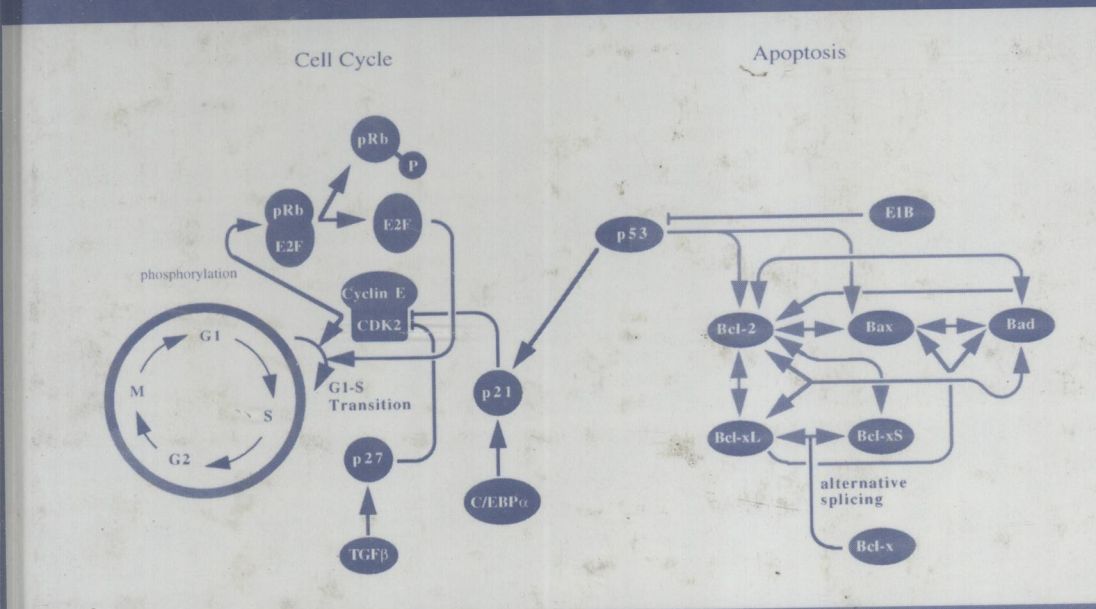
METABOLIC ENGINEERING



edited by
Sang Yup Lee
Eleftherios T. Papoutsakis

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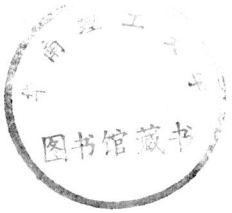

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METABOLIC ENGINEERING

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W. Courtney McGregor

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To our wives, Hyejean and Maria,
and our daughters, Gina and Ellie,
for their support and inspiration

Series Introduction

Bioprocess technology encompasses all the basic and applied sciences as well as the engineering disciplines required to fully exploit our growing knowledge of living systems and bring new or better products to the marketplace. In the era of biotechnology that began with recombinant DNA and cell fusion techniques, methods and processes have developed mostly in service of protein production. That development is documented in this series. Many protein products that derive from the technology are already marketed and more are on the way.

Now, with the rapid expansion of genomics, many new biological targets will likely be identified, paving the way for the development of an even wider array of products, mostly proteins. As knowledge of the targets develops, so will rational drug design, which may lead to development of small molecules as healthcare products. Rational genetic manipulation of cells as factories for growing products is also developing. Other examples of the application of genomics in health care include the development of gene therapy by insertion of genes into cells and the blocking of gene expression with antisense nucleotides. In such new directions, nucleotides and other small molecules, as well as protein products, will evolve. Technologies will also develop in parallel.

Transgenic technology, in which the genome of an organism is altered by inclusion of foreign genetic material, is also just beginning to develop. Recombinant protein products can already be made, for example, in the milk of transgenic animals as an alternative to conventional bioreactors. However, newer applications for transgenic technology in agriculture may take time to develop. Meanwhile, questions continue to be raised about the long-term environmental consequences of such manipulation.

W. Courtney McGregor

Preface

Metabolic engineering (ME) of (micro)organisms is emerging as a distinct subfield of genetic engineering. The development of recombinant DNA technologies starting in the 1970s enabled the development of the first generation of recDNA technologies aimed at producing heterologous or homologous proteins in a number of host cells. ME aims to alter the properties of cells and/or their metabolic reactions in order to achieve a desirable objective beyond the expression of a single gene for the production of a protein or polypeptide. Such an objective includes, but is not limited to: the production of a desirable product at higher rates or, with improved selectivity, the formation of new products not normally produced by a cell, and the coordinate expression of multiple genes that impart a desirable trait to the cells. The coining of the term and history of metabolic engineering have been discussed in the recent literature (1–3). While ME is now widely accepted as a term by both the bioengineering and the life sciences communities, the term is not accurately descriptive of a number of cases in which the properties of a cell have been beneficially modified (for a desirable objective) while, strictly speaking, the metabolic networks of the cells have not been affected. Perhaps "cellular engineering" (CE) would be a more accurate term, but one could argue that CE is too general and does not communicate the clear difference between the first generation of recDNA processes for the production of proteins and what this newer activity is meant to deliver. Thus, ME has established itself as the most suitable term for this research field.

The impact of ME is or will be felt in the production of valuable bulk or specialty chemicals (e.g., organic acids, alcohols, amino acids, vitamins, antibiotics, chiral compounds, and biopolymers), toxic-waste treatment, and therapeutic or diagnostic proteins as well as in gene therapy, as a large body of literature in the last 10 years and the chapters of this new book suggest. Despite the publication of a large number of research and review papers on ME, unique or global strategies or methodologies have yet to emerge. In terms of systematics, theoretical and modeling aspects of ME—the metabolic control analysis (MCA) and the metabolic flux analysis (MFA)—appear to reach a sense of maturity and universal acceptance. However, are there any other systematics and heuristics? Perhaps some strategies that can be applied to a variety of objectives and organisms can

now be identified, but no such strategy has been identified to date. Part of the reason is that the literature is very fragmented and differences in the physiology and genetics of the various organisms make it difficult to identify such common strategies. One of the chief objectives of this volume is to bring together, in a single source, a large number of cellular systems in which ME has been applied in order to facilitate comparisons and the emergence of global rules and strategies. Another core objective is to review the applications of the key analytical and modeling tools. Finally, we aimed to have as complete as possible a collection of cellular systems in which ME has been applied in order to review the successes and outline the goals achieved thus far. Although not all cellular systems are covered in this volume, the chapters cover all the important industrial systems (production of commodity and specialty chemicals, biopolymers, antibiotics, plant-cell derived products, and proteins, as well as bioremediation) involving virtually all cellular systems (bacteria, yeasts, plant cells, and mammalian cells).

Notable in this collection is the diversity of disciplines and countries from which the authors of the chapters have been drawn—a truly international and multidisciplinary effort! This effort represents the contributions of 36 scientists (including many well-known senior investigators whose work has defined this emerging field) whose combined expertise complements the knowledge and expertise of the editors. The contributions represent an intellectual effort of the highest order commensurate with the diversity, demands, and complexity of ME. Previously, only very limited efforts had been made to present ME as an integral inter- and multidisciplinary effort. These include a collection of papers in *Biotechnology & Bioengineering* (1998; 58(2) and (3): 119–344) and a recent book (4), both of which appeared while this book was in preparation. However, this volume has very little overlap in scope with either of these.

Who should use this book? It is intended as a companion of lasting value both to the research scientist (in the biotechnology industries as well as in academia) and to graduate courses on the subject matter. It will be of lasting value because the analytical and modeling principles covered here will "age" well and remain valid for years to come, only to be enriched and improved upon with future efforts and applications. Additionally, the early successes and efforts with various cellular systems discussed here will form the basis of things to come, and are thus likely to remain valid and useful for identifying the early developmental patterns of ME. Dare we entertain the idea that this collection will eventually reach classic status? Only time will tell. We have used earlier forms of our own book chapters as a basis of two or three regular lectures in advanced courses in microbial biotechnology and metabolic engineering. Using additional material from primary papers and review articles, most chapters can be used to support a number of lectures, and thus this book can be used as a text for an advanced course on ME.

We hope that this collection captures the excitement, anticipation, and po-

tential that many of us feel ME holds for the future of biotechnology, and technology as a whole. The dreams of efficient, environmentally friendly, and sustainable technologies, of new antiviral and antibacterial molecules for human and animal therapies, of effective gene and somatic-cell therapies, and of better crops and better food sources may eventually be fulfilled through metabolic engineering–based technologies. Intellectually, the potential is certaintly there. Clearly, much work is needed to make these dreams a reality!

Sang Yup Lee
Eleftherios T. Papoutsakis

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Introduction

EMERGENCE AND EVOLUTION OF METABOLIC ENGINEERING: FROM SPONTANEOUS MUTATION TO GENE THERAPY

Genetic improvement of organisms used by mankind is an ancient practice, relying originally on selection of variants with improved characteristics that could then, by classical breeding methods, be crossed into related organisms or combined with other useful traits emerging from independent lines of development. As the inner workings of cells were elucidated, specific molecular targets for performance-enhancing mutations were identified, perhaps best exemplified by development in Japan of bacteria providing very high titers of amino acids and nucleotides. These primal examples of directed genetic design remind us that success can depend much more on modification of *regulation*—in these cases, elimination of feedback inhibition of biosynthesis enzymes by desired products—and on knockouts of undesired activities (here, those that consume product) than on *amplification* of a pathway-limiting enzyme activity.

Discovery of technologies for gene cloning and stable incorporation of cloned genes into homologous and heterologous hosts in a context suitable for their expression gave birth to the first-generation modern biotechnology industry. Here this powerful technology found its most obvious application: transfer of genes encoding human proteins into bacteria, yeast, and mammalian cells to manufacture the corresponding proteins as human pharmaceuticals (and, a bit later, making industrial enzymes in genetically engineered bacteria and fungi). However, biologists and biochemical engineers who were already viewing the cell as a complex chemical factory soon recognized the power of genetic engineering to effect specific, targeted changes in cell function by expressing chosen activities in the cell from the corresponding cloned genes. This new application was called "molecular breeding" in the East and "metabolic engineering" in the West.

The first successful practical applications of metabolic engineering occurred in bacterial systems, enabling creation of *Escherichia coli* strains for amino acid production, which displaced other bacterial fermentations and chemi-

cal synthesis processes. Other microbial applications ensued, and development of vector systems and bioprocess technology provided necessary foundations for extension of metabolic engineering to mammalian cell culture. Metabolic engineering has also influenced agriculture, contributing to design of new transgenic plants that have had major impact in the marketplace (and, unfortunately, in the political arena in some parts of the world, delaying significant human, environmental, and economic benefits to those areas).

Absence of an essential protein activity causes various genetic diseases in humans. This has defined the first, and most obvious, goals for gene therapy (which, according to an early definition, belongs to the realm of metabolic engineering): knock out the bad gene, or put in a good gene to complement the effect of a bad one. Someday, for certain, this will be viewed as we now regard single-gene expression to manufacture a protein—as a necessary and useful first step, but also as a relatively primitive precursor to multigene reprogramming of defective functions.

PARADIGMS AND RESOURCES FOR METABOLIC DESIGN

The "Rational" Engineering Science Paradigm

One approach to metabolic engineering takes the chemical-factory concept of a cell literally, seeking to analyze and model cells by means of concepts and tools akin to those used in chemical engineering to achieve similar ends for manmade chemical process units and systems. In this realm "metabolic flux analysis" has emerged, using basic steady-state approximation and material balance concepts to achieve major insights into intracellular activities based on metabolic structure and stoichiometry. If such a picture of the cells' inner workings is augmented by kinetic descriptions, the "cell factory" model can be extended to a level useful for design and optimization calculations.

Implementation of design concepts suggested by such analyses, or by more intuitive reasoning, is increasingly feasible as resources and technologies for precise genetic modification in microbes. By defining what activities are possibly present in a cell, and also providing explicit sequence targets for vector integration or for PCR production of a needed gene or other fragment, genome data promise a revolution in metabolic engineering opportunities.

Letting Nature Be Our Guide: Inverse Metabolic Engineering and Directed Evolution

Although the engineering science approach is intellectually appealing, and also relatively comfortable for most chemical engineers, it has major deficiencies deriving from a central fact: functioning of all aspects of even the simplest living

cell is far too complex to describe in quantitative terms or even to describe qualitatively with much confidence. First, we do not know, even for *E. coli*, what all the proteins in the cell do. Next, we do not understand the response of expression of these genes to growth conditions or genetic variables, and we do not understand the extent of specificity of many activities, nor their kinetics. Proteome analysis (described in the literature only a short time ago under the pedestrian but more informative term "two-dimensional electrophoresis") shows, for example, that putting only a cloning vector into *E. coli* modifies expression of many important enzymes in many domains of metabolism, a phenomenon that must be assumed to be quite widespread, greatly complicating clear, rigorous demonstration of cause and effect in metabolic engineering. Thus, strategies other than the engineering science approach for finding genetic strategies to effect desired metabolic response are also needed.

Many responses to unusual situations—extremes in pH and temperature, oxygen deprivation, competition from more rapidly growing cells, errors in DNA replication, infection—have evolved in nature. *Inverse metabolic engineering* is the mobilization of a natural biological response mechanism to address applied goals. Here, a gene (in the future, entire genetic networks) conferring a useful phenotype found in one organism can be transferred to others via modern genetic technologies. Although there is no guarantee that a heterologous gene will exert the same effect in a different genetic background, this approach has been successfully applied to discover otherwise obscure, but highly effective, metabolic engineering strategies.

Many definitions of metabolic engineering allude specifically to recombinant DNA manipulations, in part to differentiate this field from prior "traditional" mutagenesis and screening practices. However, restricting one's resources for problem solving or technology creation is a foolish practice. Traditional tools, such as chemostat selection, are extremely powerful and can be integrated with modern tools such as proteome analysis and inverse genetics to discover genes that significantly improve metabolic activity under restrictive conditions.

A specific gene, intensively modified by means of PCR mutagenesis and combined with an efficient screen, can drive a "directed evolution" of function with effectiveness far surpassing that of conventional protein engineering approaches. Gene shuffling technology can increase the mutation rate and extend the approach to mixing of gene fragments, genes, and entire genomes.

Again, genome databases are an important resource, as are expressed sequence data and their offspring, hybridization arrays, which allow extremely rapid readout of which genes are expressed and which not, under particular environmental conditions or in response to genetic modification. Unfortunately, knowing what enzymes are present is far short of defining information about metabolic function: enzyme kinetics, especially activation and inhibition of enzyme activities, also exert critical influence on metabolism.

THE UNIQUE CAPABILITY OF METABOLIC ENGINEERING: CREATING NOVEL METABOLIC SYSTEMS

Traditional mutagenesis can never endow a cell with genes from other organisms, nor can it impart their functions. Recombinant DNA technology makes such work easy. This basic functionality is often essential to the inverse metabolic engineering approach, but its implications are much more profound. By expressing new combinations of activities in cells, we can induce those cells to synthesize products never before created. Process improvements can be achieved in many ways, including metabolic engineering. More precious, potentially, is this unique route to new complex chemicals—novel polyketides, glycoproteins, and later biopolymers and more.

MILESTONES OF METABOLIC ENGINEERING

Since the first research to exploit recombinant DNA techniques to modify metabolism, and the first reviews that codified the field, metabolic engineering has become increasingly prominent as a focal point for contemporary biotechnology research and development. A few subsequent milestones might be mentioned. The journal *Biotechnology Progress* introduced a section entitled "Applied Cellular Physiology and Metabolic Engineering" in 1994. The first Engineering Foundation Conference on Metabolic Engineering was held in 1996 (the proceedings of this conference were recently published; see *Biotechnology and Bioengineering*, vol. 58, nos. 2 and 3), and the second such conference, in what now is intended to be an ongoing series, took place in 1998. In 1997 the Keystone Symposia sponsored a conference entitled "Metabolic Engineering in Transgenic Plants." Finally, this book brings together an outstanding collection of prominent researchers in the field to provide a rich, broad set of informative reviews. A brief synopsis of this book's contents follows.

Bacteria are a natural target for the first wave of metabolic engineering development—they have numerous important applications, genetic tools are the most well developed, and generation times are shortest, accelerating experimentation and development. These properties also make bacteria a preferred model system for study of experimental methods, mathematical approaches, and design concepts. Therefore most of this book concerns bacterial systems, but seen from several different perspectives. Besides the themes already mentioned, details on the development of new vectors, transformation protocols, cloning, and expression systems are presented. In a number of cases bioscience research has bypassed organisms important industrially, necessitating this infrastructure construction.

Many industrial processes, ranging from production of baker's yeast in mixed carbon source media to biodegradation, depend critically on the range of

substrates a microorganism can import and catabolize, and on how uptake and subsequent utilization of nutrients are regulated. Thus, metabolic engineering has been mobilized to expand the range of utilizable substrates for a variety or organisms.

Product biosynthesis pathways are attractive targets for metabolic engineering because of potential for high leverage effects on production rates and also because regulation can be much simpler than in central metabolism, reducing the probability of unforeseen cell responses. Secondary metabolite synthesis by fungi and plants are therefore addressed here, as is the important general theme mentioned earlier of creating new secondary metabolite synthesis pathways that generate new chemical entities.

Metabolic engineering of mammalian cells is of immediate interest relative to biopharmaceutical production, and here modification of the basic cell "platform," in terms of growth and death characteristics, has emerged as a special theme, as has modification of product glycosylation to provide novel, more effective glycoprotein therapeutics. Here, as investigators discovered at the outset in connection with early development of amino acid overproducing *Corynebacteria*, genetic engineering to reprogram regulation of key cellular processes is a dominant theme.

THE FUTURE OF METABOLIC ENGINEERING

This volume clearly articulates the great progress achieved in the founding years of metabolic engineering. The bases are now established for expanding impact in more efficient processes for consuming unwanted substrates, for generating useful cells, and for synthesizing important industrial products. As the chemical industry moves steadily toward products that are higher in value, hence more chemically complex, and as greater concern for the environment and for sustainable technologies renders wasteful, polluting chemical processes unacceptable, biological routes to production become increasingly preferred. Metabolic engineering will undoubtedly be a key contributor to the elimination of several central limitations in reaching this goal.

Feeding the world, and making best use of available agricultural resources to produce food and chemicals of greatest value, are crucial global issues that are already being addressed by metabolic engineering of plants to alter their responses to stress, to pests, and to agricultural chemicals, and to change the properties of the plant and its products. This trend, which will expand in the future, may lead to the most significant impact of metabolic engineering in both human and economic terms. Revolutionary developments in cloning of mammals and in simultaneous incorporation of transgenes also promise dramatic new venues for productive applications of metabolic engineering.

The prospect of gene therapy for human disease has engendered tremendous excitement. Here metabolic engineering concepts and methodology will become essential, to decipher and then to treat effectively diseases with complex dependencies on multiple genetic loci. Likewise, control of gene expression, including that of installed cloned genes, will eventually be a pivotal technology for *in vitro* preparation of cells and tissues for therapeutic applications.

Indeed, the scope of future possibilities for metabolic engineering is so vast that perhaps the greatest challenge is to identify directions for future research that most creatively and productively disclose the most exciting possibilities.

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