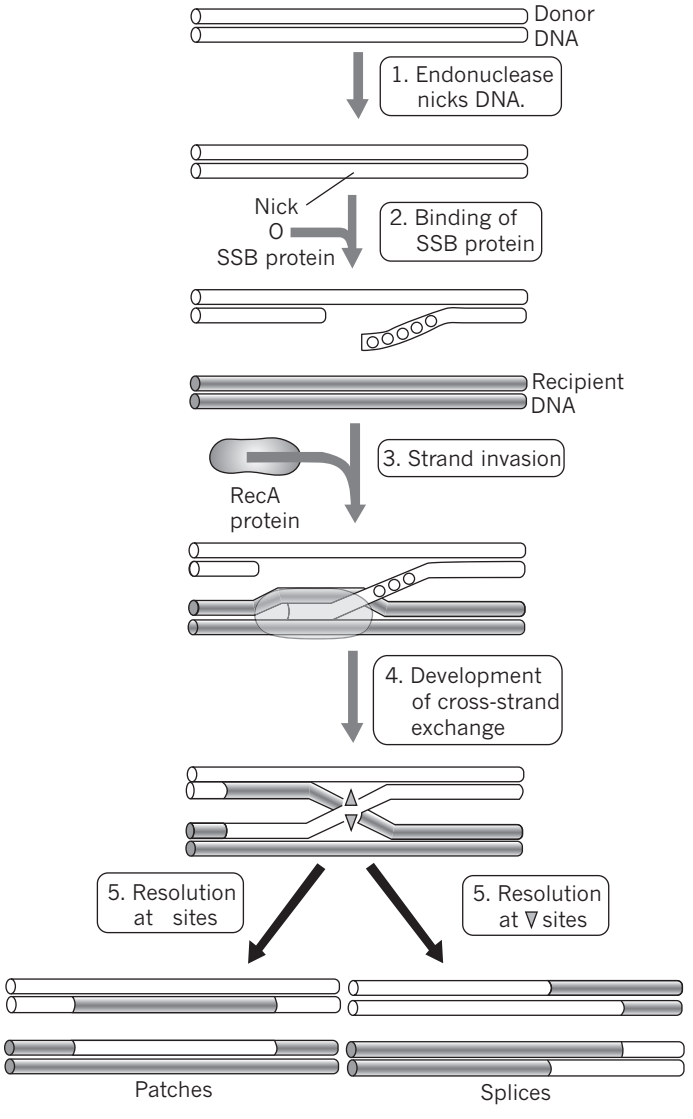


Figure 8.4. A simplified version of homologous recombination. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

†*Illegitimate recombination* between nonhomologous regions of DNA is possible, but rare. See later discussion on transposons.

cut a DNA molecule at a particular predetermined nucleotide sequence. A cell marks its own DNA (e.g., through methylation of certain purine or pyrimidine bases) to distinguish it from foreign DNA. These modifications block the action of a cell's own restriction endonucleases on its own DNA. Under natural conditions, gene transfer is effective only if the donor DNA is from the same or closely related species.

Let us now consider some details of how donor DNA can enter a cell.

8.2.2. Transformation

Transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (e.g., DNA) from its surroundings through the cell membrane(s). Transformation is a naturally occurring process in some bacteria, but it cannot be performed by all genera of bacteria. Even within transformable genera, only certain strains are transformable *(competent)*. Competent cells have a much higher capacity for binding DNA to the cell surface than do noncompetent cells. Competency can depend on the physiological state of the cell (current and previous growth conditions). Even in a competent population, not all cells are transformable. Typically, about 0.1% to 1.0% are transformable.

Transformation can also be implemented by artificial means in other cells. For example, *E. coli* cells are not normally competent, but their importance to microbial genetics has led to the development of empirical procedures to induce competency. This procedure involves treating *E. coli* with high concentrations of calcium ions coupled with temperature manipulation. The competency of treated cells varies among strains of *E. coli* but is typically rather low (about one in a million cells becomes successfully transformed). With the use of selective markers, this frequency is still high enough to be quite useful.

Transformation is useful only when the information that enters the cell can be propagated. When performing transformation, we typically use a vector called a *plasmid*. This element forms the basis for most industrially important fermentations involving recombinant DNA. A plasmid is a double-stranded, circular DNA molecule that is physically separated from chromosomal DNA (i.e., extrachromosomal) and can replicate independently (i.e., self-replicating). Some plasmids are maintained as low-copy-number plasmids (only a few copies per cell), and others have a high copy number (20 to 100 copies per cell). These plasmids differ in their mechanisms for partitioning at cell division and in the control of their replication. Plasmids encode genes typically for proteins that are nonessential for growth but that can confer important advantages to their host cells under certain environmental circumstances. For example, most plasmids encode proteins that confer resistance to specific antibiotics. Such antibiotic resistance is very helpful in selecting for cells that contain a desired plasmid.

8.2.3. Transduction

Transduction is the process by which DNA is transferred from one bacterium to another by a vector, which is usually a virus, and certainly plays an important role in nature. In the most common type of transduction, *generalized transduction*, infection of a recipient cell results in fragmentation of the bacterial DNA into 100 or so pieces. One of these fragments can become packaged randomly into a phage particle. The altered phage particle then injects bacterial DNA into another cell, where it can

recombine with that cell's DNA. With generalized transduction, any bacterial gene may be transferred.

Another method of transfer, which is far more specific with respect to the genes that are transferred, is *specialized transduction*. Here the phage incorporates into specific sites in the chromosome, and the frequency of transduction of a gene is related to its distance away from the site of incorporation. This process is summarized in Figure 8.5. A *lysogenic cell* is one carrying a prophage or phage DNA

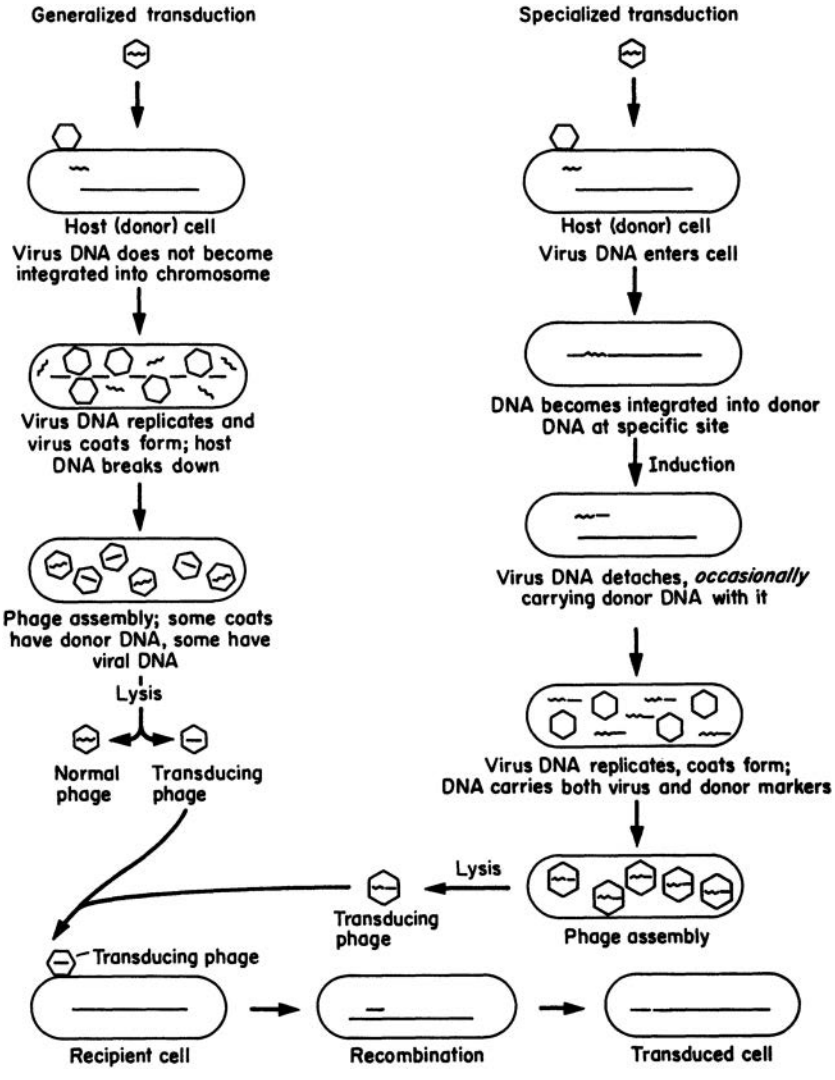


Figure 8.5. Transduction, the transfer of genetic material from donor to recipient via virus particles. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

incorporated into chromosomal DNA. Phage lambda is an example of such a *temperate phage* (a phage that can either lyse a cell or become incorporated into the chromosome). Such phages almost invariably insert at a specific site in the chromosome. The conversion of a *prophage* (the phage DNA in the chromosome) into the lytic cycle is normally a rare event (10^{-4} per cell division), but it can be induced in almost the whole culture upon exposure to UV light or other agents that interfere with DNA replication.

8.2.4. Episomes and Conjugation

A third type of gene transfer involves another genetic element. This element is called an *episome*. It is a DNA molecule that may exist either integrated into the chromosome or separate from it. When it exists separately from the chromosome (i.e., extrachromosomally), it is essentially a plasmid. A well-known episome is the F or *fertility factor*. Such factors are responsible for the process known as *conjugation*.

Most experiments with conjugation are done with the F factor, which is present in low copy number. Direct cell-to-cell contact is required. This DNA molecule encodes at least 13 genes involved in its self-transfer from one cell to another.

A population of *E. coli* frequently includes some cells with the F plasmid, which are termed F^+ (male). Other cells are F^- (female). F^+ cells encode proteins to make a *sex pilus*. When F^+ and F^- cells are mixed together, the sex pilus connects an F^+ to an F^- cell (see Figure 8.6). The sex pilus may act as a conduit for transferring a copy of the F plasmid to the F^- cell. The actual process of transfer involves replication of the F plasmid.

This process is more typical and does not involve transfer of chromosomal genes or recombination. A less common event is when the F plasmid has been integrated into the chromosome itself to form a single, large, circular molecule.

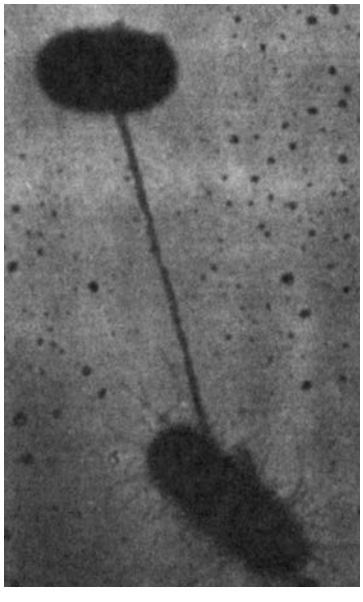


Figure 8.6. Direct contact between two conjugating bacteria is first made via a pilus. The cells are then drawn together for the actual transfer of DNA. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

Thirteen sites for integration are known. Such cells are termed Hfr (for high-frequency recombination).

When transfer is initiated, the F plasmid moves not only itself but also the attached chromosome to the recipient cell. The time required to transfer a whole *E. coli* chromosome is 100 min. If contact between the two cells is broken during the transfer process, only a proportional amount of the chromosome will have been transferred (i.e., at 50 min, about 50%). Since the transfer begins at a known point, Hfr cells can be used to map the location of genes on the chromosome. This technique for gene mapping is being replaced by methods for directly sequencing nucleotide sequences in DNA. If F^+ and F^- cells differ in properties (e.g., the ability to make lysine), conjugation can be used to alter the properties of the F^- cell.

Conjugation, transduction, and transformation all represent forms of gene transfer from one cell to another. However, gene transfer can occur within a cell.

8.2.5. Transposons: Internal Gene Transfer

Previously, we discussed the presence of IS elements on the chromosome. A closely related phenomenon is a *transposon*, which refers to a gene or genes that have the ability to "jump" from one piece of DNA to another or to another position on the original piece of DNA. The transposon integrates itself into the new position independently of any homology with the recipient piece of DNA. Transposons differ from IS elements in that they code for proteins. Transposons appear to arise when a gene becomes bounded on both sides by insertion sequences. Many of the transposons encode antibiotic resistance.

Transposons are important because they can induce mutations when they insert into the middle of a gene, they can bring once-separate genes together, and in combination with plasmid- or viral-mediated gene transfer, they can mediate the movement of genes between unrelated bacteria (e.g., multiple antibiotic resistance on newly formed plasmids). Transposon mutagenesis can be a very powerful tool in altering cellular properties usually by insertional inactivation of chromosomal genes.

8.3. GENETICALLY ENGINEERING CELLS

Our description of DNA replication, mutation, and selection and the natural mechanisms for gene transfer covered all the tools necessary to genetically engineer a cell. The purposeful, predetermined manipulation of cells at the genetic level, an idea that was farfetched prior to 1970, is easily within the grasp of beginning students.

Genetic engineering is a set of tools and not a scientific discipline. Although difficult to define precisely, it involves the manipulation of DNA outside the cell to create artificial genes or novel combinations of genes with predesigned control elements. Because many of these manipulations can be done outside the cell, we can circumvent species limitations that constrain the age-old techniques of mutation and selection and breeding (e.g., we can express a human protein in *E. coli*). Learning how to use these tools is the basis of modern biotechnology.

8.3.1. Basic Elements of Genetic Engineering

An overview of the strategy typically employed in genetic engineering is given in Figure 8.7. The strategy makes use of *recombinant DNA* techniques, the ability to isolate genes from one organism and recombine the isolated gene with other DNA that can be propagated in a similar or unrelated host. Most of our discussion is drawn from approaches for genetically engineering bacteria.

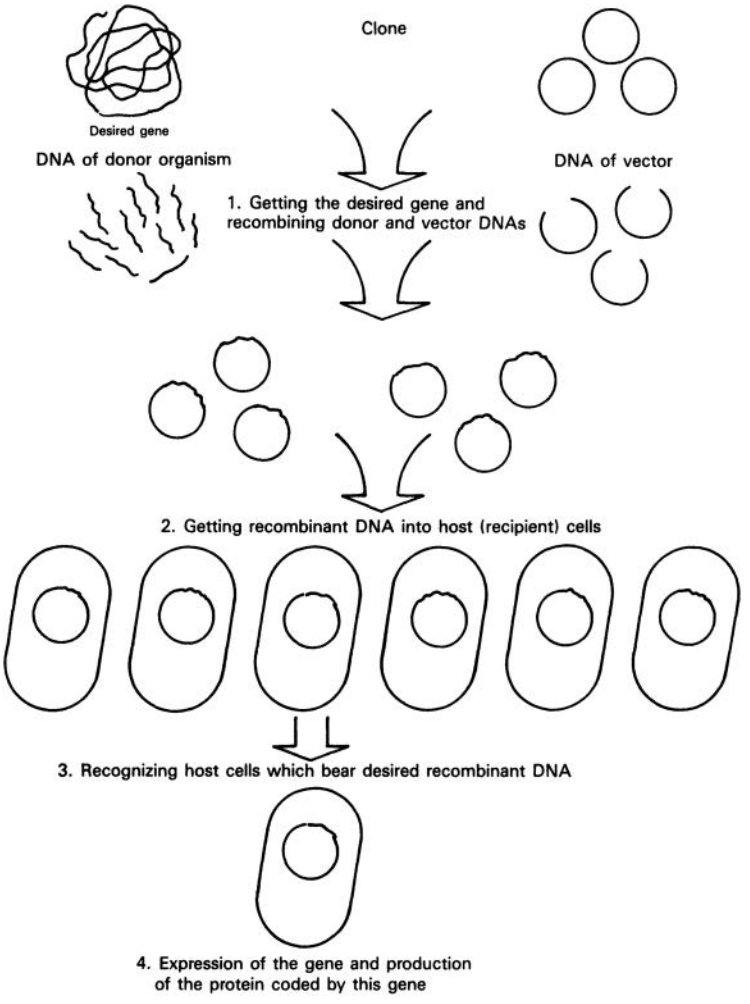


Figure 8.7. Overview of the essential steps in genetic engineering: moving a gene from one organism to another. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

8.3.1.1. Obtaining the gene of interest. The first step is obtaining the gene of interest. A simple, brute-force approach is *shotgun cloning*. Here the DNA from the donor organism is cut into fragments using *restriction enzymes*. If an efficient screening procedure is available, large numbers of host cells with random fragments of DNA can be screened for those exhibiting a property related to the desired gene.

Most often, such a shotgun approach is very inefficient. More specific approaches use *hybridization*. A *probe* can be synthesized chemically to be complementary to a portion of the gene. The probe is usually much shorter than the gene but sufficiently long that it is unlikely for other genes to have the same complementary DNA sequence. The construction of the probe requires some knowledge of either the nucleotide sequence of the desired gene or a partial amino acid sequence for the desired gene. Since the genetic code is degenerate, the deduction of the actual nucleotide sequence is ambiguous. This ambiguity requires that a variety of probes be generated. Hybridization reactions require the donor DNA to be both fragmented and converted into single strands that can react with the single-stranded probes.

An alternative to hybridization is total *chemical synthesis* of a gene that corresponds to the desired protein. This approach requires knowledge of the amino acid sequence of the desired protein and commonly involves column-based, solid-phase phosphoramidite chemistry to synthesize DNA. While gene-length DNA molecules of up to ~200 nucleotide (nt) residues can be routinely synthesized by this method, the cost and error rate can be prohibitive for sequences >200 nt. Thus, a number of methods have emerged to address the cost of making longer DNA molecules.

One approach uses chemical synthesis to make small sets of shorter oligos (typically 5–50 oligos) that are subsequently assembled into larger synthetic fragments (typically 200–3,000 nt) via a variety of methods that leverage biological mechanisms such as DNA ligase-based annealing, DNA polymerase-based assembly, or homologous recombination. More recent methods leverage *DNA microarray technology* (see Section 8.4.1), whereby standard mask-based photolithographic techniques are used to spatially localize DNA oligo synthesis on surfaces using light-activated chemistries. Microarray-derived oligos are subsequently cleaved and harvested as one "oligo pool," and the pool of oligos are assembled by a variety of techniques to yield full-length genes. Together, these methods allow us to produce specifically modified natural proteins or potentially totally human-designed proteins. Moreover, an artificial gene may be created that codes for exactly the same protein as in nature but with a sequence of nucleotides on the artificial gene that is not identical to the natural gene. For example, strategic use of *synonymous mutations* in the coding gene can optimize the expression of a recombinant gene in the target organism, a process known as *codon optimization* or *codon harmonization*. This approach takes advantage of natural codon biases that exist between organisms. Because the genetic code is degenerate (i.e., many amino acids are encoded by more than one codon), the genes of different organisms are often biased toward using one of the several codons that encode the same amino acid over the others.

Another method to obtain the desired gene is particularly useful for genes with *introns*. Since bacteria lack the cellular machinery to cut out introns and perform mRNA splicing, eucaryotic genes with introns cannot be directly placed in bacteria to make a desired protein. Often, we wish to make these proteins in bacteria, since

the bacteria grow much more rapidly and are much easier and cheaper to culture. Often the processed mRNA for the desired gene can be isolated directly from the donor organism's cytoplasm (using hybridization probes that are complementary to the poly[A] tails found on eucaryotic mRNA for total mRNA isolation). Once the mRNA is isolated, the enzyme *reverse transcriptase* (see Chapter 4) can be used to synthesize a DNA molecule with the corresponding nucleotide sequence; this molecule is called *complementary DNA* (cDNA).

Large amounts of a target gene can be generated by a technique called the *polymerase chain reaction* (PCR). PCR is the preferred method to amplify DNA. This technique uses a target sequence of interest on double-stranded DNA (dsDNA) such as cDNA or genomic DNA (gDNA).[†] In addition, the technique requires that two short primer sequences (<20 nt) on either side of the target be known and chemically synthesized. If heat is applied, the complementary strands of dsDNA separate. While the strands are separated, two pieces of chemically synthesized DNA (the primers) are added. Each primer binds to complementary sequences. A heat-stable DNA polymerase from a bacterium that grows in hot springs (the Taq polymerase) is added and quickly synthesizes from the primers the complementary DNA strands using the four nucleotides (A, G, T, and C) added to the reaction mixture. At the end of the cycle, there are two copies of the original gene. If the cycle is repeated, those two copies become four. Thirty cycles can be done over the course of a few hours. Thus, from a single gene copy, 2^{30} copies of the gene can be generated, representing more than a billion-fold increase. Thermal cyclers and PCR kits are commercially available.

It should be noted that although our focus is on amplifying the number of genes as a basis for producing target proteins or altering pathways, gene cloning is often done to obtain many copies of a particular gene. The amplification of the gene number facilitates gene sequencing and analysis. This amplification is particularly important for mapping genomes (e.g., the human genome project), for diagnosis of disease-causing organisms (both microbial and viral), for biologists studying evolution, and for forensic scientists.

8.3.1.2. Inserting the gene into DNA. Once the desired gene is isolated or made, it can be inserted into a small piece of carrier DNA called a *vector*. Typically, vectors are plasmids, although temperate viruses can be used. The process for preparing the donor DNA and vector for recombination and the actual joining of the DNA segments usually requires special enzymes (see Figure 8.8). We discussed these enzymes in our previous consideration of DNA replication and genetic recombination. A wide variety of *restriction enzymes* exist that will cut DNA at a different prespecified site. Most vectors have maps showing the various restriction sites; important examples are EcoR1 from *E. coli* and Bam H1 from *Bacillus amyloliquefaciens*. Many restriction enzymes leave "sticky ends," a few nucleotides of single-stranded DNA projecting from the cut site. Pieces of DNA with complementary sticky ends naturally associate, and in a mixture of cut vector and donor DNA,

[†]For forensic studies or phylogenic studies on relationships of organisms, a nonprotein coding sequence may be the target.

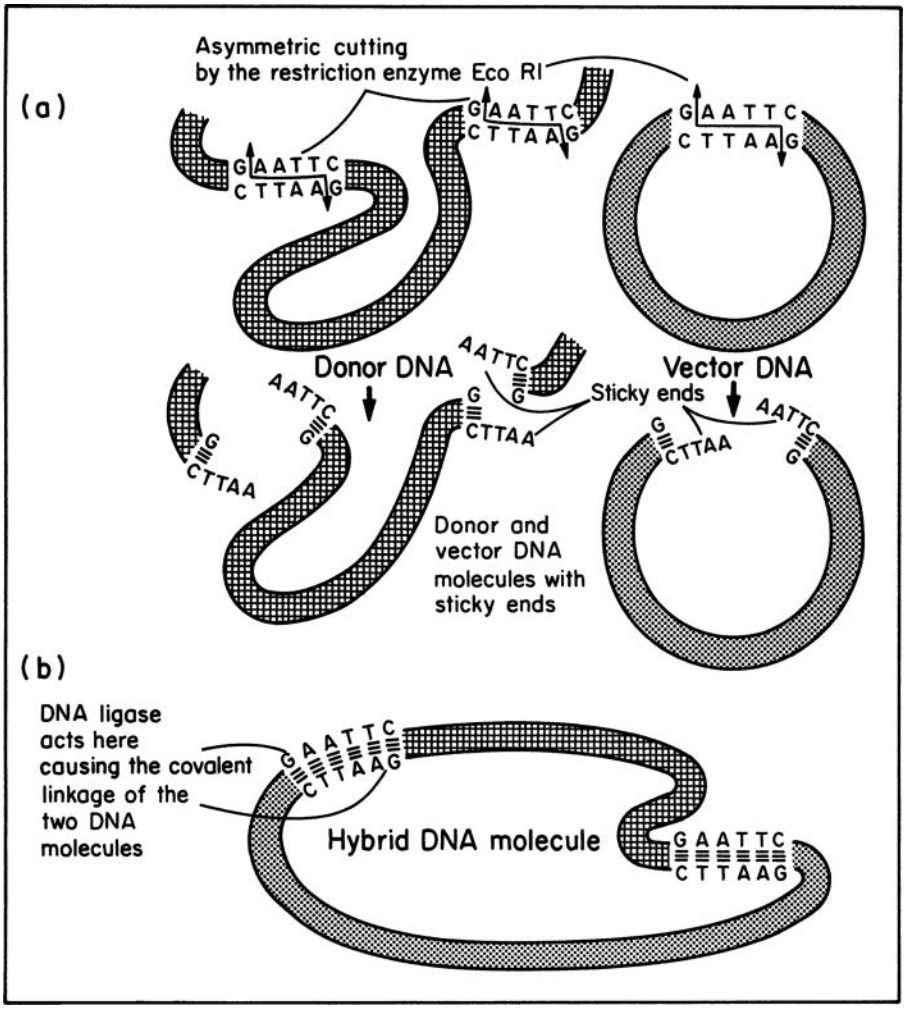


Figure 8.8. Use of special enzymes in genetic engineering. (a) The specific cutting of DNA by a restriction enzyme results in the formation of ends that contain small, single-stranded complementary sequences ("sticky ends"). (b) The enzyme DNA ligase links pieces of DNA that have become associated by their sticky ends. (Brock, Thomas D., Brock, Katherine M., Ward, David M., *Basic Microbiology with Applications*, 3rd ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

some pieces of donor DNA will associate with vector DNA. *DNA ligase* can permanently join these ends.

The mixture bearing the desired vector–donor combinations is then moved into the recipient or host cell. In most cases, this is done by transformation, although other techniques can be used if transformation of the host is difficult. Note that the construction of the desired vector–donor DNA usually results in a mixture (e.g., some vector molecules may be opened and rejoined without donor DNA being inserted,

or multiple copies of donor DNA may be inserted, or DNA contaminants of the donor DNA mixture may become inserted into the vector). Consequently, an efficient method to screen transformants for those with the desired vector–donor DNA combination is important.

Most vectors contain selectable markers such as antibiotic resistance or the genes to make essential growth factors that have been removed by mutation from the host cell. In the latter case, growth in minimal medium is possible only in the presence of the plasmid. These selectable markers allow the isolation of genetic *clones* that have been successfully transformed. A further screening step is then necessary to ensure that the donor DNA is present and being *expressed* (i.e., a functional protein is being made from the donor DNA).

8.3.1.3. Alternative strategies for gene cloning. Over the years, a number of molecular cloning strategies have emerged that provide alternatives to the "classic" restriction enzyme-cloning method previously described. Usually, several different approaches can be used for a specific cloning project; however, for any given project, there may be an ideal approach. The choice of approach may be based on speed, cost, availability of starting materials, or just personal preference.

One of these approaches, *TOPO cloning* (or *TA cloning*) takes advantage of the fact that the Taq polymerase enzyme (the same one used in PCR reactions, as discussed earlier) leaves a single adenosine (A) overhang on the 3′ end of PCR products. Using commercially available TOPO vectors, the PCR products are efficiently hybridized between the 3′ A overhang of the PCR product and the 5′ T overhang of the TOPO backbone vector. The major disadvantage of this approach is that very few plasmid backbones are available TOPO ready, and it is difficult to create a TOPO vector yourself.

Another strategy, Gateway cloning, is a bacterial recombination–based method whereby a fragment of DNA is first cloned into a "donor" plasmid, a process that is still most often achieved by traditional restriction enzyme cloning. The DNA fragment in the donor plasmid can then be rapidly shuttled into any compatible Gateway "destination" vector. The advantage of this method is that you can clone your gene of interest one time by restriction enzyme cloning into a donor plasmid, and then leverage bacterial recombination to easily move it into a series of plasmids that allow you to do many different molecular biology techniques (such as fusing it with different tags, putting it under a variety of promoters and into backbones with different selection cassettes).

Isothermal cloning, more commonly known as Gibson assembly, is a method that uses three common molecular biology enzymes—5′ exonuclease, polymerase, and ligase—to reliably assemble multiple fragments of DNA in the chosen orientation. Consequently, there is no need for any unwanted sequence at the junctions (such as restriction enzyme or Gateway recombination sites). The method works with any dsDNA fragments, PCR products, or synthesized oligos with appropriately designed overhangs (i.e., DNA extensions that will hybridize with a target sequence) that can be efficiently ligated into any plasmid backbone.

A particular type of restriction endonuclease, known as a *type IIS enzyme*, has unique properties that have been harnessed to create molecular cloning methods such

as Golden Gate, GreenGate, and modular cloning (MoClo). In these methods, the type IIS enzyme cuts dsDNA at a specified distance from the recognition sequence, which creates custom overhangs, something that is not possible using traditional restriction enzymes. One advantage of this system is that the entire cloning step (digestion and ligation) is carried out in a single pot containing a single restriction enzyme, which is possible because the resulting overhangs are distinct and maintain the directionality of the cloning reaction. Another advantage is that all recognition sequences are removed from the final product (i.e., no undesired sequence or scar remain).

Ligation independent cloning (LIC) employs the $3' \rightarrow 5'$ exonuclease activity of T4 DNA polymerase to create overhangs with complementarity between the vector and insert. Because of the relatively long stretches of base pairing in the hybridized product, ligation is rendered unnecessary. The product may be transformed directly into *E. coli*, where the *nicks* (discontinuity in the dsDNA molecule) are repaired by the normal replication process.

A final method is yeast-mediated cloning, which is very similar in principle to Gibson cloning, but rather than an in vitro assembly reaction using purified enzymes, it takes advantage of the native recombination abilities of yeast. Like Gibson cloning, this method efficiently assembles two (or more) fragments of dsDNA that have overlapping homology. The advantage of this method over others is that much larger final products can be generated (up to 100 kb), which is significant because cloning in bacterial hosts can become progressively more difficult for plasmids larger than 10 kb.

8.3.1.4. Expression of cloned genes. Obtaining good expression from the donor DNA is often a difficult challenge. Careful selection of stably propagating vectors and of promoters, checks to ensure that the correct reading frame is being used, and the selection of host cell backgrounds that do not interact unfavorably with the "foreign" protein are all important considerations. Discussions of how to obtain and maintain high levels of expression occupy much of Chapter 14, "Utilizing Genetically Engineered Organisms." We can screen for the expression of donor DNA, for example, if the product confers a selectable marker itself (e.g., the ability to grow on a substrate not normally utilized by the host). Also, antibodies to the target protein when tagged with a radiolabel or fluorescent label can be used to identify colonies expressing the target protein.

An important tool in working with both proteins and DNA fragments or plasmids is *electrophoresis*. In protein electrophoresis, an electric field is applied to a solution containing proteins placed at the top of a gel (typically made of polyacrylamide). The proteins migrate through the porous structure of the gel in a direction and at a speed that reflect both the size and net charge of the molecule. The gel reduces the effects of convection, although thermally induced convection can be problematic in large gels. For proteins, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is commonly used. In this technique, the proteins are denatured by heating with SDS and mercaptoethanol. Mercaptoethanol is a reducing agent used to break disulfide bonds. Individual polypeptide chains form a complex with SDS, which is negatively charged. The negatively charged complex then migrates through the gel at a rate that reflects the molecular weight of the polypeptide. In a typical SDS-PAGE gel, one lane is loaded with a standard that contains polymers

of known molecular weight. Other lanes will have samples with unknown proteins. After a defined period of time (typically a few hours), the process is stopped and the gel is examined. Each protein forms a band. With some types of electrophoresis, the band is highlighted by use of stains such as Coomassie blue or silver stain. Smaller molecules travel a greater distance. The molecular weight of a protein band in the unknown sample can be estimated by comparing to the lane with the molecular weight standard. The relative amount of a protein can be determined by the intensity of the band.

Various forms of gel electrophoresis can be used to determine if a protein from a clone is being produced and at what relative level. A particularly useful procedure is an *immunoblot* or *Western blot*. Here, a nondenaturing gel is run to separate proteins, they are blotted onto nitrocellulose paper, and the protein identified by binding to a specific antibody (primary antibody) followed by a secondary antibody that binds specifically to the first antibody. This secondary antibody is usually conjugated with a radioactive label or enzyme (e.g., horseradish peroxidase, HRP) that permits detection of the target protein band. A similarly conjugated primary antibody can be used instead, which eliminates the need for a secondary antibody but at the expense of the signal amplification that comes from the second antibody labeling step. The band is visualized by exposure of the nitrocellulose paper to x-ray film when a radioactive label is used or by reaction with a substrate that, when oxidized by HRP using hydrogen peroxide as the oxidizing agent, yields a characteristic change that is detectable by spectrophotometric methods. HRP catalyzes the conversion of chromogenic substrates into colored products and produces light when acting on chemiluminescent substrates (The use of phosphoimaging is replacing the use of x-ray film). Because of the high degree of specificity of antibodies, a positive band is good confirmation that the protein band represents a target product rather than another protein of similar molecular weight.

Similarly, DNA molecules can be separated by gel electrophoresis using agarose or polyacrylamide. DNA is negatively charged. DNA fragments (e.g., from digestion with restriction enzymes) can be separated by molecular weight as well as plasmids from larger DNA elements. The DNA separates on the basis of molecular weight. The DNA can be recovered by simply cutting out the part of the gel corresponding to the band for the desired DNA and eluting. The DNA is invisible unless stained. One approach is to expose DNA to an intercalating dye that fits itself in between base pairs of DNA and fluoresces when under ultraviolet light (e.g., ethidium bromide, propidium iodide).

With shotgun cloning (which produces a large *gene library* or *gene bank*), a desired colony can be isolated using radiolabeled RNA or DNA probes complementary to the cloned gene. Such a procedure involves transferring colonies to nitrocellulose filter paper, where they are lysed. After lysis, the DNA is released and binds to the filter paper. The paper is flooded with the probe; the probe only binds to the DNA with a complementary sequence (hybridization), and excess probe is washed away. If the filter is covered with x-ray film, radioactivity will expose the film (visible as a black spot), identifying which colonies contained the donor DNA. This procedure identifies colonies that have been transformed with the desired DNA; expression has to be established via separate experiments.

Another approach to using a gene library to find genes that express proteins with certain functions is the use of display technologies. Most common among these are *phage display* and *cell-surface display* (e.g., bacterial display, yeast display). For example, a bacteriophage or bacterium may display on its exterior surface proteins encoded from genes derived from the library. Each cell may display at most only a few of these gene products. An example of the use of such a system is the isolation of a cell producing a gene product that will bind to a particular molecule *(ligand)*. The ligand can be bound to a surface, and only those cells expressing a protein on their surface that binds to that ligand can "stick" to the surface. These cells that stick can be recovered and propagated to make more copies of the gene and protein.

This simple summary does not cover all the intricacies investigators often face in obtaining industrially useful clones. However, the procedures discussed here are applicable in most cases where plasmids are used as vectors to transform bacteria such as *E. coli.*

8.3.2. Genetic Engineering of Higher Organisms

The direct genetic engineering of higher organisms can be a great deal more difficult than for bacteria because of a lack of good effective techniques to introduce foreign DNA and lack of an understanding of host cell genetics. Be aware of the techniques being developed to work with some of these host systems. The introduction of foreign DNA into higher organisms is usually termed *transfection*.

Some plants are subject to infection with the bacterium *Agrobacterium tumefaciens*. *A. tumefaciens* contains a plasmid that contains a section known as T-DNA. This T-DNA can integrate into the plant chromosome. If genes are inserted into the T-DNA region, they can be incorporated ultimately in the plant chromosome. Unfortunately, most cereal plants are not readily susceptible to *Agrobacterium* infection.

The *biolistic process* for obtaining plant transformation is to coat small (1-μm diameter) projectiles (e.g., of tungsten) and shoot them into cells at high velocity. Results with this approach have been remarkably successful, and this technique is fairly general.

Another fairly general approach is *electroporation*, which involves a brief high-voltage electric discharge that renders cells permeable to DNA. Electroporation can be used with animal, plant, fungal, and bacterial systems. The formation of *protoplasts* can enhance transfection but is not essential. A protoplast is a cell in which the outer cell envelope has been removed so that only the cytoplasmic membrane remains.

Chemically or electrically mediated *protoplast fusion* is another technique for transferring genetic information from one cell to another. Such approaches have been particularly useful with some fungi for which few or no plasmids have been identified. Protoplast fusion can be interspecies and can result in stable hybrids with desired properties due to recombination events between the two genomes or extrachromosomal pieces of DNA.

For most animal cells, genetic manipulation can be accomplished by modifying viruses to become vectors. For example, in the insect cell system, a *baculovirus* can be modified so as to place a desired gene under the control of a very strong promoter at the expense of a gene product that is unessential for viral replication in cell culture.

Although the basic conceptual approach to genetic engineering is rather straightforward, its implementation can vary widely in difficulty. The level of

difficulty depends on the nature of the gene product and its corresponding gene, as well as the character of the desired host cell. Techniques to improve the ease of obtaining desirable genetic modification will undoubtedly continue to be developed. The ultimate limitation will be human imagination and wisdom.

8.3.3. Genome Engineering

Genome engineering, or *genome editing*, refers to the collection of strategies and techniques developed in recent years for the targeted, specific modification of the genetic information—or genome—of living organisms. Genome engineering has the potential to impact a wide range of applications, particularly in the areas of human health, agricultural and industrial biotechnology, and research tool development. Examples include the inactivation or modification of a specific gene for understanding its function, the correction of a gene carrying a harmful mutation, the production of therapeutic proteins, the elimination of persistent viral sequences, and the development of new generations of genetically modified crops. A number of techniques have been developed for these purposes, several of which are discussed in this section.

Multiplex automated genome engineering (MAGE) is a method for introducing targeted modifications directly into the chromosome of *E. coli* using synthetic single-stranded DNA (ssDNA). The process is cyclical and begins with transformation of ssDNA by electroporation followed by outgrowth, during which bacteriophage homologous recombination enzymes mediate annealing of ssDNAs to their genomic targets. With MAGE, many independent or combinatorial mutations can be made in genes, gene networks, or full genomes. MAGE can generate combinatorial genetic diversity in a cell population either by targeting one ssDNA per site *(locus)* in the genome (i.e., many target sites, single genetic mutations) or by targeting more than one ssDNA per locus (i.e., single target site, many genetic mutations). Using MAGE, researchers have engineered *E. coli* strains that produce five times the normal amount of lycopene, an antioxidant normally found in tomato seeds and linked to anticancer properties. The iterative approach took only 3 days and about $1000 in materials.

Several genome engineering techniques seek to efficiently eliminate a gene using a "molecular scissors" approach, with the goal of generating breaks in dsDNA along the sequence to be modified. Most standard restriction enzymes recognize very short (1 to 10 base pairs) and generally palindromic sequences, which often occur at several sites in the genome of an organism. As a result, restriction enzymes are likely to cut the DNA molecule multiple times. To gain more precise targeting for genome engineering/editing applications requires enzymes that recognize and interact with DNA sequences that are sufficiently long so as to occur only once, with high probability, in any given genome. In this way, the DNA modification (e.g., cutting) is narrowly restricted to the site of the target sequence. Because they recognize sites that are greater than 12 base pairs, meganucleases, zinc finger nucleases (ZFN), and transcription activator-like effector nuclease (TALEN) fusions offer a level of precision that is suitable for genome engineering/editing.

A drawback of these methods is the significant effort required for each new genome engineering target. Typically, a technique known as *protein engineering* (see Section 14.9) is used to produce a custom meganuclease, ZFN, or TALEN

that recognizes the sequence of interest. The need for engineering customized proteins can be circumvented using CRISPR (clustered regularly interspaced short palindromic repeat) technology. CRISPR is a naturally occurring procaryotic immune system that functions to identify and destroy foreign DNA such as bacteriophage viruses and plasmids. However, the CRISPR mechanism can be leveraged for genome engineering/editing by delivering the CRISPR-associated endonuclease (Cas9) protein and appropriate guide RNAs (gRNAs) into a target cell. Once internalized, the gRNAs direct Cas9 to the target sequence where it unwinds and cleaves the dsDNA, enabling an organism's genome to be specifically cut at any desired location. An advantage of the technique is that users need to design only gRNAs that target their DNA sequence of interest; no laborious protein engineering is required. Another advantage is that CRISPR technology has been adapted such that genome engineering/editing can be performed in cell types throughout the three domains of life. CRISPR technology has enormous potential in a number of application areas, most notably (and perhaps controversially) in altering the germline of humans, animals, and other organisms and modifying the genes of food crops.

8.4. GENOMICS

Genomics is the set of experimental and computational tools that allow the genetic blueprints of life to be read. A *genome* is an organism's total inheritable DNA. Most important, we have the genomic sequence for humans. This sequence information is simply a string of nucleotide letters. *Functional genomics* is the process of relating genetic blueprints to the structure and behavior of an organism. To completely relate physiological behavior to this sequence of nucleotides is an extremely challenging problem—one to which bioengineers can and have made significant contributions.

Much of the progress in molecular biology has been due to a reductionist approach in which a subcomponent has been isolated and studied in detail. This approach has been fruitful in learning about the detailed mechanisms at the heart of living systems. The detailed sequence information now available is the ultimate limit in reduction in biology. There is increasing interest in asking how the individual subcellular components work together. Function arises from the complex interactions of the components. A systems engineering approach allows one to integrate component parts into a functional whole. This integrative approach is often known as *systems biology*.

Whereas genetic information is linear and static, cellular systems are highly nonlinear, dynamic systems that respond to their environment and regulate gene expression. Over the next decades, a focus of biochemical engineering, in conjunction with other disciplines, will be relating this linear sequence information to those nonlinear dynamical systems.

The role of the bioengineer is twofold. One role is as an enabler, by making better tools for rapid analysis of DNA sequences, of expression of mRNAs, and of a cell's total proteins *(proteomics)*. A second role is as an interpreter and organizer of genetic information; this role usually involves mathematical modeling.

8.4.1. Experimental Techniques

The primary tools of genomics are used for DNA sequencing, detecting which mRNAs are expressed, and determining which proteins are present in a cell or tissue. Historically, the nucleotide sequence of DNA fragments was determined on a sequencing gel by a process called *Sanger sequencing*, named after its inventor Frederick Sanger. The key to this method is the use of dideoxyribonucleoside triphosphates, which are derivatives of the natural deoxyribonucleoside triphosphates and lack the hydroxyl (OH) group at the 3′ position. For example, 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP) is the derivative deoxyadenosine triphosphate (dATP). If a strand of DNA is being replicated and if ddATP is inserted into the position normally occupied by dATP, replication is stopped. The OH group at the 3′ position is essential for continued replication. The basic process for this type of sequencing is shown in Figure 8.9.

It is easiest to imagine reactions in four separate tubes labeled A, T, C, and G. To each tube is added the DNA fragment to be sequenced; DNA polymerase; a stoichiometric excess of dATP, deoxythymidine triphosphate (dTTP), deoxycytidine

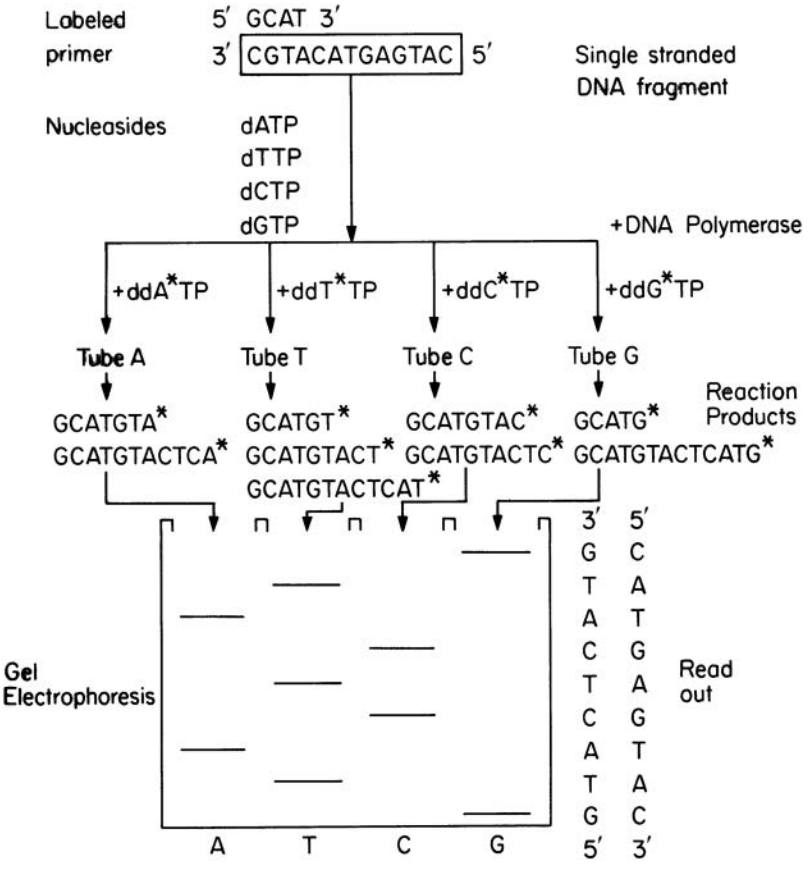


Figure 8.9. Example of a sequencing gel to obtain the nucleotide sequence of a DNA fragment. See text for details.

triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP); and an oligonucleotide primer for the DNA polymerase to use. The reaction is analogous to the first step in the PCR reaction. Only one strand of the DNA fragment to be sequenced is read. In addition to these reactants, in each tube a small amount of ddNTP is added where N is any of the four nucleotides. For example, in tube A, ddATP is added; in tube T, ddTTP is added; and so on. Since the amount of ddNTP added is small in tube N, several reaction products are formed. In tube N, the first time dNTP or ddNTP must be added to the growing copy of the original DNA fragment, there is a high probability that dNTP will be added and the chain can be extended. However, there is also a finite probability (determined by the ratio of ddNTP to dNTP) that ddNTP will be added and the chain extension will be terminated. At the second position where N is required, either ddNTP or dNTP will be added. Again, some chains will be terminated, and others will continue to extend. This reaction continues, and tube N will generate fragments of different sizes, all of which end in the letter N. These fragments are separated by gel electrophoresis, and the sequence of the DNA fragment can be read directly from the gel (as shown in Figure 8.9). This technique is limited to relatively short DNA fragments (a few hundred nucleotides).

For sequencing genomes, the Sanger technique is too slow and must be modified and automated. The basic approach to sequence a large genome is to cut it into millions of overlapping fragments of 2000 to 10,000 base pairs in length. Each fragment is ligated into a plasmid, which is transformed into *E. coli*. The *E. coli* form a living genomic library. These colonies are robotically picked, identified with a barcode, and placed in a 384-well plate. The amount of the cloned DNA fragment is amplified using PCR. From each end of the fragment, 500 letters are replicated using the ddNTP approach previously described. For the purposes of automation, a fluorescent dye is used for each ddNTP. Four different dyes are used to distinguish A, T, C, and G. These labeled fragments are then read in automated sequencing machines. These machines rely on a large number (>100) of glass capillaries; capillary electrophoresis is used to separate the fragments by size. As the fragments exit the capillary, a laser beam detects the color of the dye at the end of the fragment. The information is fed to a computer, and the 500-letter sequence is determined. Reading 100 million letters of DNA sequence is possible. These sequences are stored in a computer; computer algorithms are then used to align the overlapping sequences.

Using the capillary-electrophoresis-based Sanger sequencing method, the Human Genome Project took more than 10 years to complete and cost nearly $3 billion. In contrast, next-generation sequencing (NGS), also known as high-throughput sequencing, is a catch-all term that describes a number of recently developed sequencing methods, such as Illumina sequencing and Roche 454 sequencing that both leverage the *sequencing by synthesis (SBS)* principle. These and other related technologies make large-scale whole-genome sequencing accessible and practical for the average researcher and as such have revolutionized the study of genomics and molecular biology.

Another approach, which increases throughput, is the use of *nanopores*. A strand of DNA is forced to pass through a nanopore as a long string using, for example, electrophoresis. As each nucleotide passes through the pore, it obstructs the passage of electrical current. Each base (A, T, G, C) blocks current in its own characteristic manner, enabling a direct reading of the DNA sequences.

With recent advances in several technologies, the cost of sequencing a complete human genome decreased dramatically, and the cost to sequence the genome of a microbe and the time necessary to do so are low enough that such an effort is now routine.

Another technology that is having a large impact on genomics is *microarrays* for measuring which genes are being expressed by measuring the corresponding mRNA levels. These microarrays are high-density oligonucleotide arrays. These oligonucleotides hybridize with the corresponding mRNA. For a known gene or protein, an oligonucleotide can be synthesized that binds the corresponding mRNA. Using photolithography, the manufacturing of arrays containing 280,000 individual oligonucleotides on glass substrates of 1.64 cm^2 is now routine. *Transcriptomics* describes the measurement of all the transcripts in a cell. Such arrays can simultaneously analyze expression from 6000 to 10,000 genes. For bioprocesses, the array can be used to determine which genes are upregulated or downregulated in response to a process change (e.g., temperature).

An increase in mRNA levels does not immediately correspond to a change in protein level. Because different mRNAs have different rates of degradation, efficiency of translation, and so on, changes in mRNA expression may not translate directly into changes in the protein content of a cell or tissue. Because the proteins are the primary components responsible for biological activity, a knowledge of the protein content of a cell is highly valuable. Because proteins cannot be easily amplified, as can nucleic acids, measurements of all of the proteins in a cell (i.e., proteomics) is very difficult. Also, proteins vary greatly in properties such as hydrophobicity. Additionally, changes in splicing and posttranslational processing alter protein properties significantly.

The traditional technique for identifying proteins in a complex mixture has been two-dimensional gel electrophoresis. This technique is derived from the SDS-PAGE technique described earlier in this chapter. For two-dimensional gel electrophoresis, two methods are combined. The first is to separate proteins in one direction (say, the x-axis) according to their *isoelectric point*—the pH at which the protein has no net charge. Using a special set of buffers, a pH gradient can be established in a polyacrylamide gel. When the proteins are subjected to an electric field, they will move to the pH equal to their isoelectric point and remain there. After equilibration with an anionic surfactant, the proteins are subjected to an electric field in the other direction (say, the y-axis). The proteins will remain at the same pH but move down the y-axis according to their molecular weight. By separating protein mixtures using these two parameters (isoelectric point and molecular weight), a complex protein mixture can be resolved into separate spots. Often, those "spots" can be removed and analyzed by tandem mass spectrometry to identify the protein's sequence.

The technique of two-dimensional gel electrophoresis is time consuming and expensive. Improvements in techniques in mass spectrometry have had a large impact, although it still depends on off-line two-dimensional gel electrophoresis to prepare samples for analysis. Significant improvements will be necessary for routine analysis of cell and tissue protein content. Promising approaches combine microtechnology, liquid chromatographies, and mass spectrometry techniques. The engineering challenges in making a practical device are significant.

8.4.2. Computational Techniques

The wealth of data emerging from these new experimental techniques is overwhelming. Generally, DNA sequence information could be used to identify which sequences are genes that encode for proteins, what these proteins are, what their biological function is, and ultimately how these genes are regulated.

Deciding what nucleotide sequence corresponds to a gene is not always a clear-cut process. Investigators look for *open reading frames*, or *ORFs*. An ORF is a nucleotide sequence without a "stop" signal that encodes some minimal number of amino acids (about 100). In procaryotes, identifying ORFs is fairly straightforward. In eucaryotes, because of introns and exons, assignment of ORFs is complicated. Some computer programs can recognize probable (consensus) intron–exon boundaries.

When a prospective gene is identified, we need to know the function of the corresponding protein. In some cases, the function can be deduced by comparison to databases with amino acid sequence information on known proteins. Occasionally, the amino acid sequence and function of a protein is conserved across species.

With a highly conserved protein, we might find similar amino acid sequences—for example, in the fruit fly and humans. In this case, we would determine how *homologous* the two amino acid sequences were. We then might infer that the human gene encodes a protein with similar function. Conservation of amino acids near a catalytic or binding site is particularly critical. Efficient computer algorithms to do such searches are under development. *BLAST*, or *basic local alignment search tool*, is an example of an algorithm for the comparison of amino acid sequence information.

Ideally, one would like to predict protein structure and function solely from the amino acid sequence. Heroic efforts have been made to accomplish this goal, but there is no good general solution. Understanding the folding of proteins into their three-dimensional configuration is a computationally difficult problem.

Even with well-studied organisms, such as *E. coli*, we generally can suggest the function of about 70% of the genes. Clearly, knowing the full genome sequence has told us how little we really understand. The process of identifying single genes and the function of the corresponding genes has been the focus of *bioinformatics*.

Even if we knew the identity of every gene in a cell and the function of each corresponding protein, we would have an incomplete understanding of function and cellular physiology. A list of the proteins and their functions needs to be supplemented by an understanding of cellular structure and regulation. A combination of proteins can form metabolic pathways, and there will be a corresponding genetic circuit. Relating the cell's "parts list" to its dynamic, physiological state is an unmet challenge that provides exciting, long-term opportunities for bioengineers.

In particular, models of cells and metabolic circuits, as discussed in Chapter 6, provide tools with which to organize data and to understand biological function. Simple stoichiometric models of central metabolism have been used with good success to identify which genes are essential to a cell in a particular environment. Models that also incorporate kinetics and regulatory structure are in development. Such models will be key to relating cellular physiology to the genome. Such an understanding will provide important guidance to development of future bioprocesses.

8.5. SUMMARY

A cell's *genotype* represents the cell's genetic potential, whereas its *phenotype* represents the expression of a culture's potential. The genotype of a cell can be altered by *mutations*. Examples of mutations are *point mutations*, *deletions*, and *additions*. Additions are usually the result of *insertion sequences* that "jump" from one position to another.

Mutations may be *selectable* or *unselectable*. The rate of mutation can be enhanced by the addition of chemicals called *mutagens* or by radiation. *Auxotrophs* are of particular use in genetic analysis and as a basis for some bioprocesses. Another useful class of mutants is *conditional* mutants.

Gene transfer from one cell to another augments genetic information in ways that are not possible through mutation only. *Genetic recombination* of different DNA molecules occurs within most cells. Thus, genetic information transferred from another organism may become a permanent part of the recipient cell. The three primary modes of gene transfer in bacteria are *transformation*, *transduction*, and *conjugation*. Self-replicating, autonomous, extrachromosomal pieces of DNA called *plasmids* play important roles in transformation. *Episomes*, which are closely related to plasmids, are the key elements in conjugation. Bacteriophages are critical to *generalized transduction*, and *temperate phages* are key to *specialized* transduction. Internal gene transfer can occur due to the presence of *transposons*, which probably also play a role in the assembly of new plasmids.

We can use gene transfer in conjunction with *restriction enzymes* and *ligases* to genetically engineer cells. In vitro procedures to recombine isolated donor DNA genes with *vector* DNA (e.g., plasmids, temperate phages, or modified viruses) are called *recombinant DNA techniques*. Once the vector with the DNA donor insert has been constructed, it can be moved to a recipient cell through any natural or artificial method of gene transfer. Although transformation is the most common technique in bacteria, a large variety of artificial methods have been developed (e.g., *biolistic process*, *electroporation*, modification of infective agents, and *protoplast fusion*) to insert foreign DNA into a host cell.

Genomics is the set of experimental and computational tools that allows the genetic blueprints of a whole organism to be read. *Functional genomics* is the process of relating genetic blueprints to the behavior and structure of an organism.

SUGGESTIONS FOR FURTHER READING

Many of the references cited at the end of Chapter 4 have selections dealing with mutation and selection, gene transfer, and genetic engineering.

General Textbooks

Alberts, B., D. Bray, K. Hopkin, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, *Essential Cell Biology*, 4th ed., Garland Science, New York, 2014.

Hardin, J., G. Bertoni, and L. J. Kleinsmith, *Beckers World of the Cell*, 9th ed., Pearson Education, New York, 2015.

Madigan, M. T., J. M. Martinko, K. S. Bender, D. H. Buckley, and D. A. Stahl, *Brock Biology of Microorganisms*, 14th ed., Pearson Education, New York, 2014.

Basic Techniques

Glick, B. R., J. J. Pasternak, and C. L. Patten, *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, 4th ed., ASM Press, Washington, D.C., 2010.

Green, M. R., and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2012.

Articles

Burns, M. A., and Others, "An Integrated Nanoliter DNA Analysis Device," *Science* 282: 484–487, 1998.

Cipriany, B. R., and Others, "Real Time Analysis and Selection of Methylated DNA by Fluorescence-Activated Single Molecule Sorting in a Nanofluidic Channel," *PNAS* 109: 8477–8482, 2012.

Dutt, M. J., and K. H. Lee, "Proteomic Analysis," *Curr. Opin. Biotechnol.* 11: 176–179, 2000.

PROBLEMS

8.1. Would a cell with a point mutation or a deletion be more likely to revert back to the original phenotype? Why?

8.2. Consider the metabolic pathway based on aspartic acid shown in Chapter 4, Section 4.8, Figure 4A.1. Describe the procedure you would use to obtain a methionine overproducer. Use mutation-selection procedures, detailing the experiments to be done and their sequence.

8.3. You wish to isolate temperature-sensitive mutants (e.g., those able to grow at 30°C but not at 37°C). Describe experiments to isolate such a cell.

8.4. An important method for screening for carcinogens is called the *Ames test*. The test is based on the potential for mutant cells of a microorganism to revert to a phenotype similar to the nonmutant. The rate of reversion increases in the presence of a mutagen. Many compounds that are mutagens are also carcinogens, and vice versa. Describe how you would set up an experiment and analyze the data to determine if nicotine is mutagenic.

8.5. How many different hybridization probes must you make to ensure that at least one corresponds to a set of four codons encoding the amino acid sequence val-leu-trp-lys?

8.6. You wish to develop a genetically engineered *E. coli* producing a peptide hormone. You know the amino acid sequence of the peptide. Describe the sequence of steps you would use to obtain a culture expressing the gene as a peptide hormone.

8.7. You wish to produce a small protein using *E. coli*. You know the amino acid sequence of the protein. The protein converts a colorless substrate into a blue product. You have access to a high-copy-number plasmid with a penicillin-resistant gene and normal reagents for genetic engineering. Describe how you would engineer *E. coli* to produce this protein. Consider the source of donor DNA, regulatory elements that need to be included, how the donor and vector DNA are combined, how the vector DNA is inserted, and how you would select for a genetically engineered cell to use in production.

8.8. You wish to express a particular peptide in *E. coli* using a high-copy-number plasmid. You have the amino acid sequence for the peptide.

a. Explain the experimental process for generating and selecting the genetically engineered *E. coli* using restriction enzymes, ligase, *E. coli*, plasmid with neomycin resistance, and the known amino acid sequence.

b. What control elements would you place on the plasmid to regulate expression and to prevent read-through?

8.9. There are three primary methods for obtaining donor DNA when doing genetic engineering.

a. What are those methods (two- to six-word descriptions of each are acceptable)?

b. You need to produce a protein from humans in *E. coli*. You do not know the primary amino acid sequence. You suspect that introns are present. Which method will you use to obtain the donor DNA?

8.10. What is the difference between *transduction* and *transformation* when discussing genes transfer to bacteria?

8.11. You wish to isolate a thymidine auxotrophic mutant of *E. coli*. Describe briefly what experiments you would do to accomplish this.

8.12. For the DNA sequence TAGGATCATAAGCCA and using a primer ATCC, sketch what the corresponding sequencing gel should look like.

PART 2
Engineering Principles for Bioprocesses

9

Operating Considerations for Bioreactors for Suspension and Immobilized Cultures

So far, we have discussed what cells are, how they work, and how to describe their growth in simple reactors. We now begin our discussion of how to use these cells in industrial processes. This chapter explores some complicated reactor strategies and why they might be considered for use in real processes. Chapter 10, "Selection, Scale-Up, Operation, and Control of Bioreactors," gives more details on reactor design, and Chapter 11, "Recovery and Purification of Products," details how to recover products from these reactors. These chapters provide an understanding of how real bioprocesses can be assembled.

An important decision for constructing any process concerns the configuration the reactor system should take. The choice of reactor and operating strategy determines product concentration, number and types of impurities, degree of substrate conversion, yields, and whether sustainable, reliable performance can be achieved. Unlike many traditional chemical processes, the reactor section represents a major component (usually >50%) of the total capital expenditures. Choices at the reactor level and of the biocatalyst determine the difficulty of the separation. Thus, our choice of reactor must be made in the context of the total process: biocatalyst, reactor, and separation and purification train.

9.1. CHOOSING THE CULTIVATION METHOD

One of the first decisions is whether to use a batch or continuous cultivation scheme. Although a simple batch and continuous-flow stirred-tank reactor (CFSTR) represent extremes (we will soon learn about other reactors with intermediate characteristics), consideration of these two extreme alternatives will clarify some important issues in reactor selection.

First, we can consider productivity. The simplest case is for the production of cell mass or a primary product. For a batch reactor, four distinct phases are present: lag phase, exponential growth phase, harvesting, and preparation for a new batch (e.g., cleaning, sterilizing, and filling). Let us define t_l as the sum of the times required for the lag phase, harvesting, and preparation. The value for t_l will vary with size of the equipment and the nature of the fermentation but is normally in the range of several hours (3 to 10 h). Thus, the total time to complete a batch cycle (t_c) is

$$t_c = \frac{1}{\mu_m} \ln \frac{X_m}{X_0} + t_l \tag{9.1}$$

where X_m is the maximal attainable cell concentration and X_0 is the cell concentration at inoculation.

The total amount of cell mass produced comes from knowing the total amount of growth-extent-limiting nutrient present and its yield coefficient:

$$X_m - X_0 = Y_{X/S} S_0 \tag{9.2}$$

The rate of cell mass production in one batch cycle (r_b) is

$$r_b = \frac{Y_{X/S} S_0}{(1/\mu_m) \ln(X_m/X_0) + t_l} \tag{9.3}$$

As discussed in Chapter 6, "How Cells Grow," the maximum productivity of a chemostat is found by differentiating DX with respect to D and setting dDX/dD to zero. The value for D optimal when simple Monod kinetics apply is given by equation 6.90, and the corresponding X can be determined to be

$$X_{\text{opt}} = Y_{X/S} \left\{ S_0 + K_s - \sqrt{K_s (S_0 + K_s)} \right\} \tag{9.4}$$

Thus, the best productivity that could be expected from a chemostat where Monod kinetics apply is $D_{\text{opt}} \cdot X_{\text{opt}}$:

$$D_{\text{opt}} X_{\text{opt}} = Y_{X/S} \mu_m \left[1 - \sqrt{\frac{K_s}{K_s + S_0}} \right] \left[S_0 + K_s - \sqrt{K_s (S_0 + K_s)} \right] \tag{9.5}$$

Under normal circumstances, $S_0 >> K_s$, so the rate of chemostat biomass production, r_c, is approximately

$$r_{c,\text{opt}} = D_{\text{opt}} X_{\text{opt}} = \mu_m Y_{X/S} S_0 \tag{9.6}$$

The ratio for rates of biomass formation is

$$\frac{r_{c,\text{opt}}}{r_b} = \ln \frac{X_m}{X_0} + \mu_m t_l \tag{9.7}$$

Most commercial fermentations operate with $X_m/X_0 \approx 10$ to 20. Thus, we would expect continuous systems to always have a significant productivity advantage for primary products. For example, an *E. coli* fermentation with $X_m/X_0 = 20$, $t_l = 5$ h, and $\mu_m = 1.0\ h^{-1}$ would yield $r_{c,\text{opt}}/r_b = 8$.

Based on this productivity advantage, we might be surprised to learn that most commercial bioprocesses are batch systems. Why? There are several answers.

The first is that equation 9.7 applies only to growth-associated products. Many secondary products are not made by growing cells; growth represses product formation. Under such circumstances, product is made only at very low dilution rates, far below those values optimal for biomass formation. For secondary products, the productivity in a batch reactor may significantly exceed that in a simple chemostat.

Another primary reason for the choice of batch systems over chemostats is *genetic instability*. The biocatalyst in most bioprocesses has undergone extensive selection. These highly "bred" organisms often grow less well than the parental strain. A chemostat imposes strong selection pressure for the most rapidly growing cell. Back-mutation from the productive specialized strain to one similar to the less productive parental strain (i.e., a genetic revertant) is always present. In a chemostat, the less productive variant will become dominant, decreasing productivity. In a batch culture, the number of generations available (<25 from slant cultures to a commercial-scale fermenter) for the revertant cell to outgrow the more productive strain is more limited. Cells at the end of the batch are not reused. These considerations of genetic stability are very important for cells with recombinant DNA and are discussed in detail in Chapter 14, "Utilizing Genetically Engineered Organisms."

Another consideration is operability and reliability. Batch cultures can experience great variability from one run to another. Variations in product quality and concentration create problems in downstream processing and are undesirable. However, long-term continuous culture can be problematic; pumps may break, controllers may fail, and so on. Maintenance of sterility (absence of detectable foreign organisms) can be difficult to achieve for periods of months, and the consequences of a loss of sterility are more severe than with batch culture.

One other factor determining reactor choice is market economics. A continuous system forms the basis of a dedicated processing system—dedicated to a single product. Many fermentation products are required in small amounts, and demand is difficult to project. Batch systems provide much greater flexibility. For example, the same reactor can be used for two months to make product A and then for the next three for product B and the rest of the year for product C.

Most bioprocesses, particularly biopharmaceuticals, are based on batch reactors. Continuous systems are used to make single-cell protein, and modified forms of continuous culture are used in waste treatment; in ethanol production; and for some other large-volume, growth-associated products such as lactic acid. Because of the interest in the use of renewable resources for large-scale chemical and energy production using biological processes, it may be anticipated that large-scale continuous

systems are poised for increased development. With indications of a more flexible attitude on potential approval of continuous processes by the FDA and increasing competition from generics, pharmaceutical companies are actively considering continuous production. Several pharmaceutical companies have continuous manufacturing facilities scheduled to go on line before 2018.

Let us consider some modifications to these reactor modes.

9.2. MODIFYING BATCH AND CONTINUOUS REACTORS

Practical considerations require the use of reactor schemes that differ from the standard batch reactor or chemostat. These considerations typically seek to minimize total reactor volume and/or maximize product titer (or concentration) so as to reduce reactor cost per unit of product. These considerations are particularly important when the product is not growth associated.

9.2.1. Chemostat with Recycle

Microbial conversions are autocatalytic, and the rate of conversion increases with cell concentration. To keep the cell concentration higher than the normal steady-state level in a chemostat, cells in the effluent can be recycled back to the reactor. Cell recycle increases the rate of conversion (or volumetric productivity) and also increases the stability of some systems (e.g., wastewater treatment) by minimizing the effects of process perturbation. Cells in the effluent stream are either centrifuged, filtered, or settled in a conical tank for recycling. Since part of the cells are recycled back to the reactor, the cells (or solids) retention time (SRT) is much larger than the hydraulic residence time (HRT) of the nutrient medium. The life span of a typical bacterial cell is around 3 to 4 days. That is, when the cells remain in a system used for waste treatment for a longer period than their life span, the system may contain a significant fraction of dead cells.

For this reason, part of the cells should be removed from the system to keep the SRT at a desired level that favors an active and young microbial population in the system. Cells can be removed from the system either from the reactor or from the cell recycle stream.

Consider the chemostat system with cell recycle and solids (cells) removal from the reactor as depicted in Figure 9.1. A material balance on biomass (cell) concentration around the reactor yields

$$FX_0 + \alpha FCX_1 - (1+\alpha)FX_1 + V\mu_{net}X_1 = V\frac{dX_1}{dt} \tag{9.8}$$

where α is the recycle ratio based on volumetric flow rates, C is the concentration factor or ratio of cell concentration in the cell recycle stream to the cell concentration in the reactor effluent, F is nutrient flow rate, V is culture volume, X_0 and X_1 are cell concentrations in feed and recycle streams, and X_2 is cell concentration in effluent from the cell separator.

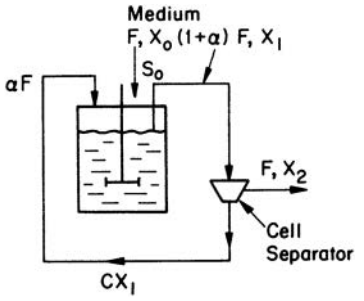


Figure 9.1. Chemostat with cell recycle. The cell separator could be a sedimentation tank, a centrifuge, or a microfiltration device.

At steady state, and if $dX_1/dt = 0$ and $X_0 = 0$ (i.e., sterile feed), then equation 9.8 becomes

$$\mu_g\text{-}k_d = \mu_{net} = (1+\alpha-\alpha C)D = [1+\alpha(1-C)]D \tag{9.9}$$

Since $C > 1$ and $\alpha(1 - C) < 0$, then $\mu_{net} < D$. That is, a chemostat can be operated at dilution rates higher than the specific growth rate when cell recycle is used. This allows a higher cell density to be achieved, which reduces required reactor size and is beneficial for products that are not strictly growth associated. In the case of wastewater treatment, cell recycle encourages endogenous metabolism, reducing the residual mass of cells that must be removed.

A material balance for growth-limiting substrate around the fermenter yields

$$FS_0 + \alpha FS - V\frac{\mu_g X_1}{Y^M_{X/S}} - (1+\alpha)FS = V\frac{dS}{dt} \tag{9.10}$$

At steady state, $dS/dt = 0$ and

$$X_1 = \frac{D}{\mu_g} Y^M_{X/S}(S_0 - S) \tag{9.11}$$

Substitution of equation 9.9, when $k_d = 0$, into equation 9.11 yields

$$X_1 = \frac{Y^M_{X/S}(S_0 - S)}{(1+\alpha-\alpha C)} \tag{9.12}$$

Therefore, the steady-state cell concentration in a chemostat is increased by a factor of $1/(1 + \alpha - \alpha C)$ by cell recycle. The substrate concentration in the effluent is determined from equation 9.9 and the Monod equation 6.32 (see Chapter 6), where endogenous metabolism is neglected:

$$S = \frac{K_s D(1+\alpha-\alpha C)}{\mu_m - D(1+\alpha-\alpha C)} \tag{9.13}$$

Then equation 9.12 becomes

$$X_1 = \frac{Y^M_{X/S}}{(1+\alpha-\alpha C)}\left[S_0 - \frac{K_s D(1+\alpha-\alpha C)}{\mu_m - D(1+\alpha-\alpha C)}\right] \tag{9.14}$$

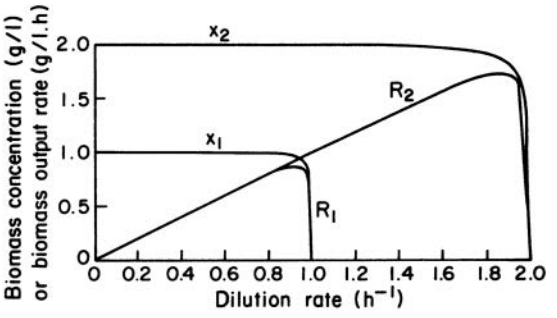


Figure 9.2. Comparison of biomass concentrations and volumetric production rates in steady states of chemostat cultures ($k_d = 0$) with and without recycle. X_1 = biomass concentration in chemostat without recycle; X_2 = biomass concentration in chemostat culture with recycle; R_1 = biomass output rate per unit volume without recycle; R_2 = biomass output rate of chemostat with recycle; $\mu_m = 1.00\ h^{-1}$; $S_r = 2.0$ g/l; $K_S = 0.010$ g/l; $Y_{X/S} = 0.5$ g/g; concentration factor, $C = 2.0$; and recycle rate, $\alpha = 0.5$.

Effluent cell concentrations and productivities in a chemostat with and without cell recycle are compared in Figure 9.2. Cell concentrations and volumetric productivities are higher with cell recycle, resulting in higher volumetric rates of substrate consumption. Systems with cell recycle are used extensively in waste treatment and are finding increasing use in ethanol production.

The application of cell recycle reactors in waste treatment is detailed in Chapter 16, "Multiple Species in Culture," and differs from this treatment in that the goal is minimization of cell mass and residual carbon source and not maximization of a secondary product. The equations in Chapter 16 differ from the previous case (equations 9.13 and 9.14) due to the inclusion of a nonzero term for endogenous metabolism (i.e., k_d).

Example 9.1

In a chemostat with cell recycle, as shown in Figure 9.1, the feed flow rate and culture volumes are $F = 100$ ml/h and $V = 1000$ ml, respectively. The system is operated under glucose limitation, and the yield coefficient, $Y^M_{X/S}$, is 0.5 gdw cells/g substrate. Glucose concentration in the feed is $S_0 = 10$ g glucose/l. The kinetic constants of the organisms are $\mu_m = 0.2\ h^{-1}$, $K_s = 1$ g glucose/l. The value of C is 1.5, and the recycle ratio is $\alpha = 0.7$. The system is at steady state.

a. Find the substrate concentration in the recycle stream (S).
b. Find the specific growth rate (μ_{net}) of the organisms.
c. Find the cell (biomass) concentration in the recycle stream.
d. Find the cell concentration in the centrifuge effluent (X_2).

Solution Using equation 9.9, we determine μ_{net}:

$$\mu_{net} = [1 + \alpha(1 - C)]D = [1 + (1 - 1.5)0.7](0.1) = \mu_g$$
$$= 0.065\ h^{-1}$$

Then we calculate the residual substrate concentration and the amount of biomass in the reactor:

$$S = \frac{K_{s_{net}}}{\mu_m - \mu_{net}} = \frac{(1)(0.065)}{0.2 - 0.065} = 0.48\ g/l$$

$$X_1 = \frac{D(S_0 - S)Y^M_{X/S}}{\mu_g} = \frac{(0.1)(10 - 0.48)0.5}{0.065}$$
$$= 7.3\ g/l$$

A biomass balance around the cell separator yields

$$(1+\alpha)X_1 = \alpha C X_1 + X_2$$
$$X_2 = (1+\alpha)X_1 - \alpha C X_1$$
$$= (1.7)(7.3) - (0.7)(1.5)(7.3)$$
$$= 4.8\ \text{g/l}$$

9.2.2. Multistage Chemostat Systems

In some fermentations, particularly for secondary metabolite production, the growth and product formation steps need to be separated, since optimal conditions for each step are different. Conditions such as temperature, pH, and limiting nutrients may be varied in each stage, resulting in different cell physiology and cellular products in multistage systems.

An example of a multistage system that may be beneficial is in the culture of genetically engineered cells. To improve genetic stability, a recombinant DNA plasmid usually uses an inducible promoter to control production of the target protein (see Chapter 8, "How Cellular Information Is Altered"). In the uninduced state, a plasmid-containing cell grows at nearly the same rate as a cell that loses the plasmid (a revertant), so the plasmid-free cell holds little growth advantage over the plasmid-containing cell. However, if the inducer is added, the plasmid-containing cells will make large quantities of the desired protein product but will have greatly reduced growth rates. Thus, a single-stage chemostat would not be suitable for the production of the target protein because of resulting problems in genetic stability. A multistage system can circumvent this problem. In the first stage, no inducer is added and the plasmid-containing cell can be maintained easily (usually an antibiotic is added to kill plasmid-free cells; see Chapter 14 for a more complete discussion). In the second stage, the inducer is added and large quantities of product are made. Cells defective in product synthesis should not overtake the culture (at least not completely), because fresh, genetically unaltered cells are being continuously fed to the reactor. Thus, the two-stage system permits stable continuous production of the target protein, which would otherwise be impossible in a simple chemostat.

Perhaps an easier situation to consider is the production of a secondary product (e.g., ethanol or an antibiotic). Here we worry not so much about a mixture of subpopulations but that conditions that promote growth completely repress product formation. Although commercial use of multistage continuous systems is limited, one very large-scale, five-stage system for ethanol production is currently in use. With renewed interest in production of biofuels, multistage continuous culture is poised for increased commercial utilization. A multistage system of CFSTR approaches plug flow reactor (PFR) behavior. A PFR mimics the batch system, where space time (the time it takes the culture fluid to reach a specific location in the PFR) replaces culture time. A multistage system is much like taking the batch growth curve and dividing it into sections, with each section being "frozen" in a corresponding stage of the multistage system. As in the batch reactor, the culture's physiological state progresses from one stage to the next.

The mathematical analysis of the multistage system that we present here is imperfect. Growth in the second and subsequent stages is intrinsically unbalanced growth, even though it is steady-state growth. New cells entering the second or subsequent stage are continuously adapting to the new conditions in that stage. Consequently, unstructured models are not expected to give completely accurate predictions. However, we use unstructured models here because of their simplicity and to illustrate at least some aspects of multistage systems.

A two-stage chemostat system is depicted in Figure 9.3. Biomass and substrate balances on the first stage yield the following equations (ignoring endogenous metabolism):

$$S_1 = \frac{K_s D_1}{\mu_m - D_1} \tag{9.15}$$

$$X_1 = Y^M_{X/S}(S_0 - S_1) \tag{9.16}$$

The biomass balance for the second stage is

$$FX_1 - FX_2 + \mu_2 V_2 X_2 = V_2 \frac{dX_2}{dt} \tag{9.17}$$

At steady state, equation 9.17 becomes

$$\mu_2 = D_2\left(1 - \frac{X_1}{X_2}\right) \tag{9.18}$$

where $X_1/X_2 < 1$ and $\mu_2 < D_2$.

Following is the substrate balance for the limiting substrate in the second stage:

$$FS_1 - FS_2 - \frac{\mu_2 X_2}{Y^M_{X/S}} V_2 = V_2 \frac{dS_2}{dt} \tag{9.19}$$

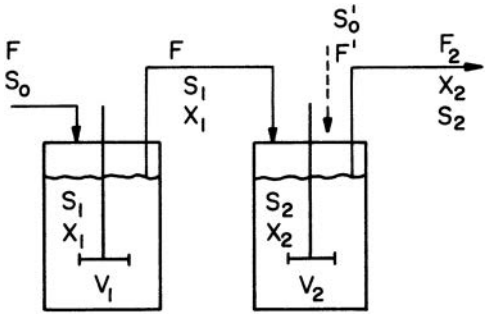


Figure 9.3 Two-stage chemostat system.

At steady state, equation 9.19 becomes

$$S_2 = S_1 - \frac{\mu_2}{D_2}\frac{X_2}{Y^M_{X/S}} \tag{9.20}$$

where

$$D_2 = F/V_2 \text{ and } \mu_2 = \frac{\mu_m S_2}{K_s + S_2}$$

Equations 9.18 and 9.20 can be solved simultaneously for X_2 and S_2 by substituting $\mu_2 = \mu_m S_2/(K_s + S_2)$ in both equations or any other functional form that describes μ_2.

When a feed stream is added to the second stage, the design equations change. The second feed stream may contain additional nutrients, inducers, hormones, or inhibitors. Biomass balance for the second stage in this case is

$$F_1X_1 + F'X' - (F_1 + F')X_2 + V_2\mu_2X_2 = V_2\frac{dX_2}{dt} \tag{9.21}$$

At steady state, when $X' = 0$, equation 9.21 becomes

$$\mu_2 = D'_2 - \frac{F_1}{V_2}\frac{X_1}{X_2} \tag{9.22}$$

where

$$D'_2 = \frac{F_1 + F'}{V_2} \text{ and } \mu_2 = \frac{\mu_m S_2}{K_s + S_2}$$

Substrate balance for the second stage yields

$$F_1S_1 + F'\,S'_0 - (F_1 + F')S_2 - \frac{V_2\mu_2X_2}{Y^M_{X/S}} = V_2\frac{dS_2}{dt} \tag{9.23}$$

Equations 9.22 and 9.23 need to be solved simultaneously for X_2 and S_2.

We can generalize these equations for a system with no additional streams added to second or subsequent units. If we do a balance around the nth stage on biomass, substrate, and product, we find

$$r_{x,n}(X_x, S_n) = D_n(X_n - X_{n-1}) \tag{9.24a}$$

or

$$\theta_n = \frac{1}{D_n} = \frac{X_n - X_{n-1}}{r_{x,n}(X_n, S_n)} \tag{9.24b}$$

$$r_{s,n} = \frac{1}{Y_{X/S}} r_{x,n}(X_n, S_n) = D_n(S_n - S_{n-1}) \tag{9.25a}$$

or

$$\theta_n = \frac{1}{D_n} = \frac{S_n - S_{n-1}}{r_{s,n}} \tag{9.25b}$$

$$r_{p,n}(X_n, S_n, \ldots) = D_n(P_n - P_{n-1}) \tag{9.26a}$$

or

$$\theta_n = \frac{1}{D_n} = \frac{P_n - P_{n-1}}{r_{p,n}(X_n, S_R, \ldots)} \tag{9.26b}$$

where $D_n = F/V_n$, θ_n is the mean residence time in the nth stage, and $r_{x,n}$, $r_{s,n}$, and $r_{p,n}$ all represent rates of reaction in the nth stage.

The preceding set of equations lends itself to machine calculations. However, graphical approaches to multistage design can also be used and have the advantage that the functional form of the growth or production rate need not be known. All that is required is a batch growth curve. However, the transfer of the information from batch growth curve to predictions of the multistage system still requires the assumption of balanced growth. Hence, the analysis must be used with caution. In at least one case (the production of spores by *Bacillus*), this approach has made experimentally verifiable predictions of the performance of a six-stage system.

The graphical approaches make use of equations 9.24 to 9.26. One approach is to use a plot of $1/(dX/dt)$ versus X or $1/(dP/dt)$ versus P derived from batch growth curves. This corresponds to using equations 9.24b and 9.26b. The size of the required reactor is determined by the area of the rectangle described with sides $X_n - X_{n-1}$ and $1/(dX/dt)$ or $P_n - P_{n-1}$ and $1/(dP/dt)$. The area of the rectangle is θ, and if F is known, V can be calculated. An alternative approach avoids some trial-and-error solutions that are necessary with the first approach. This second approach requires plots of dX/dt versus X and dP/dt versus P. The intersection of the reaction curve with a line from the mass balance equation (e.g., equation 9.24a) determines the exit concentration of X or P, while the slope of the line determines D, and if F is known, V can be found. We illustrate the use of these approaches in Example 9.2.

Example 9.2.

Data for the production of a secondary metabolite from a small-scale batch reactor are shown in Figure 9.4. Assume that two reactors, each with 700 l working volume, are available. You will use exactly the same culture conditions (medium, pH, temperature, and so on) as in the batch reactor. If the flow rate is 100 l/h, predict the outlet concentration of the product. Compare that outlet concentration to the value predicted if a single 1400 l reactor was used. Use both graphical approaches.

Solution We chose to use graphical approaches here rather than a computational approach, as we believe that the visualization of the solution is a stronger learning tool. In practice, a computational approach would likely be used. The first step in

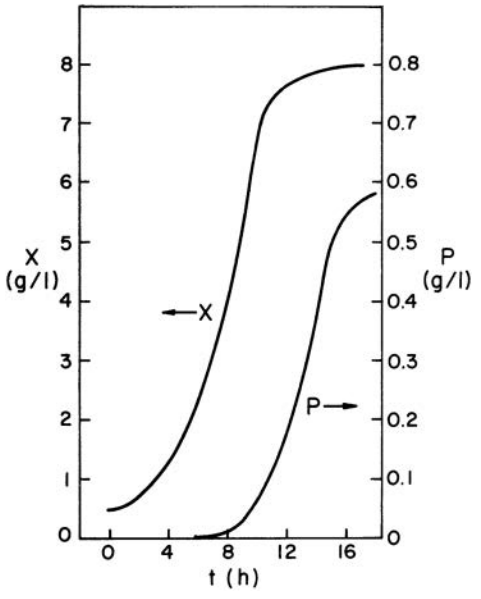


Figure 9.4. Data for Example 9.2. Data are for the production of a secondary product in batch culture.

using either graphical approach is to differentiate the data in the batch growth curve to yield dX/dt and dP/dt. The differentiation of experimental data can magnify errors present in the original data, so the values of dX/dt and dP/dt must be interpreted cautiously.

For the graphical approach illustrated in Figure 9.5, we have plotted $1/(dX/dt)$ versus X and $1/(dP/dt)$ versus P, which corresponds to equations 9.24b and 9.26b. For $\theta_1 = 7$ h (i.e., 700 l/100 l/h), we must determine what value of X_1 will satisfy

$$\theta_1 = (X_1 - X_0)\left(\frac{1}{dX/dt}\right)\Bigg|_{X_1}$$

or

$$7\text{ h} = X_1\left(\frac{1}{dX/dt}\right)\Bigg|_{X_1}$$

Since a sterile feed is to be used, $X_0 = 0$.

By trial and error, we find on the graph that $X_1 = 7.2$ g/l corresponds to $1/(dX_1/dt) = 0.95$ h/g/l or 7.2 g/l · 0.95 h/g/l = 6.84 h.

Given the accuracy with which Figure 9.5 can be read, this is an acceptable solution. The product concentration that corresponds to $X_1 = 7.2$ g/l is determined from the batch growth curve. As illustrated, $X_1 = 7.2$ g/l is achieved at 9.4 h after inoculation; at the same time, the value for P_1 is 0.08 g/l.

The effect of the second stage on the process is determined by using equation 9.26b and noting that again $\theta_2 = 7$ h:

$$\theta_2 = 7\text{ h} = (P_2 - P_1)\frac{1}{dP_1/dt}$$

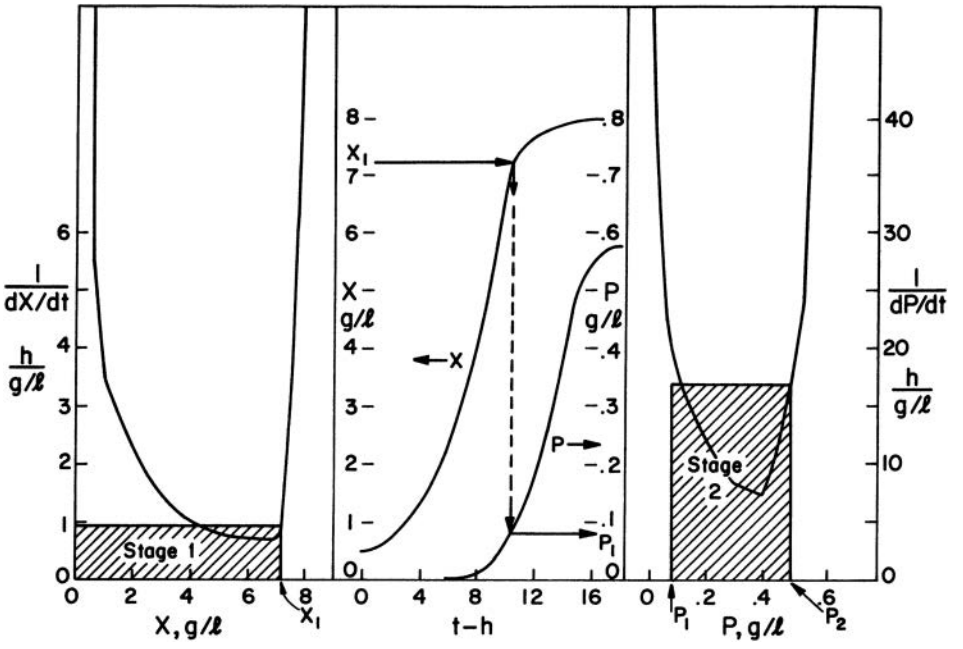


Figure 9.5. Solution of Example 9.2 for two-stage system, each with $\theta = 7$ h.

By trial and error, we find that at $P_2 = 0.49$ g/l, which corresponds reasonably closely to 7 h:

$$\theta_2 = (0.49 \text{ g/l} - 0.08 \text{ g/l})(17 \text{ h/g/l})$$
$$= 6.97 \text{ h}$$

Note that for the first stage in this solution, only solutions that exist for X_1 greater than the value of X for which $1/(dX/dt)$ is a minimum are practically obtainable. Washout occurs if θ_1 is too small.

We can compare the result to a single-stage system with the same total volume as the two-stage system (Figure 9.6). Here the trial-and-error approach indicates that for $X_1 = 7.35$ g/l we get

$$7.35 \text{ g/l} \cdot 1.9 \text{ h/g/l} = 13.97 \text{ h} \approx 14 \text{ h}$$

The value of P_1 that corresponds to $X_1 = 7.35$ g/l is 0.10 g/l. Thus, the use of the two-stage system in this case increased product concentration from 0.10 to 0.49 g/l.

An alternative graphical approach that eliminates the trial-and-error aspect of the first approach is shown in Figure 9.7. Here equations 9.24a and 9.26a have been used. Thus, realizing that $D_1 = 1/\theta$, we find that $D_1 = 0.143 \text{ h}^{-1}$. The intersection of the reaction curve with the straight line determined by $D_1(X_1 - X_0) = D_1 X_1$ is the solution to equation 9.24a. For the second stage, we consider the production phase and use equation 9.26a. The predicted values of X_1 and P_2 are the same as in the first approach. Note that the dP/dt-versus-P curve is displaced in time from the dX/dt-versus-X curve. Consequently, we use the dX/dt plot before using the dP/dt plot.

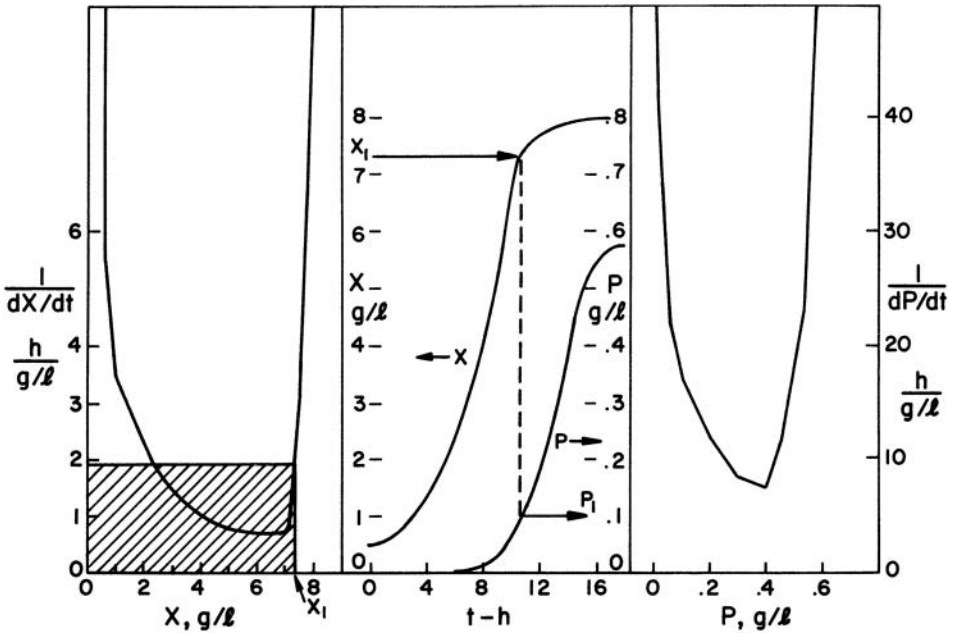


Figure 9.6. Solution of Example 9.2 with a single stage, where $\theta = 14$ h.

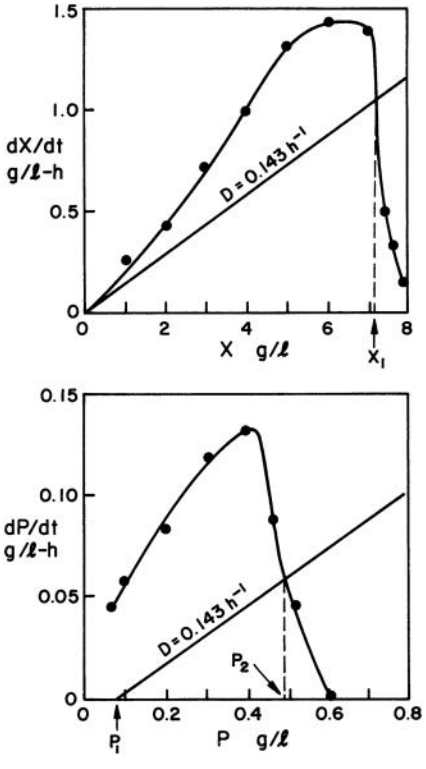


Figure 9.7. Solution to Example 9.2 using alternative graphical approach.

9.2.3. Fed-Batch Operation

Because of inefficient use of substrate, buildup of metabolic waste products, and catabolic repression, normal batch culture usually restricts the cell density and product concentration (or titer) . A fed-batch reactor can circumvent these limitations and allow a high cell density to be realized along with high product concentration, which usually facilitates product recovery. Additionally, a fed-batch system works particularly well when high concentrations of the substrate become toxic or the substrate has low solubility. Further, the system is well adapted for products that are not growth associated. The obvious disadvantage is that the growth rate in a fed-batch system is somewhat lower than in batch. Generally, the advantages outweigh disadvantages and fed-batch systems are often chosen for commercial operation.

In fed-batch culture, nutrients are continuously or semicontinuously fed, while effluent is removed discontinuously (Figure 9.8). Operation is initially batchwise with a small volume. After most of the substrate is consumed, a concentrated feed stream is introduced at a rate that allows substrate availability to match the desired growth rate of the cells. Fed-batch culture is usually used to adjust metabolic rates and to overcome substrate inhibition or catabolite repression by controlled feeding of the substrate. If the substrate is inhibitory, controlled addition of the substrate improves the productivity of the fermentation by maintaining a low substrate concentration. Fed-batch operation is also called *semicontinuous* or *variable-volume continuous culture*.

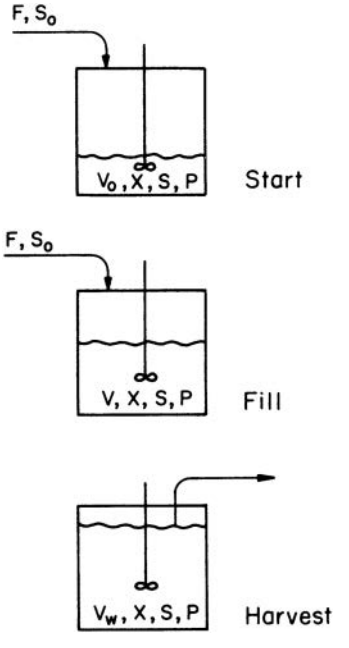


Figure 9.8. Schematic of a fed-batch culture.

Consider a batch culture where the concentration of biomass at a certain time is given by

$$X = X_0 + Y_{X/S}^{M}(S_0 - S) \tag{9.27}$$

where S_0 is the initial substrate concentration, $Y_{X/S}^{M}$ is the yield coefficient, and X_0 is the initial biomass concentration. This equation assumes no endogenous metabolism ($k_d = 0$). This assumption is satisfactory if the fed-batch system is operated at growth rates slightly reduced from the maximum values (say 80% of μ_m) and for production of pharmaceuticals such growth rates would typically be used.

When biomass concentration reaches its maximum value (X_m), the substrate concentration is very low, $S << S_0$, and also $X_0 << X$. That is, $X_m \approx Y_{X/S}^{M} S_0$. Suppose that at $X_m \cong Y_{X/S}^{M} S_0$, a nutrient feed is started at a flow rate F with the substrate concentration S_0. The total amount of biomass in the vessel is $X^t = VX$, where V is the culture volume at time t. The rate of increase in culture volume is

$$\frac{dV}{dt} = F \tag{9.28}$$

Integration of equation 9.28 yields

$$V = V_0 + Ft \tag{9.29}$$

were V_0 is the initial culture volume (l).

The biomass concentration in the vessel at any time t is

$$X = X^t/V \tag{9.30}$$

The rate of change in biomass concentration is

$$\frac{dX}{dt} = \frac{V(dX^t/dt) - X^t(dV/dt)}{V^2} \tag{9.31}$$

Since $dX^t/dt = \mu_{net}X^t$, $dV/dt = F$, and $F/V = D$, equation 9.31 becomes

$$\frac{dX}{dt} = (\mu_{net} - D)X \tag{9.32}$$

When the substrate is totally consumed (or substrate concentration is negligible as compared to S_0), $S \approx 0$, and if $k_d = 0$, then $X = X_m = Y_{X/S}^{M} S_0$. Furthermore, since nearly all the substrate in a unit volume is consumed, then $dX/dt = 0$. This is an example of a quasi-steady state. A fed-batch system operates at quasi-steady state when nutrient consumption rate is nearly equal to nutrient feed rate. Note that achieving such operation can be challenging in practice but is possible with feedback control of substrate concentration and intermittent addition of the feed. Since $dX/dt = 0$ at quasi-steady state, then

$$\mu_{net} = D \tag{9.33}$$

If maintenance energy can be neglected,

$$\mu_{\text{net}} = \mu_m \frac{S}{K_s + S}$$

then

$$S \cong \frac{K_s D}{\mu_m - D} \tag{9.34}$$

The balance on the rate-limiting substrate without maintenance energy ($k_d \to 0$) is

$$\frac{dS^t}{dt} = FS_0 - \frac{\mu_{\text{net}} X^t}{Y^M_{X/S}} \tag{9.35}$$

where S^t is the total amount of the rate-limiting substrate in the culture and S_0 is the concentration of substrate in the feed stream. The assumption of $k_d \to 0$ may become problematic if the feed rate of substrate is too slow, resulting in a value of μ_g much less than μ_m.

At quasi-steady state, $X^t = VX_m$ and the residual substrate is much less than S_0, so no significant level of substrate can accumulate. Therefore, we get

$$FS_0 = \frac{\mu_{\text{net}} X^t}{Y^M_{X/S}} \tag{9.36}$$

Also, differentiating $X' = VX_m$ at quasi-steady state with X_m being constant yields

$$\frac{dX^t}{dt} = X_m \left(\frac{dV}{dt} \right) = X_m F = FY^M_{X/S} S_0 \tag{9.37}$$

Integration of equation 9.37 from $t = 0$ to t with the initial amount of biomass in the reactor being X^t_0 yields

$$X^t = X^t_o + FY^M_{X/S} S_0 t \tag{9.38}$$

That is, the total amount of cells in the culture increases linearly with time (which is experimentally observed) in a fed-batch culture. Dilution rate, and therefore μ_{net}, decrease with time in a fed-batch culture. Since $\mu_{\text{net}} = D$ at quasi-steady state (if $k_d = 0$), the growth rate is controlled by the dilution rate. The use of unstructured models is an approximation, since μ_{net} is a function of time.

Product profiles in a fed-batch culture can be obtained by using the definitions of $Y_{P/S}$ or q_P. When the product yield coefficient $Y_{P/S}$ is constant, at quasi-steady state with $S << S_0$,

$$P \cong Y_{P/S} S_0 \tag{9.39}$$

or the potential product output is

$$FP \approx Y_{P/S} S_0 F \tag{9.40}$$

When the specific rate of product formation q_P is constant,

$$\frac{dP^t}{dt} = q_P X^t \tag{9.41}$$

where P^t is the total amount of product in culture.

Substituting $X^t = (V_0 + Ft)X_m$ into equation 9.41 gives

$$\frac{dP^t}{dt} = q_P X_m (V_0 + Ft) \tag{9.42}$$

Integration of equation 9.42 results in

$$P^t = P_0^t + q_P X_m \left(V_0 + \frac{Ft}{2} \right) t \tag{9.43}$$

In terms of product concentration, equation 9.43 can be written as

$$P = P_0 \frac{V_0}{V} + q_P X_m \left(\frac{V_0}{V} + \frac{Dt}{2} \right) t \tag{9.44}$$

Figure 9.9 depicts the variation of V, $\mu(= D)$, X, S, and P with time at quasi-steady state in a single cycle of a fed-batch culture.

In some fed-batch operations, part of the culture volume is removed at certain intervals, since the reactor volume is limited. This operation is called the *repeated fed-batch culture*. The culture volume and dilution rate ($= \mu_{net}$) undergo cyclical variations in this operation.

If the cycle time t_w is constant and the system is always at quasi-steady state, then the product concentration at the end of each cycle is given by

$$P_w = \gamma P_0 + q_P X_m \left(\gamma + \frac{D_w t_w}{2} \right) t_w \tag{9.45}$$

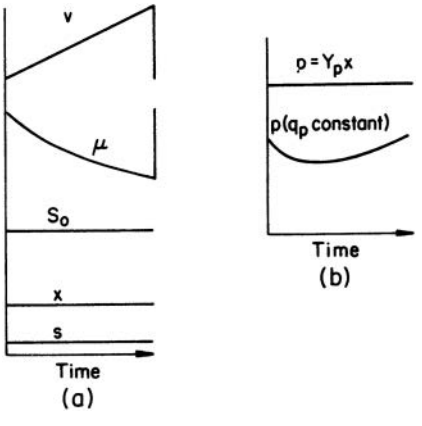


Figure 9.9. (a) Variation of culture volume (V), specific growth rate (μ), cell (X), and substrate (S) concentration with time at quasi-steady state. (b) Variation of product (P) concentration with time at quasi-steady state in a single cycle of a fed-batch culture.

where $D_w = F/V_w$, V_w is the culture volume at the end of each cycle, V_0 is the residual culture volume after removal, γ is the fraction of culture volume remaining at each cycle ($= V_0/V_w$), and t_w is the cycle time.

The cycle time is defined as

$$t_w = \frac{V_w - V_0}{F} = \frac{V_w - \gamma V_w}{F} = \frac{1-\gamma}{D_w} \tag{9.46}$$

Substitution of equation 9.46 into equation 9.45 results in

$$P_w = \gamma P_0 + \frac{q_P X_m}{2D_w}(1-\gamma^2) \tag{9.47}$$

For cases where product formation is not growth associated, it may have a more complicated response than the two limiting cases considered here. Further, it may not always be possible to match the consumption rate, and the resulting S may be sufficiently large to be measurable and possibly variable. An example of fed-batch culture is its use in some antibiotic fermentations, where a glucose solution is intermittently added to the fermentation broth to avoid the repression of pathways for the production of secondary metabolites caused by high initial glucose concentrations. The fed-batch method can be applied to other secondary metabolite fermentations such as lactic acid and other plant cell and mammalian cell fermentations, where the rate of product formation is maximal at low nutrient concentrations.

Fed-batch culture is important for *Escherichia coli* fermentations in which protein products are made from recombinant DNA technology. To make a high concentration of product, it is desirable to grow the culture to very high cell density before inducing production of the target protein. If *E. coli* has an unlimited supply of glucose, it will grow at a maximal rate but produce organic acids (e.g., acetic acid) as by-products. The accumulation of these by-products inhibits growth. If glucose is fed at a rate that maintains the growth rate at a level slightly less than maximal, *E. coli* will use the glucose more efficiently, making less inhibitory by-product. When these issues are accounted for, very high cell densities (50 to 100 g/l) can be achieved. Fed-batch culture may further benefit from active process control. For example, the feed rate of glucose can be controlled by measuring glucose concentration in the medium, the CO_2 evolution rate using a feedback controller, or control of pH.

Example 9.3

In a fed-batch culture operating with intermittent addition of glucose solution, values of the following parameters are given at time $t = 2$ h, when the system is at quasi-steady state:

$V = 1000$ ml $\qquad F = \dfrac{dV}{dt} = 200$ ml/h

$S_0 = 100$ g glucose/1 $\qquad \mu_m = 0.3\ \text{h}^{-1}$

$K_s = 0.1$ g glucose/1 $\qquad Y^M_{X/S} = 0.5$ gdw cells/g glucose

$X^t_0 = 30$ g

a. Find V_0 (the initial volume of the culture).
b. Determine the concentration of growth-limiting substrate in the vessel at quasi-steady state.
c. Determine the concentration and total amount of biomass in the vessel at $t = 2$ h (at quasi-steady state).
d. If $q_P = 0.2$ g product/g cells, $P_0 = 0$, determine the concentration of product in the vessel at $t = 2$ h.

Solution

a. $V = V_0 + Ft$

$V_0 = 1000 - 200(2) = 600$ ml

b. $D = F/V = 0.2\ \text{h}^{-1}$

$$S \approx \frac{K_s D}{\mu_m - D} = \frac{(0.1)(0.2)}{0.3 - 0.2} = 0.2 \text{ g glucose/l}$$

c. $X^t = X_0^t + FY_{X/S}^M S_0 t$

$= 30 + (0.2)(0.5)(100)(2) = 50\ g$

d. $P = P_0 \frac{V_0}{V} + q_P X_m \left(\frac{V_0}{V} + \frac{Dt}{2} \right) t$

$$= 0 + (0.2)(50)\left(\frac{600}{1000} + \frac{(0.2)(2)}{2} \right)(2)$$

$= 16\ g/1$

9.2.4. Perfusion Systems

An alternative to fed-batch culture is a perfusion system. Such systems are used most often with animal cell cultures (see Chapter 12, "Bioprocess Considerations in Using Animal Cell Cultures") particularly for production of monoclonal antibodies. Sustained operation over several weeks to months is possible. The basic characteristic is constant medium flow, cell retention, and in some cases selective removal of dead cells. High cell density can be achieved. Cell retention is usually achieved by membranes, screens, or a centrifuge capable of selective cell removal. When a membrane is used, the system has characteristics of an immobilized cell system (see Section 9.3) except that the cells are usually maintained in suspension and mixed. With a selective removal/recycle, the system approaches the cell recycle reactor discussed earlier in this chapter. Figure 9.10 depicts one type of perfusion system. In some cases, due to differences in density or aggregation, the cell separator (e.g., centrifugation) can selectively recycle live cells and send cell debris to waste. When the cells aggregate, as is common in waste treatment, an internal or external gravity sedimentation unit is utilized.

The potential advantages of a perfusion system is removal of cell debris and inhibitory by-products, removal of enzymes released by dead cells that may destroy

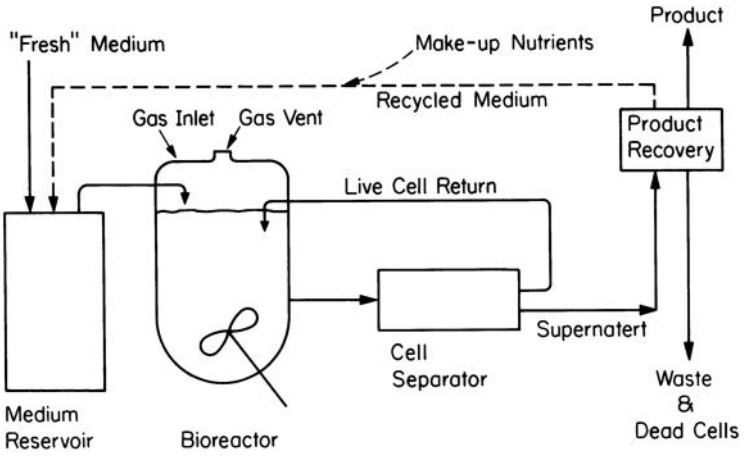


Figure 9.10. Schematic of a perfusion system with external centrifugation and return of cells. Internal retention of cells is also possible. Return of spent medium is optional.

product, shorter exposure time of product to potentially harsh production conditions (compared to batch or fed-batch operation), high per-unit volumetric productivity (due to high cell density and metabolism), and a rather constant environment.

The primary disadvantage is that a large amount of medium is typically used and the nutrients in the medium are less completely utilized than in batch or fed-batch systems. High medium usage is expensive, owing not only to the high cost of raw materials but also to the costs to prepare and sterilize the medium. Additionally, costs for waste treatment increase due to larger liquid volumes. Typically, the bioprocess engineer must consider the trade-off of improved product quality and reactor productivity with the extra costs associated with a more complex reactor system (membranes, pumps, centrifugal separator, etc.) and increased medium usage. The best choice depends on the specific situation.

9.2.5. Membrane Bioreactors

Membrane bioreactors are usually used for simultaneous product or cell separations from the fermentation media and also for controlled substrate supply to the biotic phase:

- *Membrane bioreactors for cell separations:* This type of membrane reactor is usually used in wastewater treatment. The membrane cartridge is placed inside the aeration tank where biological wastewater treatment takes place. Wastewater diffuses inside the membrane cartridge and is removed as treated water with the aid of a pump. The biomass remains inside the aeration tank and is removed in desired amounts to adjust the mean residence time of cells in the bioreactor. The membrane system acts in place of a sedimentation tank, providing much more efficient solid–liquid separation. Like a sedimentation tank, a membrane unit may be placed after the aeration tank, where

the separated solids are recycled back to the reactor and the clean wastewater is removed as the filtrate. Planar or spiral membrane configurations may be used. Membrane fouling is a major problem, reducing the performance of the membrane. Fouling can be partly eliminated by vigorous agitation and aeration in the aeration tank or by periodic back washing. Usually, microfilters or ultrafilters with pore sizes smaller than 1 μm are used for cell separation purposes.

- *Membrane bioreactors for product removal:* Product inhibition is a widely encountered phenomena in biological processing. Simultaneous removal of products during fermentation is widely used in eliminating product inhibition. Membrane separation is one of the methods used for simultaneous product removal to reduce product inhibition. Usually, ultrafiltration or dialysis membranes are used to remove products from fermentation media. Ethanol, acetone–butanol fermentations, and animal cell cultures (lactate and ammonium) produce some inhibitory products, which can be removed by using proper membranes. Some of those membranes are also used for cell separation along with product removal. Again, membranes can be placed inside or outside the reactors and should be cleaned periodically. Crossflow membrane operations may be used to partially overcome membrane fouling.
- *Membrane bioreactors for controlled supply of substrate:* Substrate supply rate to the fermentation media is one of the key factors affecting the metabolic rate. To avoid substrate inhibition due to high substrate concentrations, biotic and abiotic phases can be separated by a membrane whereby the substrate is slowly provided to the biotic phase and the products are removed simultaneously. Hollow-fiber tubular membrane cartridges can be used for this purpose where the nutrient solution passes through the tube side while the cells are immobilized on the shell side.

The following configurations are available for membrane bioreactors:

- *Internal membrane bioreactors:* Membranes are placed inside the reactor where cell or product separations take place. The filtrate is continuously removed from the other side of the membrane. Planar membrane cartridges or coiled tube membranes can be used for this purpose. Membrane surface area and hydrodynamical conditions are the most important parameters affecting the performance of the membranes.
- *External membrane bioreactors:* The membrane unit is placed outside the reactor where the reactor effluent is fed to the membrane unit for cell or product separation. A vacuum may be applied to the filtrate side of the membrane to facilitate the separation. The filtrate-containing product is sent to the recovery operations, and the liquid phase containing a high density of cells can be partially recycled back to the reactor.

Figure 9.11 depicts internal and external membrane reactor configurations.

In membrane bioreactors used for controlled substrate supply and simultaneous product removal, biological reaction rate in the bioreactor is limited by the rate

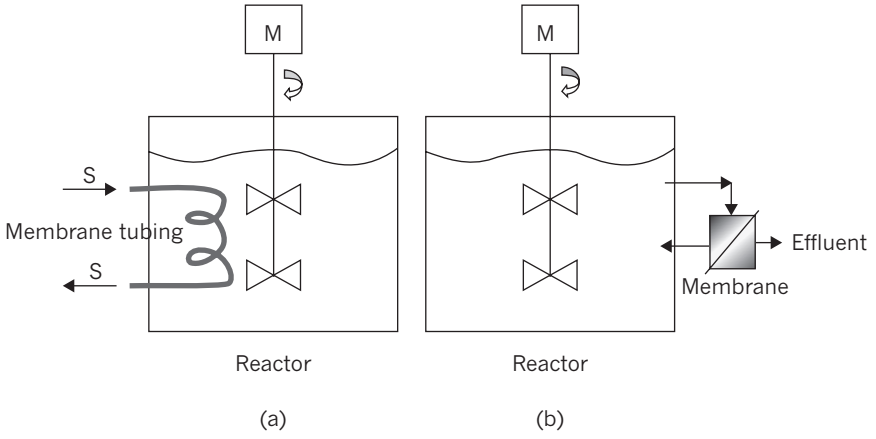


Figure 9.11. Membrane bioreactor configurations: (a) internal membrane, (b) external membrane.

of substrate transfer through the membrane. For the reactor configuration shown in Figure 9.12, cells convert substrate, S, into more cells, X, and product, P.

The rate of change in the rate-limiting substrate concentration for batch operation can be written as

$$dS/dt = K_M a(S_o - S) - \left(\mu_m / Y^M_{x/s}\right) XS/(K_S + S) = K_M a\,(S_o - S) - k\,X\,S/(K_S + S) \quad (9.48)$$

Following is the rate of change in the product concentration in the reactor:

$$dP/dt = Y^M_{p/x}\mu_m X S/(K_S + S) - K'_M a\,(P - P_e) = q_{pm} X S/(K_S + S) - K'_M a\,(P - P_e) \quad (9.49)$$

Finally, the rate of change in the biomass concentration is

$$dX/dt = \mu_m X\,S/(K_S + S) - k_d X \quad (9.50)$$

In equations 9.48, 9.49, and 9.50, K_M is the substrate mass transfer coefficient through the membrane; a is the membrane surface area per unit reaction volume (A_m/V); S_o and S are the substrate concentrations in the feed reservoir and the reactor; μ_m is the maximum specific growth rate; K_s is the saturation constant; X is the biomass concentration in the reactor; $Y^M_{x/s}$ is the maximum growth yield coefficient; k is the maximum substrate consumption rate constant ($\mu_m/Y^M_{x/s}$); $Y_{p/x}$ is the product yield constant; K'_M is the product mass transfer coefficient through the membrane; q_{pm} is the maximum specific product formation rate ($Y_{p/x}\,\mu_m$); P and P_e are the product concentrations in the reactor and in the effluent tank; and k_d is the death rate constant for the biomass in the reactor. k_d is often neglected for operations lasting less than 3 days.

In batch operation of such a membrane bioreactor, equations 9.48, 9.49 and 9.50 need to be solved simultaneously to obtain variations of the biomass, substrate, and

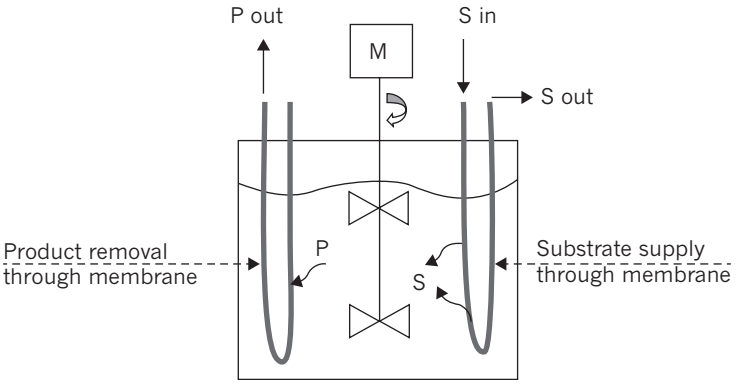


Figure 9.12. Membrane bioreactor with substrate supply and product removal membranes.

product concentrations with time. The system never reaches steady state during batch operation. However, when the bioconversion rate is limited by the rate of substrate transfer, the substrate concentration in the reactor becomes very low and measurable fluctuations become small. In this case, we can assume $dS/dt = 0$ and $dP/dt = 0$.

Then equations 9.48 and 9.49 take the following forms when substrate transfer through the membrane is the rate-limiting (i.e., the slowest) step:

$$K_M a \left(S_o - S\right) = k\ X\ S/(K_S + S) \tag{9.51}$$

$$q_{pm} X\ S/\left(K_S + S\right) = K'_M\ a\ \left(P - P_e\right) = q_p X \tag{9.52}$$

Since we assume $dS/dt = 0$ and a constant yield coefficient, the biomass concentration must be constant in the reactor and equations 9.51 and 9.52 can be solved to determine the substrate and the product concentrations in the reactor.

In case of severe substrate transfer limitations where $S << S_o$ (negligible S), the bioconversion rate can be approximated to first-order kinetics, and the rates of substrate transfer and consumption (RST) can be written as follows:

$$RST = K_M a\ S_o = k\ X\ S/\left(K_S + S\right) = k\ X\ S/K_S \tag{9.53}$$

Following are the rate of product formation (RPF) and transfer:

$$RPF = Yp/s\ K_M a\ S_o = K'_M\ a\ \left(P - P_e\right) = q_{pm} X\ S/K_S \tag{9.54}$$

Equations 9.53 and 9.54 are used to determine the substrate and product concentrations in the reactor in the case of severe membrane transfer limitations.

9.3. IMMOBILIZED CELL SYSTEMS

Immobilization of cells as biocatalysts is a strategy much like enzyme immobilization. Immobilization is the restriction of cell mobility within a defined space. Immobilized cell cultures have the following potential advantages over suspension cultures:

- Immobilization provides high cell concentrations.
- Immobilization provides cell reuse and eliminates the costly processes of cell recovery and cell recycle.
- Immobilization eliminates cell washout problems at high dilution rates.
- The combination of high cell concentrations and high flow rates (no washout restrictions) allows high volumetric productivities.
- Immobilization may also provide favorable microenvironmental conditions (i.e., cell–cell contact, nutrient product gradients, pH gradients) for cells, resulting in better performance of the biocatalysts (e.g., higher product yields and rates).
- In some cases, immobilization improves genetic stability.
- For some cells, protection against shear damage is important.

The major limitation of immobilization is that the product of interest should be excreted by the cells. A further complication is that immobilization often leads to systems for which diffusional limitations are important. In such cases, the control of microenvironmental conditions is difficult, owing to the resulting heterogeneity in the system. With living cells, growth and gas evolution present significant problems in some systems and can lead to significant mechanical disruption of the immobilizing matrix.

In Chapter 3, "Enzymes," we discussed enzyme immobilization. Figure 3.16 provides a useful summary of immobilization strategies. Many of the ideas in enzyme immobilization have a direct counterpart in whole cell immobilization. However, the maintenance of a living cell in such a system is more complex than maintaining enzymatic activity. The primary advantage of immobilized cells over immobilized enzymes is that immobilized cells can perform multistep, cofactor-requiring, biosynthetic reactions that are not practical using purified enzyme preparations.

9.3.1. Active Immobilization of Cells

Active immobilization is entrapment or binding of cells by physical or chemical forces. The two major methods of active immobilization are entrapment and binding.

Physical entrapment within porous matrices is the most widely used method of cell immobilization. Various matrices can be used for the immobilization of cells. Among these are porous polymers (e.g., agar, alginate, κ-carrageenan, polyacrylamide, chitosan, gelatin, collagen), porous metal screens, polyurethane, silica gel, polystyrene, and cellulose triacetate.

Polymer beads should be porous enough to allow the transport of substrates and products in and out of the bead. They are usually formed in the presence of cells and can be prepared by one of the following methods:

- *Gelation of polymers:* Gelatin and agar beads may be prepared by mixing the liquid form of these polymers with cell suspensions and using a template to form beads. Reduction of temperature in the templates causes solidification of the polymers with the cells entrapped. Gel beads are usually soft and mechanically fragile. However, we can use a hard core (i.e., glass, plastic) and a soft gelatin shell with entrapped cells to overcome some mechanical problems associated with polymer beads. Because of diffusional limitations, the inner core of such beads is often not active, so this approach does not necessarily decrease the amount of product made per bead.
- *Precipitation of polymers:* Cells are dispersed in a polymer solution. The polymer can be precipitated by changing the pH or the solvent entrapping cells. The starting solution of the polymer has to be prepared with an organic solvent or a water–solvent mixture. Ethanol and acetone are examples of water-miscible solvents. Polymers used for this purpose are polystyrene, cellulose triacetate, and collagen. The direct contact of cells with solvents may cause inactivation and even death of cells.
- *Ion-exchange gelation:* Ion-exchange gelation takes place when a water-soluble polyelectrolyte is mixed with a salt solution. Solidification occurs when the polyelectrolyte reacts with the salt solution to form a solid gel. The most popular example of this kind of gelation is the formation of Ca-alginate gel by mixing Na-alginate solution with a solution of $CaCl_2$. Some other polymers obtained by ion-exchange gelation are Al-alginate, Ca/Al carboxymethyl cellulose, Mg pectinate, κ-carrageenan, and chitosan polyphosphate. Alginate and κ-carrageenan are the most widely used polymers for cell-immobilization purposes. Ionic gels can be further stabilized by covalent cross-linking.
- *Polycondensation:* Epoxy resins are prepared by polycondensation and can be used for cell immobilization. Polycondensation produces covalent networks with high chemical and mechanical stability. Usually, liquid precursors are cured with a multifunctional component. Functional groups usually are hydroxy, amino, epoxy, and isocyanate groups. Some examples of polymer networks obtained by polycondensation are epoxy, polyurethane, silica gel, gelatin–glutaraldehyde, albumin–glutaraldehyde, and collagen–glutaraldehyde. Severe reaction conditions (e.g., high temperature, low or high pH values) and toxic functional groups may adversely affect the activity of cells.
- *Polymerization:* Polymeric networks can be prepared by cross-linking copolymers of a vinyl group containing monomers. Polyacrylamide beads are the most widely used polymer beads, prepared by copolymerization of acrylamide and bisacrylamide. Several different monomers can be used for polymer formation; acrylamide, methacrylamide, and 2-hydroxyethyl methacrylate are the most widely used. Cross-linking is usually initiated by copolymerization with a divinyl compound, such as methylenebis–acrylamide.

- *Three-dimensional printing:* The use of three-dimensional (3D) printing of living cells is a rapidly developing technology. A 3D cell printer (effectively a type of industrial robot) utilizes the same basic approach used for ink jet printers. While common ink jet printers are two dimensional, it is possible to print in 3D through an additive process in which an object is built up, layer upon layer. In 3D bioprinting, the "ink" is a mixture of living cells in a biocompatible matrix material (usually a polymer that can be chemically or photo-cross-linked). The system is programmed to deposit a specific "ink" droplet in a predefined location. A 3D object is made by positioning of droplets, layer by layer, with great spatial control of placement. The droplets are small (on the order of 50 to 100 μm). Multiple "inks" can be used (as in a color printer) to build 3D structures with many cell types. An early example was the printing of a human ear as a replacement tissue. Techniques other than "ink jet" type printers, such as microextrusion and laser-assisted deposition, are also applied in 3D printing. While 3D printing has been applied primarily to mammalian cells and medical applications, it can serve to build useful models and systems for bioprocess and environmental engineering. For example, 3D printing can be used to build detailed, highly designable models to probe the interactions of microbial communities.

Immobilization by polymerization is a simple method. The polymerizing solution is mixed with the cell suspension, and polymerization takes place to form a polymeric block, which is pressed through a sieve plate to obtain regular-shaped particles. Suspension or emulsion polymerization can also be used to form polymeric beads for cell entrapment.

Encapsulation is another method of cell entrapment. Microcapsules are hollow, spherical particles bound by semipermeable membranes. Cells are entrapped within the hollow capsule volume. The transport of nutrients and products in and out of the capsule takes place through the capsule membrane. Microcapsules have certain advantages over gel beads. More cells can be packed per unit volume of support material into capsules, and intraparticle diffusion limitations are less severe in capsules due to the presence of liquid cell suspension in the intracapsule space. Various polymers can be used as capsule membranes. Among these are nylon, collodion, polystyrene, acrylate, polylysine–alginate hydrogel, cellulose acetate–ethyl cellulose, and polyester membranes. Different membranes (i.e., composition and molecular weight cutoff) may need to be used for different applications in order to retain some high-molecular-weight products inside capsules and provide passage to low-molecular-weight nutrients and products.

Another form of entrapment is the use of macroscopic membrane-based reactors, as detailed in Section 9.2.5. Several commercial reactors for animal cell cultivation use membrane entrapment.

In addition to entrapment or encapsulation, cells can be bound directly to a support. Immobilization of cells on the surface of a support material can be achieved by physical adsorption or covalent binding.

Adsorption of cells on inert support surfaces has been widely used for cell immobilization. The major advantage of immobilization by adsorption is direct contact between nutrient and support materials. High cell loadings can be obtained

using microporous support materials. However, porous support materials may cause intraparticle pore diffusion limitations at high cell densities, as is also the case with polymer-entrapped cell systems. Also, the control of microenvironmental conditions is a problem with porous support materials. A ratio of pore to cell diameter of 4 to 5 is recommended for the immobilization of cells onto the inner surface of porous support particles. At small pore sizes, accessibility of the nutrient into inner surfaces of pores may be the limiting factor, whereas at large pore sizes, the specific surface area may be the limiting factor. Therefore, there may be an optimal pore size, resulting in the maximum rate of bioconversion.

Adsorption capacity and strength of binding are the two major factors that affect the selection of a suitable support material. Typical adsorption capacity can vary between 2 mg/g (porous silica) to 250 mg/g (wood chips). Porous glass carriers provide adsorption capacities (10^8 to 10^9 cells/g) that are less than or comparable to those of gel-entrapped cell concentrations (10^9 to 10^{11} cells/ml). The binding forces between the cell and support surfaces may vary, depending on the surface properties of the support material and the type of cells. Electrostatic forces are dominant when positively charged support surfaces (e.g., ion-exchange resins, gelatin) are used. Cells also adhere on negatively charged surfaces by covalent binding or H bonding. The adsorption of cells on neutral polymer support surfaces may be mediated by chemical bonding, such as covalent bonding, H bonds, or van der Waals forces. Some specific chelating agents may be used to develop stronger cell-surface interactions. Among the support materials used for cell adsorption are porous glass, porous silica, alumina, ceramics, gelatin, chitosan, activated carbon, wood chips, polypropylene ion-exchange resins (e.g., DEAE–Sephadex, carboxymethyl cellulose [CMC]), and Sepharose.

Adsorption is a simple, inexpensive method of cell immobilization. However, limited cell loadings and rather weak binding forces reduce the attractiveness of this method for microbial systems. Hydrodynamic shear around adsorbed cells should be very mild to avoid the removal of cells from support surfaces. With animal cells, well-defined porous materials, either ceramic or polymeric, have been used successfully both in bioprocess and medical applications. Animal cell attachment to surfaces with the correct chemistry can be quite strong. Entrapment in gels or within hollow microporous spheres can also be used with animal cells.

Covalent binding is the most widely used method for enzyme immobilization. However, it is not as widely used for cell immobilization. Functional groups on cell and support material surfaces are not usually suitable for covalent binding. Binding surfaces need to be specially treated with coupling agents (e.g., glutaraldehyde or carbodiimide) or reactive groups for covalent binding. These reactive groups may be toxic to cells. A number of inorganic carriers (e.g., metal oxides such as titanium and zirconium oxide) have been developed that provide satisfactory functional groups for covalent binding.

Covalent binding forces are stronger than adsorption forces, resulting in more stable binding. However, with growing cells, large numbers of cell progeny must be lost. Support materials with desired functional groups are rather limited. Among the support materials used for covalent binding are CMC plus carbodiimide; carriers with aldehyde, amine, epoxy, or halocarbonyl groups; Zr(IV) oxide; Ti(IV) oxide; and cellulose plus cyanuric chloride. Support materials with —OH groups are treated with CNBr, materials with $—NH_2$ are treated with glutaraldehyde, and supports

with COOH groups are treated with carbodiimide for covalent binding with protein groups on cell surfaces.

The direct cross-linking of cells by glutaraldehyde to form an insoluble aggregate is more like cell entrapment than binding. However, some cells may be cross-linked after adsorption onto support surfaces. Cross-linking by glutaraldehyde may adversely affect the cell's metabolic activity and may also cause severe diffusion limitations. Physical cross-linking may also be provided by using polyelectrolytes, polymers such as chitosan, and salts [e.g., $CaCl_2$,$Al(OH)_3$,$FeCl_3$]. Direct cross-linking is not widely used because of the aforementioned disadvantages.

A good support material should be rigid and chemically inert, should bind cells firmly, and should have high loading capacity. In the case of gel entrapment, gels should be porous enough and particle size should be small enough to avoid intraparticle diffusion limitations.

Some examples of cell immobilization by entrapment and by binding are summarized in Tables 9.1 and 9.2, respectively.

TABLE 9.1. Examples of Cell Immobilization by Entrapment

Cells	Support Matrix	Conversion
Saccharomyces cerevisiae	κ-Carrageenan or polyacrylamide	Glucose to ethanol
Enterobacter aerogenes	κ-Carrageenan	Glucose to 2,3-butanediol
Escherichia coli	κ-Carrageenan	Fumaric acid to aspartic acid
Trichoderma reesei	κ-Carrageenan	Cellulose production
Zymomonas mobilis	Ca-alginate	Glucose to ethanol
Acetobacter sp.	Ca-alginate	Glucose to gluconic acid
Morinda citrifolia	Ca-alginate	Anthraquinone formation
Candida tropicalis	Ca-alginate	Phenol degradation
Nocardia rhodocrous	Polyurethane	Conversion of testosterone
Escherichia coli	Polyurethane	Penicillin G to G-APA
Catharanthus roseus	Polyurethane	Isocitrate dehydrogenase activity
Rhodotorula minuta	Polyurethane	Menthyl succinate to menthol
Mammalian cells	Alginate-polylysine	Drug delivery

TABLE 9.2. Examples of Cell Immobilization by Surface Attachment

Cells	Support Surface	Conversion
Lactobacillus sp.	Gelatin (adsorption)	Glucose to lactic acid
Clostridium acetobutylicum	Ion-exchange resins	Glucose to acetone, butanol
Streptomyces	Sephadex (adsorption)	Streptomycin
Animal cells	DEAE-Sephadex/Cytodex (adsorption)	Hormones
Escherichia coli	Ti(IV) oxide (covalent binding)	
Bacillus subtilis	Agarose–carbodiimide (covalent binding)	
Solanum aviculare	Polyphenylene oxide-glutaraldehyde (covalent binding)	Steroid glycoalkaloids formation

9.3.2. Passive Immobilization: Biological Films

Biological films are the multilayer growth of cells on solid support surfaces. The support material can be inert or biologically active. Biofilm formation is common in natural and industrial fermentation systems, such as biological wastewater treatment and mold fermentations. The interaction among cells and the binding forces between the cell and support material may be very complicated.

In mixed-culture microbial films, the presence of some polymer-producing organisms facilitates biofilm formation and enhances the stability of the biofilms. Microenvironmental conditions inside a thick biofilm vary with position and affect the physiology of the cells.

In a stagnant biological film, nutrients diffuse into the biofilm and products diffuse out into liquid nutrient medium. Nutrient and product profiles within the biofilm are important factors affecting cellular physiology and metabolism. A schematic of a biofilm is depicted in Figure 9.13. Biofilm cultures have almost the same advantages as those of the immobilized cell systems over suspension cultures, as listed in the previous section.

The thickness of a biofilm is an important factor affecting the performance of the biotic phase. Thin biofilms have low rates of conversion due to low biomass concentration, and thick biofilms may experience diffusionally limited growth, which may or may not be beneficial depending on the cellular system and objectives. Nutrient-depleted regions may also develop within the biofilm for thick biofilms. In many cases, an optimal biofilm thickness resulting in the maximum rate of bioconversion exists and can be determined. In some cases, growth under diffusion limitations may result in higher yields of products as a result of changes in cell physiology and cell–cell interactions. In this case, improvement in reaction stoichiometry (e.g., high yield) may overcome the reduction in reaction rate, and it may be more beneficial to operate the system under diffusion limitations. Usually, the most sparingly soluble nutrient, such as dissolved oxygen, is the rate-limiting nutrient within the biofilm. A typical variation of dissolved oxygen concentration within the biofilm is depicted in Figure 9.14.

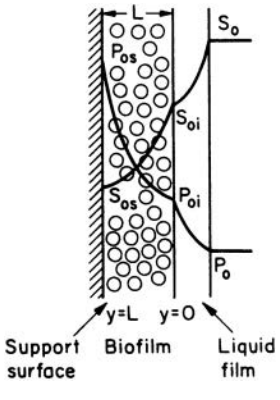


Figure 9.13. Schematic representation of a biofilm.

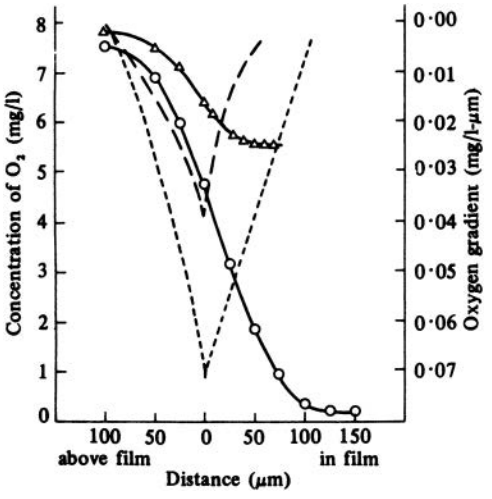


Figure 9.14. Dissolved-oxygen profiles and oxygen gradients in a microbial slime bathed in flowing medium: -Δ-Δ- oxygen profile for 20 ppm nutrient broth, 27.5°C; – – oxygen gradient for this profile; -O-O- oxygen profile for 500 ppm nutrient broth, 26°C; --- oxygen gradient for this profile. (With permission, from H. R. Bungay and others, *Biotechnol. Bioeng*. 11:765, 1969, John Wiley & Sons, Inc., New York.)

9.3.3. Diffusional Limitations in Immobilized Cell Systems

Immobilization of cells may cause extra diffusional limitations as compared to suspension cultures. The presence and significance of diffusional limitations depend on the relative rates of bioconversion and diffusion, which can be described by the Damköhler number (Da) (see equation 3.52 also):

$$\text{Da} = \frac{\text{maximum rate of bioconversion}}{\text{maximum rate of diffusion}} = \frac{r_{\max}}{(D_e/\delta)S_0} \tag{9.55}$$

Here $r_{\max}$ is the maximum rate of bioconversion (mg S/l-h), D_e is the effective diffusivity of the rate-limiting substrate, δ is the thickness of diffusion path (or liquid film), and S_0 is the bulk substrate concentration in liquid phase. When the film-theory model applies, D_e/δ is the mass transfer coefficient (i.e., $k_L = D_e/\delta$).

If Da >> 1, the rate of bioconversion is diffusion limited; for Da << 1, the rate is limited by the rate of bioconversion; and for Da ≈ 1, the diffusion and bioreaction rates are comparable. It is desirable to keep Da < 1 to eliminate diffusion limitations when the productivity of a cell population does not improve upon immobilization due to cell–cell contact and nutrient gradients.

Diffusional limitations may be external (i.e., between fluid and support surface in adsorption and covalent binding), intraparticle (i.e., inside particles in entrapment, encapsulation, or immobilization in porous particles), or both. If the external mass transfer is limiting, an increase in liquid-phase turbulence should result in an increase in the reaction rate. In case of intraparticle mass-transfer limitations, a reduction in particle size or an increase in the porous void fraction of the support material should result in an increase in the rate of the bioreaction.

In Chapter 3, we discussed in reasonable detail a mathematical model of the interaction of diffusion and reaction for surface immobilized or entrapped biocatalysts. These models apply directly to immobilized cells when the kinetics of bioconversion are described by a Michaelis–Menten type of kinetic expression.

Another interesting case is to consider biofilms where we allow cell growth. Models for immobilized enzymes have no terms for biocatalyst replication, so this case presents a new problem.

The thickness of a biofilm or the size of microbial floc increases with time during the growth phase. A *microbial floc* is an aggregation of many cells, and in some processes these aggregates can be more than 1 mm in diameter. However, since the rate of increase in biofilm thickness is much slower than the rate of substrate uptake, the system can be assumed to be at quasi-steady state for relatively short periods. The simplest case is to assume that the system is at quasi-steady state and all the cells inside the biofilm are in the same physiological state. In this situation, we write a steady-state substrate balance within the biofilm by using average kinetic constants for the biotic phase (living cells).

A differential material balance for the rate-limiting substrate within the biofilm (see Figure 9.11) yields the following at steady state:

$$D_e \frac{d^2S}{dy^2} = \frac{1}{Y_{X/S}} \frac{\mu_m S}{K_s + S} X \tag{9.56}$$

Here D_e is the effective diffusivity (cm^2/S) and $Y_{X/S}$ is the growth yield coefficient (g cells/g substrate).

Following are the boundary conditions:

$$S = S_{0i} \qquad \text{at } y = 0$$
$$\frac{dS}{dy} = 0 \qquad \text{at } y = L$$

Here L is the thickness of biofilm.

If it is also assumed that the liquid nutrient phase is vigorously agitated and the liquid film resistance is negligible, then $S_0 \approx S_{0i}$. By defining a maximum rate of substrate utilization as $r_m = \mu_m X / Y_{X/S}$ (g subs/cm^3 h), we rewrite equation 9.46 as

$$D_e \frac{d^2S}{dy^2} = \frac{r_m S}{K_s + S} \tag{9.57}$$

In dimensionless form, equation 9.51 can be written as

$$\frac{d^2\overline{S}}{d\overline{y}^2} = \frac{\phi^2 \overline{S}}{1 + \beta \overline{S}} \tag{9.58}$$

where

$$\overline{S} = \frac{S}{S_0}, \quad \overline{y} = \frac{y}{L}, \quad \beta = \frac{S_0}{K_s}$$

and

$$\phi = L \sqrt{\frac{\mu_m X}{Y_{X/S} D_e K_s}} = L \sqrt{\frac{r_m}{D_e K_s}} \tag{9.59}$$

Equation 9.58 can be solved numerically. An analytical solution can be derived for the limiting cases of zero or first-order reaction rates.

The maximum rate of substrate flux in the absence of diffusion limitations is given by

$$N_s A_s = -A_s D_e \left.\frac{dS}{dy}\right|_{y=0} = \frac{r_m S_0}{K_s + S_0}(LA_s) \tag{9.60}$$

where A_s is a surface area of biofilm available for substrate flux, N_s is the substrate flux, and L is the thickness of the biofilm.

In the presence of diffusion limitation, the rate of substrate consumption or flux is expressed in terms of the effectiveness factor:

$$N_s = -D_e \left.\frac{dS}{dy}\right|_{y=0} = \eta\left(\frac{r_m S_0}{K_s + S_0}\right)L \tag{9.61}$$

where η is the effectiveness factor, defined as the ratio of the rate of substrate consumption in the presence of diffusion limitation to the rate of substrate consumption in the absence of diffusion limitation. In the absence of diffusion limitations, $\eta \cong 1$, and in the presence of diffusion limitations, $\eta < 1$. The effectiveness factor is a function of ϕ and β. Figure 9.15 is a plot of η versus β for various values of ϕ. The ϕ value should be low ($\phi < 1$) to eliminate diffusion limitations. As the biofilm grows (slowly), the value of ϕ will gradually increase. If shear forces cause a portion of the film to detach, then ϕ will decrease abruptly.

The effectiveness factor (η) can be calculated as

$$\eta = 1 - \frac{\tanh\phi}{\phi}\left(\frac{\omega}{\tanh\omega} - 1\right), \quad \text{for } \omega \le 1 \tag{9.62}$$

$$\eta = \frac{1}{\omega} - \frac{\tanh\phi}{\phi}\left(\frac{1}{\tanh\phi} - 1\right), \quad \text{for } \omega \ge 1 \tag{9.63}$$

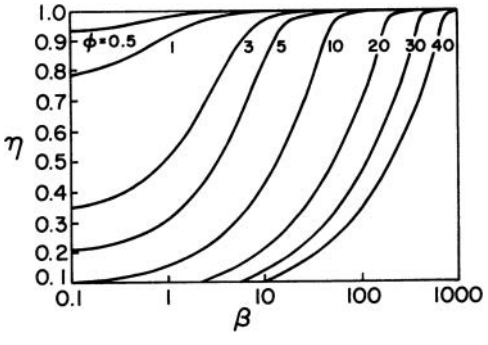


Figure 9.15. Effectiveness factor for a flat biofilm as a function of β, the dimensionless initial substrate concentration, and ϕ, the Thiele modulus. (Redrawn from B. Atkinson, *Biochemical Reactors*, Pion Ltd., London, 1974, p. 81.)

where ω is the modified Thiele modulus and is given by

$$\omega = \frac{\phi\,(S_0 / K_s)}{\sqrt{2}\left(1+\dfrac{S_0}{K_s}\right)}\left[\frac{S_0}{K_s} - \ln\left(1+\frac{S_0}{K_s}\right)\right]^{-1/2} \tag{9.64}$$

Some cells, such as molds (*Aspergillus niger*), form pellets in a fermentation broth, and substrates need to diffuse inside pellets to be available for microbial consumption. Cells may form biofilms on spherical support particles, as depicted in Figure 9.16. Similar equations need to be solved in spherical geometry in this case to determine the substrate profile within the floc and the substrate consumption rate. The dimensionless substrate transport equation within the microbial floc is

$$\frac{d^2\overline{S}}{d\overline{r}^2} + \frac{2}{r}\frac{d\overline{S}}{d\overline{r}} = \frac{\phi^2\overline{S}}{1+\overline{S}/\beta'} \tag{9.65}$$

where

$$\overline{S} = \frac{S}{S_0}, \quad \overline{r} = \frac{r}{R}, \quad \beta' = \frac{S_0}{K_s}$$

and

$$\phi = R\sqrt{\frac{\mu_m X}{Y_{X/S} D_e K_s}} = R\sqrt{\frac{r_m}{D_e K_s}} \tag{9.66}$$

The boundary conditions are

$$\overline{S} = 1, \qquad \text{at } \bar{r} = 1$$

$$\frac{d\overline{S}}{d\bar{r}} = 0, \qquad \text{at } \bar{r} = 0$$

For nonspherical particles, a characteristic length is defined as

$$L = \frac{V_P}{A_P} \tag{9.67}$$

where V_P and A_P are the volume and surface area of microbial pellet.

The rate of substrate consumption by a single microbial floc is

$$N_S A_P = -A_P D_e \left.\frac{dS}{dr}\right|_{r=R} = \eta\frac{r_m S_0}{K_s + S_0} V_P \tag{9.68}$$

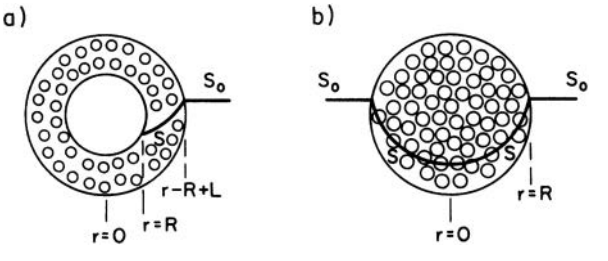


Figure 9.16. (a) Microbial film on inert spherical support particle. (b) Spherical microbial floc.

The effectiveness factor (η) is a function of ϕ and β. Variation of η with ϕ and β is similar to that of Figure 9.15. However, η values for spherical geometry are slightly lower than those of rectangular geometry for intermediate values of ϕ ($1 < \phi < 10$). An analytical solution to equation 9.65 is possible for first- and zero-order reaction kinetics.

The reaction rate can be approximated to first order at low substrate concentrations:

$$r_s = \frac{\mu_m S}{Y_{X/S} K_s} X = \frac{r_m}{K_s} S \tag{9.69}$$

where $r_m = (\mu_m / Y_{X/S})X$. The effectiveness factor in this case is given by

$$\eta = \frac{1}{\phi}\left[\frac{1}{\tanh 3\phi} - \frac{1}{3\phi}\right] \tag{9.70}$$

where

$$\phi = \frac{V_P}{A_P}\sqrt{\frac{r_m / K_s}{D_e}}$$

The rate of bioreaction can be approximated to zero order at values of $S >> K_S$. Because K_S is often very small, the zero-order limit usefully describes many systems of practical interest:

$$r_s = \frac{\mu_m X}{Y_{X/S}} = r_m \tag{9.71}$$

The solution to equation 9.57 in this case is

$$S = S_0 - \frac{r_m}{6D_e}(R^2 - r^2) \tag{9.72}$$

Substrate concentration may be zero at a certain radial distance from the center of the floc. This distance is called the *critical radius* (r_{cr}) and is determined by setting $S = 0$ at r_{cr} in equation 9.72:

$$\left(\frac{r_{cr}}{R}\right)^2 = 1 - \frac{6D_e S_0}{r_m R^2} \tag{9.73}$$

When $r_{cr} > 0$ (i.e., $R > (6D_e S_0 / r_m)^{1/2}$), then the concentration of the limiting substrate is zero for $0 < r < r_{cr}$. In this case, the limiting substrate is consumed only in the outer shell of the floc, and the effectiveness factor is given by

$$\eta = \frac{r_m \frac{4}{3}\pi(R^3 - r_{cr}^3)}{\frac{4}{3}\pi R^3 \cdot r_m} = 1 - \left(\frac{r_{cr}}{R}\right)^3 \tag{9.74}$$

or

$$\eta = 1 - \left(1 - \frac{6D_e S_0}{r_m R^2}\right)^{3/2} \tag{9.75}$$

9.3.4. Bioreactor Considerations in Immobilized Cell Systems

Various reactor configurations can be used for immobilized cell systems. Since the support matrices used for cell immobilization are often mechanically fragile, bioreactors that minimize particle collisions, such as packed-column, are preferred. Mechanically agitated fermenters can be used for some immobilized cell systems if the support matrix is strong and durable. Any of these reactors can usually be operated in perfusion mode by passing nutrient solution through a column of immobilized cells. Schematic diagrams of immobilized-cell packed-column and fluidized-bed reactors are depicted in Figure 9.17. These reactors can be operated in batch or continuous mode.

Consider the reactors shown in Figure 9.17. When the fluid recirculation rate is high, the system approaches CFSTR behavior. One commercial fluidized-bed, immobilized-animal-cell bioreactor system requires high recirculation to maintain uniform conditions in the reactor. The models we have discussed so far can be applied to such systems. The other extreme involves some waste-treatment systems where the rate of fluid recirculation is low or even zero. In the latter case, the system cannot be modeled as a CFSTR but must be treated as a PFR. To analyze such a system, consider a material balance on the rate-limiting substrate over a differential element:

$$-F\,dS_0 = N_S a A\,dz \tag{9.76}$$

where S_0 is the bulk liquid-phase substrate concentration (mg S/cm^3) and is a function of height, F is the liquid nutrient flow rate (cm^3/h), N_s is flux of substrate into the biofilm (mg S/cm^2 h), a is the biofilm or support particle surface area per unit reactor volume (cm^2/cm^3), A is the cross-sectional area of the bed (cm^2), and dz is the

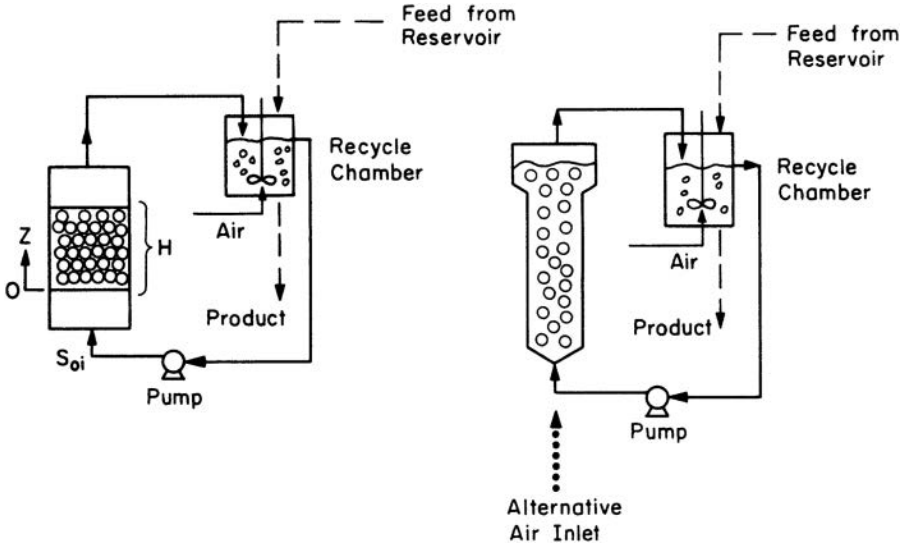


Figure 9.17. Schematics of a packed-bed and a fluidized-bed biofilm or immobilized cell bioreactors. In batch operation, only the streams with solid lines exist. In continuous operation, the streams shown by dashed lines are added. For the fluidized bed, fluidization can be accomplished by liquid recirculation only by a mixture of liquid and gas flows.

differential height of an element of the column (cm). Substituting equation 9.63 into equation 9.76 yields

$$-F\frac{dS_0}{dz} = \eta\frac{r_m S_0}{K_s + S_0} LaA \tag{9.77}$$

Integration of equation 9.77 yields

$$K_s \ln\frac{S_{0\mathrm{i}}}{S_0} + (S_{0\mathrm{i}} - S_0) = \frac{\eta r_m LaA}{F} H \tag{9.78}$$

where $S_{0\mathrm{i}}$ is the inlet bulk substrate concentration, L is the biofilm thickness or the characteristic length of the support particle ($L = V_P/A_P$), and H is the total height of the packed bed.

For low substrate concentrations in the feed, the rate of substrate consumption is first order, and equation 9.78 has the following form:

$$\ln\frac{S_0}{S_{0\mathrm{i}}} = -\frac{\eta r_m LaA}{FK_s} H \tag{9.79}$$

Substrate concentration drops exponentially with the height of the column in this case, and a plot of ln S_0 versus H results in a straight line. Equation 9.77 or 9.78 can be used as the design equation for immobilized–biofilm column reactors to determine the height of the column for a desired level of substrate conversion.

Example 9.4.

Glucose is converted to ethanol by immobilized *S. cerevisiae* cells entrapped in Ca-alginate beads in a packed column. The specific rate of ethanol production is $q_P = 0.2$ g ethanol/g cell h, and the average dry-weight cell concentration in the bed is $\overline{X} = 25$ g/l bed. Assume that growth is negligible (i.e., almost all glucose is converted to ethanol) and the bead size is sufficiently small that $\eta \cong 1$. The feed flow rate is $F = 400$ l/h, and glucose concentration in the feed is $S_{0i} = 100$ g glucose/l. The diameter of the column is 1 m, and the product yield coefficient is $Y_{P/S} \approx 0.49$ g ethanol/g glucose.

a. Write a material balance on the glucose concentration over a differential height of the column and integrate it to determine $S = S(z)$ at steady state.
b. Determine the column height for 98% glucose conversion at the exit of the column.
c. Determine the ethanol concentration in the effluent.

Solution

a. A material balance on the glucose concentration over a differential height of the column yields

$$-F\,dS_0 = \frac{q_P\overline{X}}{Y_{P/S}} dV = \frac{q_P\overline{X}}{Y_{P/S}} A\,dz$$

Integration yields

$$-F\int_{S_{0i}}^{S_0} dS_0 = \frac{q_P \overline{X}}{Y_{P/S}} A \int_0^H dz$$

$$S_{0i} - S_0 = \frac{q_P \overline{X}}{Y_{P/S}} \frac{A}{F} H$$

This equation differs from the form of equation 9.79 because S_{0i} is high and the reaction rate is effectively zero order.

b. $S_0 = 0.02(100) = 2$ g glucose/l. Substituting the given values into the previous equation yields

$$(100 - 2) = \frac{(0.2)(25)}{0.49} \frac{(\pi/4)(10)^2}{400} H$$

$$H = 49 \text{ dm} = 4.9 \text{ m}$$

c. $P = Y_{P/S}(S_{0i} - S_0) = 0.49(98) = 48$ g/l.

9.4. HYBRID BIOREACTORS: ATTACHED AND SUSPENDED CELLS

In most biofilm reactors, such as the packed or fluidized beds, cells detach from the support particle surfaces and grow in the form of suspended cells or microbial flocs as well as biofilm. When the total amount of suspended cells exceeds 10% of the cells in the form of biofilm, then the contribution of suspended or floc-forming cells cannot be neglected. In this case, both the biofilm and suspended cells need to be included in the kinetic analysis of the system.

Consider a hybrid column bioreactor containing both biofilm on support particles and suspended cells where their amounts are comparable.

A substrate balance over a differential volume yields

$$FdS = R_f dV_f + R_s dV \tag{9.80}$$

where F is the flow rate of the nutrient solution; dS is the differential difference in the substrate concentration within the volume dV; R_f is the rate of conversion of substrate by the biofilm organisms; dV_f is the differential volume of the biofilm within dV; R_s is the rate of conversion by the suspended cells; dV is the differential reaction volume, which includes both the biofilm and the suspended cells.

R_f and R_s can be described by the following equations:

$$R_f = \frac{k_f X_f S_f}{K_{sf} + S_f} = \eta \frac{k_f X_f S}{K_{sf} + S} \tag{9.81}$$

$$R_s = \frac{k_s X_s S}{K_{ss} + S} \tag{9.82}$$

where k_f and k_s are the maximum specific rates of substrate consumption by the biofilm and the suspended cells; X_f and X_s are the biofilm and suspended cell concentrations; S_f and S are the rate limiting substrate concentrations in the biofilm and in the

fermentation medium; K_{sf} and K_{ss} are the saturation constants for the biofilm and suspended cells. Due to diffusion limitations within the biofilm the limiting substrate concentration in the biofilm (S_f) will be lower than the bulk substrate concentration (S). Effectiveness factor (η) is used to include the diffusion limitations and to express the R_f in terms of bulk substrate concentration (S).

Furthermore, we have the following:

$$dV_f = L\, dA_f = L\left(dA_f / dV\right) dV = L\, a_f dV \quad and \quad dV = A_o dZ \tag{9.83}$$

where L is the average biofilm thickness (m) which is assumed to be constant; dA_f is the differential biofilm surface area (m^2); a_f is the biofilm surface area per unit reaction volume (m^2/m^3); A_o is the cross-section area of the column (m^2); and dZ is the differential height of the column (m).

Substitution of equations 9.81, 9.82, and 9.83 to equation 9.80 yields

$$-FdS = \eta \frac{k_f X_f S}{K_{sf} + S} La_f dV + \frac{k_s X_s S}{K_{ss} + S} dV \tag{9.84}$$

Assuming K_{sf} is approximately equal to K_{ss} ($K_{ss} = K_{sf} = K_s$), since the suspended cells are derived from the attached cells, equation 9.84 takes the following form:

$$-FdS = (\eta k_f X_f La_f + k_s X_s) \frac{S}{K_s + S} dV \tag{9.85}$$

The definition of $R_{mf} = k_f X_f$ and $R_{ms} = k_s X_s$, equation 9.85 becomes

$$-FdS = (\eta R_{mf} La_f + R_{ms}) \frac{S}{K_s + S} dV \tag{9.86}$$

where R_{mf} and R_{ms} are the maximum rates of substrate conversion by the biofilm and the suspended organisms.

Assuming that the biofilm and suspended cell concentrations change slowly and can be assumed to be constant, integration of equation 9.86 yields

$$K_s Ln \frac{S_i}{S_e} + (S_i - S_e) = (\eta R_{mf} La_f + R_{ms})\theta_H = (\eta R_{mf} La_f + R_{ms}) \frac{A_0 H}{F} \tag{9.87}$$

where S_i and S_e are the feed and the effluent substrate concentrations; θ_H is the hydraulic residence time; and H is the column height. The effectiveness factor (η) is determined by using either Figure 9.15 or equations 9.62 and 9.63.

For the first-order reaction rate equation 9.87 reduces to

$$K_s Ln \frac{S_i}{S_e} = (\eta R_{mf} La_f + R_{ms})\theta_H = (\eta R_{mf} La_f + R_{ms}) \frac{A_0 H}{F} \tag{9.88}$$

The substrate concentration decreases with the height of the column exponentially and faster than if the reactor contained only the biofilm or the suspended culture alone.

If the reactor is completely mixed and contains both the biofilm and the suspended cells or flocs, the substrate balance takes the following form:

$$F(S_i - S) = R_f V_f + R_s V = \eta \frac{k_f X_f S}{K_s + S} V_f + \frac{k_s X_s S}{K_s + S} V \tag{9.89}$$

With the following definitions,

$$V_f = LA_f = L(A_f / V)V = La_f V \quad \text{and} \quad V = A_o H \tag{9.90}$$

equation 9.89 takes the following form

$$F(S_i - S) = (\eta k_f X_f La_f + k_s X_s) \frac{S}{K_s + S} V \tag{9.91}$$

or

$$F(S_i - S) = (\eta R_{mf} La_f + R_{ms}) \frac{S}{K_s + S} V \tag{9.92}$$

where $R_{mf} = k_f X_f$ and $R_{ms} = k_s X_s$ which are the maximum rates of substrate removal by the biofilm and the suspended cells, respectively.

With the definition of $R_s = F(S_i - S)/V$, which is the actual rate of substrate removal, equation 9.92 reduces to

$$Rs = \frac{F(S_i - S)}{V} = (\eta R_{mf} La_f + R_{ms}) \frac{S}{K_s + S} = R_m \frac{S}{K_s + S} \tag{9.93}$$

where $R_m = \eta R_{mf} La_f + R_{ms}$ and is the maximum substrate removal rate by the biofilm and the suspended cells. The effectiveness factor (η) is determined by using Figure 9.15 or equations 9.62, 9.63, and 9.64. In an experimental study, the data can be plotted in the form of $1/Rs$ versus $1/S$ and the constants determined from the slope and the intercept of the line.

9.5. SOLID-STATE FERMENTATIONS

Solid-state fermentations (SSF) are fermentations of solid substrates at low moisture levels or water activities. The water content of a typical submerged fermentation is more than 95%. The water content of a solid mash in SSF often varies between 40% and 80%. SSFs are usually used for the fermentation of agricultural products or foods, such as rice, wheat, barley, corn, and soybeans. The unique characteristic of SSFs is operation at low moisture levels, which provides a selective environment for the growth of mycelial organisms, such as molds. In fact, most SSFs are mold fermentations producing extracellular enzymes on moist agricultural substrates. Since bacteria and yeasts cannot tolerate low moisture levels (water activities), the chances of contamination of fermentation media by bacteria or yeast are greatly reduced in SSF. Although most SSFs are mold fermentations, SSFs based on bacteria and yeast

operating at relatively high moisture levels (75% to 90%) are also used. SSFs are used widely in Asia for food products, such as tempeh, miso, and soy sauce fermentations, and also for enzyme production, and they show promise for preparing wastes for bioenergy production.

The major advantages of SSFs over submerged fermentation systems are the small volume of fermentation mash or reactor volume, resulting in lower capital and operating costs; a lower chance of contamination due to low moisture levels; easy product separation; energy efficiency; and the allowing of the development of fully differentiated structures, which is critical in some cases to product formation. Examples include the formation of fungal spores for use as biocontrol agents, which develop more fully in SSF than in submerged fermentations. The major disadvantage of SSFs is the heterogeneous nature of the media due to poor mixing characteristics, which results in control problems (pH, DO, temperature) within the fermentation mash. To eliminate these control problems, fermentation media are usually mixed either continuously or intermittently. For large fermentation mash volumes, the concentration gradients may not be eliminated at low agitation speeds, and mycelial cells may be damaged at high agitation speeds. Usually, a rotating-drum fermenter is used for SSF systems, and the rotational speed needs to be optimized for the best performance. A key development in the last decade has been improved mathematical models to guide the operation and design of such bioreactors.

Solid-substrate fermentations imply a more general method of fermentations in which moisture content may not need to be low, but the substrate is in the form of submerged solid particles in liquid media. Bacterial ore leaching (i.e., growth and microbial oxidation on surfaces of mineral sulfide particles) and fermentation of rice in a packed column with circulating liquid media are examples of solid-substrate fermentations. Solid-state (or solid-phase) fermentations are a special form of solid-substrate fermentations for which the substrate is solid and the moisture level is low.

Four primary types of bioreactors for SSF have been described. A tray bioreactor, as used in production of many traditional SSF food processes (e.g., tempeh, miso, and soy sauce), is essentially an incubator with trays of grain or other substrate inoculated with mold spores. A second form is the packed-bed bioreactor in which air is typically circulated with forced aeration, and often a mode of heat removal is included. A third is the rotating-drum design whereby gas and solid are mixed as the drum is rotated. The fourth is the mixed and forcefully aerated bioreactor, which entails mechanical mixing of the contents, such as in a rotating drum, but with air supplied by forced convection. With each of these designs, the fragility of the mold may be a limiting factor, especially when mold differentiation is required.

The *koji process* is an SSF system that employs molds *(Aspergillus*, *Rhizopus)* growing on grains or foods (wheat, rice, soybean). A typical SSF process involves two stages. The first and primary stage is an aerobic, fungal SSF of grains called the *koji*. The second stage is an anaerobic submerged fermentation with a mixed bacterial culture called the *moromi*. Listed in Table 9.3 are the products of aerobic SSF, the koji process. Fermentation in the second stage (moromi) may be realized by using the natural flora or, usually, with externally added bacteria and yeasts. Some strains of *Saccharomyces*, *Torulopsis*, and *Pediococcus* are used as flavor producers in soy sauce manufacture. The moromi is usually fermented for 8 to 12 months. However, the

TABLE 9.3. Some Traditional Food Fermentations

Product	Primary Genus	Common Substrate	Thermal Processing		Initial Moisture (%)	Incubation		Further Processing
			Temp. (°C)	Time (min)		Time (h)	Temp. (°C)	
Soy sauce	*Aspergillus*	Soybean, wheat	110	30	45	72	30	Yes
Miso	*Aspergillus*	Rice, soybean	100	40	35	44	30	Yes
Tempeh	*Rhizopus*	Soybean	100	30	40	22	32	No
Hamanatto	*Aspergillus*	Soybean, wheat				36		Yes
Tofu	*Actinomucor*	Soybean	100	10	74		15	Yes

With permission from R. E. Mudgett, Solid-state fermentations, in A. L. Demain and N. A. Solomon, eds., *Manual of Industrial Microbiology and Biotechnology*, ASM Publications, Washington, D.C., 1986.

processing time can be reduced to 6 months by temperature profiling. The final product is pressed to recover the liquid soy sauce and is pasteurized, filtered, and bottled.

The major industrial use of the koji process is for the production of enzymes by fungal species. Fungal amylases are produced by SSF of wheat bran by *Aspergillus oryzae* in a rotating-drum fermenter. Wheat bran is pretreated with formaldehyde, and the initial pH of the bran is adjusted to pH = 3.5 to 4.0 to reduce the chance of contamination. Usually, perforated pans, rotating drums, or packed beds with air ventilation are used. A typical rotary-drum type of koji fermenter is depicted in Figure 9.18. Enzymes other than amylases, such as cellulase, pectinase, protease, and lipases, can also be produced by koji fermentations. *Trichoderma viride* species have been used for the production of cellulases from wheat bran in a rotary-drum fermenter.

Some secondary metabolites, such as antibacterial agents, are produced by *Rhizopus* and *Actinomucor* species in some koji processes. Certain mycotoxins, such as aflatoxins, were produced by SSF of rice (40% moisture) by *Aspergillus parasiticus*. Ochratoxins were also produced by *Aspergillus* species on wheat in a rotary-drum koji fermenter. Microbial degradation of lignocellulosics can also be accomplished by SSF for waste-treatment purposes and in biopulping of wood chips for use in paper manufacture. Spores from some molds have found use as insecticides. Proper spore formation is difficult to obtain in submerged culture, and SSF must be used.

Major process variables in SSF systems are moisture content (water activity), inoculum density, temperature, pH, particle size, and aeration/agitation. Optimization of these parameters to maximize product yield and rate of product formation is the key in SSF systems and depends on the substrate and organism used. Most natural substrates (e.g., grains) require pretreatment to make the physical structure of substrates more susceptible to mycelial penetration and utilization. Solid substrates are usually treated with antimicrobial agents, such as formaldehyde, and are steamed in an autoclave. Nutrient media addition, pH adjustment, and the adjustment of moisture level are realized before inoculation of the fermentation mash. Koji fermentations are usually realized in a controlled-humidity air environment with air ventilation and agitation. Many solid-state mycelial fermentations are shear sensitive due to disruption of the mycelia

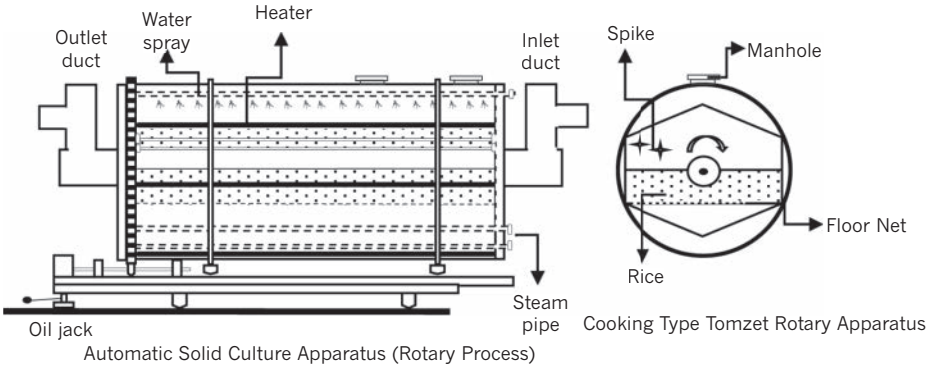


Figure 9.18. Schematic of a rotary-drum type solid-state fermenter including all operations (Koji process).

at high agitation/rotation speeds. At low agitation rates, oxygen transfer and CO_2 evolution rates become limiting. Therefore, an optimal range of agitation rate or rotation speed needs to be determined. Similarly, there is a minimum level of moisture content (~30% by weight) below which microbial activity is inhibited. At high moisture levels (>60%), solid substrates become sticky and form large aggregates. Moreover, moisture level affects the metabolic activities of cells. Optimal moisture level needs to be determined experimentally for each cell–substrate system. For most of the koji processes, the optimal moisture level is about 40% ± 5%. Particle size should be small enough to avoid any oxygen–CO_2 exchange or other nutrient transport limitations. Porosity of the particles can be improved by pretreatment to provide a larger intraparticle surface to volume ratio for microbial action.

Most of the SSF processes are realized using a rotary-tray type of reactor in a temperature and humidity-controlled chamber where controlled-humidity air is circulated through stacked beds of trays containing fermented solids. Rotary-drum fermenters are used less frequently than rotary-tray fermenters because of the shear sensitivity of mycelial cells.

The biggest challenge for the design and operation of SSF systems is the intrinsic heterogeneity in such a system. Because of the localized nature of growth and lack of complete mixing, significant pH and temperature gradients as well as variations in oxygen transfer can be generated. Increased mixing as in a rotary-drum fermenter, which provides a more uniform environment, is not always possible due to the shear sensitivity of such systems and costs for mixing. Small trays (of order 30 cm by 60 cm) are labor intensive and still do not provide uniform culture conditions.

9.6. SUMMARY

Bioreactors using suspended cells can be operated in many modes between a batch reactor and a single-stage chemostat. Although a chemostat has potential productivity advantages for primary products, considerations of genetic instability, process flexibility, and process reliability have limited the use of chemostat units. The use of cell recycle with a CFSTR increases volumetric productivity and has found use

in large-volume, low-product-value processes (e.g., waste treatment and fuel-grade ethanol production). Multistage continuous systems improve the potential usefulness of continuous processes for the production of secondary metabolites and for the use of genetically unstable cells. The fed-batch system is widely used in commercial plants and combines the features of continuous culture and batch that allow the manufacturer to maintain flexibility and ease of intervention. The perfusion system is another option that is particularly attractive for animal cells.

Immobilized cell systems offer a number of potential processing advantages, and the commercialization of such systems has occurred in cases where cell culture is expensive and difficult (e.g., animal cell tissue culture). Physical entrapment or encapsulation is used in most cases, although adsorption onto surfaces or covalent binding of cells to surfaces is possible.

In some cases, self-immobilization on surfaces is possible, and a biofilm is formed. Biofilm reactors can apply to tissue culture, mold, and bacterial systems. Biofilm-based reactors are very important in waste-treatment applications and in natural ecosystems. The analysis of immobilized cell reactors is analogous to that for immobilized enzyme reactors except for the feature of biocatalyst replication.

Solid-state fermentations share some characteristics with immobilized cell systems but differ in that no discernible liquid is present. SSFs have found important uses in the production of some traditional fermented foods and may have use in upgrading agricultural or forest materials and in the production of mold products requiring full mold differentiation.

The reactor options described in this chapter are many. The best choice of reactor systems will ultimately be determined by the choice of biocatalyst and the requirements for product recovery and purification.

SUGGESTIONS FOR FURTHER READING

Bailey, J. E., and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill, New York, 1986.

Bhargav, S., B. P. Panda, M. Ali, and S. Javed, "Solid-State Fermentation: An Overview," *Chem. Biochem. Eng. Q.* 22(1): 49–70, 2008.

Blanch, H. W., and D. S. Clark, *Biochemical Engineering*, Marcel Dekker, New York, 1996.

Chang, H. N., K. Jung, J. Choi, J. C. Lee, and H. Woo, "Multi-stage Continuous High Cell Density Culture Systems: A Review," *Biotechnol. Adv. 32:*514–525, 2014.

Connell, J. L., E. T. Ritschdorff, M. Whitley, and J. B. Shear, "3D Printing of Microscopic Bacterial Communities," *PNAS* 110(46): 18380–18385, 2013.

Guisan, J. M, *Immobilization of Enzymes and Cells*, 3d ed., Humana Press, Totowa, NJ, 2013.

Kargi, F., and M. Moo-Young, "Transport Phenomena in Bioprocesses," in M. Moo-Young, ed., *Comprehensive Biotechnology*, Vol. 2, Pergamon Press, Elmsford, NY, pp. 5–55, 1985.

Kim, B. S., S. C. Lee, S. Y. Lee, Y. K. Chang, and H. N. Chang, "High Cell Density Fed-Batch Cultivation of *Escherichia coli* Using Exponential Feeding Combined with pH-stat," *Bioprocess. Biosys. Eng.* 26(3): 147–150, 2004.

Krishna, C, "Solid-State Fermentation Systems—An Overview," *Crit. Rev. Biotechnol.* 25(1–2): 1–30, 2005.

Mantzaris, N. V., P. Daoutidis, F. Srienc, and A. G. Fredrickson, "Growth Processes in a Cascade of Bioreactors: Mathematical Models," *AIChE J.* 45(1):164–176, 1999.

Moulton, G. G., *Fed-batch Fermentation: A Practical Guide to Scalable Recombinant Protein Reduction in Escherichia coli*, Elsevier, New York, 2014.

Murphy, S. V., and A. Atala, "3D Bioprinting of Tissues and Organs," *Nat. Biotechnol.* 32(8): 773–785, 2014.

PROBLEMS

9.1. Consider a 1000l CFSTR in which biomass is being produced with glucose as the substrate. The microbial system follows a Monod relationship with $\mu_m = 0.4\ h^{-1}$, $K_s = 1.5$ g/l (an unusually high value), and the yield factor $Y_{X/S} = 0.5$ g biomass/g substrate consumed. Normal operation is with a sterile feed containing 10 g/l glucose at a rate of 100 l/h.

a. What is the specific biomass production rate (g/l-h) at steady state?

b. If recycle is used with a recycle stream of 10 l/h and a recycle biomass concentration five times as large as that in the reactor exit, what would be the new specific biomass production rate?

c. Explain any difference between the values found in parts a and b.

9.2. In a two-stage chemostat system, the volumes of the first and second reactors are $V_1 =$ 500 l and $V_2 = 300$ l, respectively. The first reactor is used for biomass production and the second is for a secondary metabolite formation. The feed flow rate to the first reactor is $F = 100$ l/h, and the glucose concentration in the feed is $S = 5.0$ g/l. Use the following constants for the cells:

$$\mu_m = 0.3\ h^{-1}, \qquad K_S = 0.1\ g/l, \qquad Y_{X/S} = 0.4\ \frac{\text{g dw cells}}{\text{g glucose}}$$

a. Determine cell and glucose concentrations in the effluent of the first stage.

b. Assume that growth is negligible in the second stage and the specific rate of product formation is $q_p = 0.2$ g P/g cell h, and $Y_{P/S} = 0.6$ g P/g. Determine the product and substrate concentrations in the effluent of the second reactor.

9.3. Consider the following batch growth data:

Time h	X g/l	P g/l	dX/dt g/l-h	dP/dt g/l-h
0	0.3	<0.01	—	—
3	1.0	<0.01	0.30	—
6	2.3	<0.01	0.55	—
8	4.0	0.010	1.0	0.005
9	5.1	0.025	1.3	0.010
10	6.5	0.060	1.4	0.045
10.5	7.0	—	1.4	—
11	7.4	0.10	0.60	0.059
12	7.7	0.17	0.20	0.072
13	7.8	0.26	0.02	0.105
14	—	0.36	—	0.130
15	8.0	0.47	~0	0.087
16	8.0	0.54	~0	0.042
17	—	0.58	—	0.021
18	—	0.60	—	0.005

You have available three tanks of different volumes: 900, 600, and 300 l. Given a flow rate of 100 l/h, what configuration of tanks would maximize product formation?

9.4. Two CFSTR in series are operated for fermentation of glucose to ethanol. The reactors are operated for a certain degree of conversion with a known feed substrate concentration (i.e., S_0 and S_2 are fixed). Assume a first order fermentation kinetics ($R_s = k_1 S$) in both reactors.

- **a.** Write down the design equations for both reactors (θ_H as function of S) and the total hydraulic residence time as function of substrate concentrations.
- **b.** Prove that total θ_H is minimum when $S_1 = (S_0.S_2)^{1/2}$.
- **c.** Prove that $\theta_{H1} = \theta_{H2}$ or the volumes of the reactors would be the same for the constant feed flow rate (F) when total θ_H or the total volume is minimum.
- **d.** Express the hydraulic residence times for each reactor and the total hydraulic residence time in terms of k_1, S_o, and S_2.

9.5. Two-stage chemostat system is used for fermentation of glucose to ethanol using *Saccharomyces cerevisiae*. The feed flow rate and glucose concentrations are 1 l/h and S_0 = 40 g/l, respectively. The following kinetic constants are known: $t_d = 5$ h, $K_s = 1$ g /l, $Y_{X/S}$ = 0.1 gX/gS, $Y_{P/S} = 0.5$ g EtOH/g glucose. Assuming 80% conversion in every reactor, determine the following:

- **a.** Substrate and product concentrations in the effluent of reactors
- **b.** Biomass concentrations in both reactors
- **c.** Volume of each reactor
- **d.** Productivity of biomass (DX) and product (DP) for each reactor
- **e.** Specific rates of product formation (q_p) and substrate consumption (q_s) for each reactor

9.6. Penicillin is produced by *Penicillium chrysogenum* in a fed-batch culture with the intermittent addition of glucose solution to the culture medium. The initial culture volume at quasi-steady state is $V_0 = 500$ l, and glucose-containing nutrient solution is added with a flow rate of $F = 50$ l/h. Glucose concentration in the feed solution and initial cell concentration are $S_0 = 300$ g/l and $X_0 = 20$ g/l, respectively. The kinetic and yield coefficients of the organism are $\mu_m = 0.2\ h^{-1}$, $K_S = 0.5$ g/l, and $Y_{X/S} = 0.3$ g dw/g glucose.

- **a.** Determine the culture volume at $t = 10$ h.
- **b.** Determine the concentration of glucose at $t = 10$ h at quasi-steady state.
- **c.** Determine the concentration and total amount of cells at quasi-steady state when $t = 10$ h.
- **d.** If $q_P = 0.05$ g product/g cells h and $P_0 = 0.1$ g/l, determine the product concentration in the vessel at $t = 10$ h.

9.7. The bioconversion of glucose to ethanol is carried out in a packed-bed, immobilized cell bioreactor containing yeast cells entrapped in Ca-alginate beads. The rate-limiting substrate is glucose, and its concentration in the feed bulk liquid phase is S_{0i} = 5g/l. The nutrient flow rate is F = 2 l/min. The particle size of Ca-alginate beads is $D_P = 0.5$ cm. The surface area of the alginate beads per unit volume of the reactor is $a = 25\ cm^2/cm^3$, and the cross-sectional area of the bed is $A = 100\ cm^2$.

Following are the rate constants for this conversion:

$$r_m = 100 \text{ mg } S/\text{cm}^3 \cdot \text{h}$$
$$K_S = 10 \text{ mg } S/\text{cm}^3$$

for the following rate expression:

$$r_s = \frac{r_m S}{K_S + S}$$

Assuming a first-order reaction-kinetics (e.g., relatively low substrate concentrations), determine the required bed height for 80% conversion of glucose to ethanol at the exit stream. *Hint:* To calculate the effectiveness factor, we can use the following equations:

$$\eta = \frac{1}{\phi}\left[\frac{1}{\tanh 3\phi} - \frac{1}{3\phi}\right]$$

where

$$\phi = \frac{V_P}{A_P}\sqrt{\frac{k_1}{D_S}}$$

and

$$k_1 = \frac{r_m}{K_S}, \qquad D_S = 10^{-6}\ \text{cm}^2/\text{s}$$

9.8. A fluidized-bed, immobilized cell bioreactor is used for the conversion of glucose to ethanol by *Zymomonas mobilis* cells immobilized in κ-carrageenan gel beads. The dimensions of the bed are 10 cm (diameter) by 200 cm. Since the reactor is fed from the bottom of the column and because of CO_2 gas evolution, substrate and cell concentrations decrease with the height of the column. The average cell concentration at the bottom of the column is $X_0 = 45$ g/l, and the average cell concentration decreases with the column height according to the following equation:

$$X = X_0(1 - 0.005Z)$$

Z is the column height (cm). The specific rate of substrate consumption is $q_S = 2$ g S/g cells h. The feed flow rate and glucose concentration in the feed are 5 l/h and 160 g glucose/l, respectively.

a. Determine the substrate (glucose) concentration in the effluent.

b. Determine the ethanol concentration in the effluent and ethanol productivity (g/l-h) if $Y_{P/S} = 0.48$ g ethanol/g glucose.

9.9. Lactic acid is produced by homolactic bacteria *Streptococcus lactis* from glucose solution using fed-batch operation. The following data are available: $V_0 = 10$ l, $X_0 = 5$ g/l, $Y_{X/S} = 0.12$ g X/g S, $Y_{P/S} = 0.80$ g P/g S, $\mu_m = 0.2\ \text{h}^{-1}$, $K_S = 0.5$ g/l, $S_F = 30$ g/l, $F = 2$ l/h.

a. How long should the sytem be operated in order to obtain $S = 0.5$ g/l in the fermenter?

b. Determine the biomass and product concentrations at the end of operation.

c. What should be the feed flow rate in order to obtain $S = 0.1$ g/l after 10 h of operation?

d. Determine P and X for this operation.

9.10. A completely mixed bioreactor is used for ethanol fermentation of hydrolyzed starch solution containing 30 g/l total sugar. The following data are available: $\mu_m = 0.15\ \text{h}^{-1}$, $K_S = 0.5$ g/l, $F = 10$ l/h, $Y_{X/S} = 0.1$ g X/g S, $Y_{P/S} = 0.5$ gP/gS, $k_S = 0.1\ \text{d}^{-1}$.

a. For 95% conversion of substrate to product, determine the required reactor volume and ethanol productivity when the system was operated without cell recycle.

b. For the same degree of conversion, determine the required reactor volume and ethanol productivity when the system was operated with cell recycle. $r = 0.4$, $C = X_r/X = 3$, $\alpha = 0.1$.

9.11. In a fluidized-bed biofilm reactor, cells are attached on spherical plastic particles to form biofilms of average thickness $L = 0.5$ mm. The bed is used to remove carbon compounds from a wastewater stream. The feed flow rate and concentration of total fermentable carbon compounds in the feed are $F = 2$ l/h and $S = 2000$ mg/l. The diameter of the column is 10 cm. The kinetic constants of the microbial population are $r_m = 50$ mg S/cm^3-h and $K_S = 25$ mg S/cm^3. The specific surface area of the biofilm in the reactor is 2.5 cm^2/cm^3. Assuming first-order reaction kinetics and an average effectiveness factor of $\eta = 0.7$ throughout the column, determine the required height of the column for effluent total carbon concentration of $S_{0i} = 100$ mg/l.

9.12. Glucose is converted to ethanol by immobilized yeast cells entrapped in gel beads. The specific rate of ethanol production is $q_P = 0.2$ g ethanol/g cell-h. The effectiveness factor for an average bead is 0.8. Each bead contains 50 g/l of cells. The voids volume in the column is 40%. Assume growth is negligible (all glucose is converted into ethanol). The feed flow rate is $F = 400$ l/h, and glucose concentration in the feed is $S_{0i} = 150$ g glucose/l. The diameter of the column is 1 m, and the yield coefficient is about 0.49 g ethanol/g glucose. The column height is 4 m.

a. What is the glucose conversion at the exit of the column?

b. What is the ethanol concentration in the exit stream?

9.13. Consider the batch growth curve in Figure 9.4 and the corresponding plots of dX/dt versus X and dP/dt versus P (Figure 9.7). You are asked to design a two-stage reactor system with continuous flow that will produce product P at a concentration of 0.55 g/l. You wish to minimize total reactor volume. For a flow rate of 1000 l/h, what size reactors (and in what order) would you recommend?

9.14. Consider Figure 9.9, which applies to a fed-batch system. Assume at $t = 0$, $V = 100$ l, $X = 2$ g/l, $\mu = 1$/h, $S_0 = 4$ g/l, and $S = 0.01$ g/l. V is increased at a constant rate such that $dV/dt = 20$ l/h $= F$ (or flow rate) and X is constant at all times.

a. Derive a formula to relate μ to V and dV/dt.

b. What is μ at $t = 5$ h?

9.15. An industrial wastewater stream is fed to a stirred-tank reactor continuously, and the cells are recycled back to the reactor from the bottom of the sedimentation tank placed after the reactor. The following are given for the system: $F = 100$ l/h; $S_0 = 5000$ mg/l; $\mu_m = 0.25$ h^{-1}; $K_s = 200$ mg/l; α (recycle ratio) $= 0.6$; C (cell concentration factor) $= 2$; $Y^M_{X/S} = 0.4$. The effluent concentration is desired to be 100 mg/l.

a. Determine the required reactor volume.

b. Determine the cell concentration in the reactor and in the recycle stream.

c. If the residence time is 2 h in the sedimentation tank, determine the volume of the sedimentation tank and cell concentration in the effluent of the sedimentation tank.

9.16. A wastewater stream is treated biologically by using a reactor containing immobilized cells in porous particles. Variation of rate of substrate removal with particle size is given in the following table.

r_s (mg/l-h)	D_P(mm)
300	1
300	2
250	3
200	4
150	5
100	7
50	10

a. What are the effectiveness factors for $D_P = 4$ mm and $D_P = 7$ mm?

b. The following data were obtained for $D_P = 4$ mm at different substrate concentrations. Assuming no liquid film resistance, determine the r_m and K_s for the microbial system.

S_0 (mg/l)	r (mg/l-h)
100	85
250	200
500	360
1000	630
2000	1000

9.17. A wastewater stream of $F = 1$ m^3/h with substrate at 2000 mg/l is treated in an upflow packed bed containing immobilized bacteria in form of biofilm on small ceramic particles. The effluent substrate level is desired to be 30 mg/l. The rate of substrate removal is given by the following equation:

$$r_s = \frac{kXS}{K_s + S}$$

By using the following information, determine the required height of the column (H): $k = 0.5$ h^{-1}, $X = 10$ g/l, $K_s = 200$ mg/l, $L = 0.2$ mm, $a = 100$ m^2/m^3, $A = 4$ m^2, $\eta = 0.8$.

9.18. A packed-column biofilm reactor (biofilter) with $H/D_0 = 2.5$ is used for aerobic biological treatment of wastewater of flow rate $Q = 5$ m^3/h and $S_0 = 1000$ mg chemical oxygen demand/l to obtain $S_e = 25$ mg COD/l in the effluent. The effluent is recycled with $R = 2$. The following information are available: $\mu_m = 0.12$ h^{-1}, $K_s = 150$ mg/l, $Y = 0.35$ g X/g S, $X = 10$ g X/l, $L = 1.5$ mm, $R = 2$ (Effluent recycle ratio), $a_f = 120$ m^2/m^3, $D_e = 10^{-5}$ cm^2/sec.

a. Determine the effectiveness factor (η).

b. Determine the volume and dimensions (D_o and H) of the column.

c. For $H = 3$ m, determine biofilm area per unit reactor volume (a_f) and D_o, when $H/D_o = 2$.

10

Selection, Scale-Up, Operation, and Control of Bioreactors

Chapter 9, "Operating Considerations for Bioreactors for Suspension and Immobilized Cultures," focused on idealized descriptions of homogeneous bioreactors with regard to operating mode (batch, fed-batch, continuous, and multistage) and generalized discussions of heterogenous reactors (immobilized cell systems and solid-state fermentation [SSF]). Real processes demand greater insight into the details of reactor configuration. It would be impossible in a book of this type to provide all the information necessary to become a design engineer, but this chapter introduces some of the concepts that drive the choice of real bioreactors. Our focus is primarily on reactors for bacteria and fungi; some of the special reactor issues for animal and plant cell tissue culture are handled in Chapter 12, "Bioprocess Considerations in Using Animal Cell Cultures," and Chapter 13, "Bioprocess Considerations in Using Plant Cell Cultures."

10.1. SCALE-UP AND ITS DIFFICULTIES

Why would the performance of a fungal culture making an antibiotic be so different at 10,000 l than at 10 l? At first glance, it might be unclear why performance should change with scale. The answers lie in the difficulty of maintaining homogeneity in large systems, changes in surface-to-volume ratios, and changes in the cultures themselves due to differences in microenvironments. To understand the problems in

scale-up, we first need to describe what traditional culture vessels are and how they are operated.

10.1.1. Overview of Traditional Reactor Types

Rather than listing the large array of suggested fermenter and bioreactor designs, we restrict ourselves to considering some basic types:

- Reactors with internal mechanical agitation
- *Bubble columns*, which rely on gas sparging for agitation
- *Loop reactors*, in which mixing and liquid circulation are induced by the motion of an injected gas, by a mechanical pump, or by a combination of the two

Although *fermenter* and *bioreactor* are often used interchangeably, some professionals use "bioreactor" primarily for animal cell cultures. Here we use "fermenter" to refer to microbial or fungal cultures and "bioreactor" as a broad term incorporating any system for cultivating living cells.

All three reactor types (see Figure 10.1 for schematics) invariably are concerned with three-phase reactions (gas–liquid–solid). The reactors described here are made from materials that allow repeated use. Three-phase reactors are difficult to design, because the mass transfer of components among the three phases must be controlled. Inadequate tools for the complex fluid mechanics in such systems coupled with the complex nature of cells as reactive solids make the prediction of system performance very difficult.

10.1.2. Reactors with Internal Mechanical Agitation

The traditional fermenter is the stirred-tank reactor (Figure 10.2), the prime example of a reactor with internal mechanical agitation. The main virtues of such systems are that they are highly flexible and can provide high $k_L a$ (volumetric mass-transfer coefficient) values for gas transfer. Stirred reactors of up to 400 m^3 are used in antibiotic production, with stirrer powers of up to 5 kW/m^3. Stirred reactors can be used commercially up to viscosities of about 2000 centipoises (2 Pa sec). Stirred-tank systems for animal and plant cell culture are typically much smaller than for microbial systems. A reactor of 20 m^3 is the upper limit used for animal cells and 75 m^3 for plant cells.

Gas under pressure is supplied to the *sparger* (usually either a ring or disk with holes or a tube with a single orifice). The size of the gas bubbles and their dispersion throughout the tank are critical to reactor performance. Although a sparging ring will initially provide smaller bubble size and better gas distribution, spargers with a single discharge point are more resistant to plugging.

Gas dispersion is a function not only of the sparger but also of the *impeller*. The impeller must provide sufficiently rapid agitation to disperse bubbles throughout the tank, to increase their residence time within the liquid, and to shear larger bubbles into small bubbles. Too much stirring can be detrimental, owing to the

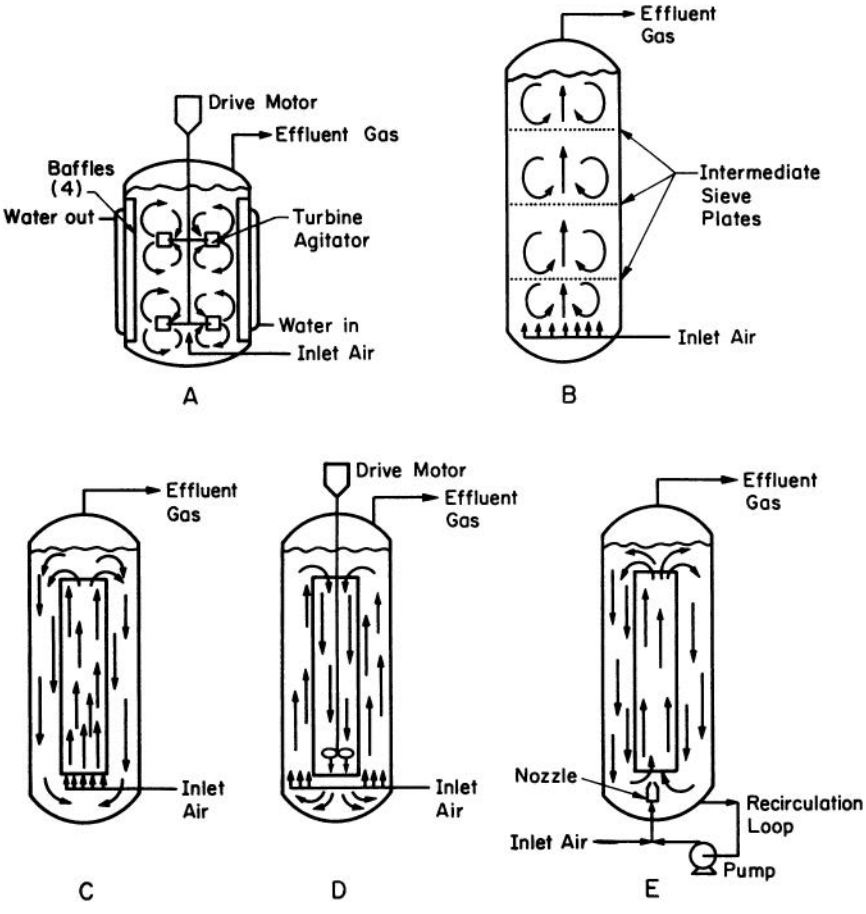


Figure 10.1. Bioreactor types. (a) Stirred-tank reactor, (b) bubble-column reactor, (c) airlift loop reactor with central draft tube, (d) propeller loop reactor, and (e) jet loop reactor. Arrows indicate fluid circulation patterns. (With permission, from D. N. Bull, R. W. Thoma, and T. E. Stinnett, *Adv. Biotechnol. Processes I*, 1, 1985, and Alan R. Liss, Inc., New York.)

shear sensitivity exhibited to varying degrees by some cells (e.g., animal cells) and the stratification of reactor contents with multiple-impeller systems. Although a wide variety of impeller designs have been proposed, the predominant choices are disk and turbine impellers, and for cellular systems with high levels of shear sensitivity, marine and paddle impellers are of particular interest.

The Rushton impeller (Figure 10.3) is a disk with typically six to eight blades designed to pump fluid in a radial direction. Until the mid-1980s, the Rushton impeller was the predominant design choice, and it is commonly found on industrial and laboratory fermenters. The axial flow hydrofoil impellers have become increasingly popular. Axial flow systems can pump liquid either down or up. They have been shown to give superior performance (compared to Rushton radial flow impellers) with respect to lower energy demands for the same level of oxygen transfer.

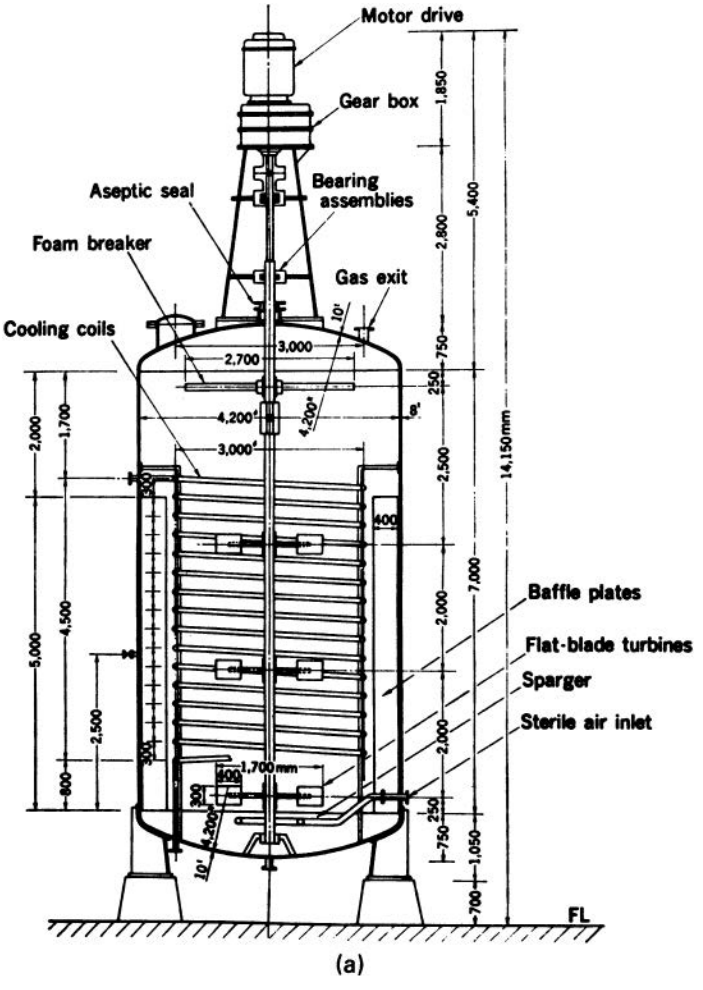


Figure 10.2. (a) Mechanically stirred 100,000-liter fermenter. (With permission, from S. Alba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, 2d ed., University of Tokyo Press, Tokyo, 1973.)

Further, they show reduced maximum shear rates, making them usable with sensitive cultures such as animal cells, while still being capable of excellent performance with viscous mycelial fermentations. Axial flow systems break up the compartmentalization often observed with multiple radial flow impellers on the same shaft. Combinations of axial flow and radial flow impeller systems are sometimes used. To augment mixing and gas dispersion, baffles are employed. A typical arrangement includes four baffles, the width of each being about 8% to 10% of the reactor diameter. With animal cell cultures, baffles cause shear damage; instead of baffles, bottom-drive axial impellers slightly offset from center can be used.

The vessel itself is almost always stainless steel. Type 316 is used on all wetted parts, type 304 on covers and jackets. With plant and animal cell tissue cultures, a

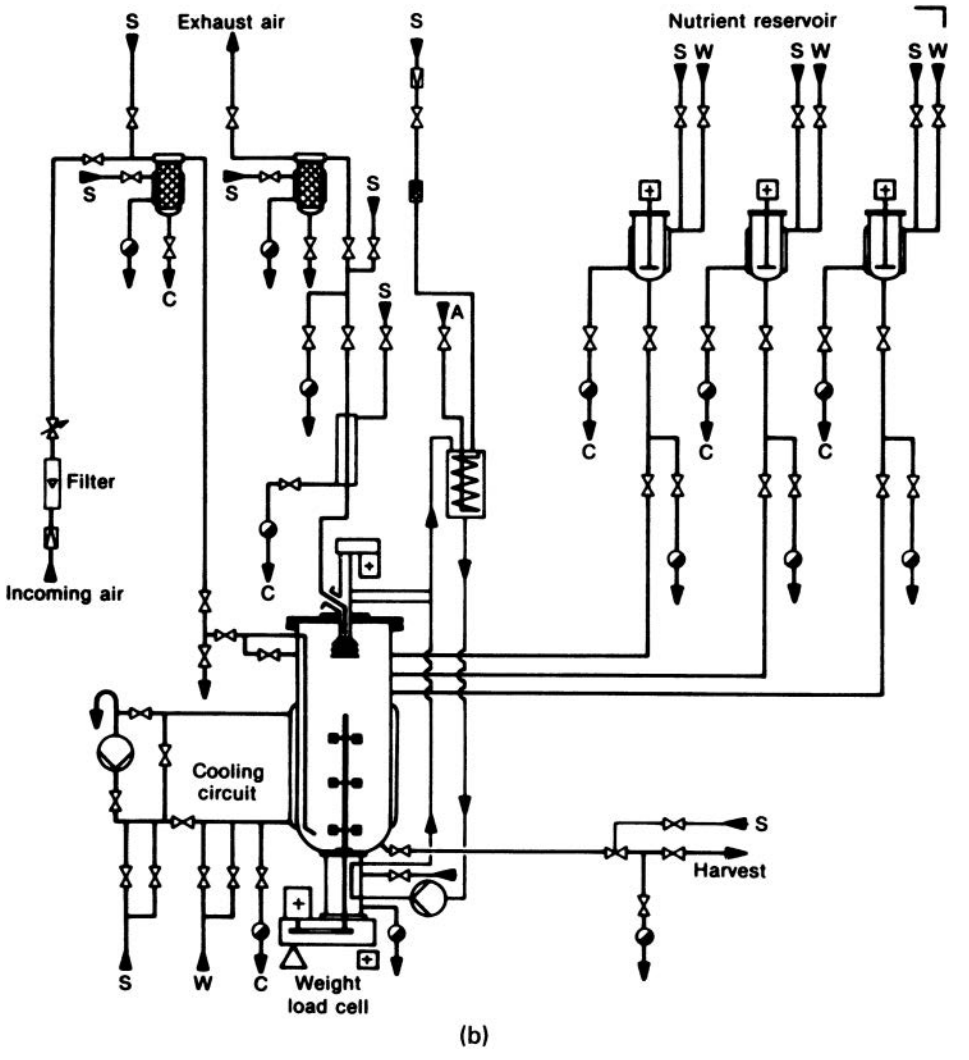


Figure 10.2. (*continued*) (b) Installation of mechanically stirred fermenter: S, steam, C, condensate, W, water, and A, air. The steam lines permit in-place sterilization of valves, pipes, and seals. The input air can be sterilized by both incineration and filtration. (With permission, from W. Crueger and A. Crueger, *Biotechnology: A Textbook of Industrial Microbiology*, R. Oldenbourg Verlag, München, Germany, 1984. With permission from Science Tech Publishers.)

low-carbon version (type 316L) is often used. Many bench-scale fermenters are glass vessels with stainless-steel cover plates. Glass fermenters are rarely used at the 50 l scale, and material considerations limit the maximum size of glass vessels to about 500 l. The use of stainless steel and glass is called for by the need to sterilize the reactor and the corrosive and/or abrasive nature of many fermentation media.

Most fermenters are built with a height-to-diameter ratio of 2 to 3, although for animal cell bioreactors this ratio tends to be closer to 1. The Rushton impeller

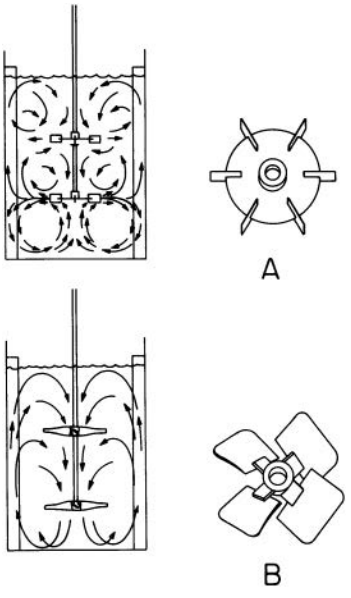


Figure 10.3. Liquid flow in bottled tanks with (A) Rushton radial flow impellers and with (B) axial flow hydrofoil impellers.

diameter is typically 30% to 40% of the tank diameter, whereas for the axial flow hydrofoil impellers it may reach 50%. In large reactors, the two main limitations on size are the abilities of the design to provide an adequate supply of oxygen and to remove metabolic heat efficiently. Large reactors usually use either internal coils for heat removal or a jacketed vessel. Although copper coils have better thermal transport characteristics, stainless-steel coils are almost always used, but this choice can depend on the nature of the media and culture. Internal coils provide advantages over jacketed vessels in terms of efficiency of heat removal, owing to the larger surface area for heat transfer. However, in many systems, the coils become rapidly fouled by microbial growth, decreasing heat transfer and often adversely affecting mixing and maintenance of sterility. In such cases, the jacketed vessels offer advantages.

Another problem often encountered in commercial fermentations is *foaming*. If foam escapes from the fermenter, it can wet filters, increasing pressure drop and decreasing gas flow. Of greater concern is that it provides a pathway for contaminating cells to enter the fermenter. For most products of commercial interest, absolute sterility is required, and contamination may cause the loss of much product, time, and money. Foam can be controlled with a mechanical foam breaker or the addition of surface-active chemical agents. Although such chemicals can be very effective in controlling foam, there are penalties. Foam-breaking chemicals usually lower $k_L a$ values, reducing the reactor's capacity to supply oxygen or other gases, and in some cases they can be inhibitory to cell growth. Foam formation is not well understood, but complex media or the formation of high levels of extracellular polymers (e.g., proteins) tend to promote foaming. Foam can limit the ultimate productivity of a fermenter. All stirred-tank fermenters provide head space for the gas to disengage from the liquid. The *working volume* (the amount of culture) in a fermenter is typically about 75% of the total fermenter volume.

Sterility is a prime design consideration for fermenter hardware. Pressurized steam is used for in-place sterilization of the reactor, seals, probes, and valves. The number of openings into the fermenter should be limited to what is essential. A trade-off is necessary between the use of many probes, which improves fermenter control, and of few probes, which improves the chances of maintaining sterility. Small openings are made leakproof with O-rings, while flat gaskets are satisfactory for larger openings. Although small fermenters can use magnetically coupled agitators, industrial-size fermenters have moving shafts that must penetrate into the fermenter. Prevention of contamination due to the entry of foreign organisms through such a shaft is a major challenge in the mechanical design of fermenters. Stuffing-box seals are common on old fermenters, while double mechanical seals are used in newer ones. All surfaces must be smooth. Crevices in the tank surface, pipes, and valves can trap large quantities of particulate organics and contaminating organisms. Such clumps of cells increase the chances for a contaminant to survive the sterilization procedure. Cleanability of all surfaces is important.

Cleaning is generally done "in place," and fermenter design includes spray balls to allow for clean-in-place (CIP) technology. Highly alkaline detergents are often used, and this factor helps dictate the selection of materials. Surfaces, especially in bioreactors for animal or plant tissue cultures, often undergo electropolishing, an electrolytic process that removes the sharp microscopic projections often resulting from mechanical polishing. All ports and valves involved in sampling and injection should be protected with steam-sterilizable closures. The application of a continuous flow of live steam to sample valves is one strategy that is often applied in antibiotic plants.

10.1.3. Bubble Column and Loop Reactor

The same concern for sterility applies to bubble columns and loop (e.g., airlift) reactors. Bubble columns offer distinct advantages for some systems. They are suitable for low-viscosity Newtonian broths; satisfactory mixing may not be possible in highly viscous broths. Bubble columns provide a higher energy efficiency than stirred-tank systems; by *energy efficiency* we mean the amount of oxygen transferred per unit of power input. An additional advantage often mentioned for bubble columns is that they provide a low-shear environment, which may be a critical consideration with some cells. Cells tend to accumulate at the bubble surface, however, and bubble bursting is highly detrimental to cells. The absence of mechanical agitation also reduces cost and eliminates one potential entry point for contaminants.

Besides having less vigorous mixing capabilities than stirred tanks, bubble-column operation is often limited by considerations of foaming and bubble coalescence. Because of bubble coalescence, bubble columns work over a rather narrow range of gas flow rates. The range of appropriate gas flows varies with the nature of the broth. Thus, bubble columns are less flexible than stirred tanks. Partial relief from the problem of coalescence can be found by using multistage columns; each stage (perforated plate) acts to redistribute gas flow. This gas redispersion, however, carries an energy penalty.

Loop reactors have intermediate characteristics between bubble columns and stirred tanks. We consider primarily the *airlift* system (Figure 10.1) in which the

motion of the gas carries fluid and cells up a draft tube. At the top, gas disengages from the liquid, and the degassed liquid (which is denser than the gassed liquid) descends in the annulus outside the tube. At the bottom of the reactor, the descending fluid again encounters the gas stream and is carried back up the draft tube. Airlift systems can generally handle somewhat more viscous fluids than bubble columns, and coalescence is not so much of a problem. The largest fermenter (1500 m^3) ever built (by ICI) is an airlift design for the production of single-cell protein. With very large fermenters (>200 m^3), the use of nonmechanically agitated designs is preferred, as high oxygen transfer rates and better cooling can be attained. With an airlift design, the interchange of material between fluid elements is small, so the transient time to circulate from the bottom of the draft tube to the top and back again is important. In the ICI design, multiple injection points for the substrate are used to prevent the cells from becoming substrate starved during circulation. The addition of mechanical stirring to a loop reactor increases flexibility (the operating range).

Although we focus our attention on agitated tanks, realize that most anaerobic fermentations (in terms of total volume) are conducted in nonstirred and nonaerated vessels. Such vessels are used often in food fermentations, such as for beer, wine, and dairy products (e.g., cheese). In anaerobic fermentations, gas evolution by the fermenting organisms can provide some mixing, but gases are not normally sparged into the vessel. However, agitated and/or aerated vessels are used on a wider variety of fermentations (in terms of number of processes), are more difficult to scale, and are more likely to be chosen for the production of new products.

10.1.4. Single-Use Bioreactors

During the last 15 years, industry has begun to use single-use (or "disposable") bioreactors instead of traditional reusable systems. The use of these systems is focused on high-value products such as those typically produced from animal cell culture where reactor size is modest. Advances that have increased per-cell productivity can often support the use of bioreactors of modest sizes (order of 100 l) and are particularly attractive for human stem cells. However, this technology has been used with microbial systems as well.

Single-use bioreactors are typically made from FDA-approved plastics (e.g., polycarbonate, polystyrene, and others). The cultivation container is delivered preassembled and sterile and is "ready-to-use." After use, the cultivation chamber is decontaminated and disposed. The advantages of this technology include rapid scale-up and implementation because sterilization/cleaning procedures and their validation are not required. Since the regulatory constraints on the pharmaceutical industry, particularly concerning cleanliness, are so demanding, single-use technology offers a major advantage. It also leads to great process flexibility and responsiveness to market changes, reduced costs (particularly initial capital costs are reduced several-fold), and improvements in biosafety. There are potential disadvantages in terms of issues with chemicals that may leach out of the plastic and contaminate the product and the mechanical stability of the plastic. Also limiting is the oxygen transfer capability of disposable systems as well as the degree of mixing, which limits the effective cell concentration attainable. Sensor technology for use in disposables lags and is another

limitation. The maximum size is order of 2 m^3 (2000 l), although efforts are being made to increase the maximum useable size.

Disposable bioreactors typically are mechanically driven either by external or internal means. One example of an externally driven system is the use of a culture bag on a rocker platform (e.g., WAVE Bioreactor). The rocker can impact oscillatory movement, resulting in a "wave." Other motions, such as rotary, vibrating, or "shaken" platforms, are possible. One category of internally driven systems involves the use of a culture bag with an internal rotating stirrer magnetically coupled to the external platform or a rigid cylindrical vessel with a mechanically coupled stirrer. Another category is vessels with pneumatically driven stirring. Hybrid systems with mechanical and prepneumatic-driven mixing also exist. While most of these systems are used primarily for animal cells, some (e.g., Saltus Bioreactor) use an oscillating hollow shaft that generates high shear conditions and cannot be used with shear-sensitive animal cells.

Disposable technology is being developed not only for bioreactors but for media and buffer preparation, filtration, and even chromatography. This trend is driven, in part, by the same issues as for the development of disposable bioreactors.

The principles for reactor design, based primarily on aeration, agitation, and heat transfer, are the same for disposable and permanent bioreactors, although details may vary due to differences in geometry and details of how oxygen enters and carbon dioxide exits the reactor. In the next section, we address these issues.

10.1.5. Considerations in Aeration, Agitation, and Heat Transfer

The basic equations describing oxygen transfer, carbon dioxide evolution, and heat generation by microbial growth are given in Chapter 6, "How Cells Grow." For industrial-scale fermenters, oxygen supply and heat removal are the key design considerations.

The severity of the oxygen requirements depends on the choice of organism. Equation 6.21 can be rewritten as

$$\text{OUR} = X \cdot q_{O_2} = k_L a(C^* - C_L) \tag{10.1}$$

where q_{O_2} is the specific uptake rate of oxygen (mol O_2/g-h). Typical values of q_{O_2} are given in Table 10.1. The value of q_{O_2} (or oxygen uptake rate OUR) is the demand side of the equation; typical requirements in large-scale systems are 40 to 200 mmol O_2/l-h, with most systems in the 40 to 60 mmol O_2/l-h range.

On the supply side, the critical parameter is $k_L a(h^{-1})$, the volumetric oxygen transfer coefficient. A wide range of equations has been suggested for the estimation of $k_L a$. References at the end of this chapter detail some of these correlations. A typical correlation has the form

$$k_L a = k(P_g/V_R)^{0.4}(v_s)^{0.5}(N)^{0.5} \tag{10.2a}$$

where k is an empirical constant, P_g is the power requirement in an aerated (gassed) bioreactor, V_R is bioreactor volume, v_s is the superficial gas exit speed, and N is the

TABLE 10.1. Typical Respiration Rates of Microbes and Cells in Culture

Organism	q_{O_2} (mmol O_2/g dw-h)
Bacteria	
Escherichia coli	10–12
Azotobacter sp.	30–90
Streptomyces sp.	2–4
Yeast	
Saccharomyces cerevisiae	8
Molds	
Penicillium sp.	3–4
Aspergillus niger	ca. 3
Plant cells	
Acer pseudoplatanus (sycamore)	0.2
Saccharum (sugar cane)	1–3
Animal cells	$0.4\frac{\text{mmol } O_2/1-\text{h}}{10^6 \text{ cells/ml}}$
HeLa	
Diploid embryo WI-38	$0.15\frac{\text{mmol } O_2/1-\text{h}}{10^6 \text{ cells/ml}}$

rotational speed of the agitator. With equations of the form of equation 10.2a, all the parameters must be in the units that are required by the correlation. Following is an example of one of the original correlations of this type (when partial pressures are used):

$$k_L a(\text{mmol/1-h-atm}) = 0.6[P_g/V_R(hp/10001)]^{0.4}[v_s(\text{cm/min})]^{0.5}[N(\text{rpm})]^{0.5} \quad (10.2b)$$

A value of P_g can be estimated from other correlations, such as the following:

$$P_g = K\left(\frac{P_u^2 \cdot N \cdot D_i^3}{Q^{0.56}}\right)^{0.45} \quad (10.3)$$

where K is a constant based on reactor geometry, P_u is the power required in the ungassed fermenter, D_i is the impeller diameter, and Q is the aeration rate (volume of gas supplied per minute divided by the liquid volume in the reactor).

The preceding correlations are not very good for Newtonian systems and are even worse for non-Newtonian or highly viscous systems. They also neglect the effects of medium components on $k_L a$. The presence of salts and surfactants can significantly alter bubble size and liquid film resistance around the gas bubble. These factors also can affect *oxygen solubility* (C^*). Temperature and pressure also affect $k_L a$ and (C^*). Finally, on the supply side, it should be noted that C_L is maintained at a value above the critical oxygen concentration (see Chapter 6) but at a low enough value to provide good oxygen transfer. For many bacterial fermentations, a C_L of about 1 mg/l provides a good margin of safety if mixing is incomplete and yet allows a good rate of oxygen transfer.

Although k_La is difficult to predict, it is a measurable parameter and can be measured for microreactors as well as large industrial fermenters. Four approaches are commonly used: unsteady state, steady state, dynamic, and sulfite methods. The way in which these methods are applied depends on whether the methods are used in the presence or absence of cells.

A new reactor, prior to operation, can be filled with pure water or a medium in which C^* can be accurately measured. Oxygen is removed from the system by sparging with N_2. With the *unsteady-state method*, air is then introduced and the change in dissolved oxygen (DO) is monitored until the solution is nearly saturated. In this case,

$$\frac{dC_L}{dt} = k_L a(C^* - C_L) \tag{10.4a}$$

or

$$\frac{-d(C^* - C_L)}{dt(C^* - C_L)} = k_L a \tag{10.4b}$$

Integration of equation 10.4b from $t = 0$ to t and $C_L = C_{Lo}$ to C_L results in equation (10.5):

$$\ln\left(\frac{(C^* - C_L)}{(C^* - C_{Lo})}\right) = -k_L a \tag{10.5}$$

As shown in Figure 10.4, a plot of $\text{Ln}\left(\frac{(C^* - C_L)}{(C^* - C_{Lo})}\right)$ versus time will give an estimate of k_La.

Another approach is the *sulfite method*. In the presence of CU^{2+}, the sulfur in sulfite (SO_3^{2-}) is readily oxidized to sulfate (SO_4^{2-}) by a zero-order reaction. This reaction is very rapid, and consequently, C_L approaches zero.

The rate of sulfate formation is monitored and is proportional to the rate of oxygen consumption ($\frac{1}{2}$ mol of O_2 is consumed to produce 1 mol of SO_4^{2-}). Thus,

$$\tfrac{1}{2} dC_{so_4}/dt = k_L a C^* \tag{10.6}$$

where C_{SO4} is the concentration of SO_4^{2-}. The factor, $\frac{1}{2}$, requires that C_{SO4} and C^* be expressed in terms of moles. Oxygen solubility, C^*, is a constant dependent on medium composition, temperature, and pressure and can be measured separately:

$$k_L a = \frac{\text{OUR}}{C^* - C_L} \tag{10.7}$$

OUR is the oxygen uptake rate. The sulfite method probably overestimates k_La. Note that if the chemical reaction takes place in the liquid film around the gas bubble, the apparent oxygen transfer will be greater than that for a system with no chemical

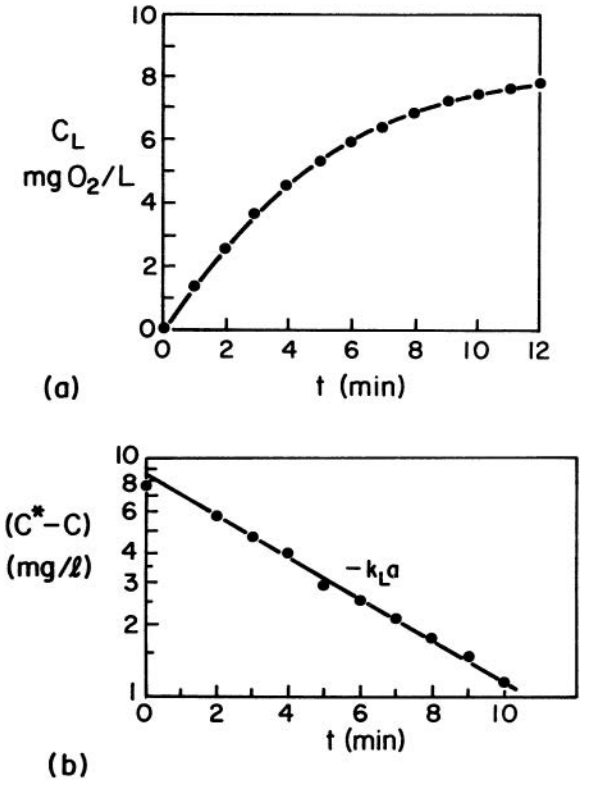


Figure 10.4. (a) Typical data for unsteady-state accumulation of oxygen in a large stirred tank. (b) Assuming C^* = 8.65 mg O_2/l, a value of k_La = 11.5 h^{-1} is determined. (D. W. Sundstrom, H. E. Klei, *Wastewater Treatment* © 1979.)

reaction in the liquid film. (An unresolved question is whether respiring organisms can enhance oxygen transfer by a similar method.)

Perhaps the best way to determine k_La is the *steady-state* method, in which the whole reactor is used as a respirometer. Such an approach requires the accurate measurement of oxygen concentration in all gas exit streams and a reliable measurement of C_L. A mass balance on O_2 in the gas allows the rate of O_2 uptake, OUR, to be calculated:

$$k_La = \frac{\text{OUR}}{C^* - C_L} \tag{10.8}$$

OUR could also be estimated with off-line measurements of a sample of the culture in a respirometer, but using information from the actual fermenter is ideal. However, one complication in this (and other methods) is determining what value of C^* to use. In a large fermenter, gas is sparged under significant pressure (due at least in part to the liquid height in an industrial fermenter). C^* is proportional to pO_2, which depends on total pressure as well as the fraction of the gas that is oxygen. At the sparger point, pO_2 will be significantly higher than at the exit, due to both higher pressure and the decrease in the fraction of the gas that is O_2 because of consumption by respiration. In a bubble column, the log mean value of C^* based on pO_2 at the entrance and exit would be a justifiable choice. In a perfectly mixed vessel, the

composition of gas in the exit stream should be the same as bubbles dispersed anywhere in the tank, and consequently C^* would be based on pO_2 at the exit. In an actual tank, a knowledge of the residence time distribution of gas bubbles is necessary to estimate a volume averaged value of C^*.

The *dynamic* method shares similarities with the steady-state method in that it uses a fermenter with active cells. It is simpler in that it requires only a DO probe and a chart recorder rather than off-gas analyzers as well as the DO probe required in the steady-state method. Following is the governing equation for DO levels:

$$dC_L/dt = \text{OTR} - \text{OUR} \tag{10.9a}$$

or

$$dC_L/dt = k_L a(C^* - C_L) - q_{O_2} X \tag{10.9b}$$

The dynamic method requires that the air supply be shut off for a short period (eq <5 min) and then turned back on. The anticipated result is shown in Figure 10.5. Since there are no gas bubbles when the gas is off, $k_L a$ will be zero. Hence,

$$dC_L/dt = -q_{O_2} X \tag{10.10}$$

The slope of the descending curve will give the OUR, or $-q_{O_2}X$. The lowest value of C_L obtained in the experiment must be above the critical oxygen concentration (recall Chapter 6) so that q_{O_2} is independent of C_L. Further complications can arise due to the measurement lag in the DO probe (about 30 to 45 s) and the dissolution of oxygen from the headspace gas into the liquid (which may be significant primar-

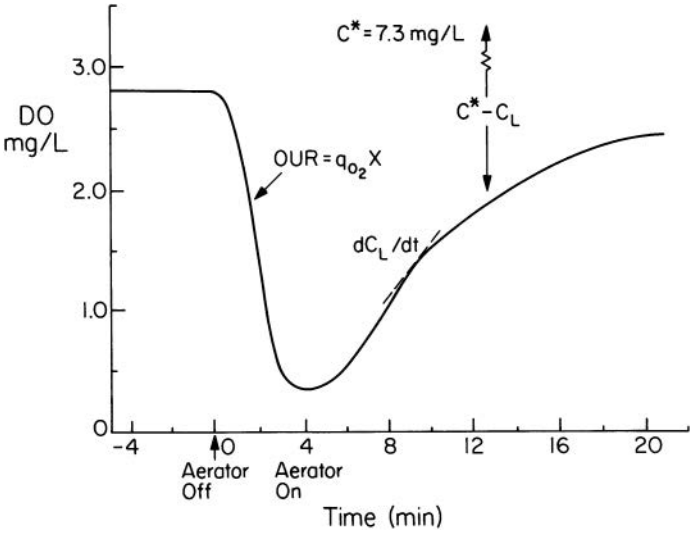


Figure 10.5. Example of response of dissolved oxygen in a fermenter when stopping and then restarting air flow. The dynamic method can be used to estimate oxygen uptake rate and $k_L a$.

ily in small vessels, since the ratio of surface area for gas transfer is high compared to liquid volume). When air sparging is resumed, the ascending curve can be used to calculate k_La. Values of dC_L/dt can be estimated from the slope of the ascending curve calculated at various time points. A plot of (dC_L/dt + OUR) versus ($C^* - C_L$) results in a line with a slope of k_La. The primary advantage of the dynamic method is that k_La can be estimated under actual fermentation conditions. Additionally, if q_{O_2} is known, the value of OUR can be used to estimate X.

Variations on these methods to determine k_La exist, but the general principles remain the same. However, once the oxygen transfer rate (OTR) can be estimated from either correlations or experimentally determined values k_La, it is possible to quickly estimate the rate of metabolic heat generation. Equation 6.26 provides such a relationship. Equations 6.24 to 6.26 apply in all cases and lead to an estimate of the rate of heat generation. The total amount of cooling surface (either jacket or coils) required can then be calculated, given the temperature of the cooling water, the maximum flow rates allowable, the desired temperature differential between the exiting coolant and the reactor, and the overall heat transfer coefficient. The latter presents the greatest problem, since it can vary greatly from fermentation to fermentation. Highly viscous fermentations, which decrease fluid mixing, obviously decrease the rate of heat transfer. Many fermentations will cause solids deposition on coils, greatly decreasing the heat transfer coefficient. In extreme cases, solids deposition can plug spaces between coils, greatly decreasing convective flow by coils and consequently the heat transfer coefficient. Cooling coils are usually placed so that high fluid velocities will strike the coils to promote cleaning. If a reactor is to be maintained as a highly flexible piece of equipment adaptable to a wide range of fermentations, very conservative estimates of the overall heat transfer coefficient must be made.

Example 10.1

Use the data in Figure 10.5 to estimate k_La from the dynamic method. Also, if the cell dry weight has been measured as 2 g/l, evaluate the specific respiration rate of the culture.

Solution

When the aerator is off, we can use the declining part of the curve in Figure 10.5 to estimate OUR. For the straight-line part of the curve (0.5 to 3 min after the aerator is off), we estimate OUR as follows:

$$-dC_L/dt = \text{OUR} = -\frac{2.5 - 0.5 \text{ mg/l}}{0.5 - 3 \text{ min}}$$
$$= 0.8 \text{ mg DO/l-min}$$

To estimate k_La, we use the ascending curve formed during reaeration:

$$k_La = \frac{dC_L/dt + \text{OUR}}{C^* - C_L}$$

The best method to estimate k_La is to plot dC_L/dt + OUR versus $(C^* - C_L)^{-1}$, but you can just make use of single points at individual times. In this problem, this is k_La = 0.16 min^{-1}.

The specific respiration rate is estimated as follows:

$$\text{OUR} = q_{o_2} X$$

or

$$q_{o_2} = \text{OUR/X}$$

$$q_{o_2} = \frac{0.8 \text{ mg DO/l-min}}{2\text{g cells/l}}$$

$$q_{o_2} = 0.4 \text{ mg DO/g cells-min}$$

10.1.6. Approaches to Scale-Up

The discussion in the previous sections has revealed the complex nature of industrial bioreactors. Now we consider how these complexities affect approaches to scaling bioreactors.

Generally, bioreactors for fungal or microbial culture maintain a height-to-diameter ratio of 2 or 3. However, for animal cell bioreactors, this ratio may be 1. If the height-to-diameter ratio remains constant, then the surface-to-volume ratio decreases dramatically during scale-up. This change decreases the relative contribution of surface aeration to oxygen supply and dissolved-CO_2 removal in comparison to the contribution from sparging. For traditional bacterial fermentations, surface aeration is unimportant, but for shear-sensitive cultures (e.g., animal cells), it can be critical because of restrictions on stirring and sparging.

More important in bacterial and fungal fermentations is *wall growth*. If cells adhere to surfaces, and if such adherent cells have altered metabolism (e.g., due to mass transfer limitations), then data obtained in a small fermenter may be unreliable in predicting culture response in a larger fermenter. This point is more clearly illustrated later in Example 10.2.

Perhaps even more importantly, it can be shown that the physical conditions in a large fermenter can never exactly duplicate those in a smaller fermenter if *geometric similarity* is maintained. In the case described in Table 10.2, a stirred-tank diameter has been increased by a factor of 5, resulting in a 125-fold increase in volume, since the height-to-diameter ratio was maintained constant. Four cases are treated in Table 10.2: scale-up based on constant power input (P_0/V), constant liquid circulation rate inside the vessel (pumping rate of impeller per unit volume, Q/V), constant shear at impeller tip (ND_i), and constant Reynolds number $(ND_i^2\rho/\mu)$. Note that $P \propto N^3 D_j^5, V \propto D_i^3, Q \propto ND_i^3, P/V \propto N^3 D_i^2$, and $Q/V \propto N$. Thus, fixing N and D_i fixes all the quantities in Table 10.2. Since these quantities have different dependencies on N and D_i, a change of scale *must result* in changes in the physical environment that the cells experience. When these changes alter the distribution of chemical species in the reactor, or they destroy or injure cells, the metabolic response of the culture will

TABLE 10.2. Interdependence of Scale-Up Parameters

Scale-Up Criterion	Designation	Small Fermenter, 80 l	Production Fermenter, 10,000:l			
			Constant, P_0/V	Constant, N	Constant, $N \cdot D_i$	Constant, Reynolds Number
Energy input	P_0	1.0	125	3125	25	0.2
Energy input/volume	P_0/V	1.0	1.0	25.0	0.2	0.0016
Impeller rotation number	N	1.0	0.34	1.0	0.2	0.04
Impeller diameter	D_i	1.0	5.0	5.0	5.0	5.0
Pump rate of impeller	Q	1.0	42.5	125.0	25.0	5.0
Pump rate of impeller/volume	Q/V	1.0	0.34	1.0	0.2	0.04
Maximum impeller speed (max. shearing rate)	$N \cdot D_i$	1.0	1.7	5.0	1.0	0.2
Reynolds number	$ND_i^2\rho/\mu$	1.0	8.5	25.0	5.0	1.0

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differ from one scale to another. In some cases, cells respond to modest changes in mechanical stress by changing physiological functions even when there is no visible cell injury or cell lysis. Thus, different scale-up rules (constant P/V implies constant OTR, constant Reynolds number implies geometrically similar flow patterns, constant N to give constant mixing times, and constant tip speed to give constant shear) can give very different results.

These scale-up problems are all related to transport processes. In particular, the relative time scales for mixing and reaction are important in determining the degree of heterogeneity in a fermenter. As we scale the system up, we may move from a system where the microkinetics (the cellular reactions) control the system response at small scale to one where transport limitations control the system response at large scale. When a change in the controlling regime takes place, the results of small-scale experiments become unreliable with respect to predicting large-scale performance.

One approach to predicting possible reactor limitations is the use of characteristic time constants for conversion and transport processes. Table 10.3 defines some of the important time constants, and Table 10.4 shows the application of these time constants to a 20 m^3 fermenter for the production of gluconic acid.

Processes with time constants that are small compared to the main processes appear to be essentially at equilibrium. If, for example, the oxygen transfer rate is much greater than the rate for oxygen consumption, then the broth would be saturated with oxygen. This situation occurs when $1/k_La \ll t_{O_2}$ where t_{O_2} is the time constant for O_2 consumption. If, on the other hand, consumption is of the same order of magnitude as oxygen supply (i.e., $1/k_La \approx t_{O_2}$ conversion), the dissolved oxygen concentration may be very low. This is precisely the case in Table 10.4 and Figure 10.6. The resulting experimental measurements of dissolved oxygen show the great variability in oxygen

TABLE 10.3. Important Time Constants (Equations)

Transport Process	Equation
Flow	L/v or V/Q
Diffusion	L^2/D
Oxygen transfer	$1/k_L a$
Heat transfer	$V\rho C_p/UA$
Mixing	$t_m = 4V/(1.5ND^3)$, stirred vessel
Conversion processes:	
Growth	$1/\mu$
Chemical reaction	C/r
Substrate consumption	$C_s / r_{\max}(C_s >> K_s)$
	$K_s / r_{\max}(C_s << K_s)$
Heat production	$\rho C_p \Delta_k T / r\Delta H$

With permission, from N. W. F. Kossen, in T. K. Ghose, ed., *Biotechnology and Bioprocess Engineering*, United India Press Link House, New Delhi, 1985, pp. 365–380.

TABLE 10.4. Time Constants to a 20 m^3 Fermenter

Transport Phenomenon	Time Constant(s)
Oxygen transfer	5.5 (noncoal.)–11.2 (coal.)[a]
Circulation of the liquid	12.3
Gas residence	20.6
Transfer of oxygen from a gas bubble	290 (noncoal.)–593 (coal.)
Heat transfer	330–650
Conversion processes:	
Oxygen consumption, zero order	16
First order	0.7
Substrate consumption	$5.5 * 10^4$
Growth	$1.2 * 10^4$
Heat production	350

[a]Coal. = coalescing air bubbles; noncoal. = noncoalescing air bubbles.
With permission, from N. W. F. Kossen, in T. K. Ghose, ed., *Biotechnology and Bioprocess Engineering*, United India Press Link House, New Delhi, 1985, pp. 365–380.

concentration in the reactor with some values at zero. This heterogeneity means that cells pass periodically through anaerobic regions. Since many cells have regulatory circuits to respond to changes from aerobic to anaerobic conditions, these circuits may be constantly altering cellular metabolism.

Traditional scale-up is highly empirical and makes sense only if there is no change in the controlling regime during scale-up, particularly if the system is only reaction or only transport controlled. Common scale-up rules are the maintenance of constant power-to-volume ratios, constant $k_L a$, constant tip speed, a combination

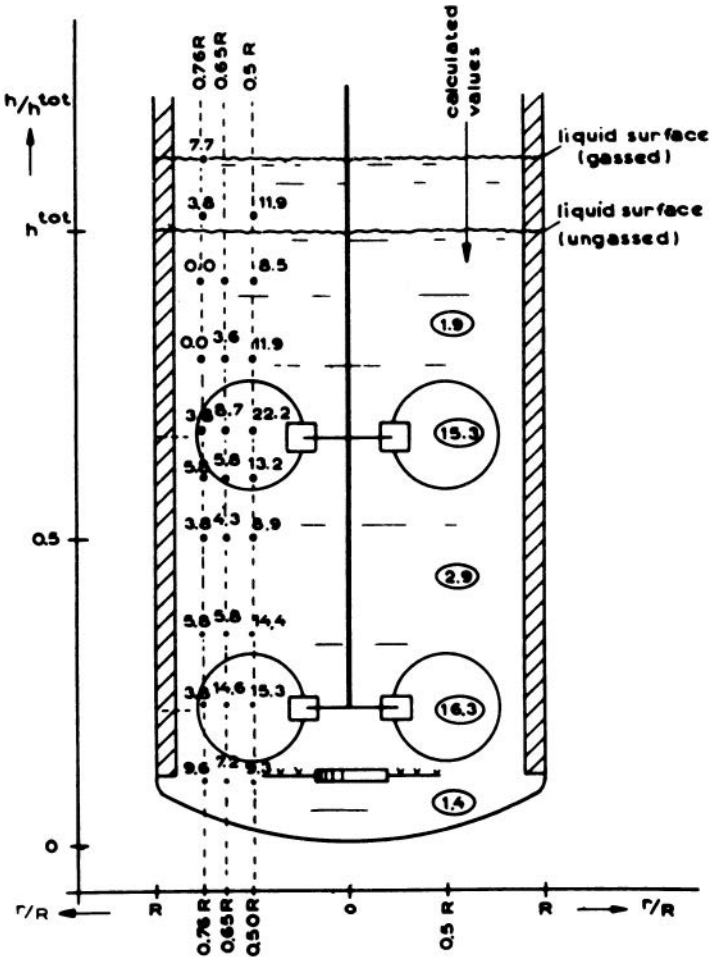


Figure 10.6. Measured oxygen concentrations in a 20 m^3 production fermenter (figures in circles are model estimates). (With permission, from N. W. F. Kossen, in T. K. Ghose, ed., *Biotechnology and Bioprocess Engineering*, United India Press Link House, New Delhi, 1985, pp. 365–380.)

of mixing time and Reynolds number, and the maintenance of a constant substrate or product level (usually DO concentration). Each of these rules has resulted in both successful and unsuccessful examples (described in several of the references at the end of the chapter). Results are more favorable with Newtonian broths than with non-Newtonian systems.

The failure of any of these rules is related to changes in the controlling regime upon scale-up. No empirical rule can satisfactorily address such situations. Advances are being made in models that predict flow distribution, mixing times, and gas dispersion in fermenters, as well as models that predict explicitly cellular responses to changes in the local environment. If these models can be integrated, they may provide a much more

fundamentally sound basis for scale-up. With the advent of improved supercomputers, the computational demands of such sophisticated models can be met. An approach to estimating mixing times in a stirred fermenter is given later in Example 10.4.

It has been shown empirically for fermenter volumes of 0.1 to 100 m^3 that mixing time can be correlated to reactor volume according to an expression such as[†]:

$$t_m = T_k \cdot V^{0.3} \tag{10.11}$$

where t_m is mixing time and V is vessel volume. The constant, T_k, is a function of impeller type, placement, and vessel design. Equation 10.11 assumes multiple Rushton-type impellers and is based on data from vessels of different sizes at practical operating conditions.

In summary, scale-up as currently practiced is an empirical, imprecise art. As long as geometric similarity is demanded at various scales, microenvironmental conditions cannot be made scale independent. Models and the use of scale-down procedures are potential approaches to improving scale-up, but these approaches are not yet fully developed.

The nature of the practical operating boundaries for an aerated, agitated fermenter can be summarized as in Figure 10.7. The exact placement of such boundaries depends on the fermentation and change as the system is scaled up or scaled down. The boundaries are fuzzy rather than sharp. Nonetheless, the bioprocess engineer must appreciate the existence of such constraints.

There are practical limits to the maximum size of a bioreactor based on both materials issues and transport limitations. Scale-up then requires the use of multiple reactors. In the pharmaceutical industry, these are often operated in parallel because batch operation is generally preferred due, in part, to regulatory issues. As described in Chapter 9, multistage or cascades of reactors are possible. Although cascades alter reaction kinetics (e.g., making the system operate more like a plug flow system than a well-mixed system), they also allow larger reaction systems to be constructed. Such multiscale systems are found in wastewater treatment and for production of chemicals such as ethanol for use as a fuel.

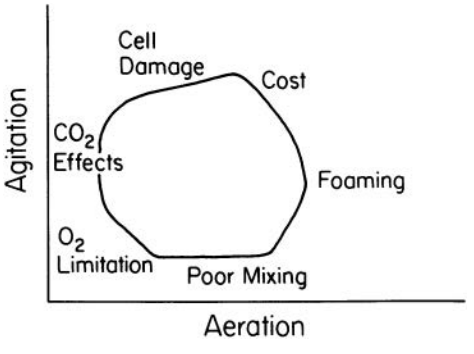


Figure 10.7. Practical operating boundaries for aerated, agitated fermenters. (After Lilly, M. D., 1983, in *Bioactive Microbial Products 2*; D. J. Winstanley and L. J. Nisbet, eds., Academic Press, London, pp. 79–89.)

[†] B. C. Buckland and M. D. Lilly, in *Biotechnology* 2d ed., H.-J. Rehm and G. Reed, Vol 3, G. Stephanopoulos, VCH, New York, 1983, pp. 12–13.

Example 10.2

After a batch fermentation, the system is dismantled and approximately 75% of the cell mass is suspended in the liquid phase (2 l), while 25% is attached to the reactor walls and internals in a thick film (ca. 0.3 cm). Work with radioactive tracers shows that 50% of the target product (intracellular) is associated with each cell fraction. The productivity of this reactor is 2 g product/l at the 2 to l scale. What would be the productivity at 20,000 l scale if both reactors had a height-to-diameter ratio of 2 to 1?

Solution

Both tanks are geometrically similar, so we can calculate the diameter and the resulting surface area and volume in both tanks:

$$V = \frac{1}{4}\pi D^2 \cdot H = \frac{1}{4}\pi D^2 \cdot 2D = \frac{1}{2}\pi D^3$$

$$S = \pi D \cdot H = \pi D \cdot 2D = 2\pi D^2$$

For the 2 l system,

$$D = 10.8 \text{ cm}$$
$$S = 738 \text{ cm}^2$$
$$V = 2000 \text{ cm}^3$$

For the 20,000 l systems,

$$D = 233.5 \text{ cm}$$
$$S = 342{,}600 \text{ cm}^2$$
$$V = 2 \times 10^7 \text{ cm}^3$$

At the bench scale (2 l), the amount of product made by surface-attached cells is

$$2\text{g/l} \cdot 2\text{l} \cdot \frac{1}{2} = 2\text{g}, \quad \text{for } 738 \text{ cm}^2 \text{ of surface area}$$

The amount of product formed at 20,000 l due to surface-attached growth is

$$342{,}600 \text{ cm}^2 / 738 \text{cm}^2 \times 2\text{g} = 928\text{g}$$

The overall yield in the 2 l system is

$$2\text{g/l} \cdot 2\text{l} = 4\text{g}$$

The overall yield in the 20,000 l system is

$$928 \text{ g} + (2 \text{ g/l} \cdot \frac{1}{2} \cdot 20{,}000 \text{ l}) = 20{,}928 \text{ g}$$

If no wall growth had been present, the 20,000 l tank would have yielded 40,000 g. Thus, wall growth, if present, can seriously alter the productivity of a large-scale system upon scale-up.

10.1.7. Scale-Down and Microbioreactors

Although scale-up models and the use of characteristic time analysis are potentially attractive, a more immediate approach to the rational scaling of reactors is *scale-down*.

Recently there has been increased interest in mini- and microbioreactors to explore factors that influence scale-up of bioprocesses more cheaply and effectively than in macro-scale bioreactors. The basic concept is to provide at a smaller scale an experimental system that duplicates exactly the same heterogeneity in environment that exists at the larger scale. In many cases, scale-up will require using existing production facilities, so it is important to mimic those production facilities at a smaller scale. At the smaller scale, many parameters can be tested more quickly and inexpensively than at the production scale. Also, such a small-scale system can be used to evaluate proposed process changes for an existing operating process and to select a range of possible operating conditions for a new process.

Figure 10.8 is a sketch of a smaller-scale apparatus to approximate the types of variations in substrate and DO concentrations that might be expected from a mixing-time analysis (or from estimates from time constants) or from actual data on residence-time distributions from a large reactor. The difference between this scale-down apparatus and a traditional smaller-scale fermenter in a pilot plant is that the type of apparatus described in Figure 10.8 is constructed specifically to mimic a known piece of equipment. It should be noted that the apparatus in Figure 10.8 would not mimic temperature heterogeneity as it might exist in the larger system. Modifications to control temperature separately in each subvessel could be made.

Such a scale-down apparatus could be used to estimate the system's response (e.g., growth rate, product formation, and formation of contaminating by-products) to changes in medium composition (e.g., a new supplier of raw materials), introduction of modified production strains, use of different inoculum preparations, new antifoam agents, and for testing for O_2 and CO_2 tolerance. Corrective protocols can be suggested also for use in the large-scale system by simulating the response

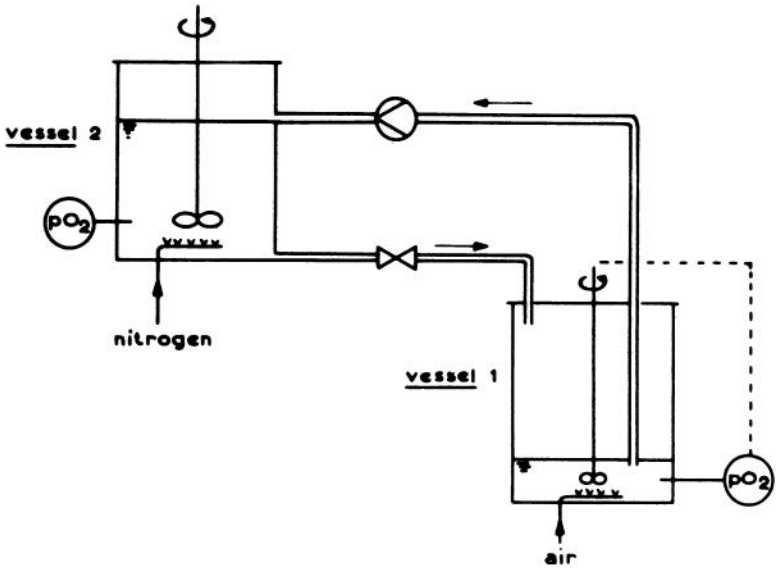


Figure 10.8. Experimental setup for scale-down experiments. (With permission, from N. W. F. Kossen, in T. K. Ghose, ed., *Biotechnology and Bioprocess Engineering*, United India Press, New Delhi, 1985, pp. 365–380.)

to pH or oxygen-probe failure or compressor failure during different phases of the fermentation.

Construction of scale-down apparatus can be a powerful complement to mathematical models, scale-up rules, and traditional pilot-plant operation.

Example 10.3

Consider the scale-up of a fermentation from a 10 l to 10,000 l vessel. The small fermenter has a height-to-diameter ratio of 3. The impeller diameter is 30% of the tank diameter. Agitator speed is 500 rpm, and three Rushton impellers are used. Determine the dimensions of the large fermenter and agitator speed for the following:

a. Constant P/V
b. Constant impeller tip speed
c. Constant Reynolds number

Assume geometric similarity and refer to Table 10.2.

Solution
Assume the vessel is a cylinder:

$$V = (\pi / 4) D_t^2 H.$$

But $H = 3\, D_t$, so

$$V = (\pi / 4)\ 3D_t^3 = 10{,}000\ \text{cm}^3$$

Solving for D_t, H, and $D_i = 0.3\, D_t$ gives

$$D_t = 16.2\ \text{cm}$$
$$H = 48.6\ \text{cm}$$
$$D_i = 4.9\ \text{cm}$$

The scale-up factor is the cube root of the ratio of tank volumes:

$$(10{,}000\ \text{l}/10\ \text{l})^{1|3} = 10$$

To maintain geometric similarity, the larger vessel will have its dimensions increased by a factor of 10:

$$D_t = 1.62\ \text{m}$$
$$H = 4.86\ \text{m}$$
$$D_i = 0.49\ \text{m}$$

a. For constant P/V to apply, $N^3 D_t^2$ must be the same in both vessels; let subscript 1 refer to the small vessel and subscript 2 to the large vessel:

$$N_2 = N_1 \left(\frac{D_{t1}}{D_{t2}} \right)^{2/3} = 500\ \text{rpm}\ (1/10)^{2/3} = 107\ \text{rpm}$$

b. For constant tip speed to apply, ND_t must be the same in both vessels:

$$N_2 = N_1\left(\frac{D_{t1}}{D_{t2}}\right) = 500 \text{ rpm } (1/10) = 50 \text{ rpm}$$

c. For constant Reynolds number to apply, ND_t^2 must be the same in both vessels:

$$N_2 = N_1\left(\frac{D_{t1}}{D_{t2}}\right)^2 = 500 \text{ rpm } (1/10)^2 = 5 \text{ rpm}$$

Scale-up on the basis of constant P/V would be the most likely choice unless the culture was unusually shear sensitive. Scale-up based on constant Reynolds number would not be used.

Example 10.4.[†]

As fermenters are scaled up, the mixing time usually increases. Mixing time, t_M^{95}, can be defined as the time it takes for the concentration of a compound to return to 95% of the equilibrium value after a local perturbation in its concentration. Mixing times are experimentally determinable by step addition of an electrolyte. The conductivity can be measured continuously at various locations distant from the injection site.

A production fermenter usually contains multiple impellers. An effective modeling approach is to divide the contents of the large tank into mixing compartments, where each compartment is perfectly mixed. As indicated in Figure 10.9, a simple

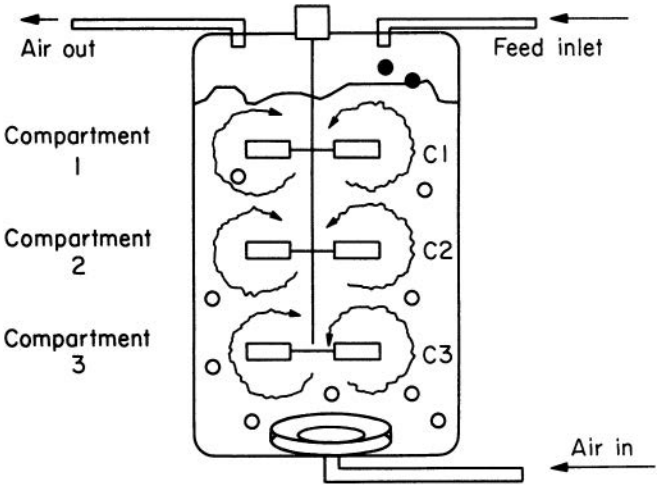


Figure 10.9. Schematic of a simple mixing model for Example 10.4. C_i = concentration of a component in the ith mixing compartment. (With permission, from J. L. Jost, in S. L. Sandler and B. A. Finlayson, eds., *Chemical Engineering Education in a Changing Environment*, Engineering Foundation, New York, 1980, pp. 21–36.)

[†] Adapted from J. Jost, Chapter 3 in S. L. Sandler and B. A. Finlayson, eds., *Chemical Engineering Education in a Changing Environment,* Engineering Foundation, New York, 1988.

model is to consider that a separate compartment is associated with each impeller. In this problem, we let H be the overall mass transfer coefficient between compartments. The transient mass balances and experimental data can be used to estimate a value of H.

Consider the case where H has been determined to be $0.43\ s^{-1}$ for a 10 l vessel and $0.075\ s^{-1}$ for a 10,000 l vessel. With *Escherichia coli* fermentations, glucose feed rates in a fed batch are adjusted to maintain a constant, relatively low concentration of glucose to prevent the formation of toxic metabolites (e.g., acetate) that would limit the ultimate cell concentration. Assume that the desired glucose concentration is 25 mg/l and that the Monod kinetics can be approximated as first order with a rate constant of about $0.05\ s^{-1}$. Assume the cell concentration changes slowly. Assume that the glucose supplemental feed is sufficiently concentrated that the total fluid volume in the reactor is constant. Also, assume that F, the mass addition rate of glucose per unit reactor volume, changes slowly in comparison to the characteristic mixing and reaction times. Compare the variation in glucose concentrations in the small and large tanks when an ideal probe (no error or lag in measurement) is used to maintain the set-point concentration at 25 mg/l in the middle compartment. Consider the response if the probe is placed in the top compartment instead of the middle compartment.

Solution

Note that F and k_1 are closely related. As the cells grow, k_1 increases, which changes the demand for glucose (F). Because F changes more slowly than the characteristic time constants for mixing, we assume a quasi-steady state.

Following Figure 10.9, we write the following equations:

$$\frac{dC_1}{dt} = 0 = H\left(C_2 - C_1\right) + F - k_1 C_1 \tag{a}$$

$$\frac{dC_2}{dt} = 0 = H\left(C_1 - C_2\right) - H\left(C_2 - C_3\right) - k_1 C_2 \tag{b}$$

$$\frac{dC_3}{dt} = 0 = H\left(C_2 - C_3\right) - k_1 C_3 \tag{c}$$

where C_i is the glucose concentration in compartment i, and k_1 is the first-order reaction constant.

Following is the solution to the preceding set of equations:

$$C_1 = \left(2 + \frac{k_1}{H} - \frac{H}{H + k_1}\right)\frac{F}{3k_1 + \left(k_1^2/H\right)} \tag{d}$$

$$C_2 = \frac{F}{3k_1 + \left(k_1^2/H\right)} \tag{e}$$

$$C_3 = \frac{H}{\left(H + k_1\right)}\frac{F}{\left(3k_1 + \left(k_1^2/H\right)\right)} \tag{f}$$

If the probe is placed in the middle compartment, then $C_2 = 25$ mg/l if the control system is perfect. Using equation e, we calculate $F = 3.9$ mg/l-s in the small tank and $F = 4.6$ mg/l-s in the large tank. Values for C_1 and C_3 are found from equations d, e, and f. The process is repeated for the second case where the probe is placed in the upper compartment.

The results are summarized as follows:

a. Probe in the middle compartment:

a1. Small tank (10 l): $F = 3.9$ mg/l-s

$$C_1 = 30.5 \text{ mg/l}; C_2 = 25 \text{ mg/1}; C_3 = 22.4 \text{ mg/1}$$

a2. Large tank (10,000 l): $F = 4.6$ mg/l-s

$$C_1 = 51.7 \text{ mg/1}; C_2 = 25 \text{ mg/1}; C_3 = 15 \text{ mg/1}$$

b. Probe in the top compartment:

b1. Small tank (10 l): $F = 3.2$ mg/l-s

$$C_1 = 25 \text{ mg/1}; C_2 = 20.5 \text{ mg/1}; C_3 = 18.4 \text{ mg/1}$$

b2. Large tank (10,000 l): $F = 2.2$ mg/l-s

$$C_1 = 25 \text{ mg/1}; C_2 = 12.1 \text{ mg/1}; C_3 = 7.3 \text{ mg/1}$$

These results illustrate the importance of the alteration of mixing time on nutrient consumption rates (F) and on the concentration gradients in a large vessel. It should also be clear that probe placement in a less than perfectly mixed vessel will have significant effects on reactions within that vessel.

Over the last decade, increasing emphasis has been placed on the use of mini-scale (5 to 50 ml) or micro-scale (1 to 5 ml) bioreactors. While used largely for animal cells, the technology is applicable to all types of cells. A large number of parameters need to be explored to determine optimal conditions for cell growth and product formation. This is particularly true for therapeutic proteins where not only titer but also the "quality" of the protein product (e.g., extent of glycosylation) is critical. Miniaturized, scaled-down bioreactors are well suited for relatively high-throughput parallel operation, saving time, reducing costs of reagents and cells, and facilitating exploration of a larger experimental space than possible with traditional bioreactors. Coupling the output of such small-scale bioreactors to advanced analytical methods is a key aspect to successful use of such systems. A challenge, however, is to ensure that predictions from these mini bioreactors reflects the performance obtained in larger-scale systems.

Although microtiter plates and flasks can be used to screen culture conditions, for scale-up to large-scale reactors, it is best to use mini-scale stirred bioreactors or nonstirred suspension reactors that are membrane based. These bioreactors provide a more controlled environment than standard tissue culture flasks and allow estimation of transport parameters necessary for scale-up. The key parameters for scale-up to commercial-size vessels are $k_L a$ and mixing time. For animal cell culture, the maximum shear rate is also critical. Stirred-tank small-scale systems typically require more volume (10 to 35ml) than the membrane-based systems (0.5 to 3ml). Figure 10.10 is a schematic of a nonmechanically stirred membrane-based system.

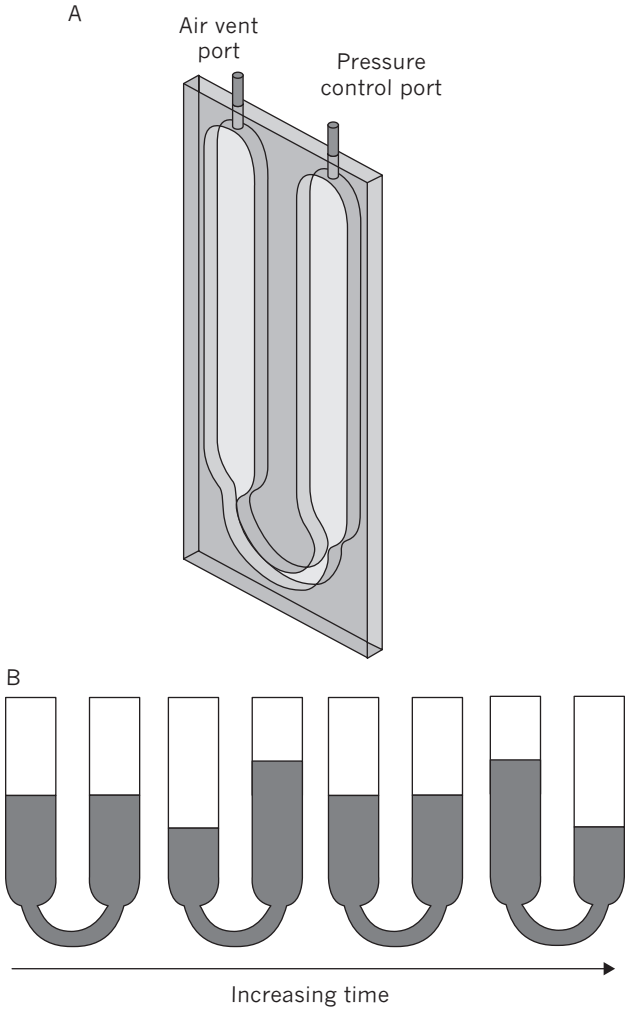


Figure 10.10. An example of a mini bioreactor with a selective membrane to facilitate O_2 and CO_2 transfer. Oscillatory air pressure causes fluid movement. (a) A schematic of the mini bioreactor holding 2.5 ml of fluid. Two 50 μm thick polymethylpenture membrane allow gas exchange while minimizing loss of water. (b) A typical fluid-mixing cycle and movement of liquid inside the channels. Fluid mixing is enhanced by fluid movement from the smaller bottom U-channel (5 mm wide) into the larger section of the system (12 mm wide). The k_La values can be readily calculated and dissolved oxygen levels directly measured. (From Kim, B. J., et al., *Biotechnol. Bioeng*. 109: 137, 2012, with permission from John Wiley & Sons, Inc.)

The system depicted depends on an oscillatory pressure wave as a motive force for fluid to move up and down in a U-tube. Published studies have shown, for several mini bioreactor systems, that it is feasible to scale up to pilot-size systems (e.g., 200 l) with good reproducibility.

Although with modern techniques, a 1 ml reactor operated in fed-batch mode can supply sufficient volume for a range of analyses, stirred vessels of order of 10 ml provide more material for analytical procedures, particularly for automated cell analysis, without disturbing reactor performance. Instrumentation for monitoring the cell state as well as traditional parameters such or pH, DO, and cell mass is now available. Several vendors offer micro-scale systems with disposable microbioreactors and independent pH and DO control.

10.2. BIOREACTOR INSTRUMENTATION AND CONTROL

The maintenance of optimal conditions for product formation in the complex environment in a bioreactor requires the control and measurement of at least a few parameters. Almost all fermenters have pH, temperature, and DO control (by control of agitator rotational speed). New probes and techniques for the measurement of various ions, substrates, and products are being developed and have been implemented in particular situations. Actual process-control strategies for bioreactors are very primitive in comparison to the petrochemical industry because of a lack of sensors for on-line measurements and of reliable, quantitative, dynamically accurate models. In this section, we summarize briefly not only the state of the art but opportunities to improve the control of bioreactors.

10.2.1. Instrumentation for Measurements of Active Fermentation

The maintenance of sterility in a fermenter imposes a severe limitation on obtaining on-line measurements of fermentation parameters. Some probes (e.g., pH and dissolved oxygen) enter the reactor through penetrations in the fermenter shell. Each penetration significantly increases the probability of contamination. Thus, the benefit in increased productivity from the use of each probe must outweigh the economic losses that result from an increased number of contamination events. The probes themselves must also be sterilizable, preferably with steam. Thus, probes must ideally be able to withstand moderately high temperatures (121°C) in the presence of 100% humidity. Chemical sterilization, which is less desirable, may allow the use of a temperature-sensitive device if it has sufficient chemical resistance. Any general technique for monitoring fermentations must be compatible with the limitations imposed by sterility requirements.

Techniques to monitor the physical environment are summarized in Table 10.5. Except for viscosity and turbidity, these parameters would be monitored on most pilot-plant fermenters and many industrial fermenters. Each parameter is generally subject to its own closed-loop control system. These individual control loops may be integrated into an overall control package, especially at the pilot-plant scale, but in many cases, higher-level control is not practiced, owing to the difficulty of measuring key parameters (e.g., product concentration) and the lack of dynamically accurate process models.

On-line measurements of concentrations beyond pH and DO are difficult, although exciting progress is being made. Table 10.6 summarizes techniques that have been considered for determining the concentrations of key components.

With insertable probes, we need to worry not only about probe performance but also about probe placement. The heterogeneity in a large fermenter means that DO and substrate levels (and to a lesser extent pH) will be position dependent (see Example 10.4). Although placement in the midsection of the vessel often gives the most representative values, mechanical design considerations may dictate placement elsewhere. Because probe fouling is a potential problem, the probe should be inserted in a region with sufficient turbulence to help keep it clean. Although the use of multiple probes would be desirable, the increased risk of contamination

TABLE 10.5. Monitoring and Control of the Physical Environment

Parameter	Measuring Device(s)	Comments
Temperature	Resistance thermometer or thermistor	Thermistors are used when small size and rapid response are required
Pressure	Diaphragm gages	Pressure is regulated by a simple back-pressure regulator in gas exit line
Agitator shaft power	Wattmeter or strain gages	Strain gages are used in bench or pilot-scale equipment
Foam	Rubber-sheathed electrode	Mechanical foam breakers are self-regulating; sensor is used to activate solenoid valve to release antifoam agent
Flow rate (gas)	Rotameters or thermal mass flow meter	Position of rotameter float is converted to an electrical signal; controller manipulates flow valve
Flow rate (liquid)	Magnetic-inductive flow meters or change in weight of additional vessels determined by load cell	Magnetic-inductive meters are good for viscous fluids or fluids with high level of particulates; controller can manipulate flow valves
Liquid level	Load cells to measure amount of liquid in vessel; liquid height, conductivity sensors; capacitance probes; ultrasound	Liquid height is a function of gas sparge rate and gas hold-up; foam can complicate measurement
Viscosity	Rotational viscometers	On-line measurement is difficult; broths with high solids content present special problems
Turbidity (to indicate cell mass)	Photometer (either as a probe into the reactor or in a slip stream)	Many problems exist, including fouling and interference from gas bubbles and suspended solids

TABLE 10.6. Approaches to Monitoring and Control of the Chemical Environment

Approach	Measuring Devices and Compounds Monitored	Comments
Insertable probes	pH electrode; H^+	Special design used to facilitate repeated autoclaving; protein fouling of diaphragm can be a problem; pH probes are used extensively
	Redox electrodes; redox potential	Measurement is fairly reliable; interpretation and use of information is often difficult
	Ion-sensitive electrodes; NH_3, NH_4, Br, Cd, Ca, Cl, Cu, CN, F, BF_4, I, Pb, NO_3, ClO_4, K, Ag, Na, S, SCN	Cross-sensitivity is a major problem; drift and calibration can be troublesome; response time can be slow; sterilizability is often poor
	O_2 probes, either galvanic or polarographic; pO_2	Measures partial pressure of O_2, not O_2 concentration; has a slow response; drift, fouling, and recalibration during extended fermentations can be a problem; O_2 probes are widely used and extremely important
	CO_2 probes; activity of dissolved CO_2	CO_2 is known to have important physiological effects, but relating these effects to dissolved CO_2 is difficult

(*continued*)

TABLE 10.6. (*continued*)

Approach	Measuring Devices and Compounds Monitored	Comments
	Fluorescence probes; NADH	Commercially available to monitor intracellular concentrations; fouling is a potential problem
	Biosensors; wide range of compounds potentially detectable (e.g., glucose, glutamic acid, ammonia, acetate, ethanol formate, cephalosporin, penicillin)	Sterilizability, enzyme cost, cofactor regeneration, and usable lifetime are all constraints on utilization of such probes; high level of development activity will lead to at least partial solution of these problems
Exit gas analyzers	Paramagnetic analyzer (O_2); thermal conductivity or long-path infrared analyzers (CO_2)	These instruments are specialized for measurements of these specific gases; important in fermentation balances; sample conditioning is important
	Flame ionization detector; low levels of organically bound carbon, especially useful for volatile organics such as ethanol or methanol	Flame ionization measures total hydrocarbon; automatic process gas chromatography equipment is available to separate into individual compounds
	Mass spectrometer; O_2, CO_2, volatile substances (and also liquid streams)	Highly specific, rapid, and accurate; expensive; has not been for complex molecules; depends on gas phase for volatile compounds
	Semiconductor gas sensors; flammable or reducing gases or organic vapors	Has been used in bioreactors primarily for ethanol measurements
Measurements from liquid slip stream	HPLC (high-pressure liquid chromatography); dissolved organics; particularly useful for proteins; auto analyzer	Highly specific; compound need not be volatile; response time is long (min); solids must be removed by filtration; membrane fouling can be a problem; guard columns must remove compounds that may foul columns; is expensive; main value is to differentiate among closely related proteins or variants of a particular protein
	Mass spectrometers; dissolved compounds that can be volatilized	Same instrument can be used also for exit gas analysis; is rapid, specific, but expensive; complex molecules present problems
	Enzymatic methods; potentially wide range, but mostly glucose	Has a slow response (min); not highly flexible, but very discriminating; potential for contamination; has limited acceptance

often argues against it. Even with accurate probe response, the interpretation of that response in a large fermenter must be done carefully.

Since many fermentations require extensive periods for completion (2 to 20 days), it is important that probe response be stable for extended periods. Industrial fermentation broths contain many proteins and other organics that have a significant tendency to adsorb onto surfaces. Many microbes also have a strong tendency to adhere to surfaces. Thus, *probe fouling* in an extended fermentation is a constant problem. Drift is a problem in some probes, and recalibration in situ is not always possible. If sterility is to be maintained, the removal and replacement of probes is practically impossible. In some cases, special designs allowing some back-flushing are

possible. However, the quality of information available tends to decrease as the length of a fermentation cycle increases.

Exit-gas measurements, particularly with mass spectrometers, are of increasing interest. Advances in building robust process instruments at lower cost have made this a more attractive proposition, particularly when such an instrument can be used for several fermenters (e.g., using a computer-controlled switching manifold). The main limitation on exit-gas analysis is that only volatile components can be monitored.

On-line high-pressure liquid chromatography (HPLC) is very powerful for measuring the levels of dissolved solutes, particularly proteins made from genetically engineered cells. Many vendors are actively developing systems for this application. However, this method, as well as most others using a liquid slipstream, requires sample preparation and has a significant time delay associated with sampling. Typically, a small sidestream is pumped from the reactor, and microfiltration or an ultrafilter is used to remove cells and particulates. The filtrate usually requires further processing (e.g., the removal of compounds that could foul the analytical-grade columns). After injection of the sample, there can be a significant time (on order of minutes) before a response is obtained. One other problem with slipstreams is the potential for contamination; the membrane filtration step is of particular concern. Nonetheless, the use of liquid slipstreams allows the relatively rapid determination of important product information. The use of HPLC is most common on laboratory- or pilot-scale systems.

Other methods not listed in Table 10.6 have the potential to significantly affect the on-line measurement of fermentation parameters. Nuclear magnetic resonance (NMR) has given important information on intracellular metabolism for off-line or small-scale growth experiments. In situ NMR has the potential for use with at least bench-scale systems, although significant technical advances must first be made. Selective fluorescence in combination with flow cytometry has given important information, off line, on the distribution of intracellular parameters (e.g., plasmid content) in a population. The adaptation of such techniques to on-line measurement is conceivable, but again, a number of technical problems must first be solved. Another analytical method that offers a good potential for on-line determination of solutes is Fourier transform infrared (FTIR) spectra analysis. Raman spectroscopy is being used with animal cell culture, enabling noninvasive analysis of cellular metabolic changes. Finally, the use of spectra analysis combined with optical fibers may offer a good technique to determine cell mass in the presence of other suspended solids.

10.2.2. Using the Information Obtained

Having information is one thing; using it wisely is another. Current fermenter design and control techniques are rather limited. We do not have a fully satisfactory approach to the effective use of information on the extracellular and intracellular chemical environments.

Computer-controlled fermenters are fairly common, particularly at the pilot-plant scale. Figure 10.11 displays an overview of the software functions for a typical antibiotic fermentation facility, and Figure 10.12 shows the relationship of primary measurements to secondary parameters.

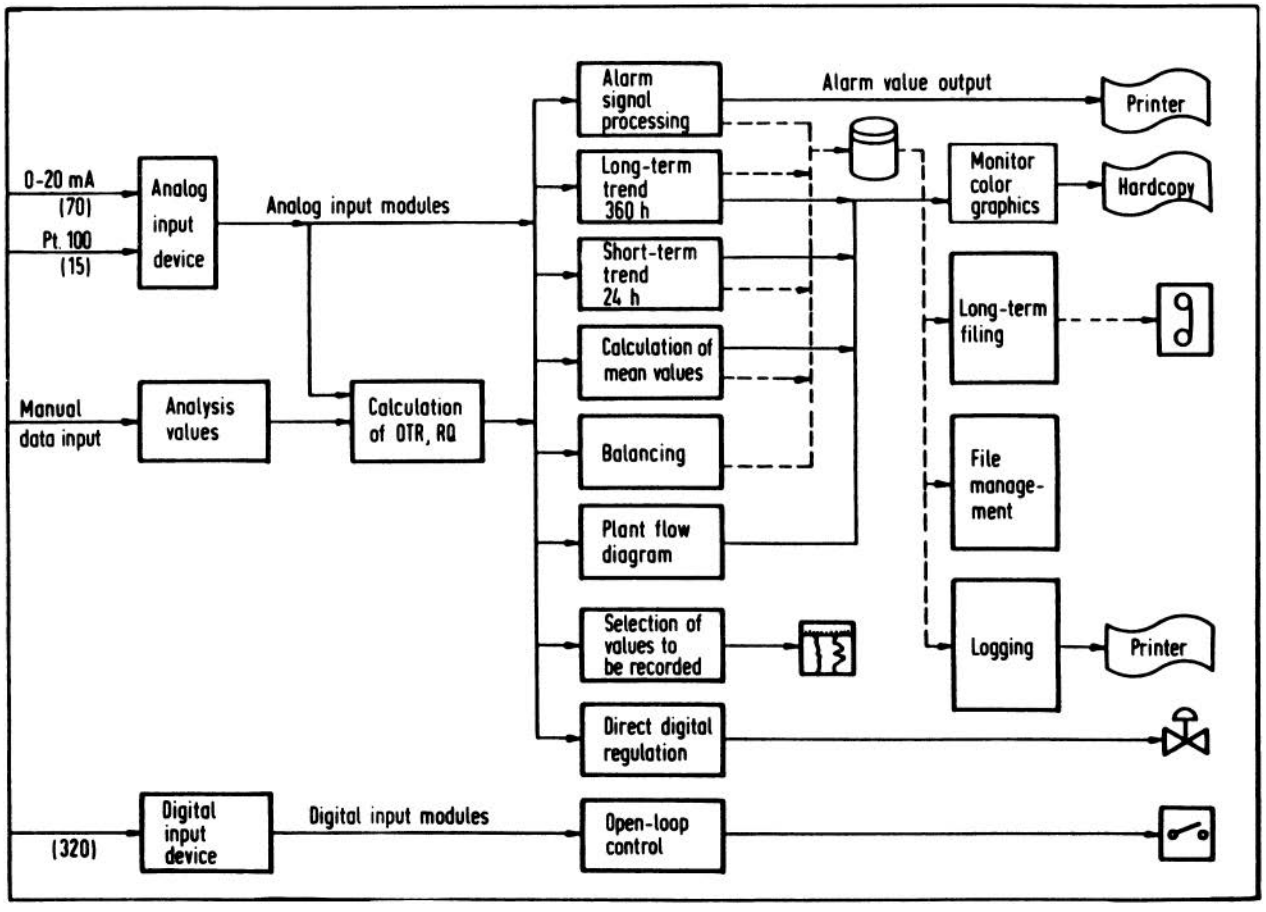


Figure 10.11. Overview of software for fermentation. (With permission, from H. Heine, J. Hahn, and A. Mangold, in *Biotechnology Focus* I, R. K. Finn and P. Prave, eds., Hanser Publishers, New York, 1988, p. 203.)

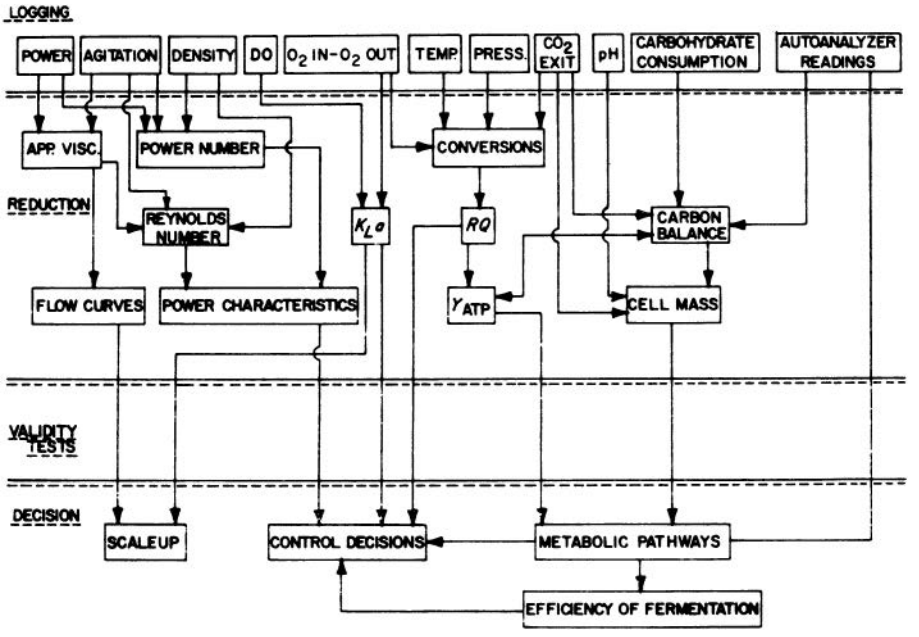


Figure 10.12. Primary measurements, shown on top, may be used to calculate many related process properties and parameters. (With permission, from L. P. Tannen and L. K. Nyiri, "Instrumentation of Fermentation Systems," p. 331, in *Microbial Technology*, 2d ed., Vol. II, H. J. Peppler and D. Perlman, eds., Academic Press, New York, 1979.)

An important function of such systems is *data logging*. This information is unusually important in the food and pharmaceutical industries. An actual record for the manufacture of each product batch is required by regulatory agencies. If the product is later found to be unsafe, such information can be used to trace the problem. Beyond the regulatory concerns, the lack of good high-level control strategies leads to a control strategy based on exactly duplicating a particular recipe. Consistency from batch to batch is an important consideration. Expert control systems may be useful in such situations.

In many cases, fermentation control schemes have begun to move beyond simple open-loop environmental control strategies. The use of computers allows the rapid manipulation of information from the data-logging operation to yield estimates of secondary parameters. For example, a secondary parameter may be cell concentration estimated from estimates of oxygen consumption or carbon dioxide evolution based on primary measurements of off-gas composition and dissolved-gas concentrations. Essentially, cell mass is estimated from mass balances using information from what have been termed *gateway sensors*. The main limitation to this approach is the accumulation of error. For example, in estimating cell mass X at time t, the value of X at time $t - \Delta t$ must be known. Any systematic errors in cell mass estimates or the inability of an algorithm to respond to an unusual perturbation can lead to significant difficulties over the length of a typical culture cycle. Data-handling procedures to mitigate this problem have been suggested (e.g., Kalman filters).

In some cases, our partial knowledge of cellular metabolism is sufficient to solve important bioreactor problems. For many years, it was thought impossible to grow *E. coli* to high cell densities (>15 or 20 g/l). An improved understanding of cell physiology led to the observation that it was the buildup of toxic metabolic by-products (primarily acetate) that inhibited growth. Acetate is formed in *E. coli* when a good carbon source, such as glucose, is available in high concentrations. Control strategies that feed glucose at a rate sufficient to maintain at least moderately good growth without forming acetate were seen as possibilities to improve reactor volumetric productivities.

Thus, several possible control strategies are immediately apparent. The simplest perhaps would be to measure acetate concentration on line and reduce nutrient feed rates in response to acetate accumulation. On-line measurement of acetate is not now routine, but improvements in sensors certainly make this a tenable approach. A strategy that is easier to implement with current sensors is based on measuring the rates of CO_2 evolution. Measurement of CO_2 in the off-gas, coupled with information on substrate concentration in the feed and nutrient flow rates, allows the glucose feed rate to be manipulated to maintain glucose concentrations at an optimal level. This indirect strategy depends on the use of mass balances and the gateway sensor concept. Another strategy is to control glucose concentration with a feedback control system if a glucose sensing system is available. The easiest approach is to use a predetermined glucose feeding schedule, although this usually results in significant periods of suboptimal or superoptimal glucose concentrations. These strategies have been used successfully. High-density *E. coli* cultures (ca. 100 g dry wt/l) have greatly increased product concentration and productivities in *E. coli*-based fermentations to make proteins from recombinant DNA. At these high densities, special strategies for oxygen supply (e.g., O_2-enriched gas) are often required. This example illustrates the important interaction between process-control strategies and cellular metabolism.

As we develop better sensors and a better understanding of metabolic pathways, it becomes more feasible to combine information from primary sensors with models of metabolism to develop better control strategies. Without direct measurement of the product, simple feedback control on the process itself is impossible. Without extremely good models for dynamic response, feedforward control strategies are ineffective. Because of the highly nonlinear nature of most culture systems, black-box techniques to develop dynamic models are usually ineffective. Good process control for fermentations awaits improved models of cultures, more sophisticated sensors, and advances in nonlinear control theory. Hierarchical bioprocess automation where control is based on interconnecting parameters is now feasible. Such an approach requires relating typical process parameters (e.g., pH, DO, nutrient concentrations, cell concentration) to quality attributes (e.g., protein folding, glycosylation patterns of proteins, impurities). Currently, semiempirical models to relate process parameters to quality attributes are possible but are not routine and are not easily utilizable.

In addition to reactor control, a computer-monitored and controlled process involves the control of medium preparation, sterilization, and some downstream recovery processes. An important factor in bioprocesses is the predominance of batch processing. The scheduling of equipment is critical, and these tasks are being done increasingly with the aid of in-house computers.

10.3. STERILIZATION OF PROCESS FLUIDS

Modern fermenters consume large amounts of media and gas. The tens of thousands of liters of medium and millions of liters of air used in a typical antibiotic fermentation must be absolutely devoid of any contaminating organism. The economic penalty for contamination is high. With bioprocesses to make proteins from recombinant DNA, the exit streams must also be treated to prevent the discharge of any living cell. The ability to ensure the destruction of viable organisms is critical.

10.3.1. The Kinetics of Death

Sterility is an absolute concept; a system is never partially or almost sterile. However, with a 100,000 l fermenter, we cannot sample every drop of fluid for a foreign organism. On a practical basis, sterility means the absence of any detectable viable organism, and a *pure culture* means that only the desired organism is detectably present.

Disinfection differs from sterilization. A disinfecting agent will greatly reduce the number of viable organisms, often a specific type of organism, to a low but nonzero value.

Fluid streams can be sterilized through the physical removal of cells and viruses or the inactivation of living particles by heat, radiation, or chemicals. If sterilization is accomplished by inactivating living cells, spores, or viruses, we need to understand the kinetics of death. *Death* in this case means the failure of the cell, spore, or virus to reproduce or germinate when placed in a favorable environment. When dealing with sterilization, the probabilistic nature of cell death cannot be ignored.

The simplest case presumes that all the viable cells or spores are identical. The *probability of extinction* of the total population, $P_0(t)$, follows:

$$P_0(t) = \left[1 - p(t)\right]^{N_0} \tag{10.12}$$

Here $p(t)$ is the probability that an individual will still be viable at time t, and N_0 is the number of individuals initially present. The expected number of individuals present at time t, $E\left[N(t)\right]$, is as follows:

$$E[N(t)] = N_0 p(t) \tag{10.13}$$

The variance about this number is

$$V[N(t)] = N_0 p(t)[1 - p(t)] \tag{10.14}$$

The specific death rate, k_d, is

$$k_d = \frac{1}{-E[N(t)]} \cdot \frac{d}{dt} E[N(t)] \tag{10.15a}$$

And if $p(t)$ is not a function of $N(t)$, then

$$k_d = -\frac{d}{dt} \ln\, p(t) \tag{10.15b}$$

The functional form for $p(t)$ depends on the organism and environment. The simplest form is to assume a first-order death model in which k_d is a constant and has units of reciprocal time:

$$p(t) = e^{-k_d t} \tag{10.16}$$

In the microbiological literature, the term *decimal reduction time* (D) is often used. It is the time for the number of viable cells to decrease tenfold. For example, with k_d as a constant, the value of D is

$$E[N(t)] = N_0 e^{-k_d t} \tag{10.17a}$$

$$0.1 = e^{-k_d D} \tag{10.17b}$$

$$D = \frac{2.303}{k_d} \tag{10.17c}$$

A plot of ln $N(t)/N_0$ versus time is called a *survival curve* (see Figure 10.13). Such a curve is implicitly deterministic; the deterministic and probabilistic approaches give essentially the same result as long as $N(t)$ is >>1 (about 10 to 100). Extrapolation of a survival curve to one organism or less is not proper.

Most real populations are not homogenous. Often, a subgroup is more resistant to the sterilization agent. Also, organisms growing in clumps tend to be more resistant to death. These factors are important in processing (see Figure 10.14 for the effects on survival curves). For example, consider a tragic error in the early development of a polio vaccine. The virus is inactivated ("killed"). The required processing time was estimated from linear extrapolation (on a semilog plot) of a survival curve. The original data for the plot did not extend to sufficiently low numbers to detect the presence of a resistant subpopulation. Consequently, some patients were inoculated with live rather than killed virus.

10.3.2. Sterilization of Liquids

The kinetics of death apply to the sterilization of liquids. Values for k_d can be determined for inactivation by chemical, thermal, or radioactive agents. Thermal inactivation is very much preferred for the economic, large-scale sterilization of equipment and liquids. However, heat-sensitive equipment must be sterilized with chemical agents or radiation.

The use of ultraviolet (UV) radiation is effective to sterilize surfaces, but UV cannot penetrate fluids easily, particularly those with high amounts of suspended material. Although x-rays can penetrate more deeply, cost and safety considerations preclude their use in large-scale systems.

A chemical agent for sterilization must leave no residue that would be toxic to the desired culture. Ethylene oxide, a gas, can be used to sterilize equipment. A 70% ethanol–water mixture acidified to a pH of 2 with HCl kills virtually all vegetative cells and many spores and can be used to sterilize equipment. A formaldehyde solution is often effective. Sodium hypochlorite solution (3%) has been used in sterilizing

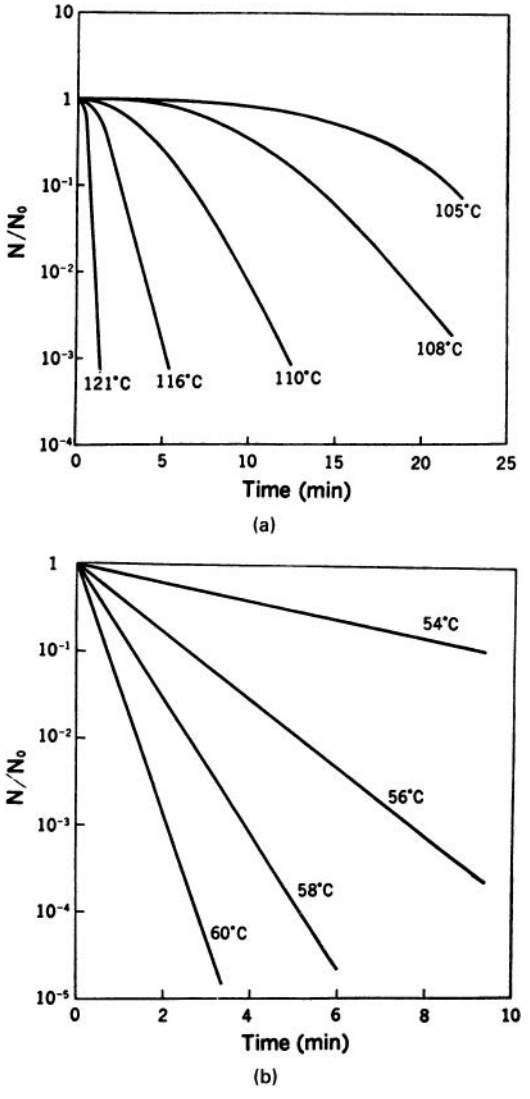


Figure 10.13. (a) Typical death-rate data for spores of *Bacillus stearothermophilus* Fs 7954 in distilled water, where N = number of viable spores at any time, N_0 = original number of viable spores. (b) Typical death rate for *E. coli* in buffer, where N = number of viable cells at any time and N_0 = original number of viable cells. (With permission, from S. Aiba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, 2d ed., University of Tokyo Press, Tokyo, 1973, p. 241.)

(or disinfecting) small-scale, heat-sensitive equipment. Some chemicals (e.g., ozone) cannot usually be used to sterilize fluids without adverse side reactions affecting medium quality.

For most large-scale equipment and liquids, thermal sterilization is used (filter sterilization is the only practical alternative for liquids). Usually, the dependence of the specific death rate on temperature is given by an Arrhenius equation:

$$k_d = \alpha e^{-E_{0d}/RT} \tag{10.18}$$

where R is the gas constant, T is absolute temperature, and E_{0d} is the activation energy for the death of the organism. Values for E_{0d} range from about 50 to 150 kcal/g-mol.

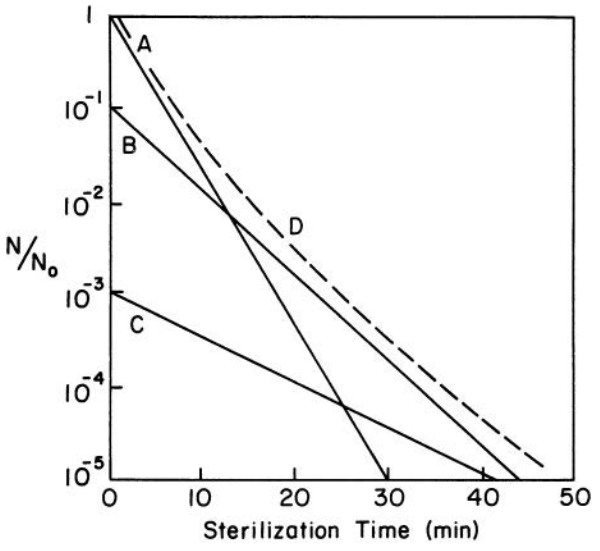


Figure 10.14. Overall survival curve (D) for a system with three distinct subpopulations (A, B, and C). Population A is normally dominant, but it is the one most sensitive to the sterilizing agent, while subpopulation C is far more resistant.

For spores of Bacillus stearothermophilus, $E_{0d} \approx 70$ kcal/g-mol, and values of 127 kcal/g-mol have been determined for *E. coli*. Most thermal sterilizations take place at 121°C. The values for k_d in such situations are very high for vegetative cells (often $>10^{10}$ min^{-1}). For spores, the values of k_d typically range from 0.5 to 5.0 min^{-1}. In most cases, we are only concerned about spores when steam-sterilizing equipment and media, since the value of k_d is so much lower with spores than vegetative cells.

The E_{0d} for vitamins and growth factors in many media is about 2 to 20 kcal/g-mol. The inactivation of viability is much more sensitive to temperature changes than the degradation of important growth factors in the medium. This factor is important to the design of sterilization equipment and protocols so as to assure complete killing of foreign organisms without the destruction of necessary growth factors in the medium.

The main factors in any sterilization protocol are the temperature, time of exposure, and initial number of organisms that must be killed. The problems of sterilization increase with scale-up. Consider the probability of an unsuccessful fermentation, $[1-P_0(t)]$, in a reactor:

$$1-P_0(t)=1-\left[1-p(t)\right]^{N_0} \tag{10.19a}$$

or with a simple model of killing in homogeneous populations:

$$1-P_0(t)=1-\left[1-e^{-k_d t}\right]^{N_0} \tag{10.19b}$$

N_0in equation 10.19 corresponds to the number of individuals in the reactor, not the concentration of organisms. Let n_0 be the concentration of particles. Now consider the probability of an unsuccessful sterilization in a 1 l and a 10,000 l reactor, where

each contains the identical solution ($n_0$is the same in both tanks), and the temperature (k_d) and time of sterilization are identical. Thus, for the 1 l tank,

$$1 - P_0(t) = 1 - \left[1 - e^{-k_d t}\right]^{1 \cdot n_0} \tag{10.20a}$$

And for the 10,000 l tank,

$$1 - P_0(t) = 1 - \left[1 - e^{-k_d t}\right]^{10{,}000 \cdot n_0} \tag{10.20b}$$

If $k_d t$ is 15 and $n_0 = 10^4$ spores/l in both cases, the probability of an unsuccessful fermentation is 0.003 in the 1 l vessel and about 1 in the 10,000 l tank ($5 \cdot 10^{-14}$ probability of extinction of the spores). Thus, the sterilization protocol that would be acceptable for a laboratory bench-scale experiment would be totally unacceptable for the larger-scale system. The larger tank would require much longer exposure to the same temperature to achieve the same degree of sterility, and the longer exposure to higher temperatures could lead to greater changes in the chemical composition of the medium.

A *sterilization chart* can be constructed from equation 10.19b. An example is depicted in Figure 10.15. To use such a chart, you need to specify the probability of failure that is acceptable (e.g., 10^{-3}) and the number of particles initially present in the fluid (n_0 total volume). For 10^{-3} and $N_0 = 10^8$, the corresponding value of $k_d t$ is about 26. If k_d = 1 min^{-1} at 121°C, then t = 26 min corresponds to the exposure time at 121°C required to ensure that 999 out of 1000 sterilizations are successful.

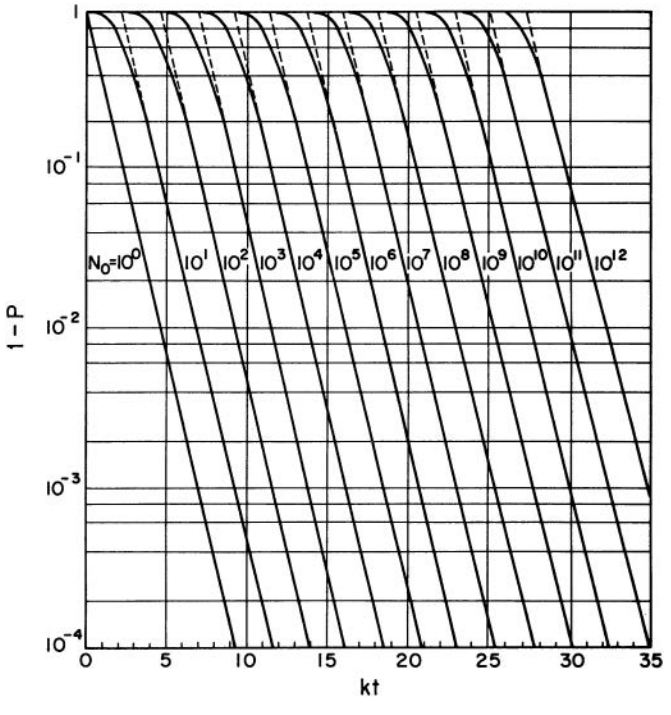


Figure 10.15. Sterilization chart. (With permission, from M. L. Shuler, *Encyclopedia of Physical Science and Technology*, Vol. 2, p. 427, Academic Press, New York, 1987.)

Steam sterilizations can be accomplished batchwise, often in situ in the fermentation vessel, or in a continuous apparatus (Figure 10.16). The greatest difficulty with batch sterilization is thermal lags and incomplete mixing. Typically, batch sterilization occurs at 121°C. The time required to heat the fluid to 121°C and to cool it back to growth temperatures (e.g., 37°C) is often much longer than the time of exposure to the desired temperature. For most spores, k_d falls very rapidly with temperatures (e.g., a tenfold decrease for k_d at T = 110°C rather than 121°C), so the heat-up and cool-down periods do little to augment spore killing. However, the elevated temperatures during heat-up and cool-down can be very damaging to vitamins and proteins, can lead to caramelization reactions for sugars, and can greatly alter medium quality.

Continuous sterilization, particularly a high-temperature, short-exposure time, can achieve complete sterilization with much less damage to the medium. Both the heat-up and cool-down periods are very rapid. Continuous sterilization is easier to control and reduces downtime in the fermenters. Two potential disadvantages of the continuous process are dilution of the medium with steam injection and foaming.

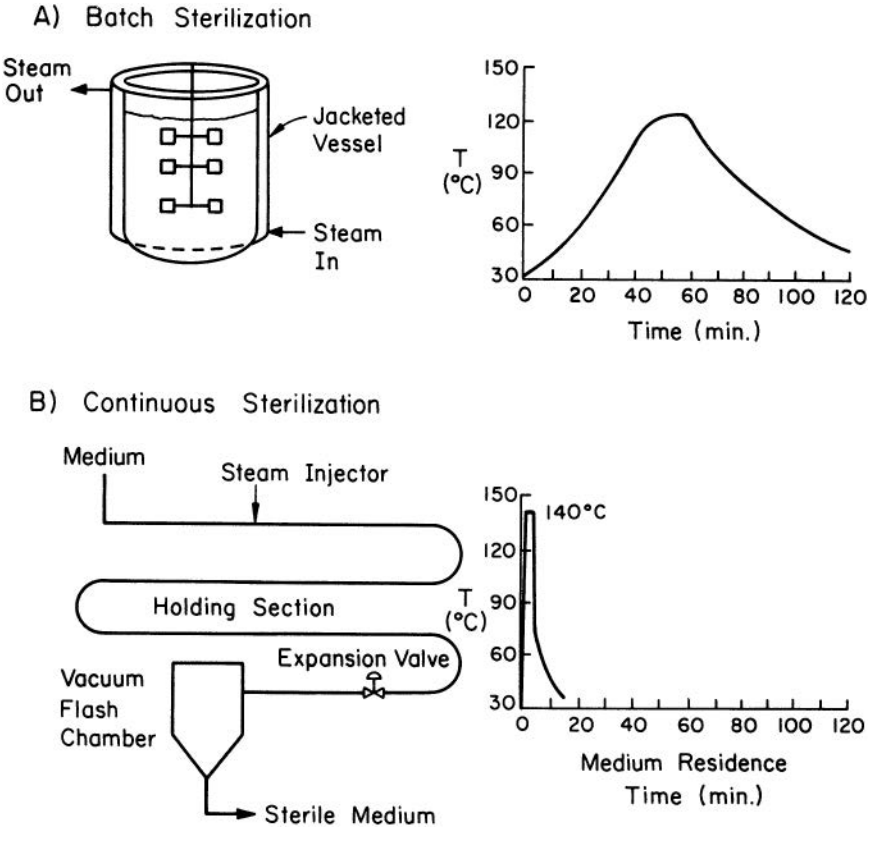


Figure 10.16. Comparison of a batch (a) with a continuous sterilization strategy (b) for the temperature profile of the medium sterilized. The continuous unit allows short-time, high-temperature sterilization.

The flow pattern inside the pipe is critical, since the fluid residence time near the wall can be different from that in the center. The average flow rate and the length of the sterilizing section should be designed to ensure high Peclet numbers (above 500), so that the velocity distribution approaches piston-like flow.

In addition to steam sterilization, process fluids can be filter sterilized. *Filter sterilization* is necessary when the medium contains heat-sensitive materials. An important example of filter sterilization in bioprocesses is the sterilization of medium to support the growth of animal cells. Microporous filters (pore sizes <0.2 μm) are typically used. The filters should be absolute filters; no pores larger than the nominal pore size must exist. A very narrow pore-size distribution is advantageous. The medium may be first prefiltered to remove large particulates that might plug the microporous filter. The actual microporous filter must be sterilized. The equipment that receives the filtered–sterilized fluid is also sterilized.

Filter sterilization is not as reliable as steam sterilization. Any defect in the membrane can lead to failure. Viruses and mycoplasma (small wall-less bacteria) can pass the filter. Consequently, filtered–sterilized medium is usually quarantined for a period of time to allow any contaminants to multiply to a detectable number. Clearly, a period of quarantine will detect only vegetative cells and not host-dependent contaminants (e.g., viruses).

Filter sterilization is used not only to filter medium but also to sterilize process air.

10.3.3. Sterilization of Gases

Aerobic fermentations require huge volumes of air. At 0.1 to 1 volume of gas per volume of liquid per minute, a 50,000 l fermenter requires $7 \cdot 10^6$ l to $7 \cdot 10^7$ l per day of air. Many fermentations are several days in duration. For a five-day fermentation, as much as $2 \cdot 10^8$ l of air may be required. This volume of air must be absolutely sterile. Typically, the concentration of microbes in air is 10^3 to $10^4/m^3$ or 1 to 10 microbes/l. Fed-batch or continuous culture systems place even more stringent demands on air sterilization, since air filters cannot be replaced during the run period.

An air supply of this magnitude requires sizable air compressors. Adiabatic compression of air can increase the air temperature (typically 150° to 220°C). Dry heat is less effective than moist heat in killing organisms. To kill spores, a temperature typically of 220°C for 30 s is required. Consequently, the adiabatic compression of process air certainly aids air sterilization. Since the exit air cools rapidly and the pipes connecting the compressor to the fermenter are difficult to maintain as absolutely sterile, an air-filtration step is almost always used. (Alternatives such as UV radiation, ozone injection, and scrubbing are not practical.)

The filtration of gases can be accomplished using either depth filters or surface filters. Historically, depth filters using glass wool were used, but *depth filters* have been almost totally replaced with membrane cartridge filters, which are *surface filters*.

Glass wool filters rely on a combination of mechanisms for the capture of particles. Possible mechanisms of removal for particles of about 1 μm diameter are direct interception, electrostatic effects, diffusion (or Brownian motion), and inertial effects. As the flowing gas approaches a fiber, the flow streamlines must curve around the fiber. If a particle had no density, it would follow the streamlines around the fiber.

Only particles whose centers are on streamlines less than a particle radius away from the fiber could be intercepted. However, for real particles, inertial effects mean that particles with mass will have a tendency to maintain a straight-line trajectory as they approach the fiber. Thus, such particles deviate from the streamline and crash into the fiber. Both interception and inertial effects are important in removing bacteria.

Diffusional effects may be important for virus removal, but bacteria are sufficiently large that diffusion is relatively unimportant. The removal of a particle by these mechanisms is probabilistic. The deeper the filter is, the smaller the probability of a particle penetrating the depth of the filter.

Depth filters using glass wool can show shrinkage and hardening upon steam sterilization. In such cases, channeling can occur, and the filter becomes far less effective than would be predicted. More recent advances in the design of fiberglass filter cartridges have overcome much of this disadvantage. Another serious problem with such fibrous filters is wetting. If a filter wets, an easy path becomes available for a contaminant to penetrate through it. A wet filter also greatly increases pressure drop. Thus, any condensation within such a filter must be avoided.

Surface filters (membrane cartridges) work using another mechanism for particle removal, a sieving effect. Figure 10.17 depicts a membrane cartridge unit and its housing. Membranes with uniformly small pores prevent the passage of particles with a radius larger than the pore radius. Such filters can be steam-sterilized many times. Also, any condensate formed on the nonsterile side cannot pass into the sterile side.

With both depth filters and membrane cartridges, pressure drop is critical. The energy input for compressed air for a commercial-scale process is significant. Air treatment can account for 25% of total production costs. Thus, the design engineer has to balance the assurance of sterility against pressure drop.

Generally, the bioprocess engineer is not involved in the design of membrane cartridge units (a number of competent vendors offer suitable products), but the testing of such units for effectiveness is important. In the United States, an example is an aerosol test using corn oil nebulized to 1.0 to 1.5 mg oil/l of air. This test is recognized by the FDA. Such an aerosol would contain particles primarily in the range of 0.2 to 1.0 μm in diameter. Aerosol in the exit gas from a filter cartridge can be monitored by a photometer. The number of sterilization cycles a cartridge can undergo before failure is an important criterion in the selection of membrane cartridge units.

Other tests for the integrity of the unit are pressure-drop-versus-flow-rate and bubble-point tests, which detect defects in the membrane and maximum pore size. *Grow-through* tests use a sterile nutrient solution on one side of the membrane and a similar nutrient solution inoculated with a test strain (e.g., *Pseudomonas diminuta*) on the nonsterile side. The greater the integrity of the filter, the longer it will take before growth on the nominally sterile side will occur. A filter should be evaluated with several different types of tests.

Because of the high costs associated with the loss of a batch due to contamination, the choice of air filter to give dependable protection for a fermentation while minimizing pressure drop is critical.

So far, we have considered only inlet gas streams. With fermentations involving pathogens (disease-causing organisms) or recombinant DNA, *all organisms* must be

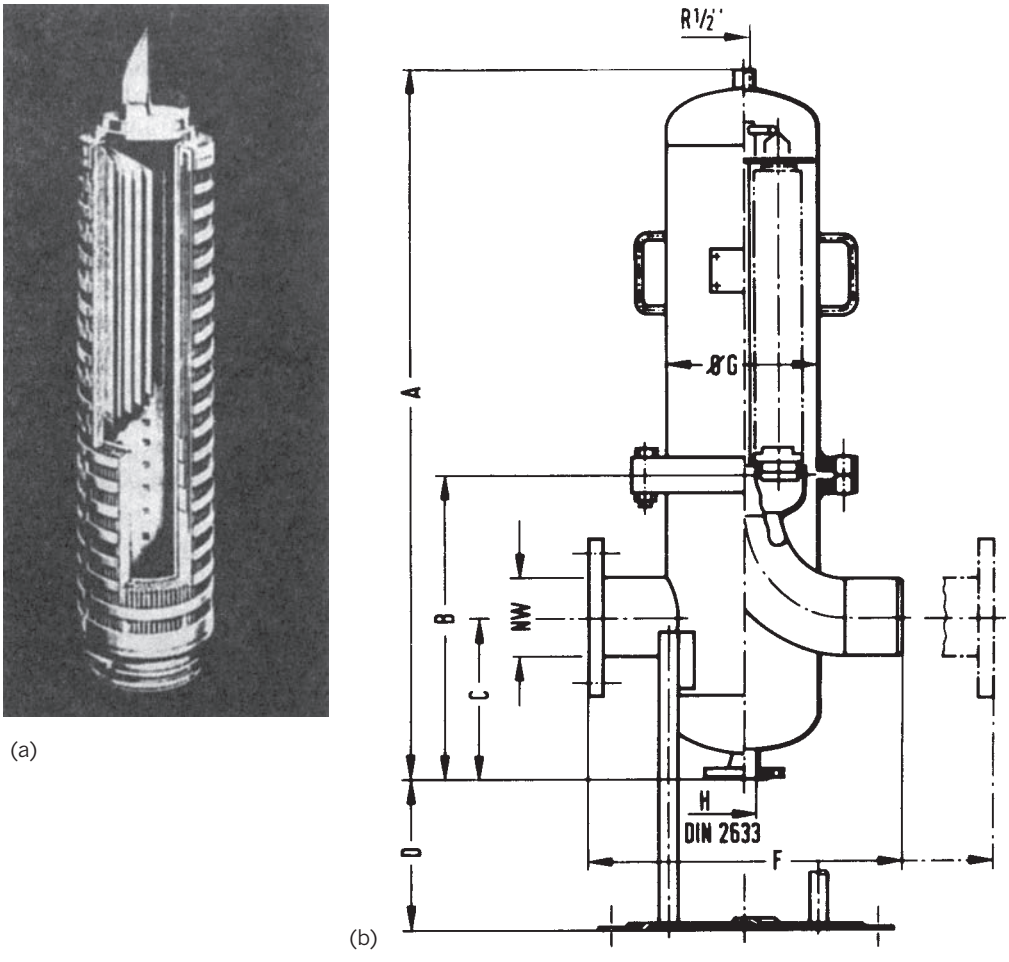


Figure 10.17. (a) A Pull-Emflon membrane cartridge for filter sterilization of air. (b) Housing for air sterilization filter. (With permission, from W. Crueger, "Sterile Techniques in Biotechnology," in R. K. Finn and P. Prave, eds., *Biotechnology Focus 2*, Hanser Publishers, New York, 1990, p. 413.)

removed from the exhaust gas. The concentration of microbes in the exit gas is far higher than in the inlet gas. Catalytically aided combustion (incineration) of the exit gas is an effective, but expensive, solution. Consequently, filtration of the exit air is of increasing importance.

10.4. SUMMARY

Scale-up of reactors is a task primarily for the bioprocess engineer. Three basic reactor types for the aerobic cultivation of suspended cells are *mechanically agitated reactors*, *bubble columns*, and *loop reactors*. Although the bubble and loop reactors offer advantages in terms of energy efficiency and reduced shear damage to cells,

the traditional stirred-tank system is more flexible and can better handle broths that become highly viscous.

The primary limitations on the size of stirred-tank bioreactors are gas supply (e.g., O_2) and heat removal. The value of $k_L a$, the *volumetric transfer coefficient*, is of prime concern. Its value depends not only on the equipment used, gas flow rates, and agitator speed, but also on the nature of the fluid (salt content, presence of surface active agents, and viscosity). The properties of the fluid can change during the fermentation. By directly monitoring oxygen concentration in the gas phase and the dissolved-oxygen level, it is possible to make on-line estimates of $k_L a$. If the rate of oxygen uptake is known, the rate of heat generation can be readily estimated in aerobic fermentations.

Scale-up is difficult, because conditions in a large vessel are much more heterogeneous than in a small vessel. If geometrically similar tanks are used, it is impossible to maintain identical values of shear, mixing times, and $k_L a$ simultaneously in both the large and small tanks. Scale-up would be simplified if good reaction models could be coupled to good transport models; since this is not yet possible, *scale-down* techniques are a good alternative.

Bioreactor instrumentation and control are less advanced than in the petrochemical industry. Improvements in sensor technology and the dynamical models of bioreactors are critical to improvements in control technology.

The large-scale bioreactor places heavy demands on processes to *sterilize* (kill or remove all organisms from) fluids entering the bioreactor. Liquid streams are thermally sterilized or filter sterilized. Steam sterilization is preferred, but the sterilization process must not damage the ability of the medium to support growth. Since longer periods of exposure to high temperatures are necessary to assure sterility in larger volumes of liquid, the sterilization process can alter the medium composition more for large-scale than for small-scale systems. This is an additional factor that can lead to differences in productivity upon scale-up. *Continuous sterilization* protects the medium components from degradation better than batch sterilization, because the heat-up and cool-down periods are greatly minimized. *Filter sterilization* of liquids is used when the medium contains particularly heat-sensitive components. Air streams are typically filter sterilized. Surface filters (membrane cartridges) are commonly used in gas sterilization.

SUGGESTIONS FOR FURTHER READING

General References

Bailey, J. E., and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill, New York, 1986.

Blanch, H. W., and D. S. Clark, *Biochemical Engineering*, Marcel Dekker, New York, 1996.

Doran, P. M., *Bioprocess Engineering Principles*, 2d ed., Academic Press, San Diego, 2013.

Specific Articles and Books

Alford, J. S., "Bioprocess Control: Advances and Challenges," *Comput. Chem. Eng.* 30: 1464–1475, 2006.

Amanullah, A., J. M. Oterro, M. Mikola, A. Hsu, J. Zhang, J. Aunins, H. B. Schreyer, J. A. Hope, and A. P. Russo, "Novel Micro-Bioreactor High Throughput Technology for Cell Culture Process Development: Reproducibility and Scalability Assessment of Fed-Batch CHO Cultures," *Biotechnol. Bioeng.* 106: 57–67, 2010.

Bull, D. N., R. W. Thoma, and T. E. Stinnett, "Bioreactors for Submerged Culture," *Adv. Biotechnol. Processes* 1: 1–30, 1983.

Bylund, F., F. Guillard, S.-O. Enfors, C. Trägardh, and G. Larsson, "Scale Down of Recombinant Protein Production: A Comparative Study of Scaling Performance," *Bioprocess Eng.* 20: 327–389, 1999.

Garcia-Ochea, F., and E. Gomez, "Bioreactor Scale Up and Oxygen Transfer Rate in Microbial Processes: An Overview," *Biotechnology Advances* 27: 153–176, 2009.

Jost, J., "Selected Bioengineering Problems in Stirred-Tank Fermenters," in S. L. Sandler and B. A. Finlayson, eds., *Chemical Engineering Education in a Changing Environment*, Engineering Foundation, New York, 1988.

Junker, B. H., M. Stanik, C. Barna, P. Salmon, E. Paul, and B. C. Buckland, "Influence of Impeller Type on Power Input in Fermentation Vessels," *Bioprocess Eng.* 18: 401–412, 1998.

Kargi, F., and M. Moo-Young, "Transport Phenomena in Bioprocesses," in *Comprehensive Biotechnology*, Vol. 2, M. Moo-Young, ed., Pergamon Press, Oxford, UK, 1985, pp. 5–55.

Kim, B. J., J. Diao, and M. L. Shuler, "Mini-scale Bioprocessing Systems for Highly Parallel Animal Cell Cultures," *Biotechnol. Progr.* 28: 595–607, 2012.

Klutz, S., J. Magnus, M. Lobedann, P. Schawn, B. Maiser, J. Niklas, M. Temming, and G. Schembecker, "Developing the Biofacility of the Future Based on Continuous Processing and Single-Use Technology," *J. Biotechnol.* 213: 120–130, 2015.

Komives, C., and R. S. Parker, "Bioreactor State Estimation and Control," *Curr. Opin. Biotechnol.* 14: 468–474, 2003.

Loffelholz, C., S. C. Kaiser, M. Kraume, R. Eibl, and D. Eibl, "Dynamic Single-use Bioreactors Used in Modern Liter and m^3-scale Biotechnological Processes: Engineering Characteristics and Scaling Up," *Adv. Biochem. Eng./Biotechnol.* 138: 1–44, 2014.

Pohlscheidt, M., S. Charaniya, C. Bork, M. Jenzsch, T. Noetzel, and A. Luebbert, "Bioprocess and Fermentation Monitoring," Chapter 69, pp. 1471–1491, in *Upstream Industrial Biotechnology: Equipment, Process Design, Sensing, Control, and cGMP Operations*, Vol. 2, M. C. Flickenger, ed., 2013.

Rameez, S., S. S. Mostafa, C. Miller, and A. Shukla, "High-Throughput Miniaturized Bioreactors for Cell Culture Process Development; Reproducibility, Scalability and Control," *Biotechnol. Progr.* 30: 718–727, 2014.

PROBLEMS

10.1. The air supply to a fermenter was turned off for a short period of time and then restarted. A value for C^* of 7.3 mg/l has been determined for the operating conditions. Use the tabulated measurements of dissolved oxygen (DO) values in the following table to estimate the oxygen uptake rate and $k_L a$ in this system.

	Time (min)	DO (mg/l)
	–1	3.3
Air off	0	3.3
	1	2.4
	2	1.3
	3	0.3
	4	0.1
	5	0.0
Air on	6	0.0
	7	0.3
	8	1.0
	9	1.6
	10	2.0
	11	2.4
	12	2.7
	13	2.9
	14	3.0
	15	3.1
	16	3.2
	17	3.2

10.2. A value of $k_L a = 30\ \text{h}^{-1}$ has been determined for a fermenter at its maximum practical agitator rotational speed and with air being sparged at 0.5 l gas/l reactor volume-min. *E. coli* with a q_{O_2} of 10 mmol O_2/g-dry wt-h are to be cultured. The critical dissolved oxygen concentration is 0.2 mg/l. The solubility of oxygen from air in the fermentation broth is 7.3 mg/l at 30°C.

a. What maximum concentration of *E. coli* can be sustained in this fermenter under aerobic conditions?

b. What concentration could be maintained if pure oxygen was used to sparge the reactor?

10.3. You are asked to design the temperature control system for a large fermenter.

a. Estimate the required cooling-water flow rate for a 100,000 l fermenter with an 80,000 l working volume when the rate of oxygen consumption is 100 mmol O_2/l-h. The desired operating temperature is 35°C. A cooling coil is to be used. The minimum allowable temperature differential between the cooling water and the broth is 5°C. Cooling water is available at 15°C. The heat capacities of the broth and the cooling water are roughly equal.

b. Estimate the required length of cooling coil if the coil has a 2.5-cm diameter and the overall heat transfer coefficient is 1420 J/s-m^2-°C.

10.4. A lethal agent (KCN) was added to the fermentation medium in the presence of aeration using diffusors. Dissolved oxygen (DO) concentration increased with time as follows when N = 100 rev/min:

t (min)	0	1	2	3	5	7	8	10
DO (mg/L)	1	1.93	2.7	3.36	4.4	5.1	5.4	5.9

Saturation DO concentration in the fermentation medium is 7.0 mg/L.

a. Determine oxygen transfer coefficient $k_L a$.

b. Determine $k_L a$ when N_i is increased to 200 rev/min.

10.5. A laboratory-scale stirred-tank fermenter of volume 5 l (Do/H = 1/2) is to be scaled up to 500 l by using the following criteria: Di/Do = 1/3, N = 100 rev/min. Determine the size of the fermenter, impeller diameter and speed for each case:

a. Constant oxygen transfer rate
b. Constant power/volume
c. Constant impeller tip speed
d. Constant mixing time

10.6. Consider Example 10.4. What would be the substrate concentrations in each compartment in the 10 l and 10,000 l tanks if the probe were placed in the bottom compartment?

10.7. Consider the 10 l and 10,000 l tanks described in Example 10.4. Suppose that fully continuous operation is to be used, F was fixed at 5 mg/l-s for both tanks, and $D = 0.2\ \text{h}^{-1}$ for each tank with fluid removal from the top. What fraction of the inlet substrate would be consumed in each tank? If the biomass yield coefficient were 0.5 g cells/g substrate and $Y_{P/X} = 0.1$ g product/g cells, what would be the effect on volumetric productivity upon scale-up?

10.8. A continuous culture system is being constructed. The fermentation tank is to be 50,000 l in size, and the residence time is to be 2 h. A continuous sterilizer is to be used. The unsterilized medium contains 10^4 spores/l. The value of k_d has been determined to be 1 min^{-1} at 121°C and 61 min^{-1} at 140°C. For each temperature (121°C and 140°C), determine the required residence time in the holding section so as to ensure that 99% of the time 4 weeks of continuous operation can be obtained without contamination (due to contaminants in the liquid medium).

10.9. Discuss the effects of mixing on sterilization in a batch fermenter.

10.10. A medium containing a vitamin is to be sterilized. Assume that the number of spores initially present is 10^5 /l. The values of the pre-Arrhenius constant and E_{0d} for the spores are

$$E_{0d} = 65 \text{ kcal/g-mol}$$

$$\alpha = 1 \cdot 10^{36}\ \text{min}^{-1}$$

For the inactivation of the vitamin, the values of E_{0d} and α are

$$E_{0d} = 10 \text{ kcal/g-mol}$$

$$\text{a} = 1 \cdot 10^{4}\ \text{min}^{-1}$$

The initial concentration of the vitamin is 30 mg/l. Compare the amount of active vitamin in the sterilized medium for 10 l and 10,000 l fermenters when both are sterilized at 121°C when we require in both cases that the probability of an unsuccessful fermentation be 0.001. Ignore the effects of the heat-up and cool-down periods.

10.11. Consider the data given in the following table on the temperature changes in a 10,000 l fermenter, which includes the heat-up and cool-down periods. Use the values for the Arrhenius parameters given in Problem 10.8 and assume an initial spore concentration of 10^5 /l and a vitamin concentration of 30 mg/l.

Time (min)	Temperature (°C)
0	30
10	40
20	54
30	70
40	95
50	121
55	121
60	121
65	106
70	98
90	75
100	64
120	46
140	32

a. What is the probability of a successful sterilization?
b. What fraction of the vitamin remains undegraded?
c. What fraction of the vitamin is degraded in the sterilization period?
d. What fraction of the vitamin is degraded in the heat-up and cool-down periods?
e. What is the fraction of spores deactivated in the heat-up and cool-down cycles?

10.12. *E. coli* has a maximum respiration rate, $q_{O_2 max}$, of about 240 mg O_2/g-dry wt-h. It is desired to achieve a cell mass of 20 g dry wt/l. The $k_L a$ is 120 h^{-1} in a 1000 l reactor (800 l working volume). A gas stream enriched in oxygen is used (i.e., 80% O_2), which gives a value of $C^* = 28$ mg/L. If oxygen becomes limiting, growth and respiration slow; following is an example:

$$q_{O_2} = \frac{q_{O_2 max} C_L}{0.2 \text{ mg/l} + C_L} \max$$

Here C_L is the dissolved oxygen concentration in the fermenter. What is C_L when the cell mass is at 20 g/l?

10.13. The temperature history of the heating and cooling of a 40,000 l tank during sterilization of medium follows: 0 to 15 min, $T = 85°C$; 15 to 40 min, $T = 121°C$; 40 to 50 min, $T = 85°C$; 50 to 60 min, $T = 55°C$; > 60 min, $T = 30°C$. The medium contains vitamins, the most fragile of the vitamins has an activation energy for destruction of 10 kcal/g-mol, and the value of α (see equation 10.18) is $1 \cdot 10^4 \text{ min}^{-1}$. Assume vitamin destruction is first order and the initial concentration is 50 mg/l. R is 1.99 cal/g-mol-K. The medium contains $2.5 \cdot 10^3$ spores/l. The spores have an $E_{0d} = 65$ kcal/g-mol, and k_d at 121°C is 1.02 min^{-1}. Estimate the probability of a successful sterilization. What fraction of the vitamin remains active?

10.14. Estimate $k_L a$ from Figure 10.5 if C^* is 35 mg/l due to the use of nearly pure oxygen rather than air.

10.15. In cultivation of baker's yeast in a stirred and aerated tank, lethal agents are added to the fermentation medium to kill the organisms immediately. Increase in dissolved oxygen (DO) concentration upon addition of lethal agents is followed with the aid of a DO analyzer and a recorder. Using the following data, determine the oxygen transfer coefficient ($k_L a$) for the reactor. Saturation DO concentration is $C^* = 9$ mg/l.

Time (min)	DO (mg/l)
1	1
2	3
2.5	4
3	5
4	6.5
5	7.2

10.16. A stirred-tank reactor is to be scaled down from 10 m^3 to 0.1 m^3. The dimensions of the large tank are D_t = 2 m; D_i = 0.5 m; N = 100 rpm.

a. Determine the dimensions of the small tank (D_t, D_i, H) by using geometric similarity.

b. What would be the required rotational speed of the impeller in the small tank if the following criteria were used?

b1. Constant tip speed

b2. Constant impeller Re number

10.17. An autoclave malfunctions, and the temperature reaches only 119.5°C. The sterilization time at the maximum temperature was 20 min. The jar contains 10 l of complex medium that has 10^5 spores/l. At 121°C, k_d = 1.0 min^{-1} and E_{0d} = 90 kcal/g-mol. What is the probability that the medium was sterile?

10.18. You are asked to design a continuous sterilizer to produce 330,000 L/d of sterile media. The system must operate for 30 days with the probability of failure to be 0.001. The initial concentration of spores in the medium is 10^4/l. The value of k_d is 1 min^{-1} at 121°C and 60 min^{-1} at 140°C.

a. Estimate the residence time in the sterilizer at 121°C necessary to achieve the desired probability of success.

b. Estimate the residence time required at 140°C.

c. What is the ratio of residence times for high-temperature, short-time sterilization to operation at 121°C?

10.19. One hundred tubes are to be sterilized. Each tube contains 10 mL of fluid. The value of k_d = 0.6 min^{-1}. The tubes are sterilized for 20 min. Ten of the tubes showed growth of contaminants. What was the concentration of spores in the tubes?

11

Recovery and Purification of Products

The recovery and purification of a fermentation product is essential to any commercial process. The difficulty entailed depends heavily on the nature of the product. Products may be the biomass itself, an extracellular component, or an intracellular component. Since the chemical nature of a fermentation or cell culture broth is quite complex and extremely high purity is required for some products (e.g., some pharmaceuticals), recovery and purification often require many processing steps and in many cases represent a manufacturing cost higher than that involved in producing the product. Since the compounds of interest are often heat or oxygen sensitive and present in dilute aqueous solution, the traditional separation techniques of the chemical engineer must be augmented with more specialized ones.

11.1. STRATEGIES TO RECOVER AND PURIFY PRODUCTS

Recovery and purification are major expenses in production of most fermentation products; often they comprise more than 50% of the total manufacturing costs, especially for an intracellular product. There is an excellent correlation

between the price of a product (on a per-kg basis) and how dilute the product is as it exits the bioreactor. For example, the concentration of citric acid is on the order of 100 g/l when it enters recovery processes and sells for order of 3 $/kg, while a therapeutic protein might be at the concentration of 0.00001 g/l and sells for 100,000,000 $/kg (although with modern production techniques most proteins now can be produced at much higher concentration). The efficiency of the production process and the difficulty and cost of the recovery and purification processes are tightly coupled.

The major unit operations used for product recovery and purification and the major principles of separations are summarized in Figure 11.1. Separation methods vary with the size and nature of the product, and a number of different methods may need to be used for a fermentation or cell culture broth containing soluble products of different molecular size. The size range of products is large (e.g., small

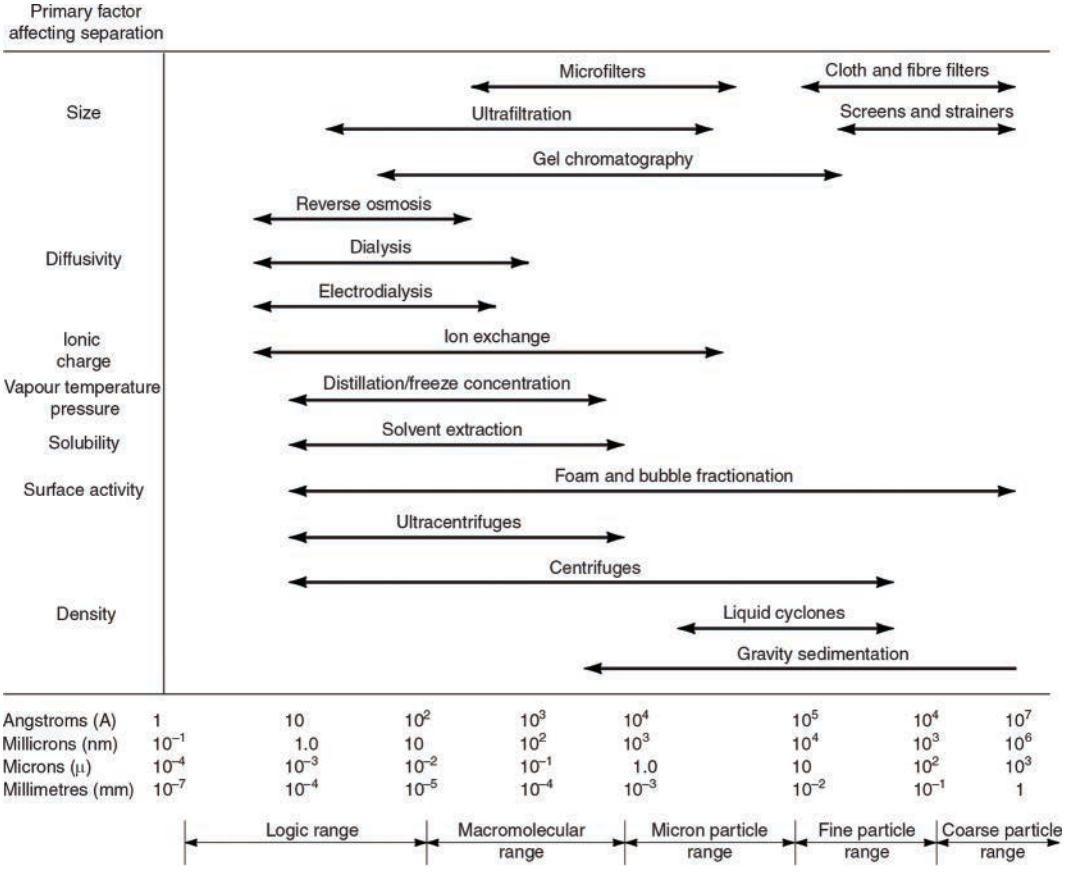


Figure 11.1. Ranges of applications of some standard unit operations. (B. Atkinson and F. Mavituna, *Biochemical Engineering and Biotechnology Handbook*, Macmillan, Inc., New York, 1983. Reproduced with permission of Palgrave Macmillan.)

molecules, enzymes, therapeutics, and clean water), and the separation process must be configured to effectively make the product. Figure 11.2 summarizes the major steps involved in the separation and purification for many small molecule and protein products.

The strategy in Figure 11.2 can be generalized to involve four primary functions: separation of insoluble products and other solids, primary isolation or concentration of product and removal of most of the water, purification or removal of contaminating chemicals, and product preparation, such as drying. Process economics dictate that water must be removed very early in the process train so that the size of the equipment in the following steps will be minimized. Because a step designed primarily to concentrate product and the steps designed to remove contaminating chemicals often remove solvent, there is no distinct separation between steps 2 and 3. The same operations to recover soluble products can apply to both steps 2 and 3. First, however, we consider those steps that remove solids, such as cells or the insoluble material.

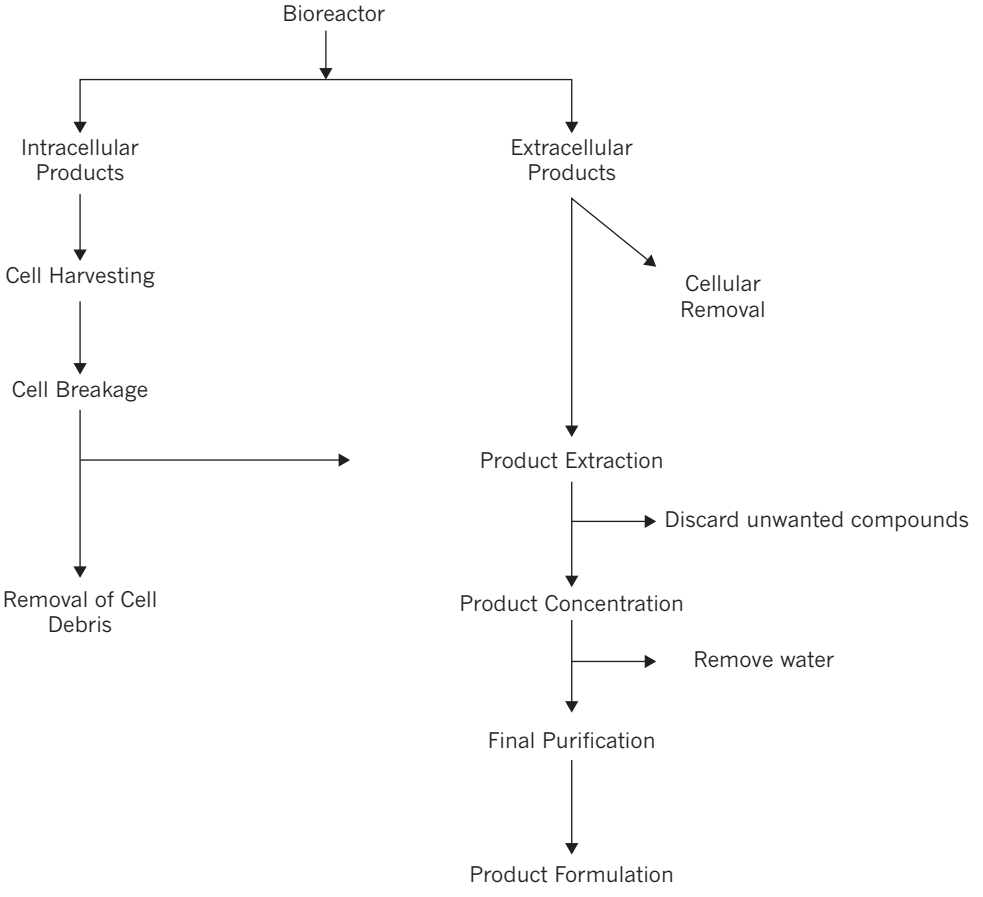


Figure 11.2. Major steps involved in the separation and purification of bioproducts.

11.2. SEPARATION OF INSOLUBLE PRODUCTS

The separation of solids such as biomass, insoluble particles, and macromolecules from the fermentation broth is usually the first step in product recovery. In some cases, the broth may need to be pretreated to facilitate solids separation. Examples of pretreatment are heat treatment, pH and ionic strength adjustment, and the addition of coagulants and flocculants. If the product is biomass, then separation of solids is the major step in product recovery, which results in a significant volume reduction. If the product is a soluble compound, solids need to be separated from liquid before the liquid is further treated to recover and purify the soluble product. For the recovery of intracellular products, the cells need to be disrupted and other cellular products need to be separated from the desired product. The major methods used for the separation of cellular material (biomass) are filtration (both rotary vacuum filtration and micro- or ultrafiltration), centrifugation, and coagulation and flocculation.

11.2.1. Filtration

Filtration is probably the most cost-effective method for the separation of large solid particles and cells from cultivation broths. The broth is passed through a filter medium, and a filter cake is formed as a result of deposition of solids on the filter surface. Continuous rotary filters or *rotary vacuum precoat filters* are the most widely used types in the fermentation industry and for wastewater treatment. The drum is covered with a layer of precoat, usually of diatomaceous earth, prior to filtration. A small amount of coagulating agent or filter aid is added to the broth before it is pumped into the filter. As the drum rotates under vacuum, a thin layer of cells adheres to the drum. The thickness of the cell layer increases in the section designed for forming the cake. The layer of solids is washed and dewatered during its passage to the discharge point, where a knife blade cuts off the cake. A vacuum maintained in the drum provides the driving force for liquid and air flow. A schematic of a continuous rotary vacuum filter is shown in Figure 11.3.

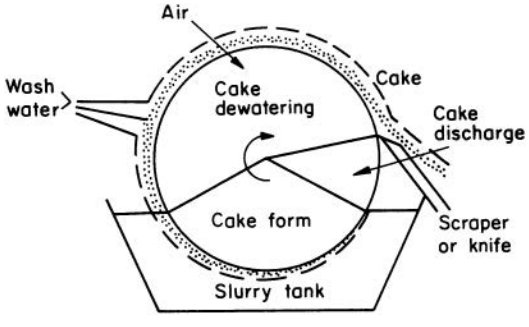


Figure 11.3. Schematic of a continuous rotary vacuum filter. A vacuum (subatmospheric pressure) is maintained within the drum, causing the pressure-driven flow of liquid (during cake formation) and air (during dewatering).

Filtration is commonly used for separating mycelium from fermentation broth in antibiotic fermentations. It is also commonly used in waste-water treatment facilities. For production of therapeutic proteins, deep-bed filtration using disposable media is typically used instead of rotary vacuum filtration.

The rate of filtration (the flow of filtrate) for a constant-pressure (vacuum) filtration operation is determined primarily by the resistance of the cake and filter medium:

$$\frac{dV}{dt} = \frac{g_c \Delta p A}{(r_m + r_c)\mu} \tag{11.1}$$

where V is the volume of filtrate, A is the surface area of the filter, Δp is the pressure drop through the cake and filter medium, μ is the viscosity of the filtrate, r_m is the resistance of the filter medium, and r_c is the resistance of the cake. The value of r_m is characteristic of the filter medium. However, the cake resistance, r_c, increases during filtration, and after a start-up period, r_c exceeds r_m. The value of r_c is given by

$$r_c = \alpha \frac{W}{A} = \alpha \frac{CV}{A} \tag{11.2}$$

where W is the total weight of the cake on filter, C is the weight of the cake deposited per unit volume of filtrate, and α is the average specific resistance of the cake.

The total weight of cake is related to the total volume of filtrate:

$$W = CV \tag{11.3}$$

Substituting equations 11.2 and 11.3 into equation 11.1 with constant A gives

$$\frac{d(V/A)}{dt} = \frac{g_c \Delta p}{\left(r_m + \alpha \frac{CV}{A}\right)\mu} \tag{11.4}$$

Integration of equation 11.4 from $V = 0$ to $V = V$ and $t = 0$ to $t = t$ yields

$$V^2 + 2VV_0 = Kt \tag{11.5}$$

where V_0 and K are defined as

$$V_0 = \frac{r_m}{\alpha C} A \text{ and } K = \left(\frac{2A^2}{\alpha C \mu}\right) \Delta p \cdot g_c$$

Equation 11.5 is known as the Ruth equation for constant-pressure filtration and can be rearranged as follows:

$$\frac{t}{V} = \frac{1}{K}(V + 2V_0) \tag{11.6}$$

A plot of t/V versus V yields a straight line with a slope of $1/K$ and intercept of $2V_0/K$, as depicted in Figure 11.4. The values for r_m and α are calculated from experimentally determined values of K and V_0.

In a rotating drum filter (like the one shown in Figure 11.3), the drum rotates at a constant speed (n rps) and only a fraction of drum-surface area is immersed in suspension reservoir (φ). The period of time during which filtration is carried out is φ/n per revolution of the drum. Equation 11.5 can be rewritten in this case as

$$\left(\frac{V'}{n}\right)^2 + 2\frac{V'}{n}V_0 = K\frac{\varphi}{n} \tag{11.7}$$

where V' = filtrate volume per unit time (volume/time) and V'/n V'/n represents the volume of filtrate filtered for one revolution of the drum.

This analysis of filtration is based on several assumptions; the primary one is an *incompressible cake*, which results in constant specific cake resistance. Usually, fermentation cakes are compressible, so α varies with Δp. The concentration of filter aid (1% to 5%) also has a significant effect on specific cake resistance. As depicted in Figure 11.5, the specific cake resistance decreases as the concentration of filter aid increases. The filterability of fermentation broth is also affected by fermentation conditions. The pH, viscosity, and composition of the medium affect the cake resistance. Specific cake resistance decreases with decreasing pH. The coagulation of mycelial protein with heat treatment improves the filterability of the fermentation broth in mycelial fermentations such as penicillin. The duration of fermentation also affects filterability and antibiotic activity in penicillin fermentations and represents an example of the interplay of upstream processing (e.g., fermentation) with downstream processing. Fermentation times of 180 to 200 h are used in penicillin fermentations to minimize the cake resistance and maximize antibiotic activity. Typical filtration conditions for streptomycin fermentations are pH = 3.6, 2% to 3% filter aid, following a heat treatment of 30 to 60 min at $T = 80°$ to 90°C.

Cell separation can also be accomplished by cross-flow filtration using ultrafiltration or microporous filtration. These systems are described in more detail in Section 11.4.7. This technique has achieved rather rapid acceptance and has replaced centrifugation and vacuum filtration in some applications. Cross-flow filtration is

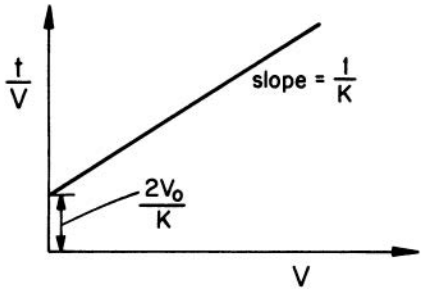


Figure 11.4. A plot of t/V versus V is used in filtration to calculate α and r_m.

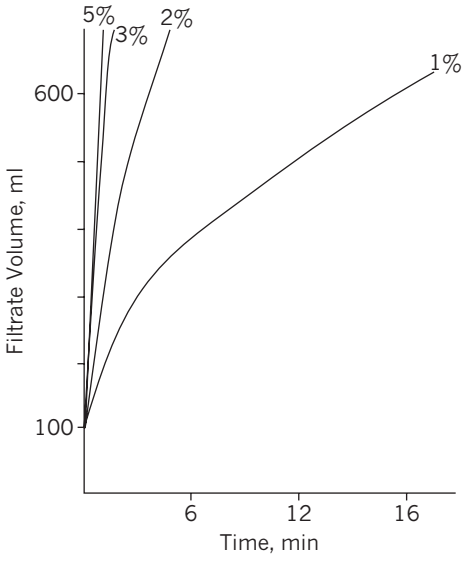


Figure 11.5. Effect of filter aid on filtration performance. Filtration rate increases by increasing the filter aid concentration from 1% to 3%.

well suited to the removal of dispersed cells such as *E. coli* and yeast, as well as larger, shear-sensitive cells from mammals. Microporous filters with pores of 0.02 and 0.2 μm are useful for bacterial separations, while larger sizes 0.2 to 2 μm can be used with yeast.

Example 11.1

The following data were obtained in a constant-pressure filtration unit for filtration of a yeast suspension:

t (min)	4	20	48	76	120
V (l filtrate)	115	365	680	850	1130

Characteristics of the filter are as follows:

$$A = 0.28\ \text{m}^2,\quad C = 1920\ \text{kg/m}^3,\quad \mu = 2.9\times10^{-3}\ \text{kg/m-s},\quad \alpha = 4\ \text{m/kg}$$

a. Determine the pressure drop across the filter.
b. Determine the filter medium resistance (r_m).
c. Determine the size of filter for the same pressure drop to process 4000 l of cell suspension in 20 min.

Solution

a. We apply equation 11.6, as illustrated in Figure 11.4:

V (l)	115	365	680	850	1130
t/V (min/l)	0.035	0.055	0.07	0.089	0.106

A plot of t/V versus V results in a straight line with a slope of 0.67×10^{-4} (min/l^2) and an intercept of 0.028 (min/l):

$$\text{slope} = \frac{1}{K} = 0.67 \times 10^{-4}, \quad K = 1.5 \times 10^4 \text{ l}^2/\text{min}$$

$$\Delta P = \frac{K\alpha C\mu}{2A^2 g_c}, \quad g_c = 9.8 \frac{kg_m}{kg_f \cdot s^2}$$

$$\Delta p = 2.3 \times 10^{-4} \text{ N/m}^2$$

b. We calculate the values of r_m from the y intercept in Figure 11.4:

$$y \text{ intercept} = \frac{2V_0}{K} = 0.028$$

$$V_0 = 2101$$

$$r_m = \frac{\alpha V_0 C}{A} = 5760 \text{ m}^{-1}$$

c. We calculate the required area from the Ruth equation (11.5):

$$V^2 + 2VV_0 = Kt$$

where $K = (2A^2/\alpha C\mu)\Delta p g_c$ and $V_0 = (r_m A/\alpha C)$. By substituting numerical values for V, Δp, and t, we obtain

$$A^2 - 25A - 66.67 = 0$$

The solution to the quadratic equation yields

$$A = 27.43 \text{ m}^2$$

11.2.2. Centrifugation

Centrifugation is used to separate particles of size between 100 and 0.1 μm from liquid by centrifugal forces. The theory of solid–liquid separations in a gravitational field should be clearly understood before centrifugal and gravity separations such as sedimentation are covered. Particle settling in a high-particle-density suspension is known as *hindered settling*, as these particles interact with each other.

The major forces acting on a solid particle settling in a liquid by gravitational forces are gravitational force (F_G), drag force (F_D), and buoyant force (F_B). When the particles reach a terminal settling velocity, forces acting on a particle balance each other, resulting in a zero net force:

$$F_G = F_D + F_B \tag{11.8}$$

where

$$F_G = \frac{\pi}{6} D_p^3 \rho_p \frac{g}{g_c} \tag{11.9}$$

$$F_B = \frac{\pi}{6} D_p^3 \rho_f \frac{g}{g_c} \tag{11.10}$$

and

$$F_D = \frac{C_D}{2g_c} \rho_f U_0^2 \, A \tag{11.11}$$

F_D is the drag force exerted by the fluid on solid particles, C_D is the drag coefficient, ρ_f is fluid density, U_0 is the relative velocity between the fluid and particle or the terminal velocity of a particle, and A is the cross-sectional area of the particles perpendicular to the direction of fluid flow; for a sphere, $A = (\pi/4)D_p^2$. For spherical particles, when $\mathrm{Re}_p < 0.3$, the drag force, F_D, is given by the Stokes equation:

$$F_D = 3\pi\mu D_p U_0 \frac{1}{g_c} \tag{11.12}$$

Substitution of equation 11.12 into equation 11.11 results in the following relationship between C_D and Re_p:

$$C_D = \frac{24}{\mathrm{Re}_p} \tag{11.13a}$$

When Re_p is between 1 and 10,000, C_D for a sphere is approximated as

$$C_D = \frac{24}{\mathrm{Re}_p} + \frac{3}{\sqrt{\mathrm{Re}_p}} + 0.34 \tag{11.13b}$$

where $\mathrm{Re}_p = D_p U_0 \rho_f / \mu$.

Substitution of equations 11.9, 11.10, and 11.12 into equation 11.8 results in

$$3\pi\mu D_p U_0 = \frac{\pi}{6} D_p^3 (\rho_p - \rho_f) g \tag{11.14}$$

or

$$U_0 = \frac{g D_p^2 (\rho_p - \rho_f)}{18\mu} \tag{11.15}$$

where D_p and ρ_p are particle diameter and density, respectively.

In a centrifugal field, the terminal separation velocity of particles, U_{0c}, is given by the following equation, where the centrifugal acceleration is substituted for the gravitational acceleration:

$$U_{0c} = \frac{r\omega^2 \cdot D_p^2 (\rho_p - \rho_f)}{18\mu} \tag{11.16}$$

or

$$U_{0c} = \frac{g Z D_p^2 (\rho_p - \rho_f)}{18\mu} = Z U_0 \tag{11.17}$$

where $Z = r\omega^2/g$ is the centrifugal factor, r is the radial distance from the central axis of rotation, and ω is angular velocity of rotation ($\omega = 2\pi N r$).

The analysis presented so far is valid only for dilute cell suspensions where particle–particle interactions are negligible. When particle concentration is high, particles interact to form a swarm, and their terminal velocity decreases from U_0 to U. This is known as *hindered settling*. The separation of cells or particles in a centrifugal field is similar to hindered settling under gravity, since particle concentration is high under centrifugation conditions.

In hindered settling, the drag force on particles is

$$F_D' = \frac{1}{g_c} 3\pi\mu D_p U \left(1 + \beta_0 \frac{D_p}{L}\right) \tag{11.18}$$

where U is the terminal velocity of the particles under hindered-settling conditions in a gravitational field, L is the average distance between adjacent particles, and β_0 is the hindered-settling coefficient. β_0 is 1.6 for a rectangular arrangement of particles. In dilute solutions, $D_p/L << 1$, so F_D' approaches F_D. Hindered settling becomes important when D_p and L are comparable.

The parameter β_0 is a function of α, the volume fraction of particles, and the shape of the particles. The following can be shown:

$$\frac{U_c}{U_{0c}} = \frac{1}{1 + \alpha' \alpha^{1/3}} \tag{11.19}$$

where α' is empirically correlated with α and depends on particle shape also. U_c is the terminal velocity of the particles of a given size and shape in the centrifugal field under conditions of hindered settling.

When designing or sizing a centrifuge, we are concerned primarily with the capacity of the centrifuge to handle a given flow rate of broth. If we know both the velocity of the particle in the centrifugal field and the distance the particle must travel to be captured, then we can proceed with the design.

Examples of two popular centrifuges are shown in Figure 11.6. Clearly, the geometry of the centrifuge influences the travel distance.

Consider a simple one-dimensional case where the following is the distance of travel of a particle:

$$y = U_{0c} t \tag{11.20}$$

Substituting equation 11.17 for U_{0c} and noting that the time of interest is $t = V_c/F_c$, we write

$$y = \frac{r\omega^2 D_p^2 (\rho_p - \rho_f)}{18\mu} \cdot \frac{V_c}{F_c} \tag{11.21}$$

where V_c is liquid volume in the centrifuge and F_c is liquid flow rate through the centrifuge.

By substituting $y = L_e/2$ and $r = r_e$ into equation 11.21, we obtain

$$F_c = 2\left[\frac{g D_p^2 (\rho_p - \rho_f)}{18\mu}\right] \frac{r_e \omega^2 V_c}{g L_e} \tag{11.22}$$

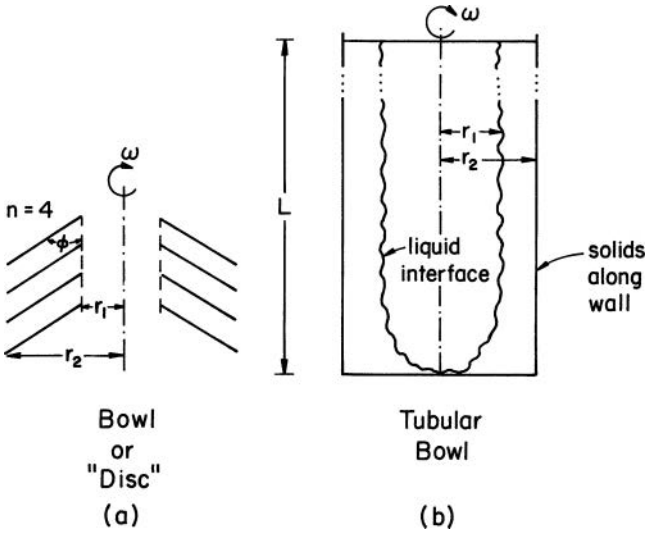


Figure 11.6. Two types of common centrifuges. (a) A disk or bowl centrifuge with multiple separator disks or bowls. The scale-up factor depends on the two radial distances, r_1 and r_2, the angle ϕ, the rotational speed, ω, and n, the number of disks or effective separator bowls. (b) A tubular bowl centrifuge can operate with continuous liquid feed with the span of continuous operation determined by the capacity of the bowl to collect solids. Key scale-up parameters are the distances r_1 and r_2, the height of the unit, L, and the rotational speed, ω.

or

$$F_c = 2U_0\Sigma \tag{11.23}$$

where $\Sigma = r_e\omega^2 V_c/gL_e$, which is known as the *centrifugation coefficient*, U_0 is the free settling velocity of the particles under gravity, L_e is the effective distance of settling ($= 2y$), and r_e is the effective radius of rotation. Σ is equal to the surface area of the gravity settling basin whose separation performance is equal to that of a particular centrifuge.

For a tubular centrifuge, Σ is

$$\Sigma = \frac{2\pi L\omega^2}{g}\left(\frac{3}{4}r_2^2 + \frac{1}{4}r_1^2\right) \tag{11.24}$$

where L is the length of cylindrical separator, and r_1 and r_2 are inner and outer radii of centrifugation distance.

For a bowl centrifuge, Σ is

$$\Sigma = \frac{2\pi n'\omega^2}{3g\tan\phi'}(r_2^3 - r_1^3) \tag{11.25}$$

where n' is the number of separator bowls, and ϕ' is the angle of inclination of the side walls.

Assuming that the free settling velocities of particles are the same, the following equation can be used for the scale-up of centrifuges:

$$\frac{F_{c_2}}{F_{c_1}} = \frac{\Sigma_2}{\Sigma_1} \tag{11.26}$$

where F_{c_1} and F_{c_2} are the flow rates through the small and large centrifuges, respectively.

Cross-flow microfiltration processes (Section 11.4.7) have replaced centrifugation in many bioprocesses, particularly when it is essential to prevent the escape of cells into the atmosphere. This consideration is particularly relevant when the organism is genetically modified. However, with recent improvements, the trend has gone back toward a combination of centrifugation and depth filtration.

11.2.3. Coagulation and Flocculation

Coagulation and flocculation are usually used to form cell aggregates before centrifugation, gravity settling, or filtration to improve the performance of these separation processes. Coagulation is the formation of small flocs from dispersed colloids using coagulating agents, which are usually simple electrolytes. Flocculation is the agglomeration of these small flocs into larger settleable particles using flocculating agents, which are usually polyelectrolytes or certain salts, such as $CaCl_2$.

Simple electrolytes used in coagulation are acids, bases, salts, and multivalent ions, which are relatively inexpensive but less effective than polyelectrolytes. Certain fine solid particles, such as clays, activated carbon, or silica, may serve as nucleation sites for coagulation. Polyelectrolytes used in flocculation are high-molecular-weight, water-soluble organic compounds, which can be anionic, cationic, or nonionic.

The analysis of gravity sedimentation is based on Stokes's and Newton's laws and is similar to the analysis presented in the centrifugation section. Sedimentation is a critical feature in the activated waste-treatment process and has been applied in some schemes to stabilize a population of genetically engineered cells. A list of widely used flocculants and their dosage ranges is given in Table 11.1. The important parameters to be considered in flocculation are flocculant–coagulant concentration, concentration of colloidal cells, kinetics of the binding, and settling phenomena. The selection criteria usually are flocculation–sedimentation rate, floc size, and the clarity of the supernatant liquid.

These processes are particularly important in waste-treatment processes or for nontherapeutic products because addition of chemicals can be problematic for production of therapeutic products, particularly proteins. However, the drive for more efficient, lower-cost production is forcing industry to reevaluate these techniques even for therapeutic proteins, as they can reduce costs.

11.3. CELL DISRUPTION

After cells are separated from liquid broth, if the desired product is intracellular, then the cells need to be disrupted to release the intracellular products. The method of disruption varies with the type of cells and the nature of intracellular products. Major methods of

TABLE 11.1. Flocculant Dosages

Agent	Flocculant Dose [g/(100 g dry cell wt)-1]				
	Glucose Broth	Hydrocarbon Broth	Resuspended Cells in Buffer	Penicillin Broth	Dilute Slurries
Polyelectrolytes					0.045–4.5
Anionic polyelectrolytes					
Polystyrene sulphate	0.2	0.1	0.06		
Polyacrylamide	Ineffective	Ineffective	Ineffective		
Cationic polyelectrolytes					
Polyethylene imine	10		7.0		
Calcium chloride				200	
Colloidal clay, bentonite	2.0	20.0	0.6		
Inorganic coagulants					0.045–4.5

B. Atkinson and F. Mavituna, *Biochemical Engineering and Biotechnology Handbook*, Macmillan, Inc., New York, 1983. Reproduced with permission of Palgrave Macmillan.

cell disruption can be classified as mechanical and nonmechanical. With small bacteria, efficient, large-capacity cell-disruption processes can be difficult to construct. Animal cells are readily disrupted, so we focus here on bacteria, yeasts, and molds.

11.3.1. Mechanical Methods

Mechanical methods can be applied to a liquid or solid medium. First, consider some methods applied to a liquid medium.

Ultrasonic vibrators (sonicators) are used to disrupt the cell wall and membrane of bacterial cells. Wave density is usually around 20 kc/s. Rods are broken more readily than cocci, and gram-negative cells are broken more easily than gram-positive cells. This method is not as effective for molds. An electronic generator is used to generate ultrasonic waves, and a transducer converts these waves into mechanical oscillations by a titanium probe immersed in a cell suspension. Intracellular compounds (enzymes, metabolites) are released into the broth upon cell disruption. Ultrasonic disruption in some cases results in denaturation of sensitive enzymes and fragmentation of cell debris. Heat dissipation is an important problem in cell disintegration, particularly if the volume subjected to sonication is large. Consequently, this method is used primarily at the laboratory scale.

The Gaulin–Manton and French presses work well on a laboratory scale. The French press is a hollow cylinder in a stainless-steel block that is filled with cell paste and subjected to a high pressure. The cylinder has a needle valve at the base, and the cells disrupt as they are extruded through the valve to atmospheric pressure. The Ribi fractionator is similar to a French press but is capable of continuous operation. It requires a preconcentrated cell paste or slurry. The Rannie high-pressure homogenizer is advertised to operate up to 1000 bar and to have a capacity of up to 40,000 l/h. A major disadvantage of this technique can be generation of heat, so these devices require effective strategies for cooling.

Other mechanical homogenizers make use of small beads (20 to 50 mesh). Some of these can be effectively used at rather large scales. The Dyno-Mill is available in sizes up to 275 l and can process 2000 kg/h of a cell suspension or about 340 kg dw/h of yeast. The Dyno-Mill can work with algae, bacteria, and fungi. The principle of operation is to pump the cell suspension through a horizontal grinding chamber filled with about 80% beads. Within the grinding chamber is a shaft with specially designed disks. When rotated at high speeds, high shearing and impact forces from millions of beads break cell walls. The broken cells are then discharged. A primary advantage of bead over pressure devices is better temperature control. Also, this device is fully enclosed, preventing release of organisms in an aerosol, which is a critical consideration with pathogenic or genetically modified organisms.

For a solid medium, such as frozen cell paste or cells attached to or within a solid matrix, different processing options are available. Ball mills can be operated on large-scale processes. More specialized pieces of equipment include the Hughes and X-presses. The Hughes press consists of a split block with a half-cylinder hollowed in each face. The frozen cell paste is placed in the hollow, and pressure is applied to it from the cylinder into channels cut in the block. In the X-press, frozen cells are forced continuously through a small hole in a disk between two cylinders at low temperature and pressure. Cell disruption takes place because of deformation of organisms embedded in the ice. Pressure levels of several hundred bars can be obtained with the X-press.

With any of these approaches, there is potential for damage to the product. Clearly, heat denaturation is a major potential problem. The release of proteases from cellular compartments can lead to enzymatic degradation of the product, so addition of protease inhibitor cocktails is often employed. Some techniques, such as bead mills, have comparatively long residence times, so that if 100% cell breakage is desired, some fragile compounds released early in the process may be damaged. Once cell breakage occurs, products are released and encounter an oxidizing environment (due to air) that can cause, for protein products, rapid denaturation and aggregation. Typically, this effect can be mitigated by addition of a reducing agent (e.g., β-mercaptoethanol) and a chelating agent (e.g., EDTA) to complex metal ions that may accelerate the oxidation process. Product quality considerations are essential to selection of a method for cell breakage.

11.3.2. Nonmechanical Methods

Osmotic shock and rupture with ice crystals are commonly used methods. By slowly freezing and then thawing a cell paste, the cell wall and membrane may be broken, releasing enzymes into the media. Changes in the osmotic pressure of the medium may result in the release of certain enzymes, particularly periplasmic proteins in gram-negative cells.[†] Cell-wall preparation of certain organisms can be made by heat-shocking. Freeze-drying after treatment with acetone, butanol, and buffers results in cell-wall disruption.

Enzymes such as lysozyme (a carbohydrase) can be used to lyse cell walls of bacteria. Gram-positive bacteria are far more susceptible to enzymatic lysis than are gram-negative bacteria. The cells may be treated with ethylenediaminetetraacetic acid (EDTA)

[†] The periplasmic space in a gram-negative cell is the space between the inner and outer membranes.

or by freezing and thawing before treatment with lysozyme. EDTA is a chelating agent; high levels of EDTA will extract divalent ions that are part of the cell envelope. Enzymatic hydrolysis is an expensive method and is not very widely used in industry.

Actively growing cells can be treated with an antibiotic, such as penicillin or cycloserine, that interferes with cell-wall synthesis and, coupled with the correct osmotic conditions, can lead to cell disruption.

After cells have been lysed and the products are released into the medium, cell debris can be separated by ultracentrifugation or ultrafiltration, and soluble products are recovered using methods discussed in the following sections. As with mechanical methods, products in the cell-free broth are subject to denaturation and aggregation, and these problems can be mitigated using the same techniques as used for broths from mechanical breakage.

11.4. SEPARATION OF SOLUBLE PRODUCTS

Most fermentation or biotechnological products, such as antibiotics, organic acids, solvents, amino acids, therapeutics, proteins, and extracellular enzymes, are soluble and extracellular. Various methods have been developed to recover such soluble products, including extraction, adsorption, ultrafiltration, and chromatography.

11.4.1. Liquid–Liquid Extraction

Liquid–liquid extraction is commonly used to separate inhibitory fermentation products such as ethanol and acetone–butanol from a fermentation broth. Antibiotics are also recovered by liquid extraction (using organic solvents such as amylacetate or isoamylacetate). Ideally, the liquid–liquid extractant should be nontoxic, selective, inexpensive, and immiscible with fermentation broth and should have a high distribution coefficient for the product. Typically liquid–liquid extraction is not used for proteins because of the danger of solvent denaturation of the protein.

The extraction of a compound from one phase to the other is based on solubility differences of the compound in one phase relative to the other. When a compound is distributed between two immiscible liquids, the ratio of the concentrations in the two phases is known as the distribution coefficient:

$$K_D = \frac{Y_L}{X_H} \tag{11.27}$$

where Y_L and X_H are concentrations of the solute in light and heavy phases, respectively. In most, but certainly not all, cases the light phase will be the organic solvent and the heavy phase will be the aqueous fermentation broth.

Assuming that K_D is constant and the solvents are totally immiscible (i.e., the mass flows of the light and heavy phases are conserved so that $L_0 = L_1 = L$ and $H_0 = H_1 = H$), a mass balance on the extracted solute yields (Figure 11.7a):

$$H(X_0 - X_1) = L Y_1 \tag{11.28}$$

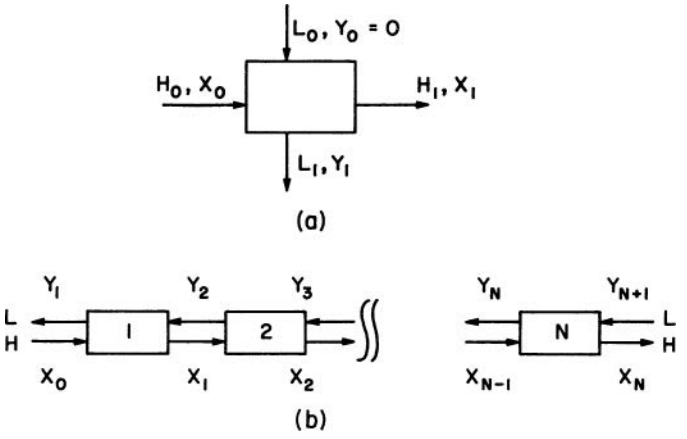


Figure 11.7. (a) Single-stage and (b) multistage countercurrent liquid extraction systems.

or

$$X_1 = X_0 - \frac{L}{H} Y_1 \tag{11.29}$$

Since $K_D = Y_1/X_1$, equation 11.29 can be written as

$$X_1 = X_0 - \frac{LK_D}{H} X_1 \tag{11.30}$$

$$\frac{X_1}{X_0} = \frac{1}{1+(LK_D/H)} = \frac{1}{1+E} \tag{11.31}$$

where $E = LK_D/H$ is the extraction factor.

For a multistage countercurrent operation, as depicted in Figure 11.7b, a material balance on extracted solute yields

$$R = \frac{E(E^n - 1)}{E - 1} \tag{11.32}$$

where R is the rejection ratio, which is the weight ratio of the solute leaving in the light phase to that leaving in the heavy phase, and n is the number of equilibrium stages.

Then the fraction of solute extracted is

$$\%\ \text{extraction} = 1 - \frac{1}{R+1} \tag{11.33}$$

When the solute enters the system in the light phase, the rejection ratio is

$$R = \frac{E^n(E-1)}{E^n - 1} \tag{11.34}$$

where n is the number of stages. Figure 11.8 depicts the relationship between E, X_n/X_0, and n, which is used to determine the number of stages (n) required for a certain degree of extraction (X_n/X_0) for a given system (E).

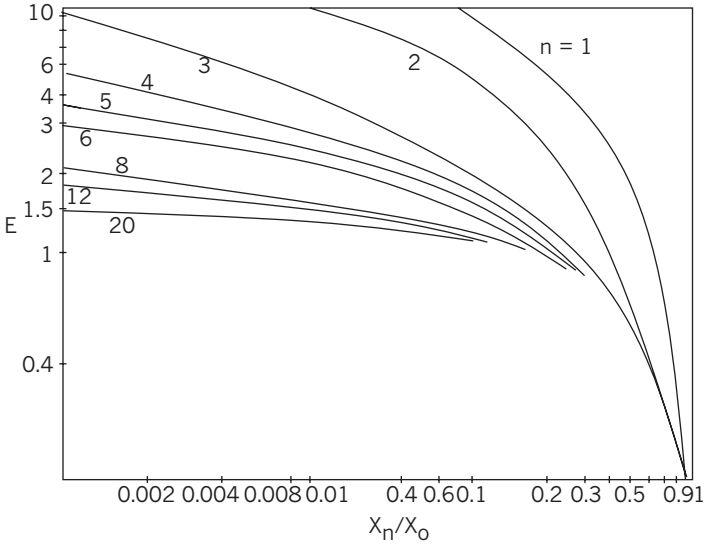


Figure 11.8. Variation of the extraction factor (E) with the degree of extraction (X_n/X_o) for different number of stages (n) in multistage continuous countercurrent extraction.

Most antibiotics are extracted from the fermentation broth by using organic solvents such as amylacetate or isoamylacetate. Usually, continuous *centrifugal Podbielniak extractors* are used for the extraction of antibiotics. Penicillin is more soluble in organic phase at low pHs (pH = 2 to 3) and is highly soluble in aqueous phase at high pHs (pH = 8 to 9). Therefore, penicillin is extracted between organic and aqueous phases several times by shifting the pH to improve the purity of the product (often known as *pH-swing extraction*).

Some products to be recovered from the fermentation broth are weak acids or weak bases. Since compounds that are not ionized are soluble in the organic phase, the pH conditions are selected such that the extracted compound is neutral and is soluble in an organic solvent. Therefore, weak bases are extracted at high pHs and weak acids are extracted at low pH values in the neutral form.

If fermentation broth contains more than one component, then the selectivity (β) of the solvent becomes an important parameter. The selectivity coefficient (β_{ij}) is defined as

$$\beta_{ij} = \frac{K_i}{K_j} \tag{11.35}$$

The ease of separation depends on the β_{ij} value: the higher the β_{ij} value, the easier the separation of i from j. In some cases, the β_{ij} value changes appreciably with a pH shift, and the separation becomes easier. For example, at low pH values, penicillins can be separated from other impurities by selective extraction into the organic solvent. However, the separation of similar compounds by extraction is difficult. If only nonionized species are soluble in both phases at extreme pH values and the solvents are immiscible, the aforementioned design equations can be used. However, at intermediate pH values, when compounds are partially ionized, the analysis of the system becomes more complicated. The extraction of ionized species (weak acid or bases) is known as *dissociation extraction*.

The apparent distribution coefficients for weak acids and bases follow:

$$\text{Weak acids:} \quad K_D^{AP} = \frac{K_D^0[H^+]}{[H^+]+K_1} \text{ or } \text{pH} - \text{p}K_1 = \log\left(\frac{K_D^0}{K_D^{AP}} - 1\right) \tag{11.36}$$

$$\text{Weak bases:} \quad K_D^{AP} = \frac{K_D^0 K_1}{K_1+[\text{H}^+]} \text{ or } \text{p}K_1 - \text{pH} = \log\left(\frac{K_D^0}{K_D^{AP}} - 1\right) \tag{11.37}$$

In equations 11.36 and 11.37, K_D^{AP} is the apparent distribution coefficient, K_D^0 is the distribution coefficient for neutral species, and K_1 is the dissociation equilibrium constant for weak acids or bases.

A particularly important device for liquid–liquid extraction for fermentation products is the Podbielniak centrifugal extractor (see Figure 11.9). Many fermentation products (e.g., penicillin) are unstable. The use of mixer–settlers can be problematic, because the residence time of the product in the pH-adjusted broth is too long. The rapid rotation of the Podbielniak extractor produces a centrifugal field that rapidly drives the two fluids countercurrent to each other, as depicted in Figure 11.9. A product can be extracted and returned to another aqueous phase (e.g., a phosphate buffer) within minutes.

Example 11.2

Penicillin is extracted from a fermentation broth using isoamylacetate as the organic solvent in a continuous countercurrent cascade extraction unit. The flow rates of organic (L) and aqueous (H) phases are $L = 10$ l/min and $H = 100$ l/min, respectively. The distribution coefficient of penicillin between organic and aqueous phases at $\text{pH} = 3$ is $K_D = Y_L / X_H = 50$. If the penicillin concentration in the feed stream is 20 g/l,

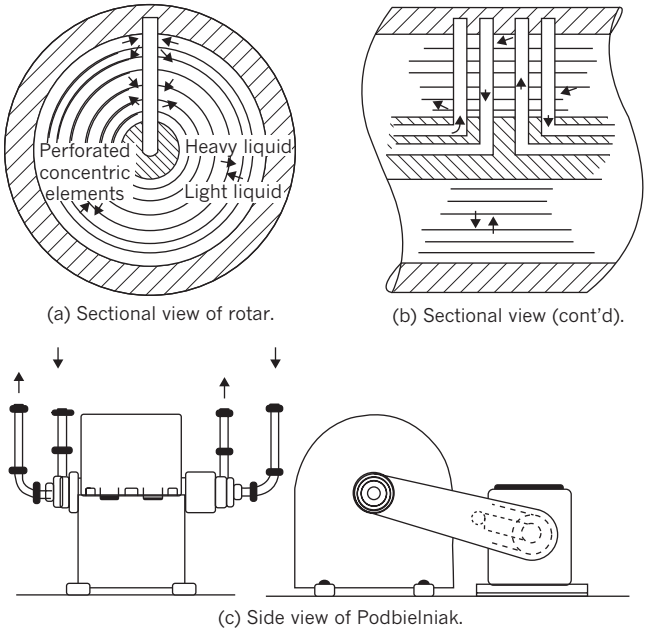


Figure 11.9. Liquid–liquid extraction equipment (Podbielniak). Bold arrow indicates heavy liquid flow; open arrow represents the flow of a mixture or light liquid. (With permission, from S. Aiba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, 2d ed., University of Tokyo Press, Tokyo, 1973.)

determine the number of stages required to reduce the penicillin concentration to 0.1 g/l in the effluent of the extraction unit.

Solution

$$E = \frac{LK_D}{H} = \frac{(10)(50)}{(100)} = 5$$

$$\frac{X_n}{X_0} = \frac{0.1}{20} = 5 \times 10^{-3}$$

Using Figure 11.8, we can obtain $n = 4$. If a Podbielniak centrifugal extractor were used, it would have to correspond to four ideal stages.

11.4.2. Aqueous Two-Phase Extraction

Aqueous two-phase extraction is an approach under active development for the extraction of soluble proteins such as enzymes between two aqueous phases containing incompatible polymers, such as polyethylene glycol (PEG) and dextran. The phases containing PEG and dextran are more than 75% water and are immiscible. Typical aqueous phases used for this purpose are PEG–water/dextran–water and PEG–water/K-phosphate–water. PEG/dextran and PEG/K phosphate are reasonably immiscible. The partition coefficient, K_p, varies with the molecular weight (MW) of the soluble protein in the form of an exponential function:

$$K_p = e^{AM/T} \tag{11.38}$$

where M is the MW of protein, T is the absolute temperature, and A is a constant.

The partition coefficient, K_p, of many enzymes between the two phases ($C_{\text{PEG}} - C_{\text{DEX}}$) varies between 1 and 3.7, resulting in poor separations in a single stage. The partition coefficient can be improved by including ion-exchange resins or certain salts, such as $(NH_4)_2SO_4$ and KH_2PO_4, in one of the phases. Figure 11.10 depicts the variation of K_p of an enzyme with a concentration of KH_2PO_4 in a PEG/$(NH_4)_2SO_4$ system. More than tenfold increases in K_p values may be achieved by increasing the concentration of KH_2PO_4 from 0.1 to 0.3 M. In PEG–salt systems, salting-out may result in protein precipitation at the interface.

Ion-exchange resins can be used to increase the value of a partition coefficient. PEG can be derivatized to yield cation- or anion-exchange properties and used in a two-phase system. Affinity ligands may also be used to increase K_p values; examples are PEG–NADH and PEG–cibacron blue systems. This method is known as *two-aqueous-phase affinity partition extraction.* Also, partial hydrolysis of dextran and PEG may result in an increase in the K_p value, since lower-MW polymers may interact with proteins more effectively. By mixing two kinds of PEGs (PEG_{400} and PEG_{4000}), the K_p value may be increased by a factor of 6 for the partition of fumarase. Two-aqueous-phase extraction can also be used for the recovery of cell debris, polysaccharides, virus or virus-like particles (VLP), and nucleic acids. The partition coefficient for whole cells and DNA is between 100 and 0.01, for proteins it is between 10 and 0.1, and for small ions it is around 1.

After the extraction step, the phases can be separated by centrifugation or decantation, and PEG can be recovered by ultrafiltration. Figure 11.11 is a block diagram of an enzyme separation unit using the two-phase partitioning method with

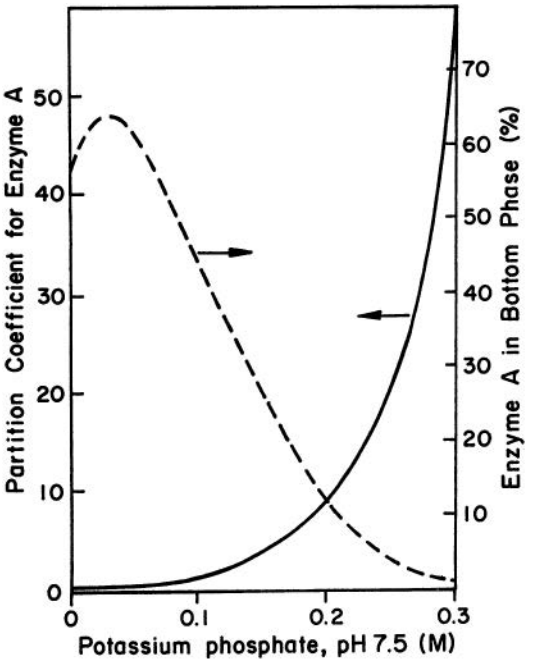


Figure 11.10. The expected behavior of an enzyme in a two-phase solution. Note the dependence of the partition coefficient of a hypothetical protein on the concentration of potassium phosphate in a polyethylene glycol 4000 ammonium sulphate system. A typical condition would be 14% PEG 4000 and 9.5% $(NH_4)_2SO_4$.

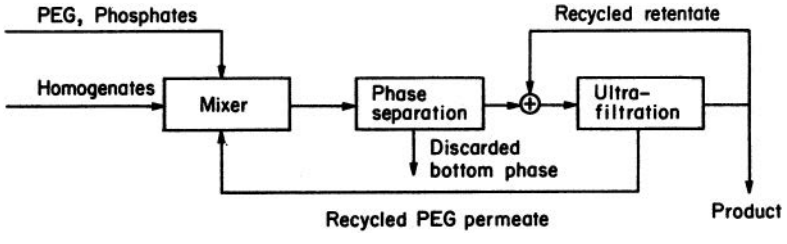


Figure 11.11. Two-phase extraction process with PEG recovery.

PEG recovery. This separation method is fast and can be operated under mild conditions of temperature, pressure, and pH. Dextran and PEG are recovered and reused, since the cost of polymers is the major economic factor.

11.4.3. Precipitation

The first step in the purification of intracellular proteins after cell disruption is often *precipitation.* Proteins in a fermentation broth (before or after cell lysis) can be separated from other components by precipitation using certain salts. Examples include streptomycin sulfate and ammonium sulfate.

The two primary methods used for protein precipitation are

- Salting-out by adding inorganic salts such as $(NH_4)_2SO_4$ at high ionic strength.
- Solubility reduction at low temperatures by adding organic solvents ($T < -5°$).

Salting-out of proteins is achieved by increasing the ionic strength of a protein-containing solution by adding salts such as $(NH_4)_2SO_4$ or Na_2SO_4. The added ions interact with water more strongly, causing protein molecules to precipitate. The solubility of proteins in a solution as a function of the ionic strength of the solution is given by

$$\log \frac{S}{S_0} = -K_S' I \tag{11.39}$$

where S is the solubility of protein in solution (g/l), S_0 is the solubility of protein when $I = 0$, I is the ionic strength of solution, and K_S' is the salting-out constant, which is a function of temperature and pH.

The ionic strength of a solution is defined as

$$I = \frac{1}{2} \sum C_i Z_i^2 \tag{11.40}$$

where C_i is molar concentration of the ionic species (mol/l), and Z_i is the charge (valence) on ions.

Figure 11.12 depicts variation of the solubility of hemoglobin with inorganic salt concentrations. At high ionic strengths, the solubility of the protein decreases logarithmically with ionic strength.

Organic solvent addition at low temperatures ($T < -5°C$) causes the precipitation of proteins by reducing the dielectric constant of the solution. The solubility of protein as a function of the dielectric constant of a solution is given by

$$\log \frac{S}{S_0} = -K'/D_s^2 \tag{11.41}$$

where D_s is the dielectric constant of the water–solvent solution.

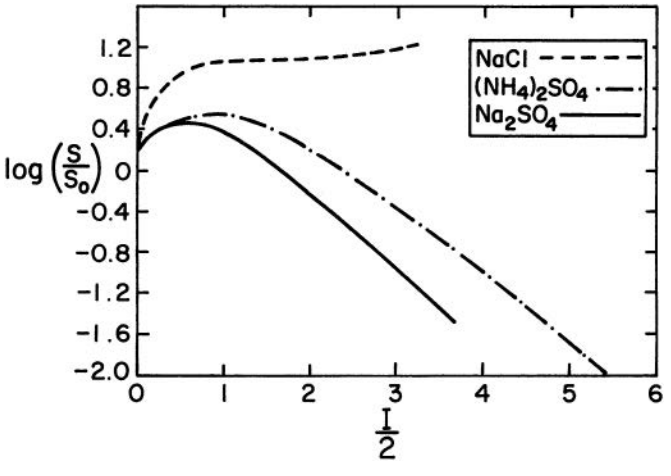


Figure 11.12. Effect of inorganic salts on solubility of a typical protein.

A reduction in the dielectric constant of a solution results in stronger electrostatic forces between the protein molecules and facilitates protein precipitation. The addition of solvents also reduces protein–water molecule interactions and therefore decreases protein solubility. Solvents may cause protein denaturation. However, denaturation of proteins in the salting-out method is less likely. *Solvent precipitation* can be used with salt addition, pH adjustment, and low temperature to improve precipitation. Among other protein precipitation methods, the following methods are the most widely used.

Isoelectric precipitation is the precipitation of proteins at their isoelectric point, which is the pH at which proteins have no net charge. The isoelectric point of a protein is defined as $\mathrm{p}I = \frac{1}{2}(\mathrm{p}K_1 + \mathrm{p}K_2)$. When $\mathrm{pH} = \mathrm{p}I$, protein becomes free of charge and precipitates. This method is effective for proteins with high surface hydrophobicity (i.e., a nonpolar surface). It is inexpensive, but low pH values may result in the denaturation of proteins.

The use of ionic polyelectrolytes, such as ionic polysaccharides, polyphosphates, and polyacrylic acids, changes the ionic strength of the media and causes protein precipitation. However, polyelectrolytes may cause protein denaturation and structural damage.

The use of nonionic polymers, such as dextrans and polyethylene glycol, reduces the amount of water available to interact with protein molecules and results in protein precipitation. High concentrations of polymers are most effective for low-MW proteins, and nonionic polymers do not interfere with further protein recovery.

11.4.4. Dialysis

Dialysis is a membrane separation operation used for the removal of low-MW solutes such as organic acids (100 da < MW < 500 da) and inorganic ions (10 da < MW < 100 da) from a solution. It is generally a small- to medium-scale operation. A well-known example is the use of dialysis membranes to remove urea (MW = 60 da) from urine in artificial kidney (dialysis) devices. In biotechnology, dialysis can be used to remove salts from a protein solution, which is often a step in resolubilizing proteins that were initially in inclusion bodies. A schematic of a dialysis membrane is depicted in Figure 11.13.

Note that the membrane is selective. The dialysis membrane separates two phases, one of which contains low-MW compounds and the other high-MW compounds. Since the MW cutoff of a dialysis membrane is very small, low-MW solutes

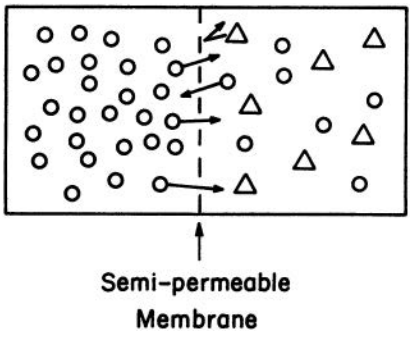


Figure 11.13. A typical dialysis membrane separation. Low-MW component 1 (○) diffuses through membrane from high- to low-concentration region. High-MW component (Δ) cannot pass.

can move from a high- to a low-concentration region across the membrane while the high-MW compounds are retained in a single compartment. At equilibrium, the chemical potentials of diffusing compounds on both sides of a membrane are equal:

$$\mu_1^{\alpha} = \mu_1^{\beta} \tag{11.42}$$

where μ is the chemical potential of the diffusing compound. In terms of concentrations, equation 11.42 can be written as

$$RT \ln C_1^{\alpha} \gamma_1^{\alpha} = RT \ln C_1^{\beta} \gamma_1^{\beta} \tag{11.43}$$

or

$$C_1^{\alpha} \gamma_1^{\alpha} = C_1^{\beta} \gamma_1^{\beta} \tag{11.44}$$

where C_1 is the concentration and γ_1 is the activity coefficient of diffusing component 1. For ideal (very dilute) solutions, $\gamma_1 \approx 1$ and

$$C_1^{\alpha} = C_1^{\beta} \tag{11.45}$$

The concentrations represented in equations 11.42 through 11.45 are concentrations of dissolved (unbound) component 1; if the compound binds to a surface, then an equilibrium relationship between bound and unbound components needs to be considered.

This dialysis equilibrium is based on the assumption of uncharged solute molecules. If macromolecules are polyelectrolytes, such as proteins and nucleic acids, then the charge equilibrium of the macromolecules must be considered. This equilibrium is known as the *Donnan equilibrium* and requires a more complete analysis.

11.4.5. Reverse Osmosis

Osmosis is the transport of water molecules from a high- to a low-concentration region (i.e., from a pure water phase to a salt-containing aqueous phase) when these two phases are separated by a selective membrane. The water passes the membrane easily, whereas the salt does not. At equilibrium, the chemical potential of water must be the same on both sides of the membrane. As the water passes into the salt solution, its pressure increases. This *osmotic pressure* can be expressed as

$$\pi = CRT(1 + B_2C + B_3C^2 + \ldots) \tag{11.46}$$

where C is the molar concentration of the solute, T is temperature, R is the gas constant, and B_2, B_3 are the virial coefficients for the solute. For very dilute, ideal solutions, $B_2 = B_3 = 0$, and

$$\pi = CRT \tag{11.47}$$

In *reverse osmosis* (RO), a pressure is applied onto a salt-containing phase, which drives water (solvent) molecules from a low- to a high-concentration region and results in the concentration of solute (salt) molecules on one side of the membrane. The pressure required to move solvent from a low- to high-concentration phase is equal to or slightly larger than the osmotic pressure. When $\Delta p > \pi$, a solvent flux takes place in the direction against the concentration gradient (see Figure 11.14).

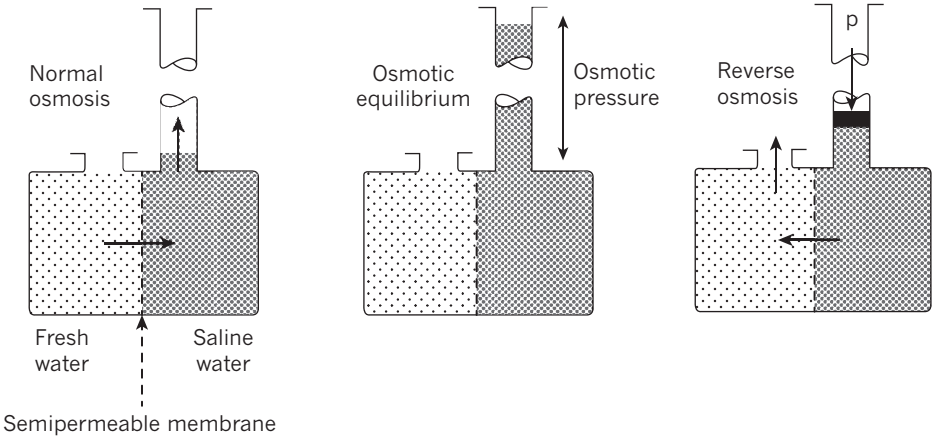


Figure 11.14. Osmotic flows across a membrane separating fresh water and saline water. (D. W. Sundstrom, H. E. Klei, *Wastewater Treatment* © 1979.)

In some RO applications, membranes may allow the passage of solute molecules along with solvent. Solute transport can be by diffusion or convection. A *reflection coefficient* (σ) for each solute can be defined as the fraction of solute molecules retained on one side of the membrane in the presence of a solvent flux. Therefore, for $\sigma = 0$, complete solute passage is obtained, and for $\sigma = 1$, no solute passage is achieved (that is, perfect reflection). Solvent and solute fluxes in RO can be expressed as

$$\text{Solvent:} \quad N_1 = K_p(\Delta P - \pi) \tag{11.48}$$

$$\text{Solute:} \quad N_2 = C(1-\sigma)N_1 + K_{p'}\,\Delta C \tag{11.49}$$

where K_p and $K_{p'}$ are permeability coefficients for solvent and solute, respectively, C is the average solute concentration in solution, and ΔC is the solute concentration difference across the membrane.

The magnitude of the required pressure varies with the concentration of solutions. Pressures on the order of 30 to 40 atm are required for a 0.6 M salt solution. The salt level in fermentation fluids may be much higher than 0.6 M, requiring high pressures for RO separations. The applications of RO in bioseparations are limited, since the method requires high pressures and is based on solvent removal. RO membranes are usually used for dewatering and concentration purposes but not for protein separations.

Another problem with RO membranes (and membranes in general) is the deposition of solute molecules on membrane surfaces, resulting in large resistances for solvent flow. This phenomenon, known as *concentration polarization*, can be overcome by increasing the degree of turbulence on the membrane surface.

The osmotic pressure for multicomponent systems is equal to the sum of the individual osmotic pressures:

$$\pi = \sum_i \pi_i = \sum_i C_i RT(1 + B_{2i}C_i + B_{3i}C_i^2 + \ldots) \tag{11.50}$$

The solvent and solute fluxes in this case are

$$N_1 = K_{pl}(\Delta P - \pi) \tag{11.51}$$

$$N_i = C_i(1 - \sigma_i)N_1 + K'_{pi}\Delta C_i; \quad i \geq 2 \tag{11.52}$$

Example 11.3

Desalination of sea water with a salt (NaCl) content of 50 g/l is realized by using an RO membrane cartridge. Saline water flow rate is 10 m^3/h, and the applied pressure is 25 atm. The filtrate is open to the atmosphere. Permeability (K_p) of the RO membrane is 0.5 m^3/m^2 atm-h. The MW of NaCl is 58.5 g/mol.

a. Determine osmotic pressure of saline water at 25°C.
b. Determine the required surface area of the RO membrane.
c. Determine the number of tubes in a multitubular RO membrane system if D_o = 0.5 cm and L = 50 cm.

Assume the desired filtrate flow rate is 90% of the feed flow rate.

Solution

a. Assuming dilute solution:

$$\pi = C\,RT = (50 \text{ g/l}/58.5 \text{ g/mol})\ (0.082 \frac{L \cdot atm}{K - mol})\ (298\ K) = 20.9 \text{ atm}$$

b. $F = K_p A\ (\Delta P - \pi)$

Note that $\Delta P = 25 - 1 = 24$ atm and $F = 0.9(10) = 9$ m^3/h

9 m^3/h = 0.5 m^3/m^2/atm-h $A(24 - 20.9)$ atm, $A = 5.80$ m^2

c. $A = N\,\pi\,D_o L$, 5.80 $m^2 = N(3.14)\ (5.10^{-3}\text{m})\ (0.5\text{m})$, solving for N,

$N = 739$ tubes

11.4.6. Ultrafiltration and Microfiltration

Membranes are widely used to concentrate or separate solute molecules such as proteins on the basis of their size and to concentrate cells from fermentation broth. Membrane separations may be used to separate proteins after precipitation to remove many of the low-MW contaminants. Membranes can serve as a molecular sieve to separate solute molecules of different molecular size. Depending on molecular-size cutoff, different membranes can be used for the separation of different MW proteins. *Microfiltration* or *microporous filtration* (MF) is used to separate cells, such as bacteria and yeast, that range from 0.1 to 10 μm in size. Ultrafilters are used for macromolecules with a molecule-weight range of 2000 to 500,000 dalton. All these membrane sieving methods (microfiltration, ultrafiltration, reverse osmosis) are based on the same driving force, namely pressure, but have some minor differences. Some *ultrafiltration* (UF) membranes have *anisotropic structure*. In an anisotropic membrane, a thin skin with small pores is formed on top of a thick, highly porous structure. The thin layer provides selectivity,

while the thicker layer provides mechanical support. With newer membrane materials available, anisotropic membranes maybe used less frequently. *Microporous filters* are usually isotropic and may have an open, tortuous path structure, which causes particle entrapment within the filter, or they may have well-defined pores of uniform size.

Although most membranes in use are made from polymers, ceramic membranes are also available. Factors that affect choice of membrane materials are interactions with proteins, mechanical stability, chemical stability (especially to cleaning agents), biocompatibility, flux rates, ease of sterilization (e.g., thermal stability), and cost. Typical polymeric materials are cellulose acetate, nylon, polytetrafluroethylene (PTFE), polyvinyldinedifluoride (PVDF), and polysulfone. Ceramic membranes have a high level of chemical resistance, can be steam sterilized, and have a much longer life time (ca. 10 yr) compared to many polymeric membranes. Such membranes have high initial cost and, while mechanically strong, are sometimes brittle. The actual choice of membrane depends on the application, the composition of the feed stream, and required product characteristics.

UF and MF membranes are widely used in the pharmaceutical, chemical, and food industries for the separation of vaccines, fermentation products, enzymes, and other proteins. UF is an energy-efficient, economical separation method used to concentrate chemicals and biologicals at a high degree of purity.

Typical UF and MF operations are pressure-driven processes in which low-MW solutes and water pass through the filter and high-MW solutes are retained near the membrane surface. Therefore, a concentration gradient builds up between the surface of the membrane and the bulk fluid. This gradient results in concentration polarization. As a result, solute diffuses back from the membrane surface to the solution (Figure 11.15). At steady state, the rate of convective transfer of solute toward

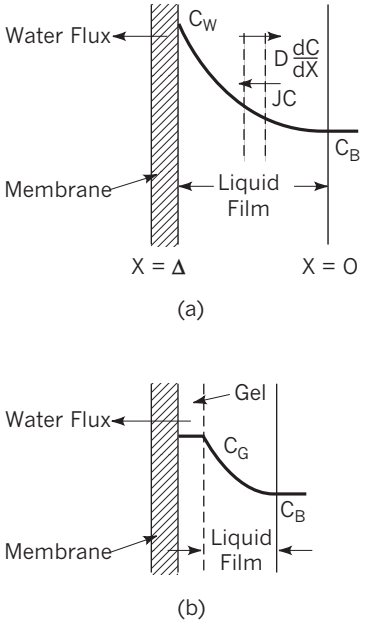


Figure 11.15. Solute transfer in a UF membrane: (a) without gel formation; (b) with gel formation.

membrane is equal to the rate of diffusion of solute in the opposite direction because of concentration polarization:

$$D_e \frac{dC}{dX} = JC \tag{11.53}$$

where D_e is the effective diffusivity of solute in liquid film (cm^2/s), J is the volumetric filtration flux of liquid ($cm^3/cm^2 s$), and C is the concentration of solute (mol/cm^3 liquid).

Integration of equation 11.53 with the boundary conditions of $C = C_B$ at $X = 0$ and $C = C_W$ at $X = \delta$ yields

$$J = \frac{D_e}{\delta} \ln \frac{C_w}{C_B} \tag{11.54}$$

or

$$J = k \ln \frac{C_w}{C_B} \tag{11.55}$$

where $k = D_e/\delta$ is the mass transfer coefficient, and δ is film thickness.

The mass transfer coefficient is a function of fluid and solute properties and flow conditions. It is embedded in the Sherwood number (Sh) and is correlated with Re (Reynolds number) and Sc (Schmidt number):

$$\mathrm{Sh} = \frac{dk}{D_e} = a\,\mathrm{Re}^b\,\mathrm{Sc}^b \tag{11.56}$$

Here $\mathrm{Re} = dv\rho/\mu$, $\mathrm{Sc} = \mu/\rho D_e$, and $\mathrm{Sh} = kd/D_e$, where v is fluid velocity. The value of a is $\frac{1}{3}$ according to the boundary-layer theory; b is approximately 0.5 for laminar flow and 1.0 for turbulent flow. That is, $k \propto v^{0.5}$ for laminar and $k \propto v$ for turbulent flow.

A gel layer is formed on the surface of the UF membrane when slowly diffusing, large macromolecules accumulate at the surface. A gel layer forms if the protein concentration in the solution exceeds 0.1%. For biological fluids, protein accumulation is usually dominant, as shown in Figure 11.15. The protein concentration in the gel, C_G, is the maximum value of C_W. In this case, the liquid flux through the filter is given by

$$J = k \ln \frac{C_G}{C_B} \tag{11.57}$$

Gel formation depends on the nature and concentration of the solute, pH, and pressure. Once gel is formed, C_G becomes constant, and liquid flux decreases logarithmically with increasing solute concentration in the bulk liquid. The gel layer causes a hydraulic resistance against flow and acts somewhat like a second membrane.

Figure 11.16 depicts the variation of liquid flux with the log of solute concentration in the absence and presence of gel formation.

11.4.7. Cross-Flow Ultrafiltration and Microfiltration

Gel formation can be partially eliminated by *cross-flow filtration*, where pressure is not applied directly perpendicular to the membrane but parallel to the membrane surface. This process is also called *tangential flow filtration.* That is, fluid flows parallel to the membrane surface, and a portion of the fluid passes through the membrane, leaving solutes in a liquid phase above the membrane. Mechanical agitation or vibration of the membrane surface can also be used to alleviate gel formation. A schematic of cross-flow filtration using a hollow fiber configuration is shown in Figure 11.17.

The pressure drop driving fluid flow in the seed flow channel is

$$\Delta P = P_i - P_0 \tag{11.58}$$

For laminar flow, using the Hagen–Poiseuille equation, we can derive the following:

$$P_i - P_0 = \Delta P = \frac{C_1 \mu L V}{d^2} = \frac{C_2 \mu L Q}{d^4} \tag{11.59}$$

where L is the length of the tube, μ is fluid viscosity, Q is the volumetric flow rate of the liquid, C_1 and C_2 are constants, and d is diameter of the tube.

For turbulent flow, the following equation is used for ΔP:

$$P_i - P_0 = \Delta P = \frac{C_3 \mu L V^2}{d} = \frac{C_4 f L Q^2}{d^5} \tag{11.60}$$

where f is the Fanning friction factor, which is a function of Re (C_3 and C_4 are empirical constants). For cross-flow filtration, turbulent flow is desired.

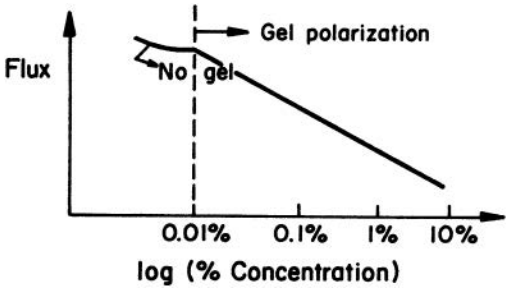


Figure 11.16. Variation of flux with solute concentration.

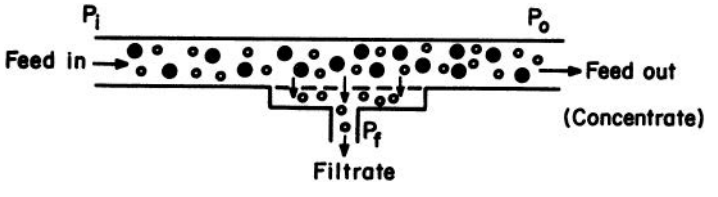


Figure 11.17. Schematic of cross-flow filtration.

The average transmembrane pressure drop is

$$\Delta P_M = \frac{P_i + P_0}{2} - P_f \tag{11.61}$$

where P_f is the filtrate pressure, which is usually near atmospheric pressure at least in laboratory systems.

Assuming that $P_f = P_{\text{atm}}$ or P_f is zero gauge pressure, we can relate ΔP_M to ΔP:

$$\Delta P_M = P_i - \tfrac{1}{2}\Delta P \tag{11.62}$$

High inlet pressure and sometimes low fluid velocities need to be used to obtain high ΔP_M.

The filtration flux (J), as a function of transmembrane pressure drop, is given by

$$J = \frac{\Delta P_M}{R_G + R_M} \tag{11.63}$$

where R_G and R_M are gel and membrane resistances, respectively. R_M is constant, and R_G varies with the solute concentration and the tangential velocity across the membrane, which can retard or eliminate gel formation. Usually, there is an optimal fluid velocity range maximizing the filtration rate. At low velocities, the mass transfer coefficient (k) is low, resulting in high gel resistance (R_G) and low filtrate flux. At high fluid velocities, ΔP is high, resulting in low ΔP_M and therefore low J values. Also, there is an optimal value of ΔP_M resulting in maximum flux because of a maximum-pressure limitation on P_i. That is, the maximum value of ΔP_M is P_i, and P_i is limited by the physical properties of the membrane. With modern membranes, especially the new ceramic membranes, it may be possible to push P_i to high levels. Therefore, at low ΔP_M values, flux increases with ΔP_M; however, at high ΔP_M values, filtration flux drops as a result of the decrease in velocity. Figure 11.18 describes optimal values of ΔP_M resulting in the maximum UF or MF rate for various solute concentrations.

In the absence of gel formation, the filtration flux increases linearly with ΔP_M. Figure 11.19 depicts the variation of filtration flux (J) with ΔP_M. At low ΔP_M values, J increases linearly with ΔP_M because of the absence of gel polarization. However, at high ΔP_M, gel formation takes place, and gel resistance (R_G) increases with increasing ΔP_M, resulting in a constant filtration flux (J) over a large range of high ΔP_M values. At higher solute concentrations, flux levels off at lower ΔP_M values.

The *rejection coefficient* of an ultrafilter is defined as

$$R = \frac{C_B - C_F}{C_B} = 1 - \frac{C_F}{C_B} \tag{11.64}$$

where C_F is the concentration of the solute in the filtrate. When $C_F = 0$, only water passes through the filter and $R = 1$, which is complete solute rejection. If $C_F = C_B$, complete solute transfer to the filtrate takes place and $R = 0$ (no rejection). Usually, $0 < R < 1$ and is closer to 1 (i.e., $R \approx 0.95$ or 0.98), as the solute size often approaches the pore size in the membrane. The value of R is a measure of the selectivity of the membrane for certain solutes. Selective separations of various compounds can be achieved using membranes with the right MW cutoff. Figure 11.20 provides an example of the variation of the rejection coefficient (R) with MW of the solute for a particular

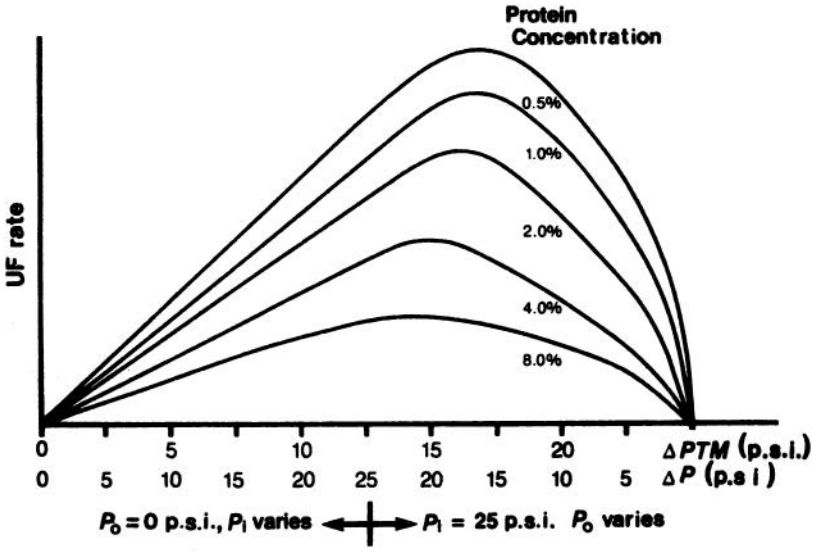


Figure 11.18. Filtration flux optimized as a function of transmembrane pressure at varying solute concentrations and fixed maximum inlet pressure. (With permission, from R. S. Tutunjian, in M. Moo-Young, ed., *Comprehensive Biotechnology*, Vol. 2, Elsevier Science, London, 1985.)

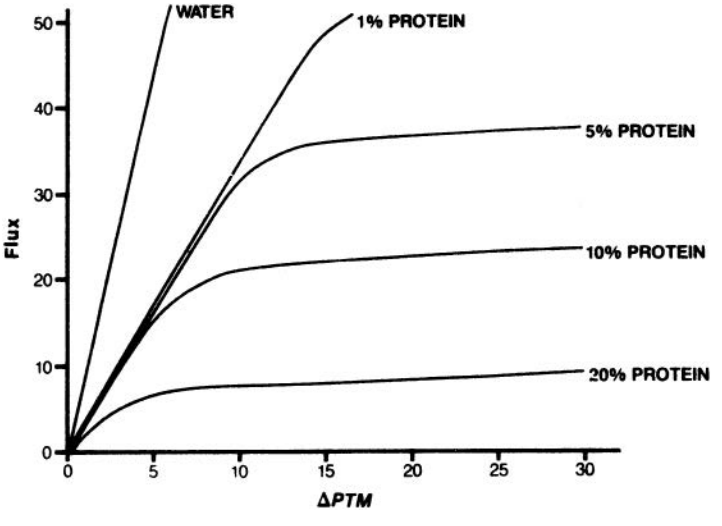


Figure11.19. Filtration flux as a function of transmembrane pressure at varying solute concentrations. (With permission, from R. S. Tutunjian, in M. Moo-Young, ed., *Comprehensive Biotechnology*, Vol. 2, Elsevier Science, London, 1985.)

membrane. For MW > 10^5 da and MW < 10^3 da, $R = 1$ and $R = 0$, respectively. That is, the membrane allows complete passage of compounds with MW < 10^3 da, retains compounds with MW > 10^5 da and partially retains compounds of 10^3 da < MW < 10^5 da.

Three major membrane configurations are depicted in Figure 11.21: hollow fibers, flat sheets, and spiral-wound cartridges. Hollow-fiber cartridges provide a large surface-to-volume ratio (as the fiber diameter is typically small). However, they

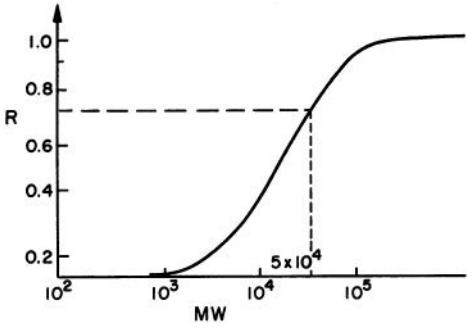


Figure 11.20. Rejection coefficient as a function of the molecular weight of the solute.

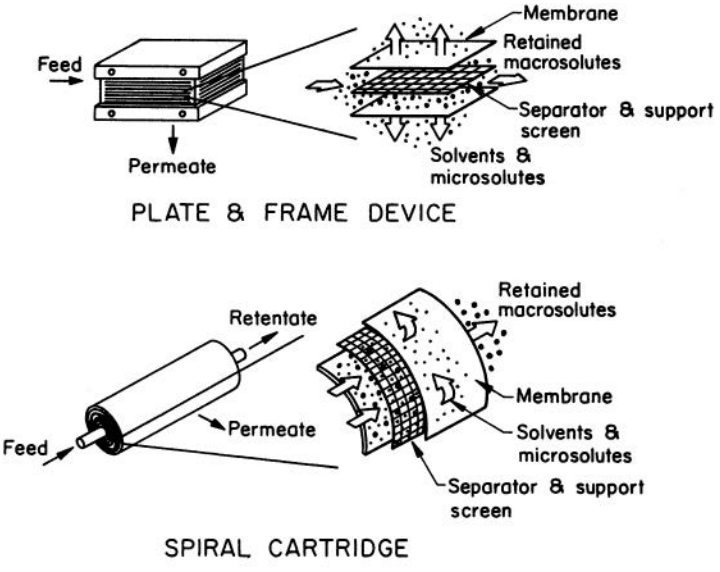


Figure 11.21. Examples of different membrane configurations. (Adapted with permission, from R. S. Tutunjian, Ultrafiltration Processes in Biotechnology, *Annals of the New York Academy of Sciences*, Vol. 413, Issue 1, December 1983, pages 238–253.)

plug more easily than the other two configurations. Flat-sheet configurations are easy to construct and allow easy membrane replacement. Originally, these types of configuration used "loose" sheets of membrane in a frame, but now most use engineered cartridges. The channel width can be altered to reduce plugging problems. Membrane support must be added to allow operation at higher pressures. Flat-sheet systems may have a low membrane surface-to-volume ratio. Spiral cartridges contain rolled membranes and are essentially flat-sheet systems configured to increase the surface-to-volume ratio. They are potentially the most economical configuration but are rarely used in biotechnology because cleaning is difficult. Most UF membranes operate with $\Delta P < 5$ to 7 atm and MF membranes at a slightly lower ΔP.

Example 11.4

Protein removal from cheese whey containing 6 g/l protein is realized by using a multitubular cross-flow ultrafiltration membrane system to reduce protein content to 0.5 g/l. Flow rate of cheese whey is 5 m^3/h, and filtrate is 90% of the feed. Desired filtrate flux is 0.5 m^3/m^2-h. The following information is available:

$R_M = 5$ atm/(m^3/m^2-h), $R_G = \frac{1}{2} R_M$, $D_e = 0.36\ 10^{-5}$ m^2/h, $P_i = 8$ atm, $P_f = 1$ atm

a. Determine cross membrane pressure drop and effluent pressure (P_0).
b. If protein concentration in the gel film is 10 g/l, determine the thickness of the gel film.
c. Determine the number of tubes in multitubular ultrafiltration cartridge if $D_0 = 1$ cm and $L = 1$ m.

Solution

a. Use equations 11.61 and 11.63:

$$J = \frac{\Delta P_M}{R_M + R_G}$$

$$\Delta P_M = 0.5(5 + 2.5) = 3.75 \text{ atm}$$

$$\Delta P_M = \frac{P_i + P_0}{2} - P_f,$$

$$3.75 = (8 + P_0)/2 - 1$$

which results in $P_0 = 1$ atm.

b. The thickness of the gel film is calculated from equation 11.54:

$$J = \frac{D_e}{\delta} \text{Ln} \frac{C_w}{C_B} \text{ or}$$

$$0.5 \text{ m}^3/\text{m}^2\text{-h} = \frac{0.36 \times 10^{-5} \text{ m}^2/\text{h}}{\delta} \text{Ln} 10/6$$

$$\text{or} \qquad \delta = 3.6 \text{ μm}$$

c. The number of tubes required is

$$A = F/J = [5 \text{ m}^3/\text{h } (0.9)]/0.5 \text{m}^3/\text{m}^2\text{-h}$$

$$= 9 \text{ m}^2$$

$$N = \frac{A}{\pi D_0 L} = \frac{9 \text{ m}^2}{(3.14)(0.01 \text{ m})(1 \text{ m})}$$

$$N = 287 \text{ tubes}$$

Three major applications of ultrafiltration are concentration, purification, and diafiltration. In concentration, water is removed from the aqueous solution using a UF membrane, and solute is concentrated. In purification, solvent and low-MW products are separated from high-MW compounds using a UF membrane. The product contains low-MW compounds and the solvent. In diafiltration, low-MW solutes such as salts, sugars, and alcohols pass through the filter, and the retained stream contains the product. The permeate that leaves the system is replaced with deionized water or another buffer.

Ultrafiltration membranes are used for the separation of proteins, enzymes, pyrogens (lipopolysaccharides from bacterial cell wall), cell debris, and viruses from fermentation media. UF separations operate under mild conditions ($T = 20°$ to 30°C, $P = 5$ to 50 psi) and are energy efficient, inexpensive, nondestructive, and easy to operate.

Microfiltration is used primarily to concentrate bacterial and yeast suspensions and provide a cell-free supernatant. Cross-flow filtration and improved membranes have been key to making this process viable. In many applications, it has replaced centrifugation. Initial devices were primarily open plate and frame, owing to possible blockages, although hollow-fiber systems as well as modern flat-sheet cartridges are now available. Application to animal cells, which are mechanically fragile, is possible but difficult owing to high levels of hydrodynamic sheer that can break cells.

11.4.8. Adsorption

Adsorption of solutes from liquid media onto solids is a common practice in separating soluble materials from fermentation or cell culture broth and removal of impurities in wastewater treatment. Various mechanisms may be involved in adsorption. In physical adsorption, weak forces, such as van der Waals forces, are dominant; however, in ion-exchange adsorption, strong ionic bonds are utilized. Solute is transferred from liquid to solid phase, and equilibrium is reached after a while in a batch operation. The type of adsorbent used depends on the particular application. The most widely used adsorbent for wastewater treatment applications is activated carbon, since it has large internal surface area per unit weight. Similarly, ion-exchange resins and other polymeric adsorbents can be used for adsorption of biotechnological products. As an example, a carboxylic acid cation-exchange resin is used to recover streptomycin. Adsorption capacity varies depending on adsorbent, adsorbate, physicochemical conditions, and the surface properties of the adsorbent and adsorbate. Usually, the exact mechanism of adsorption is not well understood, and the equilibrium data must be determined experimentally.

One typical equilibrium relationship between solute concentrations in liquid and solid phases follows:

$$C_S^* = K_F\, C_L^{*(1/n)} \tag{11.65}$$

Here C_L^* and C_S^* are equilibrium concentrations of the solute in liquid and solid phases. C_S is in units of mass of solute adsorbed per unit volume of resin. This relationship is an example of a type of Freundlich adsorption isotherm, with K_F and n being empirical parameters and n being greater than 1. Another form is a Langmuir

isotherm showing saturation as the concentration increases. The Freundlich isotherm is often better suited for real world applications.

Various types of solid–liquid contactors have been developed for the adsorption of solutes. Among these are packed-bed, moving-bed, fluidized-bed, and agitated-vessel contactors. Packed- and moving-bed contactors are the most widely used because they have a larger adsorption area per unit volume of reactor than the others. Analysis of adsorption phenomena in a *packed-bed* column is based on a differential material balance of the solution in the column:

$$U\frac{\partial C_L}{\partial Z}+\varepsilon\frac{\partial C_L}{\partial t}=-(1-\varepsilon)\frac{\partial C_S}{\partial t} \tag{11.66}$$

where U is the superficial velocity of the liquid, ε the porosity of the bed, and C_L the concentration of the solute in the liquid phase.

The first term in equation 11.66 is the convective transfer of solute in the bed, the second term represents the time course of change (transient) of the solute concentration in liquid, and the last term represents the rate of solute transfer from the liquid to the solid phase.

The rate of solute transfer from the liquid into the solid phase is

$$\frac{dC_s}{dt}=K_a(C_L-C_L^*) \tag{11.67}$$

where K_a is the overall mass transfer coefficient describing the internal and external mass transfer resistance, and C_L^* is the concentration of solute in the liquid phase, which is in equilibrium with C_S.

Substituting equation 11.67 into equation 11.68 yields

$$U\frac{\partial C_L}{\partial Z}+\varepsilon\frac{\partial C_L}{\partial t}=-(1-\varepsilon)K_a(C_L-C_L^*) \tag{11.68}$$

Equation 11.68, along with the equilibrium relationship (equation 11.65), can be solved numerically to determine the solute profile, $C_L = C_L(Z,t)$, in the column.

The preceding approach is based on plug flow conditions in the bed. However, in practice, due to back-mixing and dispersion, irregular flow profiles and channeling occur. A dispersion term $[-D_E(\partial^2 C_L/\partial Z^2)]$ is added to the right side of equation 11.68 to account for dispersion effects. A fixed-bed adsorption unit can be approximated to a large number of well-mixed tanks in series.

For very long *moving-bed columns* to which adsorbent is added continuously, the operation may be considered to be at steady state (see Figure 11.22). For the analysis, equilibrium is assumed to occur at the top of the column. If the dispersion effects are negligible, then at steady state equation 11.68 can be written as

$$U\frac{dC_L}{dZ}=-K_a(1-\varepsilon)(C_L-C_L^*) \tag{11.69}$$

with the boundary condition $C_L = C_{L0}$ at $Z = H$ for liquid feed into the top of a column.

Equation 11.69 can be integrated to determine the height of the column for a certain degree of separation:

$$-\frac{U}{K_a(1-\varepsilon)}\int_{C_L}^{C_{L0}}\frac{dC_L}{C_L-C_L^*}=\int_0^H dZ=H \tag{11.70}$$

By using an equilibrium relationship, we can integrate equation 11.70 to determine the height of the column. To complete the analysis, a relationship between the unsaturated resin and the liquid concentration is needed. This relationship is called an *operating line* (Figure 11.23) and represents a mass balance. Note that the volumetric resin flow is denoted by B and the volumetric liquid flow rate by F. Also note that

$$F = UA \tag{11.71}$$

where A is the cross-sectional area of the column.

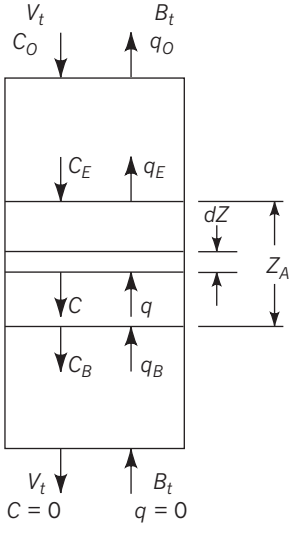


Figure 11.22. Schematic diagram of steady-state moving-bed adsorption column. (D. W. Sundstrom, H. E. Klei, *Wastewater Treatment* © 1979.)

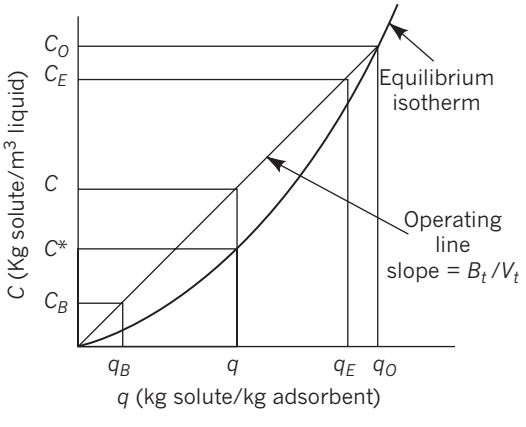


Figure 11.23. Operating line for steady-state adsorption column. (D. W. Sundstrom, H. E. Klei, *Wastewater Treatment* © 1979.)

From mass balance considerations on the solute,

$$F(C_{L0} - 0) = B(C^*_{S0} - 0) \tag{11.72}$$

or

$$\frac{B}{F} = \frac{C_L}{C_S} = \frac{C_{L0}}{C^*_{so}} \tag{11.73}$$

where C^*_{S0} is the solute concentration in the resin in equilibrium with C_{L0}. As noted in Figure 11.23, the operating line is straight.

For fixed-bed columns, the absorptive capacity is limited to a maximum level. In this view, a column would consist of three zones: a *saturated* zone, an *adsorption* zone, and a *virgin* zone. The adsorption zone moves down a column at a rate determined by the feed rate of the solute and the mass transfer characteristics of the bed. When the leading edge of the adsorption zone reaches the bottom of the column, there will be a rapid increase in the exit concentration of solute (known as "breakthrough"). Normally, the column would be shut down at this point and the resin replaced or regenerated. However, if the column were operated for a period of time equal to the time for the adsorption zone to travel its own height, we would observe the *breakthrough curve* of the adsorption zone. This process is depicted in Figure 11.24.

Under these circumstances, equation 11.70 would describe the concentration profile in the adsorption zone of a height H. If the column were of total height H_t, then the time to exhaustion of the column would be equal to the ratio of H to H_t

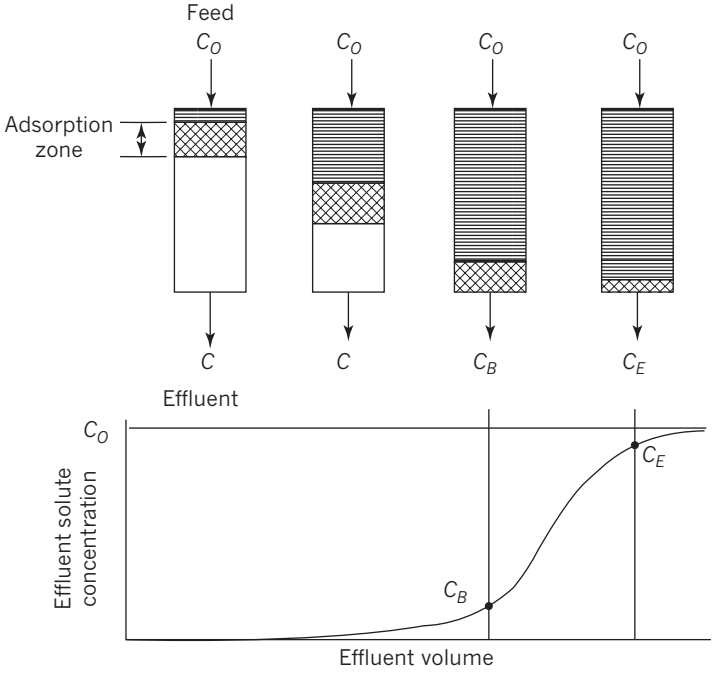


Figure 11.24. Movement of adsorption zone through a fixed-bed downflow adsorber and the corresponding breakthrough curve. (D. W. Sundstrom, H. E. Klei, *Wastewater Treatment* © 1979.)

multiplied by the velocity of the adsorption zone, plus the time required to form the adsorption zone (usually negligible).

Example 11.5

Cephalosporin is separated from fermentation broth by adsorption on an ion-exchange resin in a moving-bed column. The bed is 4 cm in diameter and contains 0.8 cm^3 resin/cm^3 bed. The density of the resin is 1.3 g/cm^3, and the feed solution contains 5 g/l of the antibiotic. If the superficial velocity of liquid is 1.5 m/h and the overall mass transfer coefficient is 15 h^{-1}, determine the height of the column for an effluent antibiotic concentration of $C_L = 0.2$ g/l. Assume an equilibrium with the following relationship:

$$C_S = 25(C_L^*)^{1/2}$$

where C_S is g solute/l resin and C_L is g solute/l solution. The ratio of volumetric flow rate of broth to resin is 10.

Solution

Equation 11.70 can be written as

$$-\frac{U}{K_a(1-\varepsilon)}\int_{C_L}^{C_{L0}} \frac{dC_L}{c_L - c_L^*} = H$$

Using the equilibrium relationship, we obtain

$$C_L^* = \frac{1}{625} C_s^2$$

By using the operating-line relationship, which is $F/B = 10$, we note that $C_S = 10C_L$:

$$C_L^* = \frac{1}{625} 100\, C_L^2 = \frac{1}{6.25} C_L^2 = 0.16C_L^2$$

Substituting this equation into equation 11.70 yields

$$-\frac{U}{K_a(1-\varepsilon)}\int_{C_L}^{C_{L0}} \frac{dC_L}{C_L - 0.16C_L^2} = H$$

Integration with the numerical values yields

$$-\frac{1.5 \text{ m/h}}{(15 \text{ h}^{-1})(0.8)}\int_{0.2}^{5} \frac{dC_L}{C_L - 0.16C_L^2} = H$$

$H = 0.6$ m.

11.4.8.1. Bed depth–service time equation for adsorption column design. A more practical approach for design of packed-bed activated carbon adsorption columns was originally developed by Thomas and later modified by Bohart and Adams[†] primarily for wastewater treatment applications. This equation is based on quasi-equilibrium between the aqueous and solid phases and relates the required bed height to duration of operation for a certain effluent adsorbate concentration (bed depth–service time equation):

$$Ln(C_o/C_e - 1) = Ln(e^{KN_0H/v_0} - 1) - KC_o t \tag{11.74}$$

[†] G. S. Bohart and E. Q. Adams, *Journal Chemical Society 42*: 523–529, 1920.

where C_o and C_e are the feed and desired effluent adsorbate concentrations (kg/m^3); K is the adsorption rate constant (m^3 bed/kg adsorbent-h); N_0 is the capacity of adsorbent (kg adsorbate/m^3 bed); H is the column height (m); v_o is linear superficial velocity of aqueous phase (m/h, F/A_o, where F and A_o are the volumetric flow rate and cross-section area of the column, respectively); and t is the service time. H/v_0 is the average hydraulic residence time (HRT) in the bed (HRT = $V_b/F = HA_o/v_0A_o = H/v_0$).

Since $e^{KN_0H/v_0} >> 1$, then equation 11.74 takes the following form:

$$t = N_0H/(C_ov_0) - (1/KC_o)Ln(C_o/C_e - 1) \tag{11.75}$$

or

$$t = N_oV_b/(C_oF) - (1/KC_o)Ln(C_o/C_e - 1) = N_oV_b/L_o - (1/KC_o)Ln(C_o/C_e - 1) \tag{11.76}$$

Here V_b is the bed volume (A_oH, m^3) and L_o is the adsorbate loading rate ($L_o = C_oF$, kg adsorbate-h). The first term is the maximum service time in the absence of transport limitations and the second term is the delay due to transfer rate of adsorbate. As indicated in equation 11.76, operation time is inversely proportional with adsorbate loading rate.

Equation 11.75 is usually written as

$$t = aH + b \tag{11.77}$$

where $a = N_0/(C_ov_0)$ and $b = -(1/KC_o)Ln(C_o/C_e - 1)$.

For a given C_o and C_e, a plot of service time (t) versus bed depth (H) yields a line with a slope of a and intercept of b, which allows determination of N_0 and K using experimental data.

The critical or minimum bed depth is defined as the bed depth at $t = 0$. Then, we get the following:

$$H_m = (v_0/KN_0)Ln(C_o/C_e - 1) = -b/a \tag{11.78}$$

The actual bed height is usually five or six times larger than H_m.

Example 11.6

The effluent of a chemical plant with a flow rate of 200 m^3/day and organics concentration of $C_o = 60$ mg/L is fed to a granular activated carbon adsorption column to obtain $C_e = 1$ mg/l in the effluent. The following data are available:

$$D_o = 0.8 \text{ m}, N_o = 70 \text{ kg adsorbate/m}^3 \text{ bed}, K = 0.9 \text{ m}^3 \text{ bed/kg adsorbent-h}$$

a. Determine the required height of the column for 6 days of operation.
b. If the void fraction in the column is $e = 0.3$, determine the volume of required activated carbon.
c. For $H = 5$ m, for how long can the column be operated to obtain effluent adsorbate C?

Solution

a. We first calculate the linear superficial velocity, v_0:

$$v_0 = F/A_o = (200\ M^3/\text{d} \div 24 \text{ h/d}) / \left(\pi/4 \cdot 0.8 \text{ m}^2\right) = 16.6 \text{ m/h}$$

We then calculate the parameters a and b:

$$a = N_O / C_O v_0 = 70\,\text{kg/m}^3 / (0.06\ \text{kg/m}^3 - 16.6\ \text{m/h})$$
$$= 70.3\ \text{h/m}$$
$$b = -(1/KC_O)\text{Ln}(C_O.C_e - 1)\ \text{ or}$$
$$= (1/(0.9\ \text{m}^3/\text{kg} \cdot 0.06\ \text{kg/m}^3)\text{Ln}(60/1 - 1)$$
$$= -75.5\ \text{h}$$
$$H = (t - b)/a = (6\ \text{d} \cdot 24\ \text{h/d} - (-75.5\ \text{h})/70.3\ \text{h/m}$$
$$= 3.1\ \text{m}$$

b. The volume of the bed is

$$V_b = (\pi/4)\ D_o^2 H = (\pi/4)(0.8)^2(3.1) = 1.56\ \text{m}^3$$
$$V_{AC} = V_b\ (1 - e) = 1.56\ (0.70) = 1.1\ \text{m}^3$$

c. We calculate the operational time using equation 11.77:

$$t = aH + b = 70.3\ (5) - 75.3 = 276\ \text{h} = 11.5\ \text{days}$$

11.4.9. Chromatography

Chromatography separates mixtures into components by passing a fluid mixture through a bed of adsorbent material. We are interested primarily in *elution chromatography*. Typically, a column is packed with adsorbent particles, which may be solid, a porous solid, a gel, or a liquid phase immobilized in or on a solid. A *mobile phase* or fluid phase with a mixture of solutes is injected. This pulse is followed by a solvent or eluent. The pulse enters as a narrow concentrated peak, but exits dispersed and diluted by additional solvent. Different solutes in the mixture interact differently with the adsorbent material *(stationary phase)*; some interact weakly and some interact strongly. Solutes that interact weakly with the matrix pass out of the column rapidly (see Figure 11.25), while those that interact strongly with the matrix exit slowly. These differential rates of migration separate the solutes into different peaks that exit at a characteristic retention time (which will change if operating conditions are altered).

Elution chromatography is similar to fixed-bed adsorption (Section 11.4.4). However, in fixed-bed adsorption (sometimes called *frontal chromatography*), the solute is captured in the adsorbent and then eluted as a concentrate; purification depends on significant differences in binding characteristics of the product and impurities. In elution chromatography, you purify the product even while it is being diluted.

When large amounts of material are being processed, *displacement chromatography* may be attractive. In this method, the column is subjected to sequential step changes in inlet conditions (e.g., nature of solvent). The feed mixture is introduced, followed by a constant infusion of displacer solution. The displacer must have a higher affinity for the stationary phase than any compound in the feed solution. The displacer "pushes" solute off the stationary phase and back into the mobile phase. If conditions are chosen correctly, the feed components are forced into adjacent square-wave-like zones of concentrated, pure solutes. These zones then break through the end of the

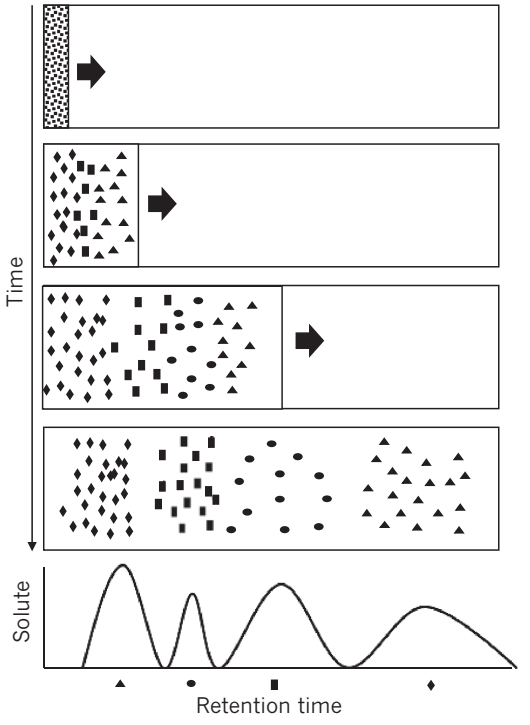


Figure 11.25. Separation by elution chromatography. The solvent with four different solutes flows through the bed and solutes are separated because of different adsorption affinities and are eluted at different times.

column with the zone having the solute with the lowest affinity for the stationary phase exiting first. The primary advantage of displacement chromatography over elution chromatography is the potential for higher throughput, but operation is more difficult, and high *resolution* (separation of solutes) can be difficult to obtain in some situations.

These chromatographic processes are almost always run as batch operations, although schemes for continuous or semicontinuous operation have been proposed. For the remainder of our discussion, we focus on elution chromatography as a batch process. Following are some important types of chromatographic methods:

- *Adsorption chromatography* (ADC) or *normal phase chromatography* is based on the adsorption of solute molecules onto solid particles, such as alumina and silica gel, by weak van der Waals forces and steric interactions.
- *Liquid–liquid partition chromatography* (LLC) is based on the different partition coefficients (solubility) of solute molecules between an adsorbed liquid phase and passing solution. Often the adsorbed liquid is nonpolar.
- *Ion-exchange chromatography* (IEC) is based on the adsorption of ions (or electrically charged compounds) on ion-exchange resin particles by electrostatic forces.
- *Gel-filtration (molecular sieving) chromatography* is based on the penetration of solute molecules into small pores of packing particles on the basis of molecular size and the shape of the solute molecules. It is also known as *size exclusion chromatography*.

- *Affinity chromatography* (AFC) is based on the specific chemical interactions between solute molecules and ligands (a functional molecule covalently linked to a support particle). Ligand–solute interaction is very specific, such as enzyme–substrate interaction, which may depend on covalent, ionic forces or hydrogen-bond formation. Affinity binding may be molecular size and shape specific.
- *Hydrophobic chromatography* (HC) or *hydrophobic interaction chromatography* (HIC) is based on hydrophobic interactions between solute molecules (e.g., proteins) and functional groups (e.g., alkyl residues) on support particles. This method is a type of *reverse phase chromatography*, which requires that the stationary phase is less polar than the mobile phase.
- *High-pressure liquid chromatography* (HPLC) is based on the general principles of chromatography, the only difference being high liquid pressure applied to the packed column. Owing to high-pressure liquid (high liquid flow rate) and dense column packing, HPLC provides fast and high resolution of solute molecules.

The choice of the stationary phase and consequently the type of chromatography depends on the nature of the solutes and process goals. Ion-exchange chromatography is widely used, particularly for recovery of proteins. This method offers good resolution of peaks, high capacity, and good speed. Hydrophobic chromatography and liquid–liquid partition chromatography share many of these attributes but are best applied when the aqueous phase is at high ionic strength. Also, organic solvents may be needed, which may denature proteins or create environmental problems. Adsorption chromatography is often relatively inexpensive, but resolution generally is not very sharp. Gel-filtration chromatography is good for buffer exchange and desalting and offers decent resolution. Because gels are compressible, capacity and throughput are often low, which typically restricts this technique to small-scale uses; new rigid packings for size exclusion chromatography allow faster flow rates and could increase throughput and scale. Affinity methods offer the possibility of very high selectivity and good capacity and speed. However, such methods can be extremely expensive, especially if based on antibodies. For real bioprocesses (e.g., recovery of proteins), multiple types of chromatography are used, since greater purity is possible if more than one basis of separation is used. Ion-exchange and affinity chromatography are the most widely used methods to recover proteins from bioprocesses.

A single chromatographic separation process may include more than one of these mechanisms. For example, adsorption chromatography using silica gel functions properly when the water content of silica gel is less than 15%. The system may operate as liquid–liquid partition chromatography when the water content exceeds 30%. In some cases, electrostatic and weak forces may be simultaneously functional.

A typical chromatographic column is shown in Figure 11.26, where a solution is flowing downward in the column and a solute is adsorbed on adsorbent solids by forming a band. As a first approximation, the solvent carries the solute downward as it moves along the axis of the column. It is assumed that a rapid equilibrium is established between the solute and adsorbent and that radial gradients are negligible.

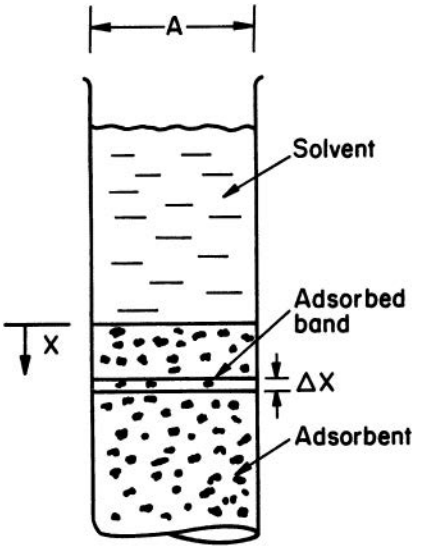


Figure 11.26. Schematic of a chromatography column.

Assume that a small volume of solvent, ΔV, is poured through the band, which carries the adsorbed solute downward in the column. A material balance on solute over a differential column height of ΔX yields

$$\begin{pmatrix}\text{total rate of}\\ \text{solute removal}\\ \text{from solvent}\end{pmatrix} = \begin{pmatrix}\text{rate of solute}\\ \text{removal in}\\ \text{void space}\end{pmatrix} + \begin{pmatrix}\text{rate of solute}\\ \text{removal in}\\ \text{solid phase}\end{pmatrix}$$

$$-\left[\left(\frac{\partial C_L}{\partial X}\Delta X\right)\right]\Delta V = \varepsilon A\,\Delta X\left(\frac{\partial C_L}{\partial V}\right)\Delta V + A\,\Delta X\left(\frac{\partial C_S'}{\partial V}\right)\Delta V \tag{11.79}$$

where C_S' is the amount of adsorbed solute per unit volume of column. Further simplification of equation 11.79 yields

$$\frac{\partial C_L}{\partial X} + A\left(\varepsilon\frac{\partial C_L}{\partial V} + \frac{\partial C_S'}{\partial V}\right) = 0 \tag{11.80}$$

In the simplest case, the amount of solute adsorbed per unit volume of column is closely related to the adsorption isotherm of the solute:

$$C_S' = Mf(C_L) \tag{11.81}$$

where M is amount of adsorbent per unit volume of column (mg/ml) and $f(C_L)$ is the adsorption isotherm or amount of adsorbed solute per unit amount of adsorbent, which is a function of C_L. By substituting the derivative of equation 11.81 into equation 11.80, we obtain

$$-\frac{\partial C_L}{\partial X} = A[\varepsilon + Mf'(C_L)]\frac{\partial C_L}{\partial V} \tag{11.82}$$

Substitution of $\partial C_L/\partial X = (\partial C_L/\partial V)(\partial V/\partial X)_C$ into equation 11.82 yields

$$\left(\frac{\partial V}{\partial X}\right)_c = A[\varepsilon + Mf'(C_L)] \tag{11.83}$$

Integration of equation 11.83 from $X = X_0$ to $X = X$ and $V = V_0$ to $V = V$ yields

$$\Delta X = \frac{\Delta V}{A[\varepsilon + Mf'(C_L)]} \tag{11.84}$$

Since ΔV, A, ε, and M are constants, ΔX is determined by $f'(C_L)$, which is the derivative of $f(C_L)$, or the adsorption isotherm. That is, the location of the solute band formed is mainly determined by the adsorption characteristics of a particular solute on an adsorbent. When the solvent contains two components, I and J, the solutes form two distinct bands at different axial distances, depending on their adsorption characteristics (see Figure 11.27).

If we define L_I as the distance traveled by solute I, L_S as the distance traveled by solvent above the adsorption column ($\Delta V/A$), and L_C as the penetration distance of the solvent into the column ($\Delta V/A\varepsilon$), then the retention factor (R) or the ratio of the distance traveled by solute I to that of solvent in the space above the column is given by

$$\frac{L_I}{L_S} = R = \frac{1}{\varepsilon + MK} \tag{11.85}$$

where $K = f'(C_L) = df(C_L)/dC_L$ = partition coefficient. Similarly, we can define R_f as

$$R_f = \frac{L_I}{L_c} = \frac{\varepsilon}{\varepsilon + MK} \tag{11.86}$$

where R_f is the ratio of the travel distance of I to that of solvent in the column.

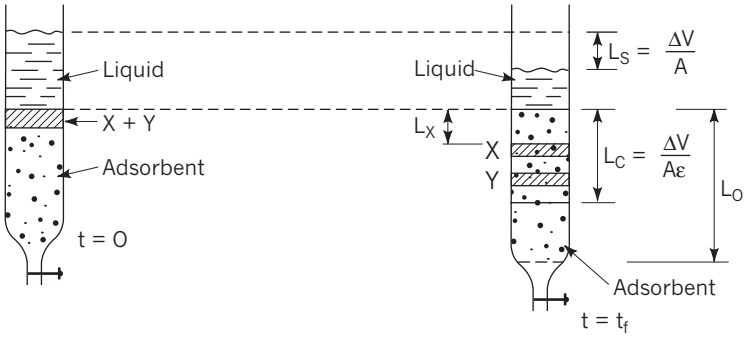


Figure 11.27. Separation of a binary mixture in a chromatographic column.

When component I is moved out of the column, then $L_I = L_0$ and $L_S = V_0/A$, where V_0 is the volume of solvent required to displace the first drop of solvent out of the packed column. Then L_S/L_I becomes

$$V_r = \frac{L_S}{L_I} = \frac{V_0}{L_0 A} = \varepsilon + MK \tag{11.87}$$

where V_r is the ratio of solvent volume to column volume.

$$\left(\frac{V'}{n}\right)^2 + 2\frac{V'}{n}V_0 = K\frac{\varphi}{n}$$

Example 11.7

In a chromatographic separation column used for the adsorption of solute A onto an adsorbent solid B, the adsorption isotherm is given by

$$C_S = k_1 C_L^3 = f(C_L)$$

where C_S is mg solute adsorbed/mg adsorbent, C_L is the solute concentration in liquid medium (mg solute/ml liquid), and k_1 is a constant. $k_1 = 0.2$ (mg solute adsorbed/mg adsorbent)/(mg solute/ml liquid)^3. The porosity (void fraction) of the packed column is $\varepsilon = 0.35$. The cross-sectional area of the column is 10 cm^2, and M is 5 g adsorbent per 100 ml column volume. The volume of the liquid added is $\Delta V = 250$ ml.

a. Determine the position (ΔX) of the solute band in the column when the solute concentration in the liquid phase at equilibrium is $C_L = 5 \times 10^{-2}$ mg/ml.
b. Find the ratio of the travel distance of solute A (L_A) to that of solvent B in the column (R_f) when $C_L = 5 \times 10^{-2}$ mg/ml (i.e., R_f).

Solution

a. $C_L = 5 \times 10^{-2}$ mg/ml

$$f(C_L) = k_1 C^3, \quad f'(C_L) = 3k_1 C_L^2$$
$$f'(C_L) = 3(0.2)(5 \times 10^{-2})^2 = 0.0015$$
$$M = 5g/100 = 50 \text{ mg/ml}$$
$$\Delta X = \frac{\Delta V}{A(\varepsilon + Mf'(c))} = \frac{250 \text{ cm}^3}{10 \text{ cm}^2[0.35 + 50(0.0015)]}$$
$$\Delta X = 58.5 \text{ cm}$$

b. $R_f = \dfrac{\varepsilon}{\varepsilon + MK} = \dfrac{0.35}{0.35 + 50(0.0015)}$

$$R_f = 0.824$$

This theory of chromatography is applicable to fixed-column adsorption, partition, ion-exchange, and affinity chromatography. The theory of *gel-filtration chromatography* is somewhat different. In gel-filtration (molecular sieving) chromatography, solute

molecules diffuse into porous structures of support particles, depending on their molecular size and shape. Small solute molecules get into the fine pore structures of the support particles and remain in contact with the solid for a "long" period, while large molecules interact with the outer surface and remain in contact with the solid for a "short" period. When the column is eluted, the largest molecules appear first in the eluent solvent, and the smallest molecules last. Different bands of solute molecules are obtained on the basis of their size and shape.

Consider the case where a buffer with the solutes of interest is added to the column, followed by sufficient washes to elute the original buffer solution.

The total volume of the buffer solution eluted from the column over a certain period of time is

$$V_e = V_0 + K_D V_i \tag{11.88}$$

where V_e is the total eluent buffer volume; V_0 is the void volume in the column ($= V\varepsilon$), which is the volume of fluid filling the void space outside the gel particles; V_i is the total void or pore volume inside the gel particles; and K_D is the partition coefficient.

For large molecules that do not penetrate inside the gel structure, $K_D = 0$; for small molecules that completely penetrate inside the gel, $K_D = 1$. Equation 11.88 can be rewritten as

$$\frac{V_e}{V_0} = 1 + K_D \frac{V_i}{V_0} \tag{11.89}$$

For a given solute mixture and gel beads, K_D and the V_i/V_0 ratio are fixed, and the V_e/V_0 ratio is approximately constant, irrespective of column geometry and V_e.

Gel-filtration chromatography can be used to determine the MW of macromolecules. A plot of V_e/V_0 versus log (MW) yields a straight line with a negative slope that is proportional with K_D.

Affinity chromatography is based on the highly specific interaction between solute molecules and ligands attached on polymeric or ceramic beads in a packed column. The concept of affinity chromatography is described in Figure 11.28. The matrix is usually agarose. However, polyacrylamide, hydroxyethyl methacrylate, cellulose, and porous glass can also be used as the matrix bead. Spacer arms between the matrix and ligand are usually linear aliphatic hydrocarbons. The use of spacer arms may reduce the steric hindrance generated by the matrix. Coupling between the matrix and ligand depends on the functional groups present on the matrix and ligand. Chemically reactive groups on the support matrix usually are the —OH, —NH_2, and —COOH groups. If the reactive group on the matrix is an —OH group (polysaccharides, glass, hydroxyalkyl methacrylate), then cyanogen bromide (CNBr) is used as a coupling agent. The cyanogen bromide–activated agarose reacts with primary amine groups present in proteins that act as ligands. After the desired solutes are bound to the ligand, elution is usually achieved by changing the pH or ionic strength in the column. Ligand-solute molecule interactions in affinity chromatography are very specific, such as enzyme–substrate or antigen–antibody interactions. That is, an

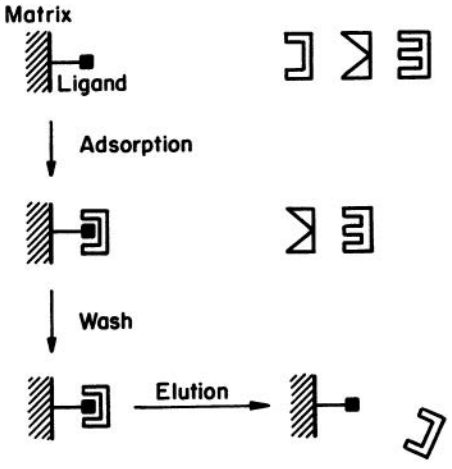


Figure 11.28. Basic principles of affinity chromatographic separations. A suitable ligand is covalently attached to an insoluble matrix. In the adsorption step, only those molecules with a specific binding site for the ligand bind to the adsorbent; molecules without the proper geometric fit pass through unaffected. In the elution step, the bound molecules are disengaged from the column and collected. (With permission, from M. L. Yarmush and C. K. Colton, in M. Moo-Young, ed., *Comprehensive Biotechnology*, Vol. 2, Elsevier Science, London, 1985.)

enzyme inhibitor or substrate may be used as a ligand in separating a specific enzyme from a mixture. Monoclonal antibodies (MAb) may be used as a ligand to separate specific antigen molecules by affinity chromatography. Of particular interest in the biotechnological industry is the use of protein A, a cell-surface protein of *Staphylococcus aureus*, which binds the heavy chain of several mammalian immunoglobins.

Another type of affinity chromatography is *immobilized-metal-affinity chromatography* (IMAC), which exploits the different affinities that solutes (especially proteins) have for metal ions chelated to a support surface. A very attractive feature is the low cost of metals and the ease of regeneration of the stationary phase, particularly in contrast to affinity chromatography using antibodies. Typically, iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) linked to an agarose or silica gel support provides attachment sites for a metal ion. A variety of target proteins have been purified using zinc, copper, and nickel chelates. Water molecules normally solvate metal ions, but in the presence of a strong Lewis base (e.g., histidine residue in a protein), the water molecules can be displaced. The bonds that form between the immobilized metal and the base (protein) result in an adsorbed protein. Factors such as the accessibility, microenvironment of the binding residue (i.e., histidine, cysteine, and tryptophan), cooperation between neighboring amino acid side groups, and local conformations play important roles in the strength of binding and thus protein retention. Subsequent destruction of the bond by lowering the pH or adding a competing ligand in the mobile phase results in the elution of the metal binding protein.

Not all proteins naturally bind metals. However, IMAC can still be used if the genetic information for the desired protein is altered to include a *tail* that has high metal-binding affinity. Typically, the structural gene encoding the target protein is extended to code for a metal-binding peptide, resulting in production of a fusion protein of the target protein and tail. The amino acid sequence of the tail is based on a sequence of amino acids known to bind metals. A common choice is six adjacent histidines. Peptide libraries have also been used to generate effective affinity tails. The biggest drawback to the method is that for many applications the tail must be removed. The system can be designed with a linker between the tail and target protein

that can be cleaved chemically. This design, however, adds additional steps to the process.

Our discussion of the theory of chromatography is based on many simplifying assumptions. Usually, the analysis of real columns is more complicated, owing to dispersion, wall effects, and lack of local equilibrium. Wall effects can be reduced by keeping the column-diameter-to-bead-diameter ratio larger than 10 ($D_c/D_b > 10$). Bed compression can occur under some operating conditions and can further complicate analysis of the system by causing flow irregularities such as viscous fingering.

Understanding these hydrodynamical and kinetic effects is also critical to scale-up of chromatographic processes. These effects alter the shape of the peaks exiting the column. In addition to these flow effects, the step controlling the rate of adsorption will alter peak shape. For a porous support internal diffusion, diffusion through the external film, or external dispersion (Taylor dispersion) in laminar flow can control the rate of mass transfer. Let us consider a more quantitative description of these ideas.

Figure 11.29 depicts the output from a chromatography column. Referring to this figure, we can define some key characteristics of the output. The *resolution* of two adjacent peaks, R_S, is

$$R_s = \frac{t_{\max,j} - t_{\max,i}}{\frac{1}{2}(t_{w,i} + t_{w,j})} \tag{11.90}$$

The yield is defined as the amount of a solute, such as i, collected between times t_1 and t_2 divided by the total amount of i injected into the column:

$$Y_{\Delta t,i} = \frac{\int_{t_1}^{t_2} y_i F \, dt}{\int_0^{\infty} y_i F \, dt} \tag{11.91}$$

where y_i is the concentration of solute, i, and F is the solvent flow rate.

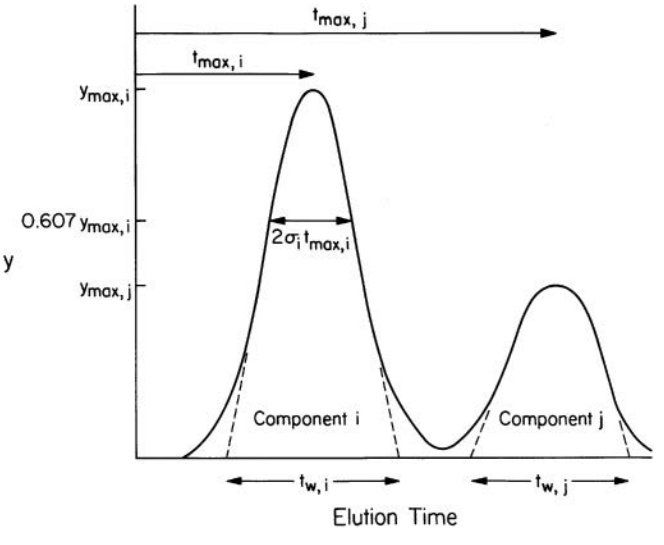


Figure 11.29. Schematic of output from a chromatography column. The relative t_{max} values are used to calculate resolution, while the standard deviation of a peak ($\sigma t_{max,i}$) is used to estimate yields, purity, and capacity and is used in scale-up.

The value of y_i can be estimated from the peak height and shape if the peak is assumed to be Gaussian. This assumption leads to

$$y_i = y_{\max,i} \exp\left[-\frac{(t - t_{\max,i})^2}{2(\sigma t_{\max,i})^2}\right] \tag{11.92}$$

where $t_{\max,i}$ is the time at which the maximum amount of i elutes, t is elution time, and $\sigma t_{\max,i}$ is the standard deviation for peak i. Then the yield can be calculated as follows:

$$Y_{\Delta t,i} = \tfrac{1}{2}\left\{\operatorname{erf}\left[\frac{(t_2 - t_{\max,i})}{\sqrt{2}\sigma t_{\max,i}}\right] - \operatorname{erf}\left[\frac{(t_1 - t_{\max,i})}{\sqrt{2}\,\sigma t_{\max,i}}\right]\right\} \tag{11.93}$$

where erf is the error function as defined by

$$\operatorname{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x e^{-u^2} du \tag{11.94}$$

The purity is defined as the amount of solute i eluted in Δt divided by the total sum of all solutes eluted in Δt:

$$P_{\Delta t,i} = \frac{\int_{t_1}^{t_2} y_i F \, dt}{\sum_k \int_{t_1}^{t_2} y_k \, F \, dt} \tag{11.95}$$

The key in this analysis is σ. The value of σ will depend on dispersion and adsorption kinetics. With a more detailed analysis, the following can be shown:

$$\sigma^2 = \frac{v}{kal} \tag{11.96}$$

where v is the superficial fluid velocity (i.e., volumetric flow rate divided by column diameter A), ka is the surface "reaction" rate consisting of a reaction rate (k) and available surface area (a), and l is column length. Note that σ^2 in equation 11.96 equals a flow rate divided by the reaction rate and is effectively a Peclet number. The value of σ^2 depends on rate-controlling step; for example,[†]

$$\text{Internal diffusion control: } \sigma^2 \propto \frac{vd^2}{l} \tag{11.97}$$

$$\text{External film control: } \sigma^2 \propto \frac{v^{1/2}/d^{3/2}}{l} \tag{11.98}$$

$$\text{Taylor dispersion: } \sigma^2 \propto \frac{vd^2}{Dl} \tag{11.99}$$

In these equations, d is the diameter of the particle used to pack the column and D is the effective diffusion coefficient of the solute.

Consider the problem of scale-up of a chromatography process to handle increasing amount of product. One approach might be to increase solute

[†] See P. A. Belter, E. L. Cussler, and W. S. Hu, *Bioseparations,* Wiley, New York, 1988.

concentration while using the same column. The danger here is that the packing would become saturated, which would reduce purity and yield. Another approach might be to increase both d and A so as to maintain a constant particle-size-to-bed-diameter ratio. Such an approach maintains flow patterns, but note from equations 11.97, 11.98, and 11.99 that σ would increase if d increased. Higher values of σ indicate broadening of peaks and reduced resolution, yield, and purity. Another approach is to fix d and increase both v and l, but maintain the ratio of v to l constant. While this approach will mathematically keep σ constant, it has practical limitations. Lengthening the column increases pressure drop. Since many packings are soft, they compress further, thereby increasing pressure drop, reducing flow, and altering flow patterns. Some chromatography columns have been made in segments, each less than 30 cm in height; segmentation reduces the bed-compression problem. Another approach is to increase A and volumetric flow to maintain a constant v and hence constant σ. This approach yields a "pancake" column. It is quite workable if the liquid can be applied uniformly over the surface of the column.

Recent advances in the design of packings (particles) for chromatography columns have also assisted in scale-up. Rigid, mechanically strong particles with macropores allow convective flow both around and through particles. The macropores are intimately connected with the micropores, so good mass transfer is maintained. These particles allow fast flow of solvent and solutes and reduce problems of bed compression, allowing column length to be increased.

Example 11.8

Consider an ion-exchange chromatography column used to purify 20 g of a particular protein. At a superficial velocity of 20 cm/h, the peak exits the column with y_{max} at 80 min. The standard deviation of the peak is 10 min.

a. Estimate how long the column must be operated to achieve 95% yield.
b. Estimate how long the column must be operated if the flow is increased to 40 cm/h and Taylor dispersion is the rate-controlling step.

Solution

a. Note the following:

$$\sigma = \frac{\sigma t_{max}}{t_{max}} = \frac{10\,\text{min}}{80\,\text{min}} = 0.125$$

From equation 11.88, the yield where $t_1 = 0$[†] is

$$Y_{\Delta t} = 0.95 = \tfrac{1}{2}\left[1 + \text{erf}\left(\frac{t - t_{max}}{\sqrt{2}\sigma t_{max}}\right)\right]$$

$$= 0.95 = \tfrac{1}{2}\left[1 + \text{erf}\left(\frac{t - 80}{\sqrt{2}\,10\,\text{min}}\right)\right]$$

or $t = 96.4$ min.

[†] Note that erf $(-x) = -\text{erf}\ x$ and erf $(-5.9) = -1$.

b. With twice the flow t_{max} becomes 40 min. Since for Taylor dispersion, $\sigma \propto v^{1/2}$,

$$\sigma_2 = \sigma_1 \left(\frac{v_2}{v_1}\right)^{1/2} = 0.125\left(\frac{40}{20}\right)^{1/2} = 0.177$$

For yield = 0.95, the time required is estimated as

$$0.95 = 1/2 + \text{erf}\left[\frac{t-40}{\sqrt{2}\cdot 0.177\cdot 40}\right]$$

or $t = 51.6$ min.

Notice that in this second case ($v = 40$ cm/h) the peak is broader and $t_{95\%}/t_{max} = 96.4/80 = 1.205$ for $v = 20$ cm/h and $t_{95\%}/t_{max} = 51.6/40 = 1.29$ for $v = 40$ cm/h.

11.4.10. Electrophoresis

Electrophoresis is used for the separation of charged biomolecules according to their size and charge in an electric field. In an electric field, the drag force on a charged particle is balanced by electrostatic forces when the particle is moving with a constant terminal velocity. A force balance on a charged particle moving with a terminal velocity in an electric field yields

$$qE = 3\pi\mu D_P V_t \tag{11.100}$$

or

$$V_t = \frac{qE}{3\pi\mu D_P} \tag{11.101}$$

where q is the charge on the particle, E is electric field intensity, D_P is particle diameter, μ is the viscosity of the fluid, and V_t is the terminal velocity of the particle.

Depending on the pH of the medium, electrostatic charges on protein molecules will be different. When pH > pI, the protein will be negatively charged; and when pH < pI, the charge on the protein will be positive. The net charge on the protein will determine the velocity of the protein. When a protein molecule is placed in a pH gradient, the electrophoretic velocity becomes zero when pH = pI, since the net charge on the protein is zero at pH = pI. Precipitation of proteins in a pH gradient at their isoelectric point is known as *isoelectric focusing*. Since the size and charge of protein molecules are different at a given pH, the terminal velocity of the proteins will be different according to equation 11.101, and therefore, proteins will be separated from each other in an electric field. Certain gels, such as agar or polyacrylamide, are used for protein separations by gel electrophoresis. Gel electrophoresis is an important analytical separation technique. Scale-up is problematic due to thermal convection resulting from electrical heating.

One analytical and micropreparative version of protein electrophoresis that has excellent resolution is called two-dimensional electrophoresis (2DE). The 2DE procedure is actually the series combination of two electrophoretic separations, which resolve protein mixtures based on two independent characteristics: charge and size. Proteins are first separated in a polyacrylamide gel matrix using isoelectric

focusing, an equilibrium separation technique that resolves proteins based on their respective isoelectric points. Proteins are subsequently coated with an anionic surfactant and separated by size in another polyacrylamide gel. Finally, proteins are detected using a chemical stain, by autoradiography, or by other methods. The result of this two-dimensional separation is a high-resolution fingerprint of protein expression that is characteristic of a particular biological system. This technique has at least two orders of magnitude better resolution than any other analytical tool for protein analysis. Separated protein spots can be cut out of the polyacrylamide gels and subjected to further microchemical analysis to determine the amino acid sequence.

11.4.11. Electrodialysis

Electrodialysis (ED) is a membrane separation method used for the separation of charged molecules from a solution by application of a direct electric current. The membranes contain ion-exchange groups and have a fixed electrical charge. Positively charged membranes (anion membranes) allow the passage of anions and repel cations; negatively charged membranes (cation membranes) allow the passage of cations and repel anions. This method is very effective in the concentration of electrolytes and proteins.

The ED process is driven by electrostatic forces and can be used to transfer salts from low to high concentration. Salt solutions can be concentrated or diluted by this method. The ED method is much faster than dialysis and is a more efficient desalting method.

Ion-exchange membranes (IEM) used in ED units are in essence ion-exchange resins in sheet form. IEMs contain mobile counterions that carry electric current. The cation-exchange membrane consists of polystyrene with negatively charged sulfonate groups bonded to phenyl groups in polystyrene. Anion membranes contain positively charged quaternary ammonium groups ($—N_3R^+$) chemically bonded to the phenyl groups in the polystyrene. Mobile counterions account for the low electrical resistance of the membranes. To control swelling of sulfonated polystyrene, some cross-linking agents such as divinyl benzene are included in the polymer. A typical ED membrane has a pore size of 10 to 20 Å with a capacity of 2 to 3 mEq/g dw resin. Membrane thickness is on the order of 0.1 to 0.6 mm, and electrical resistance is approximately 3 to 30 ohm/cm^2.

ED units can be used for concentration and dilution of salts, ion substitution, and electrolysis. A schematic of an ED unit used for concentration and dilution of salts is depicted in Figure 11.30. The unit contains a number of diluting (D^*) and concentrating (C^*) compartments separated by alternating anion–cation membranes and two electrodes, the cathode and anode. ED units can also be used for ion-substitution purposes to change the ionic composition of a process stream. If the ionic liquid stream is passed between two membranes of the same charge (two cation or two anion exchange membranes), ions will be transferred from the solution in one direction through one membrane, and equivalent ions of the same charge will be transferred into the solution through the opposite membrane from a makeup solution on the other side of the membrane. In electrolytic ED units, electrodes play an important fundamental role in the

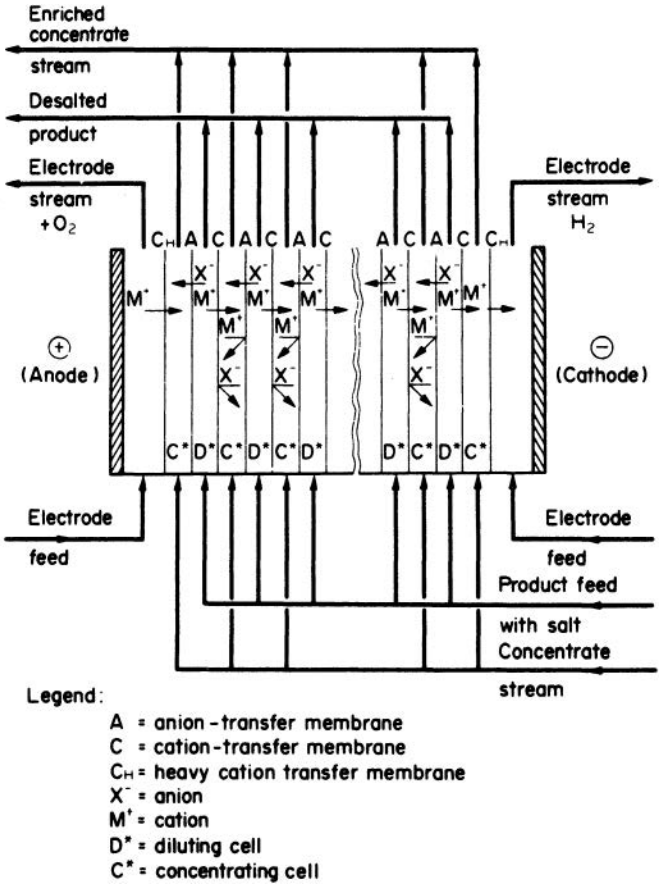


Figure 11.30. Schematic of concentrating-diluting multicell pair electrodialysis process. (With permission, from S. M. Jain and P. B. Reed, in M. Moo-Young, ed., *Comprehensive Biotechnology*, Vol. 2, Elsevier Science, London, 1985.)

process. Two electrode compartments are separated by a membrane in electrolytic units. An example of an electrolytic ED unit is a chlor-alkali cell in which an NaCl solution is electrolyzed and converted to NaOH and Cl.

11.5. FINISHING STEPS FOR PURIFICATION

The major finishing steps in fermentation-product purification are crystallization and drying, particularly for low-MW products.

11.5.1. Crystallization

Crystallization is usually the last step in producing highly purified products such as antibiotics. Crystallization operates at low temperatures to minimize thermal degradation of heat-sensitive materials. Operations are conducted at high concentrations, and therefore, unit costs are low and separation factors are high. The determination of optimal crystallization conditions is a matter of empirical experimentation, since

phase diagrams and pertinent kinetics for specific systems are usually not available. The output of crystallization is a slurry of product crystals in a liquor of solvent and uncrystallized impurities. Recovery of the solid crystals from the slurry is then needed.

High-purity crystals are recovered from the slurry by using batch Nutsche-type filters or centrifugal filters. The following equation is used for the filtration of crystal slurries, assuming the crystals are noncompressible and the resistance of the filter medium is negligible:

$$\frac{dV}{dt} = K_T \frac{A\,\Delta P}{\Delta X} \tag{11.102}$$

where ΔX is the thickness of crystal layer, t is time, V is liquid volume, ΔP is pressure drop, A is surface area, and K_T is the transfer coefficient. Substituting $W \propto V$, where W is the amount of solids in the cake, and integrating equation 11.102, we obtain

$$t = K_T \frac{W^2}{\Delta P \cdot A^2} \tag{11.103}$$

A typical centrifugal filter combines a bowl centrifuge with a filter, and the following relationship is used to determine the filtration–crystallization rate for centrifugal filters:

$$\frac{dV}{dt} = \frac{(2\pi N)^2 \rho L(r_0^2 - r_L^2)}{2\mu(\alpha W / A_m^2 + R_M / A_0)} \tag{11.104}$$

where N is the rotational speed, α is the specific cake resistance, W is the amount of solids in the cake, A_m is the log mean area, and R_M is the medium resistance.

The nature and size of crystals affect the centrifugation and washing rates; therefore, crystallization and recovery steps are interrelated and should be considered together in the optimization of crystallization operations. After washing, the crystals are discharged for drying.

11.5.2. Drying

The removal of solvent from purified wet product (crystal or dissolved solute) is usually achieved by *drying*. In selecting drying conditions, the physical properties of the product, its heat sensitivity, and the desirable final moisture or solvent content must be considered. The parameters affecting drying can be classified in four categories: physical properties of the solid–liquid system, intrinsic properties of the solute, conditions of the drying environment, and heat-transfer parameters.

Following are the major types of dryers used for drying fermentation product:

- *Vacuum-tray dryers* consist of heated shelves in a single chamber and are usually used for pharmaceutical products such as antibiotics. This is a good method for small batches of expensive materials, where product loss and heat damage must be minimized.
- *Freeze drying (lyophilization)* is a method where water is removed by sublimation (from solid ice to vapor) from the frozen solution. The freezing can be

accomplished either outside or inside the vacuum chamber prior to drying. This method is used for drugs in vials such as antibiotics, enzyme solutions, and bacterial suspensions.

- *Rotary-drum dryers* are not good for crystal solutions. Water is removed by heat conduction over a thin film of solution on the steam-heated surface of the rotating drum. The dried product is scraped from the drum with the aid of a knife at the discharge point.
- *Spray dryers* employ atomization and spraying of product solution into a heated chamber through a nozzle. Hot gas inside the chamber provides the necessary heat for evaporation of the liquid. Dried particles are separated from hot gases using cyclones. Spray dryers are expensive to purchase but are often the preferred method for heat-sensitive materials.
- *Pneumatic conveyor dryers* use a hot-air stream to suspend and transport particles. The retention time of a particle in the gas stream is short, usually a few seconds. Such systems work well when surface drying is critical, but they do not provide sufficient exposure times to dry large, porous particles where water removal is diffusion controlled. Pneumatic conveyor systems are well suited for heat-sensitive and easily oxidized materials.

11.6. INTEGRATION OF REACTION AND SEPARATION

The separate optimization of fermentation and recovery does not necessarily yield the optimal process. Traditionally, the strain development, fermentation, and recovery experts worked essentially in isolation although this has begun to change. A lack of formal training of engineers in basic biological concepts and of biologists in engineering and process concepts has made an integrated view of the bioprocesses difficult to obtain. Systems have been optimized sequentially, with only modest feedback from downstream to upstream. With improved cross-training of both engineers and biologists, it has become possible to begin to build better processes.

One form of this integration is to try to couple some aspects of recovery and purification with the bioreactor. The motivation for such approaches has come initially from a desire to relieve product inhibition and thereby increase reaction rates and/or allow the use of a more concentrated feed. However, other advantages may accrue, such as improved selectivity for the product of interest, conversion of a primarily intracellular to an extracellular product, and protection of a product from degradation.

Isolated examples of attempts at bioprocess integration stretch back to the beginning of modern biochemical engineering. In the 1970s, the energy crisis and interest in ethanol production spawned many suggested approaches to bioprocess integration. Some early approaches to such process integration include vacuum fermentation, membrane processes, addition of solid adsorbents, and liquid extractants.

One example is a reactor system that allows the use of tributylphosphate (TBP) as an extractant of ethanol from fermentation broths. Although TBP had been

recognized as an attractive solvent for this purpose, its direct use for in situ extraction was thought to be impossible because of toxicity. However, further work led to the recognition that a solvent could be toxic due to two features: a chemical or molecular level toxicity caused by solvent dissolved in aqueous broth, and a physical or phase toxicity caused by direct interaction of yeast with droplets of emulsified solvent. It turned out that TBP's toxicity was solely due to phase toxicity. One way to circumvent phase toxicity is to immobilize cells in a manner that allows entry of nutrients and exit of product while excluding droplets of solvent. A device that can do this is a *multimembrane bioreactor* (Figure 11.31).

With a hydrophobic membrane, the solvent will readily fill the pores of the membrane. If the aqueous solution is at a pressure higher than the solvent but less than the critical entry pressure of the aqueous solution into the membrane pores, then the solvent is effectively immobilized in the membrane. Such a system allows in situ solvent extraction of product while preventing the emulsification of solvent and phase toxicity. The primary advantages for this type of bioreactor are the use of more concentrated feeds, relief of feedback inhibition, and reduced distillation or separation costs (e.g., for ethanol recovery).

Not only can this integrated view be expressed through the addition of extracting agents, but it can be expressed at the molecular level. Several groups have worked on protein excretion into the extracellular compartment from *E. coli* using appropriately constructed plasmids. The motivation for this approach has been to greatly simplify recovery and purification rather than to achieve any potential increases in reactor productivity.

It is important for the bioprocess engineer to consider the whole system. The design or choice of culture, reactor configuration, and separation and purification train must be made carefully. Small changes in the upstream process can either greatly simplify or complicate the design of the downstream process.

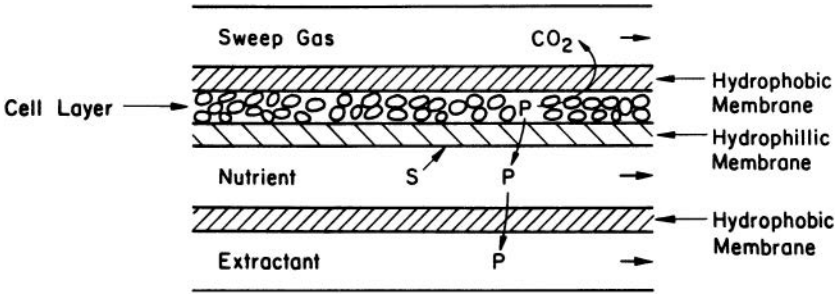


Figure 11.31. Example of a multimember reactor. The extractant fills the pores of the hydrophobic membrane but cannot pass into the nutrient solution if the pressure on the nutrient side is higher than on the extractant side. S is the substrate and P is the product. (From T. Cho and M. L. Shuler, *Biotechnology Progress* 2:1, pp. 53–60, 1986. With permission from John Wiley & Sons.)

11.7. SUMMARY

The recovery and purification of products at the end of fermentation processes is an essential step. Product separation can be accomplished either integrated with or following the fermentation step. The major categories of separation of fermentation products are separation of biomass or other insoluble products, concentration or primary isolation of product, purification, and finishing (drying and packaging).

When the product is the biomass, the separation strategy is relatively simple and involves filtration or centrifugation following coagulation–flocculation of the cells. Drying is required for a marketable biomass, such as a single-cell protein source.

For soluble products such as antibiotics, enzymes, and organic acids, the separation strategy depends on whether the products are extracellular or intracellular. Some products can be both. For intracellular products, the cells need to be disrupted for the release of the products. Mechanical, chemical, and enzymatic methods can be used for cell disruption. Products need to be separated from the other cell and medium components after cell disruption. Depending on the type and the MW of the product, separation methods involve the removal of cell debris by centrifugation or filtration, followed by extraction or precipitation (for protein products) of the product. The separation strategy for extracellular products is simpler than that for intracellular products, since cell disruption is not required. After the separation of the biomass from the fermentation medium, the product is separated from the broth by one or a combination of the following methods: liquid–liquid extraction (antibiotics), two-aqueous-phase extraction (enzymes), adsorption (antibiotics), or precipitation (proteins). Often, the product must be crystalized and dried before packaging.

Protein (enzymes, hormones, therapeutics) separations usually require a special strategy. Proteins are usually precipitated from the media either by salting-out or by the addition of organic solvents. The desired protein is crudely separated from other protein components, usually by ultrafiltration on the basis of its MW. Then some chromatographic methods, such as ion-exchange or affinity chromatography, need to be used for further purification. Depending on the chemical nature of the desired protein and the composition of the protein mixture, sometimes special protein separation methods, such as isoelectric focusing or electrophoresis (for charged proteins), may need to be used, although these are used more for research or analytic purposes.

Simultaneous separation and fermentation schemes offer special advantages over consecutive schemes, since they may overcome product inhibition and provide a more compact and economical alternative. Membrane, adsorptive, or extractive separation schemes can be utilized for the simultaneous separation of products during fermentation.

SUGGESTIONS FOR FURTHER READING

Bailey, J. E., and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill, New York, 1986.

Belter, P. A., E. L. Cussler, and W. S. Hu, *Bioseparations*, Wiley, New York, 1988.

Blanch, H. W., and D. S. Clark, *Biochemical Engineering,* Marcel Dekker, New York, 1996.

Eckenfelder, W. W., *Industrial Water Pollution Control*, 2d ed. McGraw-Hill, New York, 1989.

Flickenger, M. C., *Downstream Industrial Biotechnology: Recovery and Purification*, Wiley, New York, 2013.

Ghosh, R., *Principles of Bioseparations Engineering*, World Scientific Publishing Company, Hackensack, NJ, 2006.

Harrison, R. G., P. W. Todd, S. R. Rudge, and D. P. Petrides, *Bioseparations Science and Engineering*, 2d ed., Oxford University Press, New York, 2015.

Jungbauer, A., "Continuous Downstream Processing of Biopharmaceuticals," *Trends Biotechnol.* 8: 479–492, 2013.

Marichal-Gallardo, P. A, and M. M. Alvarez, "State-of-the-Art in Downstream Processing of Monoclonal Antibodies: Process Trends in Design and Validation," *Biotechnol. Progr.* 28: 899–916, 2012.

PROBLEMS

11.1. Yeast cells are recovered from a fermentation broth by using a tubular centrifuge. Sixty percent of the cells are recovered at a flow rate of 12 l/min with a rotational speed of 4000 rpm. Recovery is inversely proportional to flow rate.

- **a.** To increase the recovery of cells to 95% at the same flow rate, what should be the rpm of the centrifuge?
- **b.** At a constant rpm of 4000 rpm, what should be the flow rate to result in 95% cell recovery?

11.2. Gentamycin crystals are filtered through a small test filter medium with a negligible resistance. The following data were obtained:

t (sec)	10	20	30	40
V (l)	0.6	0.78	0.95	1.1

The pressure drop in this test run was 1.8 times that when water was used with a filter area of 100 cm^2. The concentration of gentamycin in solution is 5 g/l. How long would it take to filter 5000 l of gentamycin solution through a filter of 1.5 m^2, assuming the pressure drop is constant and $\mu = 1.2$ centipoise?

11.3. Streptomycin is extracted from the fermentation broth using an organic solvent in a countercurrent staged extraction unit. The distribution coefficient of streptomycin at pH = 4 is $K_D = Y_i/X_i = 40$, and the flow rate of the aqueous (H) phase is H = 150 l/min. If only five extraction units are available to reduce the streptomycin concentration from 10 g/l in the aqueous phase to 0.2 g/l, determine the required flow rate of the organic phase (L) in the extraction unit.

11.4. A new antibiotic is separated from a fermentation broth by adsorption on resin beads in a fixed bed. The bed is 5 cm in diameter and contains 0.75 cm^3 resin/cm bed. The overall mass transfer coefficient is 12 h^{-1}. If the antibiotic concentration in the feed is 4 g/l and is desired to be 0.1 g/l in the effluent in a 50 cm column, determine the liquid flow rate through the column. The equilibrium relationship is $C_S^* = 20(C_L^*)^{1/2}$, and the operating-line relationship is $C_S = 5C_L$, where C_S is g solute/l resin and C_L is g solute/l solution.

11.5. An activated carbon adsorption column is used for removal of phenolic compounds from wastewater. The feed and desired effluent adsorbate concentrations are C_o = 100 mg/l

and C_e = 1 mg/l, and the flow rate is F = 10 m^3/h. Adsorption column contains 3 m^3 activated carbon with 30% void fraction and the column diameter is D_o = 0.5 m. Adsorption capacity and rate for activated carbon are N_o = 60 kg adsorbate/m^3 bed and K = 0.8 m^3/kg-h.

a. Determine the adsorption column height and service time.

b. Using the same column characteristics, determine column height for 10 days of operation.

11.6. Refractory organic compounds are removed from aqueous phase by using an activated carbon adsorption column. For the feed flow rate of 5 m^3/h and the feed and effluent organics concentrations of 40 and 0.5 mg/L, the column can be operated for 3 days when column height was 3 m. The same column is operated for 6 days when H = 5 m. D_o = 0.6 m.

a. Determine adsorption capacity (N_o) and the rate constant (K) for the activated carbon adsorption column.

b. For the same column characteristics, determine the required column height when the flow rate doubles for 6 days of operation.

c. Determine the volume and the weight of required AC if void fraction is 35% and density of AC is 2 g/cm^3 for the column in part (b).

11.7. In a cross-flow ultrafiltration unit, a protein of MW = 3 × 10^5 da is separated from the fermentation broth by using a UF membrane. The flow rate of liquid through a tube of diameter d = 2 cm and length L = 50 cm is Q = 2 l/min. The flow regime is turbulent, f = 0.0005, and C_4 = 2 [atm (s/cm)^2]. The inlet pressure is P_i = 2 atm. Protein concentrations in the solution and on gel film are C_B = 30 mg/l and C_G = 100 g/l, respectively.

a. Determine the exit pressure (P_0).

b. Determine the transmembrane pressure drop (ΔP_M).

c. If the mass transfer coefficient (k) for protein flux is k = 5 cm/s, determine the flux of liquid through the UF membrane (J).

d. If the resistance of the filter is R_M = 0.002 atm/cm^2-s/cm^3, determine the cake resistance, R_G.

11.8. Components A and B of a binary mixture are to be separated in a chromatographic column. The adsorption isotherms of these compounds are given by

$$m_A = f_A(c) = \frac{k_1 C_A}{k_2 + C_A}$$

$$m_B = f_B(c) = \frac{k_1' C_B}{k_2' + C_B}$$

where k_1 = 0.2 mg solute A absorbed/mg adsorbent
k_2 = 0.1 mg solute/ml liquid
k'_1 = 0.05 mg solute B adsorbed/mg adsorbent
k'_2 = 0.02 mg solute/ml liquid

The bed contains 3 g of very fine support particles. The bed volume is 150 ml, bed porosity is ε = 0.35, and the cross-sectional area of the bed is A = 6 cm^2. The volume of the mixture added is ΔV = 50 ml.

a. Determine the position of each band A and B in the column, L_A and L_B (or ΔX_A and ΔX_B).

b. Determine L_A/L_B; $R_{fA} = L_A/L_c$; $R_{fB} = L_B/L_c$ when $C_A = 10^{-1}$ mg/ml and C_B = 0.05 mg/ml in liquid phase at equilibrium.

11.9. Consider the use of gel chromatography to separate two proteins, A and B. The partition coefficient (K_D) for A is 0.5 and for B is 0.15. The void volume in the column, V_o, is

20 cm^3. The void volume within the gel particles, V_i, is 30 cm^3. The total volume of the column is 60 cm^3. The flow rate of eluent is 100 cm^3/h. Ignoring dispersion and other effects, how long will it take for A to exit the column? How long for B?

11.10. Biomass present in a fermentation broth is to be separated by vacuum filtration. Following are the filter and broth characteristics:

$$A = 50 \text{ m}^2, \qquad \Delta P = 0.01 \text{ N/m}^2, \qquad C = 15 \text{ kg/m}^3$$

$$\mu = 0.003 \text{ kg/m-s}, \qquad \alpha = 2 \text{ m/kg}$$

a. If rate of filtration has a constant value of $dV/dt = 50$ l/min, determine the cake and filter resistances at $t = 30$ min.

b. Determine the filter surface area (A) required to filter 5000 l broth within 60 min with the same pressure drop across the filter.

11.11. A fermentation broth with a protein concentration of $C_0 = 100$ mg/l and flow rate of $Q = 4$ m^3/h is passed through two downflow adsorption columns connected in series. The adsorption isotherm is $q = 4C^{1/4}$. Assume the system is in equilibrium.

a. Calculate the minimum amount of activated carbon required for two days of operation if removal efficiency for the column is $E = 50\%$.

b. Protein concentration in the effluent of the second column is desired to be $C = 0.5$ mg/l. Determine the minimum amount of activated carbon required for two days of operation.

11.12. In a cross-flow ultrafiltration system used for filtration of proteins from a fermentation broth, gel resistance increases with protein concentration according to the following equation:

$$R_G = 0.5 + 0.01(C), \text{ where } C \text{ is in mg/l.}$$

Pressure at the entrance of the system is $P_i = 6$ atm and at the exit is $P_0 = 2$ atm. The shell side of the filter is open to the atmosphere, resulting in $P_f = 1$ atm. The membrane resistance is $R_M = 0.5$ atm/(mg/m^2-h), and protein concentration in the broth is $C = 100$ mg/l.

a. Determine the pressure drop across the membrane.

b. Determine the filtration flux.

c. Calculate the rejection coefficient of the membrane for effluent protein concentration of $C_f = 5$ mg/l.

11.13. A solute protein is to be separated from a liquid phase in a chromatographic column. The adsorption isotherm is given by

$$C_S = kC_L^2$$

where C_S is the solute concentration in solid phase (mg solute/mg adsorbent) and C_L is the liquid phase concentration of solute (mg solute/ml liquid). Use the following information:

$$k = 0.4, \qquad \varepsilon = 0.3, \qquad A = 25 \text{ cm}^2,$$

$$M = 10 \text{ g ads/100 ml column} = 100 \text{ mg/ml}$$

a. For $V = 400$ ml and $X = 25$ cm, determine the equilibrium solute concentrations in liquid and solid phases.

b. Determine the ratio of travel distances of solute to solvent, R_f.

11.14. Proteins A and B are to be separated using ion-exchange chromatography. When using a superficial fluid velocity of 30 cm/h, peak A exits with y_{max} at 50 min and peak B exits with y_{max} at 100 min. For peak A, the standard deviation is 12 min, and for peak B, 18 min.

a. Assuming that Taylor dispersion controls and that v is increased to 60 cm/h, what will be the values for the peak elution times and standard deviations for A and B?

b. For v = 60 cm/h, sketch the two peaks and calculate the resolution of the two peaks (R_s). (You may find Figure 11.29 to provide helpful guidance).

c. Would you recommend increasing v to 60 cm/h to increase throughput? Why or why not?

11.15. Consider the scale-up of a chromatography column for purification of a protein. A column of 40 cm length is used with a superficial velocity of 40 cm/h. The peak concentration of the target protein exits at a time of 100 min. The standard deviation of the peak is 14 min.

a. How long must you wait to collect 90% of the protein?

b. If the same column is used, but velocity is increased to 60 cm/h and external or Taylor dispersion controls, what will be the value of σ?

c. If the column is lengthened to 60 cm while the velocity is at 60 cm/h, how will σ change? Will the peak be sharper or broader than at 40 cm/h with a 40 cm column?

12

Bioprocess Considerations in Using Animal Cell Cultures

Animal cells have become important catalysts for many bioprocesses. They are capable of making a vast array of important therapeutic proteins. With recombinant DNA techniques, it is possible to achieve significant levels of production of compounds made only in minute amounts in native cells. Animal cells possess the machinery to do complex posttranslational modifications to proteins. This capacity is essential in some cases to the formation of a clinically useful product particularly for therapeutic proteins. Currently, more than 50% of new therapeutic proteins are being produced using mammalian cell tissue culture. Also, the development of stem cell technology opens the possibility that the cells themselves will become the product of interest. In this chapter, we consider some of the important constraints in using these cells.

12.1. STRUCTURE AND BIOCHEMISTRY OF ANIMAL CELLS

Animal cells vary in size (10 to 30 μm) and shape (spherical, ellipsoidal). In terms of intracellular structures, animal cells are typical eucaryotes, and we discussed their cellular structure in Chapter 2, "An Overview of Biological Basics." Figure 12.1 summarizes the structure of a typical animal cell.

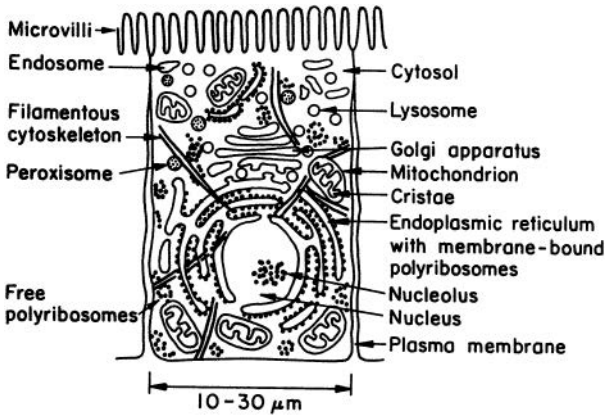


Figure 12.1. Generalized animal cell. The cytosol, endoplasmic reticulum, Golgi apparatus, endosome, nucleus, lysosome, mitochondrion, and peroxisome are distinct compartments isolated from the rest of the cell by selectively permeable membranes.

Animal cells do not have a cell wall but are surrounded by a thin and fragile plasma membrane that is composed of protein, lipid, and carbohydrate. This structure results in significant shear sensitivity. In some cells, a portion of the plasma membrane is modified to form a number of projections called *microvilli*. The microvilli increase the surface area of the cell and provide more effective passage of materials across the plasma membrane. The composition of plasma membrane is not uniform and varies in different regions of the membrane. The surface of an animal cell is negatively charged, and cells tend to grow on positively charged surfaces, such as Sephadex or collagen (anchorage-dependent cells). Many cells possess specific cell surface receptors that adhere to ligands on the surface. For example, the binding to collagen may be nonspecific or may be mediated by specific cell surface receptors. The degree of cell adhesiveness is usually greater if attachment is receptor mediated. Some animal cells such as hybridomas are nonanchorage dependent and grow in suspension culture.

Inside the cytoplasm of most animal cells is an extensive network of membrane-bounded channels called the *endoplasmic reticulum* (ER). The membranes of the ER divide the cytoplasm into two phases: the *lumenal* phase (inside the endoplasmic reticulum) and the *cytosol* (outside the rough endoplasmic membrane). Ribosomes are usually located on the outer surface of the endoplasmic reticulum. Some ribosomes are located in cytoplasm and may be interconnected by fine filaments. The ER is critical in protein synthesis and initial stages of posttranslational processing (see Chapter 4, "How Cells Work").

Mitochondria are the powerhouse of cells where respiration takes place and the bulk of the adenosine triphosphates (ATPs) are produced. Mitochondria are independent organelles in the cytoplasm containing DNA and are capable of independent reproduction. Each mitochondrion is surrounded by a double membrane: a smooth outer membrane and a highly folded inner membrane called the *cristae*. The mitochondrial matrix often contains crystallike inclusions.

Lysosomes are rather small cytoplasmic organelles bound by a single membrane, and they contain various hydrolytic enzymes, such as proteases, nucleases, and esterases. Lysosomes are responsible for the digestion of certain food particles ingested by the cell.

The *Golgi body* is a cytoplasmic organelle surrounded by a rather irregularly shaped membrane called the *cisternae*. The cisternae of a Golgi body are often stacked together in parallel rows, called *dictyosome*. The Golgi apparatus is responsible for the completion of complex glycosylation and for collecting and secreting extracellular proteins or directing intracellular protein traffic to other organelles.

Some cells contain small cytoplasmic organelles called *peroxisomes* and *glyoxysomes*. These organelles are bounded by a single membrane and contain a number of enzymes, including peroxidases (hydrolysis of H_2O_2) and glyoxalases (glyoxylic acid metabolism).

The nucleus is bounded by two nuclear membranes that form a nuclear envelope. At certain positions, the inner and outer membranes of nucleus fuse to form *pores*. Nuclear pores provide continuity between the cytoplasm and the inner part of the nucleus. The *perinuclear space* (the space between the two membranes of the nucleus) may have access to the outer cell. The nucleus contains chromosomal DNA and also some dark granular structures called *nucleoli*, which can be seen under an electron microscope. Nucleoli are not bound by a membrane and appear to be formed by ribosomal material.

Animal cells have a *cytoskeleton* or system of protein filaments that provide cell mechanical strength, control cell shape, and guide cell movement. These elements are often critical components in controlling cell response to mechanical forces such as from fluid flow or from attachment to surfaces. The three types of filaments are *actin filaments*, *intermediate filaments*, and *microtubules*. Actin filaments are relatively thin, while microtubules have the largest diameter. Microtubules are critical in cell division and cell movement. Cell division and movement require the polymerization and depolymerization of microtubules; the anticancer agent paclitaxel (Taxol) works by preventing depolymerization of microtubules and thus "freezing" the cell in a nondividing step. The *centrosome* is the primary microtubule-organizing center in the cell and is a small organelle involved in the formation of spindle poles during mitosis. Some animal cells may contain *cilia*, which are used to transport substrate across the cell surface but not for self-locomotion. Each cilium is covered by a plasma membrane and contains a specific array of microtubules.

The metabolism of nutrients by animal cells in culture is very different from that in other types of cells. A typical laboratory growth medium of an animal cell culture contains glucose, glutamine, nonessential and essential amino acids, serum (horse or calf), and mineral salts (for example, Dulbecco's modified Eagle's media, or DMEM). For commercial use of these cells to make therapeutic proteins, a serum-free medium is preferred. The major metabolic pathways for substrate uptake and utilization by animal cells in culture are depicted in Figure 12.2. Glucose is converted to pyruvate by glycolysis and also is utilized for biomass synthesis through the pentose phosphate pathway. Pyruvate is converted partly to CO_2 and H_2O by the tricarboxylic acid (TCA) cycle, partly to lactic acid and partly to fatty acids. Glucose is used as a carbon and energy source, as is glutamine. Part of the glutamine is deaminated to yield ammonium and glutamate, which is converted to other amino acids for biosynthesis purposes. Glutamine also enters into the TCA cycle to yield carbon skeletons for other amino acids and to yield ATP, CO_2, and H_2O. Animal cells are also capable of synthesizing glucose from pyruvate by the gluconeogenesis pathway. The release

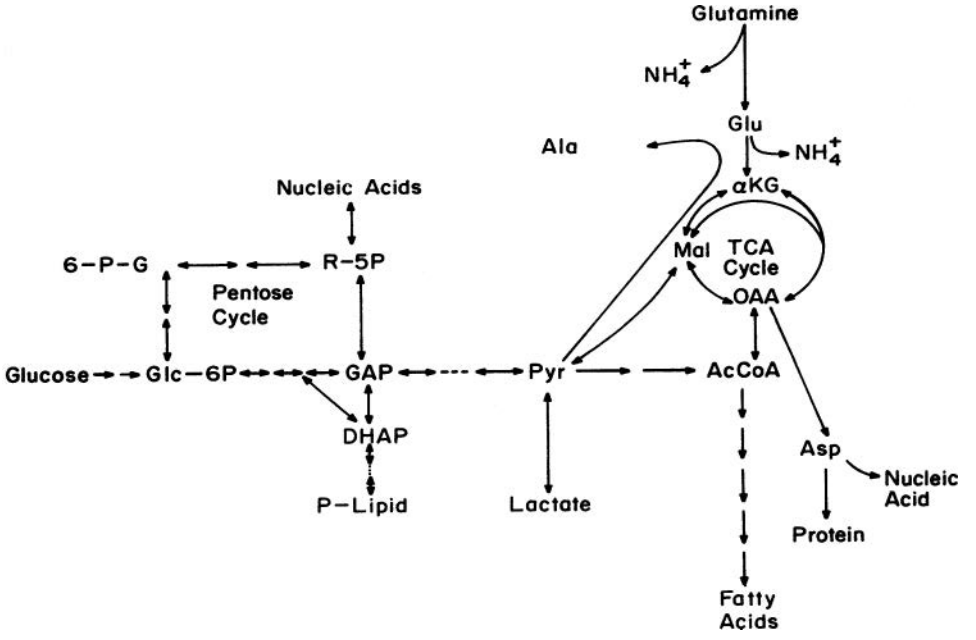


Figure 12.2. Major metabolic pathways in an animal cell. For cultured cells, glucose and glutamine are the major sources of carbon and energy. Lactate and ammonia are the primary waste products, although the release of alanine can be important. Symbols: 6-P-G is 6-P-gluconate; Glc-6P is glucose-6-phosphate; R-5P is ribose-5-phosphate; GAP is glyceraldehyde 3-phosphate; DHAP is dihydroxyacetone phosphate; PYR is pyruvate; AcCoA is acetyl-CoA; OAA is oxaloacetate; Mal is malate; αKG is α-ketoglutarate.

of lactate and ammonia as waste products of metabolism is a major problem in high-cell-density culture systems. Both lactate and ammonia at high levels are toxic to cells, primarily because they alter intracellular and lysosomal pH.

12.2. METHODS USED FOR THE CULTIVATION OF ANIMAL CELLS

The techniques used for cultivation of animal cells differ significantly from those used with bacteria, yeasts, and fungi. In this section, we discuss some of the issues in the application of these techniques and the basic growth dynamics of animal cells in simple culture systems.

12.2.1. Basic Techniques for Animal Cell Culture

Several methods can be used to culture animal cells in the context of bioprocesses. Tissues excised from specific organs of animals, such as lung and kidney, under aseptic conditions are transferred into a growth medium containing serum and small amounts of antibiotics in small T-flasks. These cells form a *primary* culture. Unlike plant cells, primary mammalian cells do not normally form aggregates but grow in the form of monolayers on support surfaces such as glass surfaces of flasks.

Using the proteolytic enzyme trypsin, individual cells in a tissue can be separated to form single-cell cultures.

To start cultures of animal cells, excised tissues are cut into small pieces (~2 mm^3) and are placed in an agitated flask containing a dilute solution of trypsin (~0.25% w/v) in buffered saline for 120 min at 37°C. The cell suspension is passed through a presterilized filter to clear the solution, and cells are washed in the centrifuge. The cells are then resuspended in growth medium and placed in T-flasks or roller bottles. Cells usually attach onto the glass surface of the bottle and grow to form a monolayer. The cells growing on support surfaces are known as *anchorage-dependent* cells. Surface attachment is necessary for these cells to assume the three-dimensional shape necessary for the alignment of internal structures in a manner allowing growth. However, some cells grow in suspension culture and are known to be *nonanchorage-dependent* cells. Often anchorage-dependent cells can be adapted to growth in suspension culture, which is desirable for industrial processes.

The cells directly derived from excised tissues are known as the *primary* culture. A cell line obtained from the primary culture is known as the *secondary* culture. Cells are removed from the surfaces of flasks using a solution of ethylenediaminetetraacetic acid (EDTA), trypsin, collagenase, or pronase. The exposure time for cell removal is 5 to 30 min at 37°C. After cells are removed from surfaces, serum is added to the culture bottle. The serum-containing suspension is centrifuged, washed with buffered isotonic saline solution, and used to inoculate secondary cultures. Many secondary lines can be adapted to grow in suspension and are nonanchorage dependent.

Most differentiated mammalian cell lines (e.g., human fibroblasts such as WI-38 and MRC-5 that are licensed for human vaccine production) are *mortal*. These cell lines undergo a process called *senescence*. Essentially, the cells, for reasons that are not completely understood, will divide only for a limited number of generations (e.g., about 30 generations for the MRC-5 cells). Cells that can be propagated indefinitely are called *continuous*, *immortal*, or *transformed* cell lines. Cancer cells are naturally immortal. All cancerous cell lines are transformed, although it is not clear whether all transformed cell lines are cancerous.

Because of the linkage of cancer to cell transformation, the FDA had been reluctant to approve products made from transformed cells. However, transformed cells usually become attachment independent and can be propagated indefinitely in suspension culture, which is highly desirable for large-scale production in bioreactors. In 1986, the FDA approved the first process for production of products, such as the therapeutic protein tissue plasminogen activator, from processes using immortalized cells. The approval of such processes has provided a major impetus for development of bioprocesses based on suspension cultures of animal cells.

Table 12.1 provides a summary of differences between nontransformed and transformed cells. One particular characteristic is *contact inhibition*. In two-dimensional culture on a surface, nontransformed cells form only a monolayer, as cell division is inhibited when a cell's surface is in contact with other cells. Transformed cells do not "sense" the presence of other cells and keep on dividing to form multilayer structures.

Although mammalian cell lines have been the primary focus of work in animal cell culture, the two are not synonymous. Insect, fish, and crustacean cell cultures are evolving technologies. In particular, insect cell culture is unusually promising for

TABLE 12.1. Comparison of Normal and Transformed Cells

Normal	Transformed
Anchorage dependent (except blood cells)	Nonanchorage dependent (i.e., suspension culture possible)
Mortal; finite number of divisions	Immortal or continuous cell lines
Contact inhibition; monolayer culture	No contact inhibition; multilayer cultures
Dependent on external growth factor signals for proliferation	May not need an external source of growth factors
Greater retention of differentiated cellular function	Typically, loss of differentiated cellular function
Display typical cell surface receptors	Cell surface receptor display may be altered

biotechnological purposes. The baculovirus that infects insect cells is an ideal vector for genetic engineering, because it is nonpathogenic to humans and has a very strong promoter that encodes for a protein that is not essential for virus production in cell culture. The insertion of a gene under the control of this promoter can lead to high expression levels (40% of the total protein as the target protein). Most cell lines are derived from ovaries or embryonic tissue, although at least one differentiated cell line (a BTI-EAA blood cell line) has been maintained in indefinite culture for more than 25 years. Insect cell lines are not transformed but are naturally continuous.

Insect cell cultures can do a significant level of posttranslational processing, but the glycoforms are not authentic with humans. However, the differences can be useful and sometimes trigger a more intense immune response or target specific cells or organs in humans. In these cases, production in insect cells is advantageous. Production of modified insect viruses have been used as insecticides. The baculovirus expression system used with insect cells has been used to make commercial levels of human and veterinary vaccines and a gene therapy agent against familial lipoprotein deficiency.

Another important category is the culture of hybridomas. Hybridoma cells are obtained by fusing lymphocytes (normal blood cells that make antibodies) with myeloma (cancer) cells. Lymphocytes producing antibodies grow slowly and are mortal. After fusion with myeloma cells, hybridomas become immortal, can reproduce indefinitely, and produce antibodies. Using hybridoma cells, highly specific, monoclonal (originating from one cell) antibodies (MAb's) can be produced against specific antigens.

To produce hybridoma cells, animals (mice) are injected (immunized) with certain antigens (see Figure 12.3). As a reaction to antigens, the animals produce antibodies. The antibody-producing cells (e.g., spleen B lymphocytes) are separated from blood sera and fused with certain tumor cells (e.g., myeloma cells with infinite capacity to proliferate and derived from lymphocytes). The resulting cells are hybrid cells (hybridomas), which secrete highly specific antibodies (MAb's) against the immunizing antigen.

Mammalian cell culture is used to produce hundreds of therapeutic proteins using genetically engineered cells. Although many host cells can be used, Chinese hamster ovary (CHO) cells are particularly popular. Most commercial CHO cell lines have been selected to be cultured in liquid suspension, although the original CHO cell lines were anchorage dependent. About 75% of proteins produced commercially from mammalian cell culture use CHO cells. Not only are high product concentrations possible from CHO cells (protein products up to 10 g/l), they are effective excreters

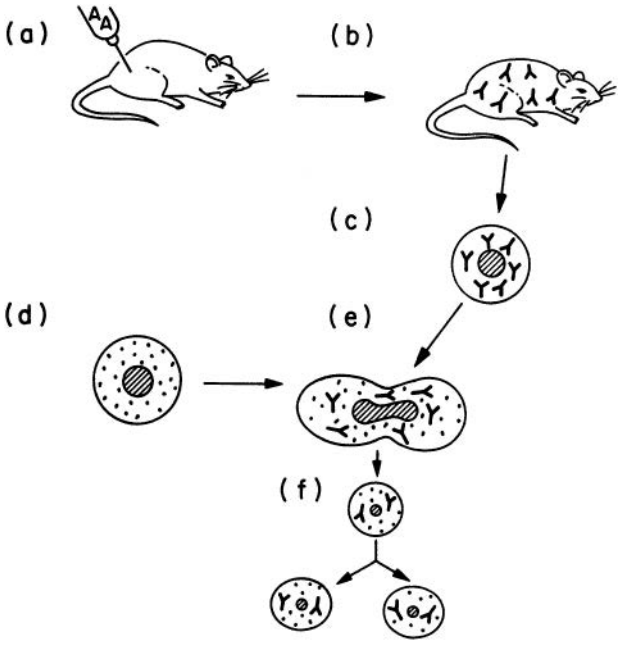


Figure 12.3. Formation of a hybridoma for making a monoclonal antibody. (a) Antigen is injected into a mouse; (b) lymphocytes in the mouse are activated to produce specific antibodies to the antigen; (c) lymphocytes are collected from the mouse; these lymphocytes grow poorly in tissue culture; (d) myeloma (cancer) cells growing in tissue culture are produced; (e) myeloma cells are fused with lymphocytes; (f) the hybrid cell grows well in tissue culture and make a single monoclonal antibody. Progeny are called *hybridomas* and can be propagated indefinitely.

of proteins simplifying product recovery. Other popular host cell lines include mouse myelomas (NSO and SP2/0), baby hamster kidney (BHK-21), human embryonic kidney (HEK-293), and human retina–derived (PerC6) cells. Human-based cell lines may have some advantages in terms of more authentic (humanlike) posttranslational processing, such as N-linked glycosylation (see Chapter 4), but there are increased concerns in using human cells due to inadvertent human viral contamination necessitating multiple viral inactivation steps. Also, the overall productivity of the human cell lines is typically somewhat lower than with CHO, but that may change with increased experience, and it is protein dependent.

12.2.2. Growth Media

A typical growth medium for mammalian cells contains serum (5% to 20%), inorganic salts, nitrogen sources, carbon and energy sources, vitamins, trace elements, growth factors, and buffers in water. Serum is a cell-free liquid recovered from blood. Examples are FBS (fetal bovine serum), CS (calf serum), and HS (horse serum). The exact composition of any serum product is not known. However, serum is known to contain amino acids, growth factors, vitamins, certain proteins, hormones, lipids, and minerals. A list of major serum components and their functions is given in Table 12.2. The major functions of serum are as follows:

- To stimulate cell growth and other cell activities by hormones and growth factors
- To enhance cell attachment by certain proteins, such as collagen and fibronectin
- To provide transport proteins carrying hormones, minerals, and lipids

TABLE 12.2. Important Components of Serum and Their Probable Role in Cell Culture

Component	Probable Function
Proteins	
Albumin	Osmoticum and buffer
	Lipid, hormone, mineral carrier
Fetuin	Cell attachment
Fibronectin	Cell attachment
α_2-Macroglobulin	Trypsin inhibitor
Transferrin	Binds iron
Polypeptides	
Endothelial cell growth factor (ECGF)	Mitogen[a]
Epidermal growth factor (EGF)	Mitogen
Fibroblast growth factor (FGF)	Mitogen
Insulin-like growth factors (IGFI and IGFII)	Mitogen
Platelet-derived growth factor (PDGF)	Mitogen and major growth factor
Hormones	
Hydrocortisone	Promotes attachment and proliferation
Insulin	Promotes uptake of glucose and amino acids
Growth hormones	Mitogen—present in fetal sera
Metabolites and Nutrients	
Amino acids	Cell proliferation
Glucose	Cell proliferation
Keto-acids (e.g., pyruvate)	Cell proliferation
Lipids (e.g., cholesterol)	Membrane synthesis
Minerals	
Iron, copper, zinc, and selenium	Enzymes and other constituents
Inhibitors	
γ-globulin	
Bacterial toxins from prior contaminants	
Chalones (tissue-specific inhibitors)	

[a]A mitogen is a substance that signals mitosis to begin.

Serum is an expensive medium component ($100 to $500/l) and may cause further complications in the cultivation and downstream separation processes. The demand for serum is increasing rapidly and periodically exceeds supply. The presence of serum proteins and peptides greatly complicates downstream processing. Serum must be filtered and sterilized, and contamination with viruses and possibly mycoplasma (a wall-less bacterium) are potential problems. Contamination by prions (agents that cause diseases such as mad cow disease) has been a real concern, and source animals cannot come from regions known to have contaminated animals. Serum-containing medium foams easily. Perhaps most disturbing from a production perspective is the intrinsic variability in serum. No one batch of serum is ever identical to another. A batch of serum will last only a year before deteriorating. Changes in serum batches require extensive testing.

Because of the aforementioned disadvantages of serum-containing media, low-serum or serum-free media have been developed. Typical compositions of two defined media are listed in Table 12.3. Serum-free medium contains basal salts (inorganic salts, carbon, nitrogen compounds), vitamins, growth factors, and hormones (e.g., insulin, transferrin, hydrocortisone, progesterone, fibronectin). By using a serum-free medium,

TABLE 12.3. Compositions of Serum-Containing and Serum-Free Defined Media

Component	Eagle's Minimum Essential Medium (mg/l)	MCDS 170 (mg/l)
Amino Acids (L-enantiomers)		
Alanine	—	8.9
Arginine-HCl	126	63.3
Asparagine	—	132.0
Aspartic acid	—	13.3
Cysteine-HCl	—	35.2
Cysteine	—	8.5
Cystine	24	—
Glutamic acid	—	14.7
Glutamine	292	292
Glycine	—	7.5
Histidine-HCl-H_2O	42	21.0
Isoleucine	52	13.1
Leucine	52	39.3
Lysine-HCl	73	36.6
Methionine	15	4.5
Phenylalanine	33	5.0
Proline	—	5.8
Serine	—	31.5
Threonine	48	35.7
Tryptophan	10	6.1
Tyrosine	36	9.1
Valine	47	35.1
Vitamins		
Biotin	—	0.0073
D-Ca pantothenate	1.0	0.48
Choline chloride	1.0	14.0
Folic acid	1.0	—
Folinic acid	—	0.0051
i-inositol	2.0	18.0
DL-α-lipoic acid	—	0.0026
Niacinamide	—	6.1
Nicotinamide	1.0	—
Pyridoxal HCl	1.0	0.06
Riboflavin	0.1	0.11
Thiamin HCl	1.0	0.34
Vitamin B12	—	0.14
Other Organics		
Adenine	—	0.135
D-glucose	1000	1440
Linoleic acid	—	0.028
Putrescine 2HCl	—	1.6×10^{-4}
Na pyruvate	—	110
Thymidine	—	0.073

(*Continued*)

TABLE 12.3. Compositions of Serum-Containing and Serum-Free Defined Media (*Continued*)

Component	Eagle's Minimum Essential Medium (mg/l)	MCDS 170 (mg/l)
Major Inorganic Salts		
$CaCl_2$	200	222
KCl	400	225
$HgSO_4$	—	180
$MgSO_4 \cdot 7H_2O$	200	—
NaCl	6800	6960
$NaH_2PO_4 \cdot H_2O$	140	—
NaH_2PO_4	—	71
Trace Elements		
$CuSO_4$	—	1.6×10^{-4}
$FeSO_4$	—	0.75
$Fe(NO_3)_3 \cdot 9H_2O$	0.10	—
H_2SeO_3	—	0.0387
$MnSO_4$	—	7.6×10^{-5}
NaS_iO_3	—	0.050
$(NH_4)_6Mo_7O_{24}$	—	0.00116
NH_4VO_3	—	5.9×10^{-4}
$NiCl_2$	—	6.5×10^{-7}
$SnCl_2$	—	9.5×10^{-7}
$ZnSO_4$	—	0.080
Buffers and Indicators		7140
HEPES ($C_8H_{18}N_2O_4S$)	—	—
$NaHCO_3$	2200	—
Phenol red	10	1.31
CO_2 (Gas Phase)	5%	2%
Serum/Serum Replacements		
Serum (e.g., fetal bovine serum, FBS)	5 to 10%	—
Phosphoethanolamine	—	15.9
Ethanolamine	—	6.1
Prostaglandin E1	—	0.01
Hydrocortisone	—	0.05
EGF	—	0.01
Insulin	—	0.5
Transferrin	—	0.5
Prolactin	—	0.5
Ovine prolactin	—	0.1

we may reduce the cost of media, eliminate some potential problems in product purification, reduce the chance of contamination, and most importantly improve the reproducibility of results. Different cell lines require different serum-free media composition. Not all cell lines have been adapted to serum-free media. In some cases, a serum-free, animal-protein-free medium is advantageous, but such media can be quite challenging to formulate.

A number of defined media have been developed. Eagle's minimal essential medium (MEM), DMEM, and rather complex media, such as Ham's F12, CMRL 1066, and RPMI 1640, are commonly used. Media such as Eagle's MEM are often supplemented with 5% to 10% (by volume) of serum when used. For serum-free media formulations, often a 1:1 (v/v) mixture of DMEM (nutrient rich) and F12 (rich in trace elements and vitamins) is used. More specialized media (e.g., MCDB 170MDS) may be used for serum-free growth of specific cell lines. A simpler serum-free medium may contain insulin, transferrin, and selenium as serum replacement components, in addition to glucose, glutamine, other amino acids, and salts. Filtered whole lymph from a cow has been used as a growth medium for some mammalian cells.

12.2.3. Growth Dynamics for Animal Cells

Mammalian cells grow at 37°C and pH ≈7.3. Typical doubling times are 12 to 20 h. Usually, 5% CO_2-enriched air is used to buffer the medium pH. A carbonate buffer ($HCO_3^{2-}/H_2CO_3^-$) is used to control pH around 7.3. Since bicarbonate is used up by the cells, CO_2-enriched air is provided to balance carbonate equilibrium to keep pH ≈7.3. The culture medium needs to be gently aerated and agitated. Other buffers such as HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) are also used to keep pH at desired level. Insect cells grow best at about 28°C and a pH of about 6.2. Fish cell lines tolerate a wide range of temperature, although temperatures below 37°C are usually preferred (25° to 35°C). Values for pH typical of mammalian cell cultures are usually satisfactory for fish cells; pH values between 7.0 and 7.5 usually give good growth.

The pattern for the kinetics of the growth of mammalian cells is similar to microbial growth. Usually, the stationary phase is relatively short, and the concentration of viable cells drops sharply thereafter, as a result of the accumulation of toxic metabolic products such as lactate and ammonium. A high level of ammonium is usually the result of glutamine metabolism, and lactate is usually a product of glucose metabolism. Controlled feeding of these nutrients to maintain a constant but lower level of nutrients, such as in fed batch, can reduce this problem. Cell concentration reaches a peak value within three to five days. However, product formation, such as MAb formation by hybridoma cells, can continue under nongrowth conditions. Most of the products of mammalian cell cultures are mixed-growth associated, and product formation takes place both during the growth phase and after growth ceases. Figure 12.4 depicts a typical variation of growth, product (MAb) formation, and glucose utilization by hybridoma cells.

The kinetics of product formation (e.g., MAb formation by hybridoma cells) can be described by a Luedeking–Piret equation:

$$\frac{1}{X}\frac{dp}{dt} = q_p = \alpha\mu + \beta \tag{6.18}$$

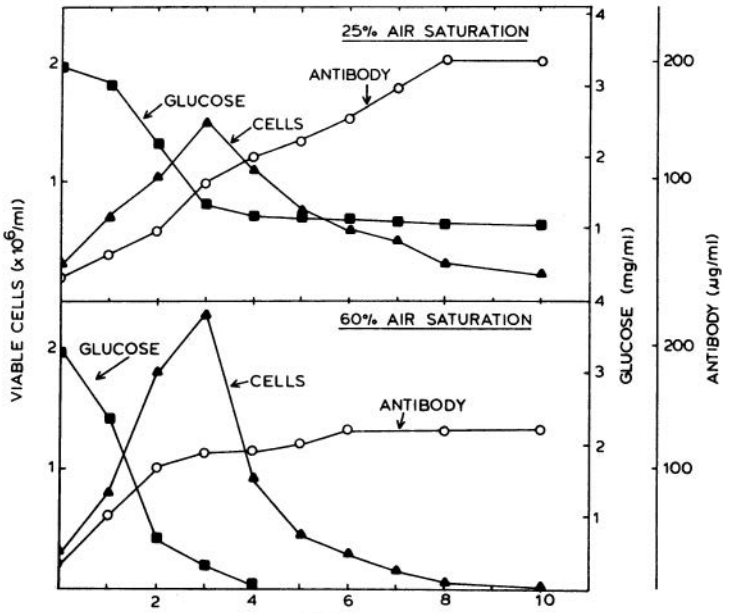


Figure 12.4. Time trajectories of hybridoma growth and monoclonal antibody production at different dissolved-oxygen concentrations in a 3-1 fermenter. (From S. Reuveay, D. Velez, J. D. Macmillian, and L. Miller, J. Factors Affecting Cell Growth and Monoclonal Antibody Production in Stirred Reactors. *Journal of Immunological Methods*, Volume 86, Issue 1, January 22, 1986, pp. 53–59. With permission from Elsevier.)

where μ is the specific growth rate. The first term in equation 6.18 is for growth-associated production, and the second term is nongrowth associated. The doubling time of mammalian cells varies between 10 and 50 h, and a typical value is 20 h. As expected, the growth rate varies depending on cell type, medium composition (including growth factors), and other environmental conditions (dissolved oxygen, carbon dioxide levels, pH, ionic strength).

Animal cells require 0.06 to 0.2×10^{-12} mol O_2/h-cell, which is about five times less than the oxygen requirements of many plant cells and much lower than for microbial cells. Typical animal cell concentration in suspension culture media is 0.1 to 1 g/l (5×10^5 to 5×10^6 cells/ml) for suspension cultures in simple batch growth. For a cell culture density of 10^6 cells/ml, the oxygen requirement is on the order of 0.1 to 0.6 mmol O_2/l-h. For shallow culture depths of 2 to 5 cm, oxygen supplied by air in the head space of the vessel should meet the oxygen requirements of cells. Forced aeration is required for submerged cultivation of denser cultures. However, animal cells are very shear sensitive, and rising air bubbles may cause shear damage to cells, particularly at the point of bubble rupture. Very small bubbles are less damaging. For this reason, special aeration and agitation systems are designed for animal cell cultures. Techniques that decouple agitation (and shear) from oxygenation are particularly attractive. Chemicals (e.g., Pluronic F-68) can be added to provide shear protection. Since the oxygen requirements of animal cells are relatively low, the oxygen transfer coefficient need not be as high as for bacterial fermentations. Typical values of k_La for suspension cultures (10^6 cells/ml) are 5 to 25 h^{-1}. At small scales animal cells are usually cultivated in spinner flasks of 5 to 1000 ml in the laboratory. Spinner flasks contain a magnetically driven impeller or spoonlike agitators that operate at 10 to 60 rpm. Aeration is usually by surface

aeration using 5% CO_2-enriched and filtered air for mammalian cell lines. Spinner flasks are set on a magnetic stirrer plate in a CO_2 incubator. However, modern commercial scale extended batch systems with medium replacement can reach cell densities of $>10^7$ cell/l. In those cases, oxygen transfer must be augmented; a typical strategy is to use pure oxygen rather than air.

Although cell lysis is the most obvious result of excess shear, sublytic levels of shear can be important. For a variety of cell lines, it has been shown that attached cells respond to shear by elongating and reorienting themselves to minimize shear stress. Also, the distribution and numbers of cell-surface receptors can be altered. These receptors interact directly with components in the medium, such as growth factors, that regulate cell metabolism. Alterations in the production of specific proteins and the rates of DNA synthesis have been observed as responses to shear. This response to sublytic shear complicates scale-up, since the same cells exposed to the same medium, but at different levels of shear, may produce the product of interest at different rates. Also processes, such as N-linked glycosylation, can be altered by shear, resulting in protein products that differ potentially in therapeutic value.

12.3. BIOREACTOR CONSIDERATIONS FOR ANIMAL CELL CULTURE

Because mammalian cells are large in size (10 to 20 μm in diameter), slow growing, and shear sensitive, bioreactors must be constructed that are "gently" agitated and aerated. In cases where anchorage-dependent cells are used, as in microcarrier cultures, additional constraints on agitation need to be considered due to liquid shear and particle collisions. Other reactor schemes use entrapped or attached systems where the medium is perfused past the cells. The properties of these cells sets certain constraints on the design of animal cell bioreactors. Following are constraints on these reactors:

- The reactor should be gently aerated and agitated. Some mechanically agitated reactors operating at higher agitation speeds and bubble-column and airlift reactors operating at high aeration rates (and large gas bubbles) may cause shear damage to cells. Shear sensitivity is strain dependent, and some cell lines are fairly shear tolerant.
- Well-controlled homogeneous environmental conditions (T, pH, DO, redox potential) and a supply of CO_2-enriched oxygen or air need to be provided.
- A large support material surface–volume ratio needs to be provided for anchorage-dependent cells.
- The removal of toxic products of metabolism, such as lactic acid and ammonium, and the concentration of high-value products, such as MAb's, vaccines, and lymphokines, need to be accomplished during cell cultivation.

The laboratory-scale cultivation of animal cells is carried out in T-flasks (25 ml to 100 ml) for anchorage-dependent cell lines and for shallow suspension cultures, spinner flasks (100 ml to 1 l) with paddle-type magnetic agitators, roller bottles

(50 ml to 5 l) rotating at about 1 to 5 rpm, and trays containing shallow liquid suspension culture. These laboratory-scale reactors are placed in a carbon dioxide incubator at 37°C (5% CO_2-containing air) for the cultivation of mammalian cells. Different steps need to be taken for anchorage-dependent and suspension cells when scaling up animal cell cultures. Reactors with a high surface–volume ratio (microcarrier systems, hollow-fiber reactors, ceramic matrix systems, weighted porous beads) are used for anchorage-dependent cells. However, modified stirred reactors and airlift or bubble-column reactors can be used for suspension cultures. Membrane bioreactors and microencapsulation methods have been developed for simultaneous cell cultivation, product concentration, and toxic product removal. Many of these systems behave as perfusion reactors (see Chapter 9, "Operating Considerations for Bioreactors for Suspension and Immobilized Cultures"). In essence, a perfusion system exists when cells are retained in the reactor, medium is added continuously or semicontinuously, and spent medium is removed. The removal of spent medium removes toxic metabolites. We can break these reactors into two major types: nonstirred and stirred systems.

12.4. BIOREACTOR SYSTEMS FOR ANIMAL CELL CULTURE

Here we describe the range of reactor systems used for animal cell cultures based on cell immobilization and type of agitation. First we look at the use of immobilized cell reactors (as described in Chapter 9). When such reactors are operated with continuous flow and cell retention, they are examples of perfusion systems, and long-term operation (several months) is possible. Several successful commercial processes are based on these techniques, as animal cell culture is quite adaptable to such systems. Standard stirred-tank systems at large scale are used extensively for production of proteins from suspension-adapted animal cells. We explore briefly these options.

12.4.1. Nonstirred Reactor Systems

The classic method for animal cell culture has been roller bottles, and they have been important components in the scale-up of processes using anchorage-dependent cells. With roller bottles, the liquid covers about 25% of the surface area. Bottles are rotated about the long axis (1 to 5 rpm). Cells adhere to the walls of the bottle and are exposed to liquid 25% of the time and to gas (e.g., 5% CO_2 and 95% air) 75% of the time. When exposed to the liquid, nutrients can be transported into the cell, and when in the gas phase, aeration takes place. The roller-bottle system has an advantage over T-flasks because of increased surface area, agitation of the liquid, and better aeration. Roller bottles are not typically used for large-scale production because of high labor requirements and bottle-to-bottle variability. However, a highly automated facility making extensive use of robots uses roller bottles for commercial production of erythropoietin (a therapeutic protein). Roller bottles are also used for production of some vaccines.

Hollow-fiber reactors have also been used to provide a high growth surface–volume ratio and, therefore, high cell concentrations. They are examples of single-use technology and are available at scales equivalent to order of a 1000 l stirred-tank system. Cells are immobilized on the external surfaces of hollow fibers, and nutrients pass through the tubes. Cell concentrations comparable to those found in tissues can be reached in hollow-fiber reactors. However, the control of microenvironmental conditions inside the reactor where cells are immobilized on fiber surfaces is very difficult because there is almost no mixing (i.e., a heterogeneous environment). Also, the quantification of growth and product formation is difficult in such a system. However, it is possible to construct the reactor using fibers of known molecular weight (MW) cutoff to control the flux into the effluent stream of products with different MWs. The accumulation of some toxic products, such as lactate and ammonium, in the fiber reactors may cause high levels of inhibition. Selection of fiber material (MW cutoff) and flow regime are critical factors for the rapid removal of toxic metabolic products. Hollow-fiber reactors have been used for the production of extracellular proteins such as MAb's from hybridoma cells. Antibody concentrations on the order of 50 mg/ml have been obtained with this reactor, since product is retained by the membrane while medium flows in through the membrane and lower molecular wastes flow out of the cell compartment. To overcome some of the difficulties with axial-flow hollow-fiber reactors, radial-flow or cross-flow hollow-fiber units have been developed. Hollow-fiber reactors are well suited to perfusion operation (see Section 9.2.5) with continuous feed. MAb production from hybridomas can be sustained for extended periods (e.g., 100 days), as stationary-phase cells still synthesize product.

Single-use disposable systems using multiple plates for growth of adherent cells operate conceptually much like roller-bottle systems, but they provide high surface areas for culture of such cells. These have generally been used more to generate cells (e.g., mesenchymal stem cells) than proteins because of difficulties in environmental control, resulting in a heterogeneous environment when scaled up to larger sizes. However, at least one commercial system has added an integrated control system for cell expansion to provide improved environmental control. Systems with surface areas greater than 10 m^2 are available.

A tubular ceramic matrix has been used for the immobilization and cultivation of mammalian cells. High cell, and therefore high extracellular, protein concentrations can be obtained using such systems. The quantification of cell concentration and the control of microenvironmental conditions within the heterogeneous cell culture in ceramic matrix are some of the major difficulties, although these matrices do provide well-defined flow channels. Scale-up of a ceramic matrix reactor may be difficult because of the heterogeneous nature of the system when long tubes are used, although operating with multiple shorter tubes in parallel is an option. These systems are also often operated in perfusion mode.

12.4.2. Systems for Entrapped Cells in Stirred Reactors

The immobilization of mammalian cells in gel beads (agar, alginate, collagen, polyacrylamide) and the use of such systems in a packed- or fluidized-bed configuration are possible. Such immobilization methods and reactor configurations will reduce or

eliminate the shear effects on cells. High cell densities make high volumetric productivities possible. However, the control of microenvironmental conditions inside bead particles and the accumulation of toxic metabolic products in beads are potential problems.

The use of microcarriers for the cultivation of anchorage-dependent mammalian cells is an attractive approach. For example, microcarriers such as DEAE-Sephadex beads, which provide large surface per unit volume of reactor (~70,000 cm^2/l), allow high cell concentrations in the medium (>10^7 cells/ml). The microcarrier beads with cells are suspended in a stirred bioreactor. Other microcarrier beads, such as polyacrylamide, polystyrene, cellulose fibers, hollow glass, and gelatin-coated dextran beads, have been developed. At present, dextran- and DEAE-based (DEAE-Sephadex, DEAE-polyacrylamide) microcarriers are the most commonly used. The surfaces of these microcarrier beads can be modified by addition of compounds, such as collagen, to promote cell adhesion and enhance cell function. Cells grow on the surfaces of microcarriers, usually in the form of monolayers and sometimes as multilayers. Microcarrier culture methods provide a large surface area for growth and a rather homogeneous growth environment. Microcarrier beads can be placed in a gently agitated reactor vessel, in a fluidized bed, or in an airlift (bubble-column) fermenter. Bead-to-bead contact and abrasion of the surfaces can be a problem, and the nonporous nature of these carriers limits the available surface area and, consequently, the maximum cell numbers achievable. Agitation of large-scale vessels with microcarrier cultures is difficult owing to balancing the needs for aeration and mixing against the shear sensitivity of cells attached to the surface of microcarriers. Shear forces are more harmful to cells attached to microcarriers than to suspended cells because attached cells cannot "tumble," and shear tends to "rip" them off the carrier surface. Macroporous microcarriers can be used; cells that enter the pores are shielded from shear effects. However, diffusional limitations and heterogeneous growth conditions may cause undesirable results. Because cells are easily retained in the bioreactor, microcarrier cultures are well suited for perfusion operation. Sometimes, weighted particles are used, particularly in fluidized bed reactors, to allow more rapid perfusion without loss of the cell-containing particles.

Instead of using a microcarrier bead, cells may be microencapsuled. Animal cells are encapsulated with a porous membrane. Typical capsule size is 300 to 500 μm, and the MW cutoff of these capsule membranes is 60 to 70 kDa. Microcapsules operate like small membrane bioreactors, in which very high cell concentrations can be reached (~10^8 cells/ml). Using the right capsule membrane with a desired MW cutoff, toxic products such as lactate and ammonium can be removed from intracapsule culture media, and high-MW products such as MAb's or lymphokines can be concentrated inside capsules. Moreover, cells are protected from hydrodynamic shear by encapsulation, providing a suitable environment for direct aeration and facilitating O_2 delivery and CO_2 removal. However, the growth and product-formation rates inside capsules may be limited by the diffusion of nutrients such as dissolved oxygen or glucose, especially at large capsule sizes. The reduction of capsule size to eliminate mass-transfer limitations (down to 200 μm) and the control of pore size to concentrate desired products within the capsule are two promising approaches to solve some of the problems associated with encapsulated cells. By varying the concentration and average MW of poly-L-lysine (PLL), the pore size of the capsule membrane can be controlled within certain limits (30 to 80 kDa). Monitoring and control of microenvironmental conditions within capsules

(pH, DO level, nutrient–product gradient) are major problems that impose limitations on growth and product formation inside capsules. The fragile nature of the microcapsule also limits the scale-up potential of this system. However, for smaller-scale systems, there is evidence that microencapsulation of CHO cells with recombinant DNA can produce about fivefold higher levels of a target protein than with suspension cultures.

12.4.3. Suspended Cultures

Although specialized bioreactors are used for mammalian cell culture, the traditional stirred-tank reactor described in Chapter 10, "Selection, Scale-Up, Operation, and Control of Bioreactors," predominates at large scale for culture of mammalian and insect cells. Many shear-protecting agents (such as serum or Pluronic F-68) work by preventing cells from accumulating at the gas–liquid interface. Gas exchange can be accomplished with membranes or silicone rubber tubing, but sparging of gas is the simplest method and is acceptable with many cell lines, particularly when shear protectants are used. Cell lines differ in shear sensitivity; CHO cells, widely used for protein production, are relatively resistant to shear damage. Gross shear damage where cells lyse *(necrosis)* is obvious, but lower levels of shear can alter cell physiology. More subtle effects of shear include changes in physiology and, possibly, induction of *apoptosis*, or programmed cell death. These more subtle changes can alter apparent growth rates, productivity, and product quality (e.g., due to release of degradative enzymes into the medium).

Conventional stirred-tank bioreactors have been modified to reduce shear rates on cells in suspension. Sail-type and axial-flow hydrofoil agitators have been developed and used for suspension cultures. The agitation rate in these reactors is on the order of 10 to 40 rpm, providing low shear rate. Some animal cells can be cultured as suspensions in bioreactors up to 20,000 l in size. Because cells are significantly smaller than the turbulent eddies in these bioreactors, cell lysis is minimized. Only cells that are at the interface of an eddy and another eddy or another surface (e.g., reactor wall) are likely to experience damage. Cells at the gas–liquid interface are particularly prone to damage. The breakage of air bubbles is particularly destructive to cells that accumulate at the interface of a gas bubble and medium.

In addition to the use of traditional stainless-steel reactors, a number of single-use, disposable bioreactors have been developed for use with animal cells. We discussed these in Chapter 10 (Section 10.1.4) and divided the reactor types on the basis of external or internal agitation. Both types are used extensively with animal cells and provide effective mixing with minimal cell damage at moderate scales (100 to 2000 l). While typically operated in fed-batch mode, operation in perfusion mode is possible by coupling with cell-separation techniques.

Overall, there are a large number of options for reactors for animal cells reflecting the diversity of constraints on these cellular production systems.

12.5. PRODUCTS OF ANIMAL CELL CULTURES

The number of commercial products made from animal cell culture, predominately from mammalian systems, exceeds 200. Table 12.4 lists the major categories of these products. Most of these products are biopharmaceuticals and generally extracellular

TABLE 12.4. Major Categories of Products of Animal Cell Cultures

Therapeutic Proteins	Hormones
Immunobiologicals	
Monoclonal antibodies	Enzymes
Lymphokines and interleukins	Insecticides
Virus vaccines	Growth factors
Cell surface antigens	Whole cells
	Stem cells
	Tissues
	Organs/mimics

proteins. Although originally made at very low levels, titers above 5 g/l have been obtained. About 40% of the products are monoclonal therapeutics. Technical improvements allow increased recirculation times in the body, making some of these protein products more cost effective than the originally formulated products. Many of them are targeted to the treatment of autoimmune diseases and for chemotherapy.

Some other products include clotting factors (for treatment of hemophilia), hormones, and enzymes (e.g., replacement therapy to treat diseases such as type 1 Gaucher disease and cystic fibrosis) and cytokines (e.g., for anticancer chemotherapy and treatment of multiple sclerosis). Most of these products are proteins that require humanlike posttranslational processing to be effective therapeutically.

Vaccines represent another major type of product that can be made from mammalian and nonmammalian cell cultures. Insecticides, typically viruses made in insect cell culture, represent another type of product. Mab's for use as diagnostics are a well-established product, and posttranslational processing is less important than for therapeutic uses.

Stem cell technology has great promise. The techniques described here have great potential for the effective large-scale production of such cells that may be used for replacement therapies and as surrogates for drug testing and development. The production of stem cells, particularly as replacement in the body, is demanding because of the need to ensure that the cell physiology is essentially identical from batch to batch. Cells are complex, and the appropriate analytical techniques to ensure equivalency are numerous and often expensive to implement.

In the last 30 years, animal cell culture has grown from a novel, small component of the bioprocess industries into a major production technique, and its growth is likely to continue.

12.6. SUMMARY

Animal cells are well suited for the production of proteins requiring extensive and accurate posttranslational processing, organized tissues (artificial skin), certain vaccines, and viruses that can be used as pesticides. Animal cells are more complex and fragile than bacterial, fungal, or yeast cultures. These characteristics require the development of unique strategies for cultivation.

Critical distinctions are *primary* cell lines (established directly from tissue) and *secondary* cell lines, which are established from primary cell lines. Normal cell lines are *mortal*; they can divide only a finite number of times. Other cell lines are *transformed* or *immortal* and divide indefinitely. Many cell lines will grow only when attached to a surface and are thus *anchorage dependent*. Some cells, particularly transformed cell lines, can grow in suspension. One particularly important type of suspension cell is the *hybridoma*. This cell is a hybrid of a mortal antibody-producing cell with a transformed cell, which results in an immortal, easily cultured cell line that produces a single type of antibody, or a *monoclonal antibody*.

The media used to grow animal cells must provide a variety of growth factors, glucose, glutamine, amino acids, specific salts, and vitamins. In some cases, serum from animals (especially mammals) is used. However, serum is expensive, increases the probability of contamination, is undefined in composition, is variable from lot to lot, and complicates the downstream recovery of protein products. In many cases, our knowledge of cellular nutrition has allowed the development of serum-free (and animal protein–free, in some cases) media, and the use of such media is of increasing importance.

Many potential bioreactor configurations are commercially available for use with animal cells. The reactor design problem centers on providing sufficient oxygen and nutrients and (for anchorage-dependent cells) surface area to achieve high cell densities while minimizing exposure to liquid shear. Also, complete medium utilization and high product concentrations are sought. Designs must minimize the formation of toxic by-products (e.g., lactate and ammonium) or effectively remove such metabolites from the microenvironment of the cells.

SUGGESTIONS FOR FURTHER READING

Al-Rubeai, M., *Animal Cell Culture*, Springer International, Switzerland, 2015.

Bandaranayake, A. D., and S. C. Almo, "Recent Advances in Mammalian Protein Production," *FEBS Letters* 588: 253–260, 2014.

Chalmers, J. J., "Animal Cell Culture: Effects of Agitation and Aeration on Cell Adaption," in *Encyclopedia of Cell Technology*, R. Spier, J. B. Griffiths, and A. H. Seragg, eds., Wiley, New York, 2000. (See also other related articles in this book.)

Dumont, J., D. Euwart, B. Mei, S. Estes, and R. Kshirsagar, "Human Cell Lines for Biopharmaceutical Manufacturing: History, Status, and Future Perspectives," *Crit. Rev. Biotechnol.* 36(6): 1110–1122, 2015.

Kim, J. Y., Y. G. Kim, and M. Lee, "CHO Cells in Biotechnology for Production of Recombinant Proteins: Current State and Further Potential," *Appl. Microbiol. Biotechnol.* 93(3): 917–930, 2012.

Nienow, A. W., "Reactor Engineering in Large Scale Animal Cell Culture," *Cytotechnol.* 50: 9–31, 2006.

Van Oers, M. M., G. P. Pijlman, and J. M. Vlak, "Thirty Years of Baculovirus-Insect Cell Protein Expression: From Dark Horse to Mainstream Technology," *J. Gen. Virol.* 96: 6–23, 2015.

Wurm, F. M., "Production of Recombinant Protein Therapeutics in Cultivated Mammalian Cells," *Nat. Biotechnol.* 11: 1393–1398, 2004.

Zhu, J., "Mammalian Cell Protein Expression for Biopharmaceutical Production," *Biotechnol. Adv.* 30: 1158–1170, 2012.

PROBLEMS

12.1. Cite the major differences between animal, plant (see Chapter 13), and bacterial cells in terms of cell structure and physiology.

12.2. Describe the role of glutamine in animal cell metabolism.

12.3. Compare serum-containing and serum-free media in terms of their advantages and disadvantages.

12.4. What are the roles of CO_2 provided in air to animal cell cultures?

12.5. What are the common features of animal cell bioreactors?

12.6. Compare the following immobilization methods used for animal cells in terms of their relative advantages and disadvantages: microcarrier (surface) culture, porous beads, encapsulation, and gel entrapment.

12.7. What do you think is the most suitable reactor type, mode of operation (batch, continuous perfusion), and cultivation method (suspended, immobilized) for animal cell cultures in general? Compare your choice with the other alternatives.

12.8. Compare various methods for the aeration of animal cell cultures. Explain the advantages and disadvantages of each method.

12.9. Describe the process for formation of hybridomas.

12.10. What value of k_La must be achieved to sustain a population of 1×10^{-7} cells/ml when the oxygen consumption is 0.1×10^{-12} mol O_2/h-cell?

12.11. Hybridoma cells immobilized on surfaces of Sephadex beads are used in a packed column for production of monoclonal antibodies (MAb). Hybridoma concentration is approximately $X = 5$ g/l in the bed. The flow rate of the synthetic medium and glucose concentration are $Q = 2$ l/h and $S_0 = 40$ g/l, respectively. The rate constant for MAb formation is $k = 1$ gX/l-d. Assume that there are no diffusion limitations and glucose is the rate-limiting nutrient.

a. Determine the volume and the height of the packed bed for 95% glucose conversion. Bed diameter is $D_0 = 0.2$ m. Neglect the growth of the hybridomas and assume first order kinetics.

b. If $Y_{p/s}$ is 4 mg MAb/g glucose, determine the effluent MAb concentration and the productivity of the system.

13

Bioprocess Considerations in Using Plant Cell Cultures

The use of plant cell cultures for production of useful compounds is an intriguing possibility, and examples of commercial processes using plant cells have emerged in the last 15 years. Effective bioreactor preparation of elite plants is another application of bioprocesses technology employed with plants. Understanding the response of plant cells and organized tissues to bioprocess choices is critical to the commercial use of plant cell tissue culture.

13.1. WHY PLANT CELL CULTURES?

The plant kingdom is very diverse. Many thousands (>200,000) of chemicals are produced only in plants, and certain chemicals are produced only by a single plant species. The biosynthetic capabilities of plant cells have been little explored. Only a small percentage of the world's plants have been scientifically named or described, and only a small fraction of these have been screened for the production of novel and useful compounds. The great genetic potential of plants to produce compounds of use to humans has been little exploited. In fact, the rapid destruction of forests worldwide is leading to the extinction of many plants without preservation of their genomes.

Even the small number of characterized plants has yielded many important compounds (e.g., over 120 prescription drugs). In the Western world, over 25% of pharmaceuticals are derived from extraction from whole plants, and a much greater fraction of medicinals are plant-derived in Asia and the rest of the world. In addition to uses as medicinals, plant products are of interest as dyes, food colors, food flavors, fragrances, insecticides, and herbicides. Peptides and proteins such as miraculin, thaumatin, and monellin are also important targets for plant cell culture technology. Some compounds of potential commercial interest are listed in Table 13.1 and shown in Figure 13.1.

TABLE 13.1. Valuable Commercial and Clinical Products Produced via Plant Cell Culture

Product	Species	Manufacturer	Use/Application
• **Secondary metabolites**			
Alpine rose–derived compounds[a]	*Rhododendron ferrugineum*	PhytoCellTec, Buchs, Switzerland[b]	Cosmetic ingredient
Anthocyanins	*Euphorbia milli* *Aralia cordata*	Nippon Paint Co., Ltd., Osaka, Japan[c]	Textile dye Coloring agents for fruit juices, wine, and other beverages
Apple-derived compounds[a]	*Malus domestica*	PhytoCellTec, Buchs, Switzerland[b]	Cosmetic ingredients
Arbutin	*Catharanthus roseus*	Mitsui Chemicals, Inc., Tokyo, Japan[c]	Whitening agent for cosmetics Pigment Antiseptic
Arctic cloudberries–derived bioactive compounds	*Rubus chamaemorus*	Lumene Cosmetics, Espoo, Finland[c]	Cosmetic ingredient
Argan-derived compounds[a]	*Argania spinosa*	PhytoCellTec, Buchs, Switzerland[b]	Cosmetic ingredient
Berberines	*Coptis japonica* *Thalictrum minus*	Mitsui Chemicals, Inc.[c]	Pharmaceutical ingredient Anticancer Antibiotic Anti-inflammatory Most commonly used for bacterial diarrhea, intestinal parasite, and eye infections
Betacyanins	*Beta vulgaris*	Nippon Shinyaku Co., Ltd., Kyoto, Japan[c]	Food dye Red to red-violet pigment
Carthamin	*Carthamus tinctorius*	Kibun Foods, Inc., Tokyo, Japan[c]	Cosmetic pigment Food colorant and dye
Cocoa polyphenols (Cocovanol)[a]	*Theobroma cacao*	Diana Plant Sciences, Portland, Oregon	Nutraceutical
Date palm–derived compounds	*Phoenix dactylifera L.*	XTEM cell, St. Malo, France[c]	Cosmetic ingredients
Docetaxel[a]	*Taxus* spp.	Phyton Biotech GmbH, Ahrensburg, Germany[d] Samyang Biopharmaceuticals Corporation, Seoul, South Korea[c]	Anticancer FDA approved for the treatment of head and neck, gastric, breast, prostate, and non-small-cell lung cancers

(*Continued*)

TABLE 13.1. (*Continued*)

Product	Species	Manufacturer	Use/Application
Echinaceae polysaccharides[a]	*Echinacea purpurea* *Echinacea angustifolia*	Diversa, Ahrensburg, Germany[c]	Pharmaceutical ingredient Immunostimulant Anti-inflammatory
Geraniol	*Gramineae* spp.	Mitsui Chemicals, Inc.	Essential oil Primary component of rose, palmarosa, and citronella oils
Ginseng saponins	*Panax ginseng* CA Meyer	CBN Biotech, Korea[c]	Dietary supplement Cosmetic ingredient Food additive
	Panax ginseng	Nitto Denko Corporation, Osaka, Japan[c]	
	Wild ginseng stem cells	Unhwa Biotech Corp., Jeonbuk, Korea[c]	
Grape-derived compounds[a]	*Vitis vinifera*	PhytoCellTec, Buchs, Switzerland[b]	UV protection for skin
Leontopodic acids A and B	*Leontopodium alpinum*	Instituto di Ricerche Biotechnologiche (IRB), Italy	Cosmetic ingredient
Paclitaxel[a]	*Taxus* spp.	Phyton Biotech GmbH[d] Samyang Biopharmaceuticals Corporation[d]	Anticancer FDA approved for the treatment of ovarian, breast, and lung cancers Largest application of commercial plant cell culture
Podophyllotoxin	*Podophyllum*-spp.	Nippon Oil, Tokyo, Japan[f]	Anticancer Starting compound for the anticancer agents etoposide and teniposide
Resistem	*Albizia julibrissin*	Sederma, Le Perray En Yvelines, FR[g]	Antiaging cosmetic ingredient
Rosmarinic acid	*Coleus blumei*	A. Nattermann & Cie. GmbH, Cologne, Germany[f]	Anti-inflammatory
Scopolamine	*Duboisia* spp.	Sumitomo Chemical Co., Ltd., Tokyo, Japan[f]	Anticholinergic Antimuscarinic Used in the treatment of motion sickness, nausea, and intestinal cramping
Shikonin	*Lithospermum erythrorhizon*	Mitsui Chemicals, Inc.[c]	Red pigment/dye Antibiotic
Symphytum[a]	*Symphytum officinale*	PhytoCellTec, Buchs, Switzerland[b]	Cosmetic ingredient
• **Heterologous Proteins** • **Vaccines**			
HN protein of Newcastle disease virus	*Nicotiana* spp.	Dow AgroSciences LLC, Indianapolis, IN[c]	Vaccine for Newcastle disease in poultry First FDA-approved plant-derived vaccine Currently not on market

(*Continued*)

TABLE 13.1. (*Continued*) Valuable Commercial and Clinical Products Produced via Plant Cell Culture

Product	Species	Manufacturer	Use/Application
• **Proteins**			
α-Galactosidase-A (PRX-102)[a]	*Daucus carota*	Protalix Biotherapeutics, Carmiel, Israel[h]	Potential Fabry disease treatment In phase I/II clinical trials
AIR DNase (deoxyribonuclease I) (PRX-110)[a]	*Daucus carota*	Protalix Biotherapeutics[h]	Potential inhalable treatment for cystic fibrosis In preclinical development
Albumin[a]	*Oryza sativa*	InVitria, Junction City, KS[i]	Cell culture additive
Antitumor necrosis factor (PRX-106)[a]	*Daucus carota*	Protalix Biotherapeutics[h]	Potential therapeutic for autoimmune diseases, such as rheumatoid arthritis In preclinical development
Human glucocerebrosidase (Elelyso/Uplyso)[a]	*Daucus carota*	Protalix Biotherapeutics[h]	Enzyme replacement therapy for Gaucher disease Licensed by Pfizer, Inc. Completed phase III trial in September 2009 Received FDA approval in May 2012
Human lysozyme[a]	*Oryza sativa*	InVitria[i]	Bacterial cell lysis agent Food and beverage preservative
Lactoferrin[a]	*Oryza sativa*	InVitria[i]	Animal-free mammalian cell culture additive
Moss-aGal (agalsidase)	*Physcomitrella patens*	Greenovation Biopharmaceuticals, Heilbronn, Germany[j]	Potential therapeutic for the treatment of Fabry disease In preclinical development
Moss-GBA (glucerase)	*Physcomitrella patens*	Greenovation Biopharmaceuticals[j]	Potential therapeutic for Gaucher disease In preclinical development
Oral human glucocerebrosidase (PRX-112)[a]	*Daucus carota*	Protalix Biotherapeutics[h]	Oral delivery of enzyme replacement therapy for Gaucher disease Carrot plant cells utilized as delivery vehicle for the therapeutic protein In phase II clinical trails
Transferrin[a]	*Oryza sativa*	InVitria[i]	Cell culture additive

[a]Verified to be commercially produced via plant cell culture as of 2015.
[b]http://www.phytocelltec.ch
[c]Meyer, H-P., and D. Schmidhalter, *Industrial Scale Suspension Culture of Living Cells*, Wiley, 2014.
[d]https://www.phytonbiotech.com
[e]https://www.samyangbiopharm.com
[f]Lehmann, N., et al. "Disposable Bioreactors for Cultivation of Plant Cell Cultures," in K.-Y. Paek, H. N. Murthy, and J.-J. Zhong, eds., *Production of Biomass and Bioactive Compounds Using Bioreactor Technology*, Springer Netherlands, 2014, pp. 17–46.
[g]http://www.sederma.com
[h]http://www.protalix.com
[i]https://www.invitria.com
[j]http://www.greenovation.com
Adapted from table presented in: Wilson, S. A., Roberts, S. C., 2012. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotechnology Journal* 10(3):249–268.

These compounds are chemically complex and are generally nonproteins. They are known as natural products, secondary metabolites (not required for growth),

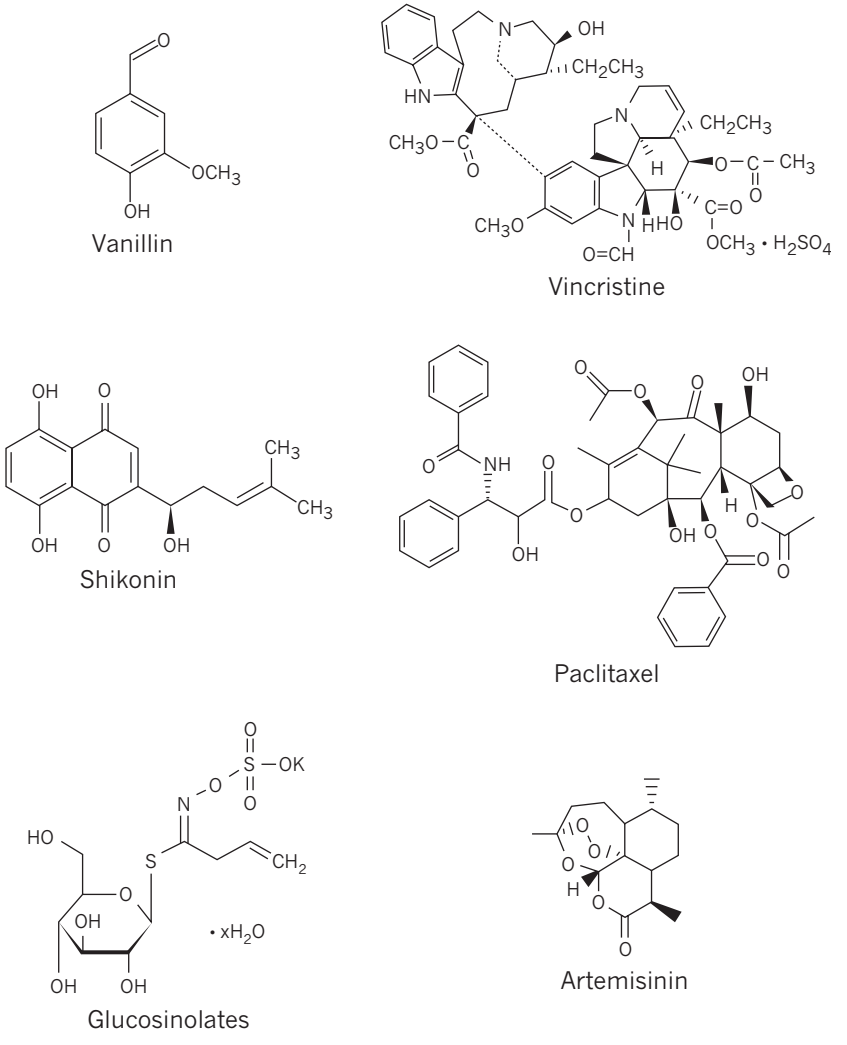


Figure 13.1. Examples of secondary metabolites from plants. (Compiled by Carolyn Lee-Parsons.)

or specialized metabolites, and their function is critical to the plant's adaptation, defense, and survival in a changing environment. The compounds most likely to be of commercial interest are those that are not synthesized by microbes and are of sufficient complexity that chemical synthesis is not a reasonable alternative. Also, these nonprotein products are often made by the condensation of intermediates derived from primary metabolism (Chapter 5, "Major Metabolic Pathways") and then modified by pathways unique to that particular plant species. Plant genetics and physiology are poorly understood in comparison to animal and bacterial systems. Because of the difficulty of finding and recovering genes from unrelated pathways, genetic engineering using more easily grown organisms, such as yeast or bacteria, to make these products is not currently an attractive alternative to plant

cell culture. However, there are emerging examples in which genetically engineered plants are useful. The enzyme human glucocerebrosidase, produced in genetically engineered carrot cells using bioreactors, is an FDA-approved product (trade name Elelyso) for treating Gaucher disease. This plant cell–derived product sells for less than the standard treatment, is said to be more reliable to produce, and avoids problems of misfolded proteins from bacteria. As the cost of transcriptomic platforms is decreasing, it will be possible to elucidate the entire biosynthetic pathway of a specialized metabolite. Such knowledge may make production in yeast and bacteria possible, although those genetic insights can be applied directly to improving plant cell cultures themselves. Plant cell tissue cultures have low volumetric productivities, so higher-value products (≥$500/kg) are desirable targets. Thus, the chemicals of greatest commercial interest must be of intermediate to high value and unique to the plant kingdom.

A good example of bioprocess development using plant cell cultures is for the production of the important anticancer agent paclitaxel (Taxol). Supply problems greatly impeded the clinical development of this drug. Initially, paclitaxel was produced by extraction from the bark of the Pacific yew tree *(Taxus brevifolia)*. It required three 100-year-old trees to supply enough paclitaxel to treat one patient. This tree is relatively uncommon, and its harvestation had significant adverse environmental impacts. Harvestation of whole trees has been replaced by a method of semisynthesis and by plant cell culture. Needles and branches of more common yews can be collected, a precursor compound extracted, and the precursor chemically converted to paclitaxel. The collection of needles and branches can be done in a way to prevent death of the plant. However, there are environmental concerns with both needle collection and disposal of solvents from the chemical processing. Production of paclitaxel in large-scale bioreactors provides a product with fewer contaminants, is highly controllable and reproducible, and is environmentally friendly. Commercial production of paclitaxel using large-scale bioreactors is now a reality. Suspension cultures in stirred-tank vessels of up to 75,000 l have been used.

The factory production of chemicals from plant cell tissue culture offers a number of important advantages:

- Control of supply of product is independent of the availability of the plant itself.
- Cultivation is under controlled and optimized conditions.
- Strain is improved with programs analogous to those used for microbial systems.
- With the feeding of compounds analogous to natural substrates, novel compounds not present in nature can be synthesized.

The production of the dye shikonin further illustrates these advantages. Japan could not grow sufficient *Lithospermum* to supply its needs, nor was it assured of a stable supply to import. The plant had to grow for at least 2 to 3 years before reaching harvestable size; at harvest, about 1% to 2% of the dry weight was shikonin. Using a combination of strain selection and optimization of reactor conditions, a commercial

process that could produce cells with 14 wt% shikonin in a 3-week batch cultivation period was established.

However, the role of bioreactors in plant cell tissue is not limited to chemical production. Transgenic plant cell cultures also can be used to produce proteins such as vaccines. An example is the production of glucocerebrosidase from carrot cells. The production of propagules or of artificial seeds may be economically important. The use of efficient submerged cultivation devices to generate elite plants may replace the current labor-intensive processes for the micropropagation of plants.

Whether the product is a chemical or a new plant, the bioprocess engineer must become familiar with some basic characteristics of plants and their implications for reactor design.

13.2. PLANT CELLS IN CULTURE COMPARED TO MICROBES

Plant cells in culture are not microbes in disguise. Aspects of plant cell structure and physiology were discussed in Chapter 2, "An Overview of Biological Basics." See Figure 13.2 for a summary of plant cell structure. The primary difference between plant cells and microbes is the ability of the cells to undergo differentiation and organization even after extended culture in the undifferentiated state. The capacity to regenerate whole plants from undifferentiated cells under appropriate environmental

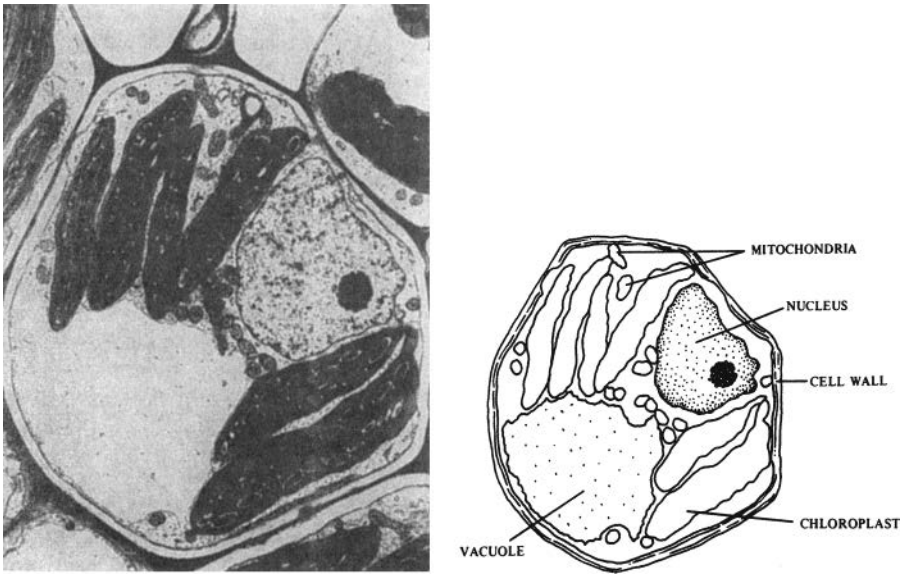


Figure 13.2. (Left) Cross section of a plant cell enlarged about 9000 times its actual size by an electron microscope. (Right) The labeled drawing identifies some of the cell's structures and organelles, those specialized cell parts that resemble and function as organs. This particular cell is a bundle sheath cell from the leaf of a *Zea mays* plant. Cells like this one are part of the veins of the leaf and completely surround the water- and food-conducting tissues (xylem and phloem) of the veins. (With permission, from Michael A. Walsh, Utah State University.)

conditions is called *totipotency*. This capacity is essential to plant micropropagation and is often associated with secondary metabolite formation. If we could do the same for animals as we can for many plants, we could remove a cell from a person's tongue and generate millions of nearly identical clones of that person's whole body!

Let us begin by considering the establishment of cell cultures for producing a particular chemical (see Figure 13.3). *Callus* and *suspension cultures* have been established from hundreds of different plants. A callus can be formed from any portion of the whole plant that contains dividing cells (Figure 13.4). The excised plant material is placed on solidified medium containing nutrients and hormones that promote rapid cell differentiation. The callus that forms can be quite large (>1 cm across and high) and has no organized structure. Although we speak of a callus as

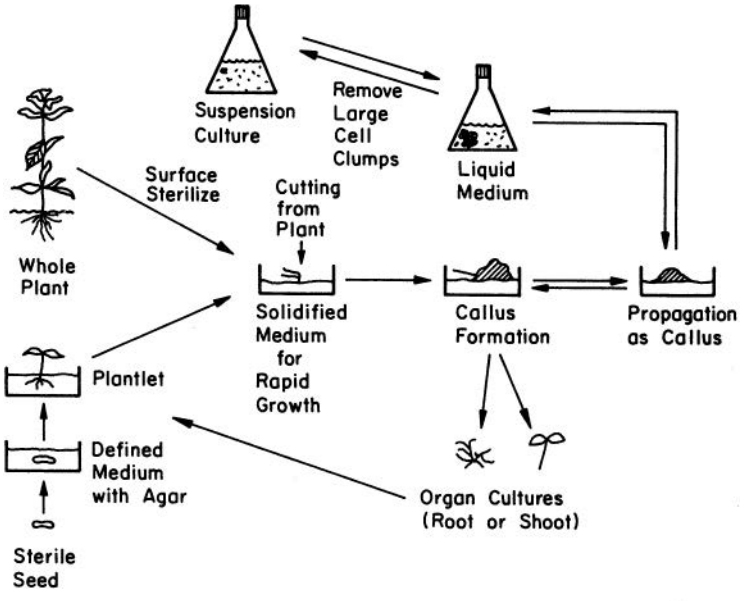


Figure 13.3. Suspension cultures can be established from many plants by establishing first callus culture and then suspension cultures; alternatively, organ cultures can be produced from callus culture. For some plants, the process can be reversed to generate whole plants from suspensions on organ cultures.

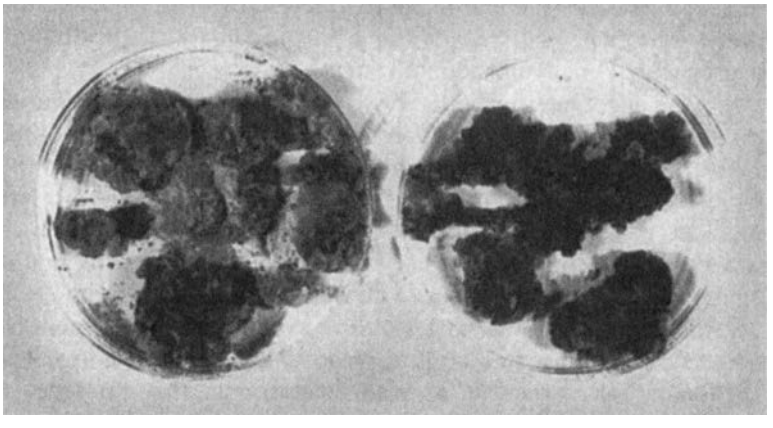


Figure 13.4. Calli of grape cultures. The plate on the left contains an unselected culture in which a few cells make high levels of reddish-purple pigments (anthocyanins). The plate on the right contains highly selected cultures in which most cells make pigment.

being *dedifferentiated tissue*, it contains a mixture of cell types. In addition to the environment conditions (e.g., plant hormones), the source and age of the initial tissue (root, shoot, and so on) can make a difference and may result in inheritable changes attributed to dedifferentiation. If we are interested in maximizing the formation of a particular compound, it is advisable to initiate the callus from a plant that is known to be a high producer. Even from the same batch of seeds, there may be considerable variation from plant to plant in their biosynthetic capacity.

For both callus and suspension cultures, a chemically defined medium is used. Typically, cultures, especially suspension cultures, are maintained in the dark. While exposure to light may be used to regulate expression of specific pathways, light is rarely used solely to support growth as most cells are incapable of sustained photoautotrophic growth. Typical media use a carbon–energy source such as sucrose. Inorganic nutrients, vitamins, and hormones (or growth regulators) are included in media. Classes of plant hormones that are growth promoters are auxins, cytokinins, and gibberellins. Most media contain at least an auxin such as naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) and a cytokinin such as kinetin or benzyladenine (BA). Ethylene is a plant hormone and is typically produced by the culture itself.

The establishment of *suspension cultures* (see Figure 13.5) from callus is generally straightforward if the callus is *friable* (easily breaks into small pieces). A piece of callus is placed in a liquid medium in a shake flask. With gentle to moderate agitation, cells or small aggregates of cells will slough off. Typical conditions are 27°C and a pH of 5.5 in the dark. These suspended cells then replicate. After 2 or 3 weeks, the suspended cells are transferred to fresh medium, and large aggregates or residual callus are discarded. Suspensions can grow to high cell densities (similar to applesauce).

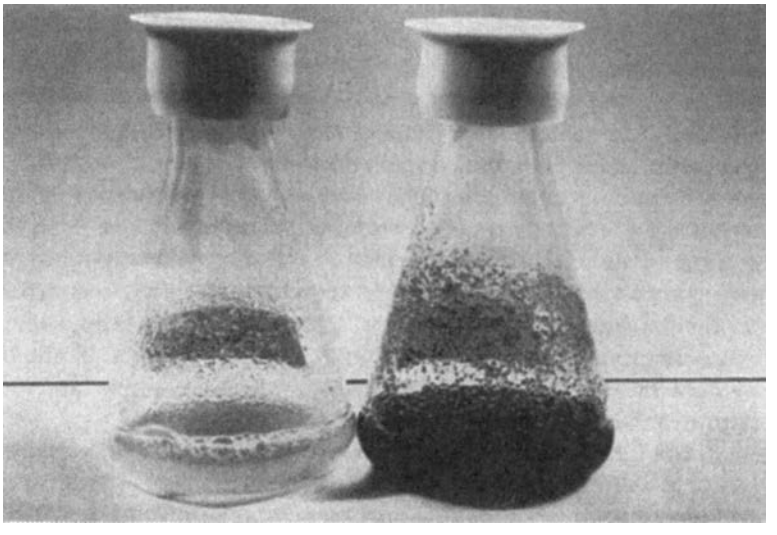


Figure 13.5. Suspension cultures of grape cells. The flask on the left is a culture with modest selection, while the culture on the right is highly selected for anthocyanin production.

All suspension cultures contain *aggregates*, but it is possible to select for fine suspensions by discarding large aggregates at each subsequent transfer. However, due to cell-to-cell communication, cell aggregates are often more productive than a dispersed cell culture.

The aggregates are due not so much to clumping as to the failure of progeny to separate. Cell-to-cell communication is very important in plants. Normally, all cells in a whole plant are connected to one another by small pores called the *plasmodesmata*. The plasmodesmata allow the interchange of lower-molecular-weight compounds (<800 da) from the cytoplasm of one cell to another. The plasmodesmata are formed principally during cell division. Cells in aggregates can communicate with one another through plasmodesmata and also through diffusable species. For example, plants generate ethylene, which acts as a hormone. In a large aggregate, there are concentration gradients of such metabolic products as well as nutrients (e.g., oxygen and hormones). These gradients lead to a wide variety of microenvironments, and as a consequence, cells in different positions in the aggregates may have greatly different biochemical and morphological structure. Although plant cell cultures are pure cultures in the sense that only one species is present (ideally, one genotype), they behave very much like a mixed culture. A mix of cell types may be necessary for the formation of some products.

Plant cells can be very large, with cell diameters typically in the range of 10 to 100 μm. They grow slowly, with doubling times typically ranging from 20 to 100 h. Growth is usually nonphotosynthetic, with sucrose or glucose supplied exogenously as the carbon and energy source. Typical respiration rates are roughly 0.5 mmol O_2/h-gDW, or about 5% to 15% of that in *Escherichia coli*. Plant cells can often be cultivated at very high densities, up to 70% of the total reactor volume as cells. Although the respiration rate is low, high cell density requires very good oxygen-transfer capabilities in a reactor.

Plant cells contain a higher percentage of water than do bacterial cells (90% to 95% versus 80%). This higher water content is due, in part, to the presence of the central vacuole, which can occupy as much as 95% of the intracellular volume in extreme cases. Plant cells tend to secrete relatively few compounds but sequester them inside the vacuole. Many of these compounds are secondary metabolites of commercial interest; many would also be cytotoxic if not removed from the cytoplasm. Their intracellular storage will have important implications in our discussions later in this chapter on bioreactor strategies.

One other biological aspect of critical importance is the role of *elicitors*. In whole plants, there are a number of defensive mechanisms that plants use to contain attacks from pathogenic fungi, bacteria, and viruses. Defense mechanisms can be activated if breakdown products of fungal or plant cell walls (oligosaccharides) are present. Other compounds, some even nonorganic, can also elicit a response. Many secondary metabolites are involved in the plant's defensive mechanisms. The exposure of suspension cultures to elicitors has led in many cases to rapid increases in the accumulation of secondary products. The use of elicitors has been an important breakthrough in improving volumetric productivities in bioreactors. The most useful elicitor is methyl jasmonate. This compound is relatively inexpensive and readily introduced into large bioreactors. It regulates expression of a wide variety of plant genes.

Cells in suspension can be made to undergo differentiation and organization if the correct environmental conditions can be found. Nutrient and hormone levels must be adjusted. Embryos, shoots, and roots can then be made from either calli or aggregates in suspension. Root cultures can also be generated through the introduction of plant hormone genes by the plant pathogenic bacteria, *Agrobacterium rhizogenes* (see Figure 13.6). Hairy root cultures can grow in the absence of added hormone and are genetically stable, an advantage over cell suspension systems. However, the type of cell or tissue culture (i.e., cell suspensions, roots, or shoots) for producing the specialized metabolite is dependent on the desired product. For certain secondary metabolites, suspension cultures can be used as the production platform in traditional bioreactors using bioprocess strategies (such as cell line selection, elicitation, in situ product removal discussed in Section 13.3), while more differentiated cultures such as root or shoot cultures are required for the production of other metabolites.

Recent advances in genomics and our understanding of plant molecular biology make it possible to consider genetically engineering the plant cell's biosynthetic pathways. It will soon be possible to redirect these toward desired chemicals.

13.3. BIOREACTOR CONSIDERATIONS

Our discussion of bioreactor design in Chapter 10 "Selection, Scale-Up, Operation, and Control of Bioreactors" laid out the principles of bioreactor design, but plant-cell cultures represent some unusual problems. Plant cells are large, they tend to form aggregates, and despite a strong cell wall they are sensitive to liquid shear. They grow slowly. In some cases, we need to cultivate fairly complex three-dimensional structures (e.g., root culture) that are sensitive to shear and create significant barriers

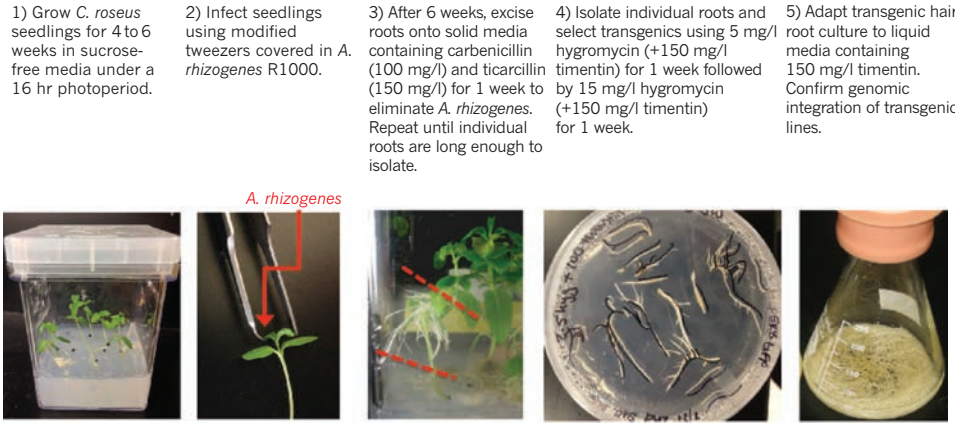


Figure 13.6. *Agrobacterium*-mediated transformation method for the generation of *Catharanthus roseus* hairy roots. (From Noreen F. Rizvi, Miglia Carnejo, Kassi Stein, Jessica Weaver, Erin J. Cram, & Carolyn W. T. Lee-Parsons [2015], *Plant Cell Tiss. Organ Cult.* 120:475–487. With permission of Springer.)

to mass transfer, especially for oxygen and carbon dioxide. Control of plant cell differentiation into complex structures remains a challenge. Appropriate selection and design of bioreactors can address many of these factors.

13.3.1. Bioreactors for Suspension Cultures

Many of the differences between plant cell cultures and microbes have direct implications for the design and scale-up of suspension culture systems (see Table 13.2). In particular, the degree of physical mixing that is desirable is important. However, the basic reactor types we discussed in Chapter 10 can usually be adapted to plant cells.

Plant cells are large, and when they are exposed to turbulent shear fields where the eddy size approaches the cell size, the cells can be exposed to a twisting motion that can damage them. Lower levels of shear appear to affect cell surface receptors and nutrient transport. Reactors with high shear must be avoided. However, plant cells can withstand far more shear than animal cells, and shear-tolerant lines can sometimes be developed. Stirred tanks designed for the culture of bacteria are not good choices, but modified stirred tanks can be suitable. Reactors up to 75000 l have been used successfully.

Plant cell cultures can achieve high cell densities and viscosities. Their reduced respiration rate, however, compensates in part for the need for vigorous agitation. Airlift reactors for low or moderate cell densities (<20 gDW/l) or paddle-type or helical-ribbon impellers for high-cell-density systems have been advocated as reactors that strike a good compromise between the need for good mixing and the shear sensitivity of plant cells.

More important than actual shear damage is the role that mixing plays in the biological response of some systems to scale-up. In at least some cases, the formation of aggregates appears to be necessary to achieving the mix of cell types essential for good secondary product formation. The degree of aggregate formation is influenced by the degree of mixing. Since the degree of mixing necessary to achieve equivalent oxygen transfer or equivalent shear changes upon scale-up, the degree of aggregation and productivity may change.

Mixing depends on a combination of sparging and mechanical agitation. Oversparging can be a problem for plant cell cultures. Even though photoautotrophic

TABLE 13.2. Differences between Plant Cells and Microbes and the Implications for Bioreactor Design

Differences	Implications for Reactor Design
• Lower respiration rate	Lower O_2 transfer rates required
• More shear sensitive	May require operation under low-shear conditions
• Cells often grow as aggregates or clumps	May have mass-transfer limitations that limit the availability of nutrients to cells within the aggregate
• Degree of aggregation may be important regard to secondary metabolism	May be an optimal aggregate size for product synthesis
• Volatile compounds may be important for cell metabolism (e.g., CO_2 or ethylene)	May need to sparge gas mixtures

With permission, from G. F. Payne and others, in B. K. Lydersen, ed., *Large Scale Cell Culture Technology*, John Wiley & Sons, Inc., New York, 1987.

growth is not important in most cultures, elevated CO_2 levels can enhance productivity. Plants make at least one volatile hormone, ethylene, and its rapid removal can affect productivity. With plant cell cultures, optimizing the gas composition, sparging rate, and degree of mechanical agitation is a difficult problem.

The low growth rates of plant cells present other problems for large-scale systems. The primary problem is maintenance of aseptic conditions for the 2 to 4 weeks often necessary to complete a batch cycle. Additionally, the low growth rates reduce volumetric productivities to levels that diminish the economic attractiveness of plant cell cultures.

A further problem is the apparent genetic instability of many cell lines. Often, suspension and callus cultures will produce a desired compound initially, but after 3 to 6 months, the capacity to make the product often decreases considerably. This loss of productivity may be due to inadequate nutrition or may have a genetic or epigenetic basis. Artificial cell culture tends to select for cells with higher ploidy levels (tetraploid instead of diploid[†]). The most frequent genetic changes include chromosome breakage and aneuploidy. With an atypical chromosome number, differentiation and regeneration become extremely difficult. Cryopreservation of cell lines is not possible in all cases, and many cell lines must be maintained through routine subculture, which may select for variants that grow better than the parental culture but are less productive.

This loss of activity can be counteracted in many cases by the active selection of productive subclones. Selection is facilitated when the product of interest is readily visible. In cases where extensive screening and selection have been done, stable, high-producing clones have been reported. A stable, high-producing cell line is essential to any commercial process. Since visible screens are not always possible, much screening is done currently with high-throughput metabolite assays.

One strategy that has proved to be useful in increasing productivity is the use of a two-phase culture. The first phase uses a medium optimized for growth, while a second phase uses a different medium optimized for product formation. The first commercial process with plant cell culture was shikonin production, which utilizes two batch reactors in series.

13.3.2. Reactors Using Cell Immobilization

Further improvements in productivity may be possible due to cell immobilization. Immobilized-cell reactors inherently follow the two-phase approach. Cells are grown and then immobilized; once immobilized, conditions for product formation are optimized. Immobilized-cell systems are advantageous when continuous operation is possible and if the product is, or can be made, extracellular. Following are some possible advantages of immobilization.[††] Probably the most important advantage is that the degree of cell-to-cell interaction can be manipulated.

- Continuous operation is facilitated.
- High cell concentrations are possible.

[†] Recall that a diploid cell has two copies of each chromosome; a tetraploid cell has four copies.

[††] G. F. Payne and others, in B. K. Lydersen, ed., *Large Scale Cell Culture Technology*, Wiley, New York, 1987.

- Cell reuse may lead to increased efficiency.
- Cells can be protected from shear.
- Once immobilized, the slow growth and strain instability of plant cells are no longer problems.
- Media can be easily changed, which is important for processes that require a series of media for optimal production.
- Continuous removal of inhibitory metabolites may enhance the overall cellular metabolism or unmask biochemical pathways.
- The biological relationships between aggregation, morphological differentiation, and secondary metabolite production may be better exploited.

Following are potential problems with immobilization:†

- Large-scale aseptic immobilization procedures must be developed.
- Mass-transfer limitations may significantly affect cell metabolism (positively and adversely).
- Products must be produced by nongrowing cells.
- Products must be released from the cell into the medium.
- Experience in the scale-up of immobilized-cell systems is limited.

Plant cells will often self-immobilize by preferentially attaching to or within a porous matrix. The resulting biofilm (if on a surface) has been shown to be very effective in a number of cases. Plant cells have also been entrapped in gels or between membranes. Immobilization generates concentration gradients that alter the biosynthetic capacity of the culture (sometimes negatively and sometimes positively). The cell-to-cell contact due to immobilization or the contact of the cell surface with the surrounding gel phase may also alter cell physiology. In some cases, immobilization has improved intrinsic production rates by more than an order of magnitude.

Plant cell cultures can be cultivated at three levels of cell-to-cell communication. In a fine suspension, aggregates are small and mass-transfer gradients are unimportant. Any diffusable species that might act as chemical messengers are diluted to a low concentration. Plasmodesmata interconnect only a small fraction of the cells. If cells are concentrated and entrapped, they form a pseudotissue; since the entrapping matrix is typically on the order of 1 to 10 mm, mass-transfer gradients become important. Diffusable chemical species can build to high local concentrations because of mass transfer resistances. However, plasmodesmata are relatively unimportant in pseudotissues. If a few cells are immobilized (say, between two membranes or in the pockets of a foam matrix) and allowed to grow in place, they will develop a tissuelike structure. Not only can diffusable species accumulate, but cells will be interconnected through the plasmodesmata. Then immobilization (and the method used for immobilization) can be used as an engineering

† G. F. Payne and others, in B. K. Lydersen, ed., *Large Scale Cell Culture Technology*, Wiley, New York, 1987.

design parameter to alter the degree and type of cell-to-cell communication. Immobilization can be coupled with other strategies to enhance product formation.

Consider the production of ajmalicine from periwinkle *(Catharanthus roseus)*. Ajmalicine is stored primarily in the vacuole in cells in suspension culture, although a small amount (10%) is excreted. Ajmalicine has a pKa of 6.3, so that at the growth pH (5.6) much of the alkaloid is in the neutral form. A neutral resin can be used to remove ajmalicine in situ. The in situ removal of the product can enhance formation of a product by relief of feedback inhibition or protection from degradation or further conversion.

Figure 13.7 shows the results of experiments involving combinations of in situ adsorption, use of a fungal elicitor, immobilization in calcium alginate gels, and use of a production medium. Using all four approaches increases extracellular concentrations almost 100-fold. The purity is high. The closely related alkaloid serpentine has a much higher pKa (10.8) and will not adsorb into the resin. The adsorption of ajmalicine increases its synthesis preferentially. Under some circumstances, the intracellular levels of ajmalicine and serpentine are nearly equal. In this system, the ratio of extracellular ajmalicine to serpentine was 60-fold, with serpentine accounting for a vanishingly small amount of the adsorbed alkaloid. Perhaps the most important result of such an experiment is that at least some normally intracellular compounds can be excreted from viable cells when environmental conditions are correctly manipulated.

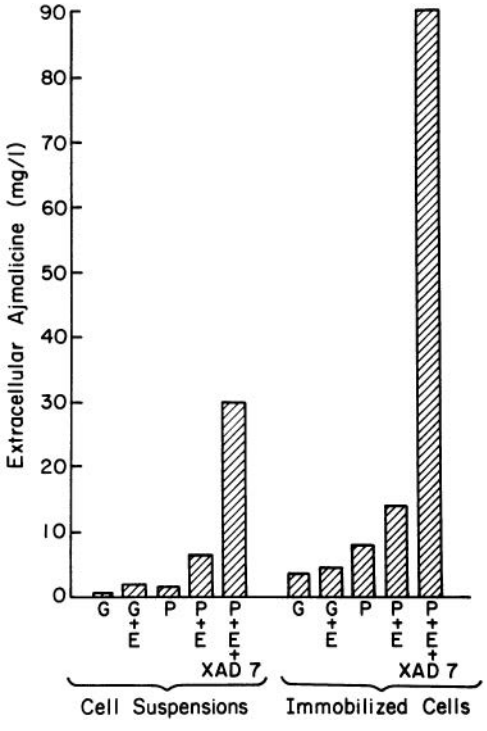


Figure 13.7. Summary of the effects of various treatments on the formation and release of ajmalicine from *Catharanthus roseus.* G stands for growth medium and P for production medium. E indicates elicitor addition (2%) of an autoclaved culture of the fungus, *Phytophthora cactorum*, for a 2-day exposure time; the neutral resin, Amberlite XAD-7, indicates the addition of resin. Samples were analyzed 23 days after inoculation. (With permission from M. Asada and M. L. Shuler, Stimulation of ajmalicine production and excretion from Catharanthus roseus: Effects of adsorption in situ, elicitors and alginate immobilization *Appl. Microbiol. Biotechnol.* 30(5):475–481, 1989. With permission of Springer.)

13.3.3. Bioreactors for Organized Tissues

In many cases, neither suspension nor immobilized-cell cultures will produce satisfactory amounts of a desired metabolite. However, organ cultures from the same plant may give good yields (Table 13.3). In addition to high yields, organ cultures have a number of distinct advantages:

- Biosynthetic capacity often returns upon organogenesis.
- Product spectrum for flavors and fragrances returns to that for parent plant.
- Product secretion is enhanced in many cases (particularly root cultures).
- Genetic stability under growth conditions is greatly improved over callus or suspension culture.
- Organ cultures are often easier to genetically engineer than whole plants or cell cultures.
- Self-immobilization provides more optimal mix of cell types.

Following are disadvantages:

- Growth rates may be lower than suspension cultures in some, but not all, cases.
- Efficient, scalable reactors for organized tissues need to be developed.
- Control of microenvironmental conditions is often more difficult.

The primary disadvantages for organ cultures have been their apparently slow growth rates and difficulties in designing effective bioreactors to handle organized tissues. Recent advances suggest that both limitations may be circumvented, and in several cases, organ cultures that grow more rapidly than their corresponding suspension cultures have been found.

Some plants (typically dicots; monocots can be infected, but it is more difficult) will respond to infection by *Agrobacterium rhizogenes* with rapid root proliferation. This infection leads to *hairy roots* (see Figure 13.6). The best doubling times for hairy root cultures approach or exceed typical values for many suspension cultures. Even in species not susceptible to *A. rhizogenes* infection, recent results indicate

TABLE 13.3. Compounds Made in Higher Yields in Organ Cultures than Suspension Cultures

Compound	Plant	Form of Culture
• Cardenolides	*Digitalis lanata*	Somatic embryos
• Digitoxin	*Digitalis purpurea*	Shoots
• Diosgenin	*Dioscorea composita*	Shoots
• Monoterpenes	*Pelargonium fragrans* (geranium)	Shoots
• Morphinan alkaloids	*Papaver somniferum* (opium poppy)	Shoots
• β-Peltatin	*Linum flavum*	Roots
• Pyrethrins	*Chrysanthemum cinerariaefolium*	Shoots
• Scopolamine	*Datura inoxia*	Roots
• Tropane alkaloids	*Hyoscyamus niger*	Roots
• Valepotriates	*Centranthus* sp.	Roots
• Withanolides	*Withania somnifera*	Shoots

that proper control of hormone content can accelerate growth rates to acceptable levels (3- to 5-day doubling times). Hairy root cultures are often easier to genetically engineer than cell cultures or whole plants. The biggest difficulty with the large-scale culture of roots is the formation of root *mats*. These mats restrict internal mass transfer, can entrap gas and float, and present significant problems in maintaining a scalable uniform environment. However, large-scale units for root culture have been built (20000 l for ginseng roots in Japan). Laboratory-scale reactors using a mist or forced convection of liquid nutrients appear promising and provide much better mass transfer in the center of root mats. Examples of commercial development of hairy root technology include Rootec (Switzerland) for key metabolites and Root Lines Technology (France) for production/secretion of complex recombinant enzymes.

One other advantage of using organ cultures over whole plants is the possibility of using precursor feeding and elicitors. For example, with species of onion and garlic, the use of precursors can greatly enhance the formation of flavor compounds. Different precursors give different levels of enhancement to particular components of the flavor spectrum. By using combinations of chemical precursors, it may be possible to custom-make flavors for specific applications.

Shoot cultures present additional problems. Light may be required for some shoot cultures, while roots can be grown easily in the dark. Shoot cultures may be *mixotrophic*, involving the exogenous supply of sugars as well as some photosynthesis. In some cases, control of the *photoperiod* (hours of exposure to light per day) is important. However, the most crucial need for light comes from the role that light often plays in cellular regulation. Exposure to light of certain wavelengths is essential to induce synthesis of some enzymes. In at least some cases, these enzymes play a crucial role in secondary metabolism. The maintenance of uniform light intensity in a large reactor is a challenging and partially unsolved problem.

One other perceived limitation on shoot cultures has been the belief that they could not be grown under totally submerged conditions. Recent experiments have shown that tobacco shoots will grow as well in submerged culture as in a standard shake flask (3-day doubling time in both cases), provided that the liquid phase is well mixed. In Israel, commercial production of plantlets using totally submerged culture has been reported.

Although organ cultures have not yet been extensively exploited, they are promising vehicles for the production of some important secondary metabolites.

13.4. ECONOMICS OF PLANT CELL TISSUE CULTURES

Far fewer commercial processes for plant cell culture have been established than for bacterial, fungal, and animal cell cultures. Currently, several large-scale systems (>1000 l) have been constructed throughout the world with the largest being 75,000 l. In North America, the possible commercial development of many products is underway (Table 13.1).

As a rule of thumb, most commercial fermentations yield revenue of more than 20¢/l-day. If the volumetric productivity of a process is known, the wholesale price necessary to achieve this level of revenue can be estimated. Table 13.4 lists the published values of the volumetric productivities reported for some of the more intensely studied cell lines. Bulk prices in excess of $220/kg would be necessary to

TABLE 13.4. Volumetric Productivities for Products Made in Plant Cell Culture

Product	Cell Line	Productivity (g/l-day)	Bulk Price to Give 20¢/l-d Revenue (¢/g)
• Anthocyanin	Grape (Bailey alicant A, *Vitis* hybrid)	0.15	133
• Berberine	*Thalictrum minus*[a] and	0.05	400
	Coptis japonica[b]	0.60	33
• Diosgenin	*Dioscorea* spp.	0.0098	2040
• Paclitaxel (Taxol)	*Taxus canadensis*	0.75	27
• Podoverine	*Podophyllum versipelle*	0.15	133
• Rosmarinic acid	*Coleus blumei*	0.91	22
• Sanguinarine	*Papaver somniferum*	0.034	588
• Shikonin	Lithospermum erythrorhizon	0.15	133

[a]Excreted into the medium, where it crystallizes.
[b]Continuous-flow system with cell retention; intracellular product.

yield 20¢/l-day for these products, and clearly prices in excess of $500/kg may be necessary for new technology.

13.5. SUMMARY

Plants produce a wide range of commercially important compounds. For some of these chemicals, plant cell tissue culture is a potential technique for their production. In addition, the bioreactor techniques that are applied to plant cell culture will be useful in the micropropagation of plants.

Since most secondary products are not made in rapidly growing cultures, two-step cultivations are employed. The first phase is used to promote growth, and a second phase is optimized for product formation. Liquid suspensions can be cultured analogously to fermenters for microbes if the special characteristics of plant cells are taken into account. Alternatively, immobilized or entrapped cell systems can be used. In either case, *elicitors* can often be used to enhance product formation. A combination of immobilization and in situ separation techniques can often dramatically improve production.

Organized tissues (shoots, roots, and embryos) hold promise as techniques to improve yields. However, bioreactor designs that supply homogeneous environments for organ cultures are only now being developed.

SUGGESTIONS FOR FURTHER READING

Doran, P. M., Design of Mixing Systems for Plant Cell Suspensions in Stirred Reactors, *Biotechnol. Progr.*15: 319–355, 1999.

Eibl, R., and D. Eibl, Design of Bioreactors Suitable for Plant Cell and Tissue Cell Cultures, *Phytochem. Rev.* 7: 593–598, 2008.

Georgiev, M. I., R. Eibl, and J. J. Zhong, Hosting the Plant Cells in Vitro: Recent Trends in Bioreactors, *Appl. Microbiol. Biotechnol.* 97: 3787–3800, 2013.

Georgiev, M. I., J. Weber, and A. Maciuk, Bioprocessing of Plant Cell Cultures for Mass Production of Targeted Compounds, *Appl. Microbiol. Biotechnol.* 83:809–823, 2009.

Huang, T. K., M. A. Plesha, and K. A. McDonald, Semicontinuous Bioreactor Production of a Recombinant Human Therapeutic Protein Using a Chemically Inducible Viral Amplicon Expression System in Transgenic Plant Cell Suspension Culture, *Biotechnol. Bioeng.* 106: 408–421, 2010.

Ketchum, R. E. B., D. M. Gibson, R. B. Croteau, and M. L. Shuler, The Kinetics of Taxoid Accumulation in Cell Suspension Cultures of *Taxus* Following Elicitation with Methyl Jasmonate, *Biotechnol. Bioeng.* 62(1): 97–105, 1999.

Mustafa, N. R., W. de Winter, F. van Iren, and R. Verpoorte, Initiation, Growth and Cryopreservation of Plant Cell Suspension Cultures, *Nat. Protoc.* 6: 715–742, 2011.

Payne, G. F., V. Bringi, C. L. Prince, and M. L. Shuler, *Plant Cell and Tissue Culture in Liquid Systems,* Hanser Publ., New York (available through Wiley), 1992.

Rizvi, N., M. Cornejo, K. Stein, J. Weaver, E. J. Cram, and C. W. T. Lee-Parsons, An Efficient Transformation Method for Estrogen-Inducible Transgene Expression in *Catharanthus roseus* Hairy Roots, *PCTOC* 120(2): 475–487, 2015.

Srivastava, S., and A. K. Srivastava, Hairy Root Cultures for Mass-Production of High-Value Secondary Metabolites, *Crit. Rev. Biotechnol.* 27: 29–43, 2007.

Weathers, P. J., M. J. Towler, and J. Xu, Bench to Batch: Advances in Plant Cell Culture for Producing Useful Products, *Appl. Microbiol. Biotechnol.* 85: 1339–1351, 2010.

Wilson, S. A., and S. C. Roberts, Recent Advances Towards Development and Commercialization of Plant Cell Culture Processes for Synthesis of Biomolecules, *Plant Biotechnol. J.*10: 249–268, 2012.

Zhong, J. J., Plant Cell Culture for Production of Paclitaxel and Other Taxanes, *J. Biosci. Bioeng.* 94: 591–599, 2002.

PROBLEMS

13.1. The uptake of the auxin indole acetic acid (IAA) by suspension cultures of *Parthenocissus* sp. is nearly zero order at 1 nmol/mg dry cell weight-min. The diffusivity of IAA in water is 5×10^{-6} cm^2/s. Beads of calcium alginate are most conveniently made as spheres with a 4 mm diameter. Assume the beads are made 25% by volume of plant cells. Assume the plant cells are 90% water and that the diffusivity of IAA in the gel is the same as in water. If the external concentration is maintained at 2 μmol, will IAA penetrate to the center of the bead?

13.2. Gel-immobilized cells of *Papaver somniferum* (opium poppy) can make codeine from codeinone. The rate of codeinone uptake is first order, with a rate constant of 3.3×10^{-8} l/g cells dry weight-s. The diffusivity of codeinone in the gel is 0.2×10^{-9} m^2/s. For a gel particle of 4 mm diameter with a 25% volume loading of cells (95% water), what will be the effectiveness factor?

13.3. The k_La of a small bubble column (2 l) has been measured as 20 h^{-1} at an airflow of 4 l/min. If the rate of oxygen uptake by a culture of *Catharanthus roseus* is 0.2 mmol

O_2/gDW-h and if the critical oxygen concentration must be above 10% of saturation (about 8 mg/l), what is the maximum concentration of cells that can be maintained in the reactor?

13.4. *C. roseus* cells immobilized in Ca-alginate beads of diameter 0.5 mm are used for production of indole alkaloids (IA) in a fluidized-bed bioreactor. The rate-limiting nutrient is glucose, and no intraparticle diffusion limitations exist. Growth is negligible and Monod kinetics is valid. Use the following data:

Flow rate of the feed:	$Q = 1$ l/h
Glucose in the feed:	$S_0 = 30$ g/l
Plant cell concentration:	$X = 6$ g/l reac.
Rate constants for IA formation:	$k = 5\ d^{-1}(\text{g/l})^{-1}$; $K_s = 0.4$ g/l
Column diameter:	$D_o = 0.15$ m

a. For 95% glucose conversion, determine required hydraulic residence time, volume, and the height of the column.

b. If $Y_{p/s}$ is 0.02 g IA/g glucose, determine IA concentration in the effluent and the productivity.

14

Utilizing Genetically Engineered Organisms

In Chapter 8, "How Cellular Information Is Altered," we discussed how cells could be genetically engineered. The techniques of genetic engineering are fairly straightforward; the design of the best production system is not. The choice of host cell, the details of the construction of the vector, and the choice of promoter must all fit into a processing strategy. That strategy includes plans not only for efficient production but also for how a product is to be recovered and purified. The development of processes for making products from genetically engineered organisms requires that many choices should be evaluated. Any choice at the molecular level imposes processing constraints. As indicated in Figure 14.1, an interdisciplinary team approach to process development is necessary to make sure that a well-designed process results.

In this chapter, we discuss some of the questions that must be considered in building processes using genetically engineered cells.

14.1. HOW THE PRODUCT INFLUENCES PROCESS DECISIONS

Genetically engineered cells can be used to make two major classes of products: proteins and nonproteins. Nonprotein products can be made by metabolically engineering cells, inserting DNA-encoding enzymes that generate new pathways or pathways with an enhanced capacity to process the precursors to a desired metabolite. Nonprotein

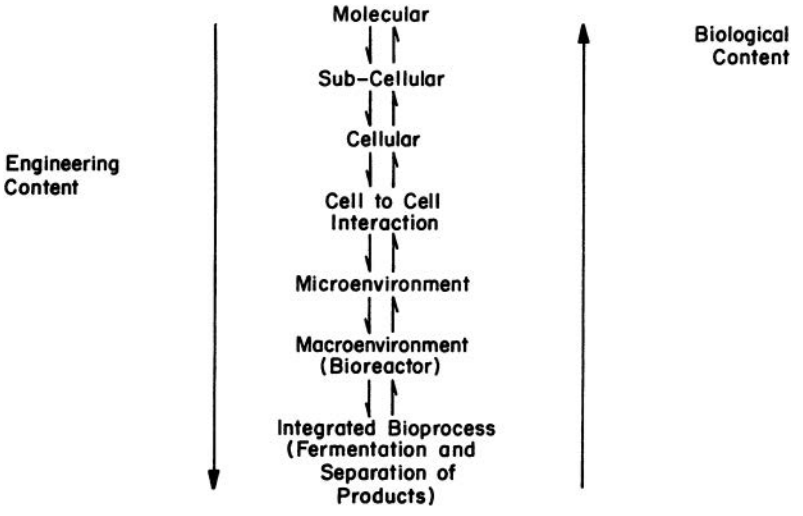


Figure 14.1. Biocatalyst and bioreactor design are strongly interconnected. Choices made at the molecular level can have profound effects on bioreactor design. A multidisciplinary approach is necessary to ensure that a well-designed process is developed.

products can also include nucleic acid- and polysaccharide-based products that are derived using recombinant DNA technology. However, most current industrial emphasis has been on proteins. Table 14.1 lists some examples. The majority of these proteins are human therapeutics, but proteins that can be used in animal husbandry, in food processing, or as industrial catalysts are also of interest. The global market for recombinant protein therapeutics, or biopharmaceuticals, which currently includes approximately 250 approved products, is forecasted to exceed $200 billion by 2020.

With therapeutic proteins that are injectable, the prime concern is the clinical efficiency of the product. Such products must be highly pure, since strong immunogenic reactions by patients or other side effects can be disastrous. The authenticity of the product is often critical. Correct or near-correct posttranslational processing of the protein (e.g., glycosylation or phosphorylation) is sometimes essential to its therapeutic action. Any variant forms of the protein (e.g., the modification of side groups on amino acids) are highly undesirable and present very difficult purification problems.

The processing challenges in making therapeutic proteins are to ensure product quality and safety. Process efficiency to reduce manufacturing cost, although important, is of less concern because these products are required in relatively small amounts, because they can command high prices, and because the selling price is mainly determined by the costs of process development and regulatory approvals, particularly clinical trials. Thus, for these products, the choice of biological system and processing equipment is dictated by the need to produce highly purified material in an absolutely consistent manner.

Other protein products are purchased strictly on an economic basis, and manufacturing costs play a much more critical role in the viability of a proposed process. In this case, regulatory demands are of less importance than in the production of

TABLE 14.1. Key Biopharmaceutical Products

Product	End Use
Hormones and Peptide Factors	
Human insulin, exenatide, liraglutide	Diabetes (hyperglycemia)
Glucagon	Diabetes (hypoglycemia)
Factor VIII-C	Hemophilia
Human growth hormone	Growth deficiency
Erythropoietin	Anemia, chronic renal failure
Interferon-alpha 2a	Hairy cell leukemia, AIDS-related cancer
Interferon-alpha 2b	Hairy cell leukemia, herpes
Interferon-beta	Cancer
Interferon-gamma	Cancer, venereal warts, infectious disease
Interleukin-2	Cancer immunotherapy, AIDS
Leuprorelin	Prostate cancer, breast cancer
Muromonab-CD3	Acute kidney transplant rejection
Granulocyte colony stimulating factor (G-CSF)	Chemotherapy effects, AIDS
Granulocyte macrophage-colony stimulating factor (GM-CSF)	Autologous bone marrow transplant
Enzymes	
Tissue plasminogen activator	Acute myocardial infarction, stroke
Prourokinase/urokinase	Heart attack
DNase	Pulmonary treatment
Glucocerebrosidase	Gaucher disease
Vaccines	
Hepatitis B	Hepatitis B vaccine
Herpes	Herpes
Monoclonal Antibodies	
Bevacizumab	Antibodies for diagnostic and therapeutic use metastatic cancer, retinopathy of prematurity
Adalimumab, infliximab	Rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis, Crohn disease, ulcerative colitis
Lucentis	Macular degeneration (wet form)
Rituximab, obinutuzumab	Lymphomas, leukemias, some autoimmune disorders
Trastuzumab	Breast cancer

therapeutic proteins. For animal vaccines or animal hormones, the products must be very pure and render favorable cost ratios. For example, the use of bovine somatropin to increase milk production requires that the increased value of the milk produced be substantially greater than the cost of the hormone and any increase in feed costs due to increased milk production.

For food use, product safety is important, but purity requirements are less stringent than for an injectable. The volume of the required product is often substantial (several metric tons per year), and price is critical because alternative products from natural sources may be available. Proteins used as specialty chemicals (e.g., adhesives and enzymes for industrial processes) usually can tolerate the presence of

contaminating proteins and compounds. The manufacturing costs for such proteins will greatly influence the market penetration.

For nonprotein products based on metabolically engineered cells, processing costs compared to costs of other routes of manufacture (usually nonbiological) will determine the success of such a process.

Note that the constraints on production can vary widely from one product class to another. These constraints determine which host cells, vectors, genetic constructions, processing equipment, and processing strategies are chosen.

14.2. GUIDELINES FOR CHOOSING HOST–VECTOR SYSTEMS

The success or failure of a process often depends on the initial choice of host organism and expression system. Such choices must be made in the context of processing strategy.

Table 14.2 summarizes many of the salient features of common host systems. The most important initial judgment must be whether posttranslational modifications of the product are necessary. If they are, then an animal cell host system is typically chosen, although the emergence of engineered yeast or bacteria that perform humanlike glycosylation reactions may also be an option. If some simple posttranslational processing is required (e.g., modification with paucimannose glycans), yeast, fungi, or insect cells may be acceptable. Whether posttranslational modifications are necessary for proper activity of a therapeutic protein cannot always be predicted with certainty, and clinical trials may be necessary.

TABLE 14.2. Characteristics of Some Host Systems for Heterologous Protein Production

	Organism				
Characteristic	*Escherichia Coli*	*Saccharomyces Cerevisiae*	*Pichia Pastoris*	*Insect*	*Mammalian*
High growth rate	E	VG	VG	P–F	P–F
Availability of genetic systems	E	G	F	F–G	F–G
Expression levels	E	VG	E	G–E	P–G
Low-cost media available	E	E	E	P	P
Protein folding	F	F–G	F–G	VG–E	E
Simple glycosylation	No	Yes	Yes	Yes	Yes
Complex glycosylation	No[a]	No	No[a]	Yes[b]	Yes
Low levels of proteolytic degradation	F–G	G	G	VG	VG
Excretion or secretion	P (normally in special cases)	VG	VG	VG	E
Safety	VG	E	VG	E	G

Note: E, excellent; VG, very good; G, good; F, fair; P, poor.

[a] Strains of *E. coli* and *P. pastoris* have been engineered to perform humanlike glycosylation.

[b] Glycosylation patterns differ from mammalian cells.

Another important consideration is whether the product will be used in foods. For example, some yeasts (e.g., *Saccharomyces cerevisiae*) are on the FDA GRAS (generally regarded as safe) list, which would greatly simplify obtaining regulatory approval for a given product. In some cases, edible portions of transgenic plants can be used to deliver vaccines or therapeutic proteins.

14.2.1. Escherichia Coli

If posttranslational modifications are unnecessary, *E. coli* is usually chosen as the initial host. The main reason for the popularity of *E. coli* is the broad knowledge base surrounding this heavily studied organism. *E. coli* physiology and its genetics are probably far better understood than any other living organism. A wide range of host backgrounds (i.e., combinations of specific mutations) is available, as well as vectors and promoters. This large knowledge base greatly facilitates sophisticated genetic manipulations. The well-defined vectors and promoters greatly speed the development of an appropriate biological catalyst.

The relatively high growth rates for *E. coli* coupled with the ability to grow *E. coli* to high cell concentrations (>50 gDW/l) and with the high expression levels possible from specific vector-promoter combinations (about 25% to 50% or more of total protein) can lead to extremely high volumetric productivities. Also, *E. coli* will grow on simple and inexpensive media. These factors give *E. coli* many economic advantages.

An important engineering contribution was the development of strategies to grow cultures of *E. coli* to high cell densities. The buildup of acetate and other metabolic by-products can significantly inhibit growth. Controlled feeding of glucose to prevent the accumulation of large amounts of glucose in the medium prevents overflow metabolism and the formation of acetate. Glucose feeding can be coupled to consumption rate if the consumption rate can be estimated on-line.

However, *E. coli* is not a perfect host. The major problems arise because *E. coli* does not normally secrete proteins. When proteins are retained intracellularly and produced at high levels, the amount of soluble active protein present is usually limited due to either proteolytic degradation or insolubilization into *inclusion bodies*.

The production of large amounts of foreign protein may trigger a *heat-shock* response. One response of the heat-shock regulon is increased proteolytic activity. In some cases, intracellular proteolytic activity results in product degradation at a rate nearly equal to the rate of production.

More often, the target protein forms an inclusion body. Although the heterologous protein predominates in an inclusion body, other cellular material is also often included. The protein in the inclusion body is misfolded. The misfolded protein has no biological activity and is worthless. If the inclusion bodies are recovered from the culture, they can be resolubilized and their activity (and value) can be restored. The difficulty of resolubilization can vary tremendously from one protein to another. When resolubilization is straightforward and recoveries are high, the formation of inclusion bodies can be advantageous, as it simplifies the initial steps of recovery and purification. It is important that during resolubilization the protein be checked by several analytical methods to ensure that no chemical modifications have occurred. Even slight changes in a side group can alter the effectiveness of the product.

Other consequences of cytoplasmic protein production can be important. The intracellular environment in *E. coli* might not allow the formation of disulfide bridges. Also, the protein will usually start with a methionine, whereas that methionine would have been removed in normal posttranslational processing in the natural host cell. If the product is retained intracellularly, then the cell must be lysed (broken) during recovery. Lysis usually results in the release of endotoxins (or pyrogens) from *E. coli.* Endotoxins are lipopolysaccharides (found in the outer membrane) and can result in undesirable side effects (e.g., high fevers) and death. Thus, purification is an important consideration.

Many of the limitations of *E. coli* can be circumvented with protein secretion and excretion. *Secretion* is defined here as the translocation of a protein across the inner membrane of *E. coli. Excretion* is defined as release of the protein into the extracellular compartment.

About 20% of all protein in *E. coli* is translocated across the inner membrane into the periplasmic space or incorporated into the outer membrane. Recall from Chapter 4, "How Cells Work," that secreted proteins are made with a signal or leader peptide. The presence of a *signal peptide* is a necessary (but not sufficient) condition for secretion. The signal peptide is a sequence of amino acids attached to the mature protein, and the signal sequence is cleaved during secretion.

Many benefits are possible if a protein is secreted. Secretion eliminates an undesired methionine from the beginning of the protein. Secretion also often offers some protection from proteolysis. Periplasmic proteases exist in *E. coli*, but usually at a low level. They are most active at alkaline pH values, and pH control can be used to reduce target protein degradation. The environment in the periplasmic space promotes the correct protein folding in some cases (including the formation of disulfide bridges). Proteins in the periplasmic space can be released by gentle osmotic shock so that fewer contaminating proteins are present than if the whole cell were lysed.

Even more attractive would be the extracellular release of target proteins. Normally, *E. coli* excretes very few of its own proteins (colicin, hemolysin, OsmY, and YebF are a few exceptions), but a variety of schemes to obtain excretion in *E. coli* are being developed. Strategies usually involve either trying to disrupt the structure of the outer membrane or attempting to use the colicin, hemolysin, OsmY, or YebF excretion systems by constructing a fusion of the target protein with components of these excretion systems. More sophisticated strategies include genetically modifying *E. coli* with multicomponent excretion machineries (e.g., type II, type III systems) that are derived from excretion-competent bacteria. Excretion without cell lysis can simplify recovery and purification even more than secretion alone while achieving the same advantages as secretion with respect to protein processing. Excretion also facilitates the potential use of continuous immobilized cell systems. The lack of established excretion systems in *E. coli* has led to interest in alternative expression systems. Also, in some cases, patent considerations may require the use of alternative hosts.

Many of the aforementioned limitations can also be circumvented using genetically engineered host strains. For example, proteolytic degradation of intracellular proteins can typically be reduced or even eliminated by using a protease-deficient host strain of *E. coli* in which one or more of the major intracellular proteases (e.g., Lon protease) are deleted from the genome. The need for disulfide bonds in cytoplasmic

proteins has prompted the development of redox-engineered host strains in which cytoplasmic enzymes that keep cysteines in their reduced form (e.g., thioredoxin reductase TrxB, glutathione reductase Gor) are deleted from the genome. These strains have been used to produce a wide range of valuable human protein products, including tissue plasminogen activator and monoclonal antibodies that require multiple disulfide bonds for proper folding. Likewise, the need for protein glycosylation has prompted the development of glyco-engineered *E. coli* in which a synthetic pathway is introduced for the production of eucaryotic *N*-glycans and the transfer of these *N*-glycans to target proteins. To circumvent concerns associated with endotoxin contamination, endotoxin-free strains of *E. coli* have been engineered by deleting the genes involved in producing the offending endotoxin signal that is normally part of the lipopolysaccharide while still retaining competency and protein expression capability.

14.2.2. Gram-Positive Bacteria

The gram-positive bacterium *Bacillus subtilis* is the best studied bacterial alternative to *E. coli.* Since it is gram positive, it has no outer membrane, and it is a very effective excreter of proteins. Many of these proteins, amylases, and proteases are produced commercially using *B. subtilis.* If heterologous proteins could be excreted as efficiently from *B. subtilis* as from amylases and proteases, then *B. subtilis* would be a very attractive production system.

However, *B. subtilis* has a number of problems that hinder its commercial adoption. A primary concern is that *B. subtilis* produces a large amount and variety of proteases. These proteases can degrade the product very rapidly. Mutants with greatly reduced protease activity have become available, but even these mutants may have sufficient amounts of minor proteases to be troublesome. *B. subtilis* is also much more difficult than *E. coli* to manipulate genetically because of a limited range of vectors and promoters. Also, the genetic instability of plasmids is more of a problem in *B. subtilis* than in *E. coli.* Finally, the high levels of excretion that have been observed with native *B. subtilis* proteins are not yet obtainable with heterologous protein (i.e., foreign proteins produced from recombinant DNA).

Other gram-positive bacteria that have been considered as hosts include *Lactococcus lactis* subsp. *cremoris* and *Streptomyces* sp. These other systems are less well characterized than *B. subtilis* but have also shown promise. For example, *L. lactis* has been evaluated up to phase II clinical trials as an oral anti-inflammatory protein delivery vector in situ in the human gut as treatment for inflammatory bowel disease.

Both gram-negative and gram-positive bacteria have limitations on protein processing that can be circumvented with eucaryotic cells.

14.2.3. Lower Eucaryotic Cells

The yeast *Saccharomyces cerevisiae* has been used extensively in food and industrial fermentations and is among the first organisms harnessed by humans. It can grow to high cell densities and at a reasonable rate (about 25% of the maximum growth rate of *E. coli* in similar medium). Yeasts are larger than most bacteria and can be recovered more easily from a fermentation broth.

Further advantages include the capacity to do simple glycosylation of proteins and to secrete proteins. However, *S. cerevisiae* tends to hyperglycosylate proteins, adding large numbers of mannose units. In some cases, the hyperglycosylated protein may be inactive. These organisms are also on the GRAS list, which simplifies regulatory approval and makes yeast particularly well suited to production of food-related proteins.

Generally, the limitations on *S. cerevisiae* are the difficulties of achieving high protein-expression levels, hyperglycosylation, and good excretion. Although the genetics of *S. cerevisiae* are better known than the genetics of any other eucaryotic cell, the range of genetic systems is limited, and stable high protein-expression levels are more difficult to achieve compared to *E. coli.* Also, the normal capacity of the secretion pathways in *S. cerevisiae* is limited and can provide a bottleneck on excreted protein production even when high expression levels are achieved.

The methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* are very attractive hosts for some proteins. These yeasts can grow on methanol as a carbon–energy source; methanol is also an inducer for the AOX 1 promoter, which is typically used to control expression of the target protein. Very high cell densities (e.g., up to 100 g/l) can be obtained. Due to high densities and, for some proteins, high expression levels, the volumetric productivities of these cultures can be higher than with *E. coli.* Protein folding and secretion often are also better than in *E. coli.* These yeasts do simple glycosylation and are less likely than *S. cerevisiae* to hyperglycosylate. Like many host systems, their effectiveness is often a function of the target protein. The disadvantages of the methylotrophic yeast are due to the high cell density and rate of metabolism, which create high levels of metabolic heat that must be removed and high oxygen demand. Effectively inducing expression while maintaining cell activity requires very good process control because of methanol's dual rate as growth substrate and inducer. Further, high levels of methanol are inhibitory (i.e., substrate inhibition), which also demands good process control. Scale-up to large reactors often is very challenging, since heat removal, oxygen supply, and process control are typically more difficult in large reactors with longer mixing times (see Chapter 10, "Selection, Scale-Up, Operation, and Control of Bioreactors"). Also, methanol is flammable, and handling large volumes of methanol is a safety concern. Nonetheless, these methylotrophic yeasts are of increasing importance.

Fungi, such as *Aspergillus nidulans* and *Trichoderma reesei*, are also potentially important hosts. They generally have greater intrinsic capacity for protein secretion than *S. cerevisiae.* Their filamentous growth makes large-scale cultivation somewhat difficult. However, commercial enzyme production from these fungi is well established, and the scale-up problems have been addressed. The major limitation has been the construction of expression and secretion systems that can produce extracellular heterologous proteins in amounts as large as some of the native proteins. A better understanding of the secretion pathway and its interaction with protein structure will be critical for this system to reach its potential.

All these lower eucaryotic systems are inappropriate when complex glycosylation and posttranslational modifications are necessary. Historically, in such cases, animal cell tissue culture has been employed. However, researchers have expanded the utility of the yeast *P. pastoris* by engineering it to secrete human glycoproteins

with fully complex, terminally sialylated *N*-linked glycans. This achievement involved genetic knockout of four genes to eliminate yeast-specific glycosylation, followed by introduction of 14 heterologous genes, allowing replication of the sequential steps of human glycosylation. The reported cell lines were able to produce functional recombinant erythropoietin. Recent efforts have focused on engineering humanlike *O*-linked protein glycosylation pathways to operate alongside the previously engineered *N*-linked pathways.

14.2.4. Mammalian Cells

Mammalian cell culture is chosen when the virtual authenticity of the product protein must be complete. Authenticity implies not only the correct arrangement of all amino acids but also that all posttranslational processing is identical to that in the whole animal. In some cases, the cells in culture may not do the posttranslation modifications identically to those done by the same cell while in the body. However, for bioreactor processes, mammalian cell tissue culture will provide the product closest to its natural counterpart. Another advantage is that most proteins of commercial interest are readily excreted.

Slow growth, expensive media, and low protein-expression levels all make mammalian cell culture expensive. As discussed in Chapter 12, "Bioprocess Considerations in Using Animal Cell Cultures," a wide variety of reactor systems are being used with animal cell cultures. Although many of these can improve efficiency significantly, processes based on mammalian cells remain expensive.

Several cell lines have been used as hosts for the production of proteins using recombinant DNA. The most popular hosts are probably lines of CHO (Chinese hamster ovary) cells.

In addition to the cost of production, mammalian cells face other severe constraints. Normal cells from animals are capable of dividing only a few times; these cell lines are *mortal.* Some cells are *immortal* or *continuous* and can divide continuously, just as a bacterium can. Continuous cell lines are *transformed* cells. Cancer cells are transformed also (i.e., have lost the inhibition of cell replication). The theoretical possibility that a cancer-promoting substance could be injected along with the desired product necessitates extreme care in the purification process. It is particularly important to exclude nucleic acids from the product. The use of transformed cells also requires precautions to ensure worker safety.

Additionally, the vectors commonly used with mammalian cell cultures have been derived from primate viruses. Again, there is concern about the reversion of such vectors back to a form that could be pathogenic in humans.

Most of these vectors cannot give high expression levels of the target protein in common host cells (usually <5% of total protein). However, higher levels of expression can be obtained (e.g., >1 g/l of secreted, active protein) through amplification of the number of gene copies. It may take 6 months with a CHO cell line to achieve stable, high-level expression. It is often easier to obtain high titers (or product concentration) when producing monoclonal antibodies from hybridoma cultures. However, the technology for producing proteins, in general, is improving rapidly and high-level production can be achieved for many proteins from animal cells.

The quality of the protein product, especially as determined by producing proteins with consistent and authentic posttranslational modifications, may change upon scale-up. Since animal cultures are chosen primarily as the best hosts to produce humanlike glycosylated proteins, it is critical to understand how process conditions affect protein quality. Since culture conditions (shear, glucose, amino sugars, dissolved oxygen [DO], etc.) can change upon scale-up, the efficiency of cellular protein processing can change, altering the level of posttranslational processing. Further, it has been shown that protein quality may change with harvest time in batch cultures. This change may be due to alterations in intracellular machinery, but often it is due to release of proteases and sialidase (an enzyme that removes the sialic acid cap from glycosylated proteins) from dead cells. Also, excessive levels of protein production may saturate the intracellular protein-processing organelles (i.e., endoplasmic reticulum [ER] and Golgi apparatus), leading to incompletely processed proteins. These problems can significantly impact process strategy. One well-known company has been forced to harvest 24 hours early to maintain the sialic acid/protein ratio specified for the product. Early harvest resulted in a 30% loss in protein concentration (as compared with delayed harvest).

Strategies to reduce such problems include selection of cell lines or genetic manipulation of cell lines with reduced levels of sialidase production or enhanced protein-processing capacity. Historically, this task has been difficult, but cell line engineering has become greatly simplified through targeted genome editing. The bacterial clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system enables rapid, easy, and efficient engineering of mammalian genomes. It has a wide range of applications from modification of individual genes to genome-wide screening or regulation of genes. The facile genome editing afforded by CRISPR/Cas9 along with the recent publication of CHO genome sequences should open the door to engineering high-level production of proteins and product quality attributes of interest in this important industrial cell line. Redesign of medium can be beneficial; chemicals that inhibit undesirable extracellular enzyme activity can be added or precursors added (e.g., amino sugars) to improve processing. Cell lysis can be reduced by adding genes (e.g., *bcl*-2) to the host cell that reduce apoptosis. An engineering solution is to remove the product from the medium as it is formed. For example, perfusion systems with an integrated product capture step can be used.

14.2.5. Insect Cell–Baculovirus System

A popular alternative for protein production at small (<100 l) or laboratory scale is the insect cell–baculovirus system. This system is particularly attractive for rapidly obtaining biologically active protein for characterization studies. Typical host cell lines come from the fall armyworm *(Spodoptera frugiperda)* and the cabbage looper *(Trichoplusia ni)*. The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector for insertion of recombinant DNA into the host cell.

This virus has an unusual biphasic replication cycle in nature. An insect ingests the occluded form in which multiple virus particles are embedded in a protein matrix. The protein matrix protects the virus when it is on a leaf from environmental stresses

(e.g., UV radiation). This protein matrix is from the polyhedrin protein. In the midgut of the insect, the matrix dissolves, allowing the virus to attack the cells lining the insect's gut; this is the primary infection. These infected cells release a second type of virus; it is nonoccluded (no polyhedrin matrix) and buds through the cell envelope. The nonoccluded virus (NOV) infects other cells throughout the insect (secondary infection).

In insect cell culture, only NOVs are infectious, and the polyhedrin gene is unnecessary. The polyhedrin promoter is the strongest known animal promoter and is expressed late in the infection cycle. Replacing the polyhedrin structural gene with the gene for a target protein allows high-level target protein production (up to 50% of cellular protein). Proteins that are secreted and glycosylated are often made at much lower levels than nonsecreted proteins.

In addition to high expression levels, the insect–baculovirus system offers safety advantages over mammal–retrovirus systems. The insect cell lines derived from ovaries or embryos are continuous but not transformed. The baculovirus is not pathogenic toward either plants or mammals. Thus, the insect–baculovirus system offers potential safety advantages. Another important advantage is that the molecular biology and high-level expression of correctly folded proteins can be achieved in less than a month.

This system also has the cellular machinery to do almost all the complex posttranslational modifications that mammalian cells do. However, even when the machinery is present, at least some of the proteins produced in the insect cell–baculovirus system are not processed identically to the native protein. In some cases, their slight variations may be beneficial (e.g., increased antigenic response in the development of an AIDS vaccine), while in others they may be undesirable. While complex glycoforms (including sialic acid) have been made, it is more common to observe only simple glycoforms. Production of complex forms requires special host cell lines and is sensitive to culture conditions.

The insect cell–baculovirus system is a good system to illustrate a holistic perspective on heterologous protein production. Any bioprocess for protein production is complex, consisting of the nonlinear interaction of many subcomponents. Thus, the optimal process is not simply the sum of individually optimized steps. Figure 14.2 presents a holistic view for the insect cell–baculovirus system. Because of the viral component, this system is even more complex than most other bioprocesses, as the infection process and resulting protein expression kinetics must be considered. One factor is the ratio of infectious particles to cells (e.g., multiplicity of infection, or MOI), which alters the synchrony of infection and the resulting protein expression kinetics. Another is the genetic design of the virus (which shares many of the general features of vector design). Also, the quality of the virus stock is important; if the virus stock is maintained incorrectly, mutant virus can form. One example is the formation of *defective interfering particles* (DIPs) that reduce protein expression in the culture by 90% when high MOIs are used.

Working from the bottom of Figure 14.2 toward the top should reveal the desired product quality and the product worth. Knowing these factors guides selection of bioprocess strategies to achieve the cost and quality desired. To develop that strategy requires an understanding of the basic kinetics and capabilities of the biological system. Understanding these requirements guides selection of the specific host cell line, the medium, and the molecular design of the virus. For example, the addition of serum to the medium of some *Ti ni* cell lines results in production of proteins with complex

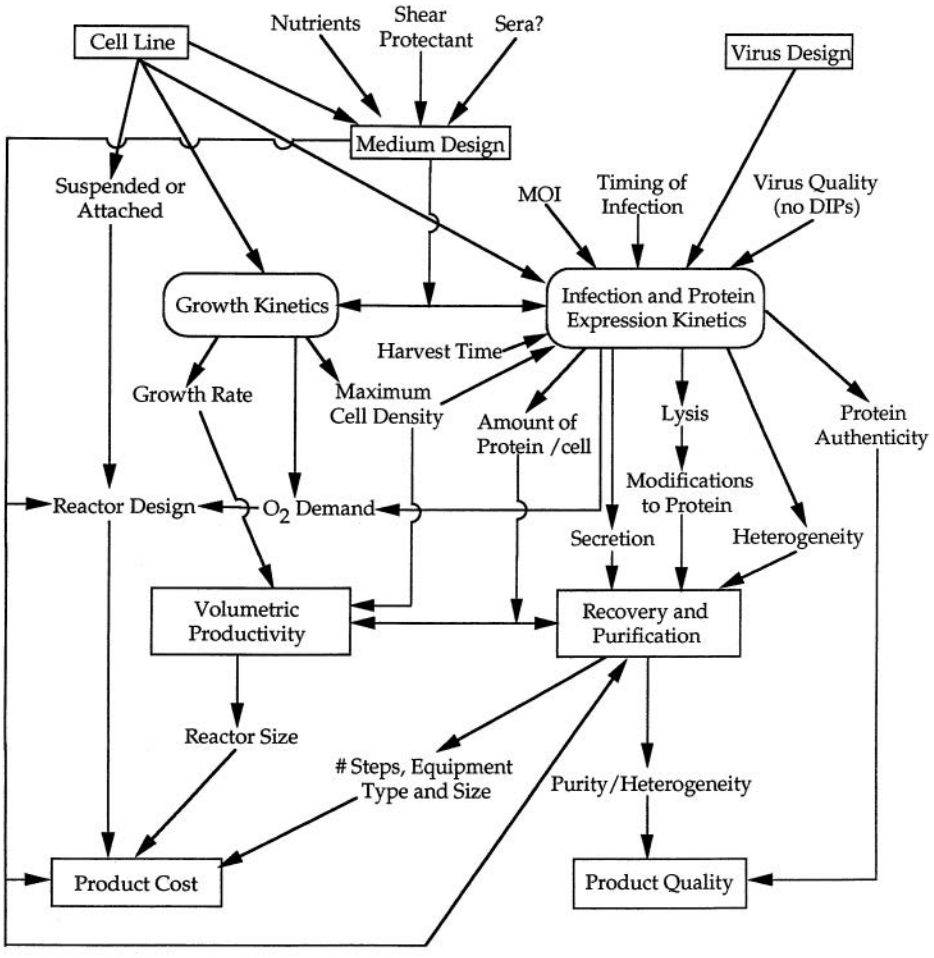


Figure 14.2. Holistic perspective on heterologous protein production with the baculovirus expression system.

N-linked glycosylation, including a sialic acid cap, which may be a requirement for product quality. However, the use of serum alters growth kinetics, expression levels (often less), and the difficulty of purification, which may alter cost. Such trade-offs need to be considered with respect to alternative approaches (e.g., development of a genetically engineered host that could perform the same reactions but in serum-free medium).

14.2.6. Transgenic Animals

In some cases, proteins with necessary biological activity cannot be made in animal cell culture. Although posttranslational protein processes, such as *N*-linked glycosylation, can be done in cell culture, other more subtle forms of posttranslational processing may not be done satisfactorily. An alternative to cell culture is the use of transgenic animals. Animals are engineered to express the protein and release it into

specific fluids, such as milk or urine. For example, transgenic goats have been engineered to produce the protein components of spider silk in milk. High concentrations of complex proteins can be achieved, and such approaches can be cost effective for complex proteins. In these cases, the role for the bioprocess engineer is in protein recovery and purification, although significant issues exist for agricultural engineers and animal scientists in devising appropriate systems to obtain the protein-containing fluid (e.g., pig milking stations). While transgenic animals can be developed from many mammals, sheep, goats, and pigs are the primary species used commercially.

There are significant limitations on this technology. In some cases, the protein of interest will cause adverse health problems in the producing animal. The use of animals raises safety concerns with respect to virus or prion transmission. The process to generate and screen for high-producing animals is inefficient and costly (e.g., $100,000 for a goat and $500,000 for a cow). Perhaps surprisingly, not all of the complex posttranslational processing steps necessary to achieve the desired product occur when the protein is expressed in milk, urine, or blood. Nonetheless, transgenic animals will be critical for production of some proteins.

14.2.7. Transgenic Plants and Plant Cell Culture

Proteins, including many complex protein assemblies, such as antibodies and virus-like particles (as vaccines), can be made inexpensively in plants. Transgenic plants offer many potential advantages in addition to cost. Since plant viruses are not infective for humans, there are no safety concerns with respect to endogenous viruses or prions. Scale-up is readily accomplished by planting more acreage. The protein can be targeted for sterile, edible compartments, either reducing the need for rigorous purification or making it an ideal vehicle for oral delivery of a therapeutic protein. Indeed, development of edible vaccines for use in developing countries is being actively pursued.

The disadvantages of transgenic plants are that expression levels are often low (1% of total soluble protein is considered good), *N*-linked glycosylation is incomplete, and some other mammalian posttranslational processing is missing. While inexpensive, with easy scale-up, it takes 30 months to test and produce sufficient seed for unlimited commercial use. Such long lead times are undesirable. Further, environmental control on field-grown crops is difficult, so the amount (and possibly quality) of the product can vary from time to time and place to place.

While many crops could be used, much of the commercial interest centers on transgenic corn. Some corn products are used in medicinals, so there exist some FDA guidelines (e.g., contamination with herbicides and mycotoxins), and there is considerable processing experience. Production costs vary with degree of desired purity. For high-purity material (95% pure) a cost of about $4 to $8/g can be estimated. For higher-purity material (99%) with full quality assurance and control, a cost of about $20 to $30/g is reasonable. At least two enzymes are being produced commercially from transgenic plants. Large-scale production of monoclonal antibodies (e.g., 500 kg/yr) for topical uses is being considered.

The use of plant cell cultures is also being explored. The primary advantage of such cultures over transgenic plants is the much higher level of control that can be exercised over the process. Plant cell cultures, compared to animal cell cultures, grow

to very high cell density, use defined media, and are intrinsically safer. See Chapter 13, "Bioprocess Considerations in Using Plant Cell Cultures," for a detailed discussion.

14.2.8. Cell-Free Protein Synthesis

Cell-free protein synthesis (CFPS) is the production of proteins in vitro without using intact, living cells. The typical components of a cell-free reaction include a cell extract, an energy source, a supply of amino acids, cofactors such as magnesium, and the DNA (linear template or circular plasmid) encoding the desired genes. Cell extracts are obtained by lysing the cell of interest and centrifuging out the cell walls, DNA genome, and other debris. The remains are the necessary cell machinery, including ribosomes, aminoacyl-tRNA synthetases, translation initiation and elongation factors, nucleases, and so on. Common cell extracts in use today are made from *E. coli*, rabbit reticulocytes, wheat germ, and insect cells.

CFPS systems have played an essential role in the discovery of the genetic code and, more recently, are used to meet the increasing demands for simple and efficient protein synthesis. Protein yields can now reach g protein/l reaction volume, batch reactions last for multiple hours, and reaction scale has reached the 100 l milestone. In one notable example, CFPS of 700 mg/l human granulocyte-macrophage colony-stimulating factor (GM-CSF) was achieved at the 100 l manufacturing scale.

There are many advantages gained when using cell-free systems. First, the open nature of the reaction allows the user to directly influence the biochemical systems of interest (e.g., protein synthesis, metabolism). As a result, new components (natural and nonnatural) can be added or synthesized, and these can be maintained at precise concentrations. Second, the chemical environment can be controlled, actively monitored, and rapidly sampled. Third, cell-free systems separate catalyst synthesis (cell growth) from catalyst utilization (protein production), circumventing a major challenge afflicting cell-based engineering efforts. Finally, there are no cell viability constraints. As a result, CFPS has found a wide range of applications from the production of pharmaceutical proteins to protein evolution. Additionally, CFPS systems can be used to test and model our understanding of biological systems.

14.2.9. Comparison of Strategies

The choice of host-vector system is complicated. The characteristics desired in the protein product and the cost are the critical factors in the choice. The dominant systems for commercial production are *E. coli* and CHO cell cultures. An interesting study of the process economics of these two systems for production of tissue plasminogen activator, tPA, has been published by Datar, Cartwright, and Rosen.[†] Although this study is dated and current CHO systems show much higher productivities and advances in making soluble protein from *E. coli* have been made, this analysis provides insight into the factors that need to be considered in comparing production strategies. Their analysis was for plants making 11 kg/yr of product. The CHO cell

[†] R. V. Darter, T. Cartwright, and C. G. Rosen, "Process Economics of Animal Cell and Bacterial Fermentations," *Bio/Technology* 11: 349, 1993.

process was assumed to produce 33.5 mg/l of product, while *E. coli* made 460 mg/l. The CHO cell product was correctly folded, biologically active, and released into the medium. The *E. coli* product was primarily in the form of inclusion bodies, and thus biologically inactive, misfolded, and insoluble. The process to resolubilize and refold the *E. coli* product into active material requires extra steps. The recovery process for the CHO cell material requires five steps, whereas 16 steps are required for the *E. coli* process. The larger the number of steps, the greater the possibility of yield loss. Total recovery of 47% with the CHO–produced material was possible compared to only 2.8% for the *E. coli*–produced material.

The extra steps in the *E. coli* process are for cell recovery, cell breakage, recovery of inclusion bodies, resolubilization of inclusion bodies, concentration, sulfonation, refolding, and concentration of the renatured protein. The difficulty of these processes depends on the nature of the protein; tPA is particularly difficult. The concentration of tPA must be maintained at 2.5 mg/l or less, and refolding is slow, requiring 48 hours. A 20% efficiency for renaturation was achieved. Many proteins can be refolded at higher concentrations (up to 1 g/l) and much more quickly. For tPA, the result is unacceptably large tanks and very high chemical usage. In this case, 5 tons of urea and 26 tons of guanidine would be necessary to produce only 11 kg of active tPA.

For tPA, the required bioreactor volumes were 14,000 l for the CHO process and 17,300 l for the *E. coli* process. The capital costs were $11.1 million for the CHO process and $70.9 million for the *E. coli* process, with 75% of that capital cost being required for the refolding tanks. Under these conditions, the unit production costs are $10,660/g for the CHO process versus $22,000/g for the *E. coli* process. The rate of return on investment (ROI) for the CHO process was 130% versus only 8% for the *E. coli* process. However, if the refolding step yield were 90% instead of 20%, the overall yield would improve to 15.4%, and the unit production cost would fall to $7,530 with an ROI of 85% for the improved *E. coli* process at production of 11 kg/yr. If the *E. coli* plant remained the original size (17,300 l fermenter) so as to produce 61.3 kg/yr, the unit production cost would drop to $4,400/g. The cost of tPA from the CHO process is very sensitive to cost of serum in the medium. If the price of media dropped from $10.5/l to $2/l (e.g., 10% to 2% serum in the medium), the cost of the CHO cell product would drop to $6,500/g. (Modern CHO processes are serum free not necessarily to save media costs but to simplify product recovery.)

A primary lesson from this exercise is the difficulty of making choices of host–vector systems without a complete analysis. The price will depend on the protein, its characteristics, and intended use. Changes in process technology (e.g., low serum medium for CHO cells or protein secretion systems in *E. coli*) can have dramatic effects on manufacturing costs and choice of the host-vector system.

14.3. PROCESS CONSTRAINTS: GENETIC INSTABILITY

There is a tension between the goal of maximal target-protein production and the maintenance of a vigorous culture. The formation of large amounts of foreign protein is always detrimental to the host cell, often lethal. Cells that lose the capacity to make the target protein often grow much more quickly and can displace the original, more productive strain, leading to *genetic instability* (see Figure 14.3).

Genetic instability can occur due to *segregational loss*, *structural instability*, *host cell regulatory mutations*, and the *growth-rate ratio* of plasmid-free or altered cells to plasmid-containing unaltered cells. Genetic instability can occur in any expression system. We illustrate this problem by considering gene expression from plasmids in bacteria.

14.3.1. Segregational Loss

Segregational loss occurs when a cell divides such that one of the daughter cells receives no plasmids (see Figure 14.4). Plasmids can be described as *high-copy-number plasmids* (>20 copies per cell) and *low-copy-number plasmids* (sometimes as low as one or two copies per cell). Low-copy-number plasmids usually have specific mechanisms to ensure their equal distribution among daughter cells. High-copy-number plasmids are usually

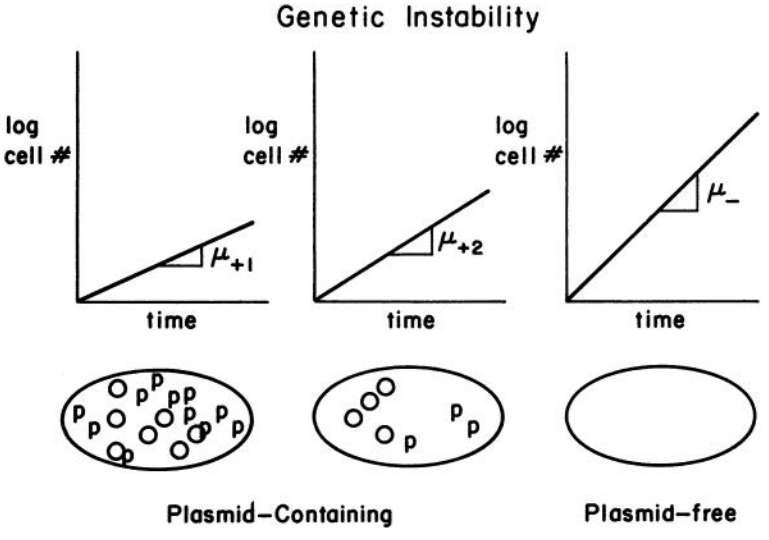


Figure 14.3. Cells that contain plasmids (O) actively making protein (p) must direct many of their cellular resources away from growth and hence grow more slowly than plasmid-free cells (that is, $\mu_{+1} < \mu_{+2} < \mu_-$).

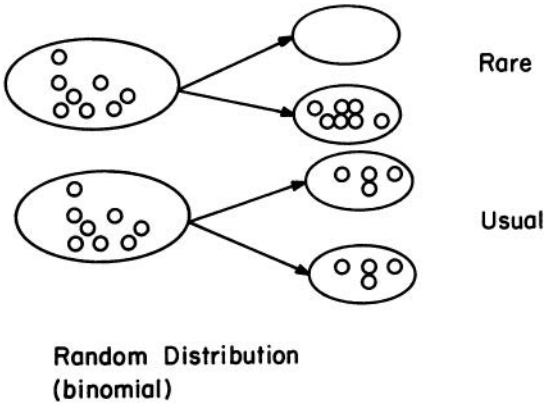


Figure 14.4. Segregational instability results when a dividing cell donates all its plasmids to one progeny and none to the other. (With permission, from G. Georgiou, Optimizing the production of recombinant proteins in microorganisms, *AIChE J.* 34(8):1233 [1988].)

distributed randomly among daughter cells following a binomial distribution. For high copy numbers, almost all the daughter cells receive some plasmids, but even if the possibility of forming a plasmid-free cell is low (one per million cell divisions), a large reactor contains so many cells that some plasmid-free cells will be present (e.g., 1000 l with 10^9 cells/ml yields 10^{15} cells and 10^9 plasmid-free cells being formed every cell generation).

The segregational loss of plasmid can be influenced by many environmental factors, such as dissolved oxygen, temperature, medium composition, and dilution rate in a chemostat. Many plasmids will also form *multimers*, which are multiple copies of the same plasmid attached to each other to form a single unit. The process of multimerization involves using host cell recombination systems. A *dimer* is a replicative unit in which two separate plasmids have been joined, and a *tetramer* is a single unit consisting of four separate monomers fused together.

Example 14.1

What fraction of the cells undergoing division will generate a plasmid-free cell for each of the following conditions:

a. All cells have 40 plasmids at division.
b. All cells have enough plasmid DNA for 40 copies, but one-half of the plasmid DNA is in the form of dimers and one-fifth in the form of tetramers.
c. Half of the cells have 10 copies of the plasmid and half have 70 copies (the average copy number is 40 as in case [a]).

Solution

a. If we assume a random distribution at division, the probability of forming a plasmid-free cell is

$$P = 2^{(1-Z)} \tag{14.1}$$

where Z is the number of plasmid replicative units. For $Z = 40$, $P = 1.8 \times 10^{-12}$ plasmid-free cells per division.

b. If the total amount of plasmid DNA is equivalent to 40 single copies, we can determine the plasmid distribution from the following:

$$M + D + T = 40 \text{ monomer plasmid equivalents} \tag{14.2a}$$

$$D = \tfrac{1}{2}(40) = 20 \text{ monomer plasmid equivalents} \tag{14.2b}$$

$$T = \tfrac{1}{5}(40) = 8 \text{ monomer plasmid equivalents} \tag{14.2c}$$

This has the following implication:

$$M = 40 - 20 - 8 = 12 \text{ monomer plasmid equivalents} \tag{14.3}$$

The number of copies of replicative units is then as follows, since a dimer consists of two monomer equivalents and a tetramer of four monomer equivalents:

$$M + D/2 + T/4 = \text{total replicative units} \tag{14.4a}$$

$$12 + 10 + 2 = 24 \text{ total replicative units} \tag{14.4b}$$

Equation 14.1 can then be applied to yield

$$P = 2^{(1-24)} = 1.2 \times 10^{-7} \tag{14.5}$$

c. When plasmids are not evenly distributed, the probability of forming a plasmid-free cell changes:

$$\begin{aligned} \tfrac{1}{2} P_{10} + \tfrac{1}{2} P_{70} &= \tfrac{1}{2}[2^{(1-10)}] + \tfrac{1}{2}[2^{(1-70)}] \\ &= 9.8 \times 10^{-4} + 8.5 \times 10^{-22} = 9.8 \times 10^{-4} \end{aligned} \tag{14.6}$$

Note that although this population has the same average copy number as case (a), the probability of forming a plasmid-free cell is 5.4×10^8 greater.

14.3.2. Plasmid Structural Instability

In addition to the problems of segregational instability, some cells retain plasmids but alter the plasmid to reduce its harmful effects on the cell (structural instability). For example, the plasmid may encode both for antibiotic resistance and for a foreign protein. The foreign protein drains cellular resources away from growth toward an end-product of no benefit to the cell. However, if the investigator has added antibiotics to the medium, the cell will benefit from retaining the gene encoding the antibiotic resistance. Normal mutations will result in some altered plasmids that retain the capacity to encode for desirable functions (e.g., antibiotic resistance) while no longer making the foreign protein. In other cases, cellular recombination systems will integrate the gene for antibiotic resistance into the chromosome. Cells containing structurally altered plasmids can normally grow much more quickly than cells with the original plasmids. A culture having undergone a change in which the population is dominated by cells with an altered plasmid has undergone structural instability.

14.3.3. Host Cell Mutations

Mutations in host cells can also occur that make them far less useful as production systems for a given product. These mutations often alter cellular regulation and result in reduced target-protein synthesis. For example, if the promoter controlling expression of the foreign protein utilizes a host cell factor (e.g., a repressor), then modification of the host cell factor may greatly modulate the level of production of the desired plasmid-encoded protein. The lac promoter (see our discussion in Chapter 4 of the lac operon) can be induced by adding chemicals; for a lac promoter lactose or a chemical analog of lactose (e.g., isopropyl-β-D-thiogalactoside [IPTG]) can be used. Such promoters are often used in plasmid construction to control the synthesis of a plasmid-encoded protein. If induction of plasmid-encoded protein synthesis from this promoter reduces cellular growth rates (as in Figure 14.2), then a mutation that

inactivates lac permease would prevent protein induction in that mutant cell. The lac permease protein is necessary for the rapid uptake of the inducer. Thus, the mutant cell would grow faster than the desired strain. Alternatively, a host cell mutation in the repressor, so that it would not recognize the inducer, would make induction impossible.

The key feature of this category of genetic instability is that a host cell mutation imparts a growth advantage to the mutant so that it will eventually dominate the culture. In this case, the mutant cell will contain unaltered plasmids but will make very little of the target plasmid-encoded protein.

14.3.4. Growth-Rate-Dominated Instability

The importance of all three of these factors (segregational loss of plasmid, structural alterations of the plasmid, and host cell mutations) depends on the growth-rate differential of the changed cell-plasmid system to the original host–vector system. If the altered host–vector system has a distinct growth advantage over the original host–vector system, the altered system will eventually dominate (i.e., genetic instability will occur).

The terms used to describe the cause of genetic instability are based on fairly subtle distinctions. For example, if genetic instability is due to segregational instability, we would infer that the rate of formation of plasmid-free cells is high. In this case, the number of plasmid-free cells would be high irrespective of whether the plasmid-free cells had a growth-rate advantage. If, on the other hand, we claimed that the genetic instability is growth-rate dependent, we would imply that the rate of formation of plasmid-free cells is low, but the plasmid-free cells have such a large growth-rate advantage that they outgrow the original host–vector system. In most cases, growth-rate-dependent instability and one of the other factors (segregational loss, structural changes in the plasmid, or host cell mutations) are important.

The growth-rate ratio can be manipulated to some extent by the choice of medium (e.g., the use of *selective pressure* such as antibiotic supplementation to kill plasmid-free cells) and the use of production systems that do not allow significant target-protein production during most of the culture period. For example, an inducible promoter can be turned on only at the end of a batch growth cycle when only one or two more cell doublings may normally occur. Before induction, the *metabolic burden* imposed by the formation of the target protein is nil, and the growth ratio of the altered to the original host–vector system is close to 1 (or less if selective pressure is also applied). This *two-phase fermentation* can be done as a modified batch system, or a multistage chemostat could be used. In a two-stage system, the first stage is optimized to produce viable plasmid-containing cells, and production formation is induced in the second stage. The continual resupply of fresh cells to the second stage ensures that many unaltered cells will be present.

The problem of genetic instability is more significant in commercial operations than in laboratory-scale experiments. The primary reason is that the culture must go through many more generations to reach a density of 10^{10} cells/ml in a 10,000 l tank

than in a shake flask with 25 ml. Also, the use of antibiotics as selective agents may not be desirable in the large-scale system owing either to cost or to regulatory constraints on product quality.

In the next section, we discuss some implications of these process constraints on plasmid design. In the section following, we discuss how simple mathematical models of genetic instability can be constructed.

14.4. AVOIDING PROCESS PROBLEMS IN PLASMID DESIGN

When we design vectors for genetic engineering, we are concerned with elements that control plasmid copy number, the level of target-gene expression, and the nature of the gene product, and we must also allow for the application of selective pressure (e.g., antibiotic resistance). The vector must also be designed to be compatible with the host cell.

Different *origins of replication* exist for various plasmids. The origin often contains transcripts that regulate copy number. Different mutations in these regulatory transcripts will yield greatly different copy numbers. In some cases, these transcripts have temperature-sensitive mutations, and temperature shifts can lead to *runaway replication* in which plasmid copy number increases until cell death occurs.

Total protein production depends on both the number of gene copies (e.g., the number of plasmids) and the strength of the promoter used to control transcription from these promoters. Increasing copy number while maintaining a fixed promoter strength increases protein production in a saturable manner. Typically, doubling copy numbers from 25 to 50 will increase protein production twofold, but an increase from 50 to 100 will increase protein production less than twofold. If the number of replicating units is above 50, pure segregational plasmid loss is very low. Most useful cloning vectors in *E. coli* have stable copy numbers from 25 to 250.

Many promoters exist. Some of the important ones for use in *E. coli* are listed in Table 14.3. An ideal promoter would be both very strong and tightly regulated. A zero basal level of protein production is desirable, particularly if the target protein is toxic to the host cell. A rapid response to induction is desirable, and the inducer should be cheap and safe. Although temperature induction is often used on a small scale, thermal lags in a large fermentation vessel can be problematic. Increased temperatures may also activate a heat-shock response and increased levels of proteolytic enzymes. Many chemical inducers are expensive or might cause health concerns if not removed from the product. Some promoters respond to starvation for a nutrient (e.g., phosphate, oxygen, and energy), but the control of induction with such promoters can be difficult to do precisely. The recent isolation of a promoter induced by oxygen depletion may prove useful because oxygen levels can be controlled relatively easily in fermenters.

Anytime a strong promoter is used, a strong transcriptional terminator should be used in the construction. Recall from Chapter 4 that a terminator facilitates the release of RNA polymerase after a gene or operon is read. Without a strong terminator, the RNA polymerase may not disengage. If the RNA polymerase reads through, it may transcribe undesirable genes or may disturb the elements

TABLE 14.3. Strong *E. coli* Promoters

Promoter	Induction Method	Characteristics[a]
lacUV5	Addition of IPTG	(about 5%)
tac	As above	Induction results in cell death (>30%)
Ipp-OmpA	As above	Suitable for secreted proteins (20%)
Ipp^{p}-5	As above	Strongest *E. coli* promoter (47%)
trp	Tryptophan starvation	Relatively weak (around 10%)
λp_L	Growth at 42°C	See text (>30%)
$\lambda p_L/cl_{trp}$	Addition of tryptophan	Easily inducible in large-scale production (24%)
att-nutL-p-att-N	10-min incubation at 42°C	No product is synthesized before induction (on/off promoter)
T7 promoter	Addition of IPTG or viral infection	As above (>35%), low basal levels
T4 promoter	Viral infection	Method of induction inhibits product degradation
phoA	Phosphate starvation	Induction in large-scale production is complicated

[a] Typical values of accumulated product as a percentage of the total protein of induced cells are given in parentheses.

With permission, from G. Georgiou, Optimizing the production of recombinant proteins in micro organisms, *AIChE J.* 34(8):1233 (1988).

controlling plasmid copy numbers. In extreme cases, this might cause runaway replication and cell death.

The nature of the protein and its localization are important considerations in achieving a good process. To prevent proteolytic destruction of the target protein, a hybrid gene for the production of a fusion protein can be made. Typically, a small part of a protein native to the host cell is fused to the sequence for the target protein with a linkage that can be easily cleaved during downstream processing. Also, fusion proteins may be constructed to facilitate downstream recovery by providing a "handle" or "tail" that adheres easily to a particular chromatographic medium.

Another approach to preventing intracellular proteolysis is to develop a secretion vector in which a signal sequence is coupled to the target protein. If the protein is secreted in one host, it will usually be excreted in another, at least if the right signal sequence is used. Replacement of the protein's natural signal sequence (e.g., from an eucaryotic protein) with a host-specific signal sequence can often improve secretion.

The secretion process is complicated, and the fusion of a signal sequence with a normally nonsecreted protein (e.g., cytoplasmic) does not ensure secretion, although several cases of secretion of normally cytoplasmic proteins have been reported. Apparently, the mature form of the protein contains the "information" necessary in the secretion process, but no general rules are available to specify when coupling a signal sequence to a normally cytoplasmic protein will lead to secretion.

To ensure the genetic stability of any construct and to aid in the selection of the desired host–vector combination, the vector should be developed to survive under selective growth conditions. The most common strategy is to include genes for antibiotic resistance. The common cloning plasmid pBR322 contains both ampicillin and

tetracycline resistance. Multiple resistance genes are an aid in selecting for human-designed modifications of the plasmid.

Another strategy for selection is to place on the vector the genes necessary to make an essential metabolite (e.g., an amino acid). If the vector is placed in a host that is auxotrophic for that amino acid, then the vector complements the host.[†] In a medium without that amino acid, only plasmid-containing cells should be able to grow. Because the genes for the synthesis of the auxotrophic factors can be integrated into the chromosome or because of reversions on the parental chromosome, double auxotrophs are often used to reduce the probability of nonplasmid-containing cells outgrowing the desired construction.

One weakness in both of these strategies is that, even when the cell loses the plasmid, the plasmid-free cell will retain for several divisions enough gene product to provide antibiotic resistance or the production of an auxotrophic factor (see Figure 14.5). Thus, cells that will not form viable colonies on selective plates (about 25 generations are required to form a colony) can still be present and dividing in a large-scale system. These plasmid-free cells consume resources without making product.

Another related problem, particularly in large-scale systems, is that plasmid-containing cells may protect plasmid-free cells from the selective agent. For example, auxotrophic cells with a plasmid may leak sufficient levels of the auxotrophic factor that plasmid-free cells can grow. With an antibiotic, the enzyme responsible for antibiotic degradation may leak into the medium. Also, the enzyme may be so effective, even when retained intracellularly, that all the antibiotic is destroyed quickly in a high-density culture, reducing the extracellular concentration to zero. Although genes allowing the placement of selective pressure on a culture are essential in vector development, the engineer should be aware of the limitations of selective pressure in commercial-scale systems.

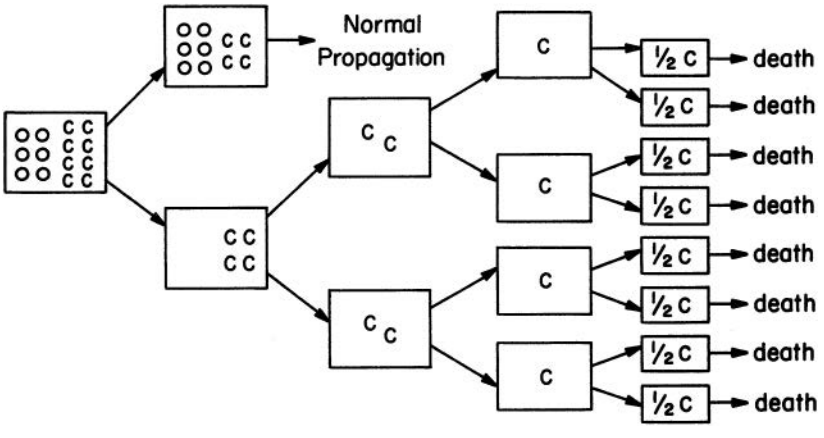


Figure 14.5. Newly born plasmid-free cells usually contain sufficient complementing factor (C) to withstand killing by a selective agent or starvation from the lack of a growth factor. In this case, the plasmid-free cell undergoes three divisions before the complementing factor is reduced to an ineffective level.

[†] Recall that an auxotrophic mutant would be unable to synthesize an essential compound on its own.

The other useful addition to plasmid construction is the addition of elements that improve plasmid segregation. Examples are the so-called *par* and *cer* loci. These elements act positively to ensure more even distribution of plasmids. The mechanisms behind these elements are incompletely understood, although they may involve promoting plasmid–membrane complexes (the *par* locus) or decreasing the net level of multimerization (the *cer* locus). Recall from Example 14.1 that multimerization decreases the number of independent, inheritable units, thus increasing the probability of forming a plasmid-free cell.

Any choice of vector construction must consider host cell characteristics. Proteolytic degradation may not be critical if the host cell has been mutated to inactivate all known proteases. Multimerization can be reduced by choosing a host with a defective recombination system. However, host cells with a defective recombination system tend to grow poorly. Many other possible host cell modifications enter into considerations of how to best construct a vector for a commercial operation.

These qualitative ideas allow us to anticipate to some extent what problems may arise in the maintenance of genetic stability and net protein expression. However, a good deal of research has been done on predicting genetic instability.

14.5. PREDICTING HOST–VECTOR INTERACTIONS AND GENETIC INSTABILITY

Many of the structured mathematical models we discussed previously can be extended to include component models for plasmid replication. Such models can then predict how plasmid-encoded functions interact with the host cell. The quantitative prediction of the growth-rate ratio and the development of plasmid-free cells due to segregational losses can be readily made. The most sophisticated models will predict the distribution of plasmids within a population and even the effects of multimerization on genetic stability. These models are too complex to warrant discussion in an introductory course.

We consider some simple models that mimic many of the characteristics we discussed with models of mixed cultures. A number of simple models for plasmid-bearing cells have been proposed. The key parameters in such models are the relative growth rates of plasmid-free and plasmid-containing cells and the rate of generation of plasmid-free cells (i.e., segregational loss). These parameters can be determined experimentally or even predicted for more sophisticated models of host–vector interactions.

Consider how a simple model may be constructed and how the parameters of interest may be determined experimentally.[†]

The simplest model considers only two cell types: plasmid free (n_-) and plasmid containing (n_+), where n_- and n_+ are the number of concentrations of plasmid-free and plasmid-containing cells, respectively. The model assumes that all plasmid-containing cells are identical in growth rate and in the probability of plasmid loss.

[†] This analysis is adapted from the paper of N. S. Cooper, M. E. Brown, and C. A. Cauleott, *Journal of General Microbiology* 133: 1871, 1987.

This assumption is the same as assuming that all cells have exactly the same copy number. As we showed in Example 14.1, the actual distribution of copy numbers can make a significant difference on plasmid loss. Also, plasmid-encoded protein production is not a linear function of copy number, so assuming that all cells have the same copy number may lead to incorrect estimates of the growth rate of plasmid-bearing cells. The assumption of a single type of plasmid-bearing cell is a weak assumption, but other assumptions result in a level of complexity inconsistent with this book's purpose. However, models that recognize the segregated nature of the plasmid population are available in the research literature.

Let us further restrict our initial considerations to a single-stage chemostat:

$$dn_+/dt = \mu_+ n_+ - Dn_+ - Rn_+ \tag{14.7}$$

$$dn_-/dt = \mu_- n_- - Dn_- + Rn_+ \tag{14.8}$$

$$dS/dt = [S_0 - S]\,D - \frac{\mu_+ n_+}{Ys_+} - \frac{\mu_- n_-}{Ys_-} \tag{14.9}$$

where R is the rate of generation of plasmid-free cells from plasmid-containing cells and Ys_+ and Ys_- are cell-number yield coefficients (i.e., the number of cells formed per unit mass of limiting nutrient consumed). R can be represented by

$$R = P\mu_+ \tag{14.10}$$

where P = probability of forming a plasmid-free cell. P can be estimated by equation 14.1 if the copy number is known or can be predicted with a more sophisticated structured–segregated model. A value for P could be estimated from an experimentally determined copy-number distribution as in Example 14.1(c), which would be more realistic than assuming a monocopy number. As we will soon see, R can be determined experimentally without a knowledge of copy number.

Equations 14.7 through 14.9 assume only simple competition between plasmid-containing and plasmid-free cells. No selective agents are present, and the production of complementing factors from the plasmid is neglected. Following is the simplest assumption for cellular kinetics:

$$\mu_+ = \mu_{+\max} \frac{S}{K_{S_+} + S} \tag{14.11a}$$

$$\mu_- = \mu_{-\max} \frac{S}{K_{S_-} + S} \tag{14.11b}$$

The situation can be further simplified if we assume that after a few generations in a chemostat with constant operating conditions the total number of cells (N^t) is constant. This approximation will be acceptable in many cases as long as the metabolic burden imposed by plasmid-encoded functions is not too drastic and D is less than

80% of either μ_{+max} or μ_{-max}. For allowable dilution rates, these assumptions allow us to decouple the substrate balance (equation 14.9) from immediate consideration. Then, addition of equations 14.7 and 14.8 yields

$$dn_+/dt + dn_-/dt = \mu_+ n_+ + \mu_- n_- - D(n_+ + n_-) \tag{14.12}$$

N^t is constant, and

$$N^t = n_+ + n_- \tag{14.13}$$

Therefore, at quasi-steady state, equation 14.12 becomes

$$0 = \mu_+ n_+ + \mu_- n_- - D(N^t) \tag{14.14a}$$

or

$$0 = \mu_+ f_+ + \mu_- f_- - D \tag{14.14b}$$

where f_+ is the fraction of the total population that is plasmid-containing cells and f_- is the fraction of plasmid-free cells. Since $f_+ + f_- = 1$,

$$D = \mu_+ f_+ + \mu_-(1 - f_+) \tag{14.15}$$

Substituting equation 14.15 into equation 14.7 after equation 14.7 is divided by N^t yields

$$df_+/dt = \mu_+ f_+ - f_+[(\mu_+ f_+) + \mu_-(1 - f_+)] - Rf_+ \tag{14.16}$$

And, after rearrangement,

$$df_+/dt = f_+^2 \Delta\mu - f_+(\Delta\mu + R) \tag{14.17}$$

$$\Delta\mu = \mu_- - \mu_+ \tag{14.18}$$

Equation 14.17 is a Bernoulli's equation. It can be solved in terms of a dummy variable $v = 1/f_+$, assuming a constant $\Delta\mu$ and R. A constant $\Delta\mu$ and R would be achieved only if the copy number distribution of plasmid-containing cells remained constant during the experiment. Also, strictly speaking, the assumption of constant $\Delta\mu$ is inconsistent with constant N^t. This analysis is best applied to situations where $\Delta\mu$ is not extremely large. The solution is

$$\frac{1}{f_+} = \frac{1}{1 - f_-} = \frac{\Delta\mu}{\Delta\mu + R} = ce^{(\Delta\mu + R)t} \tag{14.19}$$

where c is the constant of integration. The initial condition is $f_- = f_{-0}$ at $t = 0$. Then c must be

$$c = \left(\left[\frac{1}{1 - f_{-0}} \right] - \frac{\Delta\mu}{\Delta\mu + R} \right) \tag{14.20}$$

Once c is evaluated, equation 14.19 can be rearranged to yield

$$f_{-} = \frac{(f_{-0}\Delta\mu + R)e^{(\Delta\mu + R)t} - R(1 - f_{-0})}{(f_{-0}\Delta\mu + R)e^{(\Delta\mu + R)t} + \Delta\mu(1 - f_{-0})} \tag{14.21}$$

Since f_{-0} is usually $\ll 1$, equation 14.21 becomes

$$f_{-} = \frac{(f_{-0}\Delta\mu + R)e^{(\Delta\mu + R)t} - R}{(f_{-0}\Delta\mu + R)e^{(\Delta\mu + R)t} + \Delta\mu} \tag{14.22}$$

Further simplification is possible by considering three limiting cases:

Case 1: $\Delta\mu \gg R$ (growth-rate-dependent instability dominant)
Case 2: $\Delta\mu \leq R$ (segregational instability dominant)
Case 3: $\Delta\mu < 0$ and $|\Delta\mu| \gg R$ (effective selective pressure)

These limiting cases have the following yields:

Case 1: Where t is sufficiently small so that $\Delta\mu \gg (f_{-0}\Delta\mu + R)e^{(\Delta\mu t)}$:

$$f_{-} \approx (f_{-0} + R/\Delta\mu)e^{\Delta\mu t} \tag{14.23}$$

Case 2: Where $f_{-0}\,\Delta\mu$ will be $\ll R$ and using a binomial expansion:

$$f_{-} \approx 1 - (1 + \Delta\mu / R)e^{-(\Delta\mu + R)t} + \Delta\mu / R(1 + \Delta\mu / R)e^{-2(\Delta\mu + R)t} \tag{14.24}$$

Case 3: The denominator is $\approx\Delta\mu$, since $|\Delta\mu| \gg R$ and $f_{-0} \ll 1$, and we denote $\Delta\mu = -|\Delta\mu|$:

$$f_{-} \approx \frac{R}{|\Delta\mu|} + \left(f_{-0} - \frac{R}{|\Delta\mu|} \right) e^{(R - |\Delta\mu|)t} \tag{14.25}$$

For each of these cases, a straight-line portion of the data will allow estimates of $\Delta\mu$ and R if the basic assumptions for each case are met.

Figure 14.6 shows the behavior that would be expected for each case in a chemostat. The appropriate plots to estimate the parameters $\Delta\mu$ and R are also given.

In case 1 for $\Delta\mu t > 1$, we have the following:

$$\ln f_{-} = \ln(f_{-0} + R / \Delta\mu) + \Delta\mu t \tag{14.26}$$

Thus, the slope, m, gives $\Delta\mu$, and R is given by the intercept:

$$R = m(f_{-i} - f_{-0}) = \Delta\mu(f_{-i} - f_{-0}) \tag{14.27}$$

where f_{-i} is the value of f_{-} at the intercept.

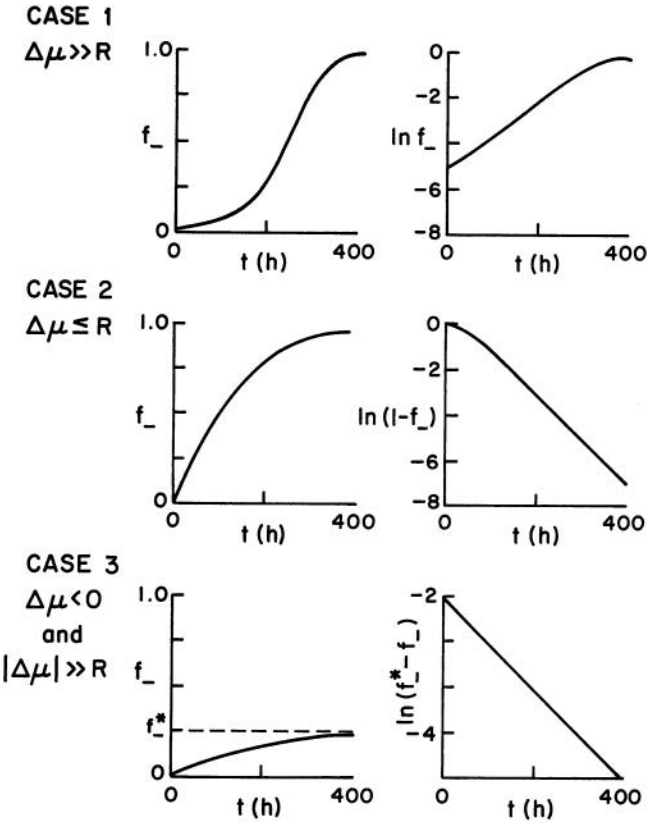


Figure 14.6. The shape of a plot of f_{-} versus t is diagnostic for limiting cases of equation 14.21. For each case, a plot with a substantial linear region can be constructed and used to evaluate $\Delta\mu$ and R.

Equation 14.27 has interesting implications in helping to quantify the conditions for which equation 14.26 is valid. That is, t must be sufficiently small when $\Delta\mu \gg R$:

$$\Delta\mu >> (f_{-0}\Delta\mu + R)e^{\Delta\mu t}$$

Thus,

$$\Delta\mu >> (f_{-0}\Delta\mu + \Delta\mu f_{-i} - \Delta\mu f_{-0})e^{\Delta\mu t} \tag{14.28}$$

or

$$1 >> f_{-i}\, e^{\Delta\mu\, t} \tag{14.29}$$

or

$$0 >> \ln f_{-i} + \Delta\mu t \tag{14.30}$$

or

$$t << \frac{-\ln f_{-i}}{\Delta\mu} \tag{14.31}$$

The analysis also assumes that $\Delta\mu t > 1$; thus, the linear region suitable for use in case 1 is

$$\frac{1}{m} < t < \frac{-\ln f_{-i}}{m} \tag{14.32}$$

In case 2, for $t > 1/(\Delta\mu + R)$,

$$f_- \approx 1 - (1 + \Delta\mu / R)e^{-(\Delta\mu + R)t} \tag{14.33a}$$

$$\ln f_+ = \ln(1 - f_-) = \ln(1 + \Delta\mu / R) - (\Delta\mu + R)t \tag{14.33b}$$

The intercept is used to evaluate f_{+i} and the slope $m = -(\Delta\mu + R)$:

$$\Delta\mu = m(f_{+i} - 1) = mf_{-i} \tag{14.34}$$

$$R = -mf_{+i} = -m(1 - f_{-i}) \tag{14.35}$$

In case 3, we note that f_- assumes a constant value between 0 and 1, which we denote as f_-^*. Equation 14.25 yields f_-^* by allowing $t \to \infty$:

$$f_-^* \approx R/\Delta\mu \tag{14.36}$$

A plot of ln $(f_-^* - f_-)$ versus time will give a slope, m:

$$m = R - |\Delta\mu| \tag{14.37}$$

Equations 14.36 and 14.37 can be combined to give

$$\Delta\mu = \frac{m}{f_-^* - 1} \tag{14.38}$$

$$R = \frac{-mf_-^*}{1 - f^*} \tag{14.39}$$

This analysis provides a method to estimate experimentally the parameters of importance in predicting genetic instability. Once these parameters are known, equation 14.21 can be used to predict chemostat performance. However, recall the large number of assumptions that went into these expressions. Case 1 is the one relevant to most commercial systems, where high expression levels are coupled with high-copy-number plasmids. Case 2 will occur only for low-copy-number plasmids that do not have a *par* locus or other stabilizing features. The results for case 3 must be applied very cautiously. For μ_- to be less than μ_+, selective pressure usually must be applied (e.g., antibiotic resistance or auxotrophic hosts). Equations 14.7 and 14.8 do not recognize the possibility of leakage of nutrients or enzymes, which would degrade the antibiotics. If there were no leakage and the antibiotic-to-cell ratio were high enough to leave an effective

residual level of antibiotic, then the analysis for case 3 could be applied if μ_- were interpreted correctly. For a newly born plasmid-free cell, there will be a carryover of the complementing factor, which will be gradually reduced by turnover and dilution (recall Figure 14.5). Thus, in the first generation after plasmid loss, μ_- may even be greater than μ_+, but μ_- will decrease from generation to generation until it becomes zero. The analysis from case 3 will give an effective average value of μ_-.

Removal of many of these assumptions to yield a more realistic analysis increases complexity and makes the development of simple analytical expressions difficult. Let us consider how this simple analysis may be used and how it can be extended.

Example 14.2

The data in Table 14.4 were obtained for *E. coli* B/r-pDW17 at two different dilution rates in glucose-limited chemostats. The average plasmid copy number for pDW17 is about 40 to 50 copies per cell. About 12% of the total protein synthesized is due to the plasmid. The proteins are retained intracellularly in soluble form. Use these data to estimate $\Delta\mu$, R, and P.

Solution

Note that data on plasmid stability are usually given in terms of the number of generations, which is usually calculated as $(\ln 2)/D$ = 1 generation. Clearly, the trend of the data in Table 14.4 is a sigmoidal dependence of f_- on time, which corresponds to growth-rate-dependent instability or a case 1 situation (see Figure 14.6). Thus, we need to plot the data as $\ln f_-$ versus time. Such a plot is given as Figure 14.7. Recall that equation 14.32, which gives the bounds on t, suggests that only data at intermediate times can be used to estimate $\Delta\mu$ and R. In this case, data for $0.01 < f_- < 0.40$ were used as a basis for the initial estimates of values of $\Delta\mu$ and R. Equation 14.32 can be used later to check the appropriate data range.

For $D = 0.3\ h^{-1}$, the slope, m, is $0.15\ gen^{-1}$ or $0.066\ h^{-1}$, and the intercept is -5.3, implying $f_{-1} = 0.005$. For $D = 0.67\ h^{-1}$, $m = 0.12\ gen^{-1}$ or $0.11\ h^{-1}$, an intercept of -5.6 gives $f_{-1} = 0.0037$. Recall that, for case 1, $m = \Delta\mu$ and $R = m(f_{-1} - f_{-0})$. The value of f_{-0} presents a problem, as an accurate value is difficult to determine experimentally when it is low (many colonies must be plated to obtain any statistical accuracy). R will be maximal if f_{-0} is assumed to be zero. If a culture is developed under strong selective pressure,

TABLE 14.4. Experimental Values for Plasmid Stability in a Glucose-Limited Chemostat

$D = 0.30\ h^{-1}$			$D = 0.67\ h^{-1}$		
Time, h	No. of Generations	f_-	Time, h	No. of Generations	f_-
0	0	≤0.002	0	0	≤0.002
11.6	5	0.010	10.3	10	0.010
23.1	10	0.030	20.7	20	0.035
46.2	20	0.13	31.0	30	0.15
57.8	25	0.22	41.4	40	0.34
69.3	30	0.39	51.7	50	0.65
80.9	35	0.76	62.1	60	0.81
92.4	40	0.88	72.4	70	0.93
104.0	45	0.97	82.8	80	0.98
115.5	50	1.00	93.1	90	1.0
138.6	60	1.00	103.5	100	1.0

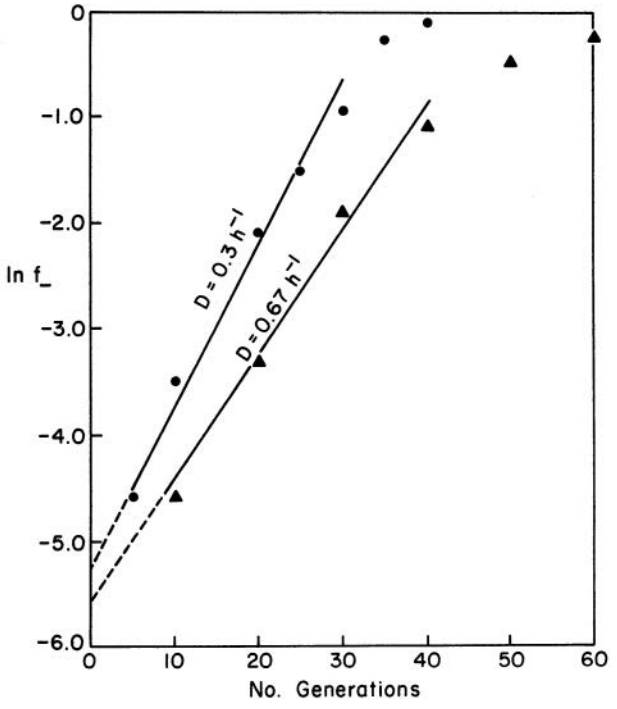


Figure 14.7. Determination of $\Delta\mu$ and R using data from Example 14.2.

with plasmid-encoded protein synthesis uninduced before the beginning of the experiment, then f_{-0} will approach zero.

Thus, we get the following data:

$m = \Delta\mu$	$D = 0.3\ h^{-1}$ 0.15 gen^{-1}	$D = 0.67\ h^{-1}$ 0.12 gen^{-1}
R max $(f_{-0} = 0)$	$7.5\times10^{-4}\ gen^{-1}$	$4.4\times10^{-4}\ gen^{-1}$
R *if* $f_{-0} = 0.002$	$4.5\times10^{-4}\ gen^{-1}$	$2.0\times10^{-4}\ gen^{-1}$
R *if* $f_{-0} = 0.003$	$3.0\times10^{-4}\ gen^{-1}$	$8.4\times10^{-5}\ gen^{-1}$
R *if* $f_{-0} = 0.0037$	$2.0\times10^{-4}\ gen^{-1}$	$0\ gen^{-1}$

To determine P, recall that $R = P\,\mu_+$ (equation 14.10). A value for μ_+ can be estimated from equation14.14b after some rearrangement to give the following:

$$D = \mu_+ + \Delta\mu f_- \tag{14.40}$$

Values of μ_+ and μ_- vary slightly during culture as a consequence of the assumption that $dN^t / dt = 0$.† Following is an average value of μ_+:

$$\mu_+ = D - \Delta\mu / 2 \tag{14.41}$$

† Physically, these changes in μ_+ and μ_- could occur due to small changes in residual substrate concentration. This observation implies that the assumption of constant $\Delta\mu$ and R used in integrating equation 14.17 would fail if $\Delta\mu$ were very large.

R, in units of gen^{-1}, is changed to h^{-1} by multiplying by $D/\ln 2$. Thus, we get the following data:

D	μ_+	R_{max}	P_{max}
$0.3\ h^{-1}$	$0.267\ h^{-1}$	$7.5\times10^{-4}\ gen^{-1}$ *or* $3.2\times10^{-4}\ h^{-1}$	1.2×10^{-3}
$0.67\ h^{-1}$	$0.615\ h^{-1}$	$4.4\times10^{-4}\ gen^{-1}$ *or* $4.2\times10^{-4}\ h^{-1}$	0.7×10^{-3}

A change in the probability of segregational losses as a function of dilution rate corresponds to the copy number, which also changes with dilution rate. The pDW17 plasmid has an *average* copy number of about 40 to 50 in exponential growth in minimal medium.

Consider equation 14.1, repeated here, which predicts the probability of plasmid loss by random segregation:

$$P = 2^{(1-z)} \tag{14.1}$$

where $Z = 40$ and $P = 1.8\times10^{-12}$, which is certainly less than the maximum value calculated in these experiments. Note from Example 14.1, however, that if the average copy number were distributed so that half the cells had 10 plasmids and half had 70, then the probability of plasmid loss would be 9.8×10^{-4}, which is very close to the maximum probabilities allowed by these experimental data.

Example 14.3

Derive equations to describe the dynamics of a plasmid-containing population when the plasmid-free host is auxotrophic for metabolite *M*. The metabolite is made and released from a plasmid-containing cell into the medium in a chemostat.

Solution

This situation is similar to that in equations 14.7, 14.8, and 14.9 (repeated here), except for the production and release of *M*.

$$dn_+/dt = \mu_+ n_+ - Dn_+ - Rn_+ \tag{14.7}$$

$$dn_-/dt = Rn_+ + \mu_- n_- - Dn_- \tag{14.8}$$

$$dS/dt = D(S_0 - S) - \frac{\mu_+ n_+}{Ys_+} - \frac{\mu_- n_-}{Ys_-} \tag{14.9}$$

The only modification is to add a mass balance for M and to alter the equations for μ_-. Let M be the concentration of M:

$$\frac{dM}{dt} = \delta\mu_+ n_+ - \frac{\mu_- n_-}{Y_{M_-}} - DM \tag{14.42}$$

where

$$\mu_+ = \mu_{+\max}\frac{S}{K_{s_+} + S} \tag{14.11a}$$

and

$$\mu_{-} = \mu_{-\max} \frac{S}{K_{S_{-}} + S} \frac{M}{K_{M_{-}} + M} \tag{14.43}$$

where δ is the stoichiometric coefficient relating the production of M to the growth of n_{+}.

This set of equations would have to be solved numerically. However, the type of analysis used in Example 14.2 could be applied if M were supplied in the medium such that the residual level of $M >> K_{M_{-}}$.

Our discussion and examples so far have been for continuous reactors. For most industrial applications, batch or fed-batch operations will be used. While continuous reactors are particularly sensitive to genetic instability, genetic instability can be a significant limitation for batch systems.

For a batch reactor, the cell-number balances are

$$dn_{+}/dt = \mu_{+} n_{+} - P\mu_{+} n_{+} \tag{14.44}$$

$$dn_{-}/dt = P\mu_{+} n_{+} + \mu_{-} n_{-} \tag{14.45}$$

where

$$@\, t = 0, \quad n_{+0} = N_{+0}, \quad n_{-0} = 0$$

Solving equation 14.44 with these initial conditions results in equation 14.46:

$$n_{+} = N_{+0} e^{(1-P)\mu_{+} t} \tag{14.46}$$

And equation 14.45, using equation 14.46, yields

$$n_{-} = \frac{P\mu_{+} N_{+0}}{(1-P)\mu_{+} - \mu_{-}} [e^{(1-P)\mu_{+} t} - e^{\mu - t}] \tag{14.47}$$

Since $1 - f_{-} = f_{+} = \dfrac{n_{+}}{n_{+} + n_{-}}$,

$$f_{+} = \frac{e^{(1-P)\mu_{+} t}}{e^{(1-P)\mu_{+} t} + \dfrac{P\mu_{+}}{(1-P)\mu_{+} - \mu_{-}} [e^{(1-P)\mu_{+} t} - e^{\mu_{-} t}]} \tag{14.48}$$

Equation 14.48 is usually recast in terms of number of generations of plasmid-containing cells (n_g), a dimensionless growth-rate ratio (α), and the probability of forming a plasmid-free cell upon division of a plasmid-containing cell (P). The mathematical definitions of n_g and α are

$$n_g = \frac{\mu_{+} t}{\ln 2} \tag{14.49}$$

$$\alpha = \frac{\mu_-}{\mu_+} \tag{14.50}$$

With these definitions, equation 14.48 becomes

$$f_+ = \frac{1-\alpha-P}{1-\alpha-P\cdot 2^{n_g(\alpha+P-1)}} \tag{14.51}$$

The use of this approach is illustrated in the following example.

Example 14.4

Estimate the fraction of plasmid-containing cells in a batch culture under the following circumstances. Cells are maintained at constant, maximal growth rate of 0.693 h^{-1} during scale-up from shake flask through seed fermenters into production fermenters. The total time for this process is 25 h. Assume that the inoculum for the shake flask was 100% plasmid-containing cells. It is known that the growth rate for a plasmid-free cell is 0.97 h^{-1}. The value of P is 0.001.

Solution

First, we calculate α and n_g:

$$\alpha = 0.97/0.693 = 1.4 \tag{14.50}$$

$$n_g = \frac{0.693\ h^{-1}\cdot 25\ h}{\ln 2} = 25\ \text{gen.} \tag{14.49}$$

Substituting into equation 14.51, we get

$$f_+ = \frac{1-1.4-0.001}{1-1.4-0.0012^{25(1.4+.001-1)}} \tag{14.51}$$

$$f_+ = 0.27$$

Consequently, 73% of the cells are plasmid-free, which would result in considerable loss of productivity.

14.6. REGULATORY CONSTRAINTS ON GENETIC PROCESSES

When genetic engineering was first introduced, there was a great deal of concern over whether the release of genetically modified cells could have undesirable ecological consequences. Reports in the popular press led to fears of "genetic monsters" growing in our sewers, on our farmlands, or elsewhere. Consequently, the use of genetic engineering technology is strictly regulated.

The degree of regulatory constraint varies with the nature of the host, vector, and target protein. For example, consider a scenario where serious harm might arise. The gene for a highly toxic protein is cloned into *E. coli* to obtain enough protein to study that protein's biochemistry. Assume that a plasmid that is

promiscuous (i.e., the plasmid will shuttle across species lines) is used. Also assume that laboratory hygiene is not adequate and a small flying insect enters the laboratory and comes into contact with a colony on a plate awaiting destruction. If that insect leaves the laboratory and returns to its natural habitat, then the target gene is accidentally released into the environment. Laboratory strains of *E. coli* are fragile and usually will not survive long in a natural environment. However, a very small probability exists that the plasmid could cross over species lines and become incorporated into a more hardy soil bacterium (e.g., *Pseudomonas* sp.). The plasmid would most certainly contain antibiotic-resistance factors as well. The newly transformed soil bacterium could replicate. Many soil bacteria are opportunistic pathogens. If they enter the body through a wound, they can multiply and cause an infection. If, in addition, the bacterium makes a toxic protein, the person or animal that was infected could die from the toxic protein before the infection was controlled. If the plasmid also confers antibiotic resistance, the infection would not respond to treatment by the corresponding antibiotic, further complicating control of the spread of the gene for the toxin.

This scenario requires that several highly improbable events occur. No case of significant harm to humans or the environment due to the release of genetically modified cells has been documented. However, the potential for harm is real.

Regulations controlling genetic engineering concentrate on preventing the accidental release of genetically engineered organisms. The deliberate release of genetically engineered cells is possible, but an elaborate procedure must be followed to obtain permission for such experiments.

The degree of containment required depends on the ability for the host to survive if released, the ability for the vector to cross species lines or for a cell to be transformed by a piece of naked DNA and then have it incorporated into the chromosome via recombination, and the nature of the genes and gene products being engineered. The National Institutes of Health (NIH) have issued guidelines that regulate the use of recombinant DNA technology. Special regulations apply to large-scale systems (defined as >10 l). There are three levels of containment: BL1-LS, BL2-LS, and BL3-LS. BL1-LS (biosafety level 1, large scale) is the least stringent. Experiments involving overproduction of *E. coli* proteins in *E. coli* using plasmids derived from wild populations are readily approved and do not require elaborate containment procedures (see Table 14.5). Experiments that would move the capacity to produce a toxin from a higher organism into bacteria or yeast would be subjected to a much more thorough evaluation, and more elaborate control facilities and procedures would be required.

Table 14.6 compares the requirements for the three containment levels.

In all cases, no viable organisms can be purposely released. Gas vented from the fermenter must be filtered and sterilized. All cells in the liquid effluent must be killed. The latter can present some operating issues, since the inactivation of the host cell must be done in such a way as not to harm what are often fragile products. Emergency plans and devices must be on hand to handle any accidental spill or loss of fluid in the fermentation area. These extra precautions increase manufacturing costs.

The issue of the regulation of cells and recombinant DNA will undoubtedly undergo further refinement with time. Both laboratory and manufacturing personnel need to keep abreast of any such changes.

TABLE 14.5. Minimum Laboratory Containment Standards for Cells with Recombinant DNA

Biosafety Level 1 (BL1)

Standard Microbiological Practices

- Access to the laboratory is limited or restricted at the discretion of the laboratory director when experiments are in progress.
- Work surfaces are decontaminated once a day and after any spill of viable material.
- All contaminated liquid or solid wastes are decontaminated before disposal.
- Mechanical pipetting devices are used; mouth pipetting is prohibited.
- Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Food may be stored in cabinets or refrigerators designated and used for this purpose only.
- Persons wash their hands after they handle materials involving organisms containing recombinant DNA molecules, and animals, and before leaving the laboratory.
- All procedures are performed carefully to minimize the creation of aerosols.
- It is recommended that laboratory coats, gowns, or uniforms be worn to prevent contamination or soiling of street clothes.

Special Practices

- Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable, leakproof container that is closed before being removed from the laboratory.
- An insect and rodent control program is in effect.

Containment Equipment

- Special containment equipment is generally not required for manipulations of agents assigned to Biosafety Level 1.

Laboratory Facilities

- The laboratory is designed so that it can be easily cleaned.
- Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.
- Laboratory furniture is sturdy. Spaces between benches, cabinets, and equipment are accessible for cleaning.
- Each laboratory contains a sink for hand-washing.
- If the laboratory has windows that open, they are fitted with fly screens.

TABLE 14.6. Physical Containment Requirements for Large-Scale Fermentations Using Organisms Containing Recombinant DNA Molecules

Description	BL1-LS	BL2-LS	BL3-LS
Closed vessel	×	×	×
Inactivation of cultures by validated procedure before removing from the closed system	×	×	×
Sample collection and addition of material in a closed system	×	×	×
Exhaust gases sterilized by filters before leaving the closed system	×	×	×
Sterilization by validated procedures before opening for maintenance or other purposes	×	×	×
Emergency plans and procedures for handling large losses	×	×	×
No leakage of viable organisms from rotating seals and other mechanical devices		×	×
Integrity evaluation procedure: monitors and sensors		×	×
Containment evaluation with the host organism before introduction of viable organisms		×	×
Permanent identification of closed system (fermenter) and identification to be used in all records		×	×

(*Continued*)

TABLE 14.6. Physical Containment Requirements for Large-Scale Fermentations Using Organisms Containing Recombinant DNA Molecules (*Continued*)

Description	BL1-LS	BL2-LS	BL3-LS
Posting of universal biohazard sign on each closed system and containment equipment when working with a viable organism		×	×
Posting of universal biohazard sign on entry doors when work is in progress			×
Operations to be in a controlled area:			×
Separate specified entry			×
Double doors, air locks			×
Walls, ceiling, and floors to permit ready cleaning and decontamination			×
Utilities and services (process piping and wiring) to be protected against contamination			×
Handwashing facilities and shower facilities in close proximity			×
Area designed to preclude release of culture fluids outside the controlled area in the event of an accident			×
Ventilation: movement of air, filtration of air			×

R. J. Giorgio and James J. Wu, Batch, fed-batch, and microcarrier cultures with CHO cell lines in a pressure-cycle driven miniaturized bioreactor. *Trends in Biotechnology* 4(8):198 (1986). With permission from John Wiley & Sons, Inc.

14.7. METABOLIC ENGINEERING

Metabolic engineering involves controlling and redirecting metabolic pathways to improve product formation rates and yields (and to suppress formation of undesired products) by using metabolic, enzymatic, and/or genetic manipulations of a host organism. Often, the tools of genetic engineering are used to generate a totally new metabolic pathway (series of enzymes), amplify an existing pathway, disable an undesired pathway, or alter the regulation of a pathway. The principle motivations for metabolic engineering are the production of specialty chemicals (e.g., secondary metabolites, amino acids, biofuels, flavorings, fragrances, polymer precursors), utilization of alternative substrates (e.g., pentose sugars from hemicellulose), or bioremediation of hazardous wastes (e.g., benzoates or trichloroethylene; see Figure 14.8 for an example). Metabolic production of chemicals is desired over chemical synthesis routes due to the high specificity of biological catalysts, mainly enzymes, to produce desired enantiomers as well as to their ability to catalyze reactions for which a chemical synthesis route is unknown or too lengthy. Additionally, metabolic synthesis can occur at more labile reaction conditions than other chemical routes and is a sustainable way of producing chemicals. Three values that are often optimized for metabolic production are final titer (g/l), yield (g/g), and specific productivity rate (g/l hr).

One of the first choices a metabolic engineer must make is whether to produce a desired compound in a native host that naturally expresses the metabolic pathway or to port the pathway into a chassis organism for heterologous expression. Due to recent advances in *synthetic biology* and *systems biology*, engineering nonmodel organisms has become easier and more widespread; however, there are still many advantages to heterologous hosts. Following are some guidelines for using each.

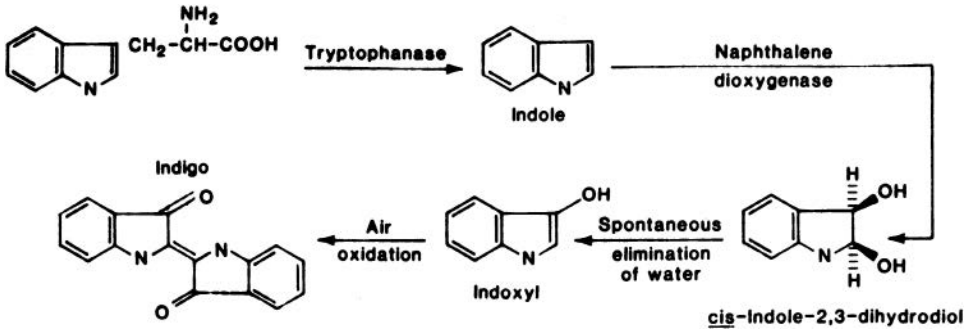


Figure 14.8. Proposed pathway for indigo biosynthesis in a recombinant strain of *E. coli.* Indole is formed from tryptophan by tryptophanase, a natural enzyme in *E. coli.* Naphthalene dioxygenase formed by the expression of the cloned *Pseudomonas* DNA oxidizes indole to indigo; *cis*-2,3-dihydroxy-2,3-dihydroindole and indoxyl have not been isolated. Their inclusion is based on the known activities of aromatic hydrocarbon dioxygenases and established mechanisms for the chemical synthesis of indigo. (With permission, from B. D. Ensley et al., Expression of naphthalene oxidation genes in Escherichia coli results in the biosynthesis of indigo, *Science* 222:167, 1983, and American Association for the Advancement of Science.)

Heterologous hosts:

- The desired metabolic pathway can be put under the control of a regulated promoter. The investigator can turn on the pathway in situations where the pathway might normally be suppressed (e.g., degradation of a hazardous compound to a concentration lower than necessary to induce the pathway in the natural isolate) as well as at desired times to not interfere with cell growth (e.g., late exponential phase to ensure enough biomass is built up and to not have the pathway interfere with cell growth). Often, the environmental signals that cause expression of pathways in native hosts are poorly understood.
- High levels of enzymes in desired pathways can be obtained with strong promoters; only low activity levels may be present in the natural isolate.
- Genetic manipulation is easier in well-understood heterologous hosts (e.g., deleting competing pathways, addition of genes to the chromosome, or production of enzymes from plasmids). Additionally, most chassis organisms are fully sequenced permitting the generation of metabolic models and prediction of knockouts.
- Transformation protocols are well established, permitting the addition of many pathway variants to the organism to select optimal performers. "Omics" (transcriptomics, proteomics, and metabolomics) techniques and data are established.
- Several pathways can be combined in a single organism by selecting enzymes from more than one organism.
- A product is toxic if it is produced in high quantities in the native host. Similarly, feedback pathway regulation may be poorly understood in the native organism.

- Pathways moved from lower eucaryotes to bacteria can be controlled by a single promoter; in lower eucaryotes, each protein has a separate promoter.
- Model organisms typically grow quickly using inexpensive media. Bioreactors are available for scale-up.
- There are safety concerns for culturing large amounts of pathogenic native hosts.
- The genetically engineered cell can be proprietary property.

Native host:

- Any of the enzymes required for the biological conversion are unknown, eliminating the ability to transfer specific genes to a heterologous host.
- The desired product is toxic to heterologous host.
- The native host has unique growth properties (e.g., utilization of unique carbon sources, necessity of specific substrate uptake to form product of interest, growth in high-temperature environments). Additionally, many organisms have noncanonical pathways, including novel reduction pathways to make them advantageous to produce specific compounds.
- The host has the ability to grow in a microbial consortia to produce the desired compound.

Equally important to the selection of host is the identification of pathway enzymes. Advances in genomic sequencing and annotation have rapidly allowed the construction of pathways never before found in nature. Increased DNA synthesis capability has permitted the use of genes from organisms that cannot be easily cultured or are pathogenic. Inexpensive gene synthesis has also enabled researchers to test many homologous enzymes for a single pathway step to find ones with optimal conversion, specificity, and solubility. Detailed metabolic maps are available through the Kyoto Encyclopedia of Genes and Genomes (KEGG), allowing for easy determination of the presence of pathways to make precursor compounds as well as competing pathways. DNA sequencing and synthesis combined with omics data has been used to elucidate novel enzyme functions for previously nonannotated enzymes, permitting their use in heterologous hosts. While addition of new reactions may be accomplished by the addition of plasmids encoding enzymes of interest, the modification or elimination of pathways in a cell requires editing of its genomic DNA. Currently, genome editing can be accomplished in many bacteria using phage-induced homologous recombination. In eucaryotes, natural homologous recombination can be induced by targeted creation of double-stranded breaks in the chromosomal DNA. This targeting can be accomplished by fusions of nucleases to DNA-binding proteins such as zinc fingers and transcription activator-like effectors (TALEs). More recently, CRISPR-Cas systems, which comprise RNA-guided nucleases, have shown remarkable versatility in targeting genomes of bacteria, yeast, plant, insect, zebrafish, and even human cell lines.

Engineering pathways require good quantitative information on the flow of metabolites in a cell. Structured models of cells can be used in conjunction with

experiments to optimize the design of new pathways. Metabolic engineering has been a fertile area for engineering contributions.

Since the first mention of metabolic engineering in the early 1990s, the field has greatly progressed due to advancements in genomic sequencing, enzyme characterization and pathway implementation. Metabolic engineers have been able to create a vast array of chemicals from those to be used as second-generation biofuels to secondary metabolites such as terpenoids, polyketides and nonribosomal peptides. One of the largest success stories of metabolic products, due to the scale of its production, is 1,3-propanediol. Biofuels such as isobutanol and *n*-butanol can now be made at large scales and high concentrations (>50 g/l for isobutanol). Engineered microbes also contribute a key step to industrial vitamin C production, which is over 110 kilotons annually. Additionally, high-value therapeutic precursors are being created, such as artemisinic acid that can be converted into antimalarial drugs and opioids such as hydrocodone. Biosynthetic routes for polymeric precursors such as glucaric acid and adipic acid have been developed. High-value fragrances and flavors such as acetoin, vanillin, and methyl ketones are possible through modification to central carbon metabolism, amino acid synthesis, and fatty acid metabolism.

14.8. SYNTHETIC AND SYSTEMS BIOLOGY

Successful implementation of genetically engineered cells for an industrial process requires exquisite control of the complex cellular environment. The discipline of *synthetic biology* arose out of the application of control principles from electrical engineering to biological systems. A primary doctrine of synthetic biology is that biological systems are composites of "parts" that can be replaced or modified to accomplish specific goals. Examples of these parts include promoters, ribosome binding sites (RBSs), terminators, and plasmid origins of replication, as well as genes of interest. For example, expression of a gene of interest might be increased by replacement of a weak promoter with a promoter of high or intermediate strength.

Another thrust in synthetic biology has been the creation of cellular circuits, whereby engineered cells can sense changes in the environment (input) and respond (e.g., by production of a reporter protein) using Boolean logic (output). Inducible promoters factor heavily into the creation of cellular circuits and repressor–promoter pairs have been assembled into multi-input NOT, AND, XOR, and other logic gates. Additionally, repressors can be layered to create feedback loops that lead to oscillatory or toggle switchlike behavior. Finally, multilayered kill-switch circuits have been developed to prevent the growth of engineered microbes outside of a designated environment.

Many efforts in synthetic biology research are focused on standardization—that is, characterization of the performance of individual parts under different conditions to reliably predict outcomes when parts are combined. These efforts seek to make construction of large, complex assemblies of parts easy for researchers to construct and implement.

Computational modeling efforts to understand the complexity of biological/metabolic/synthetic networks fall into the discipline of *systems biology*. Due to the highly nonlinear, dynamic nature of a cell and its metabolism, uninformed changes intended to improve production of a specific compound often fail to give the desired result.

The challenge of metabolic engineering and synthetic biology is to express appropriate genes in the right amount. Too much of a key enzyme may result in little change, if it is not a rate-influencing step, as there is insufficient reactant to increase the flow of substrate (or *flux*) into the reaction product. Additionally, overexpression of non-rate-limiting enzymes leads to increased metabolic burden, diverting cellular resources away from product formation to unnecessary protein expression. If it is rate influencing, the increased reaction of the substrate will alter fluxes of precursors in other pathways; sometimes these changes compromise the cell's ability to grow or to provide coreactants when needed. Metabolic engineering and synthetic biology require a delicate balance of activities and are a problem of quantitative optimization rather than simple maximization of expression of a gene. Using various computational techniques, systems biologists model reactions that contribute to particular reaction networks such as cell-signaling or blood clotting. Such networks can comprise hundreds to thousands of reactions, even up to the genome scale in microbes.

Metabolic control theory and the closely allied activity of *metabolic flux analysis* are mathematical tools that can be applied to such problems. These tools are used to predict the flux through a metabolic network, allowing determination of the right amount of enzyme expression and whether competing pathways need to be removed. A flux balance equation consists of the product of a stoichiometric matrix and an intracellular flux vector to yield an overall rate vector. Using whole genome sequencing and knowledge of pathway organization, it is possible to construct a metabolic model and the stoichiometric matrix. Typically, equations in such a model are highly underdetermined due to the fluxes being unknown and require assumptions (e.g., on energy stoichiometry) that may not be justified for a metabolically engineered cell. However, improved experimental techniques to measure the intracellular flux vector (e.g., gas chromatography or liquid chromatography combined with advanced mass spectrometry, nuclear magnetic resonance [NMR], carbon isotope labeling and tracking, high-throughput transcriptomics via RNA-Seq) coupled with genomic/proteomic information may provide the data to allow complete solution of the flux balance equations.

By assumption of steady state, the dot product of the stoichiometric matrix and flux vector is set to zero, creating a linear set of equations to be solved. A numeric solution to the underdetermined fluxes is possible when given a function to be maximized, often cell growth. Heterologous pathway enzymes and reactions can be added to the model to see how fluxes are changed upon their addition. Such models have been useful in predicting pathway knockouts to eliminate enzymes from diverting flux away from desired products as well as determining optimal expression amounts for heterologous enzymes. Additionally, these models provide detailed information about generation of endogenous precursors necessary for product formation and redox factor regeneration, which may be overlooked upon first implementation of the heterologous pathway. One caution is that analysis of pathways in isolation can be misleading. An assumption of such analysis is that the products of the pathway do not influence inputs into that pathway. Given the complexity of a cell, it is difficult to assure that such an assumption is satisfied. Due to improved experimental techniques, it is now possible to measure fluxes for hosts expressing heterologous pathways, eliminating previous concerns of its effect on endogenous metabolism. Increased computational speed, transcriptomics, proteomics, and metabolomics have

enabled a new set of kinetic-based models that do not assume steady state to provide more quantitative prediction of nonlinear metabolism.

Metabolic control theory is based on a sensitivity analysis to calculate the response of a pathway to changes in the individual steps in a pathway. This approach allows calculations of flux control coefficients, which are defined as the fractional change of flux expected for a fractional change in the amount of each enzyme. This process involves a linearization, so that flux coefficients can vary significantly if growth conditions and the cell's physiological step change. An important result of the application of this theory to many cases is that it is rare for a single enzyme step to be rate-controlling. Typically, several enzymatic steps influence rate. Maximization of flux through a particular pathway would require several enzyme activities to be altered simultaneously. The potential of such analysis to contribute to rational design of microbes is clear but not yet routinely applicable.

Quantitative modeling of metabolism with heterologous pathways has shaped the design and implementation of such pathways. Balancing enzyme expression level with flux and metabolic burden is often the first concern. Enzyme production can be varied using different transcriptional promoters, plasmid copy numbers, ribosome binding sites, transcript stability, genomic integration copies, compartmentalization, and posttranscriptional stability to achieve balance. However, these strategies provide static control of enzyme production. Dynamic production strategies make use of biosensors that provide feedback control of pathway expression to permit an even more balanced expression profile. Searching through the immense expression space is not possible for many pathways due to low-throughput titer measurements; modular metabolic engineering groups enzymes to facilitate quicker optimization of expression. Lastly, but importantly, overall pathway reaction must be considered, especially to achieve redox balance. Balancing of metabolism and expression is important to obtaining high titer product but also aids in pathway stability.

14.9. PROTEIN ENGINEERING

Not only can cells be engineered to make high levels of naturally occurring proteins or to introduce new pathways, but we can also make novel proteins. It is possible to make synthetic genes and design proteins in silico to form totally new proteins with folds and chemistries not observed in nature. Through protein crystallography, NMR, and computational modeling, we are beginning to understand the rules by which a protein's primary structure is converted into its three-dimensional form to relate a protein's shape to its functional properties, stability, and catalytic activity.

The goal of protein engineering is to endow a protein with superior characteristics from those observed in nature to facilitate their use in biotechnology. Some properties that are commonly altered include the enhancement of catalytic rate, improvement in stability (thermostability, resistance to proteases, intracellular solubility), modification in binding specificity, alteration of cofactor preference, detoxification of the protein for the desired host compatibility, modification of protein–protein interactions, alteration of posttranslational modification/regulation, and differentiation of protein localization. Protein engineering has typically followed two

paradigms: *rational design* and *directed evolution*. Rational design utilizes knowledge of how protein structure affects functional characteristics (i.e., *structure–function* relationships) in order to make specific changes to the primary sequence of the protein in a manner that alters the function in a desired way. Directed evolution involves introducing mutations randomly to the protein sequence, generating a library of protein variants that is subjected to a screen or selection to identify beneficial mutations.

In general, for rational design, the protein structure must be known from X-ray crystallography and/or NMR. Key amino acids in the structure are selected for alteration based on computer modeling and previously developed structure–function relationships. Residues in and around the active site are often mutated to change interactions of the protein with substrates to enhance catalytic rates and alter specificity. Stability improvement can occur through mutation of surface residues or introducing additional bonding pairs (e.g., noncovalent hydrogen bonds, covalent disulfide bonds). The technique used to generate genes encoding the desired changes in protein structure is called *site-directed mutagenesis*. Using this approach, any desired amino acid can be inserted precisely into the desired position. If a protein structure is not known, often related proteins are crystalized and a homology model can be generated to permit rational design.

It might not be clear why site-directed mutagenesis would be preferred to simple random mutation-selection procedures. One reason may be that mutation followed by selection for particular properties may be difficult when the alterations in protein properties are subtle and confer no advantages or disadvantages on the mutant cell. It may also prove difficult to interrogate all or most of the members in a directed evolution library (often containing 10^6 unique variants or greater), especially if the assay for characterizing the protein variants is low throughput.

A second reason is that site-directed mutagenesis can be used to generate the insertion of an amino acid in a particular location, while a random mutation giving the same result would occur so infrequently as to be unobtainable.

To make this point more evident, consider the degenerate nature of the genetic code. Each codon consists of three letters. The odds for mutation in one of these three letters is about 10^{-8} per generation. The odds that two letters would simultaneously be altered is much lower (order of 10^{-16}). The codon UAC (for tyrosine) could be altered by single-letter substitutions to give AAC (asparagine), GAC (aspartate), CAC (histidine), UCC (serine), UUC (phenylalanine), UGC (cysteine), UAA (stop signal), UAG (stop signal), and UAU (tyrosine). Random mutants in this case are very unlikely to carry substitutions for 13 of the 20 amino acids. Thus, most of the potential insertions can be generated reliably only by using site-directed mutagenesis.

It is often the case that structure–function information is not available, making rational strategies for protein engineering difficult or even impossible to implement. Under these circumstances, an alternative approach is that of *directed evolution*. This process is based on *random mutagenesis* of a gene and the subsequent isolation of proteins with desired properties. Large libraries of mutant genes must be made so that the rare beneficial variants are present. One technique to generate a library of mutants is *error-prone PCR*, where the fidelity of a DNA polymerase is decreased in a manner that permits the user to control the mutation rate and thereby generate genetic diversity. Another technique for generating gene libraries is *DNA shuffling*, whereby homologous or nonhomologous genes are randomly fragmented and

reassembled in vitro to create a library of chimeras. Once a library of mutants is generated, a high-throughput isolation step in the form of a screen or selection must be applied to identify library members that exhibit the desired characteristic(s). Screens analyze the entire protein library by coupling the mutation that leads to a desired protein property with a high-throughput assay such as flow-assisted cell sorting (FACS), cell-surface display/phage display, or colorimetric readouts. Selections impose a hard cutoff for library members by coupling the desired characteristic(s) directly to survival or death of the host cell, similar to antibiotic selection for plasmid-containing cells. Critical to the success of a directed-evolution strategy is the coupling of genotype (DNA sequence of specific variants) to phenotype (output of a screen/selection) to determine the identity of the mutations that gave rise to the desired property change and to permit an iterative approach where the improved variant is used as the template for another directed evolution cycle.

Directed-evolution and rational design are often not performed in a vacuum and are most effective when combined through what is called *semirational design*. Due to incomplete protein function–structure knowledge, often several residues must be altered to find protein variants with the desired properties. By mutating only specific residues, semirational design eliminates the need for a high-throughput screen/selection by focusing the library on variants that have a high likelihood of generating desired functional changes. Saturation mutagenesis (site directed mutagenesis where the codons for all possible amino acids are substituted), and iterative cycles of mutation and characterization are utilized to find beneficial mutations. Additionally, these techniques combined with improved biochemical methods are leading to a better understanding of the complex relationship between protein structure and function by connecting amino acid changes with altered protein characteristics.

14.10. SUMMARY

The application of recombinant DNA technology at the commercial level requires a judicious choice of the proper host–vector system. *E. coli* greatly facilitates sophisticated genetic manipulations, but process or product considerations may suggest alternative hosts. *S. cerevisiae* is easy to culture and is already on the GRAS list, simplifying regulatory approval, although productivities are low with some proteins and hyperglycosylation is a problem. *P. pastoris* can produce very high concentrations of proteins, but the use of methanol presents challenges in reactor control and safety. *Bacillus* and the lower fungi may have well-developed secretion systems that would be attractive if they can be harnessed. Animal cell culture is required when posttranslational modifications are essential. Mammalian cells offer the highest degree of fidelity to the authentic natural product. Insect cell systems potentially offer high expression levels and greater safety than mammalian cells at the cost of a potential decrease in the fidelity of posttranslational processing. Transgenic animals are good alternatives for complex proteins requiring extensive posttranslational processing. Transgenic plants and plant cell cultures are emerging as production systems for high-volume protein products. Plants are well suited to produce proteins for oral or topical delivery. The growing number of available genome sequences combined with new tools for facile genome editing (e.g., gene

knockout, gene knock-in) makes it theoretically possible to improve the utility of virtually any of these hosts (or many others) for a given application.

A host–vector system is useful only if it can persist long enough to make commercially important product quantities. *Genetic instability* is the loss of the genetic information to make the target protein. *Segregational instability* arises when a plasmid-free cell is formed during cell division. *Structured instability* results when the cell loses the capacity to make the target protein in significant quantities due to changes in plasmid structure. *Host-cell-derived instability* occurs when chromosomal mutations decrease effective plasmid-encoded protein production while the cell retains the plasmid. *Growth-rate-dependent instability* is a function of the growth-rate differential between plasmid-free and plasmid-containing cells; it is usually a determining factor in deciding how long a fermentation can be usefully maintained. Simple models to evaluate genetic instability are available.

The vector must be designed to optimize a desired process. Factors to be considered include vector copy number, promoter strength and regulation, the use of fusion proteins, signal sequences and secretion, genes providing selective pressure, and elements enhancing the accuracy of vector partitioning.

The engineer must be aware of the regulatory constraints on the release of cells with recombinant DNA. These are particularly relevant in plant design, where guidelines for physical containment must be met. Deliberate release of genetically modified cells is possible, but extensive documentation will be required.

Two increasingly important applications of genetic engineering are metabolic or pathway engineering for the production or destruction of nonproteins (along with the related fields of synthetic and systems biology) and protein engineering for the production of novel or specifically modified proteins.

SUGGESTIONS FOR FURTHER READING

Genetic Engineering for Protein Production

Baneyx, F., and M. Mujacic, Recombinant Protein Folding and Misfolding in *Escherichia coli*, *Nat. Biotechnol.* 22: 1399–1408, 2004.

Datar, R. V., T. Cartwright, and C-G. Rosen, Process Economics of Animal Cell and Bacterial Fermentations: A Case Study Analysis of Tissue Plasminogen Activator, *Nat. Biotechnol.* 11: 349, 1993.

Fernandez, J. M., and J. P. Hoeffler, *Gene Expression Systems,* Academic Press, New York, 1999.

Georgiou, G., Optimizing the Production of Recombinant Proteins in Microorganisms, *AIChE J.* 34: 1233, 1988.

Glick, B. R., J. J. Pasternak, and C. L. Patten, *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, ASM Press, Washington, DC, 2010.

Goochee, C. F., and T. Monica, Environmental Effects on Protein Glycosylation, *Nat. Biotechnol.* 8: 421, 1990.

Huang, C. J., H. Lin, and X. Yang, Industrial Production of Recombinant Therapeutics in Escherichia coli and Its Recent Advancements, *J. Ind. Microbiol. Biotechnol.* 39: 383–99, 2012.

Kunkel, J. P., D. C. Jan, M. Butler, and J. C. Jamieson, Comparisons of the Glycosylation of a Monoclonal Antibody Produced under Nominally Identical Cell Culture Conditions in Two Different Bioreactors, *Biotechnol. Progr.* 16: 462–470, 2000.

Ma, J. K., P. Drake, and P. M. Christou, Genetic Modification: The Production of Recombinant Pharmaceutical Proteins in Plants, *Nat. Rev.* 4: 794–805, 2003.

Shuler, M. L., H. A. Wood, R. R. Granados, and D. A. Hammer, *Baculovirus Expression Systems and Biopesticides*, Wiley, New York, 1995.

Velander, W. H., H. Lubon, and W. N. Drohan, Transgenic Livestock as Drug Factories, *Sci. Am.* 276: 70–74, 1997.

Aspects of Metabolic Engineering

Bailey, J. E., Towards a Science of Metabolic Engineering, *Science* 252: 1668–1675, 1991.

Keasling, J. D. Manufacturing Molecules through Metabolic Engineering, *Science* 330: 1355–1358, 2010.

Lee, S. Y., and E. T. Papoutsakis, *Metabolic Engineering,* Marcel Dekker, New York, 1999.

Moreno-Sanchez, R., E. Saavedra, S. Rodriguez-Enriquez, and V. Olin-Sandoval, Metabolic Control Analysis: A Tool for Designing Strategies to Manipulate Metabolic Pathways, *J. Biomed. Biotechnol.* 2008: 597913, 2008.

Nielsen, J., and J. D. Keasling, Engineering Cellular Metabolism, *Cell,* 164: 1185–1197, 2016.

Stephanopoulos, G., J. Nielsen, and A. Aristidou, *Metabolic Engineering: Principles and Methodologies*, Academic Press, San Diego, CA, 1998.

Sun, J., and H. S. Alper, Metabolic Engineering of Strains: From Industrial-Scale to Lab-Scale Chemical Production, *J. Ind. Microbiol. Biotechnol.* 42: 423–436, 2015.

Synthetic and Systems Biology

Barrett, C. L., T. Y. Kim, H .U. Kim, B. Ø. Palsson, and S. Y. Lee, Systems Biology as a Foundation for Genome-Scale Synthetic Biology, *Curr. Opin. Biotechnol.* 17: 488–492, 2006.

Cheng, A. A., and T. K. Lu, Synthetic Biology: An Emerging Engineering Discipline, *Annu. Rev. Biomed. Eng.* 14: 155–178, 2012.

Ideker, T., T. Galitski, and L. Hood, A New Approach to Decoding Life: Systems Biology, *Annu. Rev. Genomics Hum. Genet.* 2: 343–372, 2001.

Smanski, M. J., H. Zhou, J. Claesen, B. Shen, M. A. Fischbach, and C. A. Voigt, Synthetic Biology to Access and Expand Nature's Chemical Diversity, *Nat. Rev. Microbiol.* 14: 135–149, 2016.

Stephanopoulos, G., Synthetic Biology and Metabolic Engineering, *ACS Synthet. Biol.* 1: 514–525, 2012.

Protein Engineering

Biocatalysis: Synthesis Methods that Exploit Enzymatic Activities, *Nature Insight* 409, No. 6817, 2001. (This issue contains excellent review articles on protein engineering and the industrial use of such enzymes.)

Bloom, J. D., and F. H. Arnold, In the Light of Directed Evolution: Pathways of Adaptive Protein Evolution, *PNAS* 106: 9995–10000, 2009.

Blundell, T. L., Problems and Solutions in Protein Engineering—Towards Rational Design, *Trends Biotechnol.* 12: 145–148, 1994.

Lutz, S., Beyond Directed Evolution: Semi-Rational Protein Engineering and Design, *Curr. Opin. Biotechnol.* 21: 734–743, 2010.

Kuchner, O., and F. H. Arnold, Directed Evolution of Enzyme Catalysts, *Trends Biotechnol.* 15: 523–530, 1997.

Watters, A. L., and D. Baker, Searching for Folded Proteins In Vitro and In Silico, *Eur. J. Biochem.* 271: 1615–1622, 2004.

Regulatory Issues

Giorgiou, R. J., and J. J. Wu, Design of Large Scale Containment Facilities for Recombinant DNA Fermentation, *Trends Biotechnol.* 4: 60, 1986.

National Academy of Sciences, *Introduction of Recombinant DNA-Engineered Organisms into the Environment: Key Issue,* National Academies Press, Washington, DC, 1987.

National Institutes of Health, Guidelines for Research Involving Recombinant DNA Molecules, *Federal Register* 51 (May 7) 16958, 1987.

Modeling Genetic Instability

Aiba, S., and T. Imanaka, A Perspective on the Application of Genetic Engineering: Stability of Recombinant Plasmid, *Ann. N. Y. Acad. Sci.* 369: 1, 1981.

Cooper, N. S., M. E. Brown, and C. A. Caulcott, A Mathematical Method for Analyzing Plasmid Stability in Microorganisms, *J. Gen. Microbiol.* 133: 1871, 1987.

PROBLEMS

14.1. Calculate the probability of forming a plasmid-free cell due to random segregation for a cell with 50 plasmid monomer equivalents. Forty percent of the total plasmid DNA is in dimers and 16% in tetramers. Redo for the case where there is a population with the distribution of copy numbers per cell as follows, assuming monomers only:

≤3 Plasmids	~ 0%
4 to 8	4%
9 to 13	10%
14 to 18	25%
19 to 23	25%
24 to 28	20%
29 to 33	12%
34 to 38	4%
≥ 39	~ 0%

14.2. Assume that all plasmid-containing cells have eight plasmids; that an antibiotic is present in the medium, and the plasmid-containing cells are totally resistant; and that a newly born, plasmid-free cell has sufficient enzyme to protect a cell and its progeny for three generations. Estimate the fraction of plasmid-containing cells in the population in a batch reactor starting with only plasmid-containing cells after five generations.

14.3. Consider an industrial-scale batch fermentation. A 10,000 l fermenter with 5×10^{10} cells/ml is the desired scale-up operation. Inoculum for the large tank is brought through a series

of seed tanks and flasks, beginning with a single pure colony growing on an agar slant. Assume that a colony (10^6 plasmid-containing cells) is picked and placed in a test tube with 1 ml of medium. Calculate how many generations will be required to achieve the required cell density in the 10,000 l fermenter. What fraction of the total population will be plasmid-free cells if $\mu_+ = 1.0\ h^{-1}$, $\mu_- = 1.2\ h^{-1}$, and $P = 0.0005$?

14.4. Assume that you have been assigned to a team to produce human epidermal growth factor (hEGF). A small peptide, hEGF, speeds wound healing and may be useful in treating ulcers. A market size of 50 to 500 kg/yr has been estimated. Posttranslational processing is not essential to the value of the product. It is a secreted product in the natural host cell. Discuss what recommendations you would make to the molecular-biology team leader for the choice of host cell and the design of a reactor. Make your recommendations from the perspective of what is desirable to make an effective process. You should point out any potential problems with the solution you have proposed as well as defend why your approach should be advantageous.

14.5. Develop a model to describe the stability of a chemostat culture for a plasmid-containing culture. For some cultures, plasmids make a protein product (e.g., colicin in *E. coli*) that kills plasmid-free cells but does not act on plasmid-containing cells. Assume that the rate of killing by colicin is $k_T C n_-$, where k_T is the rate constant for the killing and C is the colicin concentration. Assume that the colicin production is first order with respect to n_+.

14.6. Consider the following data for *E. coli* B/r-pDW17 grown in a minimal medium supplemented with amino acids. Estimate $\Delta\mu$ and R. Compare the stability of this system to one with a glucose-minimal medium (Example 14.2).

$D = 0.3\ h^{-1}$			$D = 0.67\ h^{-1}$		
Time, *h*	*Generation*	f_-	*Time*, *h*	*Generation*	f_-
0	0	0.003	0	0	0.003
4.6	2	0.010	5.2	5	0.010
11.5	5	0.04	10.3	10	0.015
16.0	7	0.08	15.5	15	0.05
23.1	10	0.30	20.6	20	0.13
27.7	12	0.43	31.0	30	0.34
34.7	15	0.55	41.4	40	0.68
46.2	20	0.96	51.7	50	0.95

14.7. It has been claimed that gel immobilization stabilizes a plasmid-containing population. A factor suggested to be responsible for the stabilization is compartmentalization of the population into very small pockets. For example, the pocket may start with an individual cell and grow to a level of 200 cells per cavity. Develop a mathematical formula to compare the number of plasmid-free cells in a gel to that in a large, well-mixed tank.

14.8. You must design an operating strategy to allow an *E. coli* fermentation to achieve a high cell density ($> 50\ g/l$) in a fed-batch system. You have access to an off-gas analyzer that will measure the pCO_2 in the exit gas. The glucose concentration must be less than 100 mg/l to avoid the formation of acetate and other inhibitory products. Develop an approach to control the glucose feed rate so as to maintain the glucose level at 100 ± 20 mg/l. What equations would you use, and what assumptions would you make?

14.9. Develop a simple model for a population in which plasmids are present at division with copy numbers 2, 4, 6, 8, or 10. The model should be developed in analogy to equations

14.7 through 14.9. You can assume that dividing cells either segregate plasmids perfectly or generate a plasmid-free cell.

14.10. Assume you have an inoculum with 95% plasmid-containing cells and 5% plasmid-free cells in a 2 l reactor with a total cell population of 2×10^{10} cells/ml. You use this inoculum for a 1000 l reactor and achieve a final population of 4×10^{10} cells/ml. Assuming $\mu_+ = 0.69\ h^{-1}$, $\mu_- = 1.0\ h^{-1}$, and $P = 0.0002$, predict the fraction of plasmid-containing cells.

14.11. Assume you scale up from 1 l of 1×10^{10} cells/ml of 100% plasmid-containing cells to 20,000 l of 5×10^{9} cells/ml, at which point overproduction of the target protein is induced. You harvest 6 hours after induction. The value of P is 0.0005. Before induction, $\mu_+ = 0.95\ h^{-1}$ and $\mu_- = 1.0\ h^{-1}$. After induction, μ_+ is $0.15\ h^{-1}$. What is the fraction of plasmid-containing cells at induction? What is the fraction of plasmid-containing cells at harvest?

14.12. A metabolic pathway involves enzymes A, B, and C. Given that enzyme B is the rate-limiting enzyme, in this pathway, would you expect this enzyme to be tightly regulated? Why or why not? Name two or three strategies that could be taken to increase flux through this pathway.

14.13. You desire to change the specificity of an antibody fragment using semirational design. You have discovered a 3-amino acid stretch in complimentary determining region 3 (CDR3) that you hypothesize directs the specificity of the antibody. Your goal is to study this region by saturation mutagenesis (substituting all possible amino acids in each position).

- **a.** How large of a protein library would you need to screen if you wish to have threefold library coverage (screening three times more members than the theoretical library size that contains all possible variants)?
- **b.** After your first attempt, you decide to screen a similar 3-amino acid region in CDR1 and CDR2. How large of a library will you need to ensure threefold library coverage in all three CDRs simultaneously?
- **c.** Why is it necessary to have threefold library coverage?
- **d.** How many library members in the CDR3 library do you expect to be truncated due to the inclusion of a stop codon? Can you think of a way of biasing the library not to include stop codons in these positions?

14.14. You are a fledgling protein engineer and wish to study the diversity of every possible 40-amino acid peptide. As a first test, you want to synthesize all possible amino acid combinations for the peptide using all 20 amino acids. Ignoring the DNA needed to make each peptide, what is the mass of your peptide library if you synthesized only one molecule of each peptide variant? Are you surprised by this mass?

15

Medical Applications of Bioprocess Engineering

With increasing knowledge of cellular and molecular biology, the boundary between traditional bioprocess engineering and biomedical engineering has become increasingly fuzzy. In this chapter, we consider examples in which bioprocess principles are critical to solution of medical problems. Two important areas are tissue engineering and gene therapy using viral vectors. These are just two examples; other medical applications exist, and their number will undoubtedly increase. Indeed, we have already mentioned the use of transgenic plants as a source for edible vaccines and the techniques of microfabrication applied to make miniature process facilities for rapid DNA analysis to allow genome analysis in a physician's office.

15.1. TISSUE ENGINEERING

An important contribution to medicine of bioprocess engineering is in *regenerative medicine*. Here the techniques we have discussed in the first 14 chapters can be used to generate replacement tissues for the human body. Building such replacement tissues is a challenge, but for some simple tissues, it is a commercial and clinical success.

15.1.1. What Is Tissue Engineering?

Tissue engineering has a primary focus on developing in vitro bioartificial tissues, typically based on cells derived from donor tissue. These tissues can be used as transplants to improve biological function in the recipient. Commercial examples include living skin tissue and chondrocytes implanted in a damaged knee for production of hyaline-like cartilage. An extracorporeal (outside of the body) artificial liver employing pig liver cells has been clinically tested.

Artificial tissues/organs for transplantation that are under active development include liver, pancreas, kidney, fat (for reconstructive surgery), blood vessel, bone marrow, bone, and neurotransmitter-secreting cell constructs. An alternative form of tissue engineering is in vivo alteration of cell growth and function. An example would be the use of implanted polymeric tubes with a controlled surface chemistry to encourage and guide reconnection of damaged nerves. Another use of artificial tissue constructs is for toxicological and pharmacological testing of potential new drugs. In this case, the artificial tissue or combination of tissues acts as a surrogate, reducing the need to use animals for such testing.

The primary difference between tissue engineering and protein production from mammalian cells lies in the constraints on cell selection. For protein production we prefer continuous, transformed cell lines. A single cell type is desirable. For tissue engineering the goal is to replicate the response of a living tissue. Cells removed from a tissue and cultured as a homogenous cell type in two dimensions (e.g., on a solid surface) often lose their authentic in vivo response. Since cell transformation and cancer are closely related, transformed cells cannot be used for a product for transplantation. Reconstruction of artificial tissues requires a deep understanding of the interactions of one cell with another, control of cellular differentiation processes, and knowledge of how cells interact with surfaces to which they attach. Most often, a polymer scaffold is used to guide and organize tissue growth. Maintaining the correct ratio of cell types can be difficult, since some cell types, such as fibroblasts, can "outgrow" others. The appropriate cell types must organize themselves into the appropriate three-dimensional configuration. With such tissues, function requires appropriate structure. Ideally, the cells can be multiplied from donor tissue for at least 10 passages and then assume the fully differentiated phenotype when the correct stimulus is applied. A major challenge is the routine, reproducible culture of such cells, and bioprocess engineers are in an excellent position to contribute to this technology.

Of particular importance to many tissue-engineered constructs is the formation of extracellular matrix, the interaction of cells with one another, and the interaction with an artificial surface. Anchorage-dependent cells must attach and spread on a substrate surface to proliferate and function. Cell adhesion is mediated by extracellular matrix proteins such as fibronectin and collagen. Synthetic substrates can be modified by adding synthetic peptide sequences (three to six amino acids) to a surface at the end of a synthetic polymer. The difference in strength of cell–substrate and cell–cell adhesion can greatly alter the three-dimensional organization of the cells.

The use of simulated microgravity reactors coupled with polymer scaffolds has been useful in some cases in the development of certain tissue constructs. The use of microfabrication techniques, where the investigator has control over placement

of individual cells, is an intriguing technology for more authentic tissue constructs. An increasingly large array of tools are being developed for controlling the formation of tissue constructs with improved performance.

As noted in Chapter 9, "Operating Considerations for Bioreactors for Suspension and Immobilized Cultures," 3-D printing of tissues is an emerging technology of increasing importance, particularly for mammalian cells. It is possible to print simple tissues (typically those that do not have a high level of vascularization) that are functional in the human body. The range of materials that are biocompatible with cells and still printable is a constraint, but rapid progress is being made in developing technologies that facilitate the use of a wider range of materials. The ability to print tissue constructs in any shape, on an individual basis, can allow the personalized replacement of missing or damaged tissues. For example, a replacement ear can be printed to match rather precisely the one that was lost in an accident. Skin is another organ that has been commercially manufactured by scaling up traditional tissue engineering approaches. The technologies for producing tissue-engineered constructs are expected to become a major component of regenerative medicine. However, the development of effective tissue constructs that are biologically complex, such as the liver, is a very difficult problem, and commercial production of transplantable organs is still futuristic. However, commercial production of simple tissues, particularly ones with low vascularization has been accomplished.

The two examples of commercial production of tissue-engineered products discussed in this chapter are for skin and cartilage.

15.1.2. Tissue-Engineered Skin Replacements

Skin replacements are needed for patients with burns and pressure ulcers (usually due to diabetes) and for reconstructive surgery. Cadaver skin is undesirable because of potential disease transmission, possible immunological rejection, inflammation of the wound bed, limited availability, and high cost due to labor-intensive procurement. Animal products would be problematic due to immunological reactions. An "artificial" human skin replacement that can be taken out of the freezer and used by physicians is highly desirable. TransCyte, a human fibroblast-derived temporary skin substitute, is such a product.

Artificial human skin is made from human neonatal foreskin fibroblasts, which are obtained from routine circumcision discards. These fibroblast cells are easily available and not subject to rejection by the body; they are also capable of replication, and numbers can be expanded in a straightforward manner. Donor tissue is enzymatically digested and seeded onto a three-dimensional bioresorbable polymer scaffold. The cell-seeded scaffold is placed in a bioreactor that provides a physiologically similar environment to promote cell growth and secretion of proteins and extracellular matrix material. The cells and extracellular matrix material result in a three-dimensional tissue construct that is functionally similar to natural skin and promotes wound healing.

The manufacturing process is centered on a bioreactor system that mimics in vivo conditions through control of temperature, pH, oxygen level, nutrient supply, waste removal, and fluid hydrodynamics. It must produce thousands of individual

products of uniform and reproducible quality. The manufacturing system requires a bioreactor for product growth that also serves as final packaging. The system is sealed after assembly and remains sealed to maintain sterility. Figure 15.1 is a schematic of the process. The bioreactor is made of plastic with inlet and outlet ports. It is flat and just large enough to hold a polymer mesh about 13 by 19 cm. Twelve individual bioreactors are manifolded together. Groups of 12 are then operated in parallel to provide the desired lot size. Once the system is sterilized, it is opened only for cell seeding and nutrient replenishment. About 3 weeks are required for growth. At the end of this growth period, cryopreservative is pumped into the bioreactor module. After sealing inlet and outlet tubes, the bioreactor is packaged, frozen, and then stored or shipped. The product is thawed and used by the physician.

This manufacturing process is similar to many discussed in this book but differs in that the product is discrete and not in solution. The process must meet all of the FDA and GMP (good manufacturing practice) requirements discussed earlier.

15.1.3. Chondrocyte Culture for Cartilage Replacement

Articulate or hyaline cartilage is a thin layer of tissue found at the ends of bones. Damage to articulate cartilage in the knee is a common health problem. Cartilage consists of three primary components: collagen fibrils, proteoglycans, and cells known as *chondrocytes*. These cells are present in low numbers (1% by volume) but are responsible for synthesis and release of these other compounds, forming cartilage. When chondrocytes are cultured as two-dimensional (monolayer) cultures, the

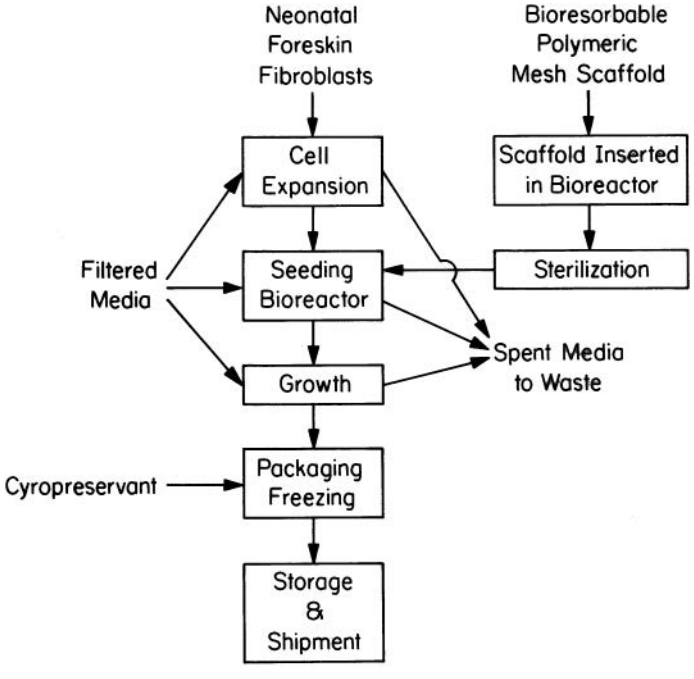


Figure 15.1. Conceptual process diagram for production of a human skin substitute.

cells are no longer differentiated and do not make normal hyaline matrix proteins. However, if cultured in three dimensions, such as a suspension in agarose, or in vivo, the cells redifferentiate and begin to manufacture hyaline-like matrix.

Because of immunological responses it is advantageous for patients to supply their own cells for expansion in number before the cells are reinjected into the knee. A manufacturing process for individual cell growth is needed. The supply and reuse of cells from an individual is termed *autologous implantation*.

The basic procedure entails biopsy of a patient to obtain a small number of chondrocytes, monolayer culture of primary chondrocytes and expansion of cell numbers, release of cells from monolayer and into suspension, assembly of cells in three dimensions, and injection into the patient's own knee. This product is known as Carticel. The implanted cells produce hyaline-like cartilage and fill in defects in the patient's knee. Patients can often reenter low-impact activities such as swimming or cycling in 6 months and vigorous physical activity in 12 months.

The primary manufacturing challenge here is the need for separate culture for each individual. To accomplish this for mass production is a challenge to any manufacturing process. There is expected to be a demand for other therapies (e.g., cancer treatment), where cells from an individual will need to be efficiently cultured and returned to the individual.

15.2. GENE THERAPY USING VIRAL VECTORS

Gene therapy is the transfer of one or more genes into cells for a therapeutic effect. It can be done ex vivo (outside the body) in tissues that are transplanted back into the patient or in vivo, where the genes, usually in a delivery vehicle such as a virus, are injected into the patient.

Gene therapy is intellectually connected to metabolic engineering. However, the complexity of humans presents an even greater challenge than the metabolic engineering of a single cell. Gene therapy is a quantitative problem that makes good use of the quantitative skills of engineers. Basically, the right gene needs to be delivered to only the right tissue target in the right amount, with the gene products being expressed at the right level at the right time for the right length of time. For gene therapy to be effective, many things have to go right. Clinical success with gene therapy has been minimal, as approaches have been qualitative and trial-and-error in nature. A rational analysis would be a useful tool.

Many methods can be used for gene delivery. These involve viral vectors, use of naked DNA, and liposome or particle-mediated gene delivery. In this chapter, we focus only on viral systems. Bioprocess technology is necessary for production of the viral vectors, and analyses arising from bioprocess studies are applicable to gene therapy.

The four primary virus vectors for gene therapy are retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (AAV). Retroviruses and lentiviruses are enveloped viruses, because they are encapsulated in a lipid bilayer membrane. The adenovirus and AAVs are nonenveloped viruses.

Typical concerns with the use of viral vectors include safety, low toxicity, genomic stability, and cell specificity. Safety to both patients and production workers is essential. Immune responses to viruses can be a significant issue and vary from

TABLE 15.1. Comparison of Major Viral Gene Delivery Systems

Characteristics	Retrovirus	Lentivirus	Adenovirus	Adeno-Associated Virus
Host cells:				
Cell line (dividing)	Yes	Yes	Yes	Yes
Primary cells (dividing)	Yes	Yes	Yes	Yes
Primary cells (nondiving)	No	Yes	Yes	Yes
Stem cells	Yes	Yes	Yes	Yes
Neuronal cells	No	Yes	Yes	Yes
Hematopoietic cells	Yes	Yes	No	No
Size of genomic insert	Moderate	Moderate	High	Low
High-level protein expression	No	No	Yes	No
Biosafety	Good (BSL-2)	Good (BSL-2)	Good (BSL-2)	Excellent (BSL-1)

patient to patient. One of the early trials, for example, with an adenoviral vector led to the death of patient. Some viral clinical vectors (e.g., lentiviruses) insert in random locations in the genome and can inactivate an essential gene in the host cell. AAV-based vectors are safer in that they insert at specific locations. AAV is not known to cause disease and elicits a mild immune response. For all of these viral vectors specificity can be broad, but targeting key receptors can increase specific delivery of genes. Some features of these viral vectors are summarized in Table 15.1.

15.2.1. Models of Viral Infection

Dee, Hammer, and Shuler[†] proposed a model for the viral trafficking of Semliki Forest virus (SFV), an enveloped RNA virus that has been considered as a vector for large-scale production of heterologous proteins. However, this analysis, which was motivated by a bioprocess application, is applicable to retrovirus vectors for gene therapy (although SFV is not appropriate for gene therapy).

As indicated in Figure 15.2, enveloped RNA viruses can enter cells through receptor-mediated endocytosis. The virus binds to specific receptor molecules on the cell's surface. It is assumed (for this analysis) that attachment is irreversible and that the number of receptor molecules is much greater than the number of virus particles present. Consider the following circumstance:

$$dV_{ex}/dt = -k_a C V_{ex} \tag{15.1}$$

Here V_{ex} is the number of extracellular viruses per cell, C is the cell concentration, and k_a is the attachment-rate constant. The value of k_a can be estimated as

$$k_a = k_f(\alpha R) \tag{15.2}$$

where k_f is the intrinsic forward rate constant for the binding of a single viral attachment protein to a receptor, α is the number of viral attachment proteins per virus,

[†] K. U. Dee, D. A. Hammer, and M. L. Shuler, *Biotechnology and Bioengineering* 46: 485–496, 1995.

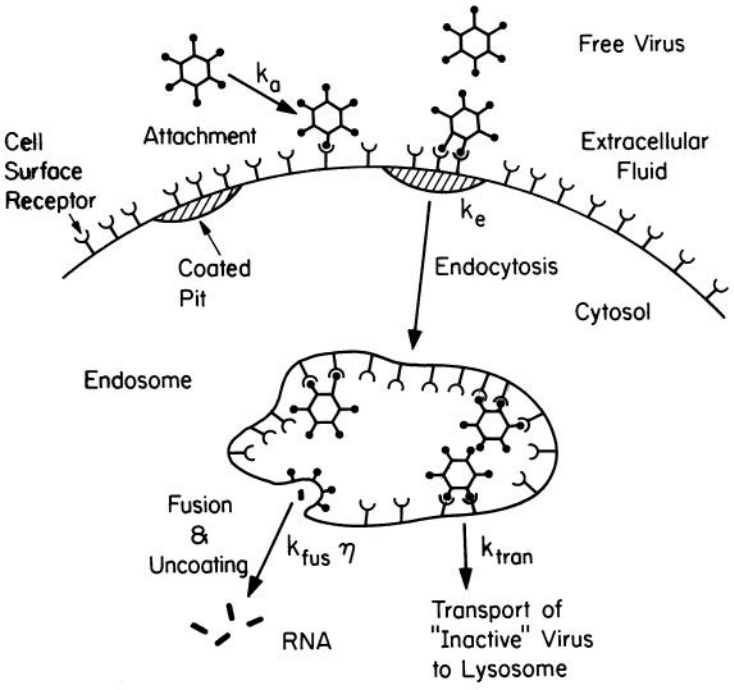


Figure 15.2. The trafficking of an enveloped RNA virus is depicted. A mathematical model of these processes is given in the text.

and R is the number of available receptors per cell. If the ratio of R to V_{ex} is large, R is approximately the total number of receptors.

Once the virus is attached, it randomly associates with "coated pits," which internalize the virus through the process of *endocytosis*. Endocytosis is a process of invagination of the plasma membrane to form a vesicle in which the receptors and anything attached to the receptors are captured within the vesicle. Such vesicles are known as *endosomes*. The rate of endocytosis is assumed to be

$$dV_i/dt = k_e V_s \tag{15.3}$$

where V_i is the number per cell of internalized virus, V_s is the surface concentration of virus (number per cell), and k_e is the endocytosis rate constant.

The amount of surface virus can be determined from combining equations 15.1 and 15.3 to give

$$dV_s/dt = k_a C V_{ex} - k_e V_s \tag{15.4}$$

Endosomes are intermediates in recycle of plasma membrane components and transport of internalized materials to lysosomes for degradation. For the virus to replicate successfully, it must escape from the endosome before it is delivered to a lysosome. Endosomes undergo a biphasic pH change. In early endosomes, pH drops from

about 7.5 to 6.0 within 5 to 10 min. In the second phase, pH drops slowly to about 5.2 in another 30 to 60 min. The pH drop is due to membrane proteins that pump hydrogen ions into the endosome.

The virus escapes through fusion of its membrane envelope with the endosome membrane. The fusion process is pH dependent. For SFV, the threshold pH for fusion is 6.2. This threshold is reached in early endosomes. The fusion process is quite rapid. However, not all virus undergoes fusion. The virus balance in the endosome is

$$dV_{ene}/dt = k_e V_s - k_{fus}\eta V_{ene} - k_{tran}V_{ene} \tag{15.5}$$

where V_{ene} is the number of virus in the early endosomes, k_{fus} is the observed fusion-rate constant, η is the fraction of virus that can successfully fuse with endosomal membrane, and k_{tran} is the rate constant for movement of inactive virus to the lysosome and degradation. The main cause of inactive virus is the fusion of virus with membrane fragments within the endosome instead of the endosomal membrane.

Virus that successfully fuses with the endosomal membrane is released into the cytosol. The protein coat is removed rapidly, and for this analysis, we assume that effectively all cytoplasmic virus is uncoated, so that the RNA is released and can be replicated.

The rate equation for uncoating is

$$dV_{cyt}/dt = k_{fus}\eta V_{ene} \tag{15.6}$$

where V_{cyt} is the cytoplasmic virus.

The amount of RNA synthesized per cell follows a saturation type response:

$$[RNA]_{cell} = \frac{k_m V_{cyt}}{K_{sv} + V_{cyt}} \tag{15.7}$$

where k_m and K_{sv} are parameters dependent on the host cell. Equation 15.7 applies only to the early parts of the infection cycle. Virus that enters after RNA replication has been completed for early-entry virus probably no longer contributes to RNA synthesis, as host cell function begins to be lost.

Equations 15.1 to 15.6 allow for an analytical solution. If we assume that only free virus is present initially ($t = 0$), then the initial conditions are $V_{ex} = V_{exo}$; $V_s = 0$; $V_i = 0$; $V_{ene} = 0$; $V_{cyt} = 0$. The solutions in this case are as follows:

$$V_{ex} = V_{exo}e^{-k_a Ct} \tag{15.8}$$

$$V_s = \frac{k_a C V_{exo}}{k_e - k_a C}(e^{-k_a Ct} - e^{-k_e t}) \tag{15.9}$$

$$V_t = \frac{V_{exo}}{k_e - k_a C}\left[k_a C(e^{-k_e t} - 1) - k_e(e^{-k_a Ct}) - 1\right] \tag{15.10}$$

$$V_{ene} = \frac{k_e k_a C V_{exo}}{k_e - k_a C}\left[\frac{1}{k_{fus}\,\eta + k_{tran} - k_a C}\left(e^{-k_a C t} - e^{-(k_{fus}\,\eta + k_{tran})t}\right)\right.$$
$$\left. - \frac{1}{k_{fus}\,\eta + k_{tran} - k_e}\left(e^{-k_e t} - e^{-(k_{fus}\eta + k_{tran})t}\right)\right] \tag{15.11}$$

$$V_{cyt} = \frac{k_{fus}\,\eta k_e k_a\, C V_{exo}}{k_e - k_a C}\left\{\frac{1}{k_{fus}\,\eta + k_{tran} - k_a C}\left[\frac{1}{k_a C}(1 - e^{-k_a C t}) + \frac{1}{k_{fus}\,\eta + k_{tran}}\left(e^{-(k_{fus}\,\eta + k_{tran})t} - 1\right)\right]\right.$$
$$\left. - \frac{1}{k_{fus}\,\eta + k_{tran} - k_e}\left[\frac{1}{k_e}(1 - e^{-k_e t}) + \frac{1}{k_{fus}\,\eta + k_{tran}}\left(e^{-(k_{fus}\eta + k_{tran})t} - 1\right)\right]\right\} \tag{15.12}$$

For wild-type SFV infecting baby hamster kidney (BHK) cells, the rate parameters have been estimated as $k_a = 5.2 \times 10^{-9}$ ml/cell-min; $k_e = 0.039$ min^{-1}; and $k_{fus}\alpha = k_{tran} = 0.14$ min^{-1} (based on 50% of internalized virus being uncoated). Such a model with these parameters fits time-course measurements from actual experiments and can also be used to make estimates of response if mutant virus forms are used.

These same equations can easily be applied to an ex vivo system for gene therapy. For in vivo use they apply in principle, but the presence of various natural compounds in the bloodstream can alter k_a (and potentially other parameters), and multiple cell types are present which may bind, but not endocytose virus. A pharmacokinetic model (to predict time-dependent virus distribution at different positions in the body) would need to be coupled with virus/cell interaction models (of the type developed here) for each major cell type. It is easy to see why ex vivo therapy would be much easier to design than an in vivo protocol. For either the ex vivo or in vivo therapies to be successful, there must be a method to efficiently produce virus.

15.2.2. Mass Production of Retrovirus

The efficient production of a highly concentrated solution of retroviruses (or the closely related lentivirus) that have been genetically modified to deliver a gene is difficult. Genetically engineered retroviruses, like all recombinant viruses, are produced by a two-part system consisting of a packaging cell line and the recombinant vector. This two-part system is necessary because the recombinant virus is unable to replicate itself. The recombinant virus is derived from a wild-type virus in which essential viral genes have been deleted and replaced with the therapeutic gene of interest. The enzyme reverse transcriptase converts RNA-encoded genes into DNA, which is transported into the nucleus and integrated into the cell's chromosomal DNA.

The packaging cell line is genetically engineered to produce the essential, structural viral genes that have been deleted from the viral genome. Because the viral genes are encoded in the packaging cell's chromosome, the resulting viral particles are incapable of causing disease but act as carriers for the desired, therapeutic genes. The resulting retrovirus vector can be used only with dividing cells, since cells must

undergo mitosis before gene integration can occur. This feature limits in vivo use to cases such as cancer suppression but is not a limitation on ex vivo systems.

Another limitation on the retrovirus system is a bioprocess limitation; the production of high-titer virus and subsequent purification and concentration without loss of infectivity have been difficult. A major limitation to the effectiveness of many gene-therapy approaches, including the use of retroviruses, is that the average number of genes delivered to target cells is too low to achieve a beneficial therapeutic effect. This low efficiency is due to the bioprocess limitation on production of a concentrated, highly infectious retrovirus preparation.

The problems in producing a highly infectious preparation derive from two factors:[†] rapid decay of virus and inhibition of viral infectivity by proteoglycans released by the packaging cell line. The proteoglycans have very high molecular weight. If the virus is concentrated by ultrafiltration, the proteoglycan is also concentrated by nearly the same factor. While the high virus concentration would be expected to increase the number of infected cells, the presence of concentrated proteoglycan leads to increased inhibition of infection, so that the number of cells infected does not increase significantly and in some cases even decreases with the concentrated viral preparation.

Another approach to increase viral titer is to produce virus at a reduced temperature (e.g., 28°C versus 37°C). While both the rate of virus production and viral decay decrease with temperature, the rate of decay is more sensitive to temperature. Thus the amount of potentially active virus is severalfold higher when produced at 28°C (e.g., 2×10^5 cfu/ml vs. 6×10^4 cfu/ml; cfu is colony-forming units and measures the number of active viruses in a dilute solution). However, the transduction efficiency did not increase, presumably due to changes in proteoglycan concentration.

These results should inspire us to consider bioreactor options beyond batch growth followed by ultrafiltration. Clearly, we would want to consider a bioreactor configuration in which the virus is removed as soon as it is produced and then purified and stored at low temperature to reduce decay. Further, a method of concentration of virus needs to be developed that does not also concentrate proteoglycan. Enzymatic digestion of proteoglycans has been suggested, but due to multiplicity of proteoglycan structures such a strategy is difficult to implement. Selective adsorption and desorption of virus is attractive, but the sensitivity of the virus to these processes is a concern. A packaging cell altered in its capability to produce proteoglycan is another possible strategy.

The primary point is that bioprocess technology is intimately connected to production of agents that can be effective in gene therapy. The solution to the biomedical problem requires a solution to the bioprocess problem.

15.3. BIOREACTORS

Mass production of cells for transplantation and the use of bioreactors as artificial hybrid organs are subjects of intense development in medicine. These are issues in which bioprocess engineers may make significant contributions.

[†] J. M. LeDoux, H. E. Davis, J. R. Morgan, and M. L. Yarmush, *Biotechnology and Bioengineering* 63: 654, 1999.

15.3.1. Stem Cells and Hematopoiesis

Some animal cells are capable of extensive replication and self-renewal. Others are highly differentiated and perform specific functions; typically, differentiated cells cannot replicate (at least, replication is very limited). A *stem cell* is an undifferentiated cell capable of continuous self-renewal that can also produce large numbers of differentiated progeny, depending on extracellular factors.

The ultimate form of a stem cell is one that is *pluripotent* and can be differentiated into any type of cell in the body. Originally, pluripotent stem cells could be derived only from embryos. For cells derived from humans, this presented ethical as well as technical challenges. In 2006, Shinaya Yamanaka (2012 Nobel Prize winner) demonstrated it was possible to convert adult, specialized cells into *induced pluripotent* stem (iPS) cells by the introduction of only four genes. In principle, these cells then can be converted into any specialized cells with the correct extracellular cues. This technology offers great promise not only for use in regenerative medicine but also for uses such as drug testing and development. Progeny from these stem cells are genetically identical to the parental cells. There are subtle differences (epigenetic), and maturation into an adult phenotype can be challenging.

The scale-up of stem cell technology for the production of large quantities of cells is a bioprocess challenge. All of the GMP standards and regulatory requirements need to be met at a large scale. While single-use technology for cell cultivation (see Chapter 10, "Selection, Scale-Up, Operation, and Control of Bioreactors") would appear to be a good option, fragile adherent cells (as stem cells are) are highly sensitive to their microenvironment, and cell harvest is a challenge. Multistack arrays based on two-dimensional growth on polystyrene plates have been proposed and may be practical, but they are still relatively inefficient. For this technology to move forward, more effective methods for mass production of cells must be sought.

Not all stem cells are pluripotent but can be precursors to a set of cell types, such as blood cells. The development of production systems for these cell types have been explored for many years, although none have been fully commercialized. Much of the early work has been focused on production of red blood cells from hematopoietic stem cells. This example requires the development of robust large-scale production systems for the process to become practical.

There are eight major types of fully mature blood cells in the human circulatory system. A hematopoietic stem cell gives rise to two types of *progenitor cells*. The progenitor cells are capable of self-replication but have a more restricted range of cells into which they may differentiate. In the hematopoietic system, one type of progenitor cell can give rise to the myeloid cells (e.g., red blood cells or erythrocytes, platelets, macrophages) and the other to lymphoid cells (e.g., T cells and B cells). The progression of differentiation depends on a large number of hematopoietic growth factors. Hematopoiesis, the process of generating these blood cells, takes place in the bone marrow. Ex vivo hematopoiesis is an alternative or supplement to bone marrow transplants. Hematopoietic stem and progenitor cells can be recovered from human blood, particularly umbilical cord blood.

Schemes for large-scale systems have been considered for 20 years. Coculture with stroma cells from the bone marrow is necessary to generate necessary growth factors, so bioreactors must accommodate adherent cell growth. Three basic reactor types are under development. *Fluidized bed reactors* with macroporous supports mimic structures much like that in bone. However, there are challenges in controlling surface chemistry and maintaining the appropriate mix of cell types. Cell sampling and control of reactor conditions can be problematic.

Flatbed reactors (e.g., modified T-flasks with continuous flow possible) can carry stroma and facilitate analysis and design, since the geometry is well defined. Direct microscopic observation of cells is possible. Automated flatbed systems have been used to generate cells for human clinical trials.

Membrane-based units, such as hollow-fiber reactors, are potential solutions. This is an efficient design that is fairly easy to characterize. However, cell observation and harvesting are considered problematic. Another alternative is the possible use of spheroid cultures (a natural aggregation of a mixed population of cells), which can be done in suspension-type bioreactors.

Bioreactors for production of other tissue types from other stem cells are a real possibility. There is far less experience with commercial-scale bioreactors for other stem cells/tissues, but the principles presented in this book should be applicable to these challenges.

15.3.2. Extracorporeal Artificial Liver

Liver failure is a major medical problem. The liver performs many metabolic functions (carbohydrate, fat, and vitamin metabolism; production of plasma proteins; conjugation of bile acids; and detoxification), of which detoxification is the most critical. In some cases, a failing liver may recover if the metabolic and detoxification demands on it can be reduced; an artificial liver may provide the respite necessary for self-repair of a liver. In other cases, an artificial liver may serve as a bridge to a transplant.

Due to the spatial and metabolic complexity of the liver, an implantable artificial liver is a distant objective. However, an extracorporeal device to serve as a temporary assist device is realistic (such a system is in clinical trials). A promising design is a hollow-fiber system using porcine (pig) hepatocytes. Such cells are relatively easy to obtain in large quantities and maintain a satisfactory level of differentiated cellular activity in regard to detoxification. A disadvantage is due to limited lifespan (and proliferative ability). The membrane that separates these cells from the blood provides protection against adverse immune reactions. The issues in the design of such hollow-fiber reactors are similar, whether the reactor is to be used in a bioprocess operation or in a biomedical application.

15.3.3. Body-on-a-Chip Systems

Preclinical drug development is a difficult process (over several billion dollars per approved drug and typically 12 to 15 years). This effort typically requires the use of

animals to test the potential safety and efficiency of drug candidates before beginning human clinical trials. However, these tests on animals are not good predictors of human response due to differences between human and animal physiology and metabolism. Consequently, about 10% of the drug candidates that pass preclinical animal testing succeed in becoming approved drugs. Because human clinical trials are very expensive, this inefficiency increases significantly the cost of drugs.

Currently, technology is being developed to construct human surrogates for use in preclinical evaluation of drugs. These surrogates are often known as *microphysiological*, *body-on-a-chip*, or *multiorgan-on-a-chip* systems. These systems depend heavily on ideas from bioreactor systems and tissue engineering or immobilized cell technology. Because drug development requires many tests done on nearly identical systems, these human surrogates are constructed on a microscale using technologies originally developed for electronics.

These systems make use of a *physiologically based pharmacokinetic* (PBPK) model of the human body to guide the development of these microscale systems. The PBPK models mathematically the uptake, distribution, metabolism, and physiological response of various organs and tissues to the parental drug and its metabolites. However, these models are limited because our understanding of drug fate and metabolism is incomplete. By replacing the differential equations in the PBPK model with living tissue/organ mimics, we can observe responses from various "organs" that would not be anticipated otherwise, thus providing a more accurate prediction of response. However, because the mathematical model and the device are easily linked and parameters can be determined relatively precisely with the device, the extrapolation of data from the device to prediction of human response is possible.

No in vitro model will ever fully capture the complexity of humans, but an improvement in preclinical testing from 10% success to 30% success would have a tremendous impact in reducing costs and increasing the number of drugs available to treat disease.

While a detailed description of this technology is beyond the scope of this book, references to further information are provided at the end of this chapter. This example demonstrates how a bioprocess knowledge of bioreactors and mass transfer can be coupled with an understanding of human physiology to make useful predictions on the potential safely and effectiveness of drug candidates (and also exposure to chemicals and cosmetics).

15.4. SUMMARY

The principles from bioprocess engineering are *not* restricted to use for biomanufacture of chemicals, pharmaceuticals, and other biological compounds. These same principles have many applications in medicine, as well. Indeed, as biomedical engineering has become more oriented to molecular and cellular systems and bioprocess engineering more toward animal cells, the boundary between these two activities has become very porous.

Important concepts in this chapter include ideas of how to produce tissue constructs on a commercial scale. Also, the efficient production of virus for use in gene

therapy is an unresolved bioprocess problem. The basic understanding of the dynamics of viral infection of cells is important both in design of biomanufacturing processes and for gene therapy. Quantitative approaches to describing such processes can lead to rational design both for the biomanufacturing process and for therapeutic strategies. Bioreactor design is also an important component of design of artificial organs and reactors to generate functional tissue from stem cells and to the development of human surrogates for drug development.

The list of examples of the intersection of bioprocessing and medicine is a growing one that will present exciting career possibilities.

SUGGESTIONS FOR FURTHER READING

Dee, K. U., D. A. Hammer, and M. L. Shuler, A Model of the Binding, Entry, Uncoating, and RNA Synthesis of Semliki Forest Virus in Baby Hamster Kidney (BHK-21) cells, *Biotechnol. Bioeng.* 46: 485–496, 1995.

Ding, Y-T., and X.-L. Shi, Bioartificial Liver Devices: Perspectives on the State of the Art, *Front. Med.* 5: 15–19, 2011.

Esch, M. B., T. L. King, and M. L. Shuler, The Role of Body-on-a-Chip Devices in Drug and Toxicity Studies, *Annu. Rev. Biomed. Eng.* 13: 55–72, 2011.

Esch, M. B., A. Smith, J. M. Prot, C. Oleaga, J. Hickman, and M. L. Shuler, How Multiorgan Microdevices Can Help Foster Drug Development, *Adv. Drug Deliv. Rev.* 69–70: 158–169, 2014.

Le Doux, J. M., H. E. Davis, J. R. Morgan, and M. L. Yarmush, Kinetics of Retrovirus Production and Decay, *Biotechnology and Bioengineering* 63: 654–662, 1999.

Lu, L., H. M. Arbit, J. L. Herrick, S. G. Segovis, A. Maran, and M. J. Yaszemski, Tissue Engineered Constructs: Perspective on Clinical Translation, *Ann. Biomed. Eng.* 43: 796–804, 2015.

Ren, S., J. I. Irudayam, D. Contreras, D. Sareen, D. Talavera-Adams, C. N. Svendsen, and V. Arumugaswami, Bioartificial Liver Device Based on Induced Pluripotent Stem Cell-Derived Hepatocytes, *J. Stem Cell Res. Ther.* 5(2): 1000263, 2015.

Singh, V. K., M. Kalsan, N. Kumar, A. Saini, and R. Chandra, Induced Pluripotent Stem Cells: Application in Regenerative Medicine, Disease Modeling, and Drug Discovery, *Front. Cell Dev. Biol.* 3: Article 1–17, 2015.

van Veen, T., and J. A. Hunt, Tissue Engineering Red Blood Cells: A Therapeutic, *J. Tissue Eng. Regen. Med.* 9: 760–770, 2015.

PROBLEMS

15.1. Many experiments for virus trafficking are done with prebound virus. Virus attaches at low temperature to suppress endocytosis, and unbound virus is washed away. Cells are then warmed to 37°C to initiate endocytosis. Derive equations in this case for the time-dependent concentration of internalized virus and virus that enters the cytosol and uncoats.

15.2. A mutant Semliki Forest virus (SFV) has values of $k_{fus}\eta = 8.3 \times 10^{-3}$ min^{-1} and $k_{tran} = 1.8 \times 10^{-2}$ min^{-1}. The wild-type virus has values of $k_{fus}\eta = 6.4 \times 10^{-2}$ min^{-1} and $k_{tran} = 1.1 \times 10^{-1}$ min^{-1}. For both wild-type and mutant SFV the value of $k_e = 0.074$ min^{-1}. Prebound virus were used (see Problem 15.1) and V_{s0} was the same for both cases. At 20 min, what is the ratio of uncoated virus for the wild-type virus to the mutant?

15.3. A value of k_aC is measured as 0.021 min^{-1}. Assume $k_{fus}\eta = k_{tran} = 0.14$ min^{-1} and $k^e = 0.04$ min^{-1}. The initial inoculum was 500 viruses/cell. At 2 hours after inoculation, unbound virus was removed. Calculate V_s, V_{cyt}, and V_{ene}.

15.4. You wish to produce active retrovirus, and you are investigating the effect of temperature on the process. Active virus is subject to decay with a rate constant, $k_d = 2.2$ day^{-1} at 37°C and 0.76 day^{-1} at 31°C. The production rate of virus from a packaging cell line is $k_p = 3.3$ virus/cell-day at 37°C and $k_p = 2.9$ virus/cell-day at 31°C. Assume that there are 1×10^6 cells, and the volume of liquid medium is 2 ml. The initial number of virus in solution is zero. How many viruses are there per ml 12 hours after initiation of virus production?

15.5. Mass-transfer limitations are often critical in design of devices such as the hollow-fiber bioartificial liver for use as an extracorporeal assist device. Consider removal (detoxification) of a slightly hydrophobic compound in a patient's blood. Draw a diagram and describe potential mass-transfer limitations on the rate of detoxification by intracellular enzymes.

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16

Bioprocesses Utilizing Mixed Cultures

The dynamics of mixed cultures (or multiple species in culture) are important considerations in some commercial fermentations. They are critical to understanding the response of many ecological systems to stress. The use of organisms with recombinant DNA has added another dimension to our consideration of how cells within a population interact with each other.

Many food fermentations, such as cheese manufacture, depend on multiple interacting species. The biological treatment of wastewaters relies on an undefined complex mixture of microorganisms. The ratio of various species in the treatment process is critical; sudden shifts in the composition of the population can lead to failure of the unit to meet its objectives.

In all natural environments, cells exist in potentially mixed populations. Understanding how these cells interact with one another is critical to understanding the natural cycles for the elements (e.g., C, N, and S), the response of ecosystems to environmental challenges (for example, acid rain), and the rate and extent of degradation of chemicals introduced into such ecosystems.

As discussed in Chapter 14, "Utilizing Genetically Engineered Organisms," many populations with recombinant DNA behave as mixed-culture systems. Some members of the population will lose or modify the inserted gene (often carried on a plasmid). Although a single species is present, the formation of mutant or plasmidless cells leads to

a distinct subpopulation. The interaction of the subpopulation with the original population follows the principles developed in this chapter for mixed populations.

16.1. MAJOR CLASSES OF INTERACTIONS IN MIXED CULTURES

The major interactions between two organisms in a mixed culture are competition, neutralism, mutualism, commensalism, amensalism, and prey–predator interactions. Table 16.1 summarizes these interactions.

Competition is an indirect interaction between two populations that has negative effects on both. In competition, each population competes for the same substrate. Two populations or microorganisms with similar nutrient requirements usually compete for a number of common, required nutrients when grown together.

TABLE 16.1. Scheme of Classification of Binary Population Interactions (Roles of *A* and *B* May Be Reversed)*

Effect of Presence of *B* on Growth Rate of *A*	Effect of Presence of *A* on Growth Rate of *B*	Qualifying Remarks	Interaction
Indirect interactions			
−	−	Negative effects caused by removal of resources	Competition
−	−	Negative effects caused by production of toxins or inhibitors	Antagonism
−	O		Amensalism
−	+	Negative effects caused by production of lytic agents; positive effects caused by solubilization of biomass	Eccrinolysis
+	O	Positive effects caused by production by *B* (host) of a stimulus for growth of *A* (commensal) or by removal by *B* of an inhibitor for growth of *A*	Commensalism
+	+	See remarks for commensalism; also, presence of both populations is not necessary for growth of both	Protocooperation
+	+	See remarks for commensalism; also, presence of both populations is necessary for growth of either	Mutualism
−	+	*B* feeds on *A*	Feeding (includes predation and suspension feeding)
Direct interactions			
−	+	The parasite (*B*) penetrates the body of its host (*A*) and converts the host's biomaterial or activities into its own	Parasitism
+	+ (or perhaps O)	*A* and *B* are in physical contact; interaction highly specific	Symbiosis
−	−	Competition for space	Crowding

*Negative growth response denoted by "–"; positive growth response by "+"; neutral response by "O."

With permission, from A. G. Fredrickson, in *Foundations of Biochemical Engineering*, American Chemical Society Symposium Series, Vol. 207, pp. 201–228, 1983.

The outcome of competition between two species for the *same growth-limiting substrate* in an open system (e.g., a chemostat) is determined by the specific growth-rate-limiting substrate concentration relationship. Two different cases can be distinguished in a mixed culture of two competing species (Figure 16.1):

- μ_a is always greater than μ_b. The organisms with the fastest growth rate will displace the others from the culture. This is known as the *exclusion principle.*
- Crossover in μ–S relationship. In this case, the faster-growing organism is determined by the dilution rate. Depending on the dilution rate, three different cases may be identified:
  - At the crossover point $D = \mu_x$; $S = S_x$, two species could be maintained in a chemostat at $D = \mu_x$. However, this is an unstable operating point.
  - If $D > \mu_x$, then $\mu_a > \mu_b$, and *B* will be washed out; *A* will dominate.
  - If $D < \mu_x$, then $\mu_a > \mu_u$, and *A* will be washed out; *B* will dominate.

In a batch system, both species would exist in culture media. The ratio of number density of species at a given time will be determined by the relative magnitudes of the specific growth rates and the initial concentrations of cells.

Neutralism is an interaction where neither population is affected by the presence of the other. That is, there is no change in the growth rate of either organism due to the presence of the other. Neutralism is relatively rare. One example of neutralism is the growth of yogurt starter strains of *Streptococcus* and *Lactobacillus* in a chemostat. The total counts of these two species at a dilution rate of 0.4 h^{-1} are quite similar whether the populations are cultured separately or together. Neutralism may occur in special environments where each species consumes different limiting substrates and neither species is affected by the end products of the other.

Mutualism and *protocooperation* are more common than neutralism and may involve different mechanisms. In both cases, the presence of each population has a positive effect on the other. For mutualism, the interaction is essential to the survival of both species. In protocooperation, the interaction is nonessential. One mechanism is the mutual exchange of required substances or the removal of toxic end products by each organism. The metabolisms of partner populations must be complementary to yield a mutualistic interaction. An example is the growth of a phenylalanine-requiring strain of *Lactobacillus* and a folic-acid-requiring strain of *Streptococcus* in a mixed culture. Exchange of the growth factors phenylalanine and folic acid produced by partner organisms helps each organism to grow in a

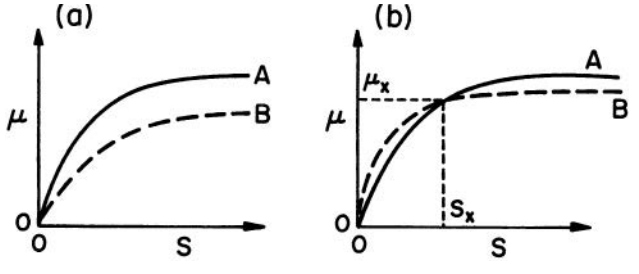


Figure 16.1. μ–S relationship for two competing species (*A* and *B*) in a mixed culture. Case (a) corresponds to $\mu_{mA} > \mu_{mB}$ and $K_{SA} \leq K_{SB}$, while case (b) corresponds to a case where $\mu_{mA} > \mu_{mB}$, but $K_{SA} < K_{SB}$. In case (b), the growth curves cross and *A* and *B* could potentially coexist at $D = \mu_x$ and $S = S_x$ in a chemostat.

mixed culture, while separate pure cultures exhibit no growth. Another example of mutualistic interaction exists between aerobic bacteria and photosynthetic algae. Bacteria use oxygen and carbohydrate for growth and produce CO_2 and H_2O. The algae convert CO_2 to carbohydrates and liberate oxygen in the presence of sunlight.

Note that *symbiosis* and mutualism are not the same. Symbiosis implies a relationship when two organisms live together. A symbiotic relationship may be mutualistic, but it may also be neutralistic, parasitic, commensalistic, and so on.

Commensalism is an interaction in which one population is positively affected by the presence of the other. However, the second population is not affected by the presence of the first population. Various mechanisms may yield a commensal interaction. Two common mechanisms are the following:

- **Type 1:** The second population produces a required nutrient or growth factor for the first population.
- **Type 2:** The second population removes a substance from the medium that is toxic to the first population.

An example of the first type of commensal interaction is the production of H_2S by *Desulfovibrio* (through the reduction of SO_4^{2-}), which is used as an energy source by sulfur bacteria (Figure 16.2).

An example of a type 2 commensal interaction is the removal of lactic acid by the fungus *Geotrichum candidum*, which allows the growth of *Streptococcus lactis.* This interaction is utilized in cheese making using *S. lactis.* Lactic acid produced by *S. lactis* inhibits the growth of the bacteria. The fungus metabolizes lactic acid and improves the growth conditions for *S. lactis.*

Amensalism is the opposite of commensalism. In amensalism, population *A* is negatively affected by the presence of the other population (*B*). However, population *B* is not affected by the presence of population *A.* Various amensal interaction mechanisms are possible. Two common mechanisms are the following:

- **Type 1:** Population *B* produces a toxic substance that inhibits the growth of population *A.*
- **Type 2:** Population *B* removes essential nutrients from the media, thus negatively affecting the growth of population *A.*

One example of the first type of amensal interaction is the production of antibiotics by certain molds to inhibit the growth of others. Some microbes excrete enzymes that decompose cell-wall polymers. Such organisms destroy their competitors and

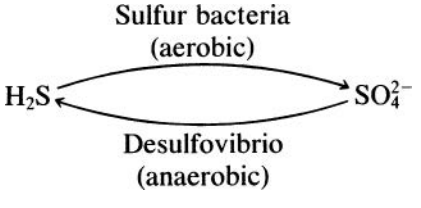


Figure 16.2. Example of a type 1 commercial interaction.

also utilize the nutrients released by the lysed cells. The microbial synthesis of organic acids reduces pH and inhibits the growth of other organisms.

Predation and *parasitism* are interactions in which one population benefits at the expense of the other. These two interactions are distinguished by the relative size of organisms and the mechanisms involved. Predation involves the ingestion of prey by the predator organism. A good example of prey–predator interaction is the ingestion of bacteria by protozoa. This interaction is common in aerobic waste-treatment reactors such as activated sludge units. In parasitism, the host, which is usually the larger organism, is damaged by the parasite. The parasite benefits from utilization of nutrients from the host. A common example of parasitism is the destruction of microorganisms by microphages. Although the physical mechanisms in predation and parasitism differ, these two phenomena have many common features in their conceptual and mathematical descriptions.

In an open system, such as a chemostat where predator–prey interactions take place, the populations of predator and prey do not necessarily reach steady state but can oscillate at certain dilution rates. At the beginning of the operation prey concentration is high, but predator concentration is low. As the predators consume prey, the number of predators increases and the prey concentration decreases. After a while, a small prey population cannot support the large predator population, and the predator population decreases while prey population increases. Depending on the dilution rate and feed substrate concentration, these oscillations may be sustained or damped or may not exist.

Finally, note that these interactions can, and often do, exist in combination. For example, A and B may compete for glucose as a nutrient, but A requires a growth factor from B to grow. In such a case, both competition and commensalism would be present.

16.2. SIMPLE MODELS DESCRIBING MIXED-CULTURE INTERACTIONS

Multiple interacting species can give rise to very complex behavior. In some cases, coexistence of species is prohibited; in others, complex sustained oscillatory behavior may be observed. Multiple steady states are possible.

Writing the appropriate equations to describe mixed populations follows the principles we discussed in Chapter 6, "How Cells Grow." In each case, a balance must be written for each species (organism, rate-limiting substrate, or product), and these balances will be the same as we have used previously. Although we may write chemically structured models for each organism, we confine our discussions to the use of unstructured models for each species. Even so, the population model is still structured in the sense that the whole biomass is divided into distinct subpopulations.

These equations are often applied to chemostats that mimic many ecosystems. When these equations are solved, they often yield multiple steady states. For each steady state we need to analyze its stability. An unstable steady state will never be realized in practice; using phase-plane portraits, we can explore the approach to the

steady state and the determination of the presence or absence of limit cycles. It is often easier to test a steady state for its local stability (linearized stability analysis) than for its global stability.

With this background, we will consider some representative examples.

Example 16.1

Competition of two species for the same growth-rate-limiting substrate is common. Determine when the two organisms may stably coexist if both *A* and *B* follow Monod kinetics.

Solution For this situation, the following equations describe the dynamic situation:

$$\frac{dX_A}{dt} = -DX_A + \frac{\mu_{mA} S}{K_{SA} + S} X_A \tag{16.1}$$

$$\frac{dX_B}{dt} = -DX_B + \frac{\mu_{mB} S}{K_{SB} + S} X_B \tag{16.2}$$

$$\frac{dS}{dt} = D(S_0 - S) - \frac{1}{Y_{X_{A/S}}} \frac{\mu_{mA} S X_A}{K_{SA} + S} - \frac{1}{Y_{X_{B/S}}} \frac{\mu_{mB} S}{K_{SB} + S} X_B \tag{16.3}$$

If both *A* and *B* coexist, we would require the following from equations 16.1 and 16.2:

$$D = \frac{\mu_{mA} S}{K_{SA} + S} = \frac{\mu_{mB} S}{K_{SB} + S} \tag{16.4}$$

Equation 16.4 can be solved to yield

$$S = \frac{\mu_{mB} K_{SA} - \mu_{mA} K_{SB}}{\mu_{mA} - \mu_{mB}} \tag{16.5}$$

Equation 16.5 is meaningful only if $S \geq 0$. Consider the two cases in Figure 16.1. In case (a), $\mu_{mA} K_{SB} > \mu_{mB} K_{SA}$ and $\mu_{mA} > \mu_{mB}$; consequently, S is always less than zero, and coexistence is impossible. In case (b), however, a value of S can be found from equation 16.5 that will allow the populations to coexist. The corresponding D for the crossover point is D_c.

Although this coexistence is mathematically obtainable, it is not physically attainable in real systems. In real systems, the dilution rate will vary slightly with time, and, in fact, the variation will show a bias. Using an analysis more sophisticated than appropriate for this text, it can be demonstrated that one competitor will be excluded from the chemostat if the intensity of the "noise" (random fluctuations) in D and the bias of the mean of D away from D_c are not both zero. Also, it is possible for either competitor to be excluded, depending on how D varies.[†]

Two competitors can coexist if we modify the conditions of the experiments. Examples of such modifications include allowing spatial heterogeneity (the system

[†] G. N. Stephanopoulos, R. Aris, and A. G. Fredrickson, *Mathematical Biosciences* 45: 99, 1979.

is no longer well mixed or wall growth is present) or another level of interaction is added (e.g., adding a predator or interchange of metabolites). Also, operation of the chemostat in a dynamic mode (D is a function of time) can sometimes lead to coexistence. It is also interesting to note that the use of other rate expressions (e.g., substrate inhibition) can lead to multiple crossover points and potentially multiple steady states.

Example 16.2

Consider a case of mutualistic growth in a chemostat. How would you write the appropriate equations for this system? Determine the effects of the addition of competition to mutualism.

Solution The physical model is for A and B as two separate species. A produces P_A as a by-product of growth and B produces P_B. Organism B requires P_A to grow, while A requires P_B. The feed to a chemostat contains all essential nutrients except for P_A and P_B, and A and B may compete for substrate, S, in the feed.

For this case, the most general description is

$$\frac{dX_A}{dt} = -DX_A + \mu_A X_A \tag{16.6}$$

$$\frac{dX_B}{dt} = -DX_B + \mu_B X_B \tag{16.7}$$

$$\frac{dP_A}{dt} = -DP_A + Y_{P_A}\mu_A X_A - \frac{1}{Y_{X_B/P_A}}\mu_B X_B \tag{16.8}$$

$$\frac{dP_B}{dt} = -DP_B + Y_{P_B}\mu_B X_B - \frac{1}{Y_{X_A/P_B}}\mu_A X_A \tag{16.9}$$

And if the growth of either A or B is limited by S, then we need to consider the following:

$$\frac{dS}{dt} = D(S_0 - S) - \frac{1}{Y_{X_{A/S}}}\mu_A X_A - \frac{1}{Y_{X_{B/S}}}\mu_B X_B \tag{16.10}$$

Note that Y_{X_B/P_A} is the biomass yield of B using P_A as substrate, and Y_{P_A} is the amount of P_A made per unit mass of A. Similar definitions apply to Y_{X_A/P_B} and Y_{P_B}. If we consider the pure mutualistic state, then we ignore equation 16.10. For a coexistent state to exist, $D = \mu_A = \mu_B$. It is also clear that the rate of production of P_A and P_B must exceed their consumption. Thus,

$$Y_{P_A}X_A > \frac{X_B}{Y_{X_B/P_A}} \tag{16.11}$$

and

$$Y_{P_B}X_B > \frac{X_A}{Y_{X_A/P_B}} \tag{16.12}$$

It then follows that

$$Y_{P_A} Y_{P_B} X_A X_B > \frac{1}{Y_{X_B/P_A}} \cdot \frac{1}{Y_{X_A/P_B}} X_A X_B \tag{16.13a}$$

or

$$Y_{P_A} Y_{P_B} > \frac{1}{Y_{X_B/P_A}} \cdot \frac{1}{Y_{X_A/P_B}} \tag{16.13b}$$

The following is also clear:

$$D < \min(\mu_{mA}, \mu_{mB}) \tag{16.14}$$

Equations 16.13b and 16.14 determine whether equations 16.6 to 16.9 allow the potential existence of a purely mutualistic steady state.

The stability of such a coexistent state has been examined, where μ_A and μ_B were represented by various growth functions. Using a linear stability analysis, it can be shown that this pure mutualistic state results in a saddle point, and the system is unstable for all physically accessible values of D. If, however, the growth-rate-limiting substrate for either A or B is S, then a stable coexistent state can be found.

Another well-known case is that of prey–predator relationships. We contrast here the classical Lotka–Volterra model with a model that explicitly includes the effect of substrate consumption. We first derive the equations for a prey–predator–substrate relationship based on chemostat culture. Consider the equations to describe growth of a bacteria on a limiting substrate such as glucose and the growth of a protozoan as a predator. This situation leads to mass balances on substrate, prey, and predator in the chemostat, as given in equations 16.15, 16.16, and 16.17:

Substrate:
$$\frac{dS}{dt} = D(S_0 - S) - \frac{1}{Y_{X_b/s}} \frac{\mu_{mb} S}{K_b + S} X_b \tag{16.15}$$

Prey:
$$\frac{dX_b}{dt} = -DX_b + \frac{\mu_{mb} S}{K_b + S} X_b - \frac{1}{Y_{X_p/b}} \frac{\mu_{mp} X_b X_p}{K_p + X_b} \tag{16.16}$$

Predator:
$$\frac{dX_p}{dt} = -DX_p + \frac{\mu_{mp} X_b X_p}{K_p + X_b} \tag{16.17}$$

In these equations, $Y_{Xb/S}$ and $Y_{Xp/b}$ are the yield coefficients for the growth of prey on substrate and the growth of predator on prey, respectively.

This model was used to describe the behavior of *Dictyostelium discoideum* and *E. coli* in a chemostat culture and was found to predict experimental results quite well. A variety of types of coexistence behavior have been revealed by stability analysis. These include no oscillations, damped oscillations, and sustained oscillations (see Figure 16.3).

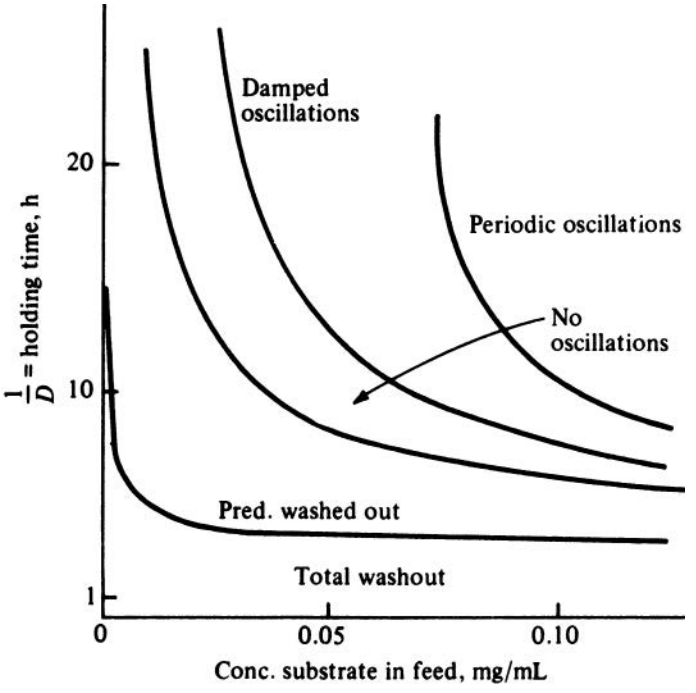


Figure 16.3. Dynamic behavior that can result from the model developed in Example 16.3. (With permission, from H. M. Tsuchiya and others, Predator-Prey Interactions of Dictyostelium discoideum and Escherichia coli in Continuous Culture, *J. Bacteriol.*, vol. 110, p. 1147, 1972. Reprinted with permission from American Society for Microbiology.)

This model, based on saturable substrate uptake, can be compared to the classical model developed by Lotka and Volterra almost a century ago. The Lotka–Volterro model does not consider that substrate influences the growth role of the prey and does not assume an open system, such as a chemostat. The equations describing this system follow:

Prey:
$$\frac{dX_b}{dt} = \mu'_b X_b - \frac{\mu'_p X_b X_p}{Y_{p/b}} \tag{16.18}$$

Predator:
$$\frac{dX_p}{dt} = -k'_d X_p + \mu'_p X_b X_p \tag{16.19}$$

The first term in equation 16.18 describes the growth of prey on substrate, and the second describes the consumption of prey by predators. The first and second terms in equation 16.19 describe the death of predator in the absence of prey and the growth of predator on prey, respectively. $Y_{p/b}$ is the yield of predators on prey (g/g), μ'_b is the specific growth rate of prey on a soluble substrate (h^{-1}), μ'_p is the growth rate of predator on prey (l/g-h), and k'_d is the specific death rate of the predator (h^{-1}).

Equations 16.18 and 16.19 allow a steady-state solution for batch growth, where dX_b/dt and $dX_p/dt = 0$. Under these conditions, $X_{bF} = k'_p/\mu'_p$ and $X_{pF} = \mu'_b Y_{p/b}/\mu'_p$.

By defining dimensionless variables such as

$$\overline{X}_b = \frac{X_b}{X_{bF}}, \overline{X}_p = \frac{X_p}{X_{pF}} \tag{16.20}$$

we can express equations 16.18 and 16.19 in terms of $\overline{X}_b$ and $\overline{X}_p$:

$$\frac{d\overline{X}_b}{dt} = \mu'_b(1-\overline{X}_p)\overline{X}_b \tag{16.21}$$

$$\frac{d\overline{X}_p}{dt} = -k'_d(1-\overline{X}_b)\overline{X}_p \tag{16.22}$$

Equations 16.21 and 16.22 can be solved with the initial conditions of $\overline{X}_b(0) = \overline{X}_{b_0}$ and $\overline{X}_p(0) = \overline{X}_{p_0}$.

The relationship between $\overline{X}_b$ and $\overline{X}_p$ can be determined by dividing equation 16.21 by equation 16.22:

$$\frac{d\overline{X}_b}{d\overline{X}_p} = \frac{\mu'_b\overline{X}_b(1-\overline{X}_p)}{-k'_d\overline{X}_p(1-X_b)} \tag{16.23}$$

Integration of equation 16.23 yields

$$\mu'_b \ln \overline{X}_p - \mu'_b\overline{X}_p + k'_d \ln \overline{X}_b - k'_d\overline{X}_b = K \tag{16.24a}$$

or

$$\left(\frac{\overline{X}_p}{e^{\overline{X}_p}}\right)^{\mu'_b}\left(\frac{\overline{X}_b}{e^{\overline{X}_p}}\right)^{k'_d} = e^K \tag{16.24b}$$

where K is an integration constant that is a function of the initial population sizes.

The phase-plane analysis of the system can be made using equation 16.24. Figure 16.4 describes the limit cycles (oscillatory trajectories) of prey–predator populations for different initial population levels.

The Lotka–Volterra model considers the exponential growth of prey species in the absence of predator and neglects the utilization of substrate by prey species

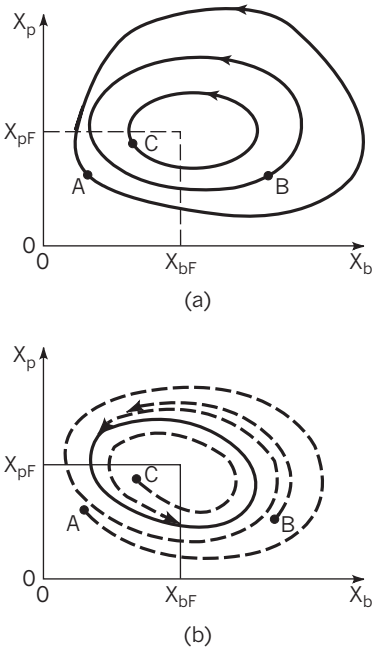


Figure 16.4. Schematics of phase-plane portraits for prey–predator interactions. (a) Limit cycles predicted by Lotka–Volterra (soft oscillations where initial conditions determine the dynamic behavior). (b) Limit-cycle prediction using the model developed in Example 16.3 (hard oscillations where the limit cycle is independent of initial conditions). The predicted steady-state point is defined by X_{pF} and X_{bF}. *A, B,* and *C* represent different initial conditions.

according to Monod form. The Lotka–Volterra oscillations depend on initial conditions and change their amplitude and frequency in the presence of an external disturbance. These types of oscillation are called soft oscillations. The other model based on Monod rate expressions (equations 16.15, 16.16, and 16.17) explains the more stable and sustained oscillations observed in nature, which are independent of initial conditions (that is, hard oscillations).

16.3. MIXED CULTURES IN NATURE

Mixed cultures of organisms are common in natural ecological systems. Microorganisms are involved in the natural cycles of most elements (e.g., carbon, nitrogen, oxygen, and sulfur). Simplified diagrams of the carbon and nitrogen cycles are presented in Figures 16.5 and 16.6. Organisms living in soil and aquatic environments actively participate in carbon and nitrogen cycles. For example, certain organisms fix atmospheric CO_2 to form carbohydrates, while others degrade carbohydrates and release CO_2 into the atmosphere. Similarly, some organisms fix atmospheric nitrogen (N_2) to form ammonium and proteins, while others convert ammonium into nitrite and nitrate (nitrification), and others reduce nitrate into atmospheric nitrogen (denitrification). Sulfur-oxidizing organisms convert reduced sulfur compounds

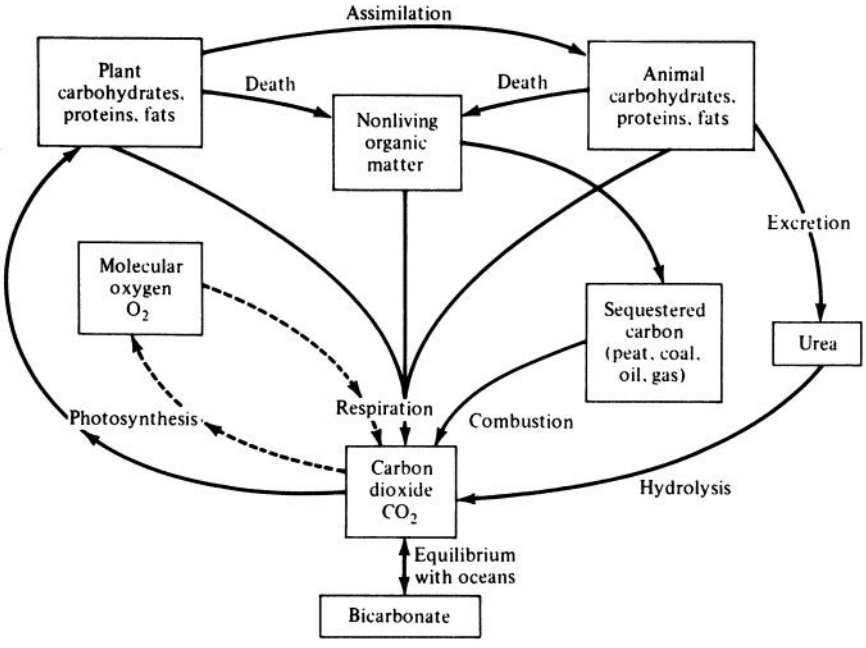


Figure 16.5. Simplified diagram of the carbon cycle. Dashed lines show the major component of the oxygen cycle, which is closely linked to the cycle of carbon. (With permission, from J. E. Bailey and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2d ed., McGraw-Hill Book Co., New York, 1986, p. 914.)

(sulfur and sulfide) into sulfate, and sulfate-reducing organisms reduce sulfate into hydrogen sulfide.

The aforementioned interactions among different species take place in natural systems in a more complicated manner. The complexity of such a system is depicted in Figure 16.7 for an anaerobic environment. Understanding such complex cycles is important to understanding the capacity of natural ecosystems to degrade organic pollutants.

16.4. INDUSTRIAL UTILIZATION OF MIXED CULTURES

Defined mixed microbial populations are commonly used in cheese making, a good example of using mixed cultures in food production. Cheeses of various types are produced by inoculating pasteurized fresh milk with appropriate lactic acid organisms. The bacteria used for lactic acid production are various species of *Streptococcus* and *Lactobacillus* in a mixed culture. Other organisms are used to develop flavor and aroma. Among these are *Brevibacterium linens, Propionibacterium shermanii, Leuconostoc* sp., and *Streptococcus diacetilactis.* After inoculation

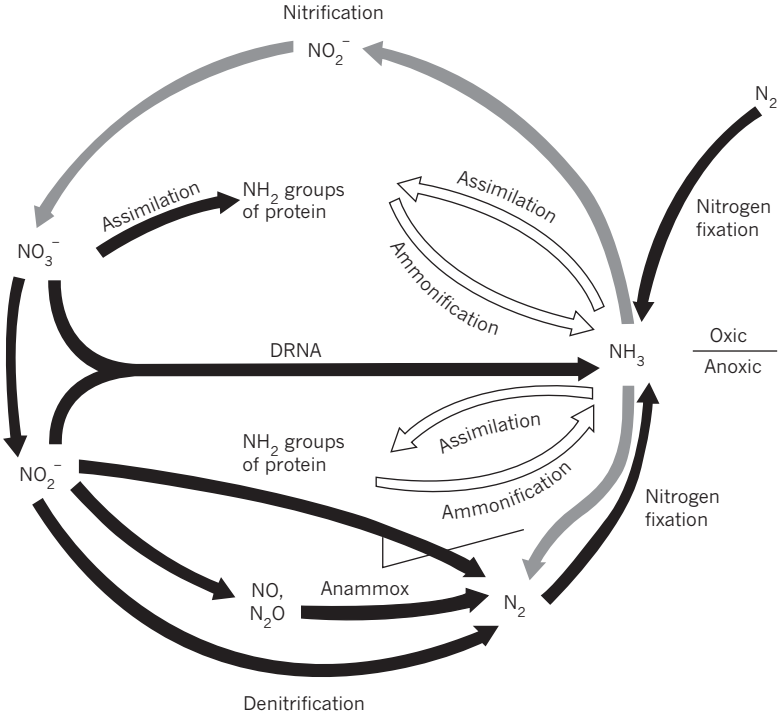


Figure 16.6. Redox cycle for nitrogen. (Brock, Thomas D.; Brock, Katherine M.; Ward, David M., *Basic Microbiology with Applications*, 14th ed., © 2015. Reprinted and electronically reproduced by permission of Pearson Education, Inc., New York, NY.)

of pasteurized milk, a protein-rich curd is precipitated by the acidity of the medium, and the liquid is drained off. The precipitated curd is allowed to age by action of bacteria or mold. Some molds used in cheese making are *Penicillium camemberti* and *Penicillium roqueforti.*

Lactic acid bacteria are also used in whiskey manufacture. *Lactobacillus* added to the yeast reduces pH and, therefore, the chance of contamination. *Lactobacillus* also contributes to the flavor and aroma of whiskey. A favorable interaction between yeast and lactic acid bacteria exists in ginger-beer fermentation.

The utilization of undefined mixed microbial cultures in waste-treatment processes is typical and unavoidable. Wastewater treatment constitutes one of the largest-scale uses of bioprocesses. Mixed cultures are also utilized in the anaerobic digestion of waste materials. Cellulase producers, acid formers, and methane producers are typical organisms involved in the anaerobic digestion of cellulosic wastes. However, attempts to encourage the growth of a particular species on waste materials have been made.

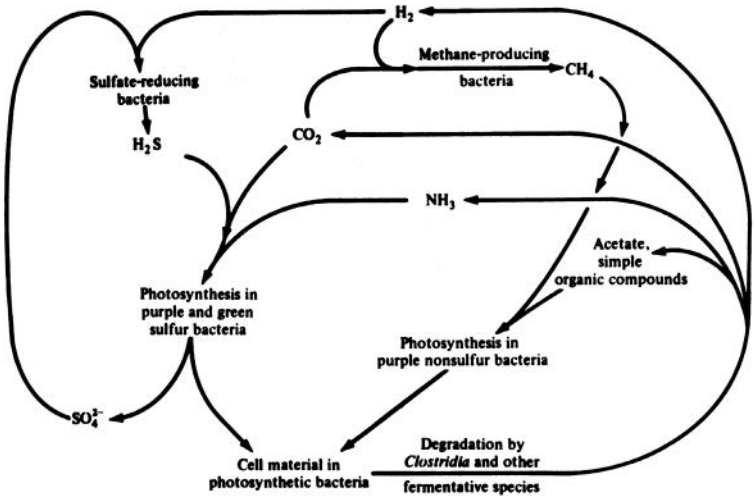


Figure 16.7. Simplified schematic of the cycles of matter in an anaerobic environment. (With permission, from J. E. Bailey and D. F. Ollis, *Biochemical Engineering Fundamentals,* 2d ed., McGraw-Hill Book Company, New York, 1986, p. 918.)

The Symba process was developed in Sweden for treating starchy wastes, particularly those from potato processing. This process utilizes *Endomycopsis fibuligera* for amylase production and a yeast, *Candida utilis*, for the utilization of sugar molecules produced from the hydrolysis of starch. Single-cell protein (SCP) is produced simultaneously with potato waste treatment.

Corn and pea wastes are also treated by a mixed culture of *Trichoderma viride* and *Geotrichum* sp. *T. viride* produces cellulase to break down cellulose into reduced sugar molecules, and *Geotrichum* produces amylases to break down starch into reduced sugar molecules. Both organisms utilize reduced sugar molecules for growth.

A mixed culture of *Candida lipolytica* and *Candida tropicalis* has been grown on hydrocarbons, *n*-paraffins, or gas oil for SCP production purposes in both laboratory- and pilot-scale operations. The utilization of a mixed culture of yeasts was proved to yield better product quality as compared to pure yeast strains.

Gaseous hydrocarbon substrates such as methane can be utilized by certain bacteria to produce SCP. Several experimental studies have shown that mixed cultures of methane-utilizing organisms grow faster than pure cultures.

Certain methane-utilizing species of *Pseudomonas* oxidize methane to methanol. However, *Pseudomonas* is inhibited by the end product, methanol. Inclusion of a methanol-utilizing bacteria such as *Hyphomicrobium* into the growth medium eliminates the problem of methanol inhibition. This relationship is mutualistic in the sense that *Pseudomonas* supplies carbon source (CH_3OH) for *Hyphomicrobium,* and *Hyphomicrobium* removes the growth inhibitor (methanol) of *Pseudomonas*:

$$CH_4 \xrightarrow{Pseudomonas} CH_3OH \xrightarrow{Hyphomicrobium} CO_2 + H_2O \qquad (16.25)$$

Feedback inhibition

16.5. BIOLOGICAL WASTE TREATMENT

Biological waste treatment is an example of the industrial utilization of mixed cultures. Waste materials generated in a society can be classified in three major categories:

- Industrial wastes are produced by various industries, and waste characteristics vary greatly from one industry to another. Industrial wastes usually contain hydrocarbons, carbohydrates, alcohols, lipids, and aromatic organics. Industrial wastes are rich in carbon compounds and usually deficient in nitrogen (high C/N ratio); therefore, the biological treatment of industrial wastes usually requires supplemental addition of nitrogen compounds and other nutrients. The presence of potentially toxic compounds must be carefully considered in devising a treatment strategy.
- Domestic wastes are treated by municipalities and derive from humans and their daily activities. They include ground garbage, laundry water, excrement, and often some industrial wastes that have been sewered into the municipal system. Domestic waste varies significantly with time in terms of flow and composition due to the periodic nature of human activity (e.g., flow decreases at night when most people sleep).
- Agricultural wastes are produced by farm animals (e.g., manure) and include waste plants, such as straws. Agricultural wastes are usually carbon rich because of high cellulosic material content, although some wastes, such as poultry manure, are high in nitrogen.

Each of these waste materials has its own characteristics, and treatment methods vary depending on these characteristics.

Three major waste treatment methods are the following:

- *Physical treatment* includes screening, flocculation, sedimentation, filtration, and flotation, which are usually used for the removal of insoluble materials.
- *Chemical treatment* includes chemical oxidations (chlorination, ozonation) and chemical precipitation using $CaCl_2$, $FeCl_2$, $Ca(OH)_2$, or $AL_2(SO_4)_3$.
- *Biological treatment* includes the aerobic and anaerobic treatment of wastewater by a mixed culture of microorganisms.

Certain characteristics of wastewater need to be known before treatment. Among them are physical characteristics, such as color, odor, pH, temperature, and solids contents (suspended and dissolved solids); and chemical characteristics, such as organic and inorganic compounds. Major carbon compounds in a typical industrial waste are carbohydrates, lipids–oils, hydrocarbons, and proteins. Other compounds, such as phenols, surfactants, herbicides, pesticides, and aromatic compounds, are usually in relatively small concentrations (<1 g/l) but are difficult to degrade by biological means. Among inorganic compounds present in wastewater are nitrogenous compounds (NH_4^+, NO_3^-), sulfur compounds (SO_4^{2-}, S^{2-}, S^0, S_3^{2-}), phosphorus

compounds (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^{1-}$), heavy metals (Ni^{2+}, Pb^{2+}, Cd^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}), and dissolved gases, such as H_2S, NH_3, and CH_4.

The carbon content (strength) of a wastewater sample can be expressed in several ways: *biological oxygen demand* (BOD), *chemical oxygen demand* (COD), and *total organic carbon* (TOC). Normally, a 5-day BOD value is reported. The BOD_5 is the amount of dissolved oxygen (DO) consumed when a wastewater sample is seeded with active bacteria and incubated at 20°C for 5 days. Since the amount of oxygen consumed is stoichiometrically related primarily to the organic content of wastewater, BOD is a measure of the strength of wastewater. This stoichiometric coefficient is not always known, since the composition of the organics is usually unknown. Also, some nitrogen-containing or inorganic compounds will exert an oxygen demand. If the only organic compound is glucose, oxygen consumption can be easily related to the carbon content of wastewater under aerobic conditions:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \tag{16.26}$$

According to the stoichiometry of this reaction, 1.07 g of oxygen is required for the oxidation of 1 g of glucose.

Samples of wastewater need to be properly diluted to obtain an accurate BOD_5 measurement, seeded with active bacteria, and incubated at 20°C for 5 days along with an unseeded blank. BOD_5 is calculated using the following equation:

$$BOD_5 = [(DO)_{t=0} - (DO)_{t=5}]_{sample} - [(DO)_{t=0} - (DO)_{t=5}]_{blank} \tag{16.27}$$

BOD measurements have some shortcomings. This method is applicable only to biodegradable, soluble organics and requires a high concentration of active bacteria preadapted to this type of waste. Moreover, if organic compounds are refractory, 5 days of incubation may not suffice, and 20 days of incubation (BOD_{20}) may be required.

COD is a measure of the concentration of chemically oxidizable organic compounds present in wastewater. Organic compounds are oxidized by a strong chemical oxidant, and using the reaction stoichiometry, the organic content is calculated. Almost all organic compounds present in wastewater are oxidized by certain strong chemical oxidants. Therefore, the COD content of a wastewater sample usually exceeds the measured BOD ($COD > BOD_5$).

A typical chemical oxidation reaction (unbalanced) is

$$C_aH_bO_c + Cr_2O_7^{2-} + H^+ \xrightarrow{\text{heat}} Cr^{3+} + CO_2 + H_2O \tag{16.28}$$

Dichromate may be used as an oxidizing agent, and by a redox balance, the amount of oxygen required to oxidize organic compounds can be calculated. This method is faster (order of 3 hours), easier, and less expensive than BOD measurements.

The TOC content of wastewater samples can be determined by using a TOC analyzer. After proper dilutions, samples are injected into a high-temperature

(900° to 950°F) furnace, and all organic carbon compounds are oxidized to CO_2, which is measured by an infrared analyzer. To determine the TOC content, wastewater samples should be acidified to remove inorganic carbon compounds (mainly carbonates). The total carbon content of wastewater can be determined before and after acidification, and the difference is inorganic carbon content.

The nitrogen content of wastewater samples is usually measured by total Kjeldahl nitrogen (TKN) determination. Other key nutrient concentrations, such as phosphate, sulfur, and toxic compounds, should be determined before waste streams are treated.

The concentration of biomass in a waste-treatment system using suspended cells is measured as mixed-liquor volatile suspended solids (MLVSS). Basically, wastewater of known volume is filtered, and the collected solids are dried and weighed to give mixed-liquor suspended solids (MLSS). This material is then *volatilized* by burning in air at 600°C. The weight of the remaining noncombustible, inorganic material is the *fixed* solids. The difference between the original mass prior to combustion and the fixed solids is the *volatile* portion. The volatile portion, or MLVSS, is assumed to be primarily microbes, although carbonaceous particles are included in the measurement.

A typical waste-treatment operation employing biological treatment includes the following steps:

1. Primary treatment includes the removal of coarse solids and suspended matter (screening, sedimentation, filtration) and conditioning of the wastewater stream by pH adjustment and nutrient additions (e.g., PO_4^{3-}, NH_4^+).
2. Secondary treatment is the major step in biological treatment; it includes biological oxidation or anaerobic treatment of soluble and insoluble organic compounds. Organic compounds are oxidized to CO_2 and H_2O by organisms under aerobic conditions. Unoxidized organic compounds and solids from aerobic treatment (e.g., cell wall material, lipids–fats) are decomposed to a mixture of CH_4, CO_2, and H_2S under anaerobic conditions. A *sludge* of undecomposed material must be purged from either system.
3. Tertiary treatment includes the removal of the remaining inorganic compounds (phosphate, sulfate, ammonium) and other refractory organic compounds by one or more physical separation methods, such as carbon adsorption, deep-bed filtration, and in some cases membrane-based techniques, such as reverse osmosis or electrodialysis.

16.5.1. Biological Waste-Treatment Processes

Biological wastewater treatment usually employs a mixed culture of organisms whose makeup varies with the nature of the waste. Biological treatment may be aerobic or anaerobic. The major aerobic processes (or reactor types) used in wastewater treatment are activated sludge, trickling filter, rotating biological contractors, and oxidation ponds.

Activated-sludge processes include a well-agitated and aerated continuous-flow reactor and a settling tank. Depending on the physical design of the tank and how

feed is introduced into the tank, it may approximate either a plug flow reactor (PFR) or continuous-flow, stirred-tank reactor (CFSTR). A long, narrow tank with single feed approaches PFR behavior; circular tanks approach CFSTR. The concentrated cells from the settling tank are recycled back to the stirred-tank reactor. Figure 16.8 is a schematic of a typical activated-sludge process. Usually, a mixed culture of organisms is utilized in the bioreactor. Some of these organisms may produce polymeric materials (polysaccharides), which help the organisms to agglomerate. Floc formation is a common phenomenon encountered in activated-sludge processes, which may impose some mass-transfer limitations on the biological oxidation of soluble organics; but good floc formation is essential to good performance of the system, since large dense flocs are required in the sedimentation step. Cell recycle from the sedimentation unit improves the volumetric rate of biological oxidation (i.e., high-density culture) and therefore reduces the residence time or volume of the sludge reactor for a given feed rate. The recycle ratio needs to be controlled to maximize BOD removal rate.

The selection of aerator and agitators is a critical factor in the design of activated-sludge processes. The aeration requirements vary depending on the strength of the wastewater and cell concentration. Oxygen requirements for a typical activated-sludge process are about 30 to 60 m^3O_2/kg of BOD removed. Various aeration devices with and without mechanical agitation can be used in activated-sludge units. Mechanical surface aerators are widely used for shallow activated-sludge units. Surface aerators consist of partially submerged impellers attached to motors mounted on fixed structures. Surface aerators spray liquid and create rapid changes at the air–water interface to enhance oxygen transfer. Pure oxygen may be used for high-strength wastewater treatment. Also, stagewise operation with pure oxygen has been found to be a very effective method of wastewater treatment. The UNOX process, first developed by Union Carbide, is based on this concept. Other forms of aeration include bubble aerators and fixed turbines similar to those we discussed in Chapter 10, "Selection, Scale-Up, Operation, and Control of Bioreactors."

The activated-sludge system faces many uncontrolled disturbances in input parameters, such as waste flow and composition. Such disturbances can lead to system failure (less-than-adequate treatment of the waste stream). One type of disturbance is *shock loading.* A shock load indicates the sudden input (pulse) of a high

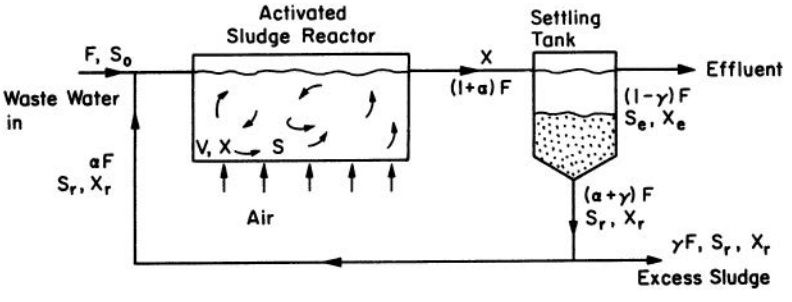


Figure 16.8. Schematic diagram of an activated-sludge unit.

concentration of a toxic compound. A CFSTR design, of course, is less affected by such inputs than a PFR design.

One response to disturbances is sludge *bulking.* A *bulking sludge* has flocs that do not settle well, and consequently cell mass is not recycled. Bulking sludge often results from a change in the composition of the microbial population in the treatment unit. For example, filamentous bacteria may dominate the normal floc-forming cells, leading to small, light flocs.

Using rate expressions for microbial growth and substrate utilization and material balances for biomass and substrate, we can determine the required volume of an activated-sludge tank for a certain degree of BOD removal. Since an activated-sludge tank contains a mixed culture of organisms, the actual kinetics of BOD removal are complicated. Usually, interactions among various species are not known. The following analysis is based on pure-culture kinetics, which is only an approximation. It is also assumed that the specific growth-rate expression is given by the Monod equation, with a death rate (or endogenous respiration rate) term:

$$\mu_{\text{net}} = \frac{\mu_m S}{K_s + S} - k_d \tag{16.29}$$

Following are steady-state material balances for biomass and rate-limiting substrates in an activated-sludge tank (Figure 16.7):

Biomass:
$$\left(\frac{\mu_m S}{K_s + S} - k_d\right) XV + \alpha F X_r = (1+\alpha) FX \tag{16.30}$$

Substrate:
$$FS_0 + \alpha F S_r = \frac{1}{Y^M_{X/s}} \left(\frac{\mu_m S}{K_s + S}\right) XV + (1+\alpha) FS \tag{16.31}$$

These equations are very similar to the case of the chemostat with recycle discussed in Chapter 9, "Operating Considerations for Bioreactors for Suspension and Immobilized Cultures." They differ in that we now include the endogenous respiration term.

Assuming no substrate utilization and cell growth in the settling tank (short residence times), material balances around the settling tank have the following yield:

Biomass:
$$(1+\alpha) FX = (1-\gamma) FX_e + (\alpha+\gamma) FX_r \tag{16.32}$$

Substrate:
$$(1+\alpha) FS = (1-\gamma) FS_e + (\alpha+\gamma) FS_r \tag{16.33}$$

where α is the ratio of sludge recycle flow rate to feed flow rate and γ is the ratio of excess sludge flow to feed flow rate.

Assuming that the substrate is not separated in the settling tank (that is, $S = S_e = S_r$), equation 16.33 can be eliminated. By rearranging equation 16.32, we can obtain

$$(1+\alpha)FX - \alpha FX_r = (1-\gamma)FX_e + \gamma FX_r \tag{16.34}$$

Substituting equations 16.34 and 16.29 into equation 16.30 yields

$$\mu_{\text{net}} VX = (1-\gamma)FX_e + \gamma FX_r \tag{16.35}$$

Defining $\mu_{\text{net}} = 1/\theta_c$, where θ_c is the cells' (solids') residence time, we obtain

$$\theta_c = \frac{1}{\mu_{\text{net}}} = \frac{VX}{F(1-\gamma)X_e + \gamma FX_r} \tag{16.36}$$

Equation 16.36 is used to calculate the cellular (solids') residence time in the sludge tank. The value of θ_c is controlled by operator choice of recycle flow rates. Hydraulic (liquid) residence time is

$$\theta_H = \frac{V}{F} = \frac{\theta_c[(1-\gamma)X_e + \gamma X_r]}{X} = \frac{(1-\gamma)X_e + \gamma X_r}{\mu_{\text{net}} X} \tag{16.37}$$

Substituting $S_r = S$ in equation 16.31 gives

$$F(S_0 - S) = \frac{1}{Y^M_{X/s}} \mu_g XV \tag{16.38}$$

or

$$V = \frac{Y^M_{X/s} F(S_0 - S)}{\mu_g X} = \frac{Y^M_{X/s} \theta_c F(S_0 - S)}{X(1 + k_d \theta_c)} \tag{16.39}$$

Equation 16.39 is used to calculate the required volume of the sludge tank for a certain degree of BOD removal ($S_0 - S$).

The resulting reactor volume can be expressed in terms of the recycle ratio by substituting equation 16.34 into equation 16.37 to yield

$$V = F\theta_c \left(1 + \alpha - \alpha \frac{X_r}{X}\right) \tag{16.40}$$

The kinetic parameters of the active organisms in the sludge tank need to be known (i.e., $\mu_m, K_s, Y^M_{X/S}$ and k_d), and other physical parameters (V, F, α, X_r, X, and S_0) need to be determined for the design (sizing, aeration requirement) of activated-sludge units. Typical hydraulic residence time for the aeration tank is 4 to 12 hours and typical sludge age is 3 to 10 days.

A summary of typical values for the kinetic parameters in biological waste treatment is presented in Table 16.2. Despite all simplifying assumptions, the pure-culture model seems to fit steady-state experimental data reasonably well, although it does not predict the dynamic performance very well.

TABLE 16.2. Model Parameters for the Activated-Sludge Process

Wastewater Composition	Kinetic Coefficients				
	$\mu_m(h^{-1})$	$K_s\,(mg/1)$	$Y^M_{X/S}\left(\frac{mg\ M}{mg\ waste}\right)$	$k_d(h^{-1})$	Basis
Domestic	0.4–0.55	50–120	0.5–0.67	$2.0-3.0\times10^{-3}$	BOD_5
Shellfish processing	0.43	96	0.58	5.8×10^{-2}	BOD_5
Yeast processing	0.038	680	0.88	3.3×10^{-3}	BOD_5
Plastic processing	0.83	167	0.30	3.3×10^{-3}	COD

D. W. Sundstrom, H. E. Klei, *Wastewater Treatment* © 1979.

Example 16.4

An industrial waste with an inlet BOD_5 of 800 mg/l must be treated to reduce the exit BOD_5 level to ≤ 20 mg/l. The inlet flow rate is 400 m^3/h. Kinetic parameters have been estimated for waste as μ_m = 0.20 h^{-1}, K_S = 50 mg/l of BOD_5, $Y^M_{X/S}$ = 0.5 mg MLVSS / mg BOD_5 and k_d = 0.005 h^{-1}. A waste treatment unit of 3200 m^3 is available. Assume a recycle ratio of 0.40 and X_e = 0. If you operate at a value of $\theta_c = 120$ h, find S and determine if sufficient BOD_5 removal is attained in a well-mixed activated-sludge process to meet specifications. What will be X and the sludge production rate from this process?

Solution Equation 16.29 can be used to estimate S:

$$\mu_{\text{net}} = \frac{1}{\theta_c} = \frac{\mu_m S}{K_s + S} - k_d \tag{16.29}$$

$$S = \frac{K_S(1 + k_d\theta_c)}{\theta_c(\mu_m - k_d) - 1}$$

$$S = \frac{(50\ \text{mg}/1)(1 + 0.005\text{h}^{-1} \cdot 120\text{h})}{120\text{h}(0.20\text{h}^{-1} - 0.005\text{h}^{-1}) - 1} = 3.57\text{mg}/1$$

Thus, these operating parameters provide more than adequate BOD_5 removal, as 3.57 mg/l is significantly less than 20 mg/l.

A value for X can be found from equation 16.39:

$$V = \frac{Y^M_{X/S}\theta_c F(S_0 - S)}{X(1 + k_d\theta_c)}$$

$$X = \frac{Y^M_{X/S}\theta_c F(S_0 - S)}{V(1 + k_d\theta_c)} \tag{16.39}$$

$$X = \frac{(0.5)(120\text{h})(400\text{m}^3/\text{h})(800 - 3.57)\text{mg}/1}{3200\text{m}^3(1 + 0.005\text{h}^{-1}120\text{h})}$$

$$X = 3733\ \text{mg}/1$$

Following is the amount of sludge produced, from either equation 16.36 or 16.37:

$$\theta_H = V/F = \frac{(1-\gamma)X_e + \gamma X_r}{\mu_{net} X} \tag{16.37}$$

Since $1/\mu_{net} = \theta_c$ and $X_e = 0$,

$$F\gamma X_r = \frac{VX}{\theta_c} = \text{sludge production rate}$$

$$F\gamma X_r = \frac{(3200\ \text{m}^3)(3733\ \text{mg/l})(10001/1\ \text{m}^3)}{120\text{h}}$$

or

$$F\gamma X_r = 9.95 \times 10^7\ \text{mg/h}$$
$$= 99.5\ \text{kg/h}$$

Although not required by the question, we could use equation 16.40 to get a value of X_r, since $\alpha = 0.40$. Once X_r is known, γ can be calculated if needed.

Modified activated sludge processes are used in order to improve performance of classical activated sludge processes depending on characteristics of wastewater:

- *Step feeding/step aeration* is used for high feed flow rates and feed COD concentrations (i.e., high COD loading rates). Long aeration tanks (large L/W ratio) are used, and the wastewater is fed to the reactor at different points along the axis of the aeration tank. Similarly, aeration is provided at different points from the bottom of the tank using finely perforated disks. Figure 16.9 depicts a schematic of the step feeding activated-sludge system. Organic loading is distributed evenly along the length of the aeration tank to avoid shock loadings. The aeration tank behaves like interconnected small tanks in series where the feeding is supplied to each section simultaneously.
- *Sludge re-aeration* is used when organic compounds are adsorbed on microbial flocs in sedimentation tank and are recycled back to the aeration tank. In order

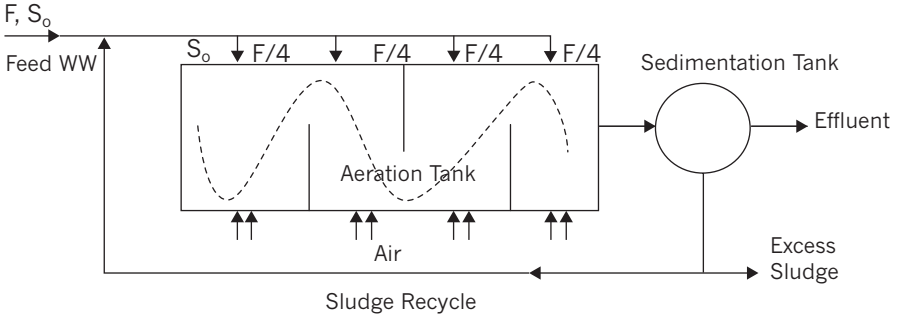


Figure 16.9. A schematic diagram of step feeding process.

to oxidize adsorbed organics and to keep the thick sludge active, sludge is aerated in the recycle stream by using a small re-aeration tank with a hydraulic residence time (HRT) of less than 1 h. Figure 16.10 depicts a schematic of an activated sludge with sludge re-aeration. This process scheme is also used for wastewaters containing insoluble organics.

- *Contact stabilization* process is commonly used for wastewaters containing insoluble organics. Instead of using a primary sedimentation tank and removing insoluble organics, a contact tank is used where the recycled sludge and the feed wastewater are mixed and insoluble organics are entrapped among microbial flocs. Insoluble organics are settled along with microbial flocs in sedimentation tank and are oxidized in an aeration tank in the sludge recycle stream. HRT of the contact tank is less than 1 h, whereas HRT of the aeration tank is between 6 and 10 h depending on the effluent COD. Figure 16.11 depicts a schematic of a contact stabilization process. The major advantage of this process is elimination of primary sedimentation tank and reduction in overall HRT or reactor volumes (space).
- *Adsorbent-added activated-sludge processes* are used for wastewaters containing refractory (nonbiodegradable) organic compounds. Usually, powdered activated carbon (PAC) is added to the aeration tank or injected to the feed

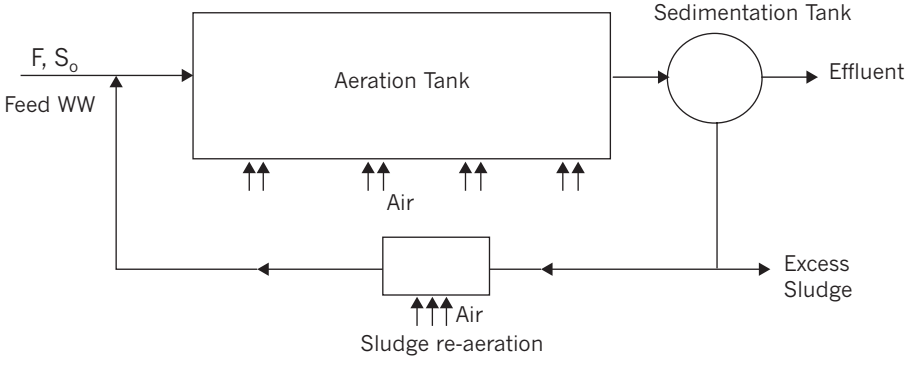


Figure 16.10. A schematic diagram of sludge re-aeration process.

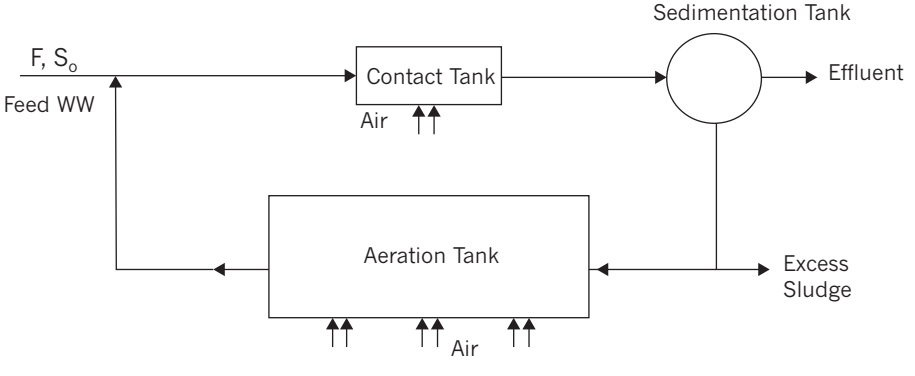


Figure 16.11. A schematic diagram of the contact stabilization process.

wastewater due to high adsorption capacity of PAC. Refractory organics are adsorbed onto PAC surfaces and are removed from wastewater by the excess sludge. PAC also stimulates floc formation, yielding better sedimentation of sludge and therefore high biomass concentrations in the recycle and also in the aeration tank. Therefore, smaller aeration tanks (low HRT) with lower aeration requirement may be used in this process as compared to the classical activated-sludge systems.

- *Oxygen-enriched air or pure oxygen* may be used for treatment of high-strength wastewaters containing refractory compounds. Use of oxygen-enriched air or pure oxygen increases solubility and diffusivity of oxygen in the aeration tank, yielding high COD removal rates. The original UNOX process uses multistage, sealed wastewater treatment tanks in series where pure oxygen is provided by using diffusors and is well distributed inside the reactors. High biomass concentrations with high COD removal rates can be achieved by using pure oxygen instead of air. Figure 16.12 depicts a schematic of the UNOX process.

Trickling biological filters consist of a packed bed of inert support particles (sand or plastics) covered with a mixed culture of microorganisms in the form of a film or slime layer and cell aggregates. The column is loosely filled with packing material to yield a void fraction of 0.4 to 0.5. The filter bed is usually arranged in the form of a rather shallow bed with a high diameter-to-height ratio ($D/H \approx 3$) to avoid possible clogging problems and axial variations. Waste-liquid is fed to the top of the bed using rotary liquid distributors. The waste-liquid flow rate should be low enough to avoid creating shear forces that would remove biofilms from the surfaces of support particles. Air enters the bed from the bottom and moves upward by natural convection. The driving force for air circulation is the temperature difference created by heat released by biological oxidations. The reaction medium is highly heterogeneous, having temperature, pH, DO, and nutrient profiles throughout the column. The thickness of the biofilm and the composition of the organisms in the biofilm may vary with the length of the column. DO limitations are likely because of the high density of cells, unfavorable hydrodynamic conditions, and diffusion barriers within the film. Pre-aeration of the feed wastewater stream (to saturate

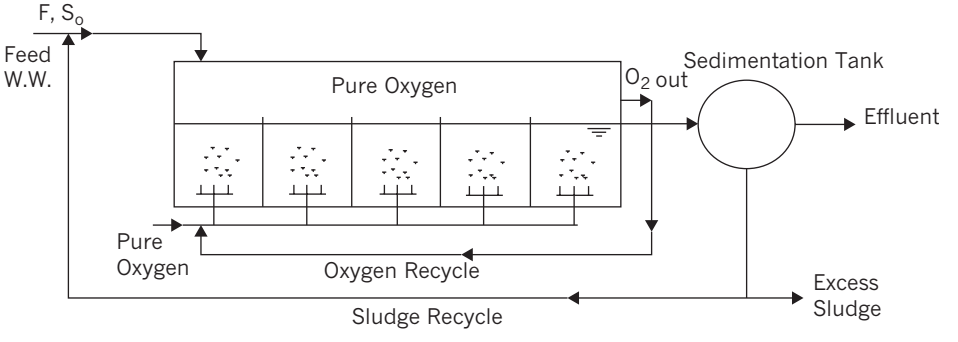


Figure 16.12. Pure oxygen multistage activated sludge process (UNOX).

with oxygen) and high liquid circulation rates may partly alleviate oxygen transfer problems.

As the liquid flows downward in the column on the surface of microbial films, organic compounds (substrates) diffuse through the microbial film and are utilized by organisms simultaneously. The liquid film over the biomass film should be thin enough to allow adequate aeration. A typical liquid film thickness is on the order of 0.01 mm, and biofilm thickness is 0.25 mm. Typical hydraulic residence times in trickling biological filters (TBF) are 0.5 to 4 h for high-rate filters using recirculation of effluent. Trickling filters are more stable against shock loads than are activated-sludge units. Trickling filters also entail lower operating costs and often give better effluent clarity compared to activated-sludge units. However, capital cost and space requirements for trickling filters are higher than for activated-sludge units, and trickling filters remove a smaller fraction of the soluble organics. Also, the maximum concentration of BOD in the influent is more constrained in trickling filters than in activated-sludge systems. A major problem with the operation of trickling filter units is poor control of conditions (T, pH, DO) due to the heterogeneous nature of the system. A comparison of TBFs with activated-sludge units is presented in Table 16.3.

The liquid effluent of TBFs is usually recycled to obtain more complete removal of BOD from wastewater streams. Figure 16.13 is a schematic of a trickling biological filter.

The substrate balance on a differential height of a trickling biological filter yields

$$-FdS_0 = N_s a A dz \tag{16.41}$$

where F is liquid flow rate (l/h), N_s is the substrate flux or rate of substrate utilization per unit surface area of biofilm (mg S/cm^2 film h), a is biofilm surface area per unit volume of the bed (cm^2 film/cm^3), and A (cm^2) and z (cm) are the cross-sectional area and the height of the bed, respectively.

TABLE 16.3. Comparison of Trickling Filter and Activated-Sludge Process

Item	Filter	Sludge Tank
Capital costs	High	Low
Operating costs	Low	High
Space requirements	High	Low
Aeration control	Partial except in enclosed forced-draft types	Good
Temperature control	Difficult	Possible; heat losses small
Sensitivity to variations in applied feed concentrations	Fairly insensitive but slow to recover if upset	More sensitive, but recovery is rapid
Clarity of final effluent	Good	Not as good
Fraction of soluble organics removed	70%–90%	85%–95%
Typical θ_H		
Low rate	6 to 48 h	4 to 6 h
High rate	0.75 to 4 h	
Fly and odor nuisance	High	Low

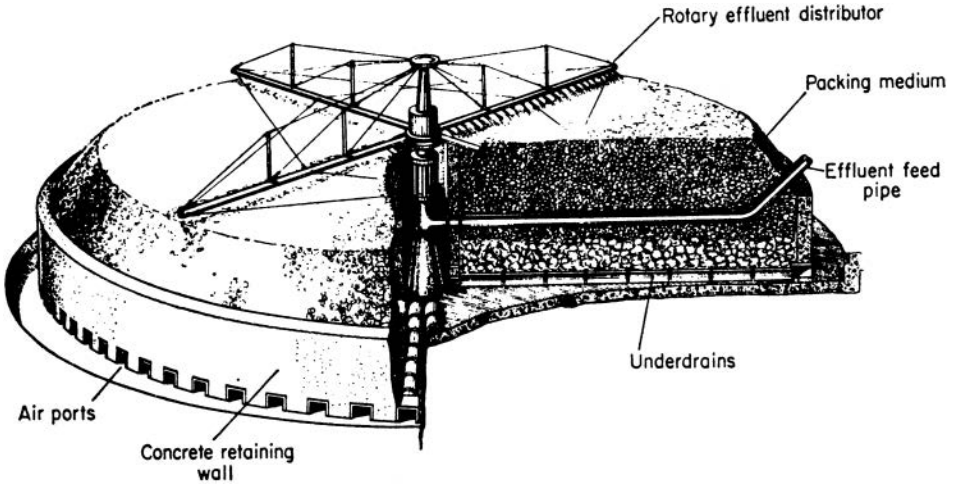


Figure 16.13. Typical trickling biological filter. (With permission, from J. W. Abson and K. H. Todhunter, in N. Blakebrough, ed., *Biochemical and Biological Engineering Science*, Vol. 1, Academic Press, New York, 1967, p. 326.)

The substrate flux or rate of substrate consumption is

$$N_s = -D_e \left.\frac{dS}{dy}\right|_{Y=L} = \eta \frac{r_m S_0}{K_s + S_0} L \tag{16.42}$$

where η is the effectiveness factor, L is the thickness of the biofilm (cm), r_m is the maximum rate of removal of substrate (mg *S*/l s), and S_0 is the substrate concentration in the bulk liquid (mg/cm^3). Typical values for r_m are 0.2 to 0.5 mg *S*/l-s. Substituting equation 16.42 into equation 16.41 yields

$$-F \frac{dS_0}{dz} = \eta \frac{r_m S_0}{K_s + S_0} LaA \tag{16.43}$$

Equation 16.43 can be integrated to yield a substrate concentration profile throughout the column. Usually, the substrate concentration in bulk liquid is very low, and the biological rate expression can be approximated to first order:

$$-F \frac{dS_0}{dz} \cong \eta \frac{r_m}{K_s} LaAS_0 \tag{16.44}$$

Integration of equation 16.44 from $z = 0$ to $z = H$ yields

$$\frac{S_0}{S_{0i}} = \exp\left[-\frac{\eta r_m LaAH}{FK_s}\right] \tag{16.45}$$

where S_{0i} is the inlet substrate concentration, and S_0 is concentration of substrate in the liquid phase at a distance z from the inlet. Experimental data of $\ln S_0/S_{0i}$ plotted versus z yield a straight line (in at least some cases), supporting the validity of this analysis.

The effectiveness factor can be calculated by using the following expressions:

$$\eta = 1 - \frac{\tanh \phi}{\phi}\left(\frac{\omega}{\tanh \omega} - 1\right) \quad \text{for } \omega \le 1 \tag{16.46}$$

$$\eta = \frac{1}{\omega} - \frac{\tanh\phi}{\phi}\left(\frac{\omega}{\tanh\omega} - 1\right), \quad \text{for } \omega \geq 1 \tag{16.47}$$

where

$$\omega = \frac{\phi(S_0/K_s)}{\sqrt{2}(1+S_0/K_s)}\left[\frac{S_0}{K_s} - \ln\left(\frac{S_0}{K_s}\right)\right]^{-1/2} \tag{16.48}$$

Here, $\phi = L\sqrt{r_m / D_e K_s}$ and $r_m = \mu_r X / Y_{X/S}$. D_e is the effective diffusivity of the substrate within the biofilm (cm^2/s), and $Y_{x/s}$ is the yield coefficient (g cells/g substrate).

Following is a similar but more empirical design equation that has been used to determine waste-treatment parameters for biological filter:

$$\frac{S_0}{S_{0i}} = \exp\left[-Ka^{1+m}\left(\frac{A}{F}\right)^n\right] \tag{16.49}$$

where a is the specific surface area of the inert support material (cm^2/cm^3), A is the cross-sectional area of the bed, F is liquid flow rate, and K is the apparent rate coefficient. In practice, K is used as an adjustable parameter in a curve-fitting procedure. Exponents m and n vary depending on system characteristics such as geometry, hydrodynamics, biological systems, and wastewater characteristics.

A schematic of a typical rotating biological contactor (RBC) is depicted in Figure 16.14, and the disks are shown in Figure 16.15. Disks are made of polystyrene, polyurethane, or PVC with 2 to 4 m diameter and are mounted on a shaft located inside a wastewater tank. The shaft is rotated (10 to 20 rpm) with the aid of a motor. Microorganisms grow in form of biofilm on surfaces of rotating disks. Aeration takes place by direct contact of biofilm with air during rotation. Part of the biofilm surface is immersed in wastewater at any time during rotation where oxidations of organic compounds take place. Biofilm thickness should be controlled in order to avoid thick biofilm formation and anaerobiosis inside the biofilm. For thin biofilms, COD removal may be limited by insufficient biomass in RBC; for thick biofilms, diffusion limitations inside biofilm may cause reductions in COD removal rates. Therefore, the system should be operated around the optimal biofilm thickness to maximize COD removal rate. Control of biofilm thickness is a major problem in this system just as in any other biofilm systems. In RBC systems, COD removal is mainly achieved by the biofilm bacteria. However, in

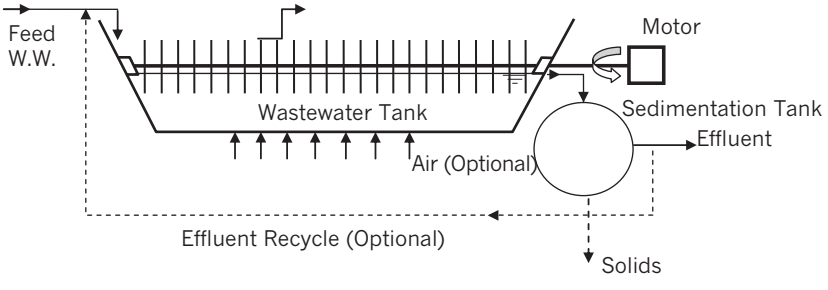


Figure 16.14. A schematic diagram of a rotating biological contactor system.

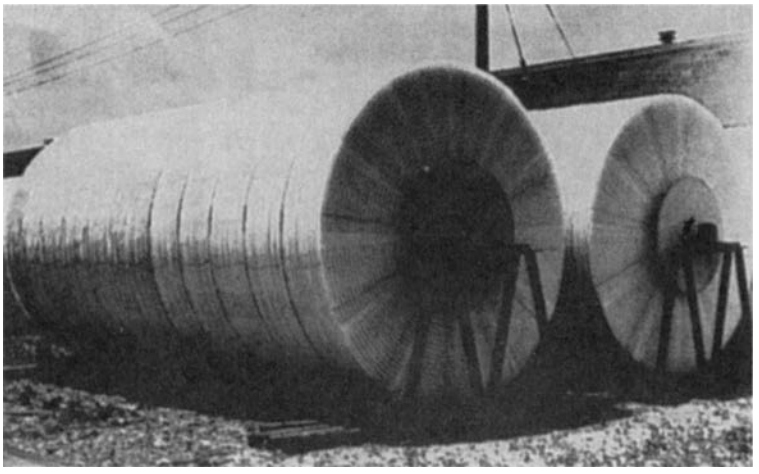


Figure 16.15. Styrofoam disks used in rotating biological contactor units (Biosystems Division, Autotrol Corporation). (With permission, from E. D. Schroeder, *Water and Wastewater Treatment*, McGraw-Hill Book Co., New York, 1977, p. 306.)

some cases, suspended bacteria may also contribute COD removal depending on relative magnitudes of total biomass in suspended culture and in biofilm.

Assuming a completely mixed aqueous phase in the wastewater tank, COD balance around the RBC tank yields

$$F(S_0 - S) = R_{sf}\, V_f + R_{ss}\, V_L = R_{sf}\, L\, A_w + R_{ss}\, V_L \tag{16.50}$$

where F is feed wastewater flow rate ($m^3\ h^{-1}$); S_0 and S are the feed and effluent COD (kgm^{-3}); R_{sf} and R_{ss} are the COD removal rates by biofilm and suspended bacteria, respectively; V_f and V_L are the biofilm and wastewater volumes (m^3) in the tank; L is biofilm thickness (assumed to be constant) and A_w (m^2) is the wet biofilm surface area on the disks.

R_{sf} and R_{ss} can be expressed as

$$R_{sf} = \frac{k_f X_f S_f}{k_{sf} + S_f} = \eta \frac{k_f X_f S}{K_{sf} + S} \text{ and } R_{ss} = \frac{k_s X_s S}{K_{ss} + S} \tag{16.51}$$

where k_f and k_s are the maximum COD removal rate constants (μ_m / Y_G) by the biofilm and suspended bacteria (kg COD kg^{-1} biomass h^{-1}); X_f and X_s are the biomass concentrations in biofilm and in wastewater (kg $X\ m^{-3}$); S_f and S are the average COD concentrations in biofilm and in wastewater (kg COD m^{-3}); K_{sf} and K_{ss} are saturation constants for biofilm and suspended bacteria (kg $S\ m^{-3}$); and η is the effectiveness factor representing diffusion limitations inside biofilm. For thin biofilms where $S_f = S$, $\eta = 1$; for thick biofilms where $S_f << S$, $\eta < 1$.

Usually, $V_L X_s << V_f X_f$ and therefore $R_{ss} << R_{sf}$. That is, COD removal by suspended cells is usually negligible as compared to that of the biofilm for low-strength

wastewaters. However, this assumption may not hold for high-strength wastewaters with liquid-phase aeration.

Therefore, with negligible R_{ss}, equation 16.50 takes the following form:

$$F(S_0 - S) = R_{sf} V_f = R_{sf} L\, A_w = \eta \frac{k_r X_r S}{K_{sf} + S} L\, A_w = \eta \frac{R_m S}{K_{sf} + S}\, L\, A_w \qquad (16.52)$$

where R_m (= $k_f X_f$) is the maximum rate of COD removal (kg COD m^{-3} h^{-1}); A_w is the wet disk surface area.

$A_w = \alpha\, A_T$ where α is the immersion ratio, and A_T is the total surface area of disks:

$$a = \frac{Aw}{A_T} = \frac{2pN\left(R_o^2 - R_u^2\right)}{2pN\, R_o^2} = 1 - \frac{R_u^2}{R_o^2} \qquad (16.53)$$

where R_o and R_u are the outer and unwetted radius of disks (m), and N is the number of disks. Immersion ratio is usually 40% to 45%, and therefore $A_w = 0.40\, A_T$.

Equation 6.52 is used to determine the required disk surface area for a certain degree of COD removal (i.e., known S_0 and S). X_f is usually difficult to determine, and for this reason $X = X_f L$ is defined as surface concentration of biomass (kg X m^{-2} disk surface), assuming constant biofilm thickness. Then equation 6.52 becomes

$$R_S = \frac{F(S_0 - S)}{A_w} = \eta \frac{R_{ms} S}{K_S + S} \qquad (16.54)$$

where $R_{ms} = R_m L$. Equation 16.54 can be used to determine the kinetic constants using experimental data obtained at different flow rates or HRTs.

For small RBC tanks with liquid-phase aeration, completely mixed reactor assumption may be valid. However, for long RBC tanks with still water, conditions may approach plug flow regime. In this case, a differential balance over the RBC tank yields

$$FdS = -R_{sf} dV_f - R_{sf} dA_f = R_{Sf} L dA_f \frac{kX_f S}{K_S + S}\, L\, dA_f \qquad (16.55)$$

Integration of equation 16.55 results in equation 16.56:

$$K_S Ln \frac{S_0}{S} + (S_0 - S) = \eta \frac{kX_f A_f}{F} = \eta \frac{kX_S}{F} A_f \qquad (16.56)$$

where A_f is the biofilm surface area (= A_w, m^2) and X_s is the surface biomass concentration (= $X_f L$, kg biomass m^{-2} surface area). Equation 16.56 can be used to determine required biofilm and disk surface area for certain degree of COD removal. The effectiveness coefficient (η) is determined by using Figure 9.15 or equations 9.62 to 9.64.

Oxidation ponds provide another inexpensive alternative to activated-sludge and TBF operations. Oxidation ponds are shallow (2 to 4 ft deep) waste-treatment reactors closely resembling natural aquatic ecosystems. Bacteria and algae grow in the same pond in a symbiotic relationship. Bacteria oxidize organic compounds by utilizing oxygen produced by algae and produce CO_2; algae utilize CO_2 produced by bacteria and produce oxygen by photosynthesis for bacterial consumption. Such ponds require large land areas, are less efficient than many other techniques, and may have

adverse environmental side effects. Toxic or hazardous materials may collect in the sediment without degradation, creating a long-term problem.

Anaerobic digestion (or biological treatment) is usually used to treat solid wastes and excess sludge produced in aerobic waste-treatment processes. Particulate waste material removed by screening or sedimentation in primary waste treatment and biomass (concentrated sludge) produced in activated-sludge units are degraded under anaerobic conditions to produce methane. Anaerobic digestion is a slow process compared to aerobic processes; typical residence times are 30 to 60 days. The microbiology and biochemistry of anaerobic digestion are very complicated. However, the major steps involved in this process are as follows:

1. *Solubilization of insoluble organics:* Waste material may contain large amounts of solids made of cellulosics (papers, agricultural wastes), starches (potato waste), and other complex insoluble organic chemicals. Solubilization of these compounds by acid or enzymatic hydrolysis (cellulases, amylases, glucoamylases, lipases, proteases) is the first step in anaerobic digestion. These compounds are not readily utilizable by microorganisms, and their hydrolysis is essential for effective microbial digestion.
2. *Formation of volatile acids:* Solubilized organic compounds are metabolized by anaerobic bacteria to produce volatile organic acids, such as acetic, butyric, formic, and propionic acids, and short-chain fatty acids. Acid-producing organisms are a mixture of facultative anaerobes, such as enteric bacteria and clostridial species, which are called *acid formers.* Alcohol formation (butanol, propanol, ethanol) also takes place to a lesser extent. The optimal temperature and pH values for this step are $T = 35°C$ and pH = 4 to 6. The partial pressure of H_2 in the reactor can greatly influence metabolism.
3. *Formation of methane:* Volatile acids and alcohols produced in the second stage are converted to methane and CO_2 by methanogenic bacteria, which are strictly anaerobic. Among methanogenic bacteria used for this purpose are *Methanobacterium* (nonspore-forming rods), *Methanobacillus* (spore-forming rods), and *Methanococcus* and *Methanosarcina* (a cocci group growing in cubes of eight cells). The optimal temperature and pH range for methanogenic bacteria are $T = 35°$ to 40°C and pH = 7 to 7.8.

The major biological reaction steps involved in a typical anaerobic digestion process can be represented as follows:

$$\text{insoluble organics} \xrightarrow[\text{enzymaticor acid}]{\text{Hydrolysis}} \text{soluble organics} \xrightarrow[\text{bacteria}]{\text{Acid-producing}}$$

$$\text{volatile acids} + \text{alcohols} + H_2 + CO_2 \xrightarrow{\text{Methanogens}}$$

$$CH_4 + CO_2 + H_2S$$

Depending on the composition of organic waste, either enzymatic hydrolysis of insolubles (e.g., cellulosics) or methane production from volatile acids is the rate-limiting step

in this reaction sequence. The concentration of H_2 is often critical in determining the rates of these processes. Hydrolysis reactions are usually carried out as a separate step. However, acid-formation and methane-formation steps can be achieved in the same reactor. Single- and two-reactor systems have been developed for anaerobic digestion. The two-step reactor scheme has been reported to result in higher reaction rates and methane yields. The operating conditions in a single-reactor system are pH = 6 to 7 and T = 35° to 40°C, which result in reasonable rates of acid formation and methane generation. Typical solids residence time in an anaerobic digester is 10 to 30 days.

Cell yields in anaerobic digesters are low, and a typical value is 0.05 g cells/g COD. Cell yield also varies with substrate. Yield on carbohydrates is larger than yield on the other carbon compounds.

Changes in temperature affect the methane-formation rate more than the acid-formation rate. Although the optimal temperature is reported to be 35° to 40°C, in some cases higher removal rates are obtained at higher temperatures (T = 55°C), depending on the composition of the organisms. The system does not function satisfactorily at temperatures below 35°C, and the temperature of the reactors needs to be controlled for stable methane generation.

The rate of methane formation is considerably lower than the rate of acid formation and is rate limiting. Sudden increases in organic load concentrations may cause volatile acid (VA) accumulation in the medium, decreasing pH and altering H_2 levels, which further depresses methane production. Typical soluble organics removal rates are on the order of 0.2 g COD/g cells day. Certain metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}) are known to be toxic to organisms of anaerobic digesters at high concentrations (>1000 mg/l), although they are stimulatory at low levels (>100 mg/l).

The composition of the product gas mixture varies, depending on the composition of waste material and other environmental factors. A typical gas composition is 70% to 75% CH_4, 20% to 25% CO_2, and 5% H_2S and other gases (NO_2, H_2, CO). Part of the methane produced in an anaerobic digestion unit is used to heat the digesters to fermentation temperature. The digester gas has a heating value of 24 to 28 million J/m^3, and the yield is 0.75 to 1.2 m^3 (std)/kg of organic material decomposed, resulting in an energy generation of 18 to 33 million J/kg of organic material. One problem associated with the use of digester gas is the H_2S content, which can cause foul odors and corrosion and needs to be removed before use.

Anaerobic digesters were originally designed as closed stationary tanks. In newer designs, reactor contents are mixed and the reactor is heated. Mixing is accomplished either by recycling a portion of the gas or by mechanical stirring. The reaction mixture is highly heterogeneous and viscous and therefore difficult to mix. Figure 16.16 is a schematic diagram of an anaerobic digester. Anaerobic digestion can be achieved in packed beds containing porous solid support particles. Bacteria grow on support surfaces and inside pores, and liquid waste may be trickled through the bed. Solids recycling schemes have been developed for use with conventional digesters that simulate activated-sludge units. Solids residence times are considerably higher than hydraulic residence times in these systems, which results in biomass accumulation and higher reaction rates. Continuous PFRs operated in upflow mode are commonly used as anaerobic digesters.

The solids content of sludge (waste) is reduced by about 50% to 60% at the end of anaerobic digestion. Digested sludge is much less biodegradable than raw sludge

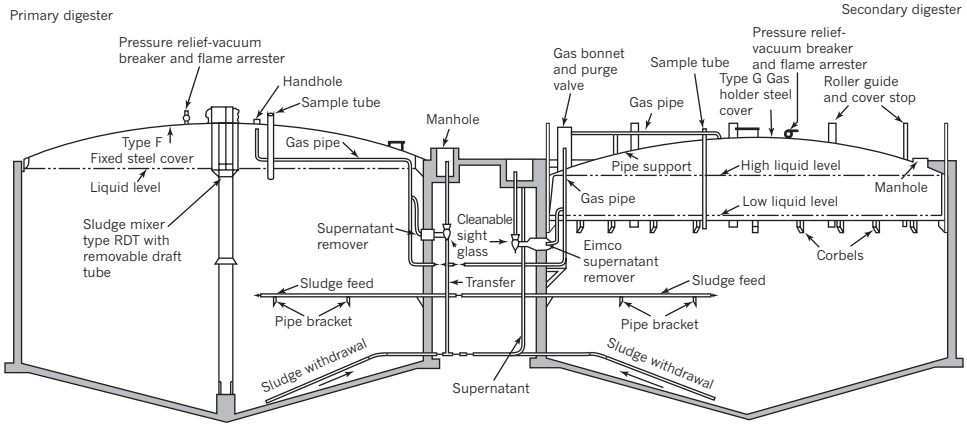


Figure 16.16. A schematic of an anaerobic digestion unit (Eimco Processing Machinery Division, Envirotech Corporation). (With permission, from E. D. Schroeder, *Water and Wastewater Treatment*, McGraw-Hill Book Co., New York, 1979, p. 327.)

and is easier to dewater. The digester effluent is dewatered by vacuum filtration and is dried. The dried solids are either incinerated or spread on land as fertilizer.

16.5.2. Advanced Wastewater Treatment Systems

Advanced wastewater treatment systems are used for removal of residual nitrogen and phosphorus along with refractory carbonaceous compounds. Examples are for nitrification and denitrification, phosphate removal processes (e.g., PhoStrip and A/O processes), and combined nitrogen and phosphate removal processes (e.g., A^2O and Bardenpho processes).

Nitrogen-containing organic compounds (e.g., amino acids and proteins) are oxidized biologically to ammonium, which is further oxidized to nitrite and nitrate by *Nitrosomonas* and *Nitrobacter*, respectively. The conversion of ammonium to nitrate by microbial catalysis is called *nitrification*:

$$NH_4^+ + \frac{3}{2}O_2 \xrightarrow{Nitrosomonas} NO_2^- + H_2O + 2H^+ \tag{16.57a}$$

$$NO_2 + \frac{1}{2}O_2 \xrightarrow{Nitro} NO_3^- \tag{16.57b}$$

Overall, combining equations 16.57a and 16.57b results in equation 16.58:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \tag{16.58}$$

Both of the aforementioned organisms fix carbon dioxide to be used for biosynthesis. Aerobic nitrification can take place during BOD removal in the presence of these organisms and increases the oxygen requirements in aerobic treatment.

These reactions are energy yielding, and the first step (equation 16.57a) is the rate-limiting one. Nitrification processes can be accomplished in a single stage with BOD removal

or as a two-stage system with separate BOD removal and nitrification units. The choice between the one-stage and two-stage processes typically depends on the ratio of carbon to nitrogen in the feed stream. Typically, this ratio is expressed as BOD_5/TKN. The growth yield of nitrifying bacteria is low (0.2 g dry wt per g N) and the fraction of the population that is nitrifying bacteria can be low. For example, at BOD_5/TKN = 10, the fraction of nitrifying bacteria is 0.02; at BOD_5/TKN = 0.5, the fraction of nitrifying bacteria rises to 0.35. For a single-stage unit with combined BOD removal and nitrification, the BOD_5/TKN ratio should be greater than 5. When it is less than 3, the two-stage process is preferred.

Nitrification is best accomplished with suspended cells, typically using a modification of the activated-sludge process. However, fixed-film processes that are well aerated can yield nitrification efficiencies over 90%. In either system, DO levels should be high (>2mg/l), and slightly alkaline solutions (7.5 to 8.5) at 20° to 30°C work best.

Under anaerobic conditions, some bacteria utilize nitrate as the final electron acceptor instead of oxygen. This process is known as *denitrification*. Two types of nitrate reduction are possible: assimilatory and dissimilatory reduction. In assimilatory reduction, nitrate is reduced to ammonia and is incorporated into cell biomass. However, in dissimilatory reduction, nitrate is reduced to nitrite and further to elemental nitrogen. An external carbon source is needed; this source must be inexpensive (e.g., waste starch, methanol, or molasses).

An example of assimilatory reduction follows:

$$NO_3^- + H_2O \longrightarrow NH_3 \tag{16.59}$$

And following is an example of dissimilatory reduction:

$$NO_3^- + \text{C compound} \longrightarrow NO^- + CO_2 + \text{cells} \tag{16.60}$$

$$NO_2^- + \text{C compound} \longrightarrow N_2 + CO_2 + \text{cells} \tag{16.61}$$

Using an average biomass composition of $C_5H_7NO_2$ and assuming that methanol is used as the carbon source, the overall balance for denitrification can be written as follows:

$$NO_3^- + 1.08\,CH_3OH + H^+ \rightarrow 0.065\,C_5H_7NO_2 + 0.47\,N_2 + 0.76\,CO_2 + 2.44\,H_2O \tag{16.62}$$

Clearly, equations such as 16.62 can be used to predict the amount of carbon source (e.g., methanol) required to remove a known amount of NO_3^-. A number of microbes can act as denitrifiers; among these are species of the genera *Pseudomonas*, *Alcaligenes*, *Arthrobacter*, and *Corynebacter*. Such bacteria grow slowly with $\mu_g = 0.5\ d^{-1}$ and with a yield coefficient of 0.8 g biomass per g nitrogen removed. Saturation constants are low (e.g., 0.1 mg/l < K_5 < 1 mg/l) for both nitrification and denitrification, so these reactions can be approximated as zero order for nitrogen concentrations above about 2 mg/l.

Activated-sludge or packed-bed biofilm type reactors may be used for anaerobic denitrification. Upflow packed-bed reactors utilizing biofilms on porous support material are reported to result in high nitrate removal rates.

A typical two-stage nitrification/denitrification process is depicted in Figure 16.17. Optimal pH and temperature values for denitrification are pH = 6.5 to 7 and T = 20° to 30°C.

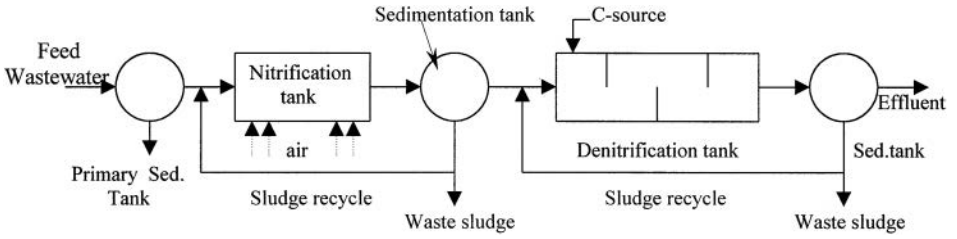


Figure 16.17. A schematic of the two-stage nitrification–denitrification process.

Phosphate removal from wastewaters by biological means can be realized either by assimilation or by "luxury" phosphate uptake. Assimilation of phosphate occurs in all aerobic/anaerobic processes, since nearly 3% of cell mass is made of phosphorus. Luxury phosphate uptake is accomplished by organisms belonging to the genera *Acinetobacter*, among which *Acinetobacter calcoaceticus* is the most widely known. *Acinetobacter* species utilize carbohydrates as carbon and energy source by the Entner–Doudoroff pathway. Those organisms utilize acetate and fatty acids under anaerobic conditions to synthesize polyhydroxybutyrate (PHB). Energy required for PHB synthesis is obtained from ATP molecules generated by breakdown of polyphosphates. Under aerobic conditions in the absence of carbon source, the organisms utilize PHB molecules as carbon–energy source to synthesize polyphosphates and store them in form of polyphosphate granules inside the cell.

An example of aerobic polyphosphate synthesis follows:

$$(PO_4)_n + ATP \longrightarrow (PO_4)_{n+1} + ADP \tag{16.63}$$

The following is an example of anaerobic polyphosphate depolymerization:

$$(PO_4)_n + H_2O \longrightarrow (PO_4)_{n-1} + H_2PO_4^{-1} \tag{16.64}$$

Consequently, the organisms remove phosphate from liquid media to synthesize and store polyphosphates under aerobic conditions and release phosphate into the media under anaerobic/anoxic conditions. *Acinetobacter* species can store polyphosphates up to 30% of their dry weight.

In phosphate removal bioprocesses, organisms are exposed to alternating anaerobic and aerobic conditions. The two major processes developed to promote luxury phosphate uptake are the A/O (anaerobic/oxic) and PhoStrip processes.

The A/O process is used for combined BOD and phosphate removal. It is a two-stage process incorporating anaerobic and oxic (aerobic) steps in sequence. In the anaerobic stage, the phosphate in the recycled sludge is released into liquid media. The released phosphate is taken up by the cells under aerobic conditions along with phosphate in the feed wastewater stream and is stored as polyphosphate granules. Polyphosphate is removed from the system by the waste sludge, which has a high value as fertilizer. When the BOD/P ratio in the feed wastewater is greater then 10, the effluent phosphate levels can be lower than 1 mg/l (as shown in Figure 16.17).

The PhoStrip process consists of an aerobic stage and an anaerobic one on the sludge recycle stream, as shown in Figure 16.18. The wastewater stream is fed

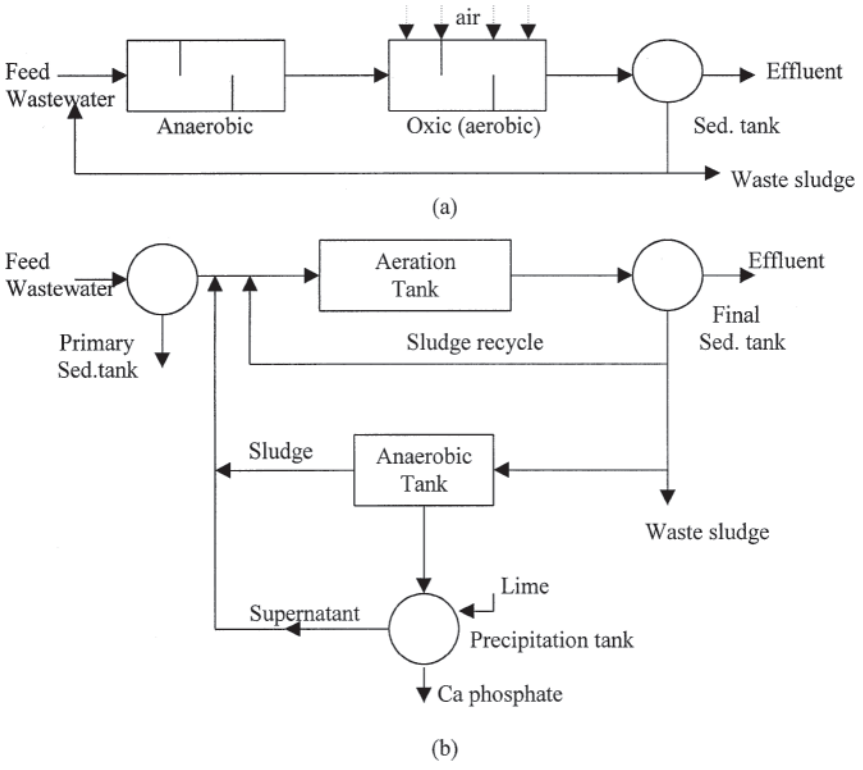


Figure 16.18. Phosphate removal processes: (a) A/O Process, (b) PhoStrip Process.

to the aerobic stage for polyphosphate synthesis, the effluent of which is fed to a sedimentation tank. The underflow of the sedimentation tank is fed to the anaerobic stage for phosphate release. The released phosphate is precipitated in a sedimentation tank with the addition of lime in form of calcium phosphate. Part of the sedimented sludge and anaerobic reactor sludge is recycled back to the aerobic stage. Phosphate is removed from the process in the form of calcium phosphate. The effluent water stream would contain less then 1.5 mg/l phosphate in a well-operating PhoStrip process.

Combined nitrogen and phosphate removal processes employ combinations of anaerobic, anoxic, and aerobic zones in different orders which accomplish simultaneous BOD removal also. The most commonly used processes for this purpose are the A^2/O and the five-stage Bardenpho processes (see Figure 16.19). Among other processes are the University of Capetown (UCT) and the Virginia Initiative Plant (VIP) processes.

The A^2/O process includes an anoxic zone for denitrification in addition to A/O process (as shown in Figure 16.19a). Hydraulic residence times in anaerobic and anoxic zones are less than 1.5 h, and in the aerobic zone 4 to 6 h. The effluent of the aerobic stage is recycled back to the anoxic zone for denitrification purposes. Phosphate release takes place in the anaerobic zone. Denitrification and BOD removal are the major functions of the anoxic zone. Phosphate removal in the form of polyphosphates, nitrification, and some BOD removal take place in aerobic zone. Phosphate is

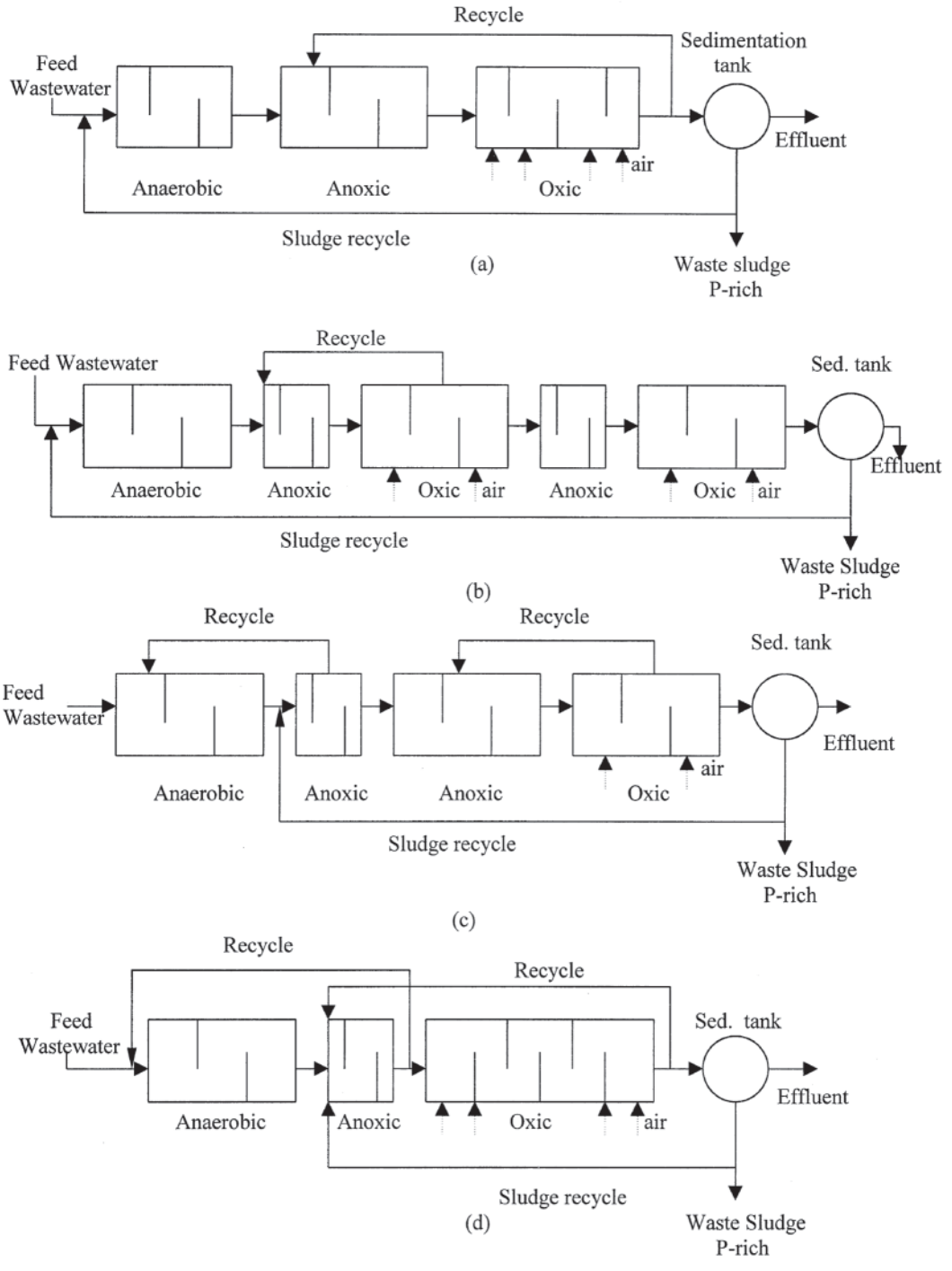


Figure 16.19. Combined biological nitrogen and phosphate removal processes: (a) A^2/O process, (b) five-stage Bardenpho process, (c) University of Capetown process, (d) Virginia Initiative Plant process.

removed from the system in form of waste sludge; nitrogen is released in form of N_2 (gas); and BOD is converted to CO_2 and H_2O.

The five-stage Bardenpho process includes two additional anoxic and aerobic zones as compared to the A^2/O process (Figure 16.19b). This process is known as the A^2/O/A/O process. The effluent of the aerobic zone is partly recycled back to the anoxic zone for denitrification purposes. The last two zones are for additional denitrification and polyphosphate removal to further reduce nitrate and phosphate levels in the effluent. Hydraulic residence times in anaerobic and anoxic zones are less than 4 h, and in the first aerobic zone, 4 to 12 h.

The UCT and VIP processes employ similar zones and recycle schemes in different orders, resulting in low nitrogen, phosphate, and BOD levels in the effluent.

In some cases, sulfur removal from wastewater is needed. Elemental sulfur in wastewater can be oxidized to sulfate by *Thiobacillus* species. Sulfate can be further reduced to sulfides by anaerobic sulfate-reducing bacteria, such as *Desulfovibrio*, and sulfides can be precipitated out of wastewater in the presence of certain metal ions, such as Fe^{2+}, Zn^{2+}, and Pb^{2+}. Sulfates can also be precipitated with the addition of limestone ($CaCO_3$) and $Ca(OH)_2$ and can be filtered out of wastewater.

16.5.3. Conversion of Wastewater to Useful Products

Some waste materials containing starch and cellulose or other easily utilizable carbonaceous compounds can easily be converted to useful products such as high-protein feedstuff or SCP, ethanol, organic acids (acetic, butyric, propionic), methane, and methanol. Agricultural wastes, waste paper, and waste wood constitute major cellulosic wastes. Major starch-containing wastes include some agricultural wastes (wheat, corn, rice, potato), some domestic solid wastes (vegetable/fruit), and some industrial wastes (food industry).

Cellulosic and starch-containing wastes are first hydrolyzed into sugar molecules, which are further converted to SCP under aerobic conditions or ethanol/organic acids under anaerobic conditions. While many organisms can be used, yeasts, particularly *Saccharomyces,* are well suited to protein production, since they already have FDA approval for use in foods. The waste must be free of toxic contaminants. To insure complete utilization of carbon, mineral nutrients may need to be added to give a C/N/P ratio of 100/15/3.

Two widely known processes for conversion of wastes to SCP are the SYMBA and the PEKILO processes. The SYMBA process converts potato wastes to SCP and consists of two stages. The first stage is for enzymatic hydrolysis of starch by *Endomycopsis* sp., and the second is for SCP production on glucose by *Saccharomyces* sp. The PEKILO process is based on growth of *Paecilomyces* sp. on waste sulfite liquor.

Such waste materials can also be converted to chemicals such as ethanol for use as a fuel, solvents such as acetone/butanol, or monomers for synthesis of polymers (lactic acid). Conversion of starches to glucose and fermentable sugars is a well-established industry that uses large quantities of enzymes such as α-amylase, β-amylase, and

glucoamylase. The economically feasible conversion of cellulosic wastes into fermentable sugars remains a bioprocess challenge. Many types of cellulases made by microbes can be used; new processes using genetically engineered microbes (e.g., *Trichoderma* sp., *Thermomonospora* sp., and some *Clostridia* sp.) may lead to less expensive cellulases. Acid hydrolysis can be used instead of enzymatic processes but is less environmentally friendly. The economical conversion of cellulosic wastes into products, such as ethanol, would be a major contribution to human welfare.

16.6. SUMMARY

Populations containing multiple species are important in natural ecosystems, well-defined processes, wastewater treatment, and systems using genetically modified cells. Some examples of interactions among these species are competition, neutralism, mutualism, protocooperation, commensalism, amensalism, predation, and parasitism. In real systems, several modes of interaction may be present. Mathematical analyses can be used to show that neither pure competition nor pure mutualism gives a stable steady state in a chemostat. However, spatial heterogeneity, dynamic fluctuations, and the addition of other interactions can lead to the sustained coexistence of species with competitive or mutualistic interactions.

One of the major process uses of mixed cultures is wastewater treatment. The activated-sludge system is commonly employed in treating wastewaters. Such a system can be considered a chemostat with cell recycle under aerobic conditions. Alternative methods of aerobic treatment include trickling-bed filters, rotating biological disks, and oxidation ponds. Anaerobic digestion can be used to directly treat wastewaters or, more commonly, to further degrade sludges from the primary and secondary treatment levels of a waste-treatment plant.

SUGGESTIONS FOR FURTHER READING

Fredrickson, A. G., Behavior of Mixed Cultures of Microorganisms, *Ann. Rev. Microbiol.* 31: 63–87, 1977.

Hammer, M. J., and M. J. Hammer, Jr., *Water and Wastewater Technology*, 7th ed., Prentice Hall, Upper Saddle River, NJ, 2011.

Schroeder, E. D., *Water and Wastewater Treatment*, McGraw-Hill, New York, 1977.

Sundstrom, D. W., and H. E. Klei, *Wastewater Treatment*, Prentice Hall, Englewood Cliffs, NJ, 1979.

Tchobanoglous, G., F. Burton, H. D. Stensel, and R. Tsuchihashi, *Wastewater Engineering: Treatment and Resource Recovery* (Metcalf & Eddy, Inc.), 5th ed., McGraw-Hill, New York, 2013.

Viessman, W., and M. J. Hammer, *Water Supply and Pollution Control*, 6th ed., Prentice Hall, Upper Saddle River, NJ, 1998.

PROBLEMS

16.1. A batch fermenter receives 1 l of medium with 5 g/l of glucose, which is the growth-rate-limiting nutrient for a mixed population of two bacteria (a strain of *E. coli* and *Azotobacter vinelandii*). *A. vinelandii* is five times larger than *E. coli.* The replication rates for the two organisms are

$$\mu_{EC} = \frac{1.0\ \text{h}^{-1}\text{s}}{0.01\ g/1 + \text{s}} - 0.05\ \text{h}^{-1}$$

and

$$\mu_{AV} = \frac{1.5\ \text{h}^{-1}\text{s}}{0.02\ g/1 + \text{s}} - 0.10\ \text{h}^{-1}$$

The yield coefficients are

$$Y_{EC} = 0.5\ \text{gDW/g glucose}$$
$$Y_{AV} = 0.35\ \text{gDW/g glucose}$$

The inoculum for the fermenter is 0.03 gDW/l of *E. coli* (1×10^8 cells/ml) and 0.15 gDW/l of *A. vinelandii* (1×10^8 cells/ml).

What will be the ratio of *A. vinelandii* to *E. coli* at the time when all of the glucose is consumed?

16.2. Consider Example 16.1, where we demonstrated that two bacteria competing for a single nutrient in a chemostat (well-mixed) could not coexist. Consider the situation where *B* can adhere to a surface but *A* cannot. Redo the balance equations, where *a* is the surface area available per unit reactor volume and the rate of attachment is first order in X_B with a rate constant k_{aB}. The sites available for attachment will be $(X^{At}{}_{BM} - X^{At}{}_{B})aV$. The attached cells can detach with a first-order dependence on the attached cell concentration ($X^{At}{}_{B}$) with a rate constant of k_{aB}. Attached cells grow with the same kinetics as suspended cells.

a. Without mathematical proofs, do you think coexistence may be possible? Why or why not?

b. Consider the following specific case and solve the appropriate balance equations for $D = 0.4\ \text{h}^{-1}$:

$$\mu_{mA} = 1.0\ \text{h}^{-1};\ \mu_{mB} = 0.5\ \text{h}^{-1}$$
$$K_{SA} = K_{SB} = 0.01\ \text{g/l}$$
$$Y_{A/S} = Y_{B/S} = 0.01\ \text{g/l}$$
$$S_0 = 5\text{g/l},\ a = 10\ \text{cm}^2/\text{cm}^3$$
$$X^{At}_{BM} = 1 \times 10^{-4}\ \text{g/cm}^2$$
$$k_{dB} = 0.5\ \text{h}^{-1},\ k_{dB} = 1000\ \text{cm}^3/\text{g} - \text{h}$$

16.3. Organism A grows on substrate S and produces product P, which is the only substrate that organism B can utilize. The batch kinetics are as follows:

$$\frac{dX_A}{dt} = \frac{\mu_A S X_A}{K_s + S}$$

$$\frac{dX_B}{dt} = \frac{\mu_B P X_B}{K_p + P}$$

$$\frac{dP}{dt} = Y_{P/A}\frac{\mu_A S X_A}{K_s + S} - \frac{\mu_B P X_B}{Y_{X_B/P}(K_p + P)}$$

$$\frac{dS}{dt} = \frac{\mu_A S X_A}{Y_{X_A/S}(K_s + S)} - \frac{Y_{P/A}}{Y_{P/S}}\frac{\mu_A S X_A}{(K_S + S)}$$

Assume the following parameter values:

$$\mu_A = 0.18\ \text{hr}^{-1}, \quad K_s = 0.42\ \text{g/l}, \quad \mu_B = 0.29\ \text{hr}^{-1}$$
$$K_P = 0.30\ \text{g/l}, \quad Y_{XA/S} = 0.3\ \text{g/g}, \quad Y_{XB/P} = 0.5\ \text{g/g}$$
$$Y_{P/S} = 1.0\ \text{g/g}, \quad Y_{P/A} = 4.0\ \text{g/g}, \quad S_0 = 10\ \text{g/l}$$

Determine the behavior of these two organisms in a chemostat. Plot S, P, X_A, and X_B versus dilution rate. Discuss what happens to organism B as the dilution rate approaches the washout dilution rate for organism A.

(Courtesy of L. Erickson, from "Collected Coursework Problems in Biochemical Engineering," compiled by H. W. Blanch for 1977 American Society for Engineering Education Summer School.)

16.4. The BOD_5 value of a wastewater feed stream to an activated-sludge unit is $S_0 = 300$ mg/l, and the effluent is desired to be $S = 30$ mg/l. The feed flow rate is $F = 2 \times 10^7$ l/day. For the recycle ratio of $\alpha = 0.5$ and a steady-state biomass concentration of $X = 5$ g/l, calculate the following:

a. Required reactor volume (V)
b. Biomass concentration in recycle (X_r)
c. Solids (cells) residence time (θ_c)
d. Hydraulic residence time (θ_H)
e. Daily oxygen requirement

Use the following kinetic parameters:

$$\mu_m = 1.5\ \text{day}^{-1}, \quad K_s = 400\ \text{mg/l}$$
$$Y^M_{X/S} = 0.5\ \text{gDW/g BOD}, \quad k_d = 0.07\ \text{day}^{-1}$$

16.5. For the activated-sludge unit shown in Figure 16.8, the specific growth rate of cells is given by the following equation:

$$\mu_{net} = \frac{\mu_m S}{K_s + S} - k_d$$

The following parameter values are known: $F = 500$ l/h, $\alpha = 0.4$, $\gamma = 0.1$, $X_e = 0$, $V = 1500$ l, $K_s = 10$ mg/l, $\mu_m = 1\ \text{h}^{-1}$, $k_d = 0.05\ \text{h}^{-1}$, $S_0 = 1000$ mg/l, $Y^M_{X/S} = 0.5$ gDW/g substrate.

a. Calculate the substrate concentration (S) in the reactor at steady state.
b. Calculate the cell concentration(s) in the reactor.
c. Calculate X_r and S_r in the recycle stream.

16.6. Wastewater with a flow rate of 6 m^3/h and COD_0 (S_0) = 1000 mg/l is treated in an activated sludge unit with aeration tank volume of 50 m^3 to obtain COD_e = 30 mg/l in the effluent. The following information are available:

$$k = 5\ d^{-1} = \mu_m/Y,\ K_s = 125\ mg/l,\ Y = 0.35\ gX/gS,\ b = k_d = 0.03\ d^{-1},\ X_r = 10\ g/l$$

a. Determine θ_c (SRT), θ_H (HRT), X and the sludge recycle ratio (r).
b. If the sludge recycle ratio is r = 0.3, what should be the flow rate of wastewater in order to obtain COD_e = 30 mg/l. Determine X and θ_H for this case.

16.7. Wastewater with a flow rate of 10 m^3/h and COD_0 = 2000 mg/l is treated in an activated-sludge unit. The following information are available:

$k = 6\ d^{-1}$, $K_s = 150$ mg/l, $Y = 0.4$ gX/gS, $b = k_d = 0.02\ d^{-1}$, $X_r = 10$ g/l, $r = 0.4$
Determine θ_{cm}, θ_c, COD_e (S), θ_H, V, X, and sludge production rate if the aeration tank is completely mixed and $\theta_c = 4\theta_{cm}$.

16.8. An aerated lagoon is an activated sludge system without sludge recycle with only a completely mixed and continuously operating aeration tank. If the wastewater flow rate is 5 m^3/h, COD_0 = 500 mg/l and the effluent COD is desired to be 30 mg/l in an aerated lagoon.
a. Determine θ_H (HRT), θ_c (SRT), biomass concentration (X), and the volume of the aeration tank (V) by using first-order kinetics.
b. Determine θ_H, θ_c, biomass concentration (X) and the volume of the aeration tank (V) by using the Monod kinetics.
c. Compare and comment on the results.

The kinetic constants are $k = 4\ d^{-1}$, $K_s = 100$ mg/l, $Y = 0.35$ gX/gS, and $b = k_d = 0$.

16.9. An activated-sludge waste treatment system is required to reduce the amount of BOD_5 from 1000 mg/l to 20 mg/l at the exit. The sedimentation unit concentrates biomass by a factor of 3. Kinetic parameters are $\mu_m = 0.2\ h^{-1}$, $K_s = 80$ mg/l, $k_d = 0.01\ h^{-1}$, and $Y^M_{X/S} = 0.5\ g$ MLVSS/g BOD_5. The flow of wastewater is 10,000 l/h, and the size of the treatment basin is 50,000 l.
a. What is the value of the solids residence time (i.e., θ_c)?
b. What value of the recycle ratio must be used?

16.10. Consider a well-mixed waste-treatment system for a small-scale system. The system is operated with a reactor of 1000 l and flow rate of 100 l/h. The separator concentrates biomass by a factor of 2. The recycle ratio is 0.7. The kinetic parameters are $\mu_m = 0.5\ h^{-1}$, $K_s = 0.2$ g/l, $Y^M_{X/S} = 0.5$ g/g, and $k_d = 0.05\ h^{-1}$. What is the exit substrate concentration?

16.11. Redo Example 16.4 if the Contois equation for growth applies. In this case, use the following equation:

$$\mu_{net} = \frac{\mu_m S}{K_{sx} X + S} - k_d$$

The values of μ_m and k_d are the same as for Example 16.4, but K_s no longer applies. Assume K_{sx} = 0.02 g BOD_5/g MLVSS.

16.12. In a trickling biological filter, the BOD value of the feed stream is S_{0i} = 500 mg/l with a feed flow of $F = 10^3$ l/h. The effluent BOD value is desired to be S_0 = 10 mg/l. The following kinetic parameters for the biocatalysts are known: r_m = 20 mgS/l-h and K_s = 200 mgS/l. The biofilm thickness is L = 0.1 mm. The cross-sectional area of the filter is A = 2 m^2, and the biofilm surface area per unit volume of the bed is a = 500 cm^2/cm^3. Assume that dissolved oxygen is the rate-limiting substrate and the diffusion coefficient of oxygen is $D_{O2} = 2 \times 10^{-5}$ cm^2/s. Determine the required height of the bed. You can assume first-order bioreaction kinetics.

16.13. Wastewater with a flow rate of $Q = 15\ m^3/h$ and $COD_0 = 1500$ mg/l is treated in a trickling biological filter with effluent recycle to obtain $COD_e = 50$ mg/l in the effluent. The following data are available:

$Y = 0.35$ gX/gS, $\mu_m = 0.1\ h^{-1}$, $X = 15$ g/l, $K_s = 150$ mg/l, $L = 1.5$ mm, $D_O/H = 4$, $H = 2.5$ m

a. Determine the effluent recycle ratio (R) in order to obtain $S_i = 500$ mg/l in the feed wastewater.
b. Determine the biofilm surface area (a_f and A_f) and particle surface area (a_p and A_p) by assuming only 65% of the particle surfaces are covered by biofilm.
c. When $a_f = 100\ m^2/m^3$, what would be the D_O and H, if $D_O/H = 4$, for the same performance of the TBF?

Use generalized design equation for TBF:

$$K_s\ L_n\ S_i/S\ +\ (S_i - S)\ =\ \frac{kXLa_f}{F(1+R)}\ Ao\ H \text{ where } k\ = \mu_m / Y$$

16.14. Wastewater with $COD_0 = 800$ mg/L is treated by using a single-stage RBC to obtain a treatment efficiency of $E = 1 - S/S_0 = 0.95$. The following information is available:

$k = 4\ d^{-1}$, $X = 100\ g/m^2$, $K_s = 120$ mg/L, $D_O = 3$ m, $N = 300$, immersion ratio is 0.40

a. What should be the flow rate of wastewater in order to obtain 95% COD removal if RBC tank is completely mixed? Determine the wet and total surface area of the disks (A_w, A_T) for this case.
b. Determine the flow rate of wastewater (F), A_w, and A_T, if the RBC tank behaves like a plug flow reactor.
c. Compare A_T/F values for both cases and comment on the results. Which reactor is better and why?

16.15. Wastewater with a flow rate of $Q = 15\ m^3/h$ and $COD_0 = 1500$ mg/l is treated by using a two stage RBC unit connected in series. The effluent of the second stage is desired to be $S_2 = 50$ mg/l. COD removal efficiencies for each stage are equal, $E_1 = E_2$. The following information is available:

$k = 3\ d^{-1}$, $X_1 = 90\ g/m^2$, $X_2 = 75\ g/m^2$, $K_s = 100$ mg/l, $D_O = 4$ m, immersion is 40%

a. Determine S_1, A_{w1}, A_{w2}, A_{WT}, A_{T1}, A_{T2}, and A_T.
b. Determine the number of disks for each stage, N_1, N_2, and N_T.

Appendix

Traditional Industrial Bioprocesses

Enzymes and microbial cells are used for production of chemicals, pharmaceuticals, foods, flavors, fragrances, and vitamins and for waste treatment. Each of these bioprocesses has unique characteristics in terms of the processing and separation technologies involved. Having covered the basics of bioprocess engineering, this appendix presents some examples of industrial bioprocesses and technologies used for production of various chemicals and pharmaceuticals. Waste-treatment aspects of bioprocess engineering are covered in Chapter 16, "Bioprocesses Utilizing Mixed Cultures."

A.1. ANAEROBIC BIOPROCESSES

Many bulk chemicals have been made industrially by anaerobic processes for a hundred years or more and represented the bulk of bioprocesses until the advert of antibiotics at the end of World War II. Although chemical processes have displaced anaerobic bioprocesses for many products, anaerobic fermentations remain important for several products. Here we review some of the most important of these processes.

A.1.1. Ethanol Production

Ethanol has many applications as a raw material, solvent, and fuel and is utilized in large quantities in the chemical, pharmaceutical, and food industries. Worldwide, 4 million tons of industrial ethanol are produced annually, 80% by fermentation. It is expected that the demand for ethanol as a fuel oxygenate will increase. An annual growth of U.S. ethanol consumption of 3.2% over the next 20 years is predicted by the Energy Information Administration.

Yeasts are the preferred organisms for industrial-scale ethanol production. Different species can be utilized, depending on the composition of the raw material utilized. *Saccharomyces cerevisiae*, particularly suitable for fermentation of hexoses, has been the major organism used so far. *Kluyveromyces fragilis* or *Candida* species can be utilized when lactose or pentoses, respectively, are the available substrates. Other alternative organisms capable of producing ethanol, such as *Zymomonas mobilis, Pachysolen* species, are not used in industrial production. However, *Zymomonas* species have significant advantages over yeast and may be used at industrial scale in the future. Other pentose and hexose fermenting organisms, such as *Clostridium hermosaccharolyticum* and *Thermoanaerobacter ethanolicus*, are thermophilic and provide significant advantages for ethanol fermentations and separation. However, they produce some undesirable end products and yield dilute ethanol solutions. Genetic engineering has transformed *Escherichia coli* into a very efficient ethanol producer, reaching ethanol concentrations up to 43% (vol/vol).

Raw materials can constitute up to 70% of the cost of ethanol production. Therefore, the selection of inexpensive materials has an important impact on process economics. The material selected should be readily and economically available in the fermentation plant. In Brazil, cane sugar is extensively used, whereas in the United States, sugar prices make sugar a nonviable raw material. Instead, corn is the most utilized material in the United States for the production of industrial ethanol. Most microorganisms require readily available sugar compounds for fermentation. Such sugar compounds are present in inexpensive raw materials, such as sugar cane, sweet sorghum, sugar beet juices, and molasses. Whey is also utilized in commercial fermentation. Starch- and cellulose-containing materials, such as grains, fruits, vegetables, wood, biomass, waste paper, and agricultural wastes, can also be used, but they need to be hydrolyzed before fermentation. Starch is enzymatically saccharified before fermentation. Acid hydrolysis of starch is not recommended, as it can result in nonfermentable or inhibitory by-products. Hydrolysis of cellulosic materials can be difficult, expensive, and inefficient. However, interest exists in increasing their utilization in an effort to recycle wastes.

Yeast converts hexoses to ethanol and carbon dioxide by glycolysis, as shown by the following reaction:

$$C_6H_{12}O \longrightarrow 2C_2H_5OH + 2CO_2 \qquad (A.1)$$

The theoretical ethanol yield over glucose is 0.51 g/g, and the growth yield over glucose is 0.12 g/g. Usually, by-products such as glycerol, succinic acid, and acetic acid are produced, and the actual yield is about 90% to 95% of the theoretical yield.

Optimal temperature and pH values for yeast are 30° to 35°C and 4 to 6, respectively. For thermophilic organisms, optimal temperature may range from 50° to 60°C. Ethanol production is triggered by anaerobic conditions. Trace amounts of oxygen (0.05 to 0.1 mm Hg) are required by yeast for lipid biosynthesis and maintenance of cellular processes.

The feed solution should be balanced in terms of nitrogen, phosphorus, minerals, and some trace elements. Glucose medium is usually supplemented with NH_4CL, KH_2PO_4, $MgSO_4$, $CaCl_2$, and yeast extract. In industry, only some ammonium and phosphate salts need to be added to diluted molasses. Glucose concentration in feed solution has an important effect on the rate and extent of ethanol production. Glucose concentrations above 100 g/l are inhibitory for yeast.

Ethanol and some of the other by-products are inhibitory to yeast above concentrations of 5% (vol/vol). Therefore, the glucose concentration in feed solution in continuous fermentation should be less than 100 g/l, resulting in ethanol concentration in the effluent below 50 g/l. Ethanol-tolerant yeast strains are being developed to avoid ethanol inhibition. Simultaneous removal of ethanol from fermentation broth is another alternative for alleviation of ethanol inhibition.

Conventional ethanol fermentations operate in batch mode under aseptic conditions. Mechanically agitated stainless-steel reactors are used for this purpose. A reactor is filled with a nutrient medium up to 70% of its volume. After pH adjustment, the reactor content is sterilized and cooled to fermentation temperature. Temperature and pH are controlled during operation, and redox potential is kept below −100mV by using reducing agents such as Na_2S. A sterile yeast culture is prepared and used for inoculation of the reactor. A batch fermentation cycle lasts nearly 30 to 40 h. Part of the yeast and aqueous medium can be recycled. Batch operation with cell recycle (the Melle–Boinot process) results in reduced fermentation times (10 h) and improved productivities of 6 g/l-h. At the end of batch operation, the reactor content is emptied and yeasts are separated by filtration or centrifugation. The liquid broth is further processed for separation of ethanol by distillation.

Continuous operation in ethanol fermentations has significant advantages over batch operation. With continuous media sterilization and aseptic operation techniques, contamination problems associated with continuous operation can be eliminated. About 95% of sugar can be converted to ethanol in continuous operation with a residence time of 21 h, as compared to batch operation time of 40 h. Under optimized conditions, the residence time for 95% conversion can be as low as 10 h. Continuous operation with cell recycle may increase fivefold the cell concentration in the reactor, resulting in faster conversion. Sedimentation tanks, centrifuges, or filters can be used for cell separation from the fermenter effluent. With cell recycle, the residence time for 95% conversion may be reduced to 1.6 h with a productivity of 30 g/l-h for a feed glucose of 100 g/l. Multistage continuous operation with cell recycle may further improve the productivity of the process. When six fermenters in series are used without cell recycle, it is possible to obtain 95 g/l in 9 h of total residence time and a productivity of 11 g/l-h.

Figure A.1 depicts the biostill process used for ethanol fermentations. This process employs continuous operation with cell recycle and a distillation column for ethanol separation. A mechanically agitated stainless-steel fermenter is used in

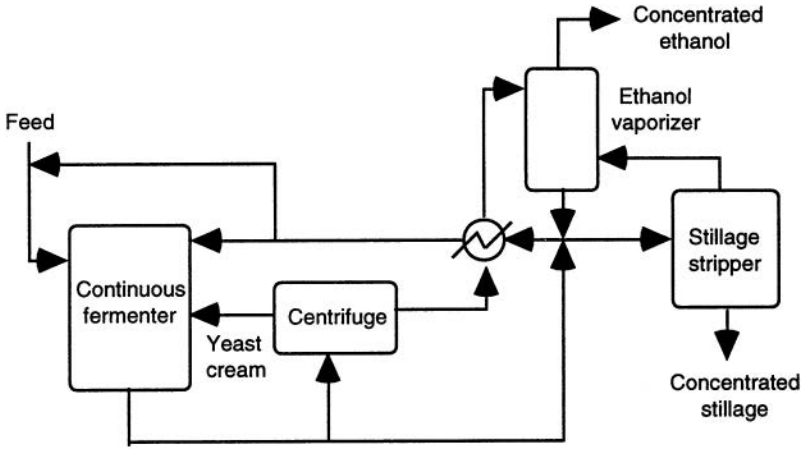


Figure A.1. Schematic for the biostill process for ethanol production.

continuous mode, and the effluent is centrifuged for yeast separation. Part of the separated yeast is recycled back to the fermenter, and the liquid medium is fed to a distillation column for separation of ethanol. Ethanol-free medum is recycled back to the fermenter. Yeast cell recycle provides high conversion rates, and liquid recycle reduces the amount of wastewater generated and dilutes the feed sugar concentration down to noninhibitory levels.

Immobilization of yeast within porous or polymeric matrices results in high cell concentrations in the reactor and, therefore, high ethanol productivities. Immobilized cell reactors may be in the form of packed columns or fluidized beds. Some flocculating yeast strains that settle rapidly may also be used in tower fermenters to obtain high cell concentrations.

A comparison of industrial processes used for ethanol production is presented in Table A.1.

Ethanol can be separated from the culture vessel during fermentation using low-temperature vacuum distillation, adsorption, or membrane separations. These methods reduce ethanol inhibition but are seldom used in industry because of operating difficulties. Separation of ethanol from fermentation broth is usually accomplished by distillation, which is energy intensive and constitutes more than 50% of the total energy consumption of the plant. The grade of industrial ethanol is usually 95% (190 proof) for chemical and pharmaceutical use, which can be obtained by using distillation columns. However, it is difficult to obtain 100% pure ethanol, since 95% ethanol in water constitutes an azeotropic mixture. A third component is added to alcohol–water mixture to break the azeotrope, but this is expensive and the third component may be toxic.

The cost of ethanol production mainly depends on the raw material and operating costs (chiefly electricity and water cooling). Total operating cost for an ethanol plant producing 190 million liters of ethanol per year is estimated to be 42 cents/l (2001 basis). Raw materials costs constitute 26 cents/l; utilities, 3.4 cents/l; labor, 6 cents/l; and fixed charges (depreciation, taxes, insurance, maintenance), 6.6 cents/l.

TABLE A.1. Comparison of Industrial Ethanol Fermentation Processes

	Total Cycle Time (h)	Ethanol Concentration (g/l)	Productivity (g/l-h)
Simple batch	36	80	2.2
Melle–Boinot	12	80	6.6
Continuous in series	8	86	11

Plant capital investment for the same size plant is estimated to be $71 million. Produced ethanol can be used as solvent, chemical intermediate (for production of acetaldehyde, acetic acid, ethylene), and fuel. In recent years, there has been an increasing demand for ethanol utilization as fuel in the form of either gasohol (10% alcohol) or pure alcohol. However, the use of ethanol as fuel can be viable only if its cost is comparable to that of oil-derived fuels.

A.1.2. Lactic Acid Production

Lactic acid was first isolated from sour milk (1780) and has two optically active forms called D- and L-lactic acids. The major use of L-lactic acid (more than 50%) is in foods as an acidulant and preservative. Lactic acid is also used as a chemical intermediate to produce other chemicals and in the pharmaceutical industry. Most lactic acid is produced by fermentation.

In industry, usually a mixture of lactic acid bacteria is used to ferment a mixture of carbohydrates. A mixture of strains may result in faster fermentation rates than pure cultures. Selected organisms should grow fast, produce lactic acid with high yields and productivities, and have low nutritional requirements. Lactic acid formation is a mixed growth–associated process that requires high growth rate and cell concentration. Lactic-acid-producing bacteria are classified in two major groups: homolactic (*Lactobacillus* sp., *Streptococcus* sp., *Pediococcus* sp.) and heterolactic bacteria (some *Streptococcus* sp., *Leuconostoc* sp.).

Homolactic species of *Lactobacillus* and *Streptococcus* are usually used for the industrial production of lactic acid. Homolactic bacteria use the Embden–Meyerhof–Parnas (EMP) pathway to generate two moles of pyruvate from one mole of glucose that are further reduced to lactic acid:

$$C_6H_{12}O_6 \longrightarrow 2C_3H_6O_3 \tag{A.2}$$

The product yield over glucose is usually above 0.9 g/g. The organisms are facultative anaerobes but generate ATP only by anaerobic fermentation. Industrially important homolactics grow at temperatures above 40°C and pH between 5 and 7. High temperature and low pH (pH <6) reduce the risk of contamination. Homolactic bacteria can produce lactic acid from pentoses as well as hexoses other than glucose. Usually, lactic and acetic acids are produced from fermentation of pentoses. Most of the lactic acid bacteria also require several B vitamins, amino acids, and phosphate. Peptides may increase cell growth rate. Fermentation yield varies, depending on substrate and the organism used.

Heterolactic fermentation is undesirable because of by-product formation. However, depending on microbial flora, energy availability, and fermentation conditions, it may occur. Heterolactic bacteria produce one mole of lactic acid, ethanol, and CO_2 from one mole of glucose:

$$C_6H_{12}O_6 \longrightarrow C_3H_6O_3 + C_2H_5OH + CO_2 \quad (A.3)$$

The theoretical lactic acid yield is 0.5 grams per gram of glucose.

Some species of *Rhizopus* (e.g., *R. oryzae*) have low nutritional requirements and can be used to produce lactic acid from carbohydrates. They have the advantage of producing stereochemically pure L-(+)-lactic acid. *Rhizopus* species can also utilize starch for the production of lactic acid.

The ideal raw material must be inexpensive, must result in high rates with high product yields and no by-product formation, should not require significant pretreatment, and should be available year round. Sucrose from sugar cane or sugar beet juice, lactose from cheese whey, and maltose or dextrose from hydrolyzed starch are used as raw materials in industry. Molasses can also be used; however, its complex nature makes separation of lactic acid problematic. Dextrose from corn starch was the most commonly used raw material in the late 1950s. Other processes based on pasteurized milk (*L. bulgaricus*), dextrose from corn, glucose (*Rhizopus*), crude sorghum extract, and potato hydrolysate have also been developed. Direct hydrolysis and fermentation of corn starch by certain *Lactobacillus* species seem to be quite promising from the economical point of view. Nitrogen sources such as malt extract, corn steep liquor, barley, and yeast extract should be added to the fermentation media to improve growth and lactic acid formation.

Industrial processes are operated batchwise. Fermenters are made of stainless steel and are equipped with heat-transfer coils. Vessels are steam-sterilized before being filled with a pasteurized medium. Slow agitation to prevent settling of calcium carbonate is provided with top-mounted mechanical stirrers. Fermentation conditions are different for each industrial producer but are usually in the range of $T = 45°$ to 60°C and pH = 5 to 6.5 for *L. delbruckii*; $T = 43$°C, pH = 6 to 7 for *L. bulgaricus*; and $T = 30°$ to 50°C, pH < 6 for *Rhizopus*. The fermentation time is 1 to 2 days for a 5% sugar source such as whey and 2 to 6 days for a 15% glucose or sucrose source. Under optimal conditions, the processing time may be reduced to 1 to 2 days. The rate of lactic acid formation depends on temperature, pH, sugar, nitrogen, and lactic acid concentrations. Temperature and pH control at optimum levels improves the rate of lactic acid formation. Produced lactic acid must be neutralized, usually by addition of calcium carbonate or calcium hydroxide. CO_2 is continuously released during fermentation, which creates anaerobic conditions in the fermenter. Lactic acid formation productivities are in the range of 1 to 3 kg/m^{3}h. The yield of lactic acid at the end of fermentation is 90% to 95% of initial sugar concentration. Cell mass yield is usually less than 15% of initial sugar concentration; however, the yield may be as high as 30%, depending on the organism and culture conditions.

Continuous, high cell density and immobilized cell reactors have been used for laboratory-scale lactic acid fermentations. Productivity and yield (100 kg/m^3) of lactic

acid formation are higher in such reactors than in batch cultures. Continuous removal of lactic acid from cultures by dialysis membranes increases production rates.

Recovery of lactic acid from the fermentation broth constitutes a significant part of production cost. The use of pure sugar solutions with minimal amounts of nitrogen simplifies product recovery. A process diagram is shown in Figure A.2. The first step is to increase the temperature of the fermenter to 80° to 100°C and the pH to 10 to 11. This procedure kills the organisms, coagulates the proteins, solubilizes calcium lactate, and degrades some of the residual sugars. The liquid is then filtered to remove biomass, and sulfuric acid is added to obtain lactic acid. Calcium sulfate is removed by filtration, and lactic acid is then concentrated. Alternatively, purification of lactic acid may be accomplished by calcium lactate precipitation. The fermenter broth is filtered and evaporated to 25% lactic acid. The calcium lactate is then crystallized and separated from the mother liquor. Several purification procedures can be performed to obtain lactic acid of different grades. These include bleaching with activated carbon, ion exchange, electrodialysis, solvent extraction, and esterification. Edible lactic acid is colorless and contains 50% to 65% lactic acid. Pharmaceutical applications require over 90% pure lactic acid. Traditionally, polymer-grade lactic acid had to be as pure as possible. However, recently polylactate has been obtained from industrial-grade lactic acid.

World demand for lactic acid was about $1.3 billion dollars (800 tons) in 2014. This is divided between fermentation and chemical synthesis where fermentation yields the highest purity lactic acid. About 30% of the market is food and beverage applications, and 10% is pharmaceuticals. The use of lactic acid to make polylactic acid is growing very quickly, as it is a biodegradable, environmentally friendly compound often used in packaging. The annual growth rate for lactic acid is projected to be greater than 15% until 2022.

Major producers of lactic acid are United States, Japan, Belgium, the Netherlands, Spain, and Brazil. China and India are also producers. The price of bulk, food-grade lactic acid is about $1.30/kg (2016). Derivatives of lactic acid such as calcium

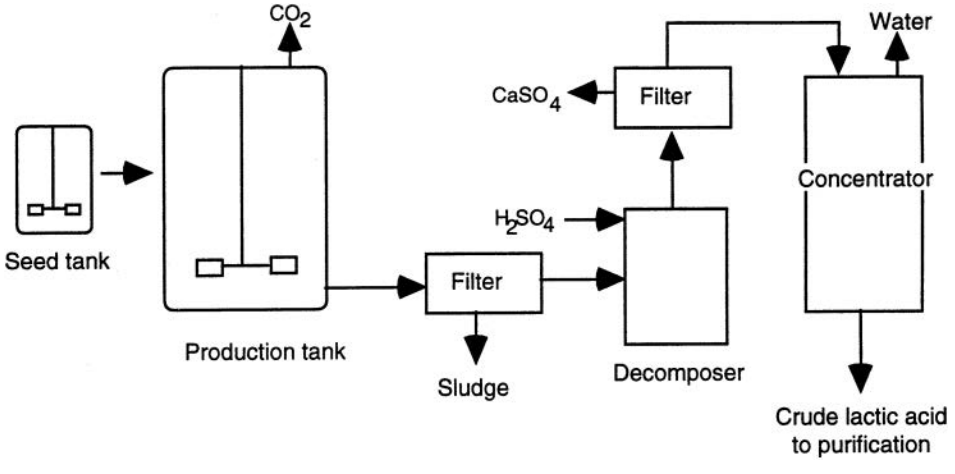


Figure A.2. Process diagram for lactic acid production.

lactate sell at a slightly higher price (by about 50%). Assuming 20% profit margin, one can estimate the manufacturing price of lactic acid as approximately $1.8/kg.

A.1.3. Acetone–Butanol Production

Acetone is used mainly as a solvent for fats, oils, waxes, resins, lacquers, and rubber plastics. Butanol is used in the production of lacquers, rayon, detergents, and brake fluids and as a solvent for fats, waxes, and resins. Butanol is a better fuel additive than ethanol because of its low vapor pressure, low solubility with water, and complete solubility with diesel fuel. Acetone and butanol (A/B) are produced from petrochemical industry intermediates. However, it is expected that the demand for cleaner processes and shortage of oil-derived products will require production by fermentation.

Clostridium species are used for A/B production. *C. acetobutylicum* is a strict anaerobe with a versatile metabolic capacity. It has amylolytic and saccharolytic enzymes to hydrolyze gelatinized starch to glucose and maltose. *C. acetobutylicum* can ferment a large number of carbohydrates such as glucose, lactose, fructose, galactose, xylose, sucrose, maltose, and starch. The fermentation products include acetone, butanol, and ethanol. Acetic and butyric acids, CO^2, and hydrogen are also produced in small amounts.

Starch, molasses, cheese whey, Jerusalem artichoke, and lignocellulosic hydrolyzates can be used as raw material for A/B fermentations. More than one of these carbon sources may be utilized simultaneously. The composition of culture medium determines the butanol-to-acetone ratio obtained and can be manipulated to obtain the desired ratio. Molasses can be used as carbon source (6% sucrose) with the addition of nitrogen and phosphate. Higher concentrations of sucrose in molasses increase the ratio of butanol to acetone and ethanol. Average solvent yields of 30% were reported when molasses was used. Utilizing a 5% to 6% starch solution derived from corn, approximately 38% solvent yield can be obtained. A butanol-to-acetone ratio of 2.75 was obtained using Jerusalem artichoke rich in inulin.

Cheese whey with nearly 6% lactose and 1% protein content can be used as carbon and nitrogen source for A/B fermentation. Somewhat different product distribution is obtained when cheese whey is used as carbon source. When corn mash was also added, the butanol-to-acetone ratio was 2. With pure glucose or pure lactose, this ratio is 2.7 and 2.9, respectively. When ultrafiltrate of cheese whey was used, butanol-to-acetone ratio of 10 was obtained without any intermediate buildup of acetic and butyric acids.

Lignocellulosics hydrolyzates (wood, paper, crop residues) contain glucose, galactose, mannose, and pentose sugars, most of which are fermentable by *C. acetobutylicum* to acetone and butanol. However, since hydrolysis of lignocellulosics is difficult, this raw material is not commonly used for A/B fermentations. Alternatively, cocultures with cellulolytic clostridia can be performed.

A schematic of the process is depicted in Figure A.3. The Weizmann process utilizes starch as raw material for A/B fermentation. *C. acetobutylicum* hydrolyzes gelatinized corn starch to glucose and maltose with amylolytic enzymes. The grain mesh is first gelatinized at 65°C for 20 min and then sterilized at 105°C for 60 min.

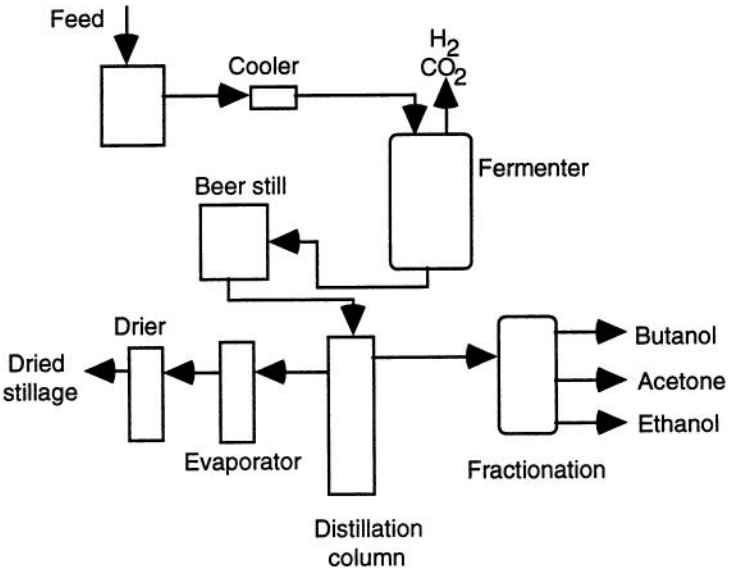


Figure A.3. Flowsheet for acetone–butanol production.

The cooked mash is cooled down to 35°C using heat exchangers and is pumped to presterilized fermenters of 250 to 2000 m^3. The fermenter is inoculated with a 5% inoculum from a 24 h culture. The final butanol-to-acetone ratio increases as the inoculum age increases. In some cases, 30% to 40% of the total volume of the mash is provided by stillage after solvents from the previous fermentation are stripped. Batch fermentation period is usually 2 to 2.5 days. First, rapid growth and production of acetic/butyric acids and carbon dioxide and hydrogen occur. The initial pH of the medium drops from 6.5 to nearly 4.5 during this phase. In a second phase, growth ceases, and the organisms convert acetic and butyric acids to neutral acetone and butanol. The acidity of the medium decreases, and gas production increases. At the end of the fermentation, the pH is approximately 5.

The South African process utilizes cane molasses as raw material and *C. acetobutylicum*. Stainless-steel fermenters of 90 m^3 are used in batch operation. The effluent contains 2% A/B, and the solvents are recovered by distillation. A/B and ethanol/isopropyl alcohol are obtained as separate fractions. The biomass, rich in riboflavin and B vitamins, is concentrated, dried, and used as animal feed supplement. Due to the high price of molasses, this process is operated intermittently. A process based on utilization of cheese whey for production of A/B has been developed. If the plant is placed near cheese manufacturers, this process can be more attractive than that based on molasses.

Different approaches have been developed to improve solvent yield in A/B fermentations. Phosphate limitation and addition of acetic and butyric acids improve butanol productivity. Calcium present in cheese whey complexes phosphate and also improves butanol yield. Hydrogen saturation in the medium at low agitation rates (25 rpm) and high head-space pressure improves butanol yield. Overall, butanol productivity may be improved by moderate to high agitation (300 rpm) during the acid

phase followed by low agitation (25 rpm) during the solvent phase. Simultaneous removal of inhibitory products (butanol, acetone) by adsorption (activated carbon) or extraction (corn oil, paraffin oil) improves fermentation productivities. Organisms can tolerate up to 1.2% (vol/vol) of butanol.

Continuous or fed-batch operations improve solvent productivities. The possibility of controlling the growth rate and environmental and nutritional conditions improves butanol productivities (2.5 g/l-h), as compared to batch cultures (0.8 g/l-h). Immobilized cultures of *C. acetobutylicum* are also used for A/B fermentations with high productivities (1.2 g/l-h). Biochemical and genetic manipulations of cells may further improve the solvent yield.

To recover A/B, at the end of the solvent phase the broth is transferred to a beer still that concentrates solvents (as shown in Figure A.3). Solvents are then separated by fractionation, and the stillage is dried. An economic evaluation was presented for A/B fermentation from cheese whey in 1982. For a plant producing 45,000 tons of solvents per year, the total capital investment including the wastewater treatment was estimated to be $28 million and the total production cost was nearly $37 million, with an annual income of nearly $53 million and an annual profit of $16 million. Nowadays, production by fermentation is not economically attractive due to low levels of product concentrations (0.7 to 1.5%) and high cost of product recovery. However, fermentation may be the preferred method for production of A/B if a shortage of oil products exists or as demands for environmentally friendly processes increase.

A.2. AEROBIC PROCESSES

Aerobic bioprocesses are widely used for the production of organic acids (citric, acetic, gluconic), vitamins, antibiotics, enzymes, flavors and fragrances, and amino acids. Owing to space limitations, only a few examples of aerobic bioprocesses are presented here.

A.2.1. Citric Acid Production

Citric acid present in citrus fruits was first crystalized from lemon juice in the form of calcium citrate. Later on, citric acid was synthesized from glycerol. Production of citric acid from sugar solutions by aerobic bioprocesses was first realized by using *Penicillium.* Due to low yields obtained from *Penicillium, Aspergillus niger* was utilized in subsequently developed processes.

Citric acid is used as an acidulant in food, confectionery, and beverages (75%). Pharmaceutical (10%) and industrial (15%) applications are also significant. Citric acid complexes with heavy metals such as iron and copper and can be used as a stabilizer of oil and fats. In the pharmaceutical industry, citric acid can be used in antacids, soluble aspirin preparations, and as a stabilizer of ascorbic acid. Metal salts of citric acid, such as trisodium citrate, are used to prevent blood clotting by complexing calcium. Trisodium citrate can be used in detergents and cleaners as a cleaning agent instead of phosphates.

A. niger is the most widely used organism for citric acid production from molasses or sugar solutions. *Candida* yeast can also be used for producing citric acid from carbohydrates or *n*-alkanes, with yields as high as 225 g/l. Beet or cane molasses can be used as source of carbohydrates for citric acid production. The concentration of heavy metals such as iron and manganese must be reduced. Typical trace-element concentrations are 0.3 ppm zinc, 1.3 ppm iron, and Mn < 0.1 ppm. High concentrations of metals can be reduced with the addition of Na- or K-ferrocyanide. Additions of nitrogen, phosphate, and other inorganic salts may not be required. Utilization of pure glucose or sucrose solutions is expensive and requires additions of nitrogen (NH_4^+), phosphate, and inorganic salts. Because of its low price and nutrient-rich nature, molasses is usually preferred to pure sugar solutions.

Citric acid production is mixed-growth associated, mainly taking place under nitrogen and phosphate limitations after growth has ceased. Since citric acid is a product of primary metabolism, it is produced in high concentrations only under very specific conditions. These include restricted growth, medium deficient in one or more essential elements, high sugar concentration, high dissolved-oxygen concentration, pH below 2, and absence of trace metals. Such conditions provoke an overflow in metabolism that results in an overproduction of citric acid. Oxalic and gluconic acids are also produced if the pH is above 2. Potassium ferrocyanide is added to reduce the concentration of metals and is a growth inhibitor that promotes the production of citric acid. The metabolic imbalance that results in citric acid production also alters the morphology of fungi. The hyphae become short, stubby, and forked in small pellets (0.2 to 0.5 mm in diameter). Limitation of certain nutrients, such as nitrogen and phosphate, stimulates citric acid formation. High dissolved-oxygen concentrations must be maintained throughout the culture. Even short interruptions in oxygen provision can result in irreversible decreases in acid production rate.

Citric acid was historically produced by surface fermentation of beet molasses, and this process is still employed by some manufacturers. It is labor intensive, but power requirements are lower than for submerged fermentation. The surface process is realized on surface-aerated trays with liquid depth of 5 to 20 cm. Sterilized diluted beet molasses, with a sugar concentration of 150 g/l and a pH of 6, is placed on trays in a temperature-controlled (30°C) and aerated clean room. Sterilized additional nutrients and alkali ferrocyanide are added into the medium. Spores of a selected strain of *A. niger* are spread over the liquid on trays. The clean chamber is aerated with filter-sterilized air to provide oxygen to the organisms and to remove fermentation heat. Mycelium forms a layer on the surface of the medium. After 7 to 10 days of incubation, the trays are emptied, the mycelium is removed, and the medium is transferred to the recovery section. The production of undesirable products such as gluconic and oxalic acids can be avoided by strain selection.

After World War II, submerged fermentation processes utilizing molasses or pure sugar solutions were developed. The submerged process is realized in deep stainless-steel vessels of 100 m^3 or larger by batch or fed-batch operation. The fermenters may be mechanically agitated or aerated towers with an internal recycle draft tube. Aeration is provided to the fermenter by air sparging (0.1 to 0.4 vvm), and temperature is controlled with cooling coils. Agitation is usually gentle (50 to 100 rpm) to avoid shear damage on molds. Diluted molasses supplemented with other nutrients is

on-line sterilized and added to the fermenter. Sterilization in the fermenter is also possible. The initial pH is adjusted at 2.5 to 3. Spores of *A. niger* are allowed to germinate in an inoculum medium before being transferred to the main fermenter. In some cases, spores are directly introduced into the fermentation media (5 to 25 × 10^6 spores/l). Since dissolved-oxygen concentration is critical for citric acid production, oxygen-enriched air may be used in some cases. About 80% of the supplied carbon is converted to citric acid in a typical fermentation. Batch operation usually results in productivities of 0.5 to 1 kg/m^3 h. Fed-batch operation can be performed to avoid substrate inhibition and to prolong the production phase 1 or 2 days after growth cessation. Typical volumetric yields of fed-batch processes are around 130 kg/m^3. When citric acid production stops, usually after 4 to 5 days, the fermenters are emptied and the biomass is separated from the broth by filtration. The liquid is transferred to the recovery section.

Precipitation is usually accomplished by addition of calcium hydroxide (lime) to the heated fermentation broth to obtain calcium citrate tetrahydrate. The precipitate is then washed and treated with dilute sulfuric acid, yielding an aqueous solution of citric acid and $CaSO_4$ (gypsum) precipitate. After bleaching and crystallization, either anhydrous or monohydrate citric acid is obtained. Solvent extraction is another option for recovery of citric acid, although it is not used commercially. Extraction avoids the use of lime and H_2SO_4 and gypsum formation; however, it may result in extraction of some impurities present in molasses. Ketones, hydrocarbons, ethers, and esters can be used as extractants. Extraction of citric acid is realized at low temperature, and the solvent is then stripped with hot water.

Almost no information is available on process economics for citric acid production because of the proprietary nature of production schemes. The global citric acid market stood at $2.6 billion in 2014 and is expected to grow to $3.6 billion by 2020.

A.2.2. Production of Baker's Yeast

Baker's yeast is essential to the modern baked goods with which we are familiar. The earliest production of baker's yeast was accomplished by a Dutch process (1781), utilizing grain mash as raw material. Later on, sugar solutions and aeration were used in Germany. The process was further improved to avoid ethanol formation and to improve biomass yield. Today, baker's yeast worldwide production is at the level of a million tons per year. The process is based on aerobic cultivation of *S. cerevisiae* on carbohydrates.

Today, the most widely used organism for baking is *S. cerevisiae.* In the past (prior to the 19th century), other strains of *Saccharomyces*, such as *S. uvarum* and *S. carlsbergensis* (brewer's yeast), had been used. However, *S. cerevisiae* is superior to other species of yeast for baking purposes, primarly because of its ability to generate gas (e.g., CO_2) in the dough. The most suitable yeast strains are selected in the laboratory and are stored by freeze drying.

Molasses is the most used carbon and energy source for production of baker's yeast. It is inexpensive, rich in nutrients (nitrogen, phosphorus, and minerals), and available year round. Other carbohydrates, such as glucose, sucrose, fructose, or hydrolyzed starch, can be used. Aqueous ammonia (NH_4OH), ammonium salts, or

urea may be used as nitrogen sources. Phosphoric acid may also be added as phosphate source. Addition of some magnesium (Mg) and ferrous (Fe) salts may be needed. Some yeast strains require B vitamins as well as Na, K, Mg, Ca, and sulfate ions for effective growth.

A material balance for baker's yeast formation can be written as follows:

$$\begin{aligned}&\text{Sucrose (200 g)} + \text{NH (10.3 g)} + \text{O}_2\ \text{(100.5 g)} + \text{Minerals (7.5 g)}\\ &\longrightarrow \text{Biomass (100 g)} + \text{CO}_2\text{(140.g)} + \text{H}_2\text{O(78 g)}\end{aligned} \tag{A.4}$$

Typical yields are about 0.5 g of cells per gram of substrate and 1 g of cells per gram of oxygen. The maximum specific growth rate of yeast is 0.6 h^{-1} (doubling time of 1.2 h). The temperature and pH of the fermentation medium are controlled at 30°C and 6 to 7, respectively. High dissolved-oxygen concentrations (above 2 mg/l) are required to promote biomass production. High carbohydrate concentrations may provoke ethanol production even in aerobic conditions (the Crabtree effect). Ethanol is inhibitory to cell growth and reduces biomass yield.

Growth of yeast results in a highly viscous culture broth. Consequently, mechanically agitated fermenters are preferred over airlifts or aerated columns. Typical reactors have a working capacity of 50 m^3 to 350 m^3 with a height-to-diameter ratio of 3. Vigorous aeration and agitation are required to provide oxygen for biomass production. Reactors are aerated in the bottom by perforated horizontal pipe spargers. Stirred tanks utilized for yeast production typically have oxygen transfer rates of 1 mole O_2/l-h and K_La of 600 h^{-1}. Temperature control is accomplished by cooling coils, and pH is controlled by addition of acid or base. Dissolved-oxygen concentration and foam are also controlled. Some plants are computerized and automatically controlled.

To avoid the Crabtree effect, production of baker's yeast is performed in fed-batch mode. The inoculum is prepared in smaller tanks and is added to the production fermenters. Multistage fermenters can be used for production. Diluted molasses, ammonium or urea, phosphoric acid, and mineral salts are stored in different tanks. The process starts batchwise after filling and sterilization of the fermenter content. Intermittent feeding of continuously sterilized nutrients starts when the initial charge of nutrients is depleted. The feeding rates of nutrients are adjusted to maximize growth rate. Dissolved-oxygen monitoring can be utilized to evaluate the metabolic activity of yeast and is the basis for feedback control systems for nutrient addition.

After 20 to 30 h of culture, the broth is transferred to centrifuges for separation of yeast. The cells are washed several times to remove inert solids. Centrifugal separation results in a light-colored cream with up to 22% solids from yeast. The cream is stored in agitated tanks at 2° to 4°C, and part is used for seeding additional fermentations. Baker's yeast can be sold in the form of cream, in compressed form (30% yeast solids), or dried (95% yeast solids). Filter press or rotary vacuum filters are used to concentrate cream. The filtered yeast may be mixed with emulsifiers prior to being extruded into yeast cakes or packaged in large paper bags. Fresh baker's yeast may be marketed as free-flowing particles by adding ingredients such as modified starch or micronized cellulose.

To produce active dry yeast, the filtered yeast cake is extruded into particles that are dried in a hot air stream. Drying temperature, drying rate, and final moisture

content of the dried yeast should be controlled during drying. The drying temperature is usually 45°C. Most of the vegetative yeast cells are killed at temperatures above 50°C. Drying time may vary from 20 min to several hours, depending on the type of drier used. The final moisture content of yeasts after drying is about 5% to 10%. Several types of driers can be used. The Roto–Louvre drier is an empty cylinder rotating at 1 to 4 rpm. Heated air at 50° to 60°C is blown to the cylinder containing yeast particles. The drying temperature is 45°C, and the duration is 10 to 15 h. In fluidized bed driers, hot air (50 to 60°C) is blown from the bottom of the column to keep the cells suspended in air. The drying temperature and time are 45°C and 1 to 2 h, respectively. Spray driers are the most widely used. A suspension with 10% to 20% yeast is atomized into a drying chamber and dried with hot air. The drying temperature and time are 50°C and 10 to 20 min, respectively. Product quality depends mainly on gassing activity of yeast. Presence of certain carbohydrates, such as trehalose or glycogenin, increases the gassing activity of yeast. Highly stable active dry yeast can be obtained from cultures with a high protein content. Fluidized bed or spray driers yield fine yeast particles of 0.2 to 2 mm with high protein content and high gassing activity.

A.2.3. Production of Penicillins

Penicillin was discovered by Alexander Fleming (see Chapter 1, "What Is a Bioprocess Engineer?"). Different penicillins are produced by different strains of *Penicillium.* Chemical structures of penicillins G and V are given in Figure A.4.

Sodium penicillin G (MW = 356.4 KDa, Activity: 1,670 U/mg) is administered parenterally, as it is degraded in acid conditions; penicillin V (MW = 372.4 KDa, Activity: 1,595 U/mg) is acid stable and is orally administered. Both forms are active against gram-positive bacteria by inhibition of cell wall synthesis. Different species of the genus *Penicillium* produce different forms of penicillin. The strain used by Fleming was *P. notatum.* Later on, different strains were used, such as *P. chrysogenum,* which is the most widely used strain in industry. Before utilization at industrial scale, considerable efforts must be spent in strain selection and development to improve the yield and activity of penicillin formation. Selected strains are stored in the form of lyophilized spores. Vegetative cells may be frozen at −70°C with glycerol as a suspending medium.

P. chrysogenum can use a variety of carbohydrates and oils as carbon and energy sources. Among those are glucose, sucrose, hydrolyzed starch, lactose, and molasses. Corn oil supplemented with lactose results in fast production of highly concentrated penicillin. Medium formulation has changed significantly with new developments.

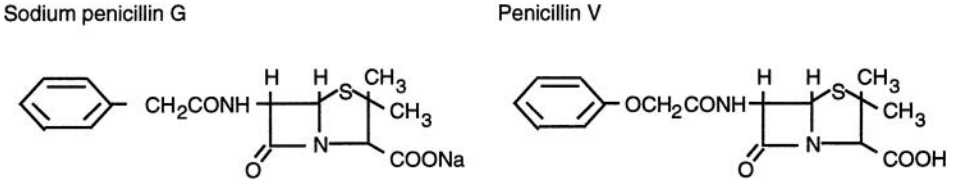


Figure A.4. Chemical structure of penicillins G and V.

The original medium (1945) contained the following compounds: lactose, 3% to 4%; corn steep liquor, 4%; $CaCO_3$, 1%; KH_2PO_4, 0.4%; and antifoam, 0.25%. Improved media resulting in higher penicillin yields have been developed. A typical composition of such media is glucose or molasses, 10%; corn steep liquor solids, 4% to 5%; phenylacetic acid (continuous feed), 0.5% to 0.8% total; and vegetable-oil antifoam, 0.5% total.

Corn steep liquor (CSL) is used as a nitrogen source, since it results in higher penicillin yields as compared to the other nitrogen sources. Some compounds in CSL are converted to phenylacetic acid or other side-chain precursors. Cottonseed flour or soybean meal may also be used as nitrogen sources; however, they are more expensive than CSL. Continuous addition of ammonium sulfate to keep the ammonium concentration around 250 to 300 mg/l is required for continued synthesis of penicillin and to avoid lysis of the mycelium.

Certain precursors of the penicillin side chain need to be added into the fermentation medium. This constitutes a major cost item. Penicillin G requires 0.47 g sodium phenylacetate and penicillin V requires 0.5 g sodium phenoxyacetate per gram of penicillin produced. Those precursors are fed continuously to avoid possible toxic effects. More than 90% of the precursors are incorporated into the structure of penicillin. Phosphorus supplied by CSL is usually sufficient, since phosphate concentration should be limiting (250 to 300 mg/l) in the media for penicillin production.

Penicillin is a secondary metabolite with a nongrowth-associated production. The process to produce penicillin involves an initial batch phase in which cell growth occurs. In the first 40 h of fermentation, rapid cell growth is achieved with a doubling time of nearly 6 h. After a high cell density has been obtained, nutrients (glucose and CSL) and precursors are added slowly or intermittently to reduce cell growth to 0.02 h^{-1} and maximize penicillin production. Oxygen, carbon, nitrogen, and phosphate concentrations should be low. A simplified diagram of the process scheme is presented in Chapter 1, Figure 1.5. Culture preparation starts with lyophilized spores and agar slant cultures. Vegetative cells are cultivated in shake flasks and then are transferred to seed fermenters (10 to 100 l). Production fermenters are agitated tanks 200 to 500 m^3 in volume made of stainless steel. Mechanical agitation is provided at a rate of 100 to 300 rpm. Temperature is controlled around 25° to 28°C (26°C optimum) by using cooling coils. Antifoam is added to reduce foam formation. Dissolved oxygen is controlled at >2 mg/l and pH at 6.5. Vigorous aeration is supplied from the bottom of fermenters by ring or tube spargers. Due to the high viscosity of the broth, oxygen transfer is a major problem in penicillin fermentations. In some cases, strains from pellets are preferred because the medium is less viscous and oxygen supply is improved. The fermentation is stopped when the oxygen uptake rate of the culture exceeds the oxygen transfer rate of the reactor, or when 80% of the fermenter is full.

The original process for the recovery of penicillin from fermentation broth was based on adsorption on activated carbon. After washing with water, the activated carbon was eluted with 80% acetone. The penicillin was concentrated by evaporation under vacuum at 20° to 30°C. The remaining aqueous solution was cooled to 2°C, acidified to pH of 2 to 3, and the penicillin extracted with amyl acetate. Penicillin was crystallized from amyl acetate with excess mineral salts at pH of 7 under vacuum. This process is uneconomical because of the high cost of activated carbon.

The current recovery process includes filtration, extraction, adsorption, crystallization, and drying. Filtration is usually achieved by using high-capacity, rotary vacuum drum filters for separation of the mycelia. The mycelia are washed on the filter and disposed. The penicillin-rich filtrate is cooled to 2° to 4°C to avoid chemical or enzymatic degradation of the penicillin. In some early processes, the filtrate was further clarified by a second filtration with the addition of alum. In recent years, macroporous filters have been used in some plants for separation of the mycelia.

Solvent extraction is accomplished at low pH such as 2.5 to 3, using amyl acetate or butyl acetate as solvent. Continuous, countercurrent, multistage centrifugal extractors (Podbielniak D-36 or Alfa–Laval ABE 216) are used for this purpose. The distribution coefficient of penicillin G or V between organic and aqueous phase depends strongly on the pH of the medium. At a pH of 3, the distribution coefficient is about 20. Penicillins G and V degrade under acidic conditions with first-order kinetics. To avoid degradation of penicillin during solvent extraction at low pH, temperature is kept around 2° to 4°C and filtration time is kept very short (1 to 2 min). Two extractors used in series result in nearly 99% penicillin recovery. Whole broth extraction (without filtration) is possible by using Podbielniak extractors in series. Due to operational difficulties, however, this approach is not used in practice. Carbon adsorption is used to remove impurities and pigments from penicillin-rich solvent after extraction. Several activated carbon columns in series can be used for this purpose.

Penicillin may be back extracted into water by addition of alkali (KOH or NaOH) or buffer at pH of 5 to 7.5. The water-to-solvent ratio in this extraction is usually between 0.1 and 0.2. A continuous, countercurrent, multistage centrifugal extractor may be used for this purpose. This step is usually omitted in the new separation schemes.

Crystallization may be performed from the solvent or aqueous phase. Na, K, and penicillin concentrations, pH, and temperature need to be adjusted for crystallization. Excess amounts of Na or K are added to the penicillin-rich solvent before crystallization in an agitated vessel. The crystals are separated by a rotary vacuum filter. The crystals may be washed and predried with anhydrous butyl alcohol to remove some impurities. Large horizontal belt filters are used for collection and drying of the crystals. Usually, warm air or radiant heat is used for drying.

Crystalline penicillins G and V are sold as an intermediate or converted to 6-APA (6-aminopenicillanic acid), which is used for production of semisynthetic penicillins. The enzyme penicillin acylase is used for cleavage of penicillin G or V to produce 6-APA. Some bacteria, *E. coli, Bacillus megaterum,* and *P. melanogenum*, as well as some molds, produce the enzyme.

Costs for penicillin production utilizing glucose as a substrate were calculated in 1982 as $19/kg. Prices of penicillin decreased to $10/kg in 2015 as a result of new producers in China and India and improvements to technology. This has put pressure on penicillin manufacturers to reduce production costs. The most important costs in penicillin production are raw materials (35%) and utilities (14%). By using cheaper raw materials, such as molasses or starch, and genetically improved strains producing higher penicillin yields, the production costs may be reduced significantly. Worldwide demand for penicillin and related beta-lactams both for medical applications and in animal feed was over $15 billion per year in 2015.

A.2.4. Production of High-Fructose Corn Syrup

High-fructose corn syrup (HFCS) is a low-calorie sweetener commonly used in beverages, desserts, and other sweet foods. Until 1935, the only syrup available was 42 DE (dextrose equivalent) acid-converted corn syrup. In 1940, enzymes were commercially available, and corn starch was hydrolyzed enzymatically to produce corn syrups. In the early 1960s, the first crystalline dextrose derived from corn was marketed. The commercial production of the enzyme glucose isomerase, which converts glucose to its sweeter (approximately 1.7 times) isomer fructose, was a major milestone. The first HFCS was produced in 1967 and contained 15% fructose. Further process improvements yielded 42% and 55% fructose-containing HFCS. The original conversion process was batch; however, immobilized enzyme technology was later used for the production of HFCS by continuous operation. U.S. production of HFCS peaked in 2000 at 9.5 million tons/yr and was about 8.5 million tons/yr in 2015.

Three major HFCS products differ by their fructose content: 42%, 55%, and 90%. HFCS containing 42% fructose is mainly used in most of the food products utilizing liquid sweeteners. HFCS with higher levels of fructose (55%) are mainly used in soft drinks as a replacement for sucrose and in jams and jellies (90%) as a low-calorie sweetener.

Production of HFCS from corn starch is an enzymatic process. The process scheme may be divided into 18 steps and five major operations (Figure A.5). Those operations are dextrose production by enzymatic hydrolysis of corn starch, primary physical and chemical treatment of dextrose syrup, isomerization of dextrose to 42% fructose, secondary refining of fructose corn syrup, and conversion of 42% fructose- to 55% fructose-containing HFCS.

First, corn starch is gelatinized by cooking at high temperatures such as 65°C and is converted to dextrose (liquefaction) by thermostable-amylase in a two-stage continuous reactor. The product is a dextrose-rich syrup containing 10 to 15 DE. The conditions in liquefaction reactors are 105°C and a 5 to 10 min holding time for the first reactor; and 95°C and a 90 to 120 min holding time for the second reactor. The feed starch slurry contains 30% to 35% solids with 0.1% to 10% enzyme at a pH of 6.5. The total and soluble protein contents of the starch slurry should be lower than 0.3% and 0.03%, respectively, to avoid color formation as a result of the Maillard reaction between amino acids and sugars at high temperatures. Saccharification of liquefied starch slurry is achieved by using the enzyme glucoamylase to produce more dextrose from branched chains of the starch. In continuous saccharification reactors, glucoamylase is added to the 10 to 15 DE liquefied starch after temperature and pH adjustment and is fed through a number of reactors in series. The conditions for this step are 60°C, pH of 4.3, and holding time of 65 to 75 h. The feed contains 30% to 35% dry substance and 1 l of glucoamylase solution per ton of dry weight of starch. The product of this step contains 94% to 96% dextrose, 2% to 3% maltose, 1% to 2% higher saccharides, and 30% to 35% dry substance. Environmental conditions must be strictly controlled during liquefaction and saccharification to obtain the 94% to 96% dextrose that is required to obtain 42% fructose HFCS.

The dextrose syrup produced by the liquefaction and saccharification step is refined to remove ash, metal ions, and proteins that may interfere with isomerization.

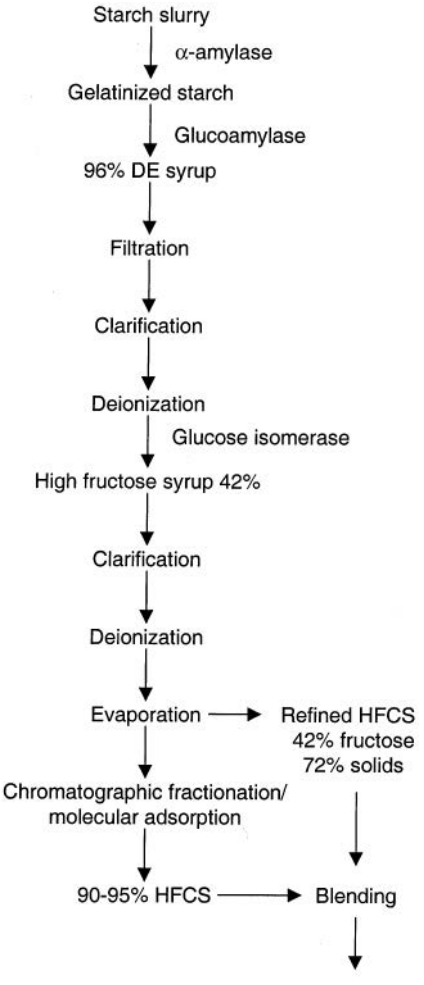


Figure A.5. Process schematic for HFCS production.

The dextrose syrup is filtered on rotary precoat vacuum filters to remove solids, proteins, and oil. The filtered liquor is then passed through several check and polish filters to remove traces of particles. The color in the filtrate is removed by granular activated carbon in columns. Carbon-treated liquor is filtered again and passed through ion-exchange columns to remove metal ions and ash. These columns are dual-pass cation–anion ion-exchange systems that also remove color. Deionized and decolorized dextrose syrup is evaporated to concentrate dextrose, and Mg ions are added to activate the isomerase.

Conversion of glucose (dextrose) to fructose by the enzyme glucose isomerize is accomplished in a packed column of immobilized enzyme. The reactor conditions are 55° to 65°C, pH of 7.5 to 8, and residence time of 0.5 to 4 h. The optimum temperature is 60°C. Temperatures higher than 60°C cause higher conversion rates and faster inactivation of the enzyme. The feed temperature may be as low as 55°C, resulting in slower conversion and enzyme inactivation rates. Microbial contamination may be a

problem at temperatures below 55°C. The optimum pH for maximum enzyme activity is 8 and for stability is between 7 and 7.5. Therefore, the operating pH is adjusted for maximum stability and activity of the enzyme. The feed syrup contains 40% to 45% dry substance, 94% to 96% dextrose, 4% to 6% higher saccharides, and 4 mM of Mg ions as activator. The enzymatic isomerization of glucose to fructose is reversible. With 96% dextrose in the feed, the equilibrium fructose concentration in the effluent is expected to be 48%. However, the exact equilibrium is not reached with 4 h of residence time, and the effluent contains 42% fructose.

The activity of the immobilized enzyme in the column drops exponentially with time. The half-life of the enzyme is 70 to 120 days. Therefore, the residence time for maximum conversion is low for new columns with unused enzymes, and the feed flow rate should be lowered at later stages of operation to obtain 42% fructose. Usually, series and parallel configurations of immobilized enzyme columns are used to compensate for activity loss of the enzyme. Parallel operation of six columns offers a good flexibility and results in good product quality. A number of process variables, such as temperature, feed pH, and flow rate, may be varied to obtain uniform product quality. Constant fructose levels are usually achieved by automatic back blending controlled by a polarimeter. Columns must be replaced two or three times a year. The cost of the enzyme is a major part of the operating cost. By improving the stability and activity of the enzyme, the cost of isomerization may be reduced significantly.

HFCS produced by isomerization of dextrose syrup can be further refined to remove color and ions by carbon treatment and ion exchange, respectively. The refined 42% HFCS is evaporated for shipment to yield 71% solids.

The HFCS from the isomerization step contains 42% fructose, 52% dextrose, and about 6% oligosaccharides. To obtain 55%- and 90%-fructose syrups, the fructose in 42% syrup needs to be concentrated. Fructose preferentially forms a complex with some cations such as calcium. This is used for concentrating fructose in HFCS. There are two commercial processes for enrichment of fructose from 42% syrup. One process utilizes an inorganic resin for selective molecular adsorption of fructose. Another process employs chromatographic fractionation using organic resins. Fructose is selectively held in fractionating columns, but dextrose is not. Deionized and deoxygenated water is used for the elution of fructose from the column. Usually, a column packed with low cross-linked fine-mesh polystyrene sulfonate-Ca cation exchange resin is used for enrichment purpose. The enriched syrup contains nearly 90% fructose and is called *very enriched fructose corn syrup* (VEFCS). The VEFCS is blended with 42%-fructose syrup to obtain the desired fructose content, such as 55%. The effluent from the isomerization step may be recycled back to the feed solution to obtain 42%-fructose syrup in the effluent of the isomerization column. The raffinate stream rich in oligosaccharides is recycled back to the saccharification step. The water used as an eluent in enrichment columns should be minimized to maximize the solids content of the syrup.

Since 1972, HFCS has replaced sucrose as a low-calorie sweetener to a large extent due to lower cost. This price difference has increased with developments in HFCS production technology. Consequently, HFCS has replaced sucrose and glucose as a low-calorie sweetener used in soft drinks, canned fruits, ice cream, and certain bakery products over the last 20 years. Low-calorie sweetener consumption is expected to level off at 130 lb per capita per year.

A.3. BIOPROCESS TECHNOLOGIES: BIOFUEL AND BIOENERGY PRODUCTION FROM BIOMASS

Renewable energy sources containing cellulose and starch constitute a vast raw material source for production of fuels, chemicals, and energy by using bioprocess technologies. Waste biomass (grains, solid–liquid wastes of food industry) provides inexpensive raw material for production of fuels and energy using bioprocess technologies. Carbohydrate-rich waste materials can be used for liquid and gas fuel production.

A general process scheme for production of biofuels from biomass is depicted in Figure A.6.

A.3.1. Production of Liquid Fuels

Ethanol and methanol are the two major fuels with production potential from waste biomass. In ethanol production, the first step is hydrolysis of cellulose or starch present in biomass by acid or enzymatic hydrolysis. Acid hydrolysis is relatively fast but requires high temperatures, high acid concentrations, and subsequent neutralization. Acid recovery is required for economic reasons. Enzymatic hydrolysis is realized by the enzymes (cellulase, amylases) produced by microorganisms under mild conditions (temperature = 30°C, pH = 7) but is relatively slow and expensive. Sugar solution obtained from hydrolysis of biomass is fermented by either yeast (*S. cerevisiae*) or bacteria (*Zymomonas mobilis*) for production of ethanol.

Anaerobic fermentation of glucose solution to ethanol takes place at 30°C and pH of 5 within 48 h. Fermentation effluent contains 5% to 10% ethanol in aqueous solution. Ethanol separation from water can be achieved by distillation, membrane separation, or selective adsorption.

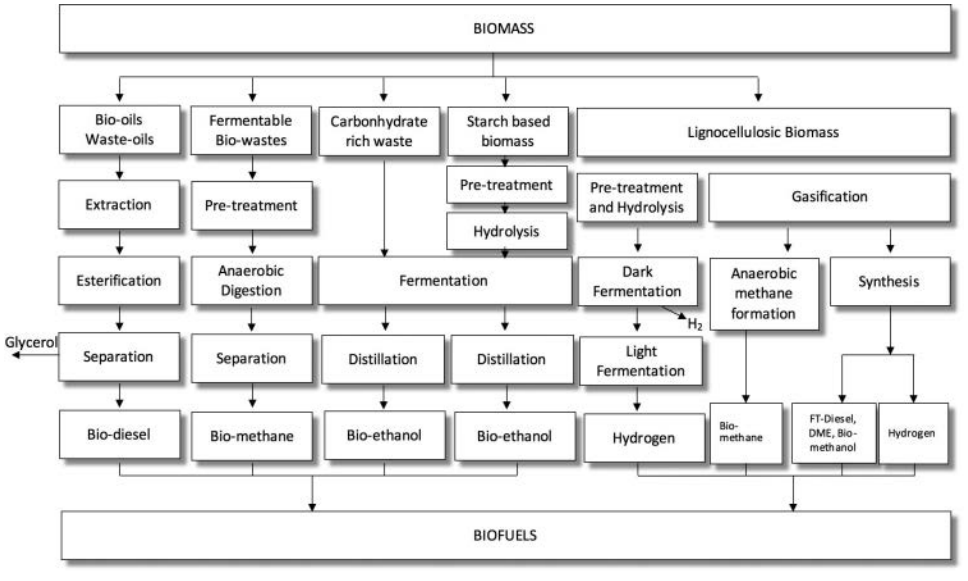


Figure A.6. Generalized process scheme for biofuels production from biomass.

Simultaneous fermentation and separation using vacuum distillation and pervaporation systems make the process economically more feasible. Figure A.7 depicts a typical bioprocess scheme for ethanol production from waste biomass.

Methanol is not an end product of any metabolic pathway. Therefore, production of methanol by fermentation alone is not possible. However, the most suitable method for methanol production is conversion of methane to methanol by enzymatic processes. Methane can be produced by anaerobic digestion of solid wastes or sludges, as described in Chapter 16, Section 16.5. Mono-oxygenase enzymes produced by some bacteria can be used to oxidize methane to methanol. Heat-treated methanotrophic bacteria (*Methlococcus* sp., *Methylomonas* sp.) with active mono-oxygenase enzyme systems can also be used for this purpose. Immobilized enzyme or cell systems are more suitable for this conversion.

A.3.2. Production of Gaseous Fuels from Biomass

Methane is the most widely known gaseous fuel produced from waste materials by anaerobic digestion. Steps involved in methane production by anaerobic digestion of waste materials are explained in Chapter 16, Section 16.5. A process scheme for methane production from waste biomass is presented in Figure A.8. The resultant biogas contains nearly 70% methane, 25% CO_2, and 5% other gases.

Hydrogen gas is a clean energy source and energy carrier with a high energy content of 122 kJ g^{-1}. Hydrogen can also be used in fuel cells for electricity generation. The major obstacle in utilization of hydrogen is its unavailability in nature. Presently, hydrogen gas is produced by steam reforming of natural gas and other hydrocarbons requiring high energy inputs. Fermentation of carbohydrate-rich raw materials for biohydrogen production offer significant advantages over costly chemical processes due to operation under mild conditions (30 to 35°C, 1 atm). Waste biomass, such as agricultural and domestic wastes, can be used as inexpensive raw materials for biohydrogen production at large scale. Biohydrogen production from carbohydrate-rich wastes requires dark and light anaerobic fermentations. The first

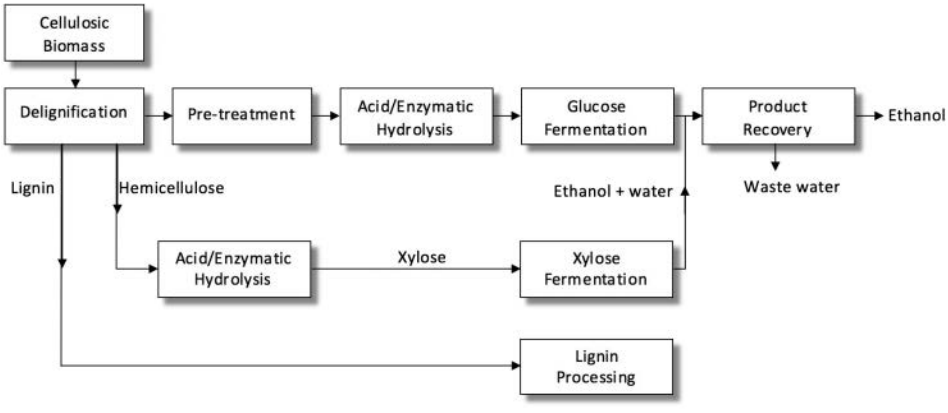


Figure A.7. A schematic diagram of ethanol production from cellulosic biomass.

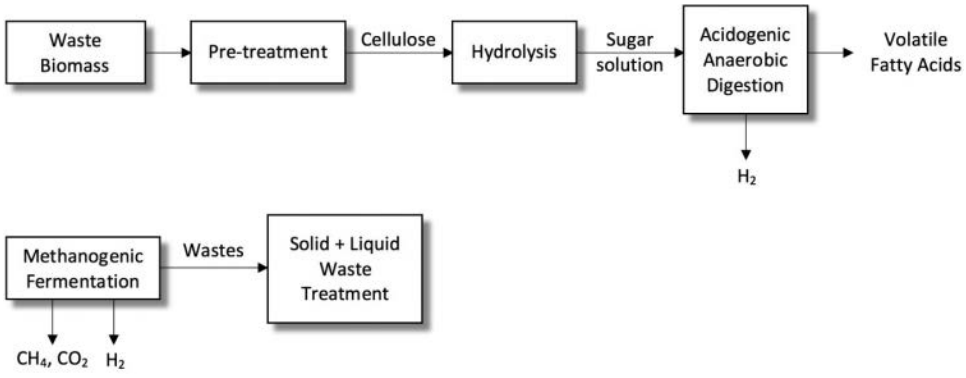


Figure A.8. A process scheme for methane production from waste biomass.

step in fermentative hydrogen production from biomass is the acid or enzymatic hydrolysis of biomass to highly concentrated sugar solution, which is followed by dark fermentation by the acetogenic-anaerobic bacteria for production of volatile fatty acids (VFA), hydrogen, and CO_2. Volatile fatty acids produced by the dark fermentation are further fermented by the photo-heterotrophic bacteria (light fermentation) such as *Rhodobacter* species to produce CO_2 and H_2.

A process scheme for biohydrogen production from waste biomass and carbohydrate-rich wastewater is depicted in Figure A.9. Starch- or cellulose-containing waste is subjected to pretreatment after grinding to make the biomass more suitable for hydrolysis. Glucose syrup obtained after hydrolysis and neutralization is fed to dark fermentation for hydrogen and VFA production. Photo-fermentation of dark fermentation effluent is used for converting VFAs to H_2 and CO_2.

A.3.3. Bioelectricity Generation from Wastes Using Microbial Fuel Cells

Electrical power generation with simultaneous wastewater treatment can be achieved by using microbial fuel cells (MFCs). A typical MFC consists of anode and cathode chambers separated by a proton exchange membrane (PEM). In an anodic chamber, anaerobic organisms degrade organic compounds present in wastewater or sludge, generating electrons and protons. Generated electrons are transferred through an external circuit, and protons diffuse through the membrane to the cathodic chamber. Electrons and protons transferred to the cathodic chamber form water in the presence of dissolved oxygen. Electron transfer through an external wire provides electrical current generated by the MFC. Anode can be graphite, carbon cloth, or copper sheet, and cathode may be platinum (Pt)-patched graphite, carbon cloth, or gold-covered copper sheet. Originally developed in two-compartment configuration, MFCs are now used in a single-chamber air-cathode configuration. Figure A.10 depicts two-compartment and single-compartment MFC configurations. The voltage generated between the two electrodes can be as high as 1 V, and power densities of 40 W/m^2

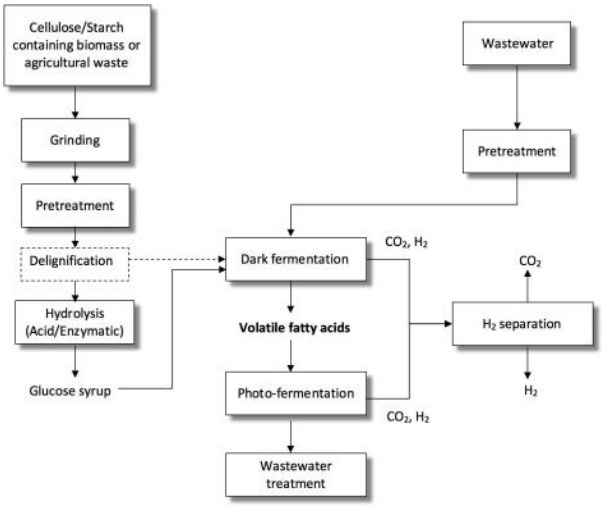


Figure A.9. A schematic diagram of biohydrogen production from waste biomass.

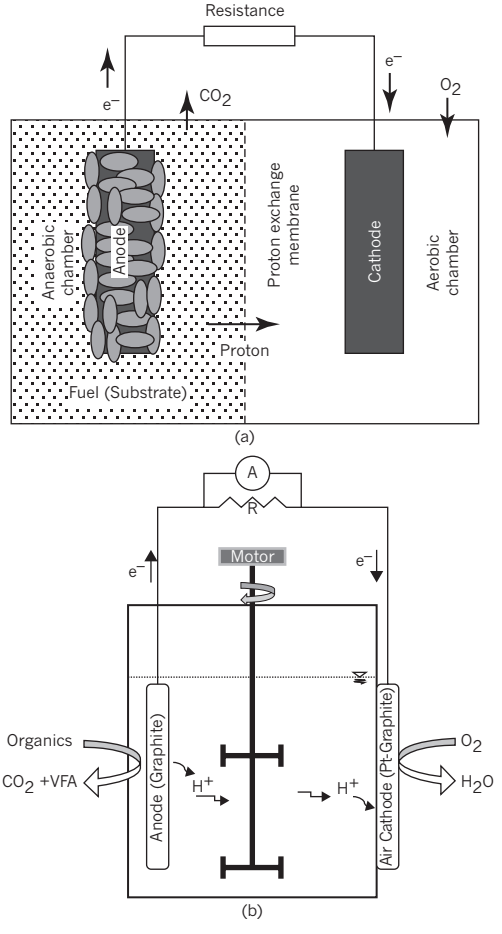


Figure A.10. (a) Two-compartment and (b) single-compartment MFC configurations.

anode were obtained by using single-chamber air cathode MFCs with considerable chemical oxygen demand (COD) removal from wastewater.

Microbial electrolysis cells (MECs) are used for hydrogen gas formation instead of electricity generation in MFCs. MECs are somewhat similar to MFCs with some differences. Cathode is not in contact with air and is completely immersed in wastewater. A low voltage (0.2 to 0.5 V) is applied to the cathode along with the generated voltage in the system (0.7 to 1 V) for hydrogen formation on cathode. Protons and electrons generated by the microorganisms growing on anode surfaces by anaerobic metabolism of organics combine with the extra electrons supplied by the external voltage source to form hydrogen gas on cathode. MFCs generate electricity; MECs produce hydrogen gas with extra energy input.

SUGGESTIONS FOR FURTHER READING

Atkinson, B., and Mavituna, F., *Biochemical Engineering and Biotechnology Handbook,* 2d ed., Stockton Press, New York, 1992.

Bailey, J. E., and Ollis, D. F., *Biochemical Engineering Fundamentals,* 2d ed., McGraw-Hill, New York, 1986.

Demain, A. L., "Small Bugs, Big Business: The Economical Power of the Microbe," *Biotechnol. Adv.* 18: 499–514, 2000.

Flickinger, M. C., and Drew, S. W., Eds., *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation,* Wiley, New York, 1999.

Logan, B. E., *Microbial Fuel Cells,* Wiley, New York, 2008.

Yang, S. T., El-Ensashy, H., and Thongchul, N., Eds., *Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels, Chemicals and Polymers*, Wiley, New York, 2013.

Moo-Young, M., Ed., *Comprehensive Biotechnology,* Vol. 3, Pergamon Press, Oxford, UK, 1985.

Index

A

A (adenine), 45, 47
A. nidulans, 478
Ab (antibody) macromodule, 34
Acetone production, 584–586
Acid formers, in waste treatment, 564
Acidophiles, 14
Actin filaments, 25, 433
Actinomycetes, 19
Activated-sludge processes, biological waste treatment, 551–553
Active cell immobilization, 298–302
Active sites, 63
Acylation, 115
ADC (adsorption chromatography), 410–411
ADC (antibody-drug conjugate), 36
Adenosine monophosphate (AMP), 45
Adenosine triphosphate (ATP). *See* ATP (adenosine triphosphate).
Adsorbent-added activated-sludge process, 557–558
Adsorption
  ADC (adsorption chromatography), 410–411
  cells, 300–301
  enzyme immobilization, 87
  Freundlich adsorption isotherm, 403–404
  immobilized cell systems, 300–301
  separation of soluble products, 403–409
Adsorption chromatography (ADC), 410–411
Adsorption zone, separation of soluble products, 405
Aeration, bioreactor scale-up, 331–337
Aerobic bioprocesses. *See also* Anaerobic bioprocesses.
  baker's yeast production, 588–590
  citric acid production, 586–588
  HFCS (high-fructose corn syrup) production, 593–595
  penicillin production, 590–592
  VEFCS (very enriched fructose corn syrup) production, 595
Aerobic organisms, 14

Aerobic respiration, glycolysis, 149
Aerotaxis, 138
AFC (affinity chromatography), 411, 414–419
Ag (antigen) macromodule, 34, 36
Aggregates in plant cell cultivation, 460
Agitation, bioreactor scale-up, 331–337
Agricultural wastes, biological waste treatment, 549
Airlift system, bioreactor scale-up, 329–330
Ajmalicine from periwinkle, 465
Aldehydes, 38
Algae
  Chlorella, 27
  definition, 27
  Dunaliella, 27
  microscopic eucaryotes, 27
  Scenedesmus, 27
  size, 27
  Spirulina, 27
Alkaliphiles, 14
Alkylation, 116
Allosteric enzymes, 71–73
Allostery, 71–73
Ameboid motion, 27
Amensalism, model of mixed culture interaction, 538–539
Amino acids. *See also* Proteins.
  in cell construction. *See* Cell construction, amino acids.
  chemical structure, 31
  a dipolar molecule, 32
  isoelectric point, 32
  natural, 31
  peptide bond, 32
  in polypeptides, 31
  proteinogenic, 31
  pyrrolysine, 31
  selenosysteine, 31
  zwitterion, 32
Aminoacyl site, 125
Aminoacyl-tRNA synthetases, 125
Amoebae, 27
AMP (adenosine monophosphate), 45
Amplifying DNA, 260. *See also* PCR (polymerase chain reaction).
Amylases, 101
Amylopectin, 41
Amylose, 41
Anabolism, definition, 146
Anaerobic bioprocesses. *See also* Aerobic bioprocesses.
  acetone production, 584–586
  butanol production, 584–586
  ethanol production, 578–581
  lactic acid production, 581–584
Anaerobic digestion, biological waste treatment, 564–566
Anaerobic metabolism
  anaerobic respiration, 161
  denitrification, 161
  ED (Entner–Doudoroff) pathway, 161
  fermentation, 161
  overview, 161–163
Anaerobic organisms, 14
Anaerobic respiration, 149, 161
Anchorage-dependent cells, 432, 435
Animal cell cultivation
  anchorage-dependent cells, 435
  basic techniques, 434–437
  cancer linkage to cell transformation, 435
  contact inhibition, 435
  continuous cells, 435
  growth dynamics, 441–443
  growth media, 437–441
  hybridoma cells, 436
  immortal cells, 435
  insect cells, 435–436
  mammalian cell culture, 436–437
  mortal cells, 435
  nonanchorage-dependent cells, 435
  primary culture, 434–435
  products of, 447–448
  secondary culture, 435
  senescence, 435
  transformed cells, 435–436
Animal cell cultivation, bioreactor considerations
  constraints, 443
  entrapped cells in stirred reactors, 445–447
  hollow-fiber reactors, 445
  nonstirred reactor systems, 444–445
  single-use disposable systems, 445, 447
  stirred-tank bioreactors, 447
  suspended cultures, 447
Animal cells
  actin filaments, 433
  anchorage-dependent cells, 432
  biochemistry, 431–434
  centrosome, 433
  cilia, 433
  cisternae, 433

cytoskeleton, 433
cytosol phase cytoplasm, 432
dictyosome, 433
ER (endoplasmic reticulum), 432
glyoxysomes, 433
Golgi body, 433
intermediate filaments, 433
lumenal phase cytoplasm, 432
lysosomes, 432
metabolism of nutrients, 433–434
microtubules, 433
microvilli, 432
mitochondria, 432
nucleoli, 433
perinuclear space, 433
peroxisomes, 433
size, 431
structure, 431–434
types of filaments, 433
Animal *vs.* plant eucaryotes, 22
Anisotropic membranes, separation of soluble products, 395
Anoxygenic photosynthetic bacteria
bacteriochlorophyll, 19
light-gathering pigments, 19
in photosynthesis, 19
Antibodies, proteins. *See* Proteins, antibodies.
Antibody (Ab) macromodule, 34
Antibody–antigen complex, 36
Antibody–antigen interaction, 36
Antibody–drug conjugate (ADC), 36
Anticancer agent (Taxol), in plant cell cultivation, 456–457
Anticodons, 125
Antigen (Ag) macromodule, 34, 36
Apoenzyme, 61
Apparent growth yield, 179–180
Aqueous two-phase affinity partition extraction, separation of soluble products, 389
Aqueous two-phase extraction, separation of soluble products, 389–390
Archaebacteria
procaryotes, 15
structure of, 20
Artificial tissue/organs, 520–521
Ascospores, 25
Asexual reproduction
eucaryotes, 23
microscopic eucaryotes, 25
Asparaginase, uses for, 102
ATP (adenosine triphosphate)
maintenance coefficient, 227–229
oxygen consumption, 227–229
phosphate bonds, 45
regularities, 229
ATP (adenosine triphosphate), yield coefficients
maintenance, 227–229
oxygen, 227–229
theoretical predictions, 235–236
Autographa californica (baculovirus), 265, 480
Autologous implantation, 523
Autotrophic metabolism, 163–165
Autotrophs, 52
Auxotrophic mutants, 250–251

B

B. subtilis
description, 19
genetic engineering, host-vector systems, 477
Bacillus (cylindrical) cells, 14
Bacillus (rod) cells, 14
Back mutations, 248–249
Bacteria. *See also* Archaebacteria; Eubacteria (true bacteria).
architecture of, 21
capsule, 20
chromatophores, 20
endospores, 20
Gram staining, 18
intracellular spores, 20
outside cell wall. *See* Capsule.
photosynthesis, 20
Bacterial microcompartments (BMCs), 20
Bacteriochlorophyll, 19
Bacteriophages. *See* Phages.
Baculovirus (*Autographa californica*), 265, 480
Baker's yeast production, 588–590
Balanced growth, 177
Balanced growth assumption, 192
Bardenpho process, biological waste treatment, 571
Basal level of proteins, 131
Basic local alignment search tool (BLAST), 271
Batch bioreactors
vs. continuous, 276–278
genetic instability, 277
market economics, 277
operability, 277
semicontinuous. *See* Fed-batch bioreactors.
variable-volume continuous culture. *See* Fed-batch bioreactors.

Batch bioreactors, modifying
chemostat with recycle, 278–281
external membrane bioreactors, 295
fed-batch operation, 288–293
internal membrane bioreactors, 295
membrane bioreactors, 294–297
multistage chemostat systems, 281–287
perfusion systems, 293–294
Batch cell growth, growth patterns
apparent growth yield, 179–180
balanced growth, 177
death phase, 179
deceleration phase, 178
decline phase, 179
diauxic growth, 177
endogenous metabolism, 178
exponential phase, 177
growth-associated products, 183
lag phase, 175–177
Luedeking–Piret equation, 183
maintenance, 180
maintenance energy, 178
microbial products, classifying, 183–184
mixed-growth-associated product formation, 183
nongrowth-associated product formation, 183
nongrowth-related functions, 181–182
phases, 175
primary metabolites, 178
secondary metabolites, 178
stationary phase, 178–179
yield factors for aerobic growth, 182
Batch cell growth, quantifying
cell concentration, quantifying, 170
cell mass concentration, determining, 171–175
cell number density, determining, 171
counting viable cells, 171
direct cell counting, 171
direct methods, 172
dry weight, determining, 171–172
hemocytometer, 171
indirect methods, 172–175
optical density, 172
packed cell volume, 172
particle counters, 171
Petroff–Hausser counting chamber, 171
plate counts, 171
turbidity, 172
viable count, 171
Batch cell growth kinetics
critical oxygen concentration, 187–188
DO (dissolved oxygen) effects, 187–188
environmental conditions, effects of, 184–189
heat generated by microbial growth, 190–191
OTR (oxygen transfer rate), 188
OUR (oxygen uptake rate), 188
pH effects, 186
redox potential, 189
temperature effects, 184–185
Batch cell growth kinetics, quantifying
balanced growth assumption, 192
batch growth, 200–201
Blackman equation, 194
carrying capacity, 197–198
cell structure, 191
chemically structured models, 202–207
Contois equation, 194
cybernetic models, 207–208
extrinsic concentration, 202
Gompertz equation, 200–201
half-velocity constant, 192–193
intrinsic concentration, 202
logistic equations, 197–200
maximum growth potential, 200–201
models for filamentous organisms (molds), 201
models for transient behavior, 201–202
models with growth inhibitors, 195–197
models with time delays, 202
Monod equation, 192–194, 197
Moser equation, 194
overview, 191–192
product formation, 200–201
product inhibition, 196
saturation constant, 192–193
segregation, 191
structured models, 191
substrate inhibition, 195
substrate-limited growth, 192–194
Tessier equation, 194
unbalanced growth, 191
unstructured nonsegregated models, 192–201
Batch growth, 200–201
Batch kinetics, 69–70
Bed depth–service time equation, separation of soluble products, 405–409
Bidirectional DNA, 117
Binding, immobilized cell systems, 300–302

Biochemical engineering
vs. bioprocess engineering, 3
bioreaction engineering, 2
bioseparations, 2
definition, 2
Biochemistry of animal cells, 431–434
Biodegradation of xenobiotics (toxic aromatic compounds), 157–158
Bioelectricity from wastes, 598–600
Bioenergetics, 146–148
Bioengineering, definition, 2
Bioengineers, definition, 2
Biofuel and bioenergy production
bioelectricity from wastes, 598–600
gaseous fuels from biomass, 597–598
hydrogen gas production, 597–598
liquid fuel production, 596–597
methane production, 597–598
MFCs (microbial fuel cells), 598–600
Bioinformatics, 271
Biolistic processes, 265–266
Biological engineering, definition, 2
Biological films, 303–304
Biological oxygen demand (BOD), 550
Biological waste treatment. *See* Mixed culture, biological waste treatment.
Biologists
research approaches, 3
working with engineers, 4–9
Biomedical engineering, definition, 2
Biomolecular engineering, definition, 2
Biopharmaceutical products from genetic engineering, 473
Bioprocess engineering
vs. biochemical engineering, 3
industrial uses for. *See* Aerobic bioprocesses; Anaerobic bioprocesses; Biofuel and bioenergy production.
origins of, 8
Bioprocess engineering, medical uses for
artificial tissue/organs, 520–521
autologous implantation, 523
bioreactor considerations. *See* Bioreactors, bioprocess engineering.
cartilage replacement, 522–523
chondrocyte culture, 522–523
endocytosis, 525
endosomes, 525
gene therapy using viral vectors, 523–528
mass production of retrovirus, 527–528
models of viral infection, 524–527
regenerative medicine, 519–523
skin replacement, 521–522
tissue engineering, 519–523
viral gene delivery systems, 524
Bioprocesses, regulatory constraints, 9–10
Bioreaction engineering, 2
Bioreactor considerations, animal cell cultivation
constraints, 443
entrapped cells in stirred reactors, 445–447
hollow-fiber reactors, 445
nonstirred reactor systems, 444–445
single-use disposable systems, 445, 447
stirred-tank bioreactors, 447
suspended cultures, 447
Bioreactor considerations, plant cell cultivation
ajmalicine from periwinkle, 465
cell immobilization, 463–465
hairy roots, 466–467
mixotrophic cultures, 467
organized tissues, 466–467
photoperiod control, 467
root mats, 467
submerged culture, 467
suspension cultures, 462–463
Bioreactor instrumentation and control
of chemical environment, 350–351
control strategies, 355
data logging, 354
exit-gas measurement, 352
FTIR (Fourier transform infrared), 352
gateway sensors, 354
HPLC (high-pressure liquid chromatography), 352
insertable probes, 349–351
measuring active fermentation, 349–352
of physical environment, 350
probe drift, 351
probe fouling, 351
recalibrating probes in situ, 351
using information from, 352–355
Bioreactor scale-up
bioreactors, definition, 324
common approaches, 337–342
fermenters, definition, 324
geometric similarity, 337
internal mechanical agitation, 324–329
overview of reactor types, 324
practical limits, 341
Rushton impeller, 325

Bioreactor scale-up, common difficulties
aeration, 331–337
agitation, 331–337
airlift system, 329–330
bubble column reactors, 329–330
cleaning, 329
dynamic method for oxygen removal, 335
energy efficiency, 329
foaming, 328
heat transfer, 331–337
internal mechanical agitation, 324–329
loop reactors, 329–330
oxygen solubility, 332–335
single-use bioreactors, 330–331
steady-state method for oxygen removal, 334
sterility, 329
unsteady-state method for oxygen removal, 333
working volume, 328
Bioreactors. *See also* Batch bioreactors; Continuous bioreactors.
attached cells, 311–313
cleaning, 329. *See also* Sterilizing process fluids.
cultivation method, choosing, 276–278
definition, 324
hybrids, 311–313
immobilized cell systems, 309–311
impellers, 324–325
microreactors, 342–348
mixed and forcefully aerated, 314
overview of reactor types, 324
packed-bed, 314
rotating drum, 314
scaling down, 342–348
for solid-state fermentation, 313–316
spargers, 324
sterilizing. *See* Sterilizing process fluids.
suspended cells, 311–313
tray, 314
Bioreactors, medical applications
body-on-a-chip systems, 530–531
extracorporeal artificial liver, 530
flatbed reactors, 530
fluidized bed reactors, 530
hematopoiesis, 529–530
iPS (induced pluripotent stem) cells, 529
membrane-based units, 530
microphysiological systems, 531
multiorgan-on-a-chip systems, 531
PBPK (physiologically based pharmacokinetic) model of the human body, 531
pluripotent stem cells, 529
progenitor cells, 529
stem cells, 529–530
Bioseparations, 2
Biosynthesis, 158–161
gluconeogenesis, 160
HMP (hexose monophosphate) pathway, 158–161
overview, 158–161
pentose–phosphate, 158–161
Biotechnology, definition, 2
Blackman equation, 194
BLAST (basic local alignment search tool), 271
Blue-green algae (cyanobacteria)
growth environment, 14
photosynthesis, 19
BMCs (bacterial microcompartments), 20
BOD (biological oxygen demand), 550
Body-on-a-chip systems, 530–531
Bottle plant, penicillin production, 6
Briggs, G. E., 66
Bubble column reactors, scaling up, 329–330
Butanol production, 584–586

C

C (cytosine), 45, 47
Cabbage looper (*Tricoplusia ni*), 480
Cake resistance, separation of insoluble products, 376–378
Callus cultures in plant cell cultivation, 458–459
Calvin cycle, 163–165
Campylobacter jejuni bacterium, 129
Cancer
bioprocesses for the anticancer agent Taxol, 456–457
linkage to cell transformation, 435
transformed cells, 479
Capsid coating, viruses, 17
Capsule, bacteria, 20
Carbohydrates
amylopectin, 41
amylose, 41
in cell construction, 37–42
cellulose, 41
deoxyribose, 39
disaccharides, 40
D-ribose, 39

glycans, 42
glycogen, 41
glycosylation, 42
lactose, 40
monosaccharides, 37–39
photosynthesis, 37
polysaccharides, 40–42
role in cells, 37
sucrose, 40
Carbon compounds, macronutrients, 52–53
Carrying capacity, 197–198
Cartilage replacement, 522–523
Catabolism
definition, 146
of glucose, 149–152
Catabolite repression, 131
Catalytic proteins, 30
cDNA (complementary DNA), 260
CDRs (complementarity determining regions), 36
Cell adsorption, 300–301
Cell concentration, quantifying, 170
Cell construction
carbohydrates, 37–42
composition of living cells, 28
fats, 42–45
fatty acids, 42–45
integral member proteins, 43
lipids, 42–45
nucleic acids, 44–51. *See also* DNA (deoxyribonucleic acid); RNA (ribonucleic acid).
peripheral member proteins, 43
PHA (polyhydroxyalkanoates), 44
PHB (polyhydroxybutyrate), 44
phosphoglycerides, 43
phospholipids, 43
scientific domain. *See* Systems biology.
Cell construction, amino acids. *See also* Proteins.
chemical structure, 31
a dipolar molecule, 32
isoelectric point, 32
peptide bond, 32
in polypeptides, 31
proteinogenic, 31
pyrrolysine, 31
selenosysteine, 31
zwitterion, 32
Cell death
batch cell growth, 179
kinetics of, 356–357
in sterilization, 356–357
and thermodynamic equilibrium, 51
Cell disruption, separation of insoluble products
mechanical, 383–385
nonmechanical, 384–385
Cell division among eucaryotes, 23, 26
Cell growth
culturing cells in a vessel. *See* Batch cell growth.
growth factors, 139
growth media, 55–56
net specific growth rate, 169–170
rate of microbial growth, 169–170
Cell growth in continuous culture
cultivation devices, 208–210
overview, 208
PFRs (plug flow reactors), 208, 210
turbidostats, 208–210
Cell growth in continuous culture, chemostats
CFSTR (continuous-flow, stirred-tank reactor), 210
description, 208–210
deviations from ideality, 218
ideal device, 210–217
maintenance coefficient, 213
mutations, 217
as a tool, 217–218
washing out a culture, 211
Cell immobilization, 463–465
Cell lysis, 18
Cell mass concentration, determining, 171–175
Cell motion, eucaryotes, 24–25
Cell nomenclature
archaebacteria, 15
by cell structure, 15. *See also* Eucaryotes; Procaryotes.
definition, 15
dual names, 15
eubacteria (true bacteria), 15
genes of a whole organism. *See* Genome.
genus, 15
Latin names, 15
for microorganisms, 15
for organizing knowledge. *See* Cell taxonomy.
within procaryotes, 15
recognized subdivisions of organisms, 16
viruses, 15
Cell number density, determining, 171

Cell nutrients
categories of, 51. *See also* Macronutrients; Micronutrients.
growth media, 55–56
intracellular composition of cells, 51–52
overview, 51–52
thermodynamic equilibrium and death, 51
Cell receptors, role of, 137–139
Cell separation. *See* Separation.
Cell shapes
cylindrical (bacillus), 14
rod (bacillus), 14
spherical, elliptical (coccus), 14
spiral (spirillum), 14
Cell taxonomy
definition, 14–15
for naming organisms. *See* Cell nomenclature.
for organizing knowledge, 14–15
Cell-free protein synthesis (CFPS), 484
Cells. *See also* DNA (deoxyribonucleic acid); RNA (ribonucleic acid).
active transport, 135–137
acylation, 115
aerotaxis, 138
alkylation, 116
cell receptors, role of, 137–139
cellular differentiation, 137–139
central dogma, 114–117
chemotaxis, 138
codons. *See* Genetic code.
cotranslational modifications, 115–116
disulfide bond formation, 115
energy-independent uptake, 135–137
estimation of elemental composition, 236–237
facilitated diffusion, 135–137
genes, 116–117. *See also* Genetic code.
genetic code. *See* Genetic code.
glycosylation, 115
group translocation, 135–137
hormones, 139
hydroxylation, 116
inteins, 116
introns, 116
lipidation, 116
macromolecules. *See* DNA (deoxyribonucleic acid); Protein; RNA (ribonucleic acid).
major groups of. *See* Eucaryotic cells; Procaryotes.
metabolic regulation. *See* Metabolic regulation.
metabolism, 137–139
mRNA splicing, 116
with or without oxygen. *See* Facultative organisms.
oxygen toxicity. *See* Anaerobic organisms.
passive diffusion, 135–137
phosphorylation, 116
phosphotransferase system, 137
phototaxis, 138
posttranslational modification, 115–116
protein splicing, 116
proton-motive forces, 136–137
replication, 114
retrotransposons, 115
reverse transcriptase, 115
reverse transcription, 115
sensing external environment, 135–139
steroid hormone receptors, 139
sulfation, 116
telomere synthesis, 115
transcription to RNA. *See* DNA transcription.
transfer of biological information, 114–117
translation into proteins, 114, 127–129
transporting small molecules across membranes, 135–137
ubiquitination, 116
ubiquitins, 116
using oxygen. *See* Aerobic organisms.
Cells and their environments
acidophiles, 14
aerobic organisms, 14
alkaliphiles, 14
anaerobic organisms, 14
cyanobacteria, 14
extreme. *See* Extremophiles.
extremophiles, 13–14, 21
facultative organisms, 14
halophiles, 14, 21
high salt concentrations. *See* Halophiles.
high temperatures. *See* Thermophiles.
low temperatures. *See* Psychrophiles.
mesophiles, 14
with minimal nutrients. *See* Cyanobacteria.
moderate temperatures. *See* Mesophiles.
neutrophiles, 14
oxygen adaptive. *See* Facultative organisms.
oxygen inhibited. *See* Anaerobic organisms.
oxygen required. *See* Aerobic organisms.
pH values 1 or 2. *See* Acidophiles.

pH values 6 to 8. *See* Neutrophiles.
pH values above 9. *See* Alkaliphiles.
psychrophiles, 14
thermoacidophiles, 21
thermophiles, 14
Cell-surface display, 265
Cellular differentiation, 137–139
Cellulases, 101
Cellulose, 41
Centrifugal Podbielniak extractors, separation of soluble products, 387–388
Centrifugation, separation of insoluble products, 378–382
Centrifugation coefficient, separation of insoluble products, 381
Centrosome, 433
Cer locus, 493
CFPS (cell-free protein synthesis), 484
CFSTR (continuous-flow, stirred-tank reactor), 210, 552
Chain, Ernst, 4
Chaperones, 125
Chelating agents, 55
Chemautotrophs, 52
Chemical agents for causing mutations, 250
Chemical environment, monitoring, 350–351
Chemical gene synthesis, 259
Chemical oxygen demand (COD), biological waste treatment, 550
Chemical synthesis, penicillin production, 5
Chemical waste treatment, 549
Chemically structured models, 202–207
Chemostat with recycle bioreactor, 278–281
Chemostats
CFSTR (continuous-flow, stirred-tank reactor), 210
description, 208–210
deviations from ideality, 218
ideal device, 210–217
maintenance coefficient, 213
mutations, 217
as a tool, 217–218
washing out a culture, 211
Chemotaxis, 138
Chlorella algae, 27
Chloroplast, 165
Chloroplasts, 24
CHO (Chinese hamster ovary) cells, 479–480, 484–485
Chondrocyte culture, 522–523
Chromatography. *See* Separation of soluble products, chromatography.
Chromatophores, 20
Chromosomes, 22
Cilia, 25, 27, 433
Ciliated organisms
definition, 25
vs. flagellated organisms, 25
Ciliates, 25, 27
CIP (clean-in-place) technology, 329
Cisternae, 433
Citric acid cycle, 149
Citric acid production, 586–588
Cleaning bioreactors, 329. *See also* Sterilizing process fluids.
Cloning
alternative strategies, 262–263
amplifying DNA, 260
cDNA (complementary DNA), 260
cell-surface display, 265
chemical gene synthesis, 259
codon harmonization, 259
codon optimization, 259
DNA microarray technology, 259
electrophoresis, 263–264
expressed DNA, 262
expression of cloned genes, 263–265
Gateway cloning, 262
gene bank, 264–265
gene library, 264–265
Gibson assembly, 262
Golden Gate cloning, 263
GreenGate cloning, 263
hybridization, 259
immunoblot testing, 264
inserting genes into DNA, 260–262
introns, 259
isothermal cloning, 262
LIC (ligation independent cloning), 263
ligands, 265
MoClo (molecular cloning), 263
obtaining the gene of interest, 259–260
overview, 258
PCR (polymerase chain reaction), 260
phage display, 265
plasmid vectors, 260
probes, 259
recombinant DNA techniques, 258
restriction endonuclease, 262–263
restriction enzymes, 259–260

Cloning *(continued)*
reverse transcriptase, 260
shotgun cloning, 259, 264
synonymous mutations, 259
TA cloning, 262
testing for proteins, 264
TOPO cloning, 262
type IIS enzyme, 262–263
Western blot testing, 264
yeast-mediated cloning, 263
Clustered regularly interspaced short palindromic repeat (CRISPR), 267, 480
Coagulation, separation of insoluble products, 382
Coccus (elliptical) cells, 14
Coccus (spherical) cells, 14
COD (chemical oxygen demand), biological waste treatment, 550
Coding DNA, 49
Coding RNA, 48
Codon harmonization, 259
Codon optimization, 259
Codons, 116–117, 123
Commensalism, model of mixed culture interaction, 538
Commonly added micronutrients, 54
Competent DNA strains, 254
Competition, 536
Competitive inhibitors, enzyme kinetics, 73–74
Complementarity determining regions (CDRs), 36
Complementary DNA (cDNA), 260
Complex glycoforms, 129
Concentration, separation of soluble products, 403
Concentration polarization, separation of soluble products, 394–395
Concerted feedback inhibition, 134
Conditional mutants, 251
Conidia, 25
Conjugation, 256–257
Constitutive exocytosis pathway, 127
Constitutive genes, 132
Contact inhibition, 435
Contact stabilization, biological waste treatment, 557
Continuous bioreactors
vs. batch, 276–278
CFSTR (continuous-flow, stirred-tank reactor), 276–278
Continuous bioreactors, modifying
chemostat with recycle, 278–281
external membrane bioreactors, 295
internal membrane bioreactors, 295
membrane bioreactors, 293–294
multistage chemostat systems, 281–287
perfusion systems, 293–294
Continuous cells, 435, 479
Continuous sterilization, 361
Continuous-flow, stirred-tank reactor (CFSTR), 210, 552
Contois equation, 194
Control sites in aerobic glucose metabolism, 154–155
Cooperative binding, 71–73
Cooperative feedback inhibition, 134
Core enzymes, 119
Cost of sequencing a complete genome, 270
Cotranslational modifications, 115–116
Cotranslational protein translation, 125
Counting viable cells, 171
Covalent binding, 301–302
Covalent bonding, 87–89
Crabtree effect, 146, 589
CRISPR (clustered regularly interspaced short palindromic repeat), 267, 480
Cristae, 23
Cross-flow filtration, separation of soluble products, 398–403
Cross-flow ultrafiltration, separation of soluble products, 398–403
Crystallization, purification process, 422–423
Cultivation devices, 208–210
Cumulative feedback inhibition, 134
Cyanobacteria (blue-green algae)
growth environment, 14
photosynthesis, 19
Cybernetic models, 207–208
Cylindrical (bacillus) cells, 14
Cytokinesis, 23
Cytoplasmic membrane, 18, 22
Cytosine (C), 45, 47
Cytoskeleton
animal cells, 433
eucaryotic cells, 25
Cytosol phase cytoplasm, 432

D

Dalton, 36
Damkohler number, 91
Dark phase of photosynthesis, 164
Data logging, 354
Death. *See* Cell death.

Deceleration phase, 178
Decline phase, 179
Degenerate code, 123
Deletion mutations, 248
Denatured enzymes, 71
Denitrification, 161
Deoxyribonucleic acid (DNA). *See* DNA (deoxyribonucleic acid).
Deoxyribonucleotides, structure of, 46
Deoxyribose, 39
Depth filters, 362–363
Diafiltration, separation of soluble products, 403
Dialysis, separation of soluble products, 392–393
Dialysis equilibrium, separation of soluble products, 393
Diauxic growth, 132, 177
Dictyosome, 433
Differentiated tissue in plant cell cultivation, 459
Dimers, 487
Diploid cells, 23
Direct cell counting, 171
Direct methods for counting viable cells, 172
Direct selection mutation, 250
Directed evolution, 512
Disaccharides, 40
Disinfection *vs.* sterilization, 356
Displacement chromatography, 409
Disposable bioreactors. *See* Single-use bioreactors.
Dissociation extraction, separation of soluble products, 387–388
Dissolved oxygen (DO) effects in batch cell growth kinetics, 187–188
Disulfide bond formation, 115
DNA (deoxyribonucleic acid). *See also* Cells; Nucleic acids; RNA (ribonucleic acid).
  amplifying, 260. *See also* PCR (polymerase chain reaction).
  building blocks of. *See* Nucleotides.
  coding sequences, 49
  competent strains, 254
  DNA methylases, 116
  donor, 253
  double helix, 48
  forming RNA molecules. *See* DNA transcription.
  inserting genes into, 260–262
  junk DNA. *See* Noncoding DNA.
  major functions of, 48
  methylation variation, 116
  noncoding DNA, 49
  plasmids, 48
  proofreading, 118
  structure of, 47
  Taq (*Thermus aquaticus*), 14
DNA gyrase enzyme, 117
DNA ligase enzyme, 118
DNA microarray technology, 259
DNA replication
  definition, 47–48
  semiconservative replication, 48–49, 117
DNA replication in *E. coli*. *See also E. coli*.
  bidirectional DNA, 117
  DNA gyrase enzyme, 117
  DNA ligase enzyme, 118
  lagging strands, 118
  leading strands, 118
  Okazaki fragments, 118
  origin of replication, 117
  process summary, 118–119
  proofreading the DNA, 118
  replication forks, 117
  RNA primer, 117–118
  unwinding enzymes, 117
DNA shuffling, 512–513
DNA transcription
  core enzymes, 119
  definition, 48
  holoenzymes, 119
  introns, 121–122
  mRNA, 121
  mRNA splicing, 122
  nonsense DNA, 122
  polyadenylation, 122
  polygenic messages, 121
  primary products of, 119
  procaryotic *vs.* eucaryotic cells, 121–122
  promoter region, 121
  reading through a terminator, 121
  reverse transcription, 115
  rho protein, 121
  RNA polymerase enzyme, 119
  rRNA, 121
  separate protein for each gene, 121
  sigma factor, 119
  stable RNA species, 121
  stop signals, 121
  strong promoter, 121

DNA transcription *(continued)*
strong terminators, 121
transcription terminators, 121
tRNA, 121
unstable RNA species, 121
weak terminators, 121
DNA translation
aminoacyl site, 125
aminoacyl-tRNA synthetases, 125
anticodons, 125
chaperones, 125
cotranslational protein translation, 125
definition, 48, 123
elongation step, 124–125
genetic code, 123–124
initiation complex, 125
initiation factors, 125
initiation step, 124–125
nonsense codons, 125
peptides from RNA, 48
peptidyl site, 125
posttranslation movement across membranes, 125
posttranslational processing, 125–129. *See also* Eucaryotic cells, posttranslational protein release; Procaryotic cells, posttranslational protein release.
primary steps, 124–125
process description, 124–125
proteins from RNA, 48
RF (release factor), 125
signal peptides, 125
stop codons, 125
termination step, 124–125
DO (dissolved oxygen) effects in batch cell growth kinetics, 187–188
Domestic wastes, biological waste treatment, 549
Donnan equilibrium, separation of soluble products, 393
Donor DNA, 253
Double helix, 48
Double-reciprocal plot, 68
D-ribose, 39
Dry weight of cells, determining, 171–172
Drying, purification process, 423–424
Dunaliella algae, 27
Dynamic method for oxygen removal, 335
Dyno-Mill homogenizer, separation of insoluble products, 384

E

E. coli
cell lysis, 18
cells breaking open. *See* Cell lysis.
conversion of aspartic acid to aspartyl phosphate, 140–142
cytoplasmic membrane, 18
description, 18
DNA replication. *See* DNA replication in *E. coli*.
genetic engineering, host-vector systems, 475–477, 484–485
inner membrane, 18
LPS (*Lipopolysaccharide*), 18
LPS removal, 18
outer membrane, 18
peptidoglycan, 18
periplasmic space, 18
strong promoters, 491
Eadie–Hofstee plot, 68–69
ED (electrodialysis), separation of soluble products, 421–422
ED (Entner–Doudoroff) pathway, 161
Effectiveness factor, enzyme immobilization, 95–96
Egg cells, 23
Elder, A. L., 5
Electron transport chain, 149, 152–154
Electrophoresis
expression of cloned genes, 263–264
separation of soluble products, 419–420
two-dimensional gel electrophoresis, 270
Electroporation, 265
Electrostatic effects on enzyme immobilization, 98
Elicitors in plant cell cultivation, 460–461
Elliptical (coccus) cells, 14
Elongation step, 124–125
Elution chromatography, 409
EMP (Embden–Meyerhof–Parnas) pathway, 149–152
Encapsulation, cell entrapment, 300
Endocytosis, 525
Endogenous metabolism, 178
Endoplasmic reticulum (ER), 23–24, 432
Endosomes, 525
Endospores, 20
Endotoxins. *See* LPS (*Lipopolysaccharide*).
End-product inhibition, 133
Energy efficiency, bioreactors, 329

Energy-independent uptake, 135–137
Engineered antibodies, 36
Engineers
research approaches, 3
working with biologists, 4–9
Enhancer regions, 131
Entner–Doudoroff (ED) pathway, 161
Entrapment, 85–87
Entrapment, active cell immobilization, 298–300, 302
Entrapped cells in stirred reactors, 445–447
Environmental conditions, batch cell growth kinetics, 184–189
Enzyme activation, 80
Enzyme immobilization
electrostatic effects, 98
steric effects, 98
Enzyme immobilization, diffusional limitations
Damkohler number, 91
effectiveness factor, 95–96
on enzymes immobilized in a porous matrix, 93–97
on reaction rate, 93
on surface-bound enzymes, 91–93
Enzyme immobilization, methods of
adsorption, 87
covalent bonding, 87–89
diffusion limitations, 87
entrapment, 85–87
enzyme leakage, 87
microencapsulation, 87
surface, 87–90
Enzyme induction, 131
Enzyme kinetics. *See also* Michaelis–Menten kinetics.
allosteric enzymes, 71–73
allostery, 71–73
cooperative binding, 71–73
enzyme activation, 80
insoluble substrates, 83–84
Langmuir–Hinshelwood kinetics, 63
mathematical model of, 63–65
multiphase enzymatic reactions, 84–85
partition coefficient, 85
pH effects, 80–82
RST (rate of substrate transfer), 85
saturation kinetics, 63–65. *See also* Michaelis–Menten kinetics.
temperature activation, 82–83
temperature inactivation, 83
thermal denaturation, 83
Enzyme kinetics, inhibited
competitive inhibitors, 73–74
inhibitors, 73
irreversible inhibitors, 73
mixed inhibition, 76
noncompetitive inhibitors, 73, 74–75
reversible inhibitors, 73
substrate inhibition, 76–79
uncompetitive inhibitors, 75–76
Enzyme kinetics, mechanistic models
quasi-steady-state assumption, 66–67
rapid-equilibrium assumption, 65–66
Enzyme leakage, 87
Enzymes. *See also specific enzymes*.
active sites, 30, 35, 63
apoenzyme, 61
classification, 62
containing protein groups, 61
denatured, 71
enzyme-substrate interaction, 63
function of, 62–63
holoenzymes, 61
induced fit model, 63
industrial uses for, 100–103
integral membrane proteins, 30
isozymes, 61
large-scale production, 98–99
lock-and-key model, 63
medical uses for, 100–103
naming, 61
orientation effect, 63
proximity effect, 63
regulatory, 30
specific activity, 70–71
structure of, 61
units, 70
Enzyme-substrate interaction, 63
Episomes, gene transfer and rearrangement, 256–257
Epitopes, 36
ER (endoplasmic reticulum), 432
Eroshin, V. K., 229
Error-prone PCR, 512
Ethanol production, 578–581
Eubacteria (true bacteria)
evolution of, 15
Gram staining, 18
photosynthesis, 19

Eucaryotes. *See also* Procaryotes.
cell division, 23, 26
cell motion, 24–25
definition, 15, 22
Eucaryotes, cell structure
actin filaments, 25
animal *vs.* plant, 22
asexual reproduction, 23
chloroplasts, 24
chromosomes, 22
cilia, 25
ciliates, 25
cristae, 23
cytokinesis, 23
cytoplasmic membrane, 22
cytoskeleton, 25
diploid cells, 23
egg and sperm cells, 23
endoplasmic reticulum, 23–24
flagella, 24–25
gametes, 23
glycans, 24
glycosylation, 24
glyoxysomes, 24
Golgi bodies, 24
haploid cells, 23
histones, 22
inner membranes, 23
intermediate filaments, 25
interphase, 23
lysosomes, 24
microtubule, 25
mitochondria, 23–24
mitosis, 23
nucleus, 22
peroxisomes, 24
photosynthesis, 24
vs. procaryotes, 22
sexual reproduction, 23
size, 22
sterols, 22
thylakoids, 24
tubulin, 25
vacuoles, 24
zygotes, 23
Eucaryotes, microscopic
algae, 27
ascospores, 25
asexual reproduction, 25
fission reproduction, 25
fungi, 25
molds, 25–27
protozoa, 27–28
yeasts, 25
Eucaryotic cells
in genetic engineering, host-vector systems, 477–479
vs. procaryotic cells, DNA transcription, 121–122
Eucaryotic cells, posttranslational protein release
Campylobacter jejuni bacterium, 129
complex glycoforms, 129
constitutive exocytosis pathway, 127
exocytosis, 127
glycans, 127
glycoforms, 127–129
glycosylation, 127
Golgi apparatus, 127
Golgi trans face, 127
hybrid glycoforms, 129
N-linked glycosylation, 129
OST (*oligosaccharyltransferase*), 129
protein release, 127
PTMs (posttranslational modifications), 127
regulated exocytosis pathway, 127
simple glycoforms, 129
transport vesicle fusion, 127
Exit-gas measurement, 352
Exocytosis, 127
Exponential growth phase, 177
Expressed DNA, 262
Expression of cloned genes, 263–265
External membrane bioreactors, 295
Extracorporeal artificial liver, 530
Extremophiles, 13–14, 21. *See also* Archaebacteria.
Extrinsic concentration, 202

F

F (fertility) factor, 256–257
Facilitated diffusion, 135–137
Facultative autotrophs, 52
Facultative organisms, 14
Fall armyworm (*Spodoptera frugiperda*), 480
Fats in cell construction, 42–45
Fatty acids
in cell construction, 42–45
definition, 42
examples, 42
fats, 42

FDA (Food and Drug Administration), 10–11
Fed-batch bioreactors, 288–293
Feedback inhibition, 133, 135
Feedback repression, 130, 135
Fermentation
  active, measuring, 349–352
  definition, 161
  glycolysis, 149
  molds, 27
  penicillin production, 5, 7
  two-phase, 489
Fermenter, 324. *See also* Bioreactor.
Fibrous proteins, 30
Filter sterilization, 361
Filtration, separation of soluble products
  cross-flow filtration, 398–403
  cross-flow ultrafiltration, 398–403
  diafiltration, 403
  MF (microporous filtration), 395–398
  microfiltration, 395–403
  rejection coefficient of an ultrafilter, 399–400
  tangential flow filtration, 398–403
  UF (ultrafiltration), 395–398, 403
Fission reproduction, microscopic eucaryotes, 25
Fixed solids, biological waste treatment, 551
Flagella, 24–25
Flagellate motion, 27
Flagellated organisms *vs.* ciliated organisms, 25
Flagellates, 27
Flat sheet membranes, separation of soluble products, 400–402
Flatbed reactors, 530
Fleming, Alexander, 4, 590
Flocculation, separation of insoluble products, 382
Florey, Howard, 4
Fluidized bed reactors, 530
Fluorescence, 165
Foaming, bioreactor scale-up, 328
Food and Drug Administration (FDA), 10–11
Fourier transform infrared (FTIR), 352
Freeze drying, purification process, 423
French presses, separation of insoluble products, 383
Freundlich adsorption isotherm, separation of soluble products, 403–404
Friable calli in plant cell cultivation, 459
Frontal chromatography, 409
FTIR (Fourier transform infrared), 352
Full inhibition, 134
Fumarate, converting to L-aspartate, 102
Functional genomics, 267
Fungi
  ascomycetes, 27
  basidiomycetes, 27
  definition, 25
  deuteromycetes, 27
  genetic engineering, host-vector systems, 478
  modes of sexual reproduction, 27
  phycomycetes, 27

G

G (guanine), 45, 47
Gametes, 23
Gaseous fuels from biomass, 597–598
Gases, sterilizing, 362–364
Gateway cloning, 262
Gateway sensors, 354
Gaulin–Manton presses, separation of insoluble products, 383
Gelation of polymers, 299
Gel-filtration chromatography, 410–411, 414–415
Gene bank, 264–265
Gene libraries, generating, 512–513
Gene library, 264–265
Gene therapy using viral vectors, 523–528
Gene transfer and rearrangement. *See also* Mutations.
  competent strains, 254
  conjugation, 256–257
  donor DNA, 253
  episomes, 256–257
  F (fertility) factor, 256–257
  generalized transduction, 254–255
  genetic recombination, 252–254
  internal gene transfer, 257
  lysogenic cells, 255
  main mechanisms, 252. *See also specific mechanisms*.
  plasmid vectors, 254
  prophage conversion, 256
  restriction endonucleases, 253
  specialized transduction, 255
  temperate phages, 256
  transduction, 254–256
  transformation, 254
  transposons, 257
Generalized transduction, gene transfer and rearrangement, 254–255
Genes, 116–117

Genetic code
anticodons, 125
codons, 116–117, 123
degenerate code, 123
description, 116–117, 123–124
nonsense codons, 123–124
Genetic engineering. *See also* Mutations.
baculovirus, 265
biolistic processes, 265–266
biopharmaceutical products, 473
cer locus, 493
directed evolution, 512
DNA shuffling, 512–513
electroporation, 265
error-prone PCR, 512
generating gene libraries, 512–513
genome engineering, 266–267
of higher organisms, 265–266
metabolic control theory, 510
metabolic engineering, 506–509
metabolic flux analysis, 510
origins of replication, 490
overview, 258
par locus, 493
plasmid design problems, 490–493
promoters, 490–491
protein engineering, 511–513
protein products *vs.* nonprotein products, 472–474
protoplast fusion, 265
protoplasts, 265
random mutagenesis, 512
rational design, 512
regulatory constraints on, 503–506
runaway replication, 490
semirational design, 513
site-directed mutagenesis, 512
synthetic biology, 509–511
systems biology, 509–511
transfection, 265–266
Genetic engineering, basic elements
alternative cloning strategies, 262–263
amplifying DNA, 260
cDNA (complementary DNA), 260
cell-surface display, 265
chemical gene synthesis, 259
codon harmonization, 259
codon optimization, 259
DNA microarray technology, 259
electrophoresis, 263–264
expressed DNA, 262
expression of cloned genes, 263–265
Gateway cloning, 262
gene bank, 264–265
gene library, 264–265
Gibson assembly, 262
Golden Gate cloning, 263
GreenGate cloning, 263
hybridization, 259
immunoblot testing, 264
inserting genes into DNA, 260–262
introns, 259
isothermal cloning, 262
LIC (ligation independent cloning), 263
ligands, 265
MoClo (molecular cloning), 263
obtaining the gene of interest, 259–260
overview, 258
PCR (polymerase chain reaction), 260
phage display, 265
plasmid vectors, 260
probes, 259
recombinant DNA techniques, 258
restriction endonuclease, 262–263
restriction enzymes, 259–260
reverse transcriptase, 260
shotgun cloning, 259, 264
synonymous mutations, 259
TA cloning, 262
testing for proteins, 264
TOPO cloning, 262
type IIS enzyme, 262–263
Western blot testing, 264
yeast-mediated cloning, 263
Genetic engineering, genetic instability
dimers, 487
growth-rate-dominated instability, 489–490
high-copy-number plasmids, 486
host cell mutations, 488–489
host-vector interactions, 493–503
low-copy-number plasmids, 486
metabolic burden, 489
multimers, 487
overview, 486
plasmid structural instability, 488
segregational loss, 486–488
selective pressure, 489
tetramers, 487
two-phase fermentation, 489

Genetic engineering, host-vector systems, 474
A. nidulans, 478
B. subtilis, 477
baculovirus (*Autographa californica*), 480
cabbage looper (*Trichoplusia ni*), 480
CFPS (cell-free protein synthesis), 484
CHO (Chinese hamster ovary) cells, 479–480, 484–485
comparison of strategies, 484–485
continuous cells, 479
CRISPR (clustered regularly interspaced short palindromic repeat), 480
E. coli, 475–477, 484–485
fall armyworm (*Spodoptera frugiperda*), 480
fungi, 478
gram-positive bacteria, 476
H. polymorpha, 478
heat shock, 475
heterologous hosts, 507–508
identification of pathway enzymes, 508–509
immortal cells, 479
inclusion bodies, 475
insect cell–baculovirus system, 480–482
L. lactis, 477
lower eucaryotic cells, 477–479
mammalian cells, 479–480
native hosts, 508
NOVs (nonoccluded viruses), 481
overview, 474–475
P. pastoris, 478
protein secretion/excretion, 476
S. cerevisiae, 477–478
signal peptides, 476
Streptomyces, 477
transformed cells, 479
transgenic animals, 482–483
transgenic plants and plant cell culture, 483–484
yeasts, 478
Genetic instability. *See* Genetic engineering, genetic instability.
Genetic recombination, gene transfer and rearrangement, 252–254
Genetic-level control, 130–133
Genome engineering. *See also* Genetic engineering; Genomics.
CRISPR (clustered regularly interspaced short palindromic repeat), 267
customizing meganucleases, 266–267
eliminating genes, 266
introducing targeted modifications into chromosomes, 266
MAGE (multiplex automated genome engineering), 266
molecular scissors, 266
protein engineering, 266–267
Genomes
definition, 15, 267
editing. *See* Genome engineering; Genomics.
Genomics. *See also* Genome engineering.
bioinformatics, 271
BLAST (basic local alignment search tool), 271
computational techniques, 271
definition, 267
functional, 267
homologous amino acid sequences, 271
ORFs (open reading frames), 271
overview, 267
proteomics, 267
systems biology, 267
Genomics, experimental techniques
cost of sequencing a complete genome, 270
high-throughput sequencing, 269
Illumina sequencing, 269
isoelectric points, 270
microarrays, 270
nanopores, 269
NGS (next-generation sequencing), 269
overview, 268–270
Sanger sequencing, 267–268
SBS (sequencing by synthesis) sequencing, 269
transcriptomics, 270
two-dimensional gel electrophoresis, 270
Genotype, definition, 248
Genus, 15
Gibson assembly, 262
Glass wool filters, 362
Globular proteins, 30
GLP (good laboratory practices), 10
Glucanases, 102
Gluconeogenesis, 160
Glucose
catabolism, 149–152
converting to fructose, 102
Glucose effect, 131
Glucose metabolism, 149–152
Glycans, 24, 36, 42, 127
Glycoforms, 127–129
Glycogen, 41

Glycolysis
aerobic respiration, 149
anaerobic respiration, 149
citric acid cycle, 149
electron transport chain, 149, 152–154
fermentation, 149
phases, 149
respiration, 149
TCA (Krebs, tricarboxylic acid), 149
Glycosylation, 24, 42, 115, 127
Glyoxysomes, 24, 433
GMP (good manufacturing process), 10
Golden Gate cloning, 263
Golgi apparatus, 127
Golgi bodies, 24, 433
Golgi trans face, 127
Gompertz equation, 200–201
Good laboratory practices (GLP), 10
Gram, Hans Christian, 18
Gram staining
bacteria, 18
colorless reaction, 18
eubacteria (true bacteria), 18
negative reaction, 18. *See also E. coli*.
no reaction, 19
positive reaction, 18. *See also B. subtilis*.
procaryotes, 20
purple reaction, 18
red reaction, 18
Gram-positive bacteria, 476
GreenGate cloning, 263
Group translocation, 135–137
Growing cells. *See* Cell growth.
Growth dynamics of animal cells, 441–443
Growth media, animal cell cultivation, 437–441
Growth-associated products, 183
Growth-rate-dominated instability, 489–490
Grow-through tests, 363
GTP (quanosine triphosphate), 45
Guanine (G), 45, 47

H

H. polymorpha, 478
Hairy roots, bioreactor considerations in plant cell cultivation, 466–467
Haldane, J. B. S., 66
Half-velocity constant, 192–193
Halophiles, 14, 21
Hanes–Woolf plot, 69–70
Haploid cells, 23
HC (hydrophobic chromatography), 411
Heat generated by microbial growth, 190–191
Heat shock, 475
Heat shock regulon, 131–132
Heat transfer, 331–337
Heatley, Norman, 4
Helical structures in proteins, 33–34
Hematopoiesis, 529–530
Hemicellulases, 102
Hemocytometer, 171
Henri, V. C. R., 63
Heterologous hosts, 507–508
Heterotrophs, 52
HFCS (high-fructose corn syrup) production, 593–595
HIC (hydrophobic interaction chromatography), 411
High-copy-number plasmids, 486
High-pressure liquid chromatography (HPLC), 352, 411
High-throughput sequencing, 269
Hindered settling, separation of insoluble products, 378–380
Histones, 22
HIV (human immunodeficiency) virus, reverse transcription, 115
HMP (hexose monophosphate) pathway, 158–161
H/O (proton/oxygen) ratio, 229
Hollow fiber membranes, separation of soluble products, 400–402
Hollow-fiber reactors, 445
Holoenzymes, 61, 119
Homologous amino acid sequences, 271
Hormones, 139
Host cell mutations, 488–489
Host-vector interactions, 493–503
HPLC (high-pressure liquid chromatography), 352, 411
Hughes press, separation of insoluble products, 384
Humoral immune response, 36
Hybrid bioreactors, 311–313
Hybrid glycoforms, 129
Hybridization, 259
Hybridoma cells, 436
Hydrocarbons, metabolism of, 156–157
Hydrogen gas production, 597–598
Hydrogen macronutrient, 53
Hydrophobic chromatography (HC), 411
Hydrophobic interaction chromatography (HIC), 411

Hydroxylation, 116
Hypervariable regions, 36
Hyphae, 25

I

IEC (ion-exchange chromatography), 410–411, 414, 419–420
IEM (ion-exchange membranes), 421
Illumina sequencing, 269
IMAC (immobilized-metal-affinity chromatography), 416–419
Immobilized cell systems
  active cell immobilization, 298–302
  binding, 300–302
  biological films, 303–304
  bioreactor considerations, 309–311
  cell adsorption, 300–301
  covalent binding, 301–302
  diffusional limitations, 304–308
  encapsulation, 300
  entrapment, 298–300, 302
  gelation of polymers, 299
  ion-exchange gelation, 299
  microbial flac, 305
  overview, 298
  passive immobilization, 303–304
  polycondensation, 299
  polymerization, 299
  precipitation of polymers, 299
  three-dimensional printing, 300
Immortal cells, 435, 479
Immunoblot testing, 264
Immunoglobulins, 34–37
Immunotherapy, 36
Impellers, 324–325
Inclusion bodies, 475
Incompressible cake, 376–378
Indirect methods of counting cells, 172–175
Indirect selection mutation, 250
Induced fit model, enzymes, 63
Induced pluripotent stem (iPS) cells, 529
Inducer exclusion, 131
Inducers, 130
Induction, 130
Industrial uses for
  enzymes, 100–103
  mixed culture biological waste treatment, 546–548
Industrial wastes, biological waste treatment, 549
Inhibitors, enzyme kinetics, 73–74
Initial-rate experiments, 67–68
Initiation complex, 125
Initiation factors, 125
Initiation step, 124–125
Inner membranes
  E. coli, 18
  eucaryotes, 23
Insect cell–baculovirus system, 480–482
Insect cells, cultivating, 435–436
Insertable probes, 349–351
Inserting genes into DNA, 260–262
Insertion-sequence (IS) elements, 248
Insoluble substrates, 83–84
Integral member proteins, 43
Integral membrane in proteins, 30
Inteins, 116
Intermediate filaments, 25, 433
Internal gene transfer, 257
Internal mechanical agitation, 324–329
Internal mechanical agitation bioreactors, 324–329
Internal membrane bioreactors, 295
Interphase, 23
Intracellular spores, 20
Intrinsic concentration, 202
Introns, 116, 121–122, 259
Ion-exchange gelation, 299
Ion-exchange membranes (IEM), 421
Ion-exchanging chromatography (IEC), 410–411, 414, 419–420
iPS (induced pluripotent stem) cells, 529
Irreversible inhibitors, enzyme kinetics, 73
IS (insertion-sequence) elements, 248
Isoelectric focusing, 420
Isoelectric points, 32, 270
Isoelectric precipitation, 390–392
Isofunctional enzymes, 134
Isothermal cloning, 262
Isozymes, 61, 134

J

Junk DNA. *See* Coding DNA.

K

Ketones, 36
Kinetics
  batch cell growth. *See* Batch cell growth kinetics; Models of cell growth kinetics.
  death, 356–357
  Michaelis–Menten. *See* Michaelis–Menten kinetics.

K_m, interpreting, 70–71
Koji process, 314
Krebs tricarboxylic acid (TCA) cycle, 149–152

L

L. lactis, 477
lacA gene, 130
Lactases, 102
Lactic acid production, 581–584
Lactose, 40
LacY enzyme, encoding, 131
lacY gene, 130
lacZ gene, 130
Lag phase, 175–177
Lagging strands, 118
Langmuir isotherm, 404
Langmuir–Hinshelwood kinetics, 63
Latin names, 15
Leading strands, 118
LIC (ligation independent cloning), 263
Ligands, 55, 265
Ligation independent cloning (LIC), 263
Light phase of photosynthesis, 164
Lineweaver–Burk plot, 68
Lipases, 101
Lipidation, 116
Lipids
  in cell construction, 42–45
  PHA (polyhydroxyalkanoates), 44
  PHB (polyhydroxybutyrate), 44
  steroids, 44–45
Lipoglycans. *See* LPS (*Lipopolysaccharide*).
Lipopolysaccharide (LPS), 18
Liquid fuel production, 596–597
Liquid–liquid extraction, 385–389
Liquids, sterilizing, 357–362
LLC (liquid–liquid partition chromatography), 410–411
LncRNA (long ncRNA), 49
Lock-and-key model, enzymes, 63
Logarithmic growth phase. *See* Exponential growth phase.
Logistic equations, 197–200
Long ncRNA (lncRNA), 49
Loop reactors, scaling up, 329–330
Lotka–Volterra model of mixed culture interactions, 542–545
Low-copy-number plasmids, 486
Lower eucaryotic cells in genetic engineering, host-vector systems, 477–479
LPS (*Lipopolysaccharide*), 18
LPS removal, 18
Luedeking–Piret equation, 183
Lumenal phase cytoplasm, 432
Lypophilization, purification process, 423–424
Lysogenic cells, gene transfer and rearrangement, 255
Lysogenic cycle of viruses, 17
Lysosomes, 24, 432
Lysozyme, uses for, 102
Lytic cycle of viruses, 17

M

Macronutrients
  autotrophs, 52
  carbon compounds, 52–53
  categories of microorganisms, 52
  chemautotrophs, 52
  definition, 51
  elements of, 53–54
  facultative autotrophs, 52
  heterotrophs, 52
  hydrogen, 53
  magnesium, 53
  mixotrophs, 52
  nitrogen, 52–53
  oxygen, 53
  phosphorus, 53
  photoautotrophs, 52
  potassium, 53
  sulfur, 53
MAGE (multiplex automated genome engineering), 266
Magnesium, macronutrient, 53
Maintenance, 180
Maintenance coefficient, 213
Maintenance energy, 178
Mammalian cell culture, 436–437
Mammalian cells in genetic engineering, host-vector systems, 479–480
Maximum growth potential, 200–201
Medical uses for
  bioprocess engineering. *See* Bioprocess engineering, medical uses for.
  enzymes, 100–103
Membrane bioreactors, 294–297
Membrane configurations, separation of soluble products, 400–402
Membrane-based units, 530
Menten, M. L., 63

Merck, producing penicillin, 5–6
Mesophiles, 14
Messenger (mRNA), 48–50
Metabolic burden, 489
Metabolic control theory, 510
Metabolic engineering
  definition, 2, 145
  heterologous hosts, 507–508
  host selection, 506–508
  identifying pathway enzymes, 508–509
  native hosts, 508
  overview, 506
Metabolic flux analysis, 510
Metabolic pathway control, 133–135
Metabolic pathways
  anabolism, definition, 146
  anaerobic metabolism, 161–163
  autotrophic metabolism, 163–165
  biodegradation of xenobiotics (toxic aromatic compounds), 157–158
  bioenergetics, 146–148
  biosynthesis, 158–161
  Calvin cycle, 163–165
  control sites in aerobic glucose metabolism, 154–155
  Crabtree effect, 146
  dark phase, 164
  degrading compounds into simpler products. *See* Catabolism.
  EMP (Embden–Meyerhof–Parnas) pathway, 149–152
  glucose metabolism, 149–152
  glycolysis, 149–152
  light phase, 164
  metabolic engineering, definition, 145
  metabolism of hydrocarbons, 156–157
  metabolism of nitrogenous compounds, 155–156
  NADH, functions in biological systems, 148
  nitrogen fixation, 156
  nitrogenase, 156
  overview, 145
  Pasteur effect, 155
  respiration, 152–154
  synthesizing complex compounds. *See* Anabolism.
  TCA cycle, 149–152
Metabolic regulation
  basal level of proteins, 131
  catabolite repression, 131
  concerted feedback inhibition, 134
  constitutive genes, 132
  cooperative feedback inhibition, 134
  cumulative feedback inhibition, 134
  diauxic growth, 132
  end-product inhibition, 133
  enhancer regions, 131
  enzyme induction, 131
  feedback inhibition, 133, 135
  feedback repression, 130, 135
  full inhibition, 134
  genetic-level control, 130–133
  glucose effect, 131
  heat shock regulon, 131–132
  inducer exclusion, 131
  inducers, 130
  induction, 130
  isofunctional enzymes, 134
  isozymes, 134
  lacA gene, 130
  LacY enzyme, encoding, 131
  lacY gene, 130
  lacZ gene, 130
  metabolic pathway control, 133–135
  operator regions, 130
  operons, 130
  permease, encoding, 131
  proteins synthesized, 130–133
  regulons, 131
  sequential feedback inhibition, 134
  unregulated genes. *See* Constitutive genes.
Metabolism
  of nutrients in animal cells, 433–434
  role of cells, 137–139
Methane production, 564, 597–598
MF (microporous filtration), 395–398
MFCs (microbial fuel cells), 598–600
Michaelis, L., 63
Michaelis–Menten kinetics
  vs. allosteric kinetics, 72
  overview, 63–65
Michaelis–Menten kinetics, determining rate parameters
  batch kinetics, 69–70
  double-reciprocal plot, 68
  Eadie–Hofstee plot, 68–69
  Hanes–Woolf plot, 69–70
  initial-rate experiments, 67–68
  interpretation of K_m and V_m, 70–71
  Lineweaver–Burk plot, 68

Microarrays, 270
Microbes, compared to plant cell cultivation, 457–462
Microbial flac, 305
Microbial products, classifying, 183–184
Microencapsulation, 87
Microfiltration, separation of soluble products, 395–403
Micronutrients
  categories, 54
  chelating agents, 55
  commonly added, 54
  definition, 51
  growth factors, 55
  ligands, 55
  rarely added, 55
  sometimes added, 54–55
Microorganisms
  categories of, 52
  naming, 15
Microphysiological systems, 531
Microporous filtration (MF), 395–398
Microreactors, 342–348
Microscopic eucaryotes. *See* Eucaryotes, microscopic.
Microtubules, 25, 433
Microvilli, 432
Minkevich, I. G., 229
miRNA (microRNA), 49
Mitochondria, 23–24, 432
Mitosis, 23
Mixed and forcefully aerated bioreactors, 314
Mixed culture, biological waste treatment
  acid formers, 564
  activated-sludge processes, 551–553
  adsorbent-added activated-sludge process, 557–558
  advanced process, 566–571
  agricultural wastes, 549
  anaerobic digestion, 564–566
  Bardenpho process, 571
  basic process, 551–566
  biological treatment, 549
  BOD (biological oxygen demand), 550
  CFSTR (continuous-flow, stirred-tank reactor), 552
  chemical treatment, 549
  COD (chemical oxygen demand), 550
  contact stabilization, 557
  converting wastewater to useful products, 571–572
  domestic wastes, 549
  fixed solids, 551
  formation of methane, 564
  formation of volatile acids, 564
  industrial uses, 546–548
  industrial wastes, 549
  MLSS (mixed-liquor suspended solids), 551
  MLVSS (mixed-liquor volatile suspended solids), 551
  modified activated sludge processes, 556–563
  nitrification, 567–568
  oxidation ponds, 563–564
  oxygen enriched air, 558
  PEKILO process, 571–572
  PFRs (plug flow reactors), 552
  physical treatment, 549
  RBC (rotating biological contactor), 561–563
  shock loading, 552–553
  sludge bulking, 553–556
  sludge re-aeration, 556–557
  solubilization of insoluble organics, 564
  stop feeding/stop aeration, 556
  SYMBA process, 571–572
  TKN (total Kjeldahl nitrogen), 551
  TOD (total organic carbon), 550
  treatment, types of, 549
  trickling biological filters, 558–561
  types of. *See also specific types*.
  typical steps, 551
  UCT process, 571
  UNOX process, 552, 558
  VIP process, 571
  volatilized material, 551
  wastes, types of, 549. *See also specific types*.
  wastewater characteristics, 549–551
Mixed culture, industrial uses for
  SYMBA process, 548
  waste treatment, 546–548
  whiskey manufacturing, 546
Mixed culture in nature, 545–546
Mixed culture interactions
  amensalism, 538–539
  commensalism, 538
  competition, 536
  mutualism, 537–538
  neutralism, 537
  parasitism, 539
  predation, 539

protocooperation, 537–538
same growth limiting substrate, 536–537
symbiosis, 538
Mixed culture interactions, models of
Lotka–Volterra, 542–545
mutualism, 541–542
overview, 539–540
prey–predator, 542–545
same growth limiting substrate, 540–541
Mixed inhibition, enzyme kinetics, 76
Mixed-growth-associated product formation, 183
Mixotrophic cultures, 467
Mixotrophs, 52
MLSS (mixed-liquor suspended solids), 551
MLVSS (mixed-liquor volatile suspended solids), 551
Mobile phase chromatography, 409
MoClo (molecular cloning), 263
Models of cell growth kinetics
for filamentous organisms (molds), 201
with growth inhibitors, 195–197
with time delays, 202
for transient behavior, 201–202
Modified activated sludge processes, 556–563
Molds
conidia, 25
definition, 25
fermentation method, 27
hyphae, 25
mycelium, 25
reproduction model, 26
size, 25, 27
uses for, 27
Molecular scissors, 266
Molecular sieving chromatography, 410, 414–415
Monod equation, 192–194, 197
Monosaccharides, 38–40
Moromi culture, 314
Mortal cells, 435
Moser equation, 194
Motion, protozoa, 27
Moving-bed columns, separation of soluble products, 404
mRNA, 48–50, 116, 121–122
Multimers, 487
Multiorgan-on-a-chip systems, 531
Multiphase enzymatic reactions, 84–85
Multiplex automated genome engineering (MAGE), 266
Multistage chemostat bioreactor, 281–287
Mutagens, 250
Mutations. *See also* Gene transfer and rearrangement; Genetic engineering.
in chemostats, 217
definition, 247–248
expressed characteristics. *See* Phenotype.
genetic construction. *See* Genotype.
genotype, 248
phenotype, 248
wild type, 248
Mutations, mechanisms of genetic change
back mutations, 248–249
deletion mutations, 248
IS (insertion-sequence) elements, 248
nonsense mutations, 248
point mutations, 248
reading frame, 248
reversions, 248–249
silent mutations, 248
suppressor mutations, 249
Mutations, selecting for desirable
auxotrophic mutants, 250–251
chemical agents, 250
conditional mutants, 251
direct selection, 250
indirect selection, 250
mutagens, 250
natural rates of mutation, 250
replica plating, 251
selectable mutations, 250
spontaneous rates of mutation, 250
unselectable mutations, 250
Mutualism, 537–538, 541–542
Mycelium, 25
Mycoplasma, 19

N

NAD (nicotinamide adenine dinucleotide), 45
NADH, functions in biological systems, 148
NADP (nicotinamide adenine dinucleotide phosphate), 45
Naming enzymes, 61
Nanopores, 269
Native hosts, 508
Natural amino acids, 31
Natural rates of mutation, 250
ncRNA (noncoding), 49, 51
Neutralism, 537
Neutrophiles, 14
Next-generation sequencing (NGS), 269

NGS (next-generation sequencing), 269
Nicotinamide adenine dinucleotide (NAD), 45
Nicotinamide adenine dinucleotide phosphate (NADP), 45
Nitrification, biological waste treatment, 567–568
Nitrogen, macronutrient, 52–53
Nitrogen fixation, 156
Nitrogenase, 156
Nitrogenous compounds, metabolism of, 155–156
N-linked glycosylation, 129
Nonanchorage-dependent cells, 435
Noncoding (ncRNA), 49, 51
Noncoding DNA, 49
Noncompetitive inhibitors, enzyme kinetics, 73, 74–75
Nongrowth-associated product formation, 183
Nongrowth-related functions, 181–182
Nonoccluded viruses (NOVs), 481
Nonprotein products *vs.* protein products, 472–474
Nonsense codons, 123–125
Nonsense DNA, 122
Nonsense mutations, 248
Nonstirred reactor systems, 444–445
Normal phase chromatography, 410
NOVs (nonoccluded viruses), 481
Nucleic acids in cell construction, 44–51
Nucleoli, 433
Nucleotides. *See also* DNA (deoxyribonucleic acid); RNA (ribonucleic acid).
  A (adenine), 45, 47
  AMP (adenosine monophosphate), 45
  ATP (adenosine triphosphate), 45
  C (cytosine), 45, 47
  G (guanine), 45, 47
  GTP (quanosine triphosphate), 45
  major components, 45
  NAD (nicotinamide), 45
  NAD (nicotinamide adenine dinucleotide), 45
  NADP (nicotinamide adenine dinucleotide phosphate), 45
  structure of, 46
  T (thymine), 45, 47
  U (uracil), 45
Nucleus, eucaryotes, 22

O

Okazaki fragments, 118
Oligosaccharyltransferase (OST), 129
Open reading frames (ORFs), 271
Operator regions, 130
Operons, 130
Optical density of cell cultures, 172
ORFs (open reading frames), 271
Organized tissues, bioreactor considerations in plant cell cultivation, 466–467
Orientation effect, 63
Osmosis, separation of soluble products, 393
Osmotic shock, separation of insoluble products, 384–385
OST (*oligosaccharyltransferase*), 129
OTR (oxygen transfer rate), 188
OUR (oxygen uptake rate), 188
Outer membrane, *E. coli*, 18
Oxidation ponds, biological waste treatment, 563–564
Oxidation-reduction half-reactions, 237–240
Oxidative phosphorylation, 152–154
Oxygen
  concentration for batch cell growth kinetics, 187–188
  enriched air biological waste treatment, 558
  macronutrient, 53
  solubility, bioreactor scale-up, 332–335
Oxygen removal
  dynamic method, 335
  steady-state method for, 334
  unsteady-state method for, 333

P

P. pastoris, 478
Pacific Yew tree, 456–457
Packed cell volume, 172
Packed-bed bioreactors, 314
Packed-bed columns, separation of soluble products, 404
par locus, 493
Parasitism, model of mixed culture interaction, 539
Particle counters, 171
Partition coefficient, 85
Passive diffusion, 135–137
Passive immobilization, 303–304
Pasteur effect, 155
Pathway enzymes, identifying, 508–509
PBPK (physiologically based pharmacokinetic) model of the human body, 531
PCR (polymerase chain reaction), 260
Pectinases, 100
PEKILO process, 571–572

Penicillin
discovery of, 4–5
WW II poster, 8
Penicillin acylase, 102
Penicillin production
aerobic bioprocesses, 590–592
bottle plant, 6
chemical synthesis, 5
fermentation, 5, 7
Merck, 5–6
Pfizer, 5–6
schematic of, 9
Squibb, 5
submerged tank process, 6
surface method, 6
USDA Northern Regional Research Laboratory, 5–6
Pentose-phosphate, 158–161
Peptide bond, 32
Peptides, forming from RNA. *See* DNA translation.
Peptidoglycan, 18
Peptidyl site, 125
Perfusion systems bioreactor, 293–294
Perinuclear space, 433
Peripheral member proteins, 43
Periplasmic space, 18
Periwinkle to ajmalicine, 465
Permease, encoding, 131
Peroxisomes, 24, 433
Petroff–Hausser counting chamber, 171
Pfizer, producing penicillin, 5–6
PFRs (plug flow reactors), 208, 210, 552
pH effects
batch cell growth kinetics, 186
on enzyme kinetics, 80–82
pH effects on cells and their environments
pH values 1 or 2. *See* Acidophiles.
pH values 6 to 8. *See* Neutrophiles.
pH values above 9. *See* Alkaliphiles.
PHA (polyhydroxyalkanoates), 44
Phage display, 265
Phages (bacteriophages), 17
PHB (polyhydroxybutyrate), 44
Phenotype, definition, 248
Phosphoglycerides in cell construction, 43
Phospholipids in cell construction, 43
Phosphorus, macronutrient, 53
Phosphorylation, 116
Phosphotransferase system, 137
Photoautotrophs, 52
Photoperiod control, 467
Photophosphorylation, 165
Photosynthesis
bacteria, 20
carbohydrates, 36
eubacteria (true bacteria), 19
eucaryotes, 24
Phototaxis, 138
Physical environment, monitoring, 350
Physical treatment, biological waste treatment, 549
Physiologically based pharmacokinetic (PBPK) model of the human body, 531
Plant cell cultivation
aggregates, 460
anticancer agent (Taxol), 456–457
callus cultures, 458–459
common products from, 452–456
compared to microbes, 457–462
differentiated tissue, 459
economics of, 467–468
elicitors, 460–461
friable cali, 459
Pacific Yew tree, 456–457
plasmodesmata, 460
suspension cultures, 458–459
totipotency, 457–461
volumetric productivities, 468
Plant cell cultivation, bioreactor considerations
ajmalicine from periwinkle, 465
cell immobilization, 463–465
hairy roots, 466–467
mixotrophic cultures, 467
organized tissues, 466–467
photoperiod control, 467
root mats, 467
submerged culture, 467
suspension cultures, 462–463
Plant *vs.* animal eucaryotes, 22
Plasmid vectors
gene transfer and rearrangement, 254
inserting genes into DNA, 260
Plasmids
definition, 48
design problems, 490–493
structural instability, 488
Plasmodesmata in plant cell cultivation, 460
Plate counts, 171
Plug flow reactors (PFRs), 208, 210, 552

Pluripotent stem cells, 529
Pneumatic conveyor dryers, purification process, 424
P/O ratio, 153–154, 229
Point mutations, 248
Polyadenylation, 122
Polycondensation, 299
Polygenic messages, 121
Polyhydroxyalkanoates (PHA), 44
Polyhydroxybutyrate (PHB), 44
Polymerase chain reaction (PCR), 260
Polymerization, 299
Polymers, precipitating, 299
Polypeptides, amino acids in, 31
Polysaccharides, 40–42
Posttranslation movement across membranes, 125
Posttranslational modification of cells, 115–116
Posttranslational modifications (PTMs), 127
Posttranslational processing, 125–129
Potassium, macronutrient, 53
Precipitation
  of polymers, 299
  separation of soluble products, 390–392
Precipitin, 36
Predation model of mixed culture interaction, 539
Prey–predator model of mixed culture interactions, 542–545
Primary culture of cells, 434–435
Primary metabolites, 178
Probability of extinction, 356
Probe drift, 351
Probe fouling, 351
Probes, 259, 349–351
Procaryotes. *See also* Eucaryotes.
  archaebacteria, 15
  BMCs (bacterial microcompartments), 20
  definition, 15
  vs. eucaryotes, 16, 22
  nomenclature, 15
  ribosomes, 20
  shape, 18
  size, 18
  spores, 20
  staining, 20
  storage granules, 20
  structures, 20
  subdivisions. *See* Archaebacteria; Eubacteria (true bacteria).
  volutins, 20
Procaryotic cells
  vs. eucaryotic cells, DNA transcription, 121–122
  posttranslational protein release, 127
Process validation, FDA, 10–11
Progenitor cells, 529
Promoter region, 121
Promoters, 490–491
Prophage conversion, 256
Prosthetic groups, 32–33
Proteases, 100
Protective proteins, 31
Protein engineering, 266–267, 511–513
Protein products *vs.* nonprotein products, 472–474
Protein release, 127
Protein secretion/excretion, 476
Protein splicing, 116
Proteinogenic amino acids, 31
Proteins. *See also* Amino acids.
  Ag (antigen) macromodule, 34, 36
  basal level, 131
  biological functions, 29
  building blocks of, 31. *See also* Amino acids.
  catalytic, 30
  definition, 29
  fibrous, 30
  forming from RNA. *See* DNA translation.
  globular, 30
  helical structures, 33–34
  humoral immune response, 36
  immunoglobulins, 34–37
  immunotherapy, 36
  integral membrane, 30
  primary structure, 33
  prosthetic groups, 32–33
  protective, 31
  quaternary structure, 33
  regulatory, 31
  secondary structure, 33
  structural, 30
  structure of, 29
  synthesized, 130–133
  tertiary structure, 33
  three-dimensional structure, 33–34
  transport, 30
Proteins, antibodies
  Ab (antibody) macromodule, 34
  ADC (antibody-drug conjugate), 36
  antibody–antigen complex, 36

antibody–antigen interaction, 36
CDRs (complementarity determining regions), 36
description, 34–36
engineered antibodies, 36
glycans, 36
hypervariable regions, 36
Proteomics, 267
Protocooperation, 537–538
Proton-motive forces, 136–137
Proton/oxygen (H/O) ratio, 229
Protoplast fusion, 265
Protoplasts, 265
Protozoa
ameboid motion, 27
amoebae, 27
cilia, 27
ciliates, 27
definition, 27–28
diseases caused by, 28
flagellate motion, 27
flagellates, 27
motion, 27
sporozoans, 27
trypanosomes, 27
wastewater treatment, 28
Proximity effect, 63
Psychrophiles, 14
PTMs (posttranslational modifications), 127
Purification
costs, 371–372
crystallization, 422–423
drying, 423–424
freeze drying, 423
lypophilization, 423–424
major steps in, 373
major units of operation, 372–373
pneumatic conveyor dryers, 424
rotary-drum dryers, 424
separation of soluble products, 403
spray dryers, 424
vacuum-tray dryers, 423
Purple and green bacteria. *See* Anoxygenic photosynthetic bacteria.
Pyrrolysine, 31

Q

Quanosine triphosphate (GTP), 45
Quasi-steady-state assumption, 66–67
Quaternary structure, proteins, 33

R

Random mutagenesis, 512
Rannie high-pressure homogenizer, 383
Rapid-equilibrium assumption, 65–66
Rarely added micronutrients, 55
Rate of substrate transfer (RST), 85
Rational design, 512
RBC (rotating biological contactor), 561–563
Reaction, integrating with separation, 424–425
Reactor. *See* Bioreactor.
Reading frame, 248
Reading through a terminator, 121
Recombinant DNA techniques, 258
Recovery. *See also* Separation.
costs, 371–372
major steps in, 373
major units of operation, 372–373
Redox potential, batch cell growth kinetics, 189
Reflection coefficient, separation of soluble products, 394
Regenerative medicine, 519–523
Registering this book, xviii
Regulated exocytosis pathway, 127
Regulatory constraints on genetic engineering, 503–506
Regulatory enzymes, 30
Regulatory proteins, 31
Regulatory RNA, 49
Regulons, 131
Rejection coefficient of an ultrafilter, 399–400
Release factor (RF), 125
Replica plating, 251
Replication
of cells, 114
DNA. *See* DNA replication.
origins of, 490
runaway, 490
Replication forks, 117
Reproduction model of molds, 26
Reproduction of viruses, 17
Research approaches, biologists *vs.* engineers, 3
Respiration
electron transport chain, 149, 152–154
glycolysis, 149
oxidative phosphorylation, 152–154
P/O ratio, 153–154
Restriction endonucleases, 253, 262–263
Restriction enzymes, 259–260
Retrotransposons, 115

Retroviruses. *See also* Viruses.
mass production of, 527–528
reverse transcription, 115
Reverse osmosis (RO), 393–395
Reverse transcriptase, 115, 260
Reverse transcription, 115
Reversible inhibitors, enzyme kinetics, 73
Reversions, 248–249
RF (release factor), 125
Rho protein, 121
Ribonucleic acid (RNA). *See* RNA (ribonucleic acid).
Ribonucleotides, structure of, 46
Ribosomal (rRNA), 49, 51
Ribosomes
definition, 49
number per cell, 20
in procaryotes, 20
Ribozymes, 61
RNA (ribonucleic acid). *See also* DNA (deoxyribonucleic acid); Nucleic acids.
building blocks of. *See* Nucleotides.
carrying genetic information, 48–49
coding, 48
definition, 49
forming peptides and proteins. *See* DNA translation.
long ncRNA (lncRNA), 49
messenger (mRNA), 48–50
microRNA (miRNA), 49
mRNA splicing, 116
noncoding (ncRNA), 49, 51
regulatory, 49
replication, 115
ribosomal (rRNA), 49, 51
ribozymes, 61
RNA silencing, 115
RNA-dependent RNA polymerases, 115
small interfering (siRNA), 49
structure of, 47
transfer (tRNA), 49–50
RNA polymerase enzyme, 119
RNA primer, 117–118
RNA silencing, 115
RNA-dependent RNA polymerases, 115
RO (reverse osmosis), 393–395
Roche 454 sequencing, 269
Rod (bacillus) cells, 14
Root mats, bioreactor considerations in plant cell cultivation, 467
Rotary vacuum precoat filters, 374
Rotary-drum dryers, purification process, 424
Rotating biological contactor (RBC), 561–563
Rotating-drum bioreactors, 314
rRNA, 49, 51, 121
RST (rate of substrate transfer), 85
Runaway replication, 490
Rupture with ice crystals, separation of insoluble products, 384–385
Rushton impeller, 325

S

S. cerevisiae, 25, 477–478
Salting out, separation of soluble products, 390–391
Same growth limiting substrate, 536–537, 540–541
Sanger, Frederick, 267
Sanger sequencing, 267–268
Saturated zone, separation of soluble products, 405
Saturation constant, 192–193
Saturation kinetics, 63–65
SBS (sequencing by synthesis) sequencing, 269
Scenedesmus algae, 27
Secondary culture of cells, 435
Secondary metabolites, 178
Segregated models, 191
Segregation, 191
Segregational loss, 486–488
Selectable mutations, 250
Selective pressure, 489
Selenosysteine, 31
Semicontinuous bioreactors. *See* Fed-batch bioreactors.
Semirational design, 513
Senescence, 435
Separation, integrating with reaction, 424–425. *See also* Recovery.
Separation of insoluble products
cake resistance, 376–378
cell disruption, mechanical, 383–385
cell disruption, nonmechanical, 384–385
centrifugation, 378–382
centrifugation coefficient, 381
coagulation, 382
Dyno-Mill homogenizer, 384
filtration, 374–378
flocculation, 382
French presses, 383

Gaulin–Manton presses, 383
hindered settling, 378–380
Hughes press, 384
incompressible cake, 376–378
osmotic shock, 384–385
Rannie high-pressure homogenizer, 383
rate of filtration, 375
rotary vacuum precoat filters, 374
rupture with ice crystals, 384–385
sonicators, 383
ultrasonic vibrators, 383
X-press, 384
Separation of soluble products
adsorption, 403–409
adsorption zone, 405
anisotropic membranes, 395
aqueous two-phase affinity partition extraction, 389
aqueous two-phase extraction, 389–390
bed depth-service time equation, 405–409
centrifugal Podbielniak extractors, 387–388
concentration, 403
concentration polarization, 394–395
cross-flow filtration, 398–403
cross-flow ultrafiltration, 398–403
diafiltration, 403
dialysis, 392–393
dialysis equilibrium, 393
dissociation extraction, 387–388
Donnan equilibrium, 393
ED (electrodialysis), 421–422
electrophoresis, 419–420
flat sheet membranes, 400–402
Freundlich adsorption isotherm, 403–404
hollow fiber membranes, 400–402
IEM (ion-exchange membranes), 421
ionized species. *See* Dissociation extraction.
isoelectric focusing, 420
isoelectric precipitation, 390–392
Langmuir isotherm, 404
liquid–liquid extraction, 385–389
membrane configurations, 400–402
MF (microporous filtration), 395–398
microfiltration, 395–403
moving-bed columns, 404
osmosis, 393
packed-bed columns, 404
precipitation, 390–392
purification, 403
reflection coefficient, 394
rejection coefficient of an ultrafilter, 399–400
RO (reverse osmosis), 393–395
salting out, 390–391
saturated zone, 405
solubility reduction, 390–391
solvent precipitation, 390–392
spiral-wound cartridge membranes, 400–402
tangential flow filtration, 398–403
UF (ultrafiltration), 395–398, 403
virgin zone, 405
Separation of soluble products, chromatography
ADC (adsorption chromatography), 410–411
AFC (affinity chromatography), 411, 414–419
definition, 409
displacement chromatography, 409
elution chromatography, 409
frontal chromatography, 409
gel-filtration chromatography, 410–411, 414–415
HC (hydrophobic chromatography), 411
HIC (hydrophobic interaction chromatography), 411
HPLC (high-pressure liquid chromatography), 411
IEC (ion-exchanging chromatography), 410–411, 414, 419–420
IMAC (immobilized-metal-affinity chromatography), 416–419
LLC (liquid–liquid partition chromatography), 410–411
mobile phase, 409
molecular sieving, 414–415
molecular sieving chromatography, 410
normal phase chromatography, 410
size exclusion chromatography, 410
stationary phase, 409
summary of methods, 410–411
typical chromatographic column, 411–414
Sequencing by synthesis (SBS), 269
Sequential feedback inhibition, 134
Sexual reproduction, eucaryotes, 23
Shock loading, biological waste treatment, 552–553
Shotgun cloning, 259, 264
Sigma factor, 119
Signal peptides, 125, 476
Silent mutations, 248
Simple glycoforms, 129
Single-use bioreactors, 330–331
Single-use disposable systems, 445, 447

siRNA (small interfering RNA), 49
Site-directed mutagenesis, 512
Size exclusion chromatography, 410
Skin replacement, 521–522
Sludge bulking, biological waste treatment, 553–556
Sludge re-aeration, biological waste treatment, 556–557
Solid-state fermentation (SSF) reactors, 313–316
Solubility reduction, separation of soluble products, 390–391
Solubilization of insoluble organics, biological waste treatment, 564
Solvent precipitation, separation of soluble products, 390–392
Sometimes added micronutrients, 54–55
Sonicators, separation of insoluble products, 383
SOP (standard operating procedures), 10
Spargers, 324
Specialized transduction, gene transfer and rearrangement, 255
Specific activity, 70–71
Sperm cells, 23
Spherical (coccus) cells, 14
Spiral (spirillum) cells, 14
Spiral-wound cartridge membranes, 400–402
Spirillum (spiral) cells, 14
Spirulina algae, 27
Spodoptera frugiperda (fall armyworm), 480
Spontaneous rates of mutation, 250
Spores of procaryotes, 20
Sporozoans, 27
Spray dryers, purification process, 424
Squibb, producing penicillin, 5
SSF (solid-state fermentation) reactors, 313–316
Stable RNA species, 121
Staining. *See* Gram staining.
Standard operating procedures (SOP), 10
Stationary phase, 178–179
Stationary phase chromatography, 409
Steady-state method for oxygen removal, 334
Stem cells, 529–530
Step feeding/step aeration, biological waste treatment, 556
Steric effects on enzyme immobilization, 98
Sterility, bioreactor scale-up, 329
Sterilization charts, 360–361
Sterilizing process fluids. *See also* Cleaning bioreactors.
  continuous sterilization, 361
  depth filters, 362–363
  vs. disinfection, 356
  filter sterilization, 361
  gases, 362–364
  glass wool filters, 362
  grow-through tests, 363
  kinetics of death, 356–357
  liquids, 357–362
  probability of extinction, 356
  sterilization charts, 360–361
  surface filters, 362–363
Steroid hormone receptors, 139
Sterols, 22
Stirred-tank bioreactors for animal cell cultivation, 447
Stoichiometric calculations
  degree of reduction, 230–235
  elemental balances, 230
Stoichiometry by oxidation-reduction half-reactions, 237–240
Stop codons, 125
Stop signals, 121
Storage granules, procaryotes, 20
Streptokinase, uses for, 102
Streptomyces, 477
Strong promoter, 121
Strong terminators, 121
Structural proteins, 30
Structured models, 191
Submerged culture, bioreactor considerations in plant cell cultivation, 467
Submerged tank process, penicillin production, 6
Substrate inhibition, 76–79, 195
Substrate-limited growth, 192–194
Sucrose, 40
Sulfation, 116
Sulfur, macronutrient, 53
Suppressor mutations, 249
Surface filters, 362–363
Surface immobilization, 87–90
Surface method of penicillin production, 6
Suspended animal cell cultures, 447
Suspended cells, 311–313
Suspension cultures
  bioreactor considerations in plant cell cultivation, 462–463
  in plant cell cultivation, 458–459
SYMBA process, 548, 571–572
Symbiosis, model of mixed culture interaction, 538

Synonymous mutations, 259
Synthetic biology, 509–511
Systems biology, 29, 267, 509–511. *See also* Genomics.

T

T (thymine), 45, 47
TA cloning, 262
Taq (*Thermus aquaticus*), 14
Taxonomy of cells. *See* Cell taxonomy.
TCA (Krebs, tricarboxylic acid) cycle, 149–152
Teichoic acids, 19
Telomere synthesis, 115
Temperate phages, 256
Temperature activation, 82–83
Temperature effects, batch cell growth kinetics, 184–185
Temperature inactivation, 83
Termination step, 124–125
Tessier equation, 194
Testing for proteins, 264
Tetramers, 487
Thermal denaturation, 83
Thermoacidophiles, 21
Thermodynamic equilibrium and cell death, 51
Thermodynamics of biological reactions, 240–242
Thermophiles, 14
Thermus aquaticus (Taq), 14
Three-dimensional printing of living cells, 300
Three-dimensional structure, proteins, 33–34
Thylakoids, 24
Thymine (T), 45, 47
Tissue engineering, 519–523
TKN (total Kjeldahl nitrogen), 551
TOD (total organic carbon), 550
TOPO cloning, 262
Total Kjeldahl nitrogen (TKN), 551
Total organic carbon (TOD), 550
Totipotency in plant cell cultivation, 457–461
Toxic aromatic compounds (biodegradation of xenobiotics), 157–158
Transcription terminators, 121
Transcriptomics, 270
Transduction, gene transfer and rearrangement, 254–256
Transfection, 265–266
Transfer (tRNA), 49–50
Transformation, gene transfer and rearrangement, 254
Transformed cells, 435–436, 479
Transgenic plants and animals, 482–484
Transport proteins, 30
Transport vesicle fusion, 127
Transposons, gene transfer and rearrangement, 257
Tray bioreactors, 314
Trickling biological filters, biological waste treatment, 558–561
Tricoplusia ni (cabbage looper), 480
tRNA, 49–50, 121
True bacteria (eubacteria)
  evolution of, 15
  Gram staining, 18
  photosynthesis, 19
Trypanosomes, 27
Trypsin, uses for, 102
Tubulin, 25
Turbidity, 172
Turbidostats, 208–210
Two-dimensional gel electrophoresis, 270
Two-phase fermentation, 489
Type IIS enzyme, 262–263

U

U (uracil), 45
Ubiquitination, 116
Ubiquitins, 116
UCT process for biological waste treatment, 571
UF (ultrafiltration), separation of soluble products, 395–398, 403
Ultrasonic vibrators, separation of insoluble products, 383
Unbalanced growth, 191
Uncompetitive inhibitors, enzyme kinetics, 75–76
Union Carbide, 552
Units, 70
UNOX process for biological waste treatment, 552, 558
Unselectable mutations, 250
Unstable RNA species, 121
Unsteady-state method for oxygen removal, 333
Unstructured nonsegregated models, 192–201
Unwinding enzymes, 117

Uracil (U), 45
USDA Northern Regional Research Laboratory, 5–6

V

Vacuoles, 24
Vacuum-tray dryers, purification process, 423
van der Waals forces, 63
Variable-volume continuous culture bioreactors. *See* Fed-batch bioreactors.
VEFCS (very enriched fructose corn syrup) production, 595
Viable cell count, 171
VIP process for biological waste treatment, 571
Viral gene delivery systems, 524
Viral infection, models of, 524–527
Virgin zone, separation of soluble products, 405
Viruses. *See also* HIV (human immunodeficiency) virus; Retroviruses.
  capsid coating, 17
  classifying, 15
  definition, 16
  infecting bacteria. *See* Phages.
  lysogenic cycle, 17
  lytic cycle, 17
  mode of reproduction, 17
  size, 16
  structure, 16–17
  that infect bacteria. *See* Phages.
V_m, interpreting, 70–71
Volatile acid formation, biological waste treatment, 564
Volatilized material, biological waste treatment, 551
Volumetric productivities in plant cell cultivation, 468
Volutins in procaryotes, 20

W

Wastes, types of, 549
Wastewater treatment. *See also* Mixed culture biological waste treatment.
  algae for, 27
  characteristics, 549–551
  converting to useful products, 571–572
  protozoa for, 28
Weak terminators, 121
Western blot testing, 264
Whiskey manufacturing, 546
Wild type mutations, 248
Working volume, bioreactor scale-up, 328

X

Xenobiotics biodegradation (toxic aromatic compounds), 157–158
X-press, separation of insoluble products, 384

Y

Yamanaka, Shinaya, 529
Yeast-mediated cloning, 263
Yeasts
  definition, 25
  in genetic engineering, host-vector systems, 478
  reproductive modes, 25
  Saccharomyces cerevisiae, 25–26
Yield coefficients
  maintenance, 227–229
  oxygen, 227–229
  theoretical predictions, 235–236
Yield factors for aerobic growth, 182

Z

Zwitterion, 32
Zygotes, 23
Zymomonas, 161

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