Biochemical Engineering

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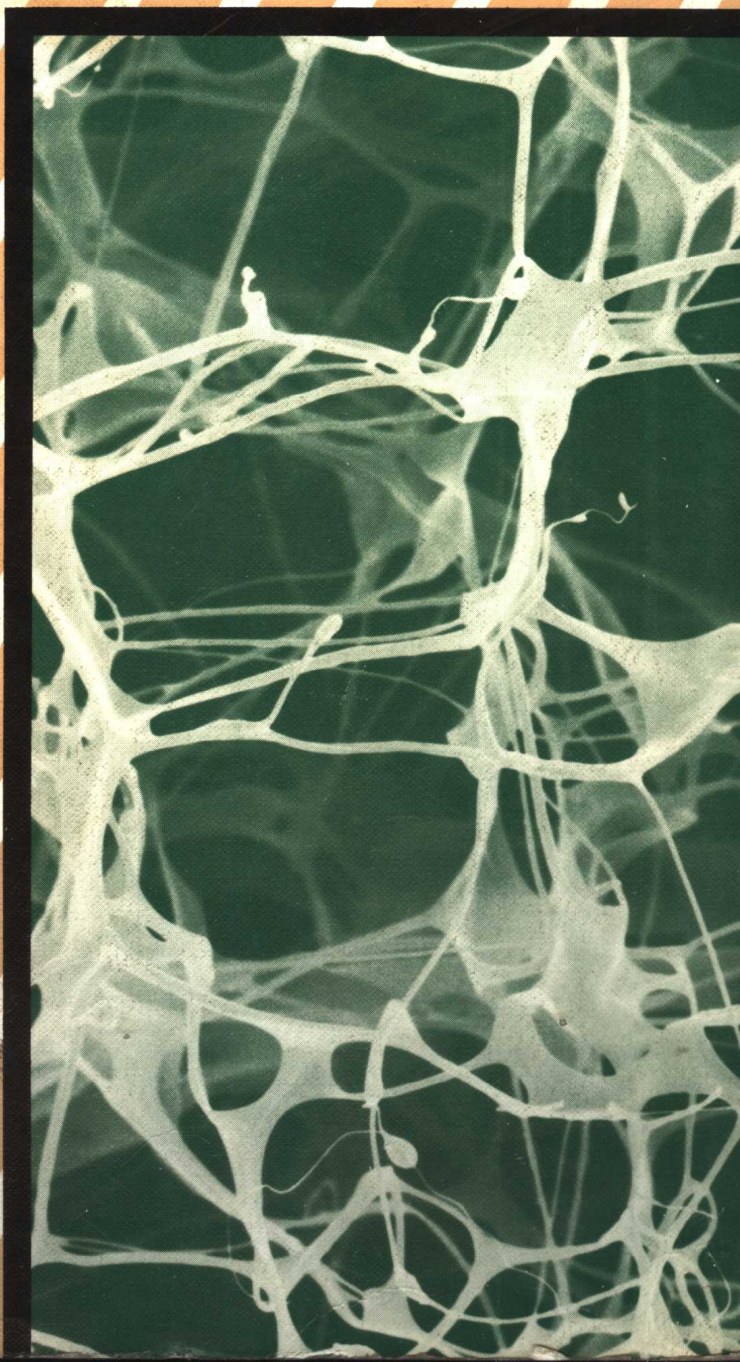

Editors
Michael L. Shuler
William A. Weigand

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BIOCHEMICAL ENGINEERING V

Edited by Michael L. Shuler and William A. Weigand

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Cover: *The cover shows the matrix structure of hybridomas (see page 137).*

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BIOCHEMICAL ENGINEERING V

Preface

This volume contains the papers presented at the Fifth Biochemical Engineering Conference held in Henniker, New Hampshire, during July 1986. The theme of the conference was Advances in Biochemical Engineering. A series of topics covering advances in both biology and engineering were covered.

The Fifth Biochemical Engineering Conference was organized by the Engineering Foundation with the financial support of the National Science Foundation and the New York Academy of Sciences. The support of these organizations is gratefully acknowledged. We are also deeply appreciative of the financial contributions made by several companies. These include: Ajinomoto Company, Amoco Corporation, Beecham Pharmaceuticals, Bio-Technical Resources, Cetus Corporation, Ciba-Geigy, H. J. Heinz Company, Kraft, Miles Laboratories, Monsanto, and Pharmacia.

The conference was organized and directed by the following: Chairman—William A. Weigand, Illinois Institute of Technology; Program Chairman—Michael Shuler, Cornell University; Executive Committee—James Bailey, California Institute of Technology; David DiBiasio, Worcester Polytechnic Institute; A. Emery, University of Birmingham; Henry C. Lim, Purdue University; Shuichi Suzuki, Saitoma Institute of Technology; K. Venkatasubramanian, H. J. Heinz Company; Wolf Vieth, Rutgers University; Christian Wandrey, Institute for Biotechnologie, Jülich; Daniel Wang, Massachusetts Institute of Technology; and Harold A. Comerer, Director, Engineering Foundation.

The session chairmen and cochairmen are as follows: R. M. Kelly, Johns Hopkins University; K. M. Ulmer, University of Maryland; D. S. Clark, Cornell University; W. M. Dean, Massachusetts Institute of Technology; H. Pederson, Rutgers University; J. Feder, Monsanto Company; A. Margaritis, University of Western Ontario; W. E. Goldstein, Miles Laboratories; D. A. Lauffenburger, University of Pennsylvania; M. Kennedy, Upjohn Company; D. DiBiasio, Worchester Polytechnic Institute; J. Fieschko, Amgen; G. Stephanopoulos, Massachusetts Institute of Technology; M. M. Domach, Carnegie-Mellon; M. Ladisch, Purdue University; and J. Hong, Illinois Institute of Technology.

In addition, Larry E. Erickson of Kansas State University and Chester Ho of SUNY Buffalo chaired a large poster session. When possible, these poster papers were placed within the sessions listed in the Table of Contents. However, in order to accommodate poster presentations that did not fit under these eight main session headings, three more new sessions were created and placed at the end of the Table of Contents. These sections are entitled as follows: Aspects of Transport Processes; Enzyme Production; and Other Aspects of Bioreactions.

W. A. Weigand
M. L. Shuler

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BIOCHEMICAL ENGINEERING V[a]

Editors and Conference Chairmen
MICHAEL L. SHULER and WILLIAM A. WEIGAND

CONTENTS

[a]The papers in this volume were presented at the Fifth Biochemical Engineering Conference, held at New England College, Henniker, New Hampshire, on July 26 to August 1, 1987. The conference was organized by the Engineering Foundation with the support of the National Science Foundation and the New York Academy of Sciences.

Part III. Bioreactors I (Tissue Cultures)

Part IV. Bioreactors II (Immobilized Biocatalysts)

Part V. Bioreactors III (Systems with Spatial Heterogeneity)

Part VI. Mixed Cultures and Genetically Unstable Populations

Part VII. Biosensors and Reactor State Estimation

Part VIII. Integration of Reaction and Recovery

Part IX. Aspects of Transport Processes

Part X. Enzyme Production

Part XI. Other Aspects of Bioreactions

Redirection of Cellular Metabolism

Analysis and Synthesis[a]

JAMES E. BAILEY, DOUGLAS D. AXE,
PAULINE M. DORAN,[b] JORGE L. GALAZZO,
KENNETH F. REARDON, ALEX SERESSIOTIS,
AND JACQUELINE V. SHANKS

Department of Chemical Engineering
California Institute of Technology
Pasadena, California 91125

INTRODUCTION

Achievement of optimal productivity and yields in bioprocesses using living cells generally requires redirection of cellular metabolic activity. Rarely is the native organism optimized with respect to process goals, thereby providing both the opportunity and the challenge of altering native metabolic function to achieve the most effective substrate utilization, cell growth, or product synthesis and release (or all of the above). The two main vehicles for metabolic manipulation—environmental control and alteration of the genetic constitution of the organism—are already evident in prior practice of bioprocessing art. However, until recently genetic manipulation was achieved primarily through random mutagenesis, and environmental manipulation was restricted to adjustment of solution composition during batch cultivation of cell suspensions.

New developments in genetic technology and in engineering systems now provide the opportunity for more substantial and more carefully controlled and characterized manipulation of cellular DNA and environment. Using contemporary cloning techniques, the metabolic structure of a cell may be modified in a precise and well-controlled fashion by adding new proteins to the cell, by inhibiting or interfering with existing enzyme activities, by altering native control of expression of protein activities, and by amplifying particular protein activities already functional in the organism (e.g., see references 1–3). Furthermore, by the use of regulated replicators and expression

[a]This work was sponsored by the Energy Conversion and Utilization Technology (ECUT) Program of the United States Department of Energy, the National Science Foundation, the Monsanto Company, and the sponsors of the Caltech Process Biocatalysis Program. J. L. Galazzo was supported by a fellowship from the National Research Council – CONICET, Argentina. These nuclear magnetic resonance experiments were made possible by the facilities and the assistance of the Southern California Regional Nuclear Magnetic Center (NSF Grant No. CHE-84-40137), and software for NMR spectral analysis was provided by the NIH Resource Laboratory at Syracuse University (Grant No. RR-01317).

[b]Current address: Department of Biotechnology, University of New South Wales, Post Office Box 1, Kensington 2033, Australia.

controls (e.g., see references 4–6), these cloned modifications of cellular metabolism may be turned up or down by adjustment of environmental conditions.

More precise and different types of environmental manipulations are made possible by advances in process control and sensors, by the advent of convenient cell retention and recycle capabilities, and by cell immobilization. In particular, cell immobilization or containment enables continuous processing without the constraint of matching process throughput with cellular growth capabilities. This thus permits decoupling of growth and product formation and also provides the opportunity for more direct control of cell environment with less influence from cellular biochemical activities.

The opportunity to adjust many aspects of cell environment as desired and to introduce virtually any new protein into the living cell over a continuum of activity levels provides important new possibilities for bioprocess innovation and improvement. Simultaneously, though, it poses new problems. The spectrum of choices available is so vast that empirical or trial-and-error procedures to search for optimal genetic and environmental conditions are almost certain to lead to a significantly suboptimal solution. In order to guide the selection of genetic and environmental conditions, the metabolic engineer requires systematic, quantitative knowledge of the structure of the metabolic reaction network and of its regulation and kinetic activities. Because such knowledge is not now available and because general methods for predicting and analyzing metabolic reaction network structure and kinetic features are not available, new research is needed to identify these systematics. Here, the capability for genetic and environmental control already mentioned plays a key role in carrying out experiments to improve understanding of the connection between different metabolic engineering strategies and corresponding consequences.

In this regard, it is important that the results of metabolic manipulation be characterized experimentally as completely and in as much detail as possible. Therefore, it is also significant that recently developed experimental techniques [including fluorescence measurements[7,8] and *in vivo* nuclear magnetic resonance (NMR) spectroscopy[9–12]] enable noninvasive, multicomponent, transient measurements of intracellular concentrations of direct physiological and biochemical interest. Used in concert with modern quantitative treatment of metabolic reaction networks, metabolic pathway kinetic models, and cellular growth models, these measurements provide a basis for necessary fundamental understanding that relates cause and effect in metabolic engineering.

The examples provided below illustrate the significant influence of immobilization on the metabolic function of yeast, explore the consequences of genetic manipulation by mutation and by the introduction of recombinant plasmids, and examine the analysis and synthesis of metabolic pathways by considering the acetone-butanol fermentation and a new computer system for identifying metabolic reaction sequences. In these examples, the role of detailed measurements of intracellular conditions and of mathematical models and structures will be illustrated as well.

ENVIRONMENTAL EFFECTS: KINETICS OF IMMOBILIZED *SACCHAROMYCES CEREVISIAE*

Most previous publications addressing the question of immobilization effects on cell kinetics have focused on reduction in overall rates of biotransformation processes

or cell growth due to either substrate or product concentration gradients (or both) arising from mass transfer limitations.[13–15] However, there is substantial evidence that immobilization (which results in cell-surface, cell-fiber, or cell-cell interactions not encountered in conventional, relatively dilute suspension cultures) can cause qualitative changes in cellular function and composition.[16–19] This type of immobilization effect is the central concern here because it is the extent to which immobilization can effect major qualitative changes in cellular function that gives cell immobilization significant potential as a means for manipulating metabolism. This summary will focus on experiments that were specifically designed to explore and to demonstrate the extent to which cell function is altered by immobilization.

Specific Rates of Suspended and Immobilized Cell Bioconversion

In these experiments, *Saccharomyces cerevisiae* (ATCC 18790) was immobilized by adsorption onto 4-mm glass beads that were coated with a thin layer of glutaralde-

TABLE 1. Comparison of Initial Specific Rates in Anaerobic Batch Ethanol Fermentation by Suspended and Immobilized *S. cerevisiae*[19]

	Suspended Cells	Immobilized Cells
Suspended cell specific growth rate (h^{-1})	0.51	0.50
Immobilized cell specific growth rate (h^{-1})	—	0.28
Initial specific glucose consumption rate (g/h cell $\times 10^{11}$)	5.0	10.5
Initial specific ethanol production rate (g/h cell $\times 10^{11}$)	2.3	3.3
Initial specific glycerol production rate (g/h cell $\times 10^{12}$)	2.7	3.5

hyde–cross-linked gelatin. The immobilized cells were studied in an anaerobic recirculation reactor system that employed a medium reservoir at controlled pH (4.5) and temperature (30 °C). The medium was circulated at high flow rates (less than 1% conversion per pass) through a packed bed reactor filled with immobilized cell beads.[19] In immobilized cell experiments, cells were inoculated onto the beads, fresh medium was added, and ethanol production was monitored until exponential increase in ethanol concentration was observed. With such a well-defined and reproducible physiological state of the cells experimentally established, the column was washed, fresh medium was added again, and samples were taken for analysis of glucose, ethanol, glycerol, and cells in suspension. Beads were removed from the reactor intermittently, and immobilized cells were removed and counted in order to directly monitor growth of the immobilized cells and to allow kinetic measurements to be evaluated on a specific (per cell) basis. Suspended cell experiments conducted for comparison involved a similar sequence of sequential cultivations and assays.[19]

TABLE 1 summarizes the resulting initial rate data and shows that immobilized

cells consume glucose at about twice the specific rate as suspended cells and produce ethanol at a specific rate around 1.5 times the suspended cell rate. Specific growth rates of suspended and immobilized cells differ by roughly a factor of two. Additional information on differences in cell-cycle regulation and macromolecular composition in suspended and immobilized cells studied by this approach is available in reference 19.

Modeling and Measuring NADH Dynamics in Suspended and Immobilized Yeast

More detailed experimental analyses have been conducted and mathematical models have been constructed and analyzed in order to probe even further into the differences between immobilized and suspended cell metabolism. Oxidation and reduction of the NADH-NAD cofactor system is a critical feature at several points in the metabolic reaction pathway from glucose to ethanol in yeast. Because of feedback interactions and nonlinear features of the fermentation reaction sequence, intracellular NADH levels measured by fluorescence in suspended cells have revealed interesting dynamic features.[20–22] For these reasons, fluorescence measurements of NADH level changes were conducted within yeast immobilized on a gelatin surface.[23] The immobilized cell surface was prepared on a glass slide, and the gelatin film supporting the immobilized yeast was subsequently removed and inserted into a quartz cuvette for

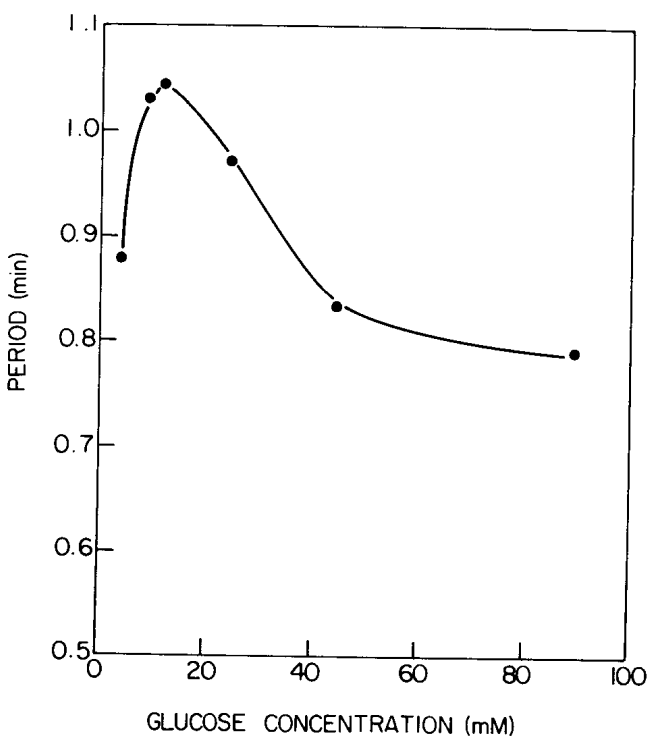

FIGURE 1. Relationship between glucose concentration and period of the NADH oscillation for suspended *S. cerevisiae*.[23]

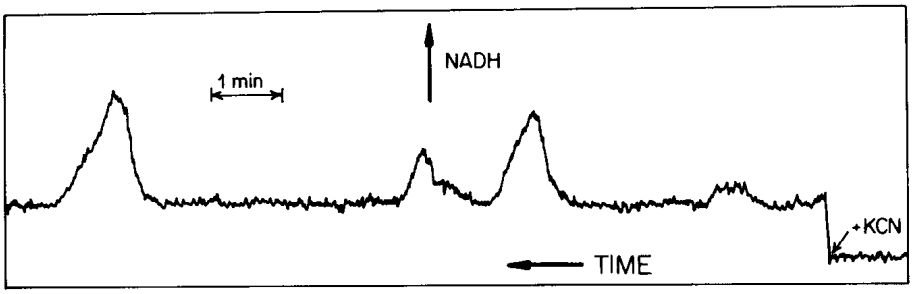

FIGURE 2. Nonlinear transient fluctuations in intracellular NADH fluorescence for immobilized cells at 25 °C after glucose addition to 11.2 mM. KCN is added to block respiration.[23]

monitoring using a fluorescence spectrophotometer. Excitation radiation was supplied at 340 nm and emitted fluorescence was monitored at 460 nm to selectively observe reduced pyridine nucleotides (as was done in numerous previous studies of suspended yeast).

In these experiments, a sample of suspended or immobilized cells was starved in buffer. Subsequently, glucose was added, and five minutes later, KCN was added to eliminate respiration. NADH transients observed after KCN addition to suspended *S. cerevisiae* were roughly sinusoidal in character, with periods that depended upon glucose concentration as indicated in FIGURE 1.

On the other hand, after applying the same protocol of starvation, glucose addition, and introduction of KCN, immobilized cells exhibited complicated and much more nonlinear oscillations.[23] One example of dynamic trajectories observed with immobilized cells following addition of KCN is shown in FIGURE 2. In addition to the difference in the character of NADH transients following glucose and KCN addition to suspended and immobilized cells, a major qualitative difference was observed for *S. cerevisiae* cells that were grown in these two configurations. In the absence of glucose, no transient-reduced pyridine nucleotide fluctuations whatsoever were observed for suspended cells. However, for immobilized cells, highly nonlinear stair-step fluctuations (which appear to be a relaxation oscillation superimposed on a mean drift of the system) are evident.

The qualitative differences observed between suspended and immobilized yeast indicate major distinctions between these two types of cells with respect to carbon catabolism and its regulation. In an attempt to gain some understanding of the basis for these different types of transient behaviors, a lumped kinetic model for the fermentation pathway was formulated.[24] In this model, steps in the fermentation pathway that are known to operate far from equilibrium or that are subject to allosteric regulation (or both) were explicitly included (TABLE 2). The rate of glucose uptake was an adjustable model parameter, and the rates of ATP hydrolysis via step 5 and of step 6 in TABLE 2 were assumed to be described by mass action kinetics. The nonequilibrium steps (nos. 2, 3, and 4 in TABLE 2) were analyzed using detailed kinetic expressions taken from the literature (V_{PFK}: reference 25; V_I: references 26 and 27; V_{PK}: reference 25). The model is written for the fluorescence measurement experimental conditions applied above in which negligible cell growth occurs. The state variables of the model

TABLE 2. Lumped Metabolic Reactions Used in the Dynamic Model for Fermentation by *S. cerevisiae*[24]

Step 1: Glucose + ATP $\xrightarrow{V_{in}}$ F6P + ADP
Step 2: F6P + ATP $\xrightarrow{V_{PFK}}$ FdP + ADP
Step 3: FdP + 2ADP + 2NAD$^+$ $\xrightarrow{V_1}$ 2PEP + 2ATP + 2NADH
Step 4: PEP + ADP + NADH $\xrightarrow{V_{PK}}$ EtOH + ATP + NAD$^+$
Step 5: ATP $\xrightarrow{V_2}$ ADP
Step 6: ATP + AMP $\underset{V_4}{\overset{V_3}{\rightleftharpoons}}$ 2ADP

are the intracellular levels of fructose 6-phosphate (F6P), fructose diphosphate (FdP), phosphoenolpyruvate (PEP), NADH, ATP, and ADP. Differential equations comprising the unsteady-state material balances on these concentrations can readily be written for a constant volume system with entry of glucose according to step 1 and exit of ethanol according to step 4.

The resulting model is highly nonlinear and requires numerical solution. In order to identify conditions under which the model will generate oscillations, methods of nonlinear bifurcation analysis were applied.[28] In this analysis, the steady state of the system is first evaluated for a given set of model parameters. Subsequently, the corresponding linearized dynamic model is evaluated by calculating the Jacobian of the original nonlinear differential equations and evaluating all partial derivatives so obtained at the steady-state conditions. Eigenvalues of this Jacobian matrix characterize the local dynamic behavior of the linear system. When model parameters are changed such that eigenvalues with the largest real parts progress from negative real part complex numbers to positive real part complex numbers, a bifurcation occurs. Accompanying the bifurcation is the loss of stability of the steady state and the appearance of new steady states or new dynamic structures such as a limit cycle. The eigenvalue configuration that crosses the imaginary axis of the complex plane (as bifurcation occurs) determines the possible types of local nonlinear dynamic behavior that result.[28]

FIGURE 3 shows stability diagrams in parameter space evaluated using this procedure.[24] The two different figures correspond to different values of the ATP consumption rate constant, k_2 (step 5). The abscissa in both diagrams is the maximum velocity of the pyruvate kinase reaction scaled by the maximum velocity of the PFK reaction, and the ordinate in both is the rate of glucose uptake similarly scaled. The indicated domains show areas of parameter values in which no steady-state solution exists, in which the steady-state solution is unstable, and in which the steady state is locally stable (all Jacobian eigenvalues have negative real parts). The boundaries separating stable from unstable regions denote parameter values for which bifurcation occurs. Through this type of analysis, it is possible to get some indications of the parameter combinations that correspond to interesting transient behavior. It is significant to note that this analysis involves only algebraic equations and that numerical integration of the full set of nonlinear equations is not necessary. However,