

1N-44  
146446

JPL Publication 88-8

DOE/CS-66001-11  
Distribution Categories  
UC-61e & UC-61f

368,

# ECUT

ENERGY CONVERSION  
AND UTILIZATION  
TECHNOLOGIES PROGRAM

---

## Biocatalysis Project Annual Report FY 1987

---

(NASA-CR-182917) ECUT (ENERGY CONVERSION AND UTILIZATION TECHNOLOGIES) PROGRAM: BIOCATALYSIS PROJECT Annual Report, FY 1987 (Jet Propulsion Lab.) 36 p CSCL 10A N88-24110 : Unclas G3/44 0146446

March 30, 1988

Sponsored by

Energy Conversion and Utilization Technologies Division  
Office of Energy Systems Research  
U.S. Department of Energy

Through an agreement with

National Aeronautics and  
Space Administration

by

Jet Propulsion Laboratory  
California Institute of Technology  
Pasadena, California

TECHNICAL REPORT STANDARD TITLE PAGE

1. Report No. JPL PUB 88-8	2. Government Accession No.	3. Recipient's Catalog No.	
4. Title and Subtitle ECUT -- Energy Conversion and Utilization Technologies Program Biocatalysis Project Annual Report Fiscal Year 1987		5. Report Date March 30, 1988	
		6. Performing Organization Code	
7. Author(s)		8. Performing Organization Report No. JPL PUB 88-8	
9. Performing Organization Name and Address JET PROPULSION LABORATORY California Institute of Technology 4800 Oak Grove Drive Pasadena, California 91109		10. Work Unit No.	
		11. Contract or Grant No. NAS7-918	
		13. Type of Report and Period Covered JPL Publication	
12. Sponsoring Agency Name and Address NATIONAL AERONAUTICS AND SPACE ADMINISTRATION Washington, D.C. 20546		14. Sponsoring Agency Code	
15. Supplementary Notes			
<p>16. Abstract</p> <p>The Annual Report presents the fiscal year (FY) 1987 research activities and accomplishments for the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Division. The ECUT Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology.</p> <p>The Biocatalysis Project's technical activities were organized into three work elements:</p> <p>The <u>Molecular Modeling and Applied Genetics</u> work element includes modeling and simulation studies to verify a dynamic model of the enzyme carboxypeptidase; plasmid stabilization by chromosomal integration; growth and stability characteristics of plasmid-containing cells; and determination of optional production parameters for hyper-production of polyphenol oxidase.</p> <p>The <u>Bioprocess Engineering</u> work element supports efforts in novel bioreactor concepts that are likely to lead to substantially higher levels of reactor productivity, product yields, and lower separation energetics.</p> <p>The <u>Bioprocess Design and Assessment</u> work element attempts to develop procedures (via user-friendly computer software) for assessing the economics and energetics of a given biocatalyst process.</p>			
17. Key Words (Selected by Author(s)) Bioengineering Conversion Techniques Materials (General) Chemical Engineering		18. Distribution Statement Unlimited/Unclassified	
19. Security Classif. (of this report) Unclassified	20. Security Classif. (of this page) Unclassified	21. No. of Pages	22. Price

# ECUT

## ENERGY CONVERSION AND UTILIZATION TECHNOLOGIES PROGRAM

---

### Biocatalysis Project Annual Report FY 1987

---

March 30, 1988

Sponsored by

Energy Conversion and Utilization Technologies Division  
Office of Energy Systems Research  
U.S. Department of Energy

Through an agreement with

National Aeronautics and  
Space Administration

by

Jet Propulsion Laboratory  
California Institute of Technology  
Pasadena, California

The Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology, for the United States Department of Energy through an agreement with the National Aeronautics and Space Administration (NASA Task RE-152, Amendment 307; DOE Interagency Agreement DE-AIO1-81CS66001).

The Biocatalysis Project focuses on resolving the major technical barriers that impede the potential use of biologically-facilitated continuous chemical production processes.

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights.

Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

---

## ABSTRACT

The Annual Report presents the fiscal year (FY) 1987 research activities and accomplishments for the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Division. The ECUT Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology.

The Biocatalysis Project's technical activities were organized into three work elements:

The Molecular Modeling and Applied Genetics work element includes modeling and simulation studies to verify a dynamic model of the enzyme carboxypeptidase; plasmid stabilization by chromosomal integration; growth and stability characteristics of plasmid-containing cells; and determination of optional production parameters for hyper-production of polyphenol oxidase.

The Bioprocess Engineering work element supports efforts in novel bioreactor concepts that are likely to lead to substantially higher levels of reactor productivity, product yields, and lower separation energetics.

The Bioprocess Design and Assessment work element attempts to develop procedures (via user-friendly computer software) for assessing the economics and energetics of a given biocatalyst process.

## FOREWORD

For further information about the ECUT Biocatalysis Project, contact:

Dr. James J. Eberhardt, Director  
Energy Conversion and Utilization  
Technologies (ECUT), CE-12  
Forrestal Bldg. 5E-066  
U.S. Department of Energy  
1000 Independence Avenue, S.W.  
Washington, D.C. 20585  
(202) 586-5377 (FTS) 896-5377

Dr. Minoo N. Dastoor, Manager  
ECUT Biocatalysis Project  
M/S 125-112  
Jet Propulsion Laboratory  
California Institute of Technology  
4800 Oak Grove Drive  
Pasadena, California 91109  
(818) 354-7429 (FTS) 792-7429

## CONTENTS

I.	EXECUTIVE SUMMARY . . . . .	1-1
II.	PROJECT DESCRIPTION . . . . .	2-1
	A. THE ECUT MISSION . . . . .	2-1
	B. HISTORICAL BACKGROUND . . . . .	2-1
	C. RELEVANCE . . . . .	2-4
	D. GOAL AND OBJECTIVE . . . . .	2-5
	E. PROJECT STRUCTURE AND ORGANIZATION . . . . .	2-5
	F. MANAGEMENT SUPPORT FUNCTION . . . . .	2-6
	G. TASK DESCRIPTIONS . . . . .	2-7
III.	FY87 TASK ACCOMPLISHMENTS . . . . .	3-1
	A. MOLECULAR MODELING AND APPLIED GENETICS . . . . .	3-1
	B. BIOPROCESS ENGINEERING . . . . .	3-3
	C. PROCESS DESIGN AND ANALYSIS . . . . .	3-9
IV.	BIBLIOGRAPHY . . . . .	4-1

## FIGURES

2-1.	ECUT Role in Energy Conservation Research and Development . .	2-2
2-2.	ECUT Program: Projects and Lead Center . . . . .	2-3

## SECTION I

### EXECUTIVE SUMMARY

The Biocatalysis Project sponsored by the Energy Conversion and Utilization Technologies (ECUT) Division of the U.S. Department of Energy (DOE) is an applied research effort focused on providing the enabling technology base for new bioprocess applications which have substantial energy implications. The primary mission of DOE-ECUT is to provide, for the nation, a bridge between basic research and industrial application for several technologies (e.g., high temperature materials, tribiology, combustion and biocatalysis) which have the potential for making positive energy contributions. Furthermore, the ECUT mission hopes to strengthen the future U.S. competitive position by conducting research directed towards exploiting appropriate advances in basic research in which the U.S. has demonstrated leadership.

As the national lead center, the Jet Propulsion Laboratory of the California Institute of Technology serves as the technical manager for the Biocatalysis Project. The primary objective of the Biocatalysis Project is to resolve the critical technical constraints (e.g., poor productivity, high separation energetics, presence of aqueous medium, lack of design tools) that impede the utilization of biocatalysis for the production of chemicals and materials. To achieve this objective, the Biocatalysis Project supports generic applied research, as well as development of predictive theoretical models (that are experimentally verifiable) which address the technological barriers to the commercial utilization of biochemical catalysis.

The ECUT Biocatalysis Project consists of three major work elements, each addressing a key technical component of the required enabling technology base. The selection, as well as the relationship between each of these three work elements, has been defined by their scale of action. The Molecular Modeling and Applied Genetics work element focuses on defining optimal microscale parameters for biocatalysis and pursuing practical applications of basic molecular biology research findings. Computer graphic models have been advanced which predict the dynamic behavior and intramolecular conformational changes of enzymes (biocatalysts), including effects of temperature and biocatalytic inhibitions. Advances in Applied Genetics have focused on the issue of biological information (plasmid) stability. Novel high-speed measurement methods employing flow cytometry have been developed, which now allow researchers to assay the fraction of plasmid-containing cells in the recombinant population in approximately three hours, more than one order of magnitude faster than present day plating methods. Results of such measurements have been used to develop a new theory of plasmid stability which recognizes the limited capacity of plasmid-free cells to grow in selective medium. The Bioprocess Engineering work element emphasizes defining the basic engineering relationships between cellular scale events and macro-level parameters. Laboratory tests using an advanced fluidized bed bioreactor possessing high cell densities ( $5-15 \times 10^{10}$  cells/ml) have demonstrated exceptionally high bioreactor productivities: 50 to 100 g/l/h (for ethanol) as compared to 5 to 10 g/l/h for current conventional processes. Similar



advances have been noted using a novel multi-membrane bioreactor design in which the chronic and ubiquitous problem of product feedback inhibition has been significantly relieved. Substantial progress, in rates and extent of synthesis, has also been reported in the technology of enzyme reactions in organic solvents (as opposed to conventional aqueous medium) for promoting appropriate chemical transformations (e.g., regiospecific oxidation of aromatics). The Process Design and Analysis work element focuses on developing user-friendly computer programs which assess the energetics and economics of biocatalyst chemical production processes. Modular computer programs have been developed which provide capabilities for rapid assessment of energy expenditures and costs for unit operations within biocatalyst processes.

## SECTION II

### PROJECT DESCRIPTION

#### A. THE ECUT MISSION

The Energy Conversion and Utilization Technologies (ECUT) Program seeks to:

- (1) Monitor advances in basic scientific research and evaluate emerging technologies for applicability to energy conservation,
- (2) Conduct exploratory development and establish feasibility of innovative or revolutionary conservation concepts,
- (3) Effect technology transfer to DOE end-use conservation programs and/or to private industry, and
- (4) Identify energy conservation research needs and feed this information back to basic research.

In fulfilling these aims, ECUT acts as a transmission in transferring the power of basic research to the wheels of industry (Figure 2-1). There are many ways to achieve these aims and since its establishment ECUT has chosen to support long-term, high-risk generic research and exploratory development for generation of more energy-efficient materials and processes. The Biocatalysis Project is one of five projects that make up the ECUT Program (Figure 2-2).

#### B. HISTORICAL BACKGROUND

The Biocatalysis Project was established in 1980 as the Chemical Processes Project with the Jet Propulsion Laboratory as the lead center. At that time the project had two work elements: Catalysis (chemical catalysis and biocatalysis), and Separation. Within these work elements, several small contracts were completed that evaluated the energy efficiencies and potential applications of relatively new separation processes, such as supercritical extraction, membrane separation, and chromatography. Research was also started at the California Institute of Technology (Caltech) on chemical catalytic behavior models and kinetics of expression in recombinant-DNA microorganisms.

In 1982, the ECUT Program was reorganized and the Chemical Processes Project was renamed as the Biocatalysis Project. As the name suggests, the project emphasis was also changed, now stressing biocatalysis as the primary research focus. The work elements were modified to include: Molecular Modeling and Applied Genetics, Bioprocess Engineering, and Process Design and Analysis. Each of these elements addresses a key technical component necessary for the development of more advanced and efficient bioprocesses. These three work elements detail a systematic progression of understanding starting with the basic understanding of biocatalytic mechanisms at the molecular level, to micro-level effects on process parameters in reactors

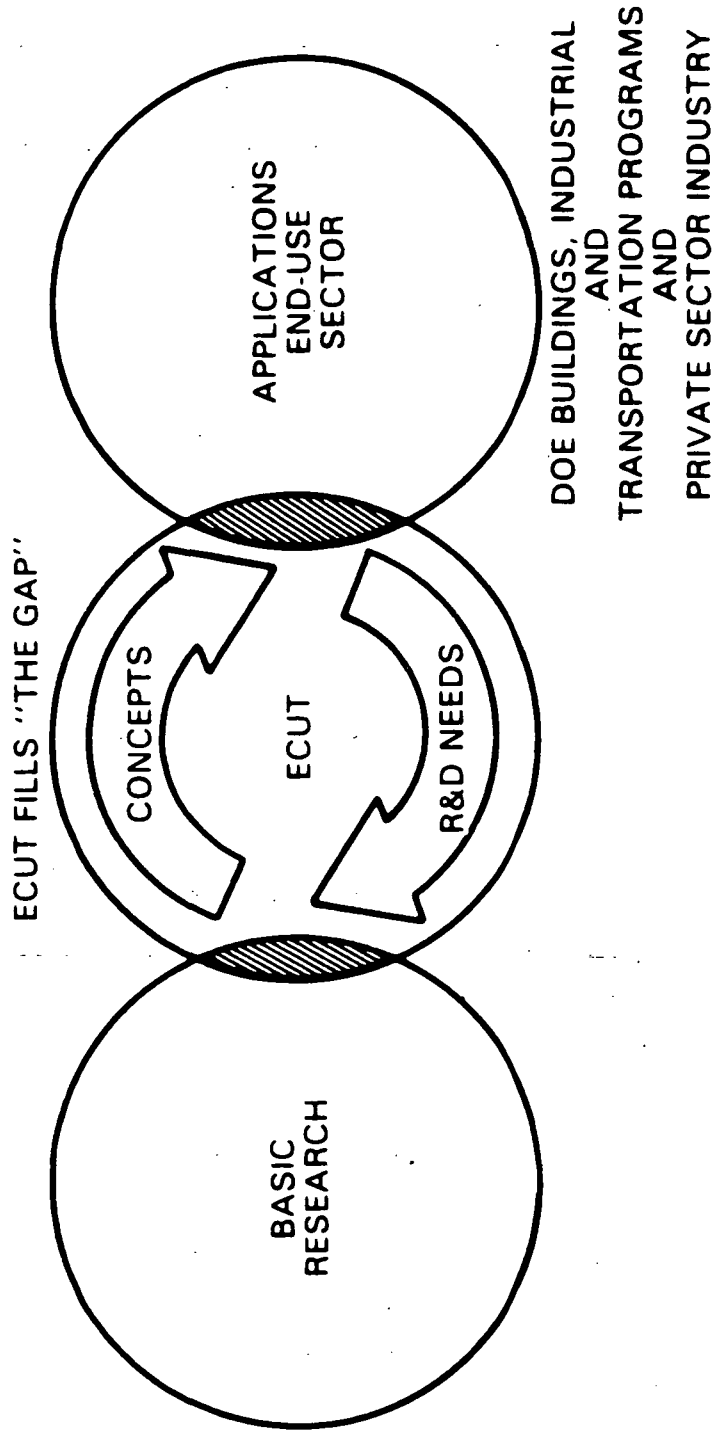


Figure 2-1. ECUT Role in Energy Conservation Research and Development

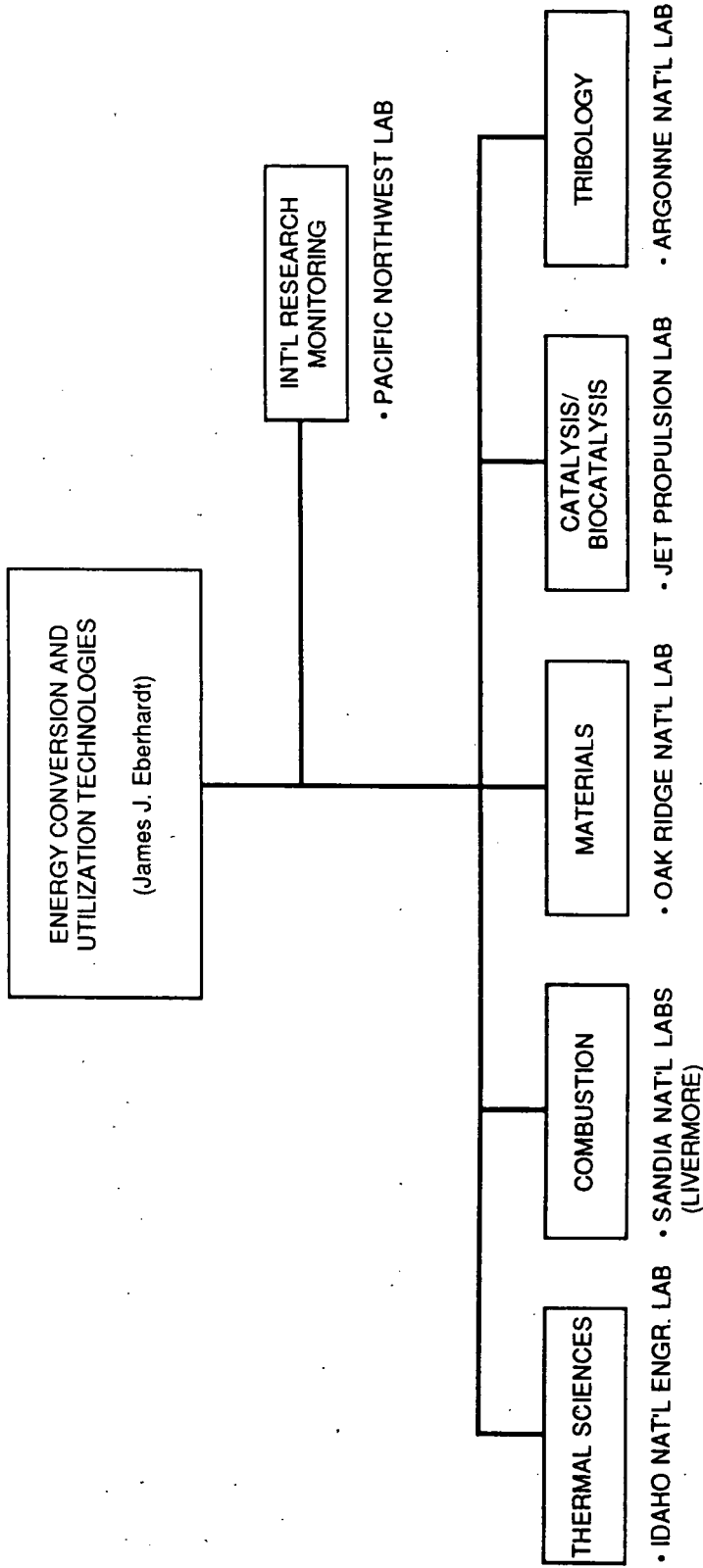


Figure 2-2: ECUT Program: Projects and Lead Center

(including effects of integrated separation processes), and finally to the development and assessment of new process concepts for technology transfer to the industrial sector. With this information the production of large-volume, low-cost, energy-intensive chemicals from renewable resources may be a practical alternative in the future.

The selection as well as the relationships between each of the work elements has been defined by their scale of action. Research activities are conducted at various scales of action or specific size dimensions. Hence, Molecular Modeling and Applied Genetics, includes research activities at a molecular and cellular level, i.e., a scale of 1  $\mu\text{m}$  and smaller. To successfully exploit the findings at the molecular and cellular level towards scale-up, Bioprocess Engineering includes research in the area of engineering kinetics and control, as well as novel concepts in reactor design. The scale in this work element is generally at the 1-meter dimension or at the reactor level. Finally, Process Design and Analysis focuses on activities that operate at the entire process level (0.4 hectares or more). A central tenet subscribed to by the ECUT Biocatalysis Project is that in the acquisition of an appropriate enabling technology, it is mandatory to integrate crucial research findings at the various levels of activity and transfer the findings to the private sector.

### C. RELEVANCE

Three main conclusions can be stated regarding the competitive status and future potential of the United States petrochemical industry: (1) Many United States industries are losing their competitive edge as the global economy is expanding; the U.S. petrochemical industry is an example of just such an industry. (2) The United States must take advantage of its competitive strengths if it is to compete effectively in the future. This includes the application of its world-renowned capabilities in basic science. Biotechnology is an example of scientific research whose application has tremendous potential for commercial application. (3) Although biotechnology is being applied towards the production of specialty chemicals by the chemical industry, its application to the far larger commodity chemical market is considered too long term and risky to attract research and development dollars. This is sensible from the perspective of each individual firm, but it is unclear whether the specialty chemical markets can sustain all of the competitors who are restructuring their operations towards specialty chemical production. A key government role in the future competitiveness of the industry may be the support of generic research which can be applied to the development of economical commercial processes.

The application of biocatalytic processes to the production of large-scale chemicals would capitalize on the three major areas in which the United States currently exerts leadership and whose successful exploitation would contribute to its global competitive position in the future. First, the United States is considered a world leader in basic research advances; our lead in molecular biology and biochemistry is unquestioned. Commercial utilization of that lead could increase the competitive advantage of our domestic chemical industries. Second, we can exert our leadership further by

using the tools of macromolecular modeling and energy optimization to design entirely novel biocatalysts and to test such catalysts by design concepts through computer simulation. Such a design strategy would be useless without a means for inexpensive synthesis; however, the newly developed techniques of site-directed mutagenesis provide the methodology to synthesize and test experimentally these design principles. The use of this design strategy would enhance our productivity in the future by significantly and intelligently reducing the time spent on experimental and empirical procedures in the laboratory. Third, the United States is one of the most efficient and productive producers of agricultural products in the world. Because biomass is often the feedstock for the biocatalyst production of organic chemicals, we have a clear competitive advantage in biomass production within our domestic agricultural sector.

#### D. GOAL AND OBJECTIVE

- (1) Goal. The goal of the ECUT/Biocatalysis Project is to exploit the United States' competitive advantage in biotechnology by facilitating the production of chemicals efficiently via biocatalytic processes. The Biocatalysis Project supports high-risk applied generic research and development aimed at advancing an enabling technology base that will allow the rational development and scale-up of large biocatalyst chemical production processes. The Biocatalysis Project does this by conducting exploratory development on, and by establishing the feasibility of, novel, innovative, or revolutionary basic research advances in biotechnology.
- (2) Objective. To meet this goal, the Biocatalysis Project has focused on developing predictive models and supporting novel bioprocessing concepts which can be utilized by commercial producers for large-scale chemical production. The strategy of the Biocatalysis Project is to reduce production costs by increasing product yields, by increasing reactor productivity, and by decreasing energy requirements for production. These problems are generic in a very large number of biocatalyst processes.

#### E. PROJECT STRUCTURE AND ORGANIZATION

The activities of the Biocatalysis Project have been incorporated as three major work elements and a Management Support function. The three major work elements and their supporting tasks are:

- (1) The Molecular Modeling and Applied Genetics work element focuses on defining optimal microscale parameters for biocatalysts and developing practical applications of basic molecular biology research findings. The primary roles of this work element are to provide a database for defining kinetic models of biocatalyst reactivity, and to develop genetically engineered solutions to the generic technical barriers that preclude widespread applications of biocatalysis.

- (a) Biocatalysis by Design.
  - (b) Chromosomal Amplification of Foreign DNA.
  - (c) Kinetics of Recombinant Cells.
  - (d) Hyperproduction and Secretion of Polyphenol Oxidase.
- (2) The Bioprocess Engineering work element emphasizes defining the basic engineering relationships between molecular scale events and macro-level parameters required for designing scaled-up biocatalyst chemical production processes. Additionally, this work element has a responsibility for establishing the technical feasibility of critical bioprocess monitoring and control subsystems.
- (a) Metabolic Engineering.
  - (b) Immobilized Cell System for Continuous Efficient Biocatalyst Processes.
  - (c) Enzyme Catalysis in Nonaqueous Solutions.
  - (d) Applications of Molecular Hydrogen in Chemical Fermentations.
  - (e) Multimembrane Bioreactor for Chemical Production.
  - (f) Multiphase Fluidized Bed Bioreactor.
  - (g) Biocatalyst Hydroxylation in Organic Solvents.
  - (h) Separation by Reversible Chemical Association.
  - (i) Bioseparation of Phosphate.
  - (j) Protein Engineering for Nonaqueous Solvents.
- (3) The Process Design and Analysis work element provides overall assessments, via energetic and economic analysis of biocatalyst chemical production processes, and initiation of technology transfer for advanced bioprocesses.
- (a) Bioprocess Synthesis, Integration, and Analysis.
  - (b) Assessment: Biotechnology and Chemical Production.

#### F. MANAGEMENT SUPPORT FUNCTION

The Management Support function has three areas of responsibility: Task Management and Planning; The Guidance and Evaluation Panel; and Industry Technology Transfer.

- (1) The Task Management and Planning area administers and coordinates the task elements of the Biocatalysis Project. These responsibilities include: developing statements of work and evaluation criteria; defining the sequence of accomplishments that will achieve the outlined objectives; monitoring and evaluating in-house and contract research progress; preparing and implementing the Annual Operating Plan (AOP); technical multi-year plans and budgets; assigning personnel; identifying new areas of research and issues to be addressed; and timely publishing of research results and developments and their dissemination to DOE headquarters, industry, and other researchers.
- (2) The Guidance and Evaluation Panel includes industrial and academic representatives who are leading authorities in the science and technology of biocatalysis. The panel is responsible for reviewing ongoing activities and future project plans, as well as evaluating the Biocatalysis Project's performance.
- (3) The Industry Technology Transfer area's task is to ensure strong, interactive relationships between the Biocatalysis Project and industry that will assist the transfer of the enabling technology required by the private sector for the production of chemicals via biocatalysis.

#### G. TASK DESCRIPTIONS

##### (1) Molecular Modeling and Applied Genetics

The Molecular Modeling and Applied Genetics work element is comprised of four research tasks: Biocatalysis by Design, Chromosomal Amplification of Foreign DNA, Kinetics of Recombinant Cells, and Hyperproduction and Secretion of Polyphenol Oxidase. The focus is on defining optimal microscale parameters for biocatalysts and developing practical applications of basic molecular biology research findings.

- (a) Biocatalysis by Design (W.A. Goddard, III, Caltech). A critical problem in the design of new biological systems (biopolymers; biocatalyst) is the prediction of the structure and properties of the new system. Thus, given a specific primary sequence of amino acids, prediction of the secondary and tertiary structures (folding) of the functional protein complex is desirable. The problem is that the conformation is determined by weak forces (van der Waals, hydrogen bonding, electrostatic), with the result that an enormous number of conformations give similar energies. A further complication is that the time scale of a system refolding from one conformation to another is slow (maybe seconds). Thus, in a molecular dynamics simulation, the system may oscillate about one conformational minimum for  $10^9$  to  $10^{15}$  time steps before it begins to unfold and refold to a better conformation. As a result, it is not



yet possible to predict the conformation (tertiary structure) of even small proteins (say, 50 amino acids or 500 atoms). However, to design new biochemical systems, it is essential that we be able to predict the structure so that the concomitant properties can be properly predicted.

Because of difficulties in predicting structure, the focus of this task was on systems where the conformation was known. The various amino acids were then modified and the molecular dynamics techniques of annealing and quenching were used to equilibrate the system. However, in designing new biosystems, it is critical to develop the ability to predict the globally minimum conformations so that more drastic changes can be utilized in designing new systems.

Three overall approaches are being considered, all of which combine random fluctuations (to allow sampling of new regions of conformational space) with molecular dynamics (allowing the forces to guide optimization of local structure): unconstrained optimization using Monte Carlo simulated annealing and simulated growth techniques combined with molecular dynamics; tube-constrained conformation searches where the centerline of the electron density from low-resolution X-ray studies is used as a conformation constraint for molecular dynamics; and pairwise-constrained conformation searches where pairwise distances from two-dimensional NMR are used.

- (b) Chromosomal Amplification of Foreign DNA (G. Bertani, JPL). Chromosomal amplification (and productivity of recombinant microorganisms, discussed below) represent new advances in the area of biological information stability. Information is a dominant scientific issue in the case of genetically engineered organisms. Since the "new" information is exogenously added to the biological system (microorganism), it is a perturbation from the natural state and, hence, the stability characteristics of the engineered information with respect to time are central for success. The objective of this research is to determine the possibility of solving the plasmid stability problem by inserting the recombinant DNA plasmid directly into the bacterial chromosome. Because the plasmid carrying the desired genetic trait would then be part of the chromosome, it could not be easily lost as the bacterium multiplies.
  
- (c) Kinetics of Recombinant Cells (J.E. Bailey, Caltech). This research employs experimental methods in concert with mathematical models at the molecular and population level for increasing the productivity of bioreactors using genetically engineered recombinant microorganisms. There are two main research areas that are necessary for successful development of this method: kinetics of DNA replication, which determines the rates of growth and

stability of desired recombinant cells; and control and monitoring of plasmids, which are the DNA elements that contain the genetic instructions needed to make desired products more efficiently and selectively.

Quantitative descriptions (kinetic expressions) of the growth and stability (plasmid retention) of recombinant cells are needed to optimize the combination of natural cellular products and components introduced to maximize the efficiency of the overall system. Although cells can be genetically engineered to increase efficiency, specificity, and product yield, the presence of recombinant DNA can have deleterious effects on the activity of cells with the desired characteristics.

- (d) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta). The objectives of this research are to: purify intracellular and extracellular polyphenol oxidases from Coriolus versicolor maintained in liquid culture; and determine if intracellular polyphenol oxidase is either synthesized de novo or activated. The approach is to: establish the time courses for the appearance of intracellular and extracellular polyphenol oxidase and subsequently quantify oxidase specific activity; and determine optimum growth conditions of C. versicolor for maximum production of polyphenol oxidase, including determination of cofactor requirements.

(2) Bioprocess Engineering

This work element consists of ten tasks: Metabolic Engineering, Immobilized Cell System for Continuous Efficient Biocatalyst Processes, Enzyme Catalysis in Nonaqueous Solutions, Applications of Molecular Hydrogen in Chemical Fermentations, Multimembrane Bioreactor for Chemical Production, Multiphase Fluidized Bed Bioreactor, Biocatalyst Hydroxylation in Organic Solvents, Separation by Reversible Chemical Association, Bioseparation of Phosphate, and Protein Engineering for Nonaqueous Solvents. They are primarily concerned with the definition of basic engineering relationships between molecular and microscale events and macro-level parameters required to design and scale-up bioprocesses.

- (a) Metabolic Engineering (J.E. Bailey, Caltech). The objective is to formulate different types of mathematical structures for analysis and synthesis of directed metabolic changes accomplished by recombinant DNA genetic engineering methods. One approach is the definition of a highly structured, single-cell model of E. coli which includes many of the mechanistic details of nutrient assimilation, cell growth, and protein synthesis.

- (b) Immobilized Cell System for Continuous Efficient Biocatalyst Processing (C.E. Scott, ORNL). The task objectives are to enhance productivity and operability of a fluidized bed reactor system containing immobilized microorganisms, and to investigate bioreactor dynamics, including the formulation and investigation of kinetic properties of biocatalyst particles. This will lead to a better understanding of reactor behavior and control predictability. The reactor consists of a column containing immobilized cells; the substrate (glucose) and nutrients are passed into the bottom of the column and the product ethanol (in water) is continuously withdrawn from the top. The primary advantage of this type of bioreactor is increased rates of fermentation resulting from the high concentration of cells in the reactor.
- (c) Enzyme Catalysis in Nonaqueous Solutions (H.W. Blanch, U.C. Berkeley). Techniques for increasing the solubility and transport rate of organics at the site of enzyme action must be found before enzymes will be widely used for production of large-scale chemicals. The approach proposed here is based on the use of a second organic liquid, in which the substrate or product is soluble to a much greater extent than in an aqueous system. A second aspect of the use of enzymes in nonaqueous environments is the potential to run many reactions "backwards." In cases where water is a reaction substrate, its high activity in aqueous solution generally results in a shift of equilibrium to favor the normal products of the reaction. In a nonaqueous system such reactions may be forced in the reverse direction, as water will be present at low concentrations. Examples of this type of reaction are esterification and synthesis of tryptophan, an important amino acid.
- (d) Applications of Molecular Hydrogen in Chemical Fermentations (P.C. Maxwell, Celgene). Glucose, derived from renewable biomass, currently has two serious limitations as a fermentation raw material: selectivity to form specific end products is often low and the variety of end products that can be made is limited. It has recently been found that if fermentation is conducted in the presence of hydrogen, the formation of oxidized coproducts is decreased so that selectivity is increased. In addition, the types of primary products formed may contain less oxygen. Two target fermentations have been selected to demonstrate these concepts: fermentation of glucose to succinic acid which will demonstrate a desirable shift in product mix composition, and fermentation of glucose to 1,3-propanediol to demonstrate the ability to extend the range of end products.

- (e) Multimembrane Bioreactor for Chemical Production (M.L. Shuler, Cornell). A four-layer, multimembrane reactor concept is being developed: one for cell entrapment, one for substrate flow, one for gas flow, and one for the flow of an extractant which selectively removes the product. Task efforts will include: proof-of-concept experiments; development of a mathematical model relating the micro-environment to reactor performance; development of a process simulator from the mathematical model; and experiments to validate the model and to monitor the interrelationship of cell physiology to immobilization.
- (f) Multiphase Fluidized Bed Bioreactor (B. Allen, Battelle Columbus). This task is directed towards the development of a novel, fluidized-bed bioreactor for the continuous production of low-value, high-volume chemicals. The concept incorporates the features required to minimize the effects of end-product inhibition, a fundamental biochemical/microbiological limitation that adversely affects the economics of current bioprocess technologies. End-product inhibition decreases biocatalyst activity which leads to low productivity, high-volume reactors, and low-product concentration, which requires capital/energy-intensive aqueous separation systems.

The bioreactor concept in this task combines the use of an immobilized biocatalyst with in-situ, nonaqueous sorbent separation to minimize end-product inhibition. This is achieved by the use of multi-phase fluidized-bed (MPFB) bioreactor process. The biocatalyst is immobilized as relatively large or dense particles (dense phase) which are fluidized in a conventional manner using the fermentation media. Either a solid or an insoluble, non-aqueous solvent (entrained phase) is then circulated through the dense bed to remove secreted products. After circulating through a separation/desorption unit to recover products, the entrained phase is regenerated.

- (g) Biocatalyst Hydroxylation in Organic Solvents (A. Klibanov, MIT). As discussed earlier, one of the major obstacles in utilization of enzymes in organic chemistry is the necessity to carry out enzymatic reactions in water, which is a poor medium for most industrial chemical processes. For example, the enzyme polyphenol oxidase has a great potential for selective hydroxylation of aromatic compounds, an often desirable but very difficult problem in organic chemistry. However, polyphenol oxidase-catalyzed oxidations cannot be conducted in water because: most phenolic substrates are only slightly soluble in water; quinones (formed as the product of the enzymatic oxidation) spontaneously polymerize in water; and during that polymerization the enzyme is chemically modified and consequently inactivated.

It has recently been discovered that several different enzymes can function in nearly anhydrous organic solvents. Under such conditions, polyphenol oxidase can quantitatively convert phenols to the corresponding o-quinones which then can be chemically reduced to catechols. The overall process affords preparative regioselective hydroxylation of a phenol. This effort includes determination of the mechanism, implications and preparative use of the above phenomenon.

- (h) Separation by Reversible Chemical Association (C.J. King, U.C. Berkeley). The objective of this task is to examine and evaluate reversible chemical association, or complexation, using organic agents as a method for separating polar organic substances from dilute aqueous solutions (e.g., bioprocess product or waste streams). The goal is to obtain sufficient understanding of underlying chemical, equilibrium, and transport behavior to enable rational selection of separating agents, and methods of implementation, as well as rational conceptual design and economic evaluation. In the production of carboxylic acids and other products, processes such as fermentation yield low concentrations of products in water. The subsequent separation and purification of the desired products is often difficult and energy intensive.
- (i) Bioseparation of Phosphate (Rogers, INEL). The phosphate industry utilizes about 0.3 quads/yr for the separation of phosphate from apatite ore. Therefore, this task is directed toward development of bioprocessing for solubilization and separation of phosphate from ore. The specific objectives of the proposed research are to: define a microbiological system which will extract phosphate from its ore; develop a basic understanding of the biochemical mechanisms involved; and, through the use of modern biotechnology, develop a bioprocessing system for transfer to the phosphate industry. If a more efficient recovery process can be developed, it could also be applied to phosphate mine waste because ore containing <26% phosphorus is currently considered waste and is used as mine backfill. The approach will be to gain a thorough understanding of the biochemical interactions which cause the microbial release of phosphate from its ore. This will be accomplished by screening microorganisms obtained from areas of high phosphate content (i.e., phosphate mines, process waste streams, fertilized agricultural lands, etc.) for their ability to solubilize phosphate. Those organisms which are positive for the desired trait will be selected for further biochemical studies. When a better understanding of the mechanism of solubilization has been established, the work will be focused on development of methods to enhance production. Enhancement could be through physical/chemical stimulation, genetic manipulations, or both.

- (j) Protein Engineering for Nonaqueous Solvents (F.H. Arnold, Caltech). The industrial applications of biocatalysts have been severely limited by constraints on the solvent environment of proteins, which normally require an aqueous medium for effective operation. With the advent of convenient methods for altering the amino acid composition and for synthesis of entirely new proteins, it is worthwhile to consider engineering proteins that would be effective in nonaqueous solvents. However, the success of a rational design procedure for constructing proteins to use in organic solvents depends on understanding relationships among various factors: amino acid sequence, secondary and tertiary protein structure, and activity and stability in nonaqueous solvents. The goal of this research is to begin to define these relationships, by implementing an integral and iterative protein engineering approach based on the model hydrophobic protein crambin.

(3) Process Design and Analysis

The Process Design and Analysis work element includes development of design and process technology for scaling up bioprocesses to the pilot-plant level and overall assessments (i.e., systems analyses) of biocatalyst chemical production processes. This work element is comprised of two research tasks: Bioprocess Synthesis, Integration, and Analysis; and Assessment: Biotechnology and Chemical Production.

- (a) Bioprocess Synthesis, Integration, and Analysis (J.D. Ingham, JPL). The purpose of this task is to assemble a series of candidate bioprocesses and systematically conduct relevant energy-economic analyses and comparisons of their projected potential for commercial development within the next decade. Bioprocesses to be assessed will include projected research advances (e.g., genetically engineered microorganisms, bioreactor modeling and verification, membrane development, and biocatalyst immobilization) for energy-economic comparative assessments. The four parameters that determine bioprocess economics and total energy requirements are cell density, productivity, product recovery energy and yield.
- (b) Assessment: Biotechnology and Chemical Production (Chem Systems Inc.). The objective of this work is to carry out techno-economic assessments of specific bioprocesses where most of the new technology has already been developed, primarily in support of technology transfer activities.

## SECTION III

### FY87 TASK ACCOMPLISHMENTS

#### A. MOLECULAR MODELING AND APPLIED GENETICS

- (1) Kinetics of Recombinant Cells (J. Bailey, Caltech). This research focuses on control and monitoring of plasmids and kinetics of DNA expression. Novel high-speed measurement methods employing flow cytometry are used to characterize the cell population in a reactor in terms of cell size and cellular plasmid concentration. These experimental methods are used in concert with mathematical models at the molecular and population level to improve understanding of plasmid stability and increasing productivity of bioreactors using genetically engineered recombinant microorganisms.

The single-cell measurement method based upon labelling now permits detection of single-plasmid copies in yeast. This is accomplished through the use of flow cytometry in which light scattering and fluorescence measurements can be made on a single cell in a flowing cell suspension. Using this experimental method, the fraction of plasmid-containing cells in the recombinant population can be determined in approximately three hours.

Results of these measurements have been used to develop a plasmid stability model for recombinant populations propagating unstable multicopy plasmids. The model incorporates plasmid replication and partition functions, as well as the effect of plasmid presence on host growth rate. It has been found that the amount of a cloned enzyme produced by a genetically engineered cell depends upon the differences between the rate of enzyme or messenger RNA (mRNA) synthesis and the rate of enzyme or mRNA degradation. The rates of mRNA and protein turnover have been found to be significantly influenced by changes in the number of plasmids per cell, the longevity of the mRNA, the physiological condition of the cell, and the frequency of plasmid segregation of the plasmid between sister cells at cellular division. For example, as the number of plasmids within the cell increases, the quantity of DNA-directed mRNA increases, which in turn produces higher quantities of gene product (protein). This increase in gene product is not gratis: costs include the additional energy to manufacture the gene, its mRNA, and its specific protein. This additional energy cost places these cells at a competitive disadvantage in the bioreactor when compared to non-plasmid-containing cells, leading to product production failure. Because plasmids are transferred between cells, there is also a chance for their loss in division. Normally the genetic content of the plasmid is adjusted such that the cell is dependent upon its presence for its survival. It is then assumed that if the plasmid is lost, the necessary material (enzyme) for survival is lost and

the cell will be lost. Yet it has now been shown that the availability of the enzyme depends on the half life of the mRNA. The longer the half life of the mRNA, the longer the cell can continue to grow without a plasmid. The cell that lacks a plasmid is not then penalized and can grow for some time without contributing to the reactor performance. This same phenomenon of continued growth without a plasmid can also occur if the quantity of mRNA is high. Thus, the overall productivity of the reactor depends on a number of variables. This model now allows workers in the field to estimate the impact of plasmid instability on bioreactor performance.

(2) Chromosomal Amplification of Foreign DNA (G. Bertani, JPL).

Another approach to obviate the intrinsic instability of plasmids, and therefore of any foreign genetic material that is built into them, is to insert a copy of the plasmid, with whatever genes are carried by it, into the chromosome of the bacterium. This approach has now been successfully demonstrated. To increase the number of copies of the genes thus introduced on the chromosome, and make the structure equivalent to that of a typical bacterium loaded with plasmids, an indirect method of selection for chromosome amplification was applied, also successfully. Such chromosomally amplified structures can be undone by normal genetic recombination, however. During the year, attempts at reducing the effects of normal genetic recombination have been made, by introducing mutations that reduce recombination in the bacterium. This work is still in progress. Another problem that is being investigated is the unexpectedly low growth rate of those strains where plasmid amplification has been produced within the chromosome.

(3) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta University). Two Ph.D students have prepared task plans that define each of their activities that will be implemented to accomplish the objectives of this research. Experimental work has been started to determine kinetics of production of extracellular oxidases from C. versicolor, establish optimum growth conditions, and compare specific activities for the enzyme utilizing different phenolic substrates.

Experiments have been completed to unequivocally establish time courses for the formation of intra- and extracellular polyphenol oxidases (PPO). Cycloheximide addition (to C. versicolor intracellular total protein) inhibited total protein levels by 57.4% and enhanced PPO activity by a factor of 2.3, to suggest that PPO is activated, rather than synthesized de novo. Protocols for the purification of extracellular PPO are being developed. Presentations of this and related work were made in St. Louis, MO and at the University of Cambridge, England.



## B. BIOPROCESS ENGINEERING

- (1) Protein Engineering for Nonaqueous Solvents (F.H. Arnold, Caltech). The goal of this research is to begin to define the relationship between the amino acid sequence and protein structure, based on the model hydrophobic protein crambin. The unusual stability and solubility of crambin in a wide range of nonaqueous solvents will be investigated. The structures, stabilities, and solubilities of crambin mutants will be measured and correlated with specific alterations in the protein amino acid sequence. This research includes studies of the properties of crambins, design of new mutant crambins by computer/molecular graphics, production of new mutants by site-directed mutagenesis, and detailed NMR studies of crambin stability and response to nonaqueous solvents. The results of this work will be used to formulate a set of criteria and a method for rational design of proteins to be used in the presence of nonaqueous solvents.

The synthetic gene for crambin has been expressed in *E. coli*. The gene was inserted into pKK223-3, containing the *tac* promoter. Crambin is expressed intracellularly at reasonably high levels. The protein produced in the bacteria is reduced and unfolded. Attempts to refold the recombinant crambin in a mixture of oxidized and reduced thiols have been successful. The resulting protein has an NMR proton spectrum very similar to the native material. The genes for five variants of crambin have been produced, including one with an additional disulfide bridge between residues 12 and 30. A preliminary set of design criteria for engineering proteins to be stable in nonaqueous solvents has been proposed and presented at the IX Enzyme Engineering Conference in Santa Barbara.

- (2) Enzyme Catalysis in Nonaqueous Solutions (H.W. Blanch, U.C. Berkeley). This research pursues conversion of cholesterol to cholestenone by using an enzyme (with and without enzyme-water encapsulation) dispersed in an organic solvent. Kinetics of enzymatic conversion in a two-phase system (toluene-water, without encapsulant) have been determined as a basis for comparison with the encapsulated system and determination of the effects of surfactant concentration and stirring rate. Oxidase activity at 25°C decreased after one day, probably because of denaturation at the solvent interface, followed by no further decrease before termination after 5 days. Although the surfactant (Span 85, >0.5 vol%, used to disperse the solvent in the aqueous phase) increased the initial rate by a factor of about five, the effect of surfactant on interfacial enzymatic activity is not well understood. Therefore, the effect of a biological surfactant, phosphatidylcholine (PC), on the rate was determined, and the relative effects of the two surfactants were rationalized on the basis of differences in their chemical structures. A model was developed to account for various effects on rates of reaction in a two-phase system, and oxidase activity assay and control of pH in the two-phase system were investigated.

A method has been developed to avoid capsule membrane swelling when the capsules are transferred from the encapsulant formation solvent (cyclohexane) to the reaction solvent (toluene or benzene), and to control pH within the microcapsules, by using cyclohexane-toluene (1:1) as a common solvent: the mixed solvent does not cause swelling and allows a higher acid concentration in the solvent. In the oxidase-catalyzed conversion of cholesterol to cholestenone within microcapsules, control of pH is required to maximize enzymatic activity. To provide for transfer of acid across the membrane and into the capsules, they are exposed to HCl with Aliquat 336 in the organic phase. A non-invasive technique has been developed to monitor acidity within the capsules by using NMR ( $^{31}\text{P}$ ) spectrometry to determine encapsulated mono- and dihydrogen phosphate. The relative amounts of each phosphate type are then used to calculate the pH inside the capsules. The pH for maximum oxidase activity was determined to be from 6.0 to 6.5.

Additional research concerns tryptophan synthesis and extractive catalysis. Although it was originally planned to study urea synthesis, attention has been redirected to tryptophan formation as a model to minimize experimental difficulties (e.g., definition of an appropriate solvent system for urea, and development of a fundamental understanding of extractive catalysis and the effect of product water on reaction equilibria). In this conversion, ammonium pyruvate or serine plus indole react to form tryptophan and water in the presence of tryptophanase. The normal reaction is hydrolysis, because of the high effective concentration of water in most physiological environments. A liquid membrane (cyclohexane) or micellar solution is used to isolate the enzyme in an aqueous environment at pH 9 from the aqueous outer phase at pH 7, into which the product is extracted, as a result of the pH gradient across the liquid organic membrane. (Micelles consist of microscopic drops of water within an organic phase, which is dispersed as an emulsion in water.) Because of limited ammonia transport across the organic phase, it was found that serine, rather than ammonium pyruvate, must be used in a micellar environment.

A model has been developed and experimentally verified to describe mass transfer kinetics for substrate and products of this system. Another significant accomplishment is a detailed description and clarification of the effects of solvents on the equilibria of enzymatic reactions in two-phase systems, which will be extremely useful in the definition of future biocatalyst conversion processes based on extractive catalysis (i.e., catalyzed reactions in two-phase systems). In this work, equilibrium equations have been derived in terms of partition coefficients and activities of the reactants; these equations can be used to predict product yields in two-phase systems. Further work on tryptophan synthesis includes initiation of studies using EPR spectrometry and spin labels to investigate water and enzyme interactions (e.g., conformational mobility of enzymes within micelles).

- (3) Biocatalyst Hydroxylation in Organic Solvents (A.M., Klibanov, MIT). The purpose of this research is to develop new methods for enzymatic oxidative catalysis in organic solvents, e.g., for selective oxidation of aromatic compounds to produce quinones and hydroxylated aromatic chemicals, for peroxidase-catalyzed depolymerization of lignin, and for potential polymerization of aromatic compounds.

Conditions for maximum enzymatic depolymerization of polyconiferyl alcohol as a model for lignin have been determined. These include utilization of a solvent consisting of 95% dioxane in water at a pH of 5.0 with 0.5 mg/ml of the enzyme (horseradish peroxidase) and 300 ug/ml of polyconiferyl alcohol. Alternative solvents were also investigated. While peroxidase-catalyzed depolymerization of synthetic lignin did not take place in aqueous solutions, significant depolymerization was accomplished in organic solvents using either horseradish or lactoperoxidases. A mechanistic evaluation of the ability of horseradish peroxidase to catalyze the cleavage of specific lignin interunit linkages using low molecular weight model compounds indicated a preference for benzylic ether cleavage over aryl-alkyl cleavage if both were present. Molecular oxygen was shown to stimulate depolymerization, and this was interpreted to be the result of formation of free radical lignin species produced by the peroxidase-initiated depolymerization reactions. The ability of horseradish peroxidase to depolymerize lignin in organic solvents may enable useful low molecular weight chemicals to be produced economically from lignin or lignin waste products.

The depolymerization of lignin has been extended to show that extracted lignins from milled wood or kraft pine can be successfully depolymerized using the same methods as for polyconiferyl alcohol. Also, milled wheat straw has been delignified with a modified, dioxane-soluble peroxidase. No depolymerization took place in aqueous solutions, to show the advantage of enzymatic reaction in organic solvents. Studies have also been initiated on horseradish peroxidase-catalyzed reactions to polymerize phenols in organic solvents as an alternative to phenol-formaldehyde resins. Two inventions developed in this work have been licensed for commercial applications: Enzymatic Reactions in Liquid and Solid Paraffins; Application for Temperature Abuse Sensors; and Method and Apparatus for Detecting Compounds Utilizing Enzyme-Catalyzed Reactions in the Gaseous Phase.

- (4) Separation by Reversible Chemical Association (C.J. King, LBL, U.C. Berkeley). In the production of carboxylic acids, processes such as fermentation yield low concentrations of carboxylic acids in an aqueous multicomponent solution. The subsequent separation, purification, and concentration of the carboxylic acids is difficult and energy intensive.

A novel processing approach for recovery of low-volatility carboxylic acids has been developed. This method involves use of two diluents that are substantially more volatile than the amine and the acid. One (e.g., chloroform) is an "active" diluent, which solvates the complex and greatly increases the equilibrium distribution coefficient. The other (e.g., n-heptane) is an "inert" diluent, which does not interact with the complex and gives lower distribution coefficients. The active diluent is present during extraction of the acid from water. It is then removed by distillation and replaced by the inert diluent, which is present during back-extraction of the acid into water. The inert diluent is then removed by distillation and replaced with the active diluent for reuse of the solvent mixture. Equilibrium measurements for extraction of succinic acid with amine present in a diluent containing different proportions of chloroform and heptane show that this approach is promising. Extraction of lactic acid by Alamine 336 in chloroform and m-cresol diluents shows very high distribution coefficients, which indicates that this system can be applied to energy-efficient recovery of lactic acid.

Fixed-bed adsorption runs have been made with Porapak Q (Waters Assoc.) to determine the effect of particle size on adsorption. Succinic acid, acetic acid, and 1,3-butanediol have been adsorbed from aqueous solutions, using the bed in both wetted and non-wetted conditions. In the latter case, separation follows the order of volatilities. This enables a separation among solutes that is not possible in the wetted state. The shapes of breakthrough curves for the non-wetted state display a number of transfer units about a factor of 10 less than expected. Preliminary indications are that this is the result of channelling, caused by preferential wetting of the wall of the glass column. The possibility of using a polycarbonate column is being investigated. Sorption properties of a large number of basic resins have also been examined experimentally.

Preliminary infrared spectroscopic measurements have been made for succinic acid dissolved in dry amine with chloroform diluent to try to confirm the presence of specific complexes. Equilibrium data have been obtained for extraction of ethanol by various phenolic compounds in different diluents to attempt to identify structural factors that lead to favorable equilibria. Many phenolics are not amenable as alcohol extractants because they are solids at practical temperatures that cannot be dissolved in diluents at high concentrations. Alkyl groups on the phenol decrease solubility loss in water, but distribution coefficients are unfavorably decreased. It was also determined that there is no significant advantage in using pentachlorophenol or dihydroxybenzenes. Sorption properties of various basic resins (e.g., polyethyleneimine, epoxy polyamines, and polyvinylpyridine) have been examined experimentally to identify attractive regenerable sorbents for carboxylic acids.

- (5) Metabolic Engineering (J. Bailey, Caltech). The objective of this study is to formulate mathematical models [i.e., the Metabolic Pathway Synthesis Program (MPS)] for analysis of directed metabolic changes accomplished by recombinant DNA methods. The MPS program can be used to predict, in a qualitative way, the effect of adding or deleting enzymatic activities to or from the cellular environment, to classify pathways with respect to cellular objectives, and to extract information about metabolic regulation. MPS can be used for the identification of appropriate genotypes or genetic modifications that will redirect metabolism towards amplified production of desirable bioproducts. These capabilities have been illustrated by synthesizing the classical conversion of glucose 6-phosphate to pyruvate (Embden-Meyerhof-Parnas, pentose phosphate, and Entner-Doudoroff pathways), and biosynthesizing L-alanine that does not necessitate the use of alanine aminotransferase from pyruvate.
- (6) Multiphase Fluidized Bed Bioreactor (B. Allen, Battelle Memorial Institute, Columbus). A Multiphase Fluidized Bed Reactor (MPFB) was built by Battelle Memorial Institute and its design and operation was tested. This effort was successful at elucidating many operational characteristics of fluidized beds used as bioreactors for the bacterium Clostridium acetobutylicum.

The bacterium C. acetobutylicum was successfully encapsulated in alginate beads with bead sizes ranging from 0.5 mm to 3 mm (and larger). The inclusion of aluminum oxide or iron was also successfully achieved to create beads with greater density, which operate more effectively as the dense phase in the fluidized bed. The bacterium was active and was viable for over 300 hours of continuous operation with active production of butanol for over 240 hours. Operationally, it was found that the small diameter beads were required for successful fluidization. The specific organism was chosen because of its acute sensitivity to product inhibition. Thus, the relief of such inhibition by the sorbent extraction in this system should be better observed.

The operational mode for the extractive or sorbent phase was examined. It was determined that a liquid sorbent which operates in a co-current mode would be best to test the concept. Several candidate sorbents were chosen based on published distribution coefficients for butanol in a biphasic liquid system and tested for toxicity towards cells and for actual distribution coefficient values. Some sorbents were found that possessed appealing distribution coefficient values. However, most were found to be toxic to the cells and the final choice was made to use oleyl alcohol. Although not the best choice for processing, it serves well to test the feasibility of this concept.

An actual working prototype of the MPFB system was built and used for cold flow model testing in order to obtain operational data. It was 3" in diameter by 6' in height with a working volume of about 7 to 8 liters. Bed expansion volumes, velocity

measurements, pumping and circulation requirements, and sorbent recirculation parameters were measured. A novel measurement tool was employed to measure bed and solvent velocities: a Fiber Optic Laser Doppler Anemometer. This instrument permitted a non-invasive measurement of fluid flows, although it had a restricted range. A major result of this work was to show that the fluidized bed operates as a reactor that is intermediate between plug flow and backmixed operation. These data will be helpful in designing larger units for scale-up.

The prototype of the MPFB was reconfigured to be sterilizable, thus permitting operation with bacterial cells. This unit will provide a unique facility for testing authentic microbial cultures with the MPFB concept. This unit has undergone initial testing and operation to eliminate "bugs" prior to the actual feasibility tests. Tests to demonstrate sorbent extraction of butanol in the fluidized bed were conducted. The results indicated successful partitioning of butanol into the oleyl alcohol from beads containing butanol (no microbial encapsulation in this case).

An independent technoeconomic analysis of the MPFB concept was conducted by the engineering firm, Bio En-Gene-Er Assoc., Inc. Their analysis was quite favorable and concluded that this fluidized bed concept could increase the effective concentration of the product ten-fold while maintaining actual concentrations near the organism below the threshold of inhibition. As currently configured, the MPFB would reduce the cost of butanol by about 34% (to yield a 30% pretax return on investment). Further optimization could reduce this cost even further. Guidelines for further research were proposed, including a need for better product stoichiometry from the microbe and an improvement in the sorbent.

- (7) Multimembrane Bioreactor for Chemical Production (M. Shuler, Cornell). The Multimembrane Bioreactor with extraction was shown to operate and exhibit improved performance over conventional batch and immobilized fermenters. In the MMB system with extraction, glucose concentration nearly approached zero, lowering production costs. In addition, ethanol productivity was increased by a factor of 5 to 7. Operational problems with this system were encountered which were overcome by defining nutrient requirements. One new concept utilizing the MMB system was attempted by operating the system with pressure cycling in the gas phase. It was anticipated that such operation would enhance mass transfer and permit increased cell densities. Experiments were conducted to test this hypothesis.

It was subsequently demonstrated that a five-fold improvement in average fermentation rate for a 200 g/l glucose feed for a pressure cycle reactor could be achieved over the normal diffusion-controlled system (8.7 g glucose consumed/hr versus 1.8 g glucose consumed/hr). The pressure cycle removes ethanol more efficiently from the cell layer (2.8 g/l/hr versus 1.1

g/l/hr). Additional characteristics of this reactor system were examined, such as the effect of high cell densities on operation and operation at high temperatures (above 40°C).

The effect of high cell densities on the reactor system were studied. The analysis evaluated growth rate as a function of cell density. The apparent growth rate at 41 g/l was roughly half of that at 9 g/l (0.16 versus 0.29/hr). The reduction appeared to be due to cell contact blocking uptake sites at high cell concentrations. The operation of the pressure cycle reactor at high temperatures was also evaluated. Previous attempts to operate such fermentations at higher temperatures in the diffusion mode showed non-performance at 40° and 45°C. In the pressure cycle mode, operation at 40°C was very effective (i.e., rapid glucose consumption), but at 45°C cell viability was lost and the fermentation became "stuck" (or inhibited). Work with the pressure cycled MMB system is now being directed towards continuous operation and/or increasing the size of the system by operating two or more units in series.

Additional areas of research involved an analysis of the separations and aeration requirements for this extractive system, and modeling of the ethanol yeast fermentation system. Preliminary experiments to evaluate separations were carried out. In a simple two-step distillation with "spent" TBP and ca. 30 g/l of ethanol and ca. 60 g/l of water in the TBP, a top product with 80 wt% ethanol and virtually no TBP was recovered. If the spent TBP was dehydrated with  $MgSO_4$ , then the top product is nearly pure ethanol. These results indicated that purification is achievable. The effects of aeration on the specific yeast strain employed were studied. This strain operated poorly under strictly anaerobic conditions, but required a level of oxygen at 2 to 3% of 100% saturation.

An initial formulation of a mathematical model for yeast growth and metabolism was completed. After considerable work and improvement, the model is considered to be one of the most complete and powerful simulators of yeast behavior available. It can accurately predict transient behavior in batch culture, a predictive behavior not found in models currently available. The model will continue to be improved until the end of this effort to better fit empirical data obtained in the laboratory.

## C. PROCESS DESIGN AND ANALYSIS

- (1) Bioprocess Synthesis, Integration, and Analysis (J. D. Ingham, JPL). The purpose of this task is to derive or synthesize a series of candidate bioprocesses and systematically conduct relevant energy-economic analyses and comparisons for added-value commodity chemicals to determine potential for process technology transfer and commercial development. These bioprocesses will be

modified to include projected research advances (e.g., genetically engineered microorganisms, bioreactor modeling and verification, membrane development, and biocatalyst immobilization) for energy-economic comparative assessments. The four parameters that determine bioprocess economics and total energy requirements are cell density, productivity, product recovery energy, and yield.

Extensive utilization of bioprocesses for the production of commodity chemicals could significantly decrease U.S. dependence on the supply of foreign and domestic non-renewable petroleum resources, but because of relatively high feedstock costs and some specific limitations of biocatalyst processes, most of them are not yet competitive with current petrochemical routes with respect to either process energy consumption or economics. Since these limitations often result in either relatively low rates of reaction and production, low product concentration, or both, assessments of a series of processes for ethanol (as a model of a high volume industrial chemical) have been completed to establish a more quantitative basis for estimation of the relative effects of production rates and product concentration in the bioreactor on process energy and economics. The results of model bioprocess simulations for ethanol show that energy consumption is sensitive to product concentration, is essentially independent of rate of production, and that the effect of production rate on economics is at least an order of magnitude less than the effect of product concentration. It is also suggested that concentration is likely to be even more important for other potential industrial chemical biocatalyst processes (e.g., for n-butanol or acetic acid) where product recovery is more energy intensive than for ethanol. It can be concluded that future research advances to improve the energy, economics, and effective synthesis of new, competitive biocatalyst processes for industrial chemicals should be directed toward significantly increasing product concentration, rather than by attempting to independently maximize only rates of production.

A computer model has been formulated where fundamental kinetic equations and intrinsic parameters (e.g., maximum specific rates, saturation constants, and yield factors) result in fixed relationships with respect to conversion, product yield, product concentration, and productivity. Calculations show that there are maximum limits for these parameters for specific bioprocesses: the highest productivity normally will occur at a relatively low yield and concentration. Therefore, investigations have been initiated to establish reactor configurations and conditions that result in minimum energy requirements and production costs. Preliminary calculations for simulated processes of rate, yield, and concentration combinations agree with previous experimental observations. This bioreactor kinetic study will be completed and used to attempt to confirm that earlier assessments are realistic and consistently applicable to potential advanced bioprocesses, to ensure that assumed reactor conditions are reasonable, and that projected improvements could be realized.



## SECTION IV

### BIBLIOGRAPHY

- Allison, J. M., and Goddard, W. A., "Oxidative Dehydrogenation of Methanol to Formaldehyde," J. Catal., Vol. 92, pp. 127-135, 1985.
- Allison, J. M., and Goddard, W. A., "Active Sites on Molybdate Surfaces, Mechanistic Considerations for Selective Oxidation, and Ammoxidation of Propene," ACS Symposium Series No. 279, pp. 23-36, 1985.
- Bertani, L. E., and Bertani, G., "Chromosomal Integration and Excision of a Multicopy Plasmid Joined to Phage Determinants for Site Specific Recombination," in Plasmids in Bacteria, D. R. Helinski, ed., Plenum Press, NY, p. 917, 1985.
- Bertani, L. E., and Six, E. W., "The P2-like Phages and Their Parasite, P4," to appear in The Viruses, editors: Wagner, R. R., and Fraenkel-Conrat, H., Plenum Publishing Corp, 1988.
- Biocatalysis Research Activity Annual Report: FY 1983, Jet Propulsion Laboratory, JPL Publication 84-27, DOE/CS-66001-4, April 15, 1984.
- Biocatalysis Project Annual Report: FY 1984, Jet Propulsion Laboratory, JPL Publication 85-31, DOE/CS-66001-8, April 15, 1985.
- Biocatalysis Project Annual Report: FY 1985, Jet Propulsion Laboratory, JPL Publication 86-32, DOE/CS-66001-9, July 30, 1986.
- Biocatalysis Project Annual Report: FY 1986, Jet Propulsion Laboratory, JPL Publication 87-11, DOE/CS-66001-10, April 30, 1987.
- Celniker, S., Sweder, K., Srienc, F., Bailey, J. E., and Campbell, J. L., "Deletion Mutations Affecting the Yeast Autonomously Replicating Sequence, ARSI," Mol. Cell. Biology, Vol. 4, p. 2455, 1985.
- Chem Systems, Inc., "Technical and Economic Assessment of Processes for the Production of Butanol and Acetone - Phase Two: Analysis of Research Advances," JPL 9950-928, DOE/CS-66001-5, Prepared for Jet Propulsion Laboratory by Chem Systems, Inc., Tarrytown, NY, August 1984.
- Chem Systems Inc., "Techno-Economic Assessment of Microbial Ammonia Production," prepared for Jet Propulsion Laboratory by Chem Systems Inc., Tarrytown, NY, February 1986.
- Chem Systems Inc., "Assessment of ORNL Immobilized Cell Ethanol Fermentation Process," prepared for Jet Propulsion Laboratory by Chem Systems Inc., Tarrytown, NY, March 1986.
- Chem Systems Inc., "Implications of ORNL Immobilization Technology on Citric Acid Production," prepared for Jet Propulsion Laboratory by Chem Systems Inc., Tarrytown, NY, June 1986.

- Cho, T., and Shuler, M. L., "Multi-Membrane Bioreactor for Extractive Fermentation," Biotech. Prog., Vol. 2, pp. 53-60, 1986.
- Davison, B. H., and Scott, C. D., "Ethanol Production on an Industrial Feedstock by Zymomonas Mobilis in a Fluidized Bed Bioreactor," Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TE, May 13-16, 1986.
- Davison, B. H., and Scott, C. D., "Fermentation Kinetics of Zymomonas mobilis under Mass Transfer Limitations," 192nd ACS National Meeting, Anaheim, CA, September 7-12, 1986.
- Eggers, D. K., Lim, D. J., and Blanch, H. W., "Enzymatic Production of l-Tryptophan in Liquid Membrane Systems," submitted to Bioprocess Eng.
- Eggers, D. K., Blanch, H. W., and Prausnitz, J. M., "Extractive Catalysis: Solvent Effects on Equilibria of Enzymatic Reactions in Two-Phase Systems," submitted to Bioprocess Eng.
- England, C., "Isothermal Separation Processes Update," JPL 9950-927, DOE/CS-66001-6, prepared for Jet Propulsion Laboratory by Engineering Research West, Santa Monica, CA, August 1984.
- Goddard III, W. A., Low, J. J., Olafson, B. D., Redondo, A., Zeiri, Y., Steigerwald, M. L., Carter, E. A., Allison, J. N., and Chang, R., "The Role of Oxygen and Other Chemisorbed Species on Surface Processes for Metals and Semiconductors; Approaches to Dynamical Studies of Surface Processes," Proceedings of the Symposium on the Chemistry and Physics of Electrocatalysis, Vol. 84-12, The Electrochemical Society, Ind., Pennington, NJ, pp. 63-95, 1984.
- Goddard III, W. A., "Theoretical Chemistry Comes Alive: Full Partner with Experiment," Science, Vol. 227, pp. 917-923, 1985.
- Hatcher, H. J., "Biocatalysis Program Bioseparations Task," Summary Report, E. G. and G. Idaho, Inc., Idaho National Engineering Laboratory, Idaho Falls, ID, February 1986.
- Ingham, J. D., Biocatalyst Processes for Production of Commodity Chemicals: Assessment of Future Research Advanced for N-Butanol Production, Jet Propulsion Laboratory, JPL Publication 84-60, July 1, 1984.
- Kertes, A. S., and King, C. J., "Extraction Chemistry of Fermentation Product Carboxylic Acids," Biotechnology and Bioengineering, Vol. 28, pp. 269-282, 1986.
- King, C. J., chapter on Chemical Association in Handbook of Separation Process Technology, R. W. Rousseau, ed., John Wiley and Sons, 1987.
- King, C. J., et al., "Non-wetting Polymeric Adsorbents for Selective Uptake of Carboxylic Acids and Glycols," presented at the Symposium, Fundamentals of Adsorption, Meeting of the American Chemical Society, Denver, Colorado, April 1987, and Foundation Conference on Advances in Separation Technology, West Germany, April 1987.

- Klibanov, A. M., "Polyphenol Oxidase - Catalyzed Hydroxylation of Aromatic Compounds in Organic Solvents," Quarterly Report No. 1, Massachusetts Institute of Technology, Cambridge, MA, March 1986.
- Kuhn, R. H., and Linden, J. C., "Effects of Temperature and Membrane Fatty Acid Composition on Butanol Tolerance of Clostridium acetobutylicum," Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TE, May 13, 1986.
- Lee, S. B., Seressiotis, A., and Bailey, J. E., "A Kinetic Model for Product Formation in Unstable Recombinant Populations," Biotechnology and Bioengineering, Vol. 27, pp. 1699-1709, 1985.
- Lee, S. B., and Bailey, J. E., "A Mathematical Model for  $\nu$  Plasmid Replication: Analysis of Wild-Type Plasmid," Plasmid, Vol. 11, No. 151, 1984.
- Lee, S. B., and Bailey, J. E., "A Mathematical Model for  $\nu$  Plasmid Replication: Analysis of Copy Number Mutants," Plasmid, Vol. 11, No. 151, 1984.
- Lee, S. B., and Bailey, J. E., "Analysis of Growth Rate Effects on Productivity of Recombinant Escherichia coli Populations Using Molecular Mechanism Models," Biotechnol. Bioeng., Vol. 26, No. 66, 1984.
- Lee, S. B., and Bailey, J. E., "Genetically Structured Model for Lac Promoter-Operator Function in the Chromosome and in Multicopy Plasmids: Lac Promoter Function," Biotechnol. Bioeng., to be published.
- Linden, J. C., and Kuhn, R. H., "Biochemistry of Alcohol Effects on Clostridia," in Ethanol Toxicity at the Cellular Level, Van Uden, N., ed., CRC Press, Cleveland, OH, in press.
- Miskowski, V. M., "The Electronic Spectra of  $\mu$ -Peroxodicobalt (III) Complexes," Comments Inorg. Chem., in press.
- Peretti, S. W., and Bailey, J. E., "A Mechanistically Detailed Model of Cellular Metabolism for Glucose-Limited Growth of Escherichia coli B/r-A," Biotechnol. Bioeng., Vol. 28, p. 1672, 1986.
- Peretti, S. W., and Bailey, J. E., "Simulations of Host-Plasmid Interactions in Escherichia coli: Copy Number, Promoter Strength, and Ribosome Binding Site Strength Effects on Metabolic Activity and Plasmid Gene Expression," Biotechnol. Bioeng., Vol. 29, p. 316, 1987.
- Scott, C. D., "Techniques for Producing Mono-dispersed Biocatalyst Beads for Use in Columnar Bioreactors," Proceedings of the Seventh Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TE, May 16, 1985.
- Scott, C. D., "Innovative Techniques for Gel Immobilization of Microorganisms and Enzymes," International Conference on Enzyme Engineering, Helsingor, Denmark, September 1985, to be published in The Annals of the New York Academy of Sciences.

- Scott, C. D., and Thompson, J. E., "Mass Transfer Properties of Biocatalyst Beads," Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TE, May 13-16, 1986.
- Seo, J. H., and Bailey, J. E., "Effects of Recombinant Plasmid Content on Growth Properties and Cloned Gene Product Formation in Escherichia coli," Biotechnology and Bioengineering, Vol. 27, pp. 1668-1674, 1985.
- Seressiotis, A., and Bailey, J. E., "Optimal Gene Expression and Amplification Strategies for Batch and Continuous Recombinant Cultures," Biotechnol. Bioeng., Vol. 29, p. 392, 1987.
- Seressiotis, A., and Bailey, J. E., "MPS: An Algorithm and Data Base for Metabolic Pathway Synthesis," Biotechnol. Left., Vol. 8, p. 837, 1986.
- Srienc, F., Campbell, J. L., and Bailey, J. E., Biotechnol. Letters, Vol 5, p. 43, 1985.
- Srienc, F., Bailey, J. E., and Campbell, J. L., "Effect of ARSI Mutations on Chromosome Stability in Saccharomyces Cerevisiae," Mol. Cell. Biology, Vol. 5, No. 7, pp. 1676-1684, 1985.
- Srienc, F., Campbell, J., and Bailey, J. E., "Flow Cytometry Characterization of Plasmid Stability in Saccharomyces cerevisiae," Third European Congress on Biotechnology, Verlag Chemie, Weinheim, Federal Republic of Germany, Vol. III, pp. 111-187, 1984.
- Srienc, F., Campbell, J. L., and Bailey, J. E., "Analysis of Unstable Recombinant Saccharomyces cerevisiae Population Growth in Selective Medium," 1984, submitted for publication.
- "System for Biotechnology Assessment," Final Report, Phase I: August 1984 - July 31, 1985, Washington University, St. Louis, MO, August 1985.
- Tamada, T. A., Kertes, A. S., and King, C. J., "Solvent Extraction of Succinic Acid from Aqueous Solutions," International Conference on Solvent Extraction, Munich, Germany, September 1986.
- The ECUT Biocatalysis Project, Jet Propulsion Laboratory, ECUT Program Bulletin, DOE, ECU-86/1, PB86-900401, Jan.-Feb., 1986.
- The ECUT Biocatalysis Project, Jet Propulsion Laboratory, submitted for Publication in the ECUT Program Bulletin, May 1987.
- Warren, L. F., "Electrocatalysis Research," Final Report for the Period August 25, 1982 through December 30, 1983, JPL 9950-926, DOE/CS-66001-7, prepared for Jet Propulsion Laboratory by Rockwell International, Science Center, Thousand Oaks, CA, August 1984.
- Wilcox, R. E., Industry, University, and Research Institute Interest in the U.S. Department of Energy ECUT Biocatalysis Research Activity, Jet Propulsion Laboratory, JPL Publication 83-90, DOE/CS-66001-3, November 1, 1983.