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ENERGY CONVERSION
AND UTILIZATION
TECHNOLOGIES PROGRAM

Biocatalysis Project Annual Report FY 1985

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16. Abstract <p>This report presents the fiscal year (FY) 1985 activities, accomplishments, and planned research efforts of the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Program. The Project's technical activities were organized as follows:</p> <p>In the Molecular Modeling and Applied Genetics work element, research focused on (1) modeling and simulation studies to establish the physiological basis of high temperature tolerance in a selected enzyme and the catalytic mechanisms of three species of another enzyme, and (2) determining the degree of plasmid amplification and stability of several DNA bacterial strains. In the Bio-process Engineering work element, research focused on (1) studies of plasmid propagation and the generation of models, (2) developing methods for preparing immobilized biocatalyst beads, and (3) developing an enzyme encapsulation method. In the Process Design and Analysis work element, research focused on (1) further refinement of a test case simulation of the economics and energy efficiency of alternative biocatalyzed production processes, (2) developing a candidate bioprocess to determine the potential for reduced energy consumption and facility/operating costs, and (3) a techno-economic assessment of potential advancements in microbial ammonia production.</p>			
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The Biocatalysis Project focuses on resolving the major technical barriers that impede the potential use of biologically-facilitated continuous chemical production processes.

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ABSTRACT

This report presents the fiscal year (FY) 1985 activities, accomplishments, and planned research efforts of the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Program. The Project's technical activities during FY 1985 were organized as follows:

In the Molecular Modeling and Applied Genetics work element, research focused on: (1) modeling and simulation studies to establish the physiological basis of high temperature tolerance in a selected enzyme and the catalytic mechanisms of three species of another enzyme, and (2) determining the degree of plasmid amplification and stability of several DNA bacterial strains where the DNA plasmid had been inserted directly into the bacterial chromosome.

In the Bioprocess Engineering work element, research focused on: (1) studies of plasmid propagation and the generation of models for plasmid replication and gene expression control in recombinant microorganisms, (2) developing methods for preparing immobilized biocatalyst beads for use in a fluidized bed reactor system, and (3) developing an enzyme encapsulation method for suspension in non-aqueous solutions.

In the Process Design and Analysis work element, research focused on: (1) further refinement of a test case simulation of the economics and energy efficiency of alternative biocatalyzed production processes, (2) developing a candidate bioprocess to determine potential for reduced energy consumption and facility/operating costs, and (3) a techno-economic assessment of potential advancements in microbial ammonia production.

The Project completed the evaluation and selection of proposals for advanced bioprocess concepts. Five contracts were awarded late in the fiscal year. Additionally, the Project supported a biocatalysis workshop and met with the Guidance and Evaluation Panel to develop technology transfer strategies.

FOREWORD

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EXECUTIVE SUMMARY

A. PROJECT DESCRIPTION

Biocatalysis research is a major component of the U. S. Department of Energy (DOE) Energy Conversion and Utilization Technologies (ECUT) Program. The Biocatalysis Project is an applied research and exploratory development effort focused on providing the enabling technology base for new industrial bioprocess applications. The Project's goal is to resolve critical bioprocess engineering problems that impede the biocatalyzed production of high-volume, energy-intensive chemicals where genetically modified and/or engineered microorganisms or derived enzymes are utilized. To achieve the Project objective, generic bioprocess scale-up and design data and experimentally verifiable predictive models for biochemical catalysis will be developed. This area of research, which ECUT has been supporting since fiscal year (FY) 1981, has been identified by the Office of Technology Assessment (OTA)¹ as one of the weakest links in the U.S. biotechnology effort.

B. RATIONALE

The recent decline in petroleum feedstock prices and the continued existence of U.S. chemical processing plant overcapacity has made the chemical processing industry (CPI) reluctant to support large-scale bioprocess research. However, within the next decade it seems certain that the United States will become severely dependent on foreign sources of petroleum and petroleum-based feedstocks for the production of chemicals. In an effort to help address this emerging vulnerability, the DOE-ECUT Biocatalysis Project is working to establish a bioprocess engineering technology base that will enable the CPI's development and utilization of alternative energy feedstocks.

The advantages that biocatalyzed processes have over conventional petrochemical processes include: (1) the lack of a requirement for energy-intensive conditions (high temperatures and pressures) and (2) the capability to use renewable resources that are indigenously available as feedstocks. However, bioprocesses have a number of technical constraints that must be resolved before they can compete with conventional petrochemical processes. Some of the major constraints include:

- (1) Relatively low productivity (mass of product per unit reactor volume per unit time).
- (2) Inhibition of product synthesis at relatively low product concentration.
- (3) Loss of desired product synthesis of recombinant microorganisms as cells grow and divide (plasmid instability).

¹ Office of Technology Assessment, Commercial Biotechnology: An International Assessment, OTA-BA-218, January 1984.

- (4) High energy consumption for product separation from dilute aqueous product streams.
- (5) Low yields, as mass of product per unit mass of feedstock.

These and related problems do not seem to be insurmountable; however, major research advances are required to raise the CPI's awareness of and interest in bioprocess applications.

C. FISCAL YEAR 1985 ACCOMPLISHMENTS OF THE BIOCATALYSIS PROJECT

The Project's FY 1985 research activities were organized into four work elements: Molecular Modeling and Applied Genetics, Bioprocess Engineering, Process Design and Analysis, and Management. Each of the first three work elements addresses a key technical component for the development of the enabling technology base to produce large volumes of energy-intensive chemicals. Nontechnical descriptions of these activities are presented in this Executive Summary. Technical discussions are presented in Section II of this report.

1. Molecular Modeling and Applied Genetics

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. In FY 1985, this work element comprised two research tasks: Enzyme Reaction Models for Biocatalysis and Chromosomal Amplification of Foreign DNA.

a. Enzyme Reaction Models for Biocatalysis. In more recent years, microbes have been increasingly developed for efficient manufacturing of high-value products such as antibiotics, vitamins, enzymes, and plant-growth factors. The latest advances in molecular genetics and recombinant-DNA technology promise a revolution in the manufacture of such high-value products as insulin and interferon. In the categories of foods, beverages, and pharmaceuticals, industrial development and applications based on the advances in fundamental research have been rapid and have attracted substantial industrial and venture capital.

Nevertheless, the development and application of biocatalysis for the production of fuel and chemical feedstocks have not advanced rapidly, especially in areas where nonbiological processes are already in production. Long-term applied research and exploratory development are needed to provide the technology base required for industrial technology development.

The following are some of the major bioengineering difficulties:

- (1) The inherently slow reaction rates of biocatalyzed processes due to their sensitivity to elevated temperatures.
- (2) Low product concentrations in aqueous media.
- (3) Process requirements for recycling expensive enzyme cofactors.

- (4) The general inability to predict and provide a reactor environment for biocatalysts that will both lengthen their lifetime and optimize their production of useful products.

Success in the development of a predictive, computer-generated microscopic reaction model for explaining the behavior of and constraints on enzyme activity will assist in addressing some of these problems and subsequently in enabling industry to design, formulate, prepare, and use biocatalysts in more efficient and productive chemical processes. Such a model will also complement the microscopic reaction models being developed for studying conventional chemical catalysis. Together, these two models could be used to develop hybrid chemical/biological catalysts.

During FY 1985, modeling techniques were used to investigate the origin of thermophilicity (ability to operate at high temperature) of the biocatalyst, thermolysin, at the molecular scale. Also, the enzyme carboxypeptidase A was simulated to confirm the modeling approach and to investigate the role of the water environment on biocatalytic processes.

b. Chromosomal Amplification of Foreign DNA. The application of genetic engineering techniques in certain microorganisms may lead to greatly increased yields of useful products such as organic chemicals. However, before this potential can be realized in large-scale industrial processes, a method must be developed for stabilizing and retaining genetically engineered traits (i.e., capabilities) in large quantities ("cultures") of microorganisms.

One of the most popular recombinant-DNA techniques for introducing new genetic traits of potential commercial value into microorganisms focuses on incorporating new DNA sequences (i.e., genetic instructions) into circular strands of DNA (called "plasmids") that commonly float freely inside bacterial cells. However, plasmids (unlike chromosomes) tend to segregate themselves during cell division, and their number and presence in a given cell population are unstable. This instability leads to the loss of the introduced DNA in the bacterial population over time. Because biocatalyzed chemical production processes based on recombinant DNA will likely be developed only if they take advantage of the economies of continuous operations (similar to conventional processes), a means for stabilizing recombinant-DNA traits must be developed.

An ongoing research activity at the Jet Propulsion Laboratory (JPL) is testing 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 bacterial chromosome, it could not be easily lost as the bacterium multiplies. (The Project's experimental work uses bacteria for convenience, but in industrial practice, yeast or other microorganisms are more likely to be used.)

During FY 1985 several bacterial strains containing multiple copies of a plasmid in their chromosomes were isolated and characterized. The degree of stability of one such structure in the absence of selection was determined.

2. Bioprocess Engineering

The Bioprocess Engineering work element emphasizes definition of the basic engineering relationships between molecular-scale events and macro-level parameters required for designing scaled-up biocatalyzed chemical production processes. Additionally, this work element has a responsibility for establishing the technical feasibility of critical bioprocess monitoring and control subsystems. In FY 1985, this work element had three research tasks: Productivity of Recombinant Microorganisms, Immobilized Cell System for Continuous Efficient Biocatalyzed Processing, and Enzyme Catalysis in Non-Aqueous Solutions.

a. Productivity of Recombinant Microorganisms. This research employs novel high-speed experimental methods in concert with mathematical models at the molecular and population level to provide a systematic method 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:

- (1) Kinetics of DNA replication, which determines the rates of growth and stability of desired recombinant cells.
- (2) Control and monitoring of plasmids, which are the DNA elements that contain the genetic instructions needed to make desired products more efficiently and selectively.

Because of the lack of quantitative descriptions ("kinetic expressions") of the growth and stability (plasmid retention) of recombinant cells as well as of the production and accumulation of enzymes and other cell products, it is not possible to optimize genetic or reactor design for biocatalyzed processes. 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. Quantitative descriptions are needed to understand and to optimize the combination of natural cellular products and components introduced by genetic engineering to maximize the efficiency of the overall system.

During FY 1985, mathematical modeling of cell processes at the molecular level has been continued, including the study of plasmid propagation in recombinant yeast (Saccharomyces cerevisiae) populations and generation of models for plasmid replication and gene expression control in recombinant E. coli.

b. Immobilized Cell System for Continuous Efficient Biocatalyzed Processing. The objectives of this task 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 to lead to a better understanding of reactor behavior and control predictability. The reactor consists of a column containing immobilized cells. The substrate (glucose sugar) and nutrients are passed into the bottom of the column and ethanol in water is continuously withdrawn from the top. The primary advantage of this type of bioreactor is increased production rate or productivity that is

obtained because the concentration of cells (biocatalyst) can be increased several times in comparison with a conventional tank reactor, resulting in corresponding higher rates of fermentation. Productivity is also increased in this specific bioreactor because the bacterium Z. mobilis is used instead of yeast. The former converts sugar to alcohol at rates that are also several times higher than yeast.

Accomplishments during FY 1985 included: (1) development of methods for preparing the small biocatalyst beads that contain the biocatalyst, including methods for increasing their density to prevent them from being washed out of the column; (2) measurement of bead characteristics, such as rates of diffusion of substrate and product into and out of the beads; and (3) successful operation of the bioreactor for greater than 400 h at productivities exceeding 200 g/(l-h) without replacement of the biocatalyst or feed sterilization.

c. Enzymes Catalysis in Non-Aqueous Solutions. The objectives of this task are:

- (1) To increase transport of substrates and products to and from biocatalyst sites by developing encapsulated enzyme systems in organic solvent solutions.
- (2) To determine the feasibility of such systems for producing commodity chemicals.

This work should provide enabling technology for the efficient production of certain chemicals that cannot be made on a large scale by biocatalyzed processes using currently available processes.

When enzymes (which are biocatalysts that can be used to increase the rates of formation of desired chemicals in biochemical processes) are used in some systems, the rates are decreased because either the organic chemical product or substrates are not sufficiently soluble in water. Rates are decreased because desired reactions are limited by the ability of the substrate or product to be rapidly transported to or from the site of enzyme action, which is in an aqueous environment.

The approach in this work is to use an organic liquid (for example, a solvent that will not dissolve in water) to increase the rates of transport to or from the active enzyme site to increase reaction rates. Such a system may also be used to favor formation of products where water is produced as a by-product because the presence of the latter tends to shift the reaction in the direction that corresponds to decreased product formation. Because the enzyme operates in an aqueous environment, it will be enclosed in small capsules to prevent deterioration of catalytic action by the organic solvent.

3. Process Design and Analysis

The Process Design and Analysis work element focuses on developing design tools and the process technology bases for scaling up bioprocesses to the pilot-plant level and providing overall assessments (i.e., system analyses) of biocatalyzed chemical production processes. In FY 1985, this work element comprised three research tasks: Software for Bioprocess Assessment; Bioprocess

Synthesis, Integration, and Analysis; and Techno-Economic Assessment of Microbial Ammonia Production.

a. Software for Bioprocess Assessment. The objective of this task is to develop a flowsheeting system that will provide a uniform basis for thermodynamic and economic assessment of alternative biotechnology processes. An existing computer program known as ASPEN was developed for DOE for assessment of synfuel processes. The approach in this task is to adapt DOE-ASPEN to function for bioprocesses by adding unit operations specific to bioprocesses, updating and extending costing routines, and extending the data base to include bacterial and cell properties. The adapted version of ASPEN is called BIOASPEN.

During FY 1985, this work was directed toward simulating a test example (i.e., production of acetone, butanol and ethanol from wood, using the ABE bioprocess). As part of this effort, a literature survey on hydrolysis of cellulose was completed to obtain data needed for the creation of new data structures. Also, the new release of ASPEN was acquired, installed, and tested; and new modules (e.g., for fermenters and other process units) were incorporated for further development of BIOASPEN. With this release, in addition to other new enhancements (including modifications to costing systems and addition of graphics capabilities) it is possible to extract on-line information on subroutines and variables that will assist in debugging and program modification.

b. Bioprocess Synthesis, Integration, and Analysis. The purpose of this task is to derive or synthesize 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.

Typical products being considered include ethyl, isopropyl and butyl esters, e.g., ethyl acetate, ethyl or butyl lactates, etc., where prices are in the range of \$0.40 to \$2.00/lb. The overall goal is to demonstrate decreased costs and energy consumption for the final product esters through new technology applications and process synthesis and integration.

Preliminary assessments conducted during FY 1985 indicate that for ethyl acetate, where ethanol and acetic acid are produced by fermentation and the final product ester is produced by chemical esterification, there is a significant reduction in energy consumption as a result of process synthesis and integration. The decrease is obtained because there is no need for complete separation and purification of the intermediates (ethanol and acetic acid) from large volumes of water and because of the opportunities for thermal energy recovery and utilization in the integrated process. Furthermore, separation and purification of the final product, ethyl acetate, requires relatively little energy because it is only slightly soluble in water; and it vaporizes at a lower temperature than either ethanol or acetic acid. As research advances progress, it may become possible to include biocatalyzed esterification to produce the final product, i.e., to develop a totally biocatalytic, energy- and cost-effective process for esters.

c. Techno-Economic Assessment of Microbial Ammonia Production.

This work was initiated late in FY 1985 to assess bioprocesses for ammonia production, including the evaluation of potential research advances. The current Haber-Bosch process for production of ammonia is energy-intensive, requiring 15 to 20 MBtu/lb of product, primarily because of high utilization and associated energy for conversion of natural gas to hydrogen synthesis gas. The final chemical synthesis step (hydrogen plus nitrogen to make ammonia) is not energy-intensive.

Most of the ammonia produced is used for fertilizer; therefore, it is an important high-volume, low-cost (\$0.10/lb) commodity chemical. Thus, if it could be made by a bioprocess that would be economically competitive with the conventional process, significant decreases in energy requirements and utilization of non-renewable resources could be realized.

4. Management

The Project's Management work element has four areas of responsibility: work element coordination (which includes conducting Project-competitive procurements), technology transfer, planning and integration, and the Guidance and Evaluation Panel.

During FY 1985, the evaluation and selection of proposals from the Project's first Request for Proposals (RFP), "Advanced Bioprocess Concepts and Design," were completed. Negotiations with the selected proposers were completed late in the fiscal year and five contracts were awarded. Each of these research contracts (listed below) addresses one or major barriers to the potential use of biocatalyzed processes for the production of chemicals.

<u>Proposal</u>	<u>Organization</u>
Application of Molecular Hydrogen in Fermentations	Celanese Research Company
Multiphase Fluidized Bed Reactor	Battelle-Columbus Laboratories
Multimembrane Bioreactor for Chemical Production	Cornell University
Membrane Modifications for Alcohol-Tolerant Bacteria	Colorado State University
Enzyme Catalysis in Non-Aqueous Solutions	University of California, Berkeley

The "Enzyme Catalysis in Non-Aqueous Solutions" task is summarized above under Bioprocess Engineering and in Section II. The objectives and approach of each of the remainder of these new efforts are summarized in Section III.

A Biocatalysis Workshop was held at Washington University, St. Louis, Missouri, December 4-5, 1984. This Workshop was planned as an additional step to promote productive interaction and communication among the diverse interests

involved. Through a combination of formal presentations and informal group discussion, the Workshop:

- (1) Elicited industry, research community, and other feedback to guide the development and future direction of ECUT biocatalysis research activities.
- (2) Reviewed results of ECUT-sponsored biocatalysis research performed to date.

The introductory part of the Workshop provided background on the ECUT program and its accomplishments to date. The major part of the Workshop focused on engineering of bioprocesses and on commercial aspects, including discussions of potential products from biomass and by-product utilization.

c. Planning for Biologically Effected Separation/Beneficiation Research and Development. The purpose of this task is to develop a detailed research plan in the area of Biological Separation for future expansion of bioprocess activities in the Biocatalysis Project to ensure that industrially relevant applications of biotechnology are included. Three areas of research are considered:

- (1) Biologically assisted leaching of metals.
- (2) Accumulation of metals from dilute solutions by microorganisms.
- (3) Separation of biologically produced chemicals from dilute process streams by microorganisms.

The plan will incorporate the assessment of technology status, definition of goals and Federal/private-sector responsibilities, program organization and budgets, industry laboratory cooperative strategies, and technology-transfer provisions.

The first part of this effort has been to complete a compilation of information on conventional separation systems used by the mining, chemical, food, and paper industries. Separation processes selected for further consideration are drying and evaporation because they are energy-intensive and are amenable to modifications using biotechnology. A second part of the task is a comprehensive literature survey, including continuing assessments, on topics relevant to bioseparation processes. This work is essentially completed, except for further additions. A third part of the work consists of contacts with relevant industrial and academic personnel through meetings, workshops, and discussions. Numerous contacts have been made, and responses from academic personnel have been promising.

d. Guidance and Evaluation Panel. The Guidance and Evaluation Panel consists of a group of leading authorities in the science and technology of biocatalysis, including representatives from industry and the academic community. The primary roles of the Panel are to review the Project's future plans and ongoing research.

The Panel met once in FY 1985 at the California Institute of Technology. The meeting focused on the Project's "technology-transfer" strategies. A general consensus was reached that:

- (1) The Project was addressing a technical area that industry was de-emphasizing.
- (2) Industry's de-emphasis of biocatalysis R&D was largely the result of falling oil prices.
- (3) In the long term, industry may relinquish its bulk chemical markets to Middle Eastern and Third World oil-producing countries.
- (4) Such a shift in world chemical production may increase the United States' vulnerability to oil and oil-products supply disruptions.
- (5) The Project's research could be viewed as a "national insurance policy" against the long-term effects of such potential disruptions.
- (6) The major challenge for the Project will be to maintain and build interest within industry for the Project's R&D findings and advances.

SECTION I

INTRODUCTION

A. PROJECT DESCRIPTION

1. The ECUT Program

The Energy Conversion and Utilization Technologies (ECUT) Program was established by the United States Department of Energy (DOE) in fiscal year (FY) 1981 as a centralized, generic research and development subprogram within the Office of Energy Systems Research. The ECUT Program has two major goals:

- (1) Evaluate new or innovative concepts for improved efficiency or alternate fuel use in energy conversion and utilization equipment.
- (2) Expand the technology base necessary for development of improved energy conversion and utilization equipment.

2. The Biocatalysis Project

The Biocatalysis Project is a component of the ECUT Program (Figure 1-1). This annual report describes the FY 1985 activities, accomplishments, and future plans of the Project.

a. Background (Prior to FY 1985). The Chemical Processes Project was established in May 1980 to investigate and develop new techniques and reaction sequences for the chemical processing industry (CPI). Concurrently, the Jet Propulsion Laboratory (JPL) was selected as the Project's lead laboratory. DOE funding for the Project began in July 1981. Prior to that date, JPL assisted the ECUT Office in evaluating and prioritizing potential Project work elements. These initial planning efforts resulted in the selection of two work elements for the Project: Catalysis (chemical catalysis and biocatalysis) and Separation.

During FY 1981, JPL started preparing biocatalysis and chemical catalysis advocacy papers and a multi-year plan for the Separation work element. Two research contracts were initiated at the California Institute of Technology (Caltech) in the Department of Chemistry and Chemical Engineering. The first contract focused on developing quantifiable relationships between biocatalysts produced by recombinant-DNA organisms and their environment. The second contract concentrated on developing efficient processes for hydrocarbon production in dilute (or waste-stream) feedstocks.

In FY 1982, the Chemical Processes Project had research activities in three areas: Catalyst Modeling, Biocatalysis, and Separation. Catalyst Modeling research concentrated on developing models for predicting and optimizing the reactivity of major heterogeneous catalysts. Biocatalysis

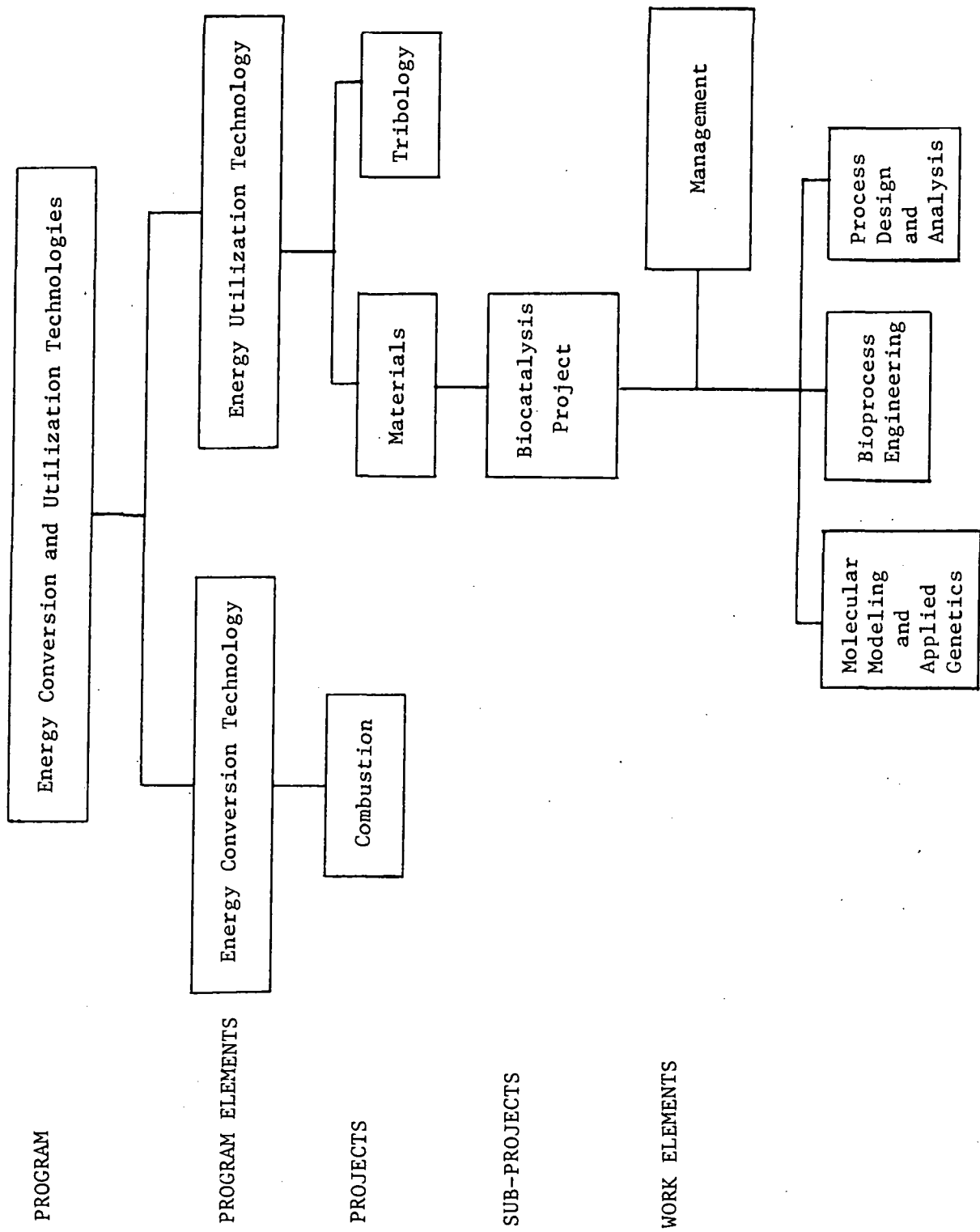


Figure 1-1. Relationship of the FY 1985 Biocatalysis Project to the Overall ECUT Program

research focused on resolving the major technical barriers that impede the potential use of biologically facilitated, continuous chemical production processes. These barriers included maintenance of stable genotypes in biocatalytically useful microorganisms, reduction of biological dependence on a water and dilute product environment, cellular-level operational requirements, process reactor-level operational requirements, and biocatalytic product separation technology optimization. Separation research focused on establishing the technical feasibility of innovative, less energy-intensive separation concepts than conventional techniques (e.g., distillation). Activities involved investigating techniques such as membrane separation, supercritical fluid extraction, and high-performance liquid chromatography.

In late 1982, the ECUT Program was reorganized. The Chemical Processes Project was consolidated and renamed the Biocatalysis Research Activity. Biocatalysis became the primary research focus and work element. Catalyst modeling activities were reorganized under a Molecular Modeling work element that emphasized establishing the technical feasibility for theoretically based design, optimization, and control of biocatalyzed and hybrid chemically/biologically catalyzed chemical production processes. The Separation work element was inactivated.

In FY 1984, the Biocatalysis Research Activity became the Biocatalysis Project. As sufficient funds became available to broaden industrial participation in the Project, the Project released its first RFP, "Advanced Bioprocess Concepts and Design." The RFP emphasized exploration of those novel processes that were generic in nature and that elucidated the relationships between events at the molecular/cellular level to macroscale events at the reactor level. Twenty-one proposals were received in response to the RFP. They came from a wide range of organizations, including private industry, universities, and research institutes. The majority of the proposals contained some form of proposed cost-sharing.

Also in FY 1984, the Project was reorganized into its current four work elements: Molecular Modeling and Applied Genetics, Bioprocess Engineering, Process Design and Analysis, and Management. Each of the first three work elements, as described below, addresses a key technical component for the development of the enabling technology base to efficiently produce large volumes of low-priced, energy-intensive chemicals.

b. Current Work Element Organization. As indicated above, the Project's R&D tasks are organized into four work elements.

Molecular Modeling and Applied Genetics. 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 application of biocatalysis.

Bioprocess Engineering. The Bioprocess Engineering work element emphasizes definition of the basic engineering relationships between molecular-scale events and macro-level parameters required for designing scaled-up biocatalyzed chemical production processes. Additionally, this work element is

responsible for establishing the technical feasibility of critical bioprocess monitoring and control subsystems.

Process Design and Analysis. The Process Design and Analysis work element focuses on developing design tools and the process technology bases for scaling up bioprocesses to the pilot-plant level and providing overall assessments (i.e., system analysis) of biocatalyzed chemical production processes.

The selection as well as the relationship between each of these three work elements has been defined by their scale of action. Development of the enabling technology base for Biocatalysis will require that research activities be conducted at various scales of action of specific size dimensions. Hence, the Molecular Modeling and Applied Genetics work element includes research activities at a molecular and cellular level (i.e., a scale of 1 micrometer and smaller). To successfully exploit the findings at the molecular and cellular level towards scale-up, the Bioprocess Engineering work element supports research in engineering kinetics and in control of state-of-the-art and novel reactor design concepts. The scale of action in this work element is generally at the 1-meter dimension or at the reactor level. Finally, the Process Design and Analysis work element focuses on activities that operate at the largest level (i.e., 1 acre or more in dimension), namely the entire process level.

Management. The Project's Management work element has four areas of responsibility: work element coordination, planning and integration, technology transfer, and the Guidance and Evaluation Panel. Work element coordination involves the administration and coordination of the Project's various work elements, including the development of work statements and evaluation criteria for Project competitive procurements. Planning and integration encompasses the technical multi-year planning activities for the Project and the monitoring and evaluation of in-house and contracted research tasks. Technology transfer entails ensuring strong, interactive relationships between the Project and industry by disseminating research results and soliciting industrial opinions regarding the critical areas for biocatalysis research. The Guidance and Evaluation Panel reviews the Project's future plans and ongoing research efforts.

3. Goal and Objective

a. Goal. The Biocatalysis Project is a mission-oriented, applied research and exploratory development activity that seeks to resolve critical bioprocess engineering problems that impede the biocatalyzed production of high-volume, energy-intensive chemicals where genetically modified and/or engineered microorganisms or derived enzymes are used.

b. Objective. To meet this goal, the Project has adopted the following objective: Establish a technical base of process design data and experimentally verifiable predictive models that will allow rational development and scale-up of large-scale biocatalyzed chemical production processes in the 1990s.

4. Rationale

The recent decline in petroleum feedstock prices and the continued existence of U.S. chemical processing plant overcapacity has presently made the CPI reluctant to significantly support large-scale bioprocess research for near-term return on investment. However, within the next decade it seems certain that the United States will become severely dependent on foreign sources of petroleum and petroleum-based feedstocks for the production of chemicals. In an effort to help address this emerging vulnerability, the DOE-ECUT Biocatalysis project is working to ensure the future availability of a bioprocess engineering technology base that would enable the development and utilization of alternative energy feedstocks by the chemical processing industry.

The advantage that biocatalyzed processes have over conventional petrochemical processes include: (1) the lack of a requirement for energy-intensive conditions (high temperatures and pressures) and (2) the capability of using renewable biomass resources, which are indigenously available. However, bioprocesses have a number of technical constraints that must be resolved before they can be considered to be competitive with conventional petrochemical processes. Some of the major constraints include:

- (1) Relatively low productivity (mass of product per unit reactor volume per unit time).
- (2) Inhibition of product synthesis at relatively low product concentration.
- (3) Loss of desired product synthesis of recombinant microorganisms as cells grow and divide (plasmid instability).
- (4) High energy consumption for product separation from dilute aqueous product streams.
- (5) Low yields, as mass of product per unit mass of feedstock.

These and related problems do not seem to be insurmountable; however, major research advances are required to raise the CPI's awareness of and interest in bioprocess applications.

B. ORGANIZATION OF THE REMAINDER OF THIS REPORT

Sections II and III of this report present, respectively, the FY 1985 accomplishments and FY 1986 plans of the Biocatalysis Project. Section IV lists reports and papers prepared or published by the Project during FY 1985.

SECTION II

FISCAL YEAR 1985 ACCOMPLISHMENTS OF THE BIOCATALYSIS PROJECT

As described in Section I, the Biocatalysis Project during FY 1985 was organized into four work elements. Table 2-1 shows the organization of the various tasks constituting these components of the Project. This section of the report describes the activities and accomplishments for each of these tasks during FY 1985.

Table 2-1. Organization of FY 1985 Activities of the Biocatalysis Project

Work Elements		
Molecular Modeling and Applied Genetics	Bioprocess Engineering	Process Design and Analysis
Enzyme reaction models for biocatalysis	Productivity of recombinant micro-organisms	Software for bioprocess assessment
Chromosomal amplification of foreign DNA	Immobilized cell system for continuous efficient biocatalyzed processing	Bioprocess synthesis, integration, and analysis
	Enzyme catalysis in non-aqueous solutions	Techno-economic assessment of microbial ammonia production
Management		
Competitive procurement		
Industry technology-transfer workshop		
R&D planning for biologically effected separation/ Beneficiation		
Guidance and Evaluation Panel		

A. MOLECULAR MODELING AND APPLIED GENETICS

As shown in Table 2-1, the Molecular Modeling and Applied Genetics work element comprised two research tasks in FY 1984-1985: Enzyme Reaction Models for Biocatalysis and Chromosomal Amplification of Foreign DNA.

1. Enzyme Reaction Models for Biocatalysis - California Institute of Technology, Department of Chemistry and Chemical Engineering

a. Description. The objective is to develop and test computer simulation methods capable of simulating the activity of enzymes in solution and enzymes immobilized on supports. This should allow design of supports for immobilizing enzymes without degradation of performance and design of enzymes for selectivity and resistance to poisons. The input data to the simulation is a set of analytic force fields describing the interactions between all atoms. These force fields were obtained by a combination of experimental and theoretical data for short-range interactions and theoretical data for long-range interactions (van der Waals, hydrogen bonding, electrostatics).

During 1985, a major thrust of the research has been to model and investigate the origin of thermophilicity of the enzyme thermolysin to establish methods of increasing the ability of enzymes to operate at higher temperatures. The active site is very rigid and hardly changes upon binding various inhibitors. This is partly because the active site is formed from two surfaces of two different domains, each with stable alpha helices that bind directly to the Zn of the active site. In addition, there are four Ca ions that organize a number of potentially hydrophilic groups into the interior region of the enzyme. Removal of some of these Ca ions leads to loss of activity (which can be reactivated by addition of Ca) and removal of all Ca leads to complete, irreversible degradation of the enzyme. The critical Ca has been identified as the one binding to residues 194 through 200. Removal of this Ca at elevated temperatures would lead to unraveling of a large region of one whole domain, eliminating part of the active site. This exposes TYR 193 to solvent. When TYR 193 is exposed, thermolysin will quickly cleave other thermolysin molecules at this residue because thermolysin is very selective for cleaving peptide bonds adjacent to tyrosine. Thus begins the process for complete degradation of the enzyme. Site-specific mutagenesis directed toward TYR 193 could result in an enzyme that remains intact at higher temperatures.

A second major thrust has been to examine another Zn-containing peptidase, Carboxypeptidase A (CPA), which is not thermophilic and which has a very different selectivity (it is an exopeptidase, that cleaves the C-terminal amino acid residue). CPA is one of the most extensively studied enzymes, and yet the basic mechanism of the catalytic process remains uncertain. Cleavage of peptide and/or ester bonds may result from H₂O attack at the carbonyl carbon, possibly promoted by GLU-270 or by direct nucleophilic attack of the glutamate. Most proposed mechanisms also invoke TYR-248 as either a proton donor or H₂O promoter. The role of the Zn²⁺ species is also questioned. It has been proposed to be involved in the polarization of the carbonyl group or as the coordination site for attacking H₂O and/or OH ligands.

As the first step of this study, three CPA species were examined for which crystal structural data are available. These data include the native form of the enzyme plus its first coordination shell of H₂O, the enzyme plus a slowly

hydrolyzed substrate bound in the active site, and the enzyme with an inhibitor bound. Investigation of these three systems allows for assessment of the amino acid residues involved in catalysis. The aromatic ring of TYR-248 is seen to move from its open/extended position in the native form to a conformation that holds any substrate into the active site cavity. The guanidinium residue of ARG-145 repositions itself in toward the active site and thus supports the concept of its electrostatic interaction with the C terminus, bringing the substrate into alignment for catalysis. The differences in conformation of the BLU-270 and neighboring H₂O molecules are not as straightforward. Their role(s) in activity are currently being studied.

b. FY 1985 Accomplishments

- (1) The origin of thermophilicity of the enzyme thermolysin was investigated, and a mechanism for high-temperature degradation of the enzyme has been formulated along with a potential approach for increasing enzyme thermophilicity.
- (2) Three species of a peptidase, Carboxypeptidase A (CPA) have been investigated to determine catalytic mechanisms at the molecular level to obtain information needed to design more selective biocatalysts and establish strategies for enzyme immobilization.

2. Chromosomal Amplification of Foreign DNA - Jet Propulsion Laboratory

a. Description. Work on this project started in FY 1983. The aim of the project is twofold: (1) to demonstrate the feasibility of a scheme that will allow routine transfer of genetic material directly to the chromosome of a microorganism and its amplification in situ and (2) to measure the genetic stability of such a structure and compare it with that of a standard genetically engineered microorganism in which the foreign genetic material is carried by plasmids. The expectation is that the new method will be far superior to the standard method in supplying stable strains of microorganisms that may be used in large-scale applications. (The Project's experimental work uses bacteria for convenience, but in industrial practice, yeast or other microorganisms are more likely to be used.)

b. FY 1985 Accomplishments

- (1) The degree of amplification was measured for the integrated plasmid in several of the tetracycline hyperresistant strains described in the FY 1984 report.² The Southern blot technique was used (see FY 1984 report) together with radioactive probe that reveals both sequences of the pBR322 plasmid (a component of the pEE240 plasmid used in this work) and of a gene of the biotin system. The latter is assumed to be present in single copy on the chromosome of

²

Biocatalysis Project Annual Report FY 1984, JPL Publication 85-31, Jet Propulsion Laboratory, Pasadena, California, April 15, 1985.

the amplified strains and is used as a reference for the amounts of probe radioactivity that hybridize with the DNA of the amplified strains. These relative amounts were determined by scanning densitometry of radioautographs of the blots, and were compared with similar data obtained for the DNA of the parental, non-amplified strain (C-4034 in the FY 1984 report). In six hyperresistant strains, independently derived from C-4034, the following copy numbers for the integrated plasmid were obtained: 3, 4, 4, 5, 13, 18. These results thus confirm and extend in a quantitative way the initial observations included in the FY 1984 report.

- (2) The sizes of the repeated DNA segments in two of the chromosomally amplified strains were estimated, using restriction enzymes and gel electrophoresis. Unique bands, corresponding to DNA fragments that are present in excess over single-copy DNA, were directly visible in stained electrophoresis gels when the DNA of such amplified strains was digested with the *Ava*I enzyme, which cuts the pEE240 plasmid only once. Bands of the following sizes were detected: for the hyperresistant C-4055 (a derivative of C-4020) strain, 7.4, 7.7, 8.3, and 9.7 kilobases, and for the hyperresistant C-4054 (a derivative of C-4034) strain, 7.7, 8.3, 9.7, and 12.5 kilobases. Thus, although the amplified segments in the two strains, as expected, give some common bands, they are clearly not identical. The calculated sizes of the repeated units for the two strains are 33.1 and 38.2 kilobases. These are minimum estimates, because smaller restriction fragments would go undetected. The two restriction fragments of 7.7 and 9.7 kilobases correspond in size to the left and right uncton fragments previously demonstrated in strains carrying one integrated copy of the plasmid (see FY 1984 report).
- (3) Amplified chromosomal structures are expected to be unstable as a result of genetic recombination. This was confirmed for the strain C-4054 when it was grown under non-selective conditions, i.e., in the absence of tetracycline. DNA preparations were made from such cultures at inoculation and after two and five passages. Each passage involves a thousandfold growth factor, i.e., about 10 cell generations. The copy number for the amplified structure was measured in each DNA preparation. At the beginning of the experiment the copy number was 18. After two passages, it was 2.9. After five passages, it was 0.9 (not significantly different from 1). The experiment was done at 40°C, where this strain is phenotypically *pola*⁻. Although these structures are clearly unstable in the absence of selection, a control performed with the corresponding strain carrying the same plasmid in the cytoplasm (i.e., non-integrated) showed an even higher instability.

- (4) The main question to be resolved in future work is whether the instability of chromosomally amplified structures can be corrected by the introduction of a mutation, recA, that inactivates the recombination systems of the bacterium. Experiments in this direction have been initiated. The combination recA⁻ polA⁻ is suspected of being non-viable. Using the ts-polA strain and doing the experiments at 30°C, recombinants have been obtained by transduction (using Phase P1 and taking advantage of the linkage between the recA⁻ and srl genes) that are presumed to be genotypically recA⁻ ts-polA⁻. These recombinants could be transferred only when the incubation temperature was further lowered to 20°C, however, and even then they grew extremely poorly. As a control, a similar experiment was done with the same strain made polA⁺.

B. BIOPROCESS ENGINEERING

The Bioprocess Engineering work element encompassed three research tasks: Productivity of Recombinant Microorganisms, Immobilized Cell System for Continuous Efficient Biocatalyzed Processing, and Enzyme Catalysis in Non-Aqueous Solutions.

1. Productivity of Recombinant Microorganisms - California Institute of Technology, Department of Chemistry and Chemical Engineering

a. Description. The objective of this work is to develop kinetic models of recombinant systems at the molecular level to explore qualitative and quantitative features provided by different genetic designs. With the extremely powerful capabilities now available for precise design of promoter and operator regions at the nucleotide sequence level, it is important to use such quantitative and systematic modeling relationships to assess complex interactions in recombinant systems and to compare the effects of many potential alternative molecular designs. Current capabilities for molecular genetic manipulation provide so many options that empirical approaches are inadequate for optimization. Instead, quantitative systematic models are necessary for rational and efficient expression-system design to optimize the genetic and environmental aspects of fermentation processes, including bioreactor configuration and control strategies. However, to verify such models, real-time experimental methods for control and monitoring of plasmids are required. As described under the summary of the Chromosomal Amplification of Foreign DNA task, recombinant-DNA microorganisms, as they reproduce, tend to lose their plasmids and, therefore their ability to make desired products. Because the amount of enzymes or other products synthesized depends upon the number of plasmids per cell, a technique is needed to monitor a large number of cells individually for the existence and level of plasmids.

A method (using flow cytometry) to accomplish this type of measurement was initially demonstrated in FY 1982. In FY 1983, the technique was developed further, including an increase in sensitivity by a factor of 10^3 and improved definition of conditions for optimized measurements and enhanced detail. Since then, resolution and applicability of the method has been further improved by

identification of new labeling reactions and by implementation of multiparameter analysis of the data using a microcomputer.

b. FY 1985 Accomplishments. Mathematical modeling and experimental measurements of plasmid concentrations in individual cells have been continued. Specific highlights are as follows:

- (1) Systematic utilization of information from the molecular biology and biochemistry literature to formulate robust, genetically structured models for plasmid replication and gene expression control systems in recombinant E. coli has been demonstrated.
- (2) Significant advances in the study of plasmid propagation in recombinant Saccharomyces cerevisiae populations include (a) increased sensitivity to allow detection of single-plasmid copies and (b) further development of the assay method for plasmid-containing cells in the recombinant population to permit measurements to be made more than an order of magnitude faster than by alternative, conventional plating methods.
- (3) Analysis of growth of unstable recombinant populations, taking into account growth of plasmid-free cells in selective medium, was completed for the case of a plasmid-containing population with relatively homogeneous plasmid content.
- (4) Studies of optimal batch reactor operating strategies (that is, when plasmid amplification should be initiated and when plasmid-gene expression should be activated) has continued, based upon the phenomenological model of unstable recombinant populations, including effects of plasmids and products on host-cell growth.
- (5) The influences of different types of continuous-flow reactor cascades, in which different conditions are used in each reactor to carry out plasmid amplification and gene expression, have also been explored by simulation studies using the recombinant population model.

2. Immobilized Cell System for Continuous Efficient Biocatalyzed Processing - Oak Ridge National Laboratories

a. Description. To decrease energy consumption and capital equipment costs, it is necessary to increase productivities of biocatalyzed processes significantly. Therefore, the goals of this task 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 to lead to a better understanding of reactor behavior and control predictability to increase reactor efficiency. The immobilized catalyst beads are prepared by forced flow of hydrocolloidal gels (consisting of up to 5 wt% of kappa-carrageen or 2 wt% calcium alginate in water) and the bacterium

Z. mobilis through a small nozzle into dilute KCL solution with imposed vibration to obtain beads about 1 mm in diameter. The incorporation of inorganic oxide powder is used to control bead density and the oxide powder plus a more dense surface film retards cell leakage. Because the bead density is higher than the glucose nutrient solution flowing into the column, the beads are retained even at high dilution rates, corresponding to high ethanol productivity.

b. FY 1985 Accomplishments

- (1) Methods have been developed for preparing immobilized biocatalyst beads containing the microorganism Z. mobilis for conversion of sugar to ethanol in a fluidized bed column bioreactor.
- (2) Measurements of substrate glucose diffusion coefficients into and from 4 wt% kappa carrageen and 1 wt% alginate beads showed the coefficients were from 3.8 to 6.3×10^{-6} cm²/s, depending on composition and bead size, in comparison with the coefficient of 6.8×10^{-6} cm²/s for distilled water; therefore, immobilization should not seriously decrease biocatalyst reaction rates.
- (3) The column bioreactor has been operated continuously for 400 h at a productivity of 243 g/(l-h) with a conversion rate of 80% and a yield of greater than 95% without feed sterilization.
- (4) A continuous stirred tank reactor (CSTR) containing biocatalyst beads has been operated at a productivity of 49 g/(l-h) using raw corn dextrose as a substrate and corn light steep water (LSW, which is normally a waste stream) as a nutrient component, which indicates that the technology transfer of techniques and knowledge in operations of immobilized Z. mobilis will not be limited by use of industrial feedstocks or nutrients.

3. Enzyme Catalysis in Non-Aqueous Solvents - University of California, Berkeley

a. Description. To permit the use of enzymes (in either free or immobilized form) to enter and, more broadly into the production of large-scale chemicals, techniques for increasing the solubility and transport rate of organics at the site of enzyme action must be found. The approach proposed here is based on the use of a second organic liquid in which the substrate or product are soluble to a much greater extent than in an aqueous system. The second organic liquid may be water-miscible or immiscible. Such systems are used currently for the enzymatic transformation of steroids. A second aspect of the use of enzymes in non-aqueous environments is the potential to run many reactions "backwards." In cases in which water is a reaction substrate, its high activity in aqueous solution usually results in a shift of equilibrium to favor the normal products of the reaction. In a non-aqueous system such reactions may be forced in the desired, reverse direction because water will be

present at low concentrations. Examples of this type of reaction include esterification, peptide synthesis, and dehydration reactions such as urea formation from ammonium carbonate $(\text{NH}_4)_2\text{CO}_3$. The objectives of this work are:

- (1) To develop an encapsulated enzyme system that will allow biocatalysis to occur in the presence of water within the capsules, with an organic solvent outside that will increase mass transport of substrates and products with low water solubility to and from biocatalyst sites.
- (2) To determine the feasibility of using a similar system for urease-catalyzed conversion of ammonium carbonate or bicarbonate to urea, as a model of the use of enzymes to synthesize a commodity chemical.

The normal function of urease in an aqueous environment is to catalyze the reverse of this reaction (conversion of urea to ammonium carbonate). The system to be developed will allow reaction reversal where water is one of the products and normally shifts the reaction equilibrium in the direction of reactants, as in condensation reactions, such as esterification or conversion of ammonium carbonate to urea.

The encapsulation procedure using 1,6-diaminohexane and terephthaloyl chloride as interfacial condensation reactants is being optimized to increase biocatalysis reaction rates and maintain stability of the microcapsules. Although the reaction discussed in the proposal is reduction of cortisone, which requires a cofactor system and has the advantage that cofactor regeneration within the microcapsules may be developed, a simpler model enzyme oxidation system that does not require cofactors is being investigated to first develop and characterize enzyme encapsulation.

b. FY 1985 Accomplishments:

- (1) The draft of the Conceptual Design Analysis and Research Methodology Report was submitted, and revisions have been initiated.
- (2) Technical work was initiated in the last quarter of the fiscal year.
- (3) A two-phase system has been developed for the oxidative enzymatic conversion of cholesterol to 4-cholesten-3-one where the conversion was increased from 10% (in the totally aqueous system) to 50% after 5 h, but enzyme activity decreased with time.
- (4) An encapsulation system using interfacial condensation of a diamino alkane with a diacid chloride has been partially developed in which conversion is about twice that of the all-aqueous system and enzyme stability is maintained for more than four days.
- (5) A reaction using the enzyme tryptophanase (TRPase) to convert indole, pyruvic acid, and ammonia to L-tryptophan (plus water) has been selected and investigated as a model

reaction to simulate the type of reaction in which ammonium bicarbonate is converted to urea and water.

- (6) For the ammonium pyruvate system a reverse micellar and liquid membrane concept has been defined in which tryptophan product should be transferred out of the micelles, with substrates transported into the micellar phase for conversion to product.

C. PROCESS DESIGN AND ANALYSIS

The Process Design and Analysis work element comprised three research tasks: Software for Bioprocess Assessment; Bioprocess Synthesis, Integration and Analysis; and Techno-Economic Assessment of Microbial Ammonia Production.

1. Software for Bioprocess Assessment - Washington University

a. Description. The objective of the proposed work is to develop a flowsheeting system that will provide a uniform basis for thermodynamic and economic assessment of alternative biotechnology processes. An existing computer program known as ASPEN was developed for DOE-ASPEN to function for bioprocesses by adding unit operations specific to such processes, updating and extending costing routines, and extending the data base to include bacterial and cell properties.

To assist in model development, work has been proceeding on an on-line documentation aid for programming ASPEN and BIOASPEN to provide information on the hierarchy of sub-routines used in the simulation of any process module. This aid is being developed because a test problem showed that ASPEN contained inadequate documentation for solids-handling modules, and there was no convenient way to upgrade the documentation. A flowsheet for the Acetone, Butanol and Ethanol (ABE) bioprocess was developed and simulated. Required modifications to ASPEN have been defined (e.g., data on physical parameters and thermodynamic behavior of species involved in bioprocesses, such as glucose, xylose and wood).

b. FY 1985 Accomplishments

- (1) A test process (acetone, butanol, and ethanol or ABE bioprocess) was simulated, and some limitations of the first version BIOASPEN were defined.
- (2) Modules and data banks for some components specific to bioprocesses, e.g., costing modules and modules for hydrolysis steps, have been developed to allow more rigorous simulations of bioprocesses using BIOASPEN.

2. Bioprocess Synthesis, Integration and Analysis - Jet Propulsion Laboratory

a. Description. This effort is conducting assessments of selected processes that are expected to be commercially viable as the required enabling

technology becomes available. The assessments will be used (1) to derive or synthesize a series of candidate bioprocesses and (2) to conduct relevant energy-economic analyses and comparisons of conventional and commodity chemical production processes. 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.

A base-case process for ethyl acetate is being synthesized and assessed because it involves biocatalytic production of two large-scale industrial chemicals and conversion to an added-value product, and because many of the research advances derived from current work (e.g., on new bioprocess concepts and designs) can be evaluated relative to this process to determine expected energy-economic improvements. In this case, less energy should be required to produce a higher value product than either ethanol or acetic acid because of inherent advantages of process synthesis and integration. For example, there is no need to separate the intermediates completely from large volumes of water, the energy-consuming step in purification of ethanol from the azeotrope is avoided, and the product ester is more volatile than either ethanol or acetic acid, which results in much lower energy consumption for product recovery.

b. FY 1985 Accomplishments

- (1) The overall ethyl acetate base-case process has been structured and subjected to preliminary assessments.
- (2) Liquid-vapor equilibrium curves showing compositions at temperatures below the boiling point of aqueous alcohol at different concentrations have been calculated to allow comparison of energy requirements for various vapor separation processes.
- (3) Reactor modeling of base-case kinetics indicates that, for this specific system, ethanol inhibition at concentrations up to 60 g/l is not a serious limitation; but the rate of fructose conversion will not allow dilution rates higher than about 0.3 h^{-1} at feedstock conversion greater than 95%, which corresponds to productivity of about 20 g/(l-h).
- (4) A survey of numerous continuous ethanol processes indicates that high productivities (greater than 20 g/(l-h) for yeast and greater than 100 g/(l-h) for Z. mobilis) have not been obtained when product concentrations exceeded 60 g/l.

3. Techno-Economic Assessment of Microbial Ammonia Production - Chem Systems Inc.

a. Description. Work has recently been initiated to assess bioprocesses for production of ammonia, including comparisons of a bioprocess base-case design and conventional Haber-Bosch technology with potential improved bioprocesses. The latter will include realistic anticipated research advances based on literature surveys and sensitivity analysis of the base case

to parameters that reflect the most important design factors, e.g., productivity, product concentration, and substrate and nutrient requirements.

Because the current overall Haber-Bosch process for ammonia is energy-intensive, and annual production of ammonia is higher than most other chemicals, availability of an improved bioprocess that would compete with the conventional process for ammonia would have a major impact on national energy requirements of the chemical processing industries.

Klebsiella pneumoiae and the Anabaena produce ammonia and have been successfully immobilized, with effective catalyst lives of 200 to 340 h and maximum product concentration of 0.02 g/l. The major difference in process design selection criteria is that Klebsiella requires a source of amino acid as well as a relatively expensive carbon and energy source, glucose, and nitrogen rather than air, whereas the prototroph Anabaena can use carbon dioxide enriched air as a carbon and nitrogen source, and water. Therefore, base-case design is proceeding on the basis of a system that will use blue-green algae (Anabaena) to fix nitrogen from air, water, and carbon dioxide, using sunlight as an energy source.

b. FY 1985 Accomplishment. The base-case (or model) microorganism has been selected, and preliminary process design characteristics have been defined for a bioprocess based on utilization of the microorganism Anabaena.

D. MANAGEMENT

Management work element activities during FY 1985 included the competitive procurement "Advanced Bioprocess Concepts and Designs," the Industry Technology Transfer Workshop, R&D Planning for Biologically Effected Separation/Beneficiation, and the Guidance and Evaluation Panel.

1. Competitive Procurement - "Advanced Bioprocess Concepts and Designs"

a. Description. An RFP was released by the Project in May 1984 for generic bioprocess concepts and designs. By the end of FY 1984, 21 proposals had been received from private industry, universities, and research institutes, the majority of which proposed some form of cost-sharing. The RFP emphasized exploration of those novel processes that were generic in nature and that elucidated the relationships between events at the molecular/cellular level to macroscale events at the reactor level. The Project expects that this integrated biological and chemical engineering approach will provide generic bioprocess conceptual designs that will stimulate the development and eventual commercialization of large-scale, efficient bioprocesses.

b. FY 1985 Accomplishment. The evaluation and selection of proposals from the RFP were completed. Negotiations with the selected proposers were completed late in the fiscal year and five contracts were awarded. Each of these research contracts (listed below) addresses one or more major barriers to the potential use of biocatalyzed processes for the production of chemicals.

<u>Proposal</u>	<u>Organization</u>
Application of Molecular Hydrogen in Fermentations	Celanese Research Company
Multimembrane Bioreactor for Chemical Production	Cornell University
Multiphase Fluidized Bed Reactor	Battelle-Columbus Laboratories
Membrane Modifications for Alcohol-Tolerant Bacteria	Colorado State University
Enzyme Catalysis in Non-Aqueous Solutions	University of California, Berkeley

The "Enzyme Catalysis in Non-Aqueous Solutions" task is summarized above under Bioprocess Engineering. The objectives and approach of each of the remainder of these new efforts are summarized in Section III.

2. Industry Technology Transfer Workshop

a. Description. Technology transfer entails ensuring strong, interactive relationships between the Project and industry by disseminating research results and soliciting industrial opinions regarding the critical areas for biocatalysis research.

b. FY 1985 Accomplishment. A biocatalysis workshop was held at Washington University, St. Louis, Missouri, December 4-5, 1984. This workshop was planned as an additional step to promote productive interaction and communication among the diverse interests involved. Through a combination of formal presentations and informal group discussion, the workshop:

- (1) Elicited industry, research community, and other feedback to guide the development and future direction of ECUT biocatalysis research activities.
- (2) Reviewed results of ECUT-sponsored biocatalysis research performed to date.

The introductory part of the Workshop provided background on the ECUT program and its accomplishments to date. The major part of the Workshop focused on engineering of bioprocesses and on commercial aspects, including discussions of potential products from biomass and by-product utilization.

3. Biologically Effected Separation/Beneficiation - Idaho National Energy Laboratory

a. Technical Description. The purpose of this task is to develop a detailed research plan in the area of Biological Separation for future expansion of bioprocess activities in the Biocatalysis Project to ensure that

industrially relevant applications of biotechnology are included. Typical research areas being considered include:

- (1) Biologically assisted leaching of metals.
- (2) Accumulation of metals from dilute solutions by microorganisms.
- (3) Separation of biologically produced chemicals from dilute process streams by microorganisms.

The plan will incorporate the assessment of technology status, definition of goals and Federal/private-sector responsibilities, program organization and budgets, industry laboratory cooperative strategies, and technology-transfer provisions. The work has been developed in three parts to present a broad view of opportunities in relation to improved energy economics through use of bioseparation methods.

Part 1 of the plan is a compilation of information on energy-intensive separation processes used by the mining, chemical, food/agriculture, and paper industries. These industries use processes that provide the best opportunities for the use of bioseparation technology and use a great deal of energy. The most energy-intensive separation systems used in these industries are distillation, evaporation, and drying. The objective of Part 1 of the project is to define present separation processes for the purpose of making comparisons with potentially applicable bioseparation processes.

Part 2 will consist of a comprehensive literature survey involving a wide variety of topics relevant to bioseparations. As new insights into the project are acquired, new sections are being added. Each section covers a specific area of bioseparation potential: polymerization, biooxidation, biodegradation, biohydrometallurgy, and coal desulfurization. Each of the main topics is divided into subtopics so that many aspects of each main topic are covered. The objective of Part 2 is to survey the literature for the purpose of determining which current biotechnical advances can be used for bioseparation processes.

In Part 3 of the task, some contacts have been made with appropriate personnel in industrial and academic institutions. The contacts are primarily being made through professional meetings. Dr. J. H. Wolfram presided over a Symposium on the Chemistry of Biotechnology. Dr. H. J. Hatcher attended the meetings of the Division of Microbial and Biochemical Technology and Division of Fuel Chemistry in Chicago, Illinois. A Research Assessment form has been sent to 62 persons to request their opinions on potential areas of research. Typical areas are: biopolymers for use as surfactants, dispersants and emulsifiers; membrane technology for water and materials recovery; biodegradable process chemical; and polymer manufacturing using enzyme technology. Most of the addressees were contacted by telephone and asked for opinions and cooperation. In all cases except two, a positive and cordial reply was given. Most of the areas listed in the form were suggested by addressees. The goal of Part 3 is to make as many contacts as possible with knowledgeable persons from industrial and academic institutions. Support for the project will be based on research initiatives that are highly regarded by the industrial and academic communities. The purpose of the contacts is to obtain their assessment of what these research initiatives should be.

b. FY 1985 Accomplishments

- (1) An overall strategy has been developed in the area of Biological Separation/Beneficiation to determine which research initiatives should be pursued and to develop a detailed research plan that will eventually minimize industrial energy consumption, specifically in separation processing.
- (2) A survey of energy aspects of conventional systems used by the mining, chemical, food, and paper industries has been completed, and a comprehensive literature and industrial survey on topics relevant to bioseparation processes has been initiated.

4. Guidance and Evaluation Panel

a. Description. The Panel consists of a group of leading authorities in the science and technology of biocatalysis, including representatives from industry and academia. The Panel's roles are to review the Project's future plans and ongoing research.

b. FY 1985 Accomplishments. The Panel met once in FY 1985 at the California Institute of Technology. The meeting focused on the Project's "technology-transfer" strategies. A consensus was reached that:

- (1) The Project was addressing a technical area that industry was de-emphasizing.
- (2) Industry's de-emphasis of biocatalysis R&D was largely the result of falling oil prices.
- (3) In the long term, industry may relinquish its bulk chemical markets to Middle Eastern and Third World oil-producing countries.
- (4) Such a shift in world chemical production may increase the United States' vulnerability to oil and oil-products supply disruptions.
- (5) The Project's research could be viewed as a "national insurance policy" against the long-term effects of such potential disruptions.
- (6) The major challenge for the Project will be to maintain and build interest within industry for the Project's R&D findings and advances.

SECTION III

FISCAL YEAR 1986 PLAN FOR THE BIOCATALYSIS PROJECT

The organization of the Project's planned FY 1986 tasks is shown in Table 3-1.

Table 3-1. Organization of FY 1986 Activities of the Biocatalysis Project

Work Elements		
Molecular Modeling and Applied Genetics	Bioprocess Engineering	Process Design and Analysis
Enzyme reaction models for biocatalysis	Productivity of recombinant micro-organisms	Software for bioprocess assessment
Chromosomal amplification of foreign DNA	Immobilized cell system for continuous efficient biocatalyzed processing	Bioprocess synthesis, integration, and analysis
	Enzyme catalysis in non-aqueous solutions	Techno-economic assessment of microbial ammonia production
	Application of molecular hydrogen in fermentations	
	Multimembrane bioreactor for chemical production	
	Multiphase fluidized bed reactor	
	Membrane modifications for alcohol-tolerant bacteria	
Management		
	R&D planning for biologically effected separation/beneficiation	
	Project multi-year planning	
	Guidance and Evaluation Panel	

This section briefly describes the FY 1986 content of the Project's work elements.

A. MOLECULAR MODELING AND APPLIED GENETICS

Both of the FY 1985 research tasks of the Molecular Modeling and Applied Genetics work element will continue in FY 1986.

1. Enzyme Reaction Models for Biocatalysis - California Institute of Technology

Future work will include modeling and simulation of CPA or related Zn^{2+} systems for the patterning of more efficient and selective catalytic sites, including the design of selective inhibitors and the establishment of concepts and strategies for immobilizing enzymes on supports in such a way that catalytic activity and stability are enhanced.

The Chromosomal Amplification of Foreign DNA research task will attempt to increase the stability of the genetic structures isolated to date through this work by the introduction in those bacteria of mutations that minimize genetic recombination.

B. BIOPROCESS ENGINEERING

All of the FY 1985 tasks in the Bioprocess Engineering Work Element will continue in FY 1986. Four additional research contracts resulting from the Project's first RFP will also be initiated: Application of Molecular Hydrogen in Fermentations, Multimembrane Bioreactor for Chemical Production, Multiphase Fluidized Bed Reactor, and Membrane Modifications for Alcohol Tolerant Bacteria.

The FY 1986 plans for each of the ongoing (from FY 1985) research tasks are summarized below, followed by a more detailed description of each of the new FY 1986 research contracts.

1. Productivity of Recombinant Microorganisms

The stability (i.e., retention) of yeast plasmids in populations grown in continuous culture will be determined to assess the long-term effects of continuous cultivation and of processing rate on stability. The processing rate will be varied by changing the dilution rate, which is the reciprocal of the residence time of the culture in the reactor. Calibration of the method for determining plasmid content in individual cells will be verified by comparisons with results from alternative methods that are much slower than the flow cytometry method used in this work. Modeling will also be extended to investigate a runaway phenomenon in which plasmid copy number increases to very large values, and which is expected to lead to decreased plasmid stability. In addition, the effects of certain promoters will be investigated by simulation methods to obtain new insights into ways to design regulatory sequences in

plasmids to obtain better control and to avoid the instability and runaway phenomena to be investigated in parallel with this research.

2. Immobilized Cell System for Continuous Efficient Biocatalyzed Processing

Additional bead characterizations will be performed, including measurements of diffusion rates into and from immobilized biocatalyst sites of higher molecular weight species, to provide data needed for optimizing bioreactor design. Further operating tests of the fluidized bioreactor will also be conducted for this effort, and the use of immobilized Z. mobilis on a crude sugar media, of the type available industrially, will be demonstrated in the fluidized bed reactor to determine if industrial feedstock can be used to make ethanol continuously at high productivity.

3. Enzyme Catalysis in Non-Aqueous Solutions

Future work on the cholesterol oxidase conversion will include: (1) determination of the rates of formation of product in aqueous and two-phase systems as well as quantitative effects of acidity and inhibition of rates by the product formed; (2) determination of the effects of substrate and product transport when the enzyme is encapsulated; and (3) complete development of efficient, robust enzyme microcapsules. In work on tryptophan synthesis, the rates of conversion, including the effects of transport rates in a reverse micelle system (or mixture of water droplets containing the enzyme in an organic solvent, stabilized by an emulsifying agent, or surfactant) will be determined; and the micelle system performance will be optimized on the basis of effects of system parameters on rates and stability.

4. Application of Molecular Hydrogen in Fermentations - Celanese Research Company

The objective of this project is to increase the versatility of glucose as a feedstock for commodity chemicals. Extending the versatility of glucose will be tested by producing succinate from glucose in the presence of hydrogen (as a co-substrate). New product production will be tested by producing 1,3-propanediol from glucose and hydrogen. Organisms will be screened from either laboratory stocks or from enrichments for their ability to use hydrogen and glucose under anaerobic conditions, producing the required products. If necessary, mutants will be constructed lacking enzymes that might interfere with the production of the desired end products.

The effort is divided into three tasks: (1) strain screening and isolation, (2) succinic acid/hydrogen experiments and strain construction, and (3) 1,3-propanediol/hydrogen experiments and strain construction.

- (1) Strain screening and isolation. Primary enrichments of either succinic acid or 1,3-propanediol producers have been made using Winogradsky columns containing hydrogen and either fumaric acid or glycerol as the sole carbon source. Secondary enrichments for succinic acid producers will be made using the chemostat. The chemostat will be operated in a fumaric acid limited fashion

supplemented with hydrogen. Following chemostat treatment, succinate producers will be isolated on medium that contains fumarate and hydrogen. These isolates will then be tested for their ability to reduce benzyl viologen in the presence of hydrogen while growing on glucose. Isolates will then be tested for their ability to shift the redox balance of the products when shifted from fumarate/hydrogen to glucose/hydrogen. For 1,3-propanediol producers secondary enrichment will consist of growth on glycerol minimal medium followed by isolation of those organisms that are not inhibited by the presence of hydrogen. Isolates will then be checked for their ability to reduce benzyl viologen while fermenting glucose in the presence of hydrogen. Positive isolates will then be checked for their ability to shift the redox balance of the end products when moved from glycerol to glucose metabolism under hydrogen.

- (2) Succinic acid/hydrogen experiments and strain construction. Organisms identified in Task 1 that perform mixed acid fermentations from glucose producing succinic acid in the presence of hydrogen will be used to assess the effect of increasing concentrations of hydrogen on their fermentation products. If strains are not found that can use hydrogen in the presence of glucose, then genetically manipulated strains need to be constructed. Mutants that lack enzymes that divert the flow of carbon from glucose to succinic acid and carbon dioxide will have to be isolated. Mutants will be isolated by defining growth conditions that show hydrogen-dependent growth. Strains will then be cycled under conditions of hydrogen-dependent and independent growth. Strains that are constitutive for hydrogen and glucose will have a growth advantage over non-constitutive strains due to their already induced hydrogenase system. If necessary, chemical mutagenesis can be used to produce constitutive hydrogen utilizers. Survivors of such treatment will be isolated on agar plates, and hydrogen utilizers will be identified by the reduction of methyl viologen.
- (3) 1,3-propanediol/hydrogen experiments and strain construction. The experimental approach for this task is similar to Task 2 except the target chemical is 1,3-propanediol instead of succinic acid. It is suggested that for the strain construction experiments the target enzymes to be eliminated will be the triose phosphate dehydrogenase and glyoxylase enzymes. These mutants will be isolated following Penicillin enrichment on semi-selective media. Mutants will be identified as small colonies on minimal agar containing limited quantities of glycerol and excess glyceraldehyde and confirmed by direct enzyme analysis.

5. Multimembrane Bioreactor for Chemical Production - Cornell University

The major objective of this work is to prove the technical feasibility of the multimembrane reactor. The membrane reactor involves a compartmentalized system of cell growth, product extraction, and waste CO₂ removal using semipermeable membranes. The primary technical advances that need to be made will be in the choice of suitable membranes, the use of a solvent extraction system, and the determination of the effects of immobilization on the physiological status of the microbial cells. A model will be

developed, and a preliminary economic assessment on the overall system will be conducted.

In FY 1986, proof-of-concept experiments will be conducted to demonstrate the performance of the Multimembrane Reactor (MMR). This will include measuring the effects of temperature and nutrient supply on the MMR performance. Also, candidate membranes will be evaluated for potential MMR applications.

6. Multiphase Fluidized Bed Reactor - Battelle-Columbus Laboratories

This effort is designed to develop a multi-phased fluidized bed (MPFB) bioreactor based on existing technology for a multi-phased chemical reactor. In the MPFB system, an immobilized cell or enzyme is mixed in the reactor with an insoluble solvent or liquid. As the cells, solvent, and liquid interact, the solvent continuously absorbs the chemical that is produced biochemically. The chemical of interest is stripped from the solvent and the solvent recycled for further use. It is this concept that must be proven in actual practice. It will be done in two stages. The first stage will use a model system (without biological reactions) to investigate the concept, followed by experiments with an actual biological process.

During FY 1986, preparation and characterization of immobilized biocatalysts for feasibility demonstrations on the MPFB bioreactor will be initiated; development of a generic database for the design and initial scale-up of MPFB bioreactor will be started; and the first "cold flow" tests of the MPFB bioreactor will be conducted.

7. Membrane Modifications for Alcohol-Tolerant Bacteria - Colorado State University

The objective of this project is to quantitate improvements in butanol tolerance and production in Clostridium acetobutylicum that are connected with cell membrane modification. Modification of the cell membrane is accomplished by the incorporation of very long chain fatty acids into the bacterial membranes. Quantitation of the effect of fatty acid incorporation is accomplished by the characterization of the active transport of glucose into modified cells.

The effort is divided into four tasks: (1) incorporation and analysis of very long chain fatty acid into cellular membranes, (2) characterization of glucose transport, (3) determination of fermentation parameters, and (4) experimentation with fatty acids from practical seed sources.

- (1) Incorporation and analysis of very long chain fatty acids into cellular membranes. C. acetobutylicum will be grown in biotin-deficient medium supplemented individually with oleic, elaidic, eicosenoic (cis and trans), erucic and bassidic acids. Cells will be adapted to growth in the medium by repeated transfer of log phase cells. Stationary phase cells from the supplemented cultures will be extracted in chloroform/methanol, purified, esterified, and analyzed by gas chromatography for fatty acid cell membrane incorporation.

- (2) Characterization of glucose transport. Kinetic studies of glucose uptake will be quantitatively determined by proton uptake as measured by pH. Exponentially growing cells will be harvested and suspended in salts solution. Rates of glucose uptake will be determined and activation energy for transport will be obtained from Arrhenius plots of test data for each membrane modified population.
- (3) Determination of fermentation parameters. Fermentation parameters using the chemostat will be determined for the culture exhibiting optimal butanol tolerance. The parameters to be determined include butanol production rates, productivity, yields, and final product concentration.
- (4) Experimentation with fatty acids extracted from practical seed sources. Very long chain fatty acids derived from various seeds will be tested for their ability to confirm butanol tolerance to cells following the procedure outlined in Task 1.

C. PROCESS DESIGN AND ANALYSIS

All three of the FY 1985 Process Design and Analysis tasks will continue in FY 1986.

1. Software for Bioprocess Assessment - Washington University

During the first quarter of FY 1986, some work will be carried out at Washington University to develop improved modules for simulation of the ABE process, including more complete databases and costing routines. After that time, this task may be funded directly from DOE to SERI where proposed follow-on work can be done to complete the development of BIOASPEN. This effort will include:

- (1) Development of generalized modules for batch and continuous bioprocesses, sterilization, various types of fermenters (e.g., gas-lift, immobilized whole cell, and membrane reactors) and downstream processing units (e.g., flocculators, centrifuges, extractors and dryers).
- (2) Development of a more extensive data bank containing additional microorganism properties, stoichiometric and yield coefficients, specific heats, compositions, solubility data, and required data on nutrients and enzymes.
- (3) Selected bioprocess analysis, including assessment of energy requirements and costs.

2. Bioprocess Synthesis, Integration and Analysis - Jet Propulsion Laboratory

Future work will include detailed modeling of reactor kinetics for the base-case system and an overall assessment of energy and cost requirements. This system will be compared with at least one conceptual

process being investigated as part of the Biocatalysis Project, where expected research advances should result in substantially decreased costs and energy requirements.

It has been observed that in continuous biocatalyzed ethanol processes, product concentrations needed for maximum productivity are often much lower than 60 g/l. Thus, productivity is increased by maximizing dilution rate at high cell concentrations. Unfortunately, such low product concentrations require high capital costs and energy requirements for product separation even though productivity is increased. This product concentration limitation has been attributed to various factors, especially inhibition within yeast cells by autogenously-produced ethanol. Although product inhibition of Z. mobilis may be less than for S. cerevisiae, highest productivity is still obtained at concentrations near 50 g/l. Therefore, kinetic modeling studies will be carried out during FY 1985 to attempt to establish relevant mechanisms and quantitative relationships between productivity and the capability to increase product concentrations. The objective is to determine research advances needed to maintain high productivity while decreasing energy requirements of biocatalyzed processes.

3. Techno-Economic Assessment of Microbial Ammonia Production - Chem Systems Inc.

A conceptual process design for the production of ammonia using photosynthetic nitrogen-fixing blue-green algae will be assessed to determine technical and economic viability of such a process. As a part of this effort, alternative processes (where possible research advances are included) will also be assessed for comparison with the base case to determine which advances should result in the greatest technical and economic advantages. At the present time it seems that significant advances will be required before an ammonia bioprocess can be developed that will be competitive with the current chemical process.

D. MANAGEMENT

During FY 1986 the Project will continue its R&D Planning for Biologically Effected Separation/Beneficiation and its Guidance and Evaluation Panel activities. A multi-year plan will also be developed for the Project.

1. R&D Planning for Biologically Effected Separation/Beneficiation

An overall strategy for development of a detailed research program plan in the area of bioseparations will be implemented, and development of the research plan will be completed. Three research areas will be assessed to determine the potential benefits to ECUT of pursuing their corresponding research initiatives: biologically assisted leaching, accumulation of metals from dilute solutions by microorganisms, and separation of biologically produced chemicals from dilute process streams by microorganisms. A Research Needs Assessment of industrial and academic institutions will be completed to acquire and evaluate information regarding new technology required, primarily for the purpose of suggesting and formulating specific research objectives in bioseparations tasks.

2. Guidance and Evaluation Panel

During FY 1986, the membership of the Project's Guidance and Evaluation Panel will be rotated and expanded. The panel will continue to review the Project's future plans and ongoing research.

3. Multi-Year Project Plan

The Project's multi-year plan will present a detailed discussion of the Project's objectives, rationale, strategy, schedule, resource requirements, and management organization. The major thrusts of this planning effort will be (1) to outline an approach for assuring that the Project's current and future research tasks are coordinated and integrated to resolve the major technical barriers constraining commercial development of large-scale biocatalysis applications and (2) to identify "technology-transfer" mechanisms that will raise industry's awareness of and interest in the potential development and application of biocatalysis technology.

SECTION IV

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