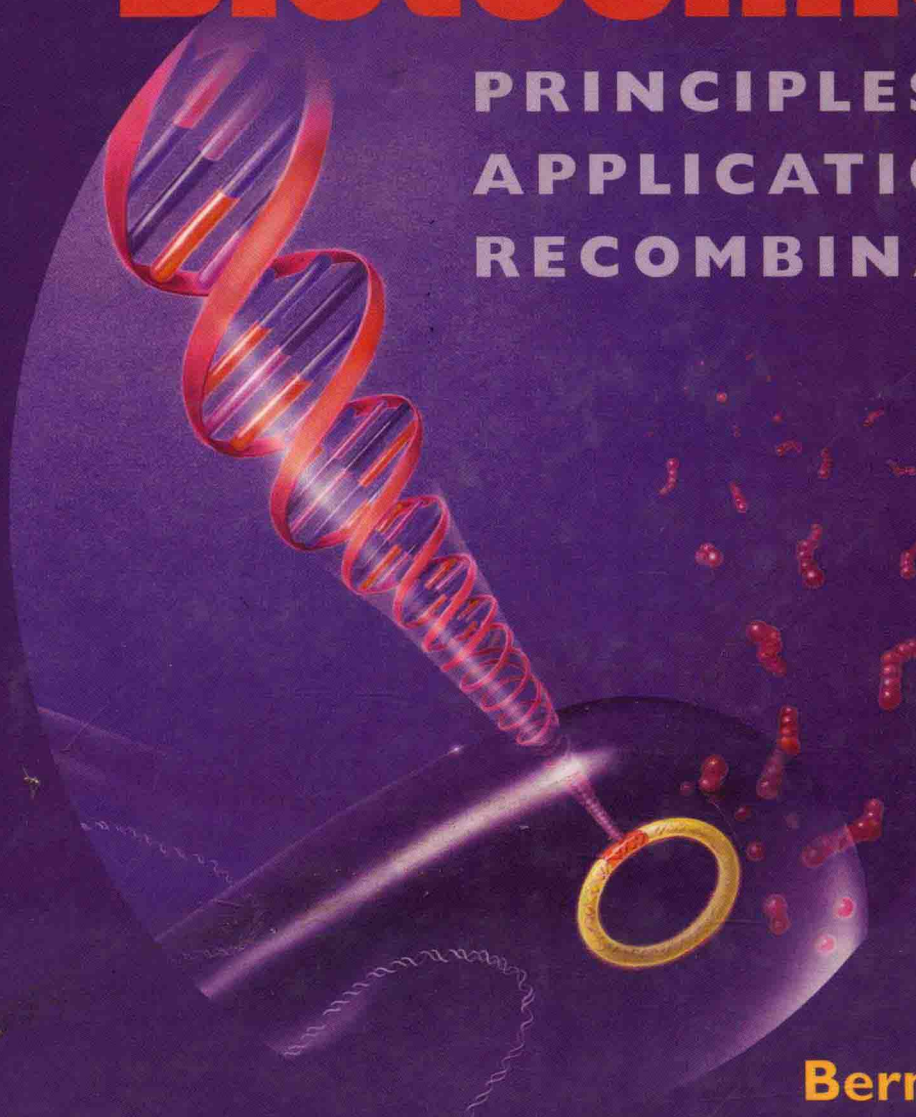


THIRD EDITION

Molecular Biotechnology

PRINCIPLES AND
APPLICATIONS OF
RECOMBINANT DNA



Bernard R. Glick
Jack J. Pasternak

THIRD EDITION

Molecular Biotechnology

PRINCIPLES AND APPLICATIONS
OF RECOMBINANT DNA

Bernard R. Glick

Jack J. Pasternak

Department of Biology, University of Waterloo
Waterloo, Ontario, Canada



ASM
PRESS

WASHINGTON, D.C.

Address editorial correspondence to ASM Press, 1752 N St. NW, Washington, DC 20036-2904, USA

Send orders to ASM Press, P.O. Box 605, Herndon, VA 20172, USA
Phone: (800) 546-2416 or (703) 661-1593
Fax: (703) 661-1501
E-mail: books@asmusa.org
Online: www.asmpress.org

Copyright © 1994, 1998, 2003 ASM Press
American Society for Microbiology
1752 N St. NW
Washington, DC 20036-2904

Library of Congress Cataloging-in-Publication Data

Glick, Bernard R.

Molecular biotechnology : principles and applications of recombinant DNA /
Bernard R. Glick and Jack J. Pasternak.—3rd ed.

p. ; cm.

Includes bibliographical references and index.

ISBN 1-55581-224-4 (hardcover : alk. paper)—ISBN
1-55581-269-4 (paperback : alk. paper)

1. Biotechnology. 2. Genetic engineering. 3. Molecular biology.

[DNLM: 1. Biotechnology. 2. Genetic Engineering. 3. Molecular Biology.
TP 248.2 G559m 2003] I. Pasternak, Jack J. II. Title.

TP248.2 .G58 2003
660'.65—dc21

2002013148

10 9 8 7 6 5 4 3 2 1

All Rights Reserved
Printed in the United States of America

Cover and interior design: Susan Brown Schmidler
Cover illustration: Terese Winslow

THIRD EDITION

Molecular
Biotechnology

PRINCIPLES AND
APPLICATIONS OF
RECOMBINANT DNA

To Marcia, for being Marcia
BRG

*To the memory of Albert and Jennie Pasternak
and to their great-grandchildren, Madelaine,
Miranda, Nina, and Maya*
JJP

Preface

MOLECULAR BIOTECHNOLOGY HAS MADE MAJOR ADVANCES since the second edition of *Molecular Biotechnology: Principles and Applications of Recombinant DNA* was published in 1998. Otherwise, a new edition wouldn't be necessary! Molecular biotechnology is no longer based on the hope that recombinant DNA technology will someday produce worthwhile "goods and services." The last 4 years has seen many molecular biotechnology products for medical, agricultural, and industrial uses become commonplace. Recombinant DNA technology has finally lived up to all the hype. Along with this maturity, new developments and innovative techniques have been perfected. In this new edition, we were able to update all aspects of *Molecular Biotechnology*, although the original framework has been maintained. A number of chapters have been extensively overhauled to accommodate new information, some chapters have been conflated with others, many new figures that illustrate concepts and principles have been added, and various recent examples replace those from the mid-1990s and earlier. And, as noted in the prefaces to the previous editions, we have tried both not to rely on confusing scientific terminology for its own sake and to present all the information as clearly as possible.

We appreciate the support and enthusiasm of Jeff Holtmeier, director of ASM Press. We thank Mary McKenney, who edited the manuscript with consummate skill and understanding. And we are especially indebted to Ken April of ASM Press, who patiently shepherded us through the entire process and, with the greatest aplomb, combined all the disparate elements to create this new edition.

Preface to the First Edition

MOLECULAR BIOTECHNOLOGY EMERGED as a new research field that arose as a result of the fusion in the late 1970s of recombinant DNA technology and traditional industrial microbiology. Whether one goes to the movies to see *Jurassic Park* with its ingenious but scientifically untenable plot of cloning dinosaurs, reads in the newspaper about the commercialization of a new “biotech” tomato that has an extended shelf life, or hears one of the critics of molecular biotechnology talking about the possibility of dire consequences from genetic engineering, there is a significant public awareness about recombinant DNA technology. In this book, we introduce and explain what molecular biotechnology actually is as a scientific discipline, how the research in the area is conducted, and how this technology may realistically impact on our lives in the future.

We have written *Molecular Biotechnology: Principles and Applications of Recombinant DNA* to serve as a text for courses in biotechnology, recombinant DNA technology, and genetic engineering or for any course introducing both the principles and the applications of contemporary molecular biology methods. The book is based on the biotechnology course we have offered for the past 12 years to advanced undergraduate and graduate students from the biological and engineering sciences at the University of Waterloo. We have written this text for students who have an understanding of basic ideas from biochemistry, molecular genetics, and microbiology. We are aware that it is unlikely that students will have had all of these courses before taking a course on biotechnology. Thus, we have tried to develop the topics in this text by explaining their broader biological context before delving into molecular details.

This text emphasizes how recombinant DNA technology can be used to create various useful products. We have, wherever possible, used experimental results and actual methodological strategies to illustrate basic concepts, and we have tried to capture the flavor and feel of how molecular biotechnology operates as a scientific venture. The examples that we have selected—from a vast and rapidly growing literature—were chosen as case studies that not only illustrate particular points but also provide the reader with a solid basis for understanding current research in specialized areas of

molecular biotechnology. Nevertheless, we expect that some of our examples will be out of date by the time the book is published, because molecular biotechnology is such a rapidly changing discipline.

For the ease of the day-to-day practitioners, scientific disciplines often develop specialized terms and nomenclature. We have tried to minimize the use of technical jargon and, in many instances, have deliberately used a simple phrase to describe a phenomenon or process that might otherwise have been expressed more succinctly with technical jargon. In any field of study, synonymous terms that describe the same phenomenon exist. In molecular biotechnology, for example, recombinant DNA technology, gene cloning, and genetic engineering, in a broad sense, have the same meaning. When an important term or concept appears for the first time in this text, it is followed in parentheses with a synonym or equivalent expression. An extensive glossary can be found at the end of the book to help the reader with the terminology of molecular biotechnology.

Each chapter opens with an outline of topics and concludes with a detailed summary and list of review questions to sharpen students' critical thinking skills. All of the key ideas in the book are carefully illustrated by the more than 200 full-color diagrams in the pedagogical belief that a picture is indeed worth a thousand words. After introducing molecular biotechnology as a scientific and economic venture in Chapter 1, the next five chapters (2 to 6) deal with the methodologies of molecular biotechnology. The chapters of Part I act as a stepping-stone for the remainder of the book. Chapters 7 to 12 in Part II present examples of microbial molecular biotechnology covering such topics as the production of metabolites, vaccines, therapeutics, diagnostics, bioremediation, biomass utilization, bacterial fertilizers, and microbial pesticides. Chapter 13 describes some of the key components of large-scale fermentation processes using genetically engineered (recombinant) microorganisms. In Part III, we deal with the molecular biotechnology of plants and animals (Chapters 14 and 15). The isolation of human disease-causing genes by using recombinant DNA technology and how, although it is in its early stages, genetic manipulation is being currently contemplated for the treatment of human diseases are presented in Chapters 16 and 17. The book concludes with coverage of the regulation of molecular biotechnology and patents in Part IV.

A brief mention should be made about the reference sections that follow each chapter. Within many of the chapters we have relied upon the published work of various researchers. In all cases, although not cited directly in the body of a chapter, the original published articles are noted in the reference section of the appropriate chapter. In some cases, we have taken "pedagogic license" and either extracted or reformulated data from the original publications. Clearly, we are responsible for any distortions or misrepresentations from these simplifications, although we hope that none has occurred. The reference sections also contain other sources that we used in a general way, which might, if consulted, bring the readers closer to a particular subject.

Acknowledgments

We express our appreciation to the following people who reviewed various parts of the manuscript as it was being developed. The comments of these expert scientists and teachers helped us immeasurably: Arthur I. Aronson, Purdue University; Ronald M. Atlas, University of Louisville; Fred Ausubel,

Massachusetts General Hospital; David R. Benson, University of Connecticut; Jean E. Brenchley, Pennsylvania State University; A. M. Chakrabarty, University of Illinois at Chicago; Stan Gelvin, Purdue University; Janet H. Glaser, University of Illinois at Urbana-Champaign; David Gwynne, Cambridge NeuroScience; George D. Hegeman, Indiana University; James B. Kaper, University of Maryland at Baltimore; Donald R. Lightfoot, Eastern Washington University at Cheney and Spokane; Cynthia Moore, Washington University; William E. Newton, Virginia Polytechnic University; Danton H. O'Day, University of Toronto in Mississauga; Richard D. Palmiter, University of Washington; David H. Persing, Mayo Clinic; William S. Reznikoff, University of Wisconsin; Campbell W. Robinson, University of Waterloo; Marc Siegel, University of Waterloo; Aaron J. Shatkin, Center for Advanced Biotechnology and Medicine at Rutgers University; Jim Schwartz, Genentech; Daniel C. Stein, University of Maryland at College Park; Dean A. Stetler, University of Kansas; and Robert T. Vinopal, University of Connecticut.

The following professionals at ASM Press worked on the book and deserve our thanks: Susan Birch, senior production editor; Ruth Siegal, developmental editor; Jodi Simpson, copy editor; Susan Schmidler, designer and art director; Peg Markow at Ruttle, Shaw & Wetherill, Inc., senior project manager; and Network Graphics, illustrators. Finally we are indebted to Patrick Fitzgerald, Director of ASM Press, who, in all possible ways, helped transform our original efforts into an acceptable final form. His encouragement as a persistent and friendly "torturer" was deeply appreciated.

Bernard R. Glick
Jack J. Pasternak

Contents

Preface xix

Preface to the First Edition xxi

I Fundamentals of Molecular Biotechnology 1

1 The Molecular Biotechnology Revolution 3

Recombinant DNA Technology 3

Emergence of Molecular Biotechnology 5

Commercialization of Molecular Biotechnology 7

Concerns and Consequences 10

SUMMARY 12

REFERENCES 13

REVIEW QUESTIONS 13

2 Molecular Biotechnology Biological Systems 14

Prokaryotic and Eukaryotic Organisms 14

Escherichia coli 15

Saccharomyces cerevisiae 17

Secretion Pathways in Prokaryotic and Eukaryotic Organisms 18

Eukaryotic Cells in Culture 21

SUMMARY 22

REFERENCES 22

REVIEW QUESTIONS 22

3 DNA, RNA, and Protein Synthesis 23

Structure of DNA 23

DNA Replication 27

Decoding Genetic Information: RNA and Protein 28
Translation 34
Regulation of mRNA Transcription in Bacteria 38
Regulation of mRNA Transcription in Eukaryotes 42
SUMMARY 45
REFERENCES 46
REVIEW QUESTIONS 46

4 Recombinant DNA Technology 47
Restriction Endonucleases 49
Plasmid Cloning Vectors 57
Plasmid Cloning Vector pBR322 58
Transformation and Selection 60
Other Plasmid Cloning Vectors 61
Creating and Screening a Library 64
Making a Gene Library 64
Screening by DNA Hybridization 67
Screening by Immunological Assay 73
Screening by Protein Activity 73
Cloning DNA Sequences That Encode Eukaryotic Proteins 75
Vectors for Cloning Large Pieces of DNA 80
Bacteriophage λ Vectors 80
Cosmids 83
High-Capacity Bacterial Vector Systems 86
Genetic Transformation of Prokaryotes 86
Transferring DNA into *E. coli* 86
Electroporation 86
Conjugation 87
SUMMARY 89
REFERENCES 89
REVIEW QUESTIONS 90

5 Chemical Synthesis, Sequencing, and Amplification of DNA 91
Chemical Synthesis of DNA 91
The Phosphoramidite Method 92
Uses of Synthesized Oligonucleotides 96
DNA Sequencing Techniques 101
Dideoxynucleotide Procedure for Sequencing DNA 102
Automated DNA Sequencing 106
Using Bacteriophage M13 as a DNA Sequencing Vector 107
Primer Walking 110
PCR 110
Gene Synthesis by PCR 115
Cycle Sequencing 115

| | |
|------------------|-----|
| SUMMARY | 118 |
| REFERENCES | 119 |
| REVIEW QUESTIONS | 120 |

6 Manipulation of Gene Expression in Prokaryotes 121

Gene Expression from Strong and Regulatable Promoters 122

| | |
|------------------------------------|-----|
| Regulatable Promoters | 122 |
| Increasing Protein Production | 126 |
| Large-Scale Systems | 126 |
| Expression in Other Microorganisms | 128 |

Fusion Proteins 130

| | |
|-----------------------------|-----|
| Cleavage of Fusion Proteins | 131 |
| Uses of Fusion Proteins | 131 |
| Surface Display | 135 |

Unidirectional Tandem Gene Arrays 136

Translation Expression Vectors 138

Increasing Protein Stability 142

Protein Folding 142

Overcoming Oxygen Limitation 145

| | |
|--|-----|
| Use of Protease-Deficient Host Strains | 145 |
| Bacterial Hemoglobin | 145 |

DNA Integration into the Host Chromosome 146

| | |
|----------------------------------|-----|
| Removing Selectable Marker Genes | 149 |
|----------------------------------|-----|

Increasing Secretion 151

| | |
|-----------------|-----|
| L-Form Bacteria | 153 |
|-----------------|-----|

Metabolic Load 155

| | |
|------------------|-----|
| SUMMARY | 159 |
| REFERENCES | 159 |
| REVIEW QUESTIONS | 161 |

7 Heterologous Protein Production in Eukaryotic Cells 163

Saccharomyces cerevisiae Expression Systems 165

| | |
|---|-----|
| <i>S. cerevisiae</i> Vectors | 166 |
| Intracellular Production of Heterologous Proteins in <i>S. cerevisiae</i> | 169 |
| Secretion of Heterologous Proteins by <i>S. cerevisiae</i> | 171 |

Pichia pastoris and Other Yeast Expression Systems 171

Baculovirus–Insect Cell Expression System 174

| | |
|--|-----|
| Baculovirus Expression Vector System | 175 |
| Increasing the Yield of Recombinant Baculovirus | 175 |
| Construction of an <i>E. coli</i> –Insect Cell Baculovirus Shuttle Vector | 177 |
| Mammalian Glycosylation and Processing of Precursor Proteins in Insect Cells | 180 |

Mammalian Cell Expression Systems 181

| | |
|--|-----|
| Selectable Marker Systems for Mammalian Expression Vectors | 185 |
|--|-----|

SUMMARY 187
REFERENCES 187
REVIEW QUESTIONS 189

8 Directed Mutagenesis and Protein Engineering 190

Directed Mutagenesis Procedures 191

Oligonucleotide-Directed Mutagenesis with M13 DNA 192
Oligonucleotide-Directed Mutagenesis with Plasmid DNA 194
PCR-Amplified Oligonucleotide-Directed Mutagenesis 195
Random Mutagenesis with Degenerate Oligonucleotide Primers 198
Random Mutagenesis with Nucleotide Analogues 201
Error-Prone PCR 201
DNA Shuffling 203
Mutant Proteins with Unusual Amino Acids 204

Protein Engineering 205

Adding Disulfide Bonds 205
Changing Asparagine to Other Amino Acids 209
Reducing the Number of Free Sulfhydryl Residues 209
Increasing Enzymatic Activity 211
Modifying Metal Cofactor Requirements 212
Decreasing Protease Sensitivity 214
Modifying Protein Specificity 215
Increasing Enzyme Stability and Specificity 218
Altering Multiple Properties 219

SUMMARY 221
REFERENCES 222
REVIEW QUESTIONS 223

II Molecular Biotechnology of Microbial Systems 225

9 Molecular Diagnostics 227

Immunological Diagnostic Procedures 228

Enzyme-Linked Immunosorbent Assay 229

Monoclonal Antibodies 230

Formation and Selection of Hybrid Cells 231
Identification of Specific Antibody-Producing Hybrid Cell Lines 232

DNA Diagnostic Systems 234

Hybridization Probes 235
Diagnosis of Malaria 236
Detection of *Trypanosoma cruzi* 236
Nonradioactive Hybridization Procedures 237
Molecular Beacons 240
DNA Fingerprinting 241

| | |
|--|------------|
| Random Amplified Polymorphic DNA | 242 |
| Bacterial Biosensors | 244 |
| Molecular Diagnosis of Genetic Disease | 245 |
| Screening for Cystic Fibrosis | 246 |
| Sickle-Cell Anemia | 246 |
| The PCR/OLA Procedure | 248 |
| Padlock Probes | 250 |
| Genotyping with Fluorescence-Labeled PCR Primers | 250 |
| SUMMARY | 253 |
| REFERENCES | 253 |
| REVIEW QUESTIONS | 254 |

10 Therapeutic Agents 256

| | |
|--|------------|
| Pharmaceuticals | 257 |
| Isolation of Interferon cDNAs | 257 |
| Human Interferons | 258 |
| Human Growth Hormone | 259 |
| Tumor Necrosis Factor Alpha | 260 |
| Optimizing Gene Expression | 261 |
| Therapeutics Produced and Delivered by Intestinal Bacteria | 262 |
| Enzymes | 263 |
| DNase I | 263 |
| Alginate Lyase | 265 |
| Phenylalanine Ammonia Lyase | 266 |
| α_1 -Antitrypsin | 267 |
| Monoclonal Antibodies as Therapeutic Agents | 267 |
| Structure and Function of Antibodies | 269 |
| Preventing Rejection of Transplanted Organs | 270 |
| Treating Brain Tumors | 270 |
| Chemically Linked Monoclonal Antibodies | 271 |
| Human Monoclonal Antibodies | 272 |
| Hybrid Human–Mouse Monoclonal Antibodies | 274 |
| Production of Antibodies in <i>E. coli</i> | 277 |
| Phage Combinatorial Libraries | 277 |
| Shuffling CDR Sequences | 280 |
| Single-Chain Antibodies | 282 |
| Nucleic Acids as Therapeutic Agents | 283 |
| Antisense RNA | 284 |
| Antisense Oligonucleotides | 284 |
| Ribozymes | 288 |
| Chimeric RNA–DNA Molecules | 291 |
| Interfering RNAs | 292 |
| Antibody Genes | 294 |
| Treating Genetic Disorders | 295 |
| Human Gene Therapy | 298 |
| Prodrug Activation Therapy | 303 |
| SUMMARY | 304 |

REFERENCES 305

REVIEW QUESTIONS 307

11 Vaccines 309

Subunit Vaccines 312

Herpes Simplex Virus 312

Foot-and-Mouth Disease 313

Peptide Vaccines 315

Genetic Immunization: DNA Vaccines 317

Attenuated Vaccines 322

Cholera 322

Salmonella Species 324

Leishmania Species 325

Herpes Simplex Virus 326

Vector Vaccines 327

Vaccines Directed against Viruses 327

Vaccines Directed against Bacteria 333

Bacteria as Antigen Delivery Systems 335

SUMMARY 336

REFERENCES 337

REVIEW QUESTIONS 338

12 Synthesis of Commercial Products by Recombinant Microorganisms 340

Restriction Endonucleases 340

Small Biological Molecules 344

Synthesis of L-Ascorbic Acid 345

Microbial Synthesis of Indigo 349

Synthesis of Amino Acids 350

Removal of Lipids 354

Antibiotics 355

Cloning Antibiotic Biosynthesis Genes 356

Synthesis of Novel Antibiotics 360

Engineering Polyketide Antibiotics 361

Improving Antibiotic Production 365

Peptide Antibiotics 367

Biopolymers 367

Engineering *Xanthomonas campestris* for Xanthan Gum Production 367

Isolation of Melanin Biosynthesis Genes 369

Synthesis of an Animal Adhesive Biopolymer in Microbial Cells 370

Microbial Synthesis of Rubber 372

Microbial Production of Polyhydroxyalkanoates 372

SUMMARY 375

REFERENCES 375

REVIEW QUESTIONS 377

13 Bioremediation and Biomass Utilization 378**Microbial Degradation of Xenobiotics 378****Genetic Engineering of Biodegradative Pathways 382**

Manipulation by Transfer of Plasmids 384

Manipulation by Gene Alteration 385

Utilization of Starch and Sugars 394

Commercial Production of Fructose and Alcohol 395

Improving Alcohol Production 396

Improving Fructose Production 398

Zymomonas mobilis 400

Silage Fermentation 404

Utilization of Cellulose 405

Components of Lignocellulose 406

Isolation of Prokaryotic Cellulase Genes 408

Isolation of Eukaryotic Cellulase Genes 410

Manipulation of Cellulase Genes 412

SUMMARY 413

REFERENCES 414

REVIEW QUESTIONS 415

14 Plant Growth-Promoting Bacteria 416**Nitrogen Fixation 417**

Nitrogenase 419

Components of Nitrogenase 419

Genetic Engineering of the Nitrogenase Gene Cluster 421

Glycogen Synthase Mutants 425

Engineering Oxygen Levels 427

Hydrogenase 427

Hydrogen Metabolism 428

Genetic Engineering of Hydrogenase Genes 428

Nodulation 430

Competition among Nodulating Organisms 430

Genetic Engineering of Nodulation Genes 431

Growth Promotion by Free-Living Bacteria 435

Decreasing Plant Stress 438

Phytoremediation 440**Biocontrol of Pathogens 442**

Siderophores 443

Antibiotics 445

Enzymes 447

Ice Nucleation and Antifreeze Proteins 447

Ethylene 449

Root Colonization 451

SUMMARY 451

REFERENCES 452

REVIEW QUESTIONS 453

15 Microbial Insecticides 455

Insecticidal Toxin of *B. thuringiensis* 456

Mode of Action and Use 456

Toxin Gene Isolation 460

Engineering of *B. thuringiensis* Toxin Genes 462

Preventing the Development of Resistance 470

Genetic Engineering for Improved Biocontrol 472

Baculoviruses as Biocontrol Agents 473

Mode of Action 473

Genetic Engineering for Improved Biocontrol 475

SUMMARY 477

REFERENCES 478

REVIEW QUESTIONS 480

16 Large-Scale Production of Proteins from Recombinant Microorganisms 481

Principles of Microbial Growth 483

Batch Fermentation 483

Fed-Batch Fermentation 485

Continuous Fermentation 486

Maximizing the Efficiency of the Fermentation Process 488

High-Density Cell Cultures 489

Bioreactors 490

Typical Large-Scale Fermentation Systems 494

Two-Stage Fermentation in Tandem Airlift Reactors 495

Two-Stage Fermentation in a Single Stirred-Tank Reactor 496

Batch versus Fed-Batch Fermentation 497

Harvesting Microbial Cells 499

Disrupting Microbial Cells 502

Downstream Processing 504

Protein Solubilization 506

Large-Scale Production of Plasmid DNA 506

SUMMARY 507

REFERENCES 507

REVIEW QUESTIONS 509

III Eukaryotic Systems 511

17 Genetic Engineering of Plants: Methodology 513

Plant Transformation with the Ti Plasmid of *Agrobacterium tumefaciens* 514

Ti Plasmid-Derived Vector Systems 517

Physical Methods of Transferring Genes to Plants 520