ECUT
ENERGY CONVERSION
AND UTILIZATION
TECHNOLOGIES PROGRAM

Biocatalysis Research Activity
Annual Report
FY 1983

April 15, 1984

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

Through an Agreement with
National Aeronautics and Space Administration

Prepared by:
Jet Propulsion Laboratory
California Institute of Technology
Pasadena, California 91109
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The Biocatalysis Research Activity is managed by the Jet Propulsion Laboratory, California Institute of Technology, for the United States Department of Energy through an agreement with the National Aeronautics and Space Administration (NASA Task RE-152, Amendment 307; DOE Interagency Agreement DE-AIO1-81CS66001).

The Biocatalysis Research Activity 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 FY 1983 activities, accomplishments, and planned research efforts of the Biocatalysis Research Activity of the U.S. Department of Energy (DOE), Energy Conversion and Utilization Technologies (ECUT) Program. The activities of the Biocatalysis Research Activity during FY 1983 were organized into the Biocatalysis and Molecular Modeling work elements and a supporting planning and analysis function.

In the Biocatalysis work element, progress was made in developing a method for stabilizing genetically engineered traits in microorganisms, refining a technique for monitoring cells that have been genetically engineered, identifying strains of fungi for highly efficient preprocessing of biomass for biocatalyzed processes, and determining molecular level conditions for optimizing the efficiency of bioreactors.

In the Molecular Modeling work element, a preliminary model of the behavior of enzymes was developed. Also, a preliminary investigation of the potential for synthesizing enzymes for use in electrochemical processes was completed.

As part of the Activity's supporting planning and analysis efforts, contact with industry and universities was made to define key biocatalysis technical issues and to broaden the range of potential participants in the Activity. Additionally, analyses were conducted to identify and evaluate potential concepts for future research funding by the Activity.
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EXECUTIVE SUMMARY

A. PROJECT DESCRIPTION

The Biocatalysis Research Activity is a sub-project of the U.S. Department of Energy (DOE) Energy Conversion and Utilization Technologies (ECUT) Program. The Activity was initially established in May 1980 as part of the Chemical Processes Project to investigate and develop new techniques and reaction sequences for the chemical processing industry (CPI). Starting in FY 1983, biocatalysis became the focus of the ECUT Program's technology-base-building research for the CPI. The California Institute of Technology's Jet Propulsion Laboratory (JPL) manages the Activity for DOE.

In fiscal year 1983, the Activity supported two major areas of research: Biocatalysis and Molecular Modeling. Biocatalysis research focuses on resolving the major technical barriers that impede the potential use of biologically facilitated, continuous chemical production processes.

Molecular Modeling research emphasizes developing theoretically based models and design parameters for controlling biocatalyzed and hybrid chemically/biologically catalyzed chemical production processes.

B. GOAL AND OBJECTIVES

The current goal of the Biocatalysis Research Activity is to sufficiently build the technical and engineering base of biocatalysis technology to enable industry to displace a significant level of nonrenewable resource requirements by the year 2000. The Activity has an objective for each work element:

- Biocatalysis. Establish the technical feasibility for continuous, efficient production of chemicals by biocatalyzed processes.
- Molecular Modeling. Establish the technical feasibility for theoretically based design, optimization, and control of biocatalyzed and hybrid chemically/biologically catalyzed chemical production processes.

C. FISCAL YEAR 1983 ACCOMPLISHMENTS OF THE BIOCATALYSIS RESEARCH ACTIVITY

The activities of the Biocatalysis Research Activity during fiscal year 1983 were organized into the Biocatalysis and Molecular Modeling work elements and a supporting planning and analysis function. Nontechnical descriptions of these activities are presented here in the Executive Summary. Technical discussions are presented in Section II of this report. A glossary of key terms and concepts is provided at the end of the Executive Summary.
1. Biocatalysis


a. Chromosomal Amplification. The application of genetic engineering techniques in certain microorganisms may lead to greatly reduced production costs for some 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.

A number of steps are required to genetically engineer a bacterium to yield a valuable product. First, the gene (instruction sequence) responsible for directing the bacterium to produce the protein of interest must be isolated and joined ("spliced") to the DNA of an appropriate plasmid. This plasmid is then introduced into a bacterium ("transformation") where the plasmid will multiply ("amplification"), and a strain of bacteria will be established that carries multiple plasmid copies in each bacterial cell. The existence of the foreign gene in numerous plasmid copies results in higher yields of the desired product than if only a single or a few plasmid copies had the desired recombinant trait. Because bacteria can be grown on relatively inexpensive feedstocks (e.g., biomass), production costs for some organic chemicals may be significantly lowered if genetically engineered processes can be cheaply scaled up.

One of the major technical barriers constraining such a production process arises from the typical characteristic of plasmids to float freely in bacterial cytoplasm, unconnected to the chromosome. Because only chromosomes, and not plasmids, consistently duplicate themselves before a bacterium physically divides and grows into two parts, the number of plasmid copies in each bacterium after division is not stable (i.e., the number may either increase or decrease). If the plasmid multiplies too much, it can overburden and kill the carrier bacterium; if it multiplies too little, the plasmid is lost as the bacterium multiplies. In fact, plasmid loss in scaled-up bacterial cultures appears to be a significant shortcoming of the general technique of plasmid splicing.

This research activity is testing the possibility of solving the plasmid stability problem by inserting the genetically engineered plasmid directly into the bacterial chromosome and amplifying it (or just the foreign gene) in place. Because the plasmid with its foreign gene of interest would then be part of the bacterial chromosome, the desired recombinant trait could not be easily lost as the bacterium multiplies. (The Activity's experimental work uses bacteria for convenience; but, in industrial practice, yeast or other microorganisms are more likely to be used.)

This new technique will require several stages. First, the plasmid must be given the capability to insert itself into the bacterial chromosome. The DNA of certain bacterial viruses already possess this capability through a special form of recombination. Plasmids can acquire this property if genes
from these bacteriophages are genetically engineered into the plasmid. Once the plasmid is inserted into the bacterial chromosome, a first level of amplification (i.e., a doubling of the introduced gene) may occur through spontaneous duplication. This rare event, still incompletely understood, is required for the bacterium to have higher yields of the desired product. (This requirement coincides with the need for multiple plasmid copies in conventional plasmid splicing systems in order to have higher yields.) After the first gene duplication "repeat," the ordinary mechanisms of the cell may cause additional repeats to be formed, or alternatively, the first repeat may be lost. It will be necessary to isolate those bacteria where multiple repeats have been formed, and by genetic means inactivate this mechanism so the chromosome's "amplified" state will be stabilized.

The stability of bacterial strains resulting from these manipulations will be compared with the stability of strains created by conventional plasmid splicing techniques. While this research is using laboratory strains of E. coli, the general principles involved could easily be applied to other types of bacteria.

During FY 1983, a number of bacterial strains carrying a special hybrid plasmid integrated in the chromosome of the host bacterium were isolated and characterized.

b. Cellulase Hyperproduction. The primary objective of this task is to identify and optimize species of fungi that produce and secrete high levels of the necessary enzymes collectively referred to as "complete cellulase," which completely catalyzes the decomposition of cellulose into glucose (a simple sugar that is the starting point for many chemical fermentations). This step is the main barrier to the efficient biological conversion of biomass into useful chemicals and fuels. It has been determined that over 50% of the cost in producing a fuel such as ethanol has been in the production of the glucose from the cellulose. This task will attempt to develop hyper-producers of the complete cellulase in fungi that have the unique property of excreting the enzymes. This property of secretion is important to the scaling up of a fermentation process, as it is a natural process for separating the product (in this case, cellulase) from the source microorganisms without the need for the energy-intensive disruption of the cells followed by complex separation procedures to extract the product. Such organisms would be able to inexpensively produce the complete cellulase in high yield, thereby reducing the overall bioconversion cost.

From 18 candidate strains of fungi, two have been selected for further development. These strains secrete substantial quantities of cellulase and are from a taxonomic group of fungi for which genetic manipulation techniques are available. Evidence shows that the genetic regulation mechanism of "catabolite repression" operates on the cellulase in these species. This means that the fungi produce cellulase in these species only when grown on cellulose, not on simple carbon sources such as glucose. Attempts are being made to alter this property by mutation so that the fungi will produce the enzymes under all nutrient conditions. Initial studies using drugs that alter secretion in animal cells have indicated that the process of secretion in the fungi is similar. Assays for the detection of secreted enzymes from fungi
grown from individual spores have been developed, and these will now be used in the detection of mutants with elevated levels of cellulase production and secretion. Eventually, strains of fungi with multiple improvements will be bred from mutant individuals with desirable properties of growth, enzyme synthesis, and secretion.

c. Techniques for Plasmid Monitoring. Using recombinant DNA technology, it is possible to make microorganisms produce desired enzymes (biocatalysts) and other products. A small ring of genetic instructions called a plasmid is introduced into the microorganism. However, when the cell divides (to reproduce) it may eventually lose these plasmids with their recombinant traits and the corresponding capability to make desired products. Therefore, bioprocess engineers and scientists require a technique to monitor a large number of cells individually for the existence and level of plasmids.

A technique (using flow cytometry) to accomplish this was initially developed in fiscal year 1982. In fiscal year 1983, the technique was developed further, including an increase in sensitivity of a factor of $10^3$ and improved definition of conditions for optimized measurements and enhanced detail.

d. Kinetics for Process Design. Recombinant DNA technology and engineering will contribute significantly to applications of biocatalysis for the production of chemicals because the catalyst systems (microorganisms and enzymes) can be designed to optimize product specificity, production rate, and yields. However, because of the lack of quantitative descriptions ("kinetic expressions") of the growth and stability of recombinant cells and accumulation of enzymes and other 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 the recombinant DNA has deleterious effects on reproduction of the cells with the desired characteristics.

The purpose of this work is to describe such interactions on a molecular level to optimize genetic design and determine conditions for maximum efficiency in bioreactors and processes utilizing recombinant microorganisms.

In fiscal year 1983, previously formulated molecular-level mathematical models were used to investigate alternative genetic designs to improve controllability of fermentation with recombinant strains. Also kinetic models were developed to calculate effects of genetic instability in different bioreactor configurations for optimizing reactor design.

2. Molecular Modeling

The Molecular Modeling work element encompassed two research activities: Enzyme Reaction Models for Catalytic Processes and Electro catalysis.
a. Enzyme Reaction Models for Catalytic Processes. Biotechnology has long been an important part of the food and beverage industries and of the drug industry. Thus, enzymes have long been utilized in manufacturing such products as cheese, bread, beer, and wine. 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. Currently, advances in molecular genetics and recombinant DNA technology promise a revolution in the manufacture of such high-value products as insulin and interferon. In these areas of foods, beverages, and pharmaceuticals, industrial development and applications based on the advances in fundamental research have been rapid and have been primarily financed by industrial and venture capital.

On the other hand, development and application of biocatalysis for the production of fuels and chemical feedstocks have not advanced rapidly, particularly in areas where nonbiological processes are already in production. The problem here is the need for long-term applied research and exploratory development in order to provide the technology base required before industrial development and exploration become feasible.

Some of the major bioengineering difficulties involve:

1. Slow rates: Biocatalysis, although selective, is often quite slow. In addition, it is often necessary to work with dilute aqueous media, and the reactions are often slowed or stopped in the presence of large amounts of product. One would like to develop systems that have intrinsically higher turnovers and that could operate at higher concentrations of catalyst and with large concentrations of substrate and product.

2. Thermosensitivity: In order to increase reaction rates, one would like to use higher temperatures, but most enzymes are denatured by 50 to 60°C. One would like to develop enzyme systems that could withstand much higher temperatures.

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Cofactors: Biocatalysis often involves a multi-step sequence of reactions, each of which requires participation of cofactors essential to the catalytic process. These cofactors are often quite expensive and difficult to retain at the active site.

Immobilization: It is often necessary to immobilize biocatalysts in a matrix or on a support in order to retain their biocatalytic activity in the reaction or to improve their lifetime. There are numerous questions concerning immobilization. Unfortunately, very little is known concerning how immobilization affects the three-dimensional structure of biocatalysts, and thus there are little data to use in trying to design more effective immobilization strategies.\textsuperscript{4,6,7}

Success with the development of a microscopic reaction model for enzyme systems will be the necessary first step in solving the above problems and subsequently enabling industry to design, formulate, prepare, and utilize biocatalysts in more efficient and productive chemical processes.

This model would complement the microscopic reaction models being developed for studying chemical catalysis. Together, these two models could be used to develop hybrid chemical/biological catalysts.

During FY 1983, a preliminary molecular model of the behavior of enzymes was developed. Advanced versions of this model will help in optimizing the design and operation of biocatalyzed and hybrid chemically/biologically catalyzed chemical production processes.

b. Electrocatalysis. Most conventional industrial chemical processes, in order to attain acceptable reaction rates, are usually carried out at high temperatures and pressures, and therefore may be energy intensive. Electrochemical processes (i.e., processes that use electricity to drive chemical reactions) can be more energy efficient than conventional processes because they do not require high pressures and temperatures. Electrocatalysis involves raising the energy efficiency of electrochemical processes by adding a catalyst to surfaces (i.e., electrodes) where chemical reactions occur. Certain forms of electrocatalysis offer the future potential for new sources of fuels (e.g., hydrogen from water, or higher hydrocarbons from simpler compounds -- perhaps even carbon dioxide).

During FY 1983, a method was developed to synthesize an inorganic analogue of the enzyme nitrogenase. Nitrogenase is the enzyme present in microorganisms that is responsible for natural nitrogen fixation (e.g., the conversion of nitrogen to ammonia). The method involves the synthesis and immobilization of a molybdenum-based inorganic complex that can bind nitrogen directly to the metal. Such catalysts could eventually be applicable to the direct manufacture of ammonia from nitrogen.


3. Planning Support and Analysis


a. Guidance and Evaluation Panel. During FY 1983, a meeting of the Panel was held during the AIChE conference at the Biltmore Hotel in Los Angeles, California. During the meeting, the Panel generated a list of key biocatalysis technical issues. These issues provided the starting point for drafting the Research Opportunity Notice.

b. Energy and Economic Analysis. The objective of this activity has been to compare biocatalyzed chemical production processes with conventional processes, including the effects of projected research advancements in biocatalysis to assess and evaluate expected relative energy consumption and economics. Following analysis of a base-case state-of-the-art fermentation process for acetone/butanol/ethanol (ABE) in fiscal year 1982, a series of potential advancements was ranked with respect to estimates of information availability, technical feasibility, energy consumption, and economics. During FY 1983, the potential advances selected and analyzed included vacuum fermentation, continuous fermentation, the Lignol process (lignin to phenol, benzene, and fuel), Baelene solvent extraction, a modified prehydrolysis and dual enzyme process, and an increased butanol concentration tolerance process. Each analysis consisted of integration of the potential advance into the base-case process for economic and energy comparisons with the latter.

Of the potential research advances assessed, increased butanol concentration tolerance was found to be the most promising. Calculations based on a small increase in tolerance show that a 30% decrease in energy consumption and a 20% decrease in selling price is possible, which could result in eventual displacement of conventional processing by this biocatalyzed process.

c. Second Law Analysis. During FY 1983, a general analytical methodology, based upon fundamental thermodynamics, was developed for determining the total energy consumption of chemical production process separation steps. The methodology was used to analyze four proposed novel separation processes, all of which were claimed to be energy efficient. Results from the analysis indicated that energy efficiencies were lower than claimed primarily because previous calculations failed to consider the energy balance of the entire chemical production process.

d. Research Opportunity Notice. In April 1983, the Activity published a Research Opportunity Notice (RON) in Commerce Business Daily. The RON solicited expressions of interest from petrochemical and chemical companies, bioengineering firms, biochemical engineering consultants, private research laboratories, and universities for participating in the Activity. The results of the RON indicate that broad interest for the Activity exists
within the nation's industry, universities, and research institutes. A significant number of the country's large chemical companies and emerging leaders in biotechnology expressed interest in responding to the Activity's forthcoming Request for Proposals (RFPs).

D. FY 1984 PLAN FOR THE BIOCATALYSIS RESEARCH ACTIVITY

1. Biocatalysis

All of the FY 1983 research activities will continue in FY 1984. Chromosomal Amplification work will isolate derivatives with multiple plasmid copies on the bacterial chromosome from the strains prepared during FY 1984. Attempts will then be made to introduce genes that will block further drift of the number of copies.

Cellulase Hyperproduction work will investigate fungi reproductive and regulatory behavior and cell physiology to better understand growth and cellulase secretion factors. Also, a search will be initiated for mutant fungi that contain favorable cellulase production/secretion characteristics.

Techniques for Plasmid Monitoring work will focus on determining the effects of different plasmid replication controls on the cell's ability to retain and use plasmid-encoded genetic information.

Kinetics for Process Design work will involve (1) calculating the impact of genetic instability on different bioreactor configurations and (2) defining strategies for optimizing the genetic and environmental aspects of biocatalyzed processes.

A competitive procurement is planned for late in the fiscal year. The RFP topics will be chosen based on the interests of the Research Opportunity Notice respondents, results of the Energy and Economic Analysis, and consultations with the Activity's Guidance and Evaluation Panel.

2. Molecular Modeling

The Enzyme Reaction Models for the Biocatalysis task will continue in FY 1984.

The Enzyme Reaction Modeling work will focus on refining the current force field model. The new model, based on a combination of theory and experiment, should be more accurate in describing the metal atoms in enzymes, for many of which there is little empirical geometry and energy data. This model will be able to calculate the structure of enzyme substrate and enzyme inhibitor complexes. The information gained from these studies will ultimately lead to the ability to select and/or modify (design) biological and other catalysts based on a knowledge of their structure.

Second Law Analysis work will further develop methods for identifying low-energy approaches for separation of fermentation intermediates and products.
3. Planning Support

The Guidance and Evaluation Panel will assist in selecting research topics for competitive solicitations.

Energy and Economic Analysis work will encompass further economic energy analysis of selected combinations of research advances. Vacuum fermentation will be assessed again because the previous study did not account for the real behavior of n-butanol-water mixtures. In addition, base-case economics will be adjusted to reflect the impact of different feedstocks.

A new Planning Support activity -- System for Biotechnology Assessment -- will be initiated during FY 1984. This work will involve modifying the computer program ASPEN (which is currently used for analyzing conventional chemical production processes) so that it can be used to evaluate biocatalyzed processes. Successful completion of this work will result in an improved capability for conducting sensitivity analyses of the energy and economic impacts of improvements to biocatalyzed processes.

E. GLOSSARY

Bacteriophage - A type of virus that attacks bacteria rather than ordinary cells. A particle of bacteriophage consists of a nucleic acid (usually DNA) molecule enclosed in a protein shell. The nucleic acid can enter a bacterium and either multiply in it to form progeny particles, or it can variously interact with the chromosome of the bacterium.

Bacterium/Bacteria - The smallest unicellular microorganisms, usually enclosed in a hard cell wall. They do not have mitosis, the complicated mechanism of animal and plant cells for the equal ripartition of chromosomes at cell division. They apparently have only one chromosome. A great many bacterial types are known, performing an enormous variety of reactions. Most bacteria are free living; some are the cause of disease.

Baelene Solvent Extraction - A (Baeol Corp.) process where fermentation products are recovered by solvent extraction with a volatile fluorocarbon solvent.

Biocatalysts - Catalysts, called enzymes, that are biological in origin and participate in the metabolic activity of cells.

Biomass - Any plant material: leaves, wood, bark, algae, etc.

Butanol Concentration Tolerance - In ABE fermentation, n-butanol stops the fermentation process when n-butanol concentration increases to 2 wt. % because it affects functioning of the microorganisms.

Catalyst - Any substance that facilitates the occurrence of a chemical reaction. In the presence of the appropriate catalyst, reactions can take place at room temperature that otherwise would occur spontaneously only at very high temperatures.
Chemical or Chemical Product - Examples are alcohols, acetone, and acids. These are small molecules that may be used as solvents, fuels, or to make other chemicals or products such as plastics or rubber.

Chromosomes - The main cell structures that carry genetic information. The main component of a chromosome is a long DNA chain. A chromosome can be visualized as a string of genes. Animal and plant cells have several chromosomes (a constant number for each species) contained in the nucleus. In a bacterium, there is only one chromosome, free in the cytoplasm of the cell.

Cosolvent - A solvent added to a different solvent to increase the ability of the latter to dissolve a product, e.g., a chemical containing chlorine added to a hydrocarbon will dissolve dried paint better than the hydrocarbon alone.

Critical Fluid Extraction - See extract.

Critical Pressure - The pressure exerted by a chemical if it is confined in a small space at its critical temperature. For carbon dioxide it is about 1100 pounds per square inch.

Critical Temperature - The temperature (characteristic of each chemical) above which the chemical as a gas cannot be changed to a liquid by increasing the pressure. It is about 31°C for carbon dioxide.

Cytoplasm - The main body of a cell, exclusive of the nucleus (in a bacterium, exclusive of the chromosome) and of the cell membrane and wall.

DNA - Deoxyribonucleic acid: the type of nucleic acid of which chromosomes are made. As a molecule, it is a linear structure, consisting of a chain (or double chain) of four types of small molecules ("bases"). DNAs differ in the sequence of such bases, a bit like messages in Morse code. The length of a DNA molecule may be from a few thousand bases to millions.

Decarboxylation - A chemical reaction where a carboxylic acid, such as acetic acid, is converted to a hydrocarbon, such as methane, by loss of carbon dioxide. (Vinegar is essentially a dilute water solution of acetic acid prepared by a biocatalyzed process; methane is the major product in natural gas.)

Distillation - A process in which a chemical can be separated from water (or another chemical) by heating the mixture. The chemical that boils at the lowest temperature is converted to a gas or vapor first when that temperature is reached. The chemical is then cooled to convert it back to a liquid, which contains less water or other chemical.

Electron Spin Resonance Spectroscopy - A sophisticated instrumental technique for measuring the number and types of certain sites on a material or catalyst where chemical changes can take place.

Element - In the chemical sense, the small components of molecules, such as carbon (symbol-C), hydrogen (H), oxygen (O), nitrogen (N), chlorine (Cl), sulfur (S), etc. The pure elements will have altogether different characteristics than the chemical products that are composed of them. For example,
sodium (Na) is a liquid metal that reacts violently with chlorine, a yellowish, poisonous gas, to give sodium chloride, or table salt.

**Enzyme** - A protein acting as a catalyst for a particular chemical reaction.

**Extract, Extraction** - To separate a chemical product from water or contaminants by selectively dissolving it in a solvent. In critical fluid extraction, the solvent (usually carbon dioxide) is under pressure. When the pressure is released, the carbon dioxide (in the form of a gas) is no longer a good solvent for the product and can itself be separated by allowing it to diffuse away from the product.

**Feedstock** - Any relatively available substance that can be converted by chemical reactions to a more useful chemical product or products. At the present time, the most common feedstocks for organic chemical production are petroleum and natural gas.

**Feedstock Energy** - The energy content of the feedstock; the energy available in Btu/lb that would be obtained if it was burned as a fuel.

**Fluorogenic Substance** - A nonfluorescent substance that can be processed into a fluorescent product.

**Gene** - In general, a segment in a DNA molecule that specifies the sequence of amino acids in a type of protein. A mutation in a gene will cause the formation of an altered, often nonfunctional protein. Hereditary traits are determined by specific genes, usually through the proteins that genes make and through the products (or "metabolites") of the reactions catalyzed by such proteins.

**Heterogeneous Catalyst** - A catalyst positioned on a support material which is not miscible in the reacting medium.

**Intermediate** - Molecular species that are in a transition between initial reactant molecules and the subsequent product species.

**Lignol Process** - A process for converting lignin (wood byproduct) to phenol, benzene, and fuel gas and oil; developed by the Hydrocarbon Research Institute.

**Membrane** - A thin film or sheet, usually plastic, that is designed to let some chemicals (or water) pass through it by a sieving action while retaining other chemicals on the feed or upstream side.

**Membrane Fouling** - Plugging of a membrane surface or pores with contaminants, which can result in a decrease in the rate of flow of the desired chemical or water through the membrane.

**Metabolite** - A general term for a biological compound that is the product of an enzymatic reaction.
Modified Prehydrolysis and Dual Enzyme - A process whereby wood is converted to fermentable sugars under modified conditions to increase the sugar yield to 100%. (All the wood is converted to sugar.)

Molecule - A group of elements combined in a specific structural way; the smallest discrete entity of a specific chemical product.

Osmosis - The spontaneous movement or diffusion of a solvent, such as water, through a membrane to a more concentrated solution from a less concentrated solution.

Permeability - The ability of a membrane to allow passage of molecules (e.g., chemicals or water) through the membrane.

Permselectivity or Permeation Selectivity - The extent to which a membrane allows retention of some components while allowing another or others to pass through it; a measure of its separation capability.

Plasmid - Any one of a variety of small, circular DNA molecules that may be found free in the cytoplasm of a cell or bacterium, replicating more or less in unison with the chromosome of the cell, but in an autonomous way. They may be extracted in the form of a DNA preparation and artificially transferred to other cells ("transformation"). Depending on the genes they carry, they may endow the host cell with new properties.

Process Energy - The amount of energy required to process or convert a feedstock to a relatively pure chemical product; may be expressed in units such as Btu/\text{lb}.

Protein - The main chemical component of living matter. A protein molecule is a chain of up to several hundred amino acids and is folded into a more or less compact structure. Because some 20 different amino acids are used by living matter in making proteins, the variety of protein types is enormous.

Recombination - In genetics, a very widespread cellular mechanism by which segments of DNA may be exchanged between two different DNA molecules. When at least one of the molecules is circular, the result may be the insertion of one molecule into the other ("integration").

Reverse Osmosis (RO) - The most widely used membrane separation process, most commonly used to separate salt water to produce water for drinking or other uses (desalination). The feed mixture is pumped at high pressure to drive the more permeable component (water in desalination) through the membrane. It is called reverse osmosis because the pressure is applied to the feed stream in a direction opposite to the pressure of normal osmosis.

Rhodium - A metallic element that is sometimes present in certain specific chemical catalysts.

Second Law Analysis - Analysis and determination of energy required to carry out a process that take into account energy losses that result because
of the second law of thermodynamics. This law limits the direction of flow of heat and the conversion efficiency of heat energy into other forms of energy, e.g., electrical or mechanical.

Vacuum Fermentation - A process by which a vacuum is applied to a fermentation reactor to selectively remove the volatile fermentation products as they are formed.
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 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 Research Activity

The Biocatalysis Research Activity is a sub-project of the ECUT Program (see Figure 1-1). This annual report describes the FY 1983 activities, accomplishments, and future plans of the Activity.

a. Background (Prior to FY 1983). The Chemical Processes Project was established in May 1980 to investigate and develop new techniques and reaction sequences for the chemical processing industry. Concurrently, JPL was selected as the Project's lead laboratory. DOE funding for the Project began in July 1981. Prior to this 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 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 research focused on resolving the major technical barriers that
Figure 1-1. Relationship of the FY 1983 Biocatalysis Research Activity to the Overall ECUT Program
impede the potential use of biologically facilitated, continuous chemical production processes. These barriers include: maintenance of stable genotype 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 involve 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. This organization of the Activity remained throughout FY 1983 and will continue in FY 1984.

3. Goal and Objectives

The current goal of the Biocatalysis Research Activity is to sufficiently build the technical and engineering base of biocatalysis technology to enable industry to displace a significant level of nonrenewable resource requirements by the year 2000. The activity has an objective for each work element:

- Biocatalysis. Establish the technical feasibility for continuous, efficient production of chemicals by biocatalyzed processes by 1990.

- Molecular Modeling. Establish the technical feasibility for theoretically based design, optimization, and control of biocatalyzed and hybrid chemically/biologically catalyzed chemical production processes by 1997.

B. ORGANIZATION OF THE REMAINDER OF THIS REPORT

Sections II and III of this report present, respectively, the FY 1983 accomplishments and FY 1984 plans of the Biocatalysis Research Activity. Section IV lists reports and papers prepared or published by the Activity during FY 1983.
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SECTION II

FY 1983 ACCOMPLISHMENTS OF THE BIOCATALYSIS RESEARCH ACTIVITY

The Biocatalysis Research Activity during FY 1983 was organized into the Biocatalysis and Molecular Modeling work elements, and a supporting planning function (see Table 2-1). This section of the report describes the tasks and FY 1983 accomplishments for each of these areas.

Table 2-1. Organization of FY 1983 Activities in the Biocatalysis Research Activity

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A. BIOCATALYSIS

As shown in Table 2-1, the Biocatalysis work element comprised four research tasks: (1) Chromosomal Amplification, (2) Cellulase Hyperproduction, (3) Kinetics for Process Design, and (4) Techniques for Plasmid Monitoring.

1. Chromosomal Amplification - Jet Propulsion Laboratory and California Institute of Technology

   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 Activity's experimental work uses bacteria for convenience; but, in industrial practice, yeast or other microorganisms are more likely to be used.)

b. FY 1983 Accomplishments

- Chromosomal integration of appropriately engineered plasmids was demonstrated by genetic methods. The plasmids carried a fragment of bacteriophage P2 DNA, comprising both the DNA site (att) and the gene (int) which are normally used by the bacteriophage for inserting its own DNA (as prophage) into the bacterial chromosome. The bacterial strain used (Escherichia coli C ts-pola2) makes a defective DNA polymerase at high (37 to 42°C) temperature. Because the plasmid requires an active DNA polymerase for autonomous replication, it was possible to eliminate the cytoplasmic copies of the plasmid by growing the bacteria at high temperature following integration.

- The stability of the strains carrying an integrated plasmid was increased (i.e., the probability of their reverting to the autonomous plasmid state was reduced) by using DNA from a bacteriophage mutant defective for integration (the P2 int150 mutation, in plasmid pEE240). The integration of this plasmid was obtained by transiently supplying the gene product required through simultaneous infection with phage.

- Sorbitol defective mutants of the polA defective strain — to be used in a later stage of the project — were isolated.

- Selection methods for the isolation of strains with chromosomally amplified plasmids were tested in preliminary experiments with promising results.

2. Cellulase Hyperproduction - Jet Propulsion Laboratory

a. Description. The overall purpose of this task is to identify and optimize genetically manipulable species of fungi that produce copious quantities of complete cellulase which degrade cellulose to glucose. The two specific objectives of the effort are (1) to genetically alter the metabolic regulation of cellulase synthesis so that it is produced during growth on all nutrients (constitutive synthesis), and (2) to augment the secretion of the enzymes as a strategy for reducing purification steps and costs.
b. **FY 1983 Accomplishments**

- From 18 candidate strains of fungi, two have been selected for further development based on levels of secreted cellulase in liquid culture and in growth on semisolid media employing acid-swollen cellulose or carboxymethyl cellulose as carbon sources.

- Zone clearing assays have been improved for detection of cellulase activity on acid-swollen cellulose, and a new plate assay for the activity of culture filtrates on carboxymethyl cellulose was implemented. This allows an assay of the endoglucanase activity independent of total cellulolytic activity. These plate assays will now be used in mutant hunts for hyper-producers.

- Evidence has been obtained that cellulase in the two promising species are subject to catabolite repression. An attempt will be made to alter this property by mutation so that the fungi will produce the enzymes under all nutrient conditions.

- Initial studies using the drug chlorpromazine, which inhibits the regulatory protein, calmodulin, and alters secretion in animal cells, have indicated that secretion in the fungi may also be under calmodulin/calcium regulation. If this is truly the case, it may provide a way to detect calmodulin binding proteins that are involved in secretion and may in addition provide a way of selecting secretion mutants that are resistant to calmodulin inhibitors.

3. **Kinetics for Process Design - California Institute of Technology, Division 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 eventually, 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 molecular 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.

b. **FY 1983 Accomplishments**

- Previously formulated detailed molecular-level mathematical models have been used to investigate alternative molecular
designs of regulation of plasmid functions to improve controllability of the fermentation with recombinant strains. Also, kinetic models have been developed, based upon a simplified version of the molecular-level models, for calculating influences of genetic instability in different bioreactor configurations. These equations can be used to optimize the design of genetic and environmental aspects of the fermentation process.

4. Techniques for Plasmid Monitoring - California Institute of Technology, Department of Chemistry and Chemical Engineering

a. Description. Synthesis of protein products encoded by plasmid genes in recombinant cells depends upon a variety of host cell constituents. Conversely, the presence of the plasmid and its protein products may have deleterious effects upon growth and macromolecular synthesis in the host cell. To understand the stability of plasmids in recombinant cells, it is necessary to develop a technique whereby a growing population can be sampled and large numbers of individual cells can be assessed for their plasmid contents. This measurement technique must prevent interference between cells containing a plasmid marker (a particular enzyme) and cells lacking the enzyme. Such interference arises by diffusion of the fluorescence marker used for the experiment from one type of cell to the other.

Development of such a technique (using flow cytometry) was carried out in fiscal years 1982 and 1983.

b. FY 1983 Accomplishments

- Methodology has been improved to enhance greatly the sensitivity and detail provided by the measurements. Instrument modifications included:
  - Higher resolution photomultiplier tube
  - New lens for light collection
  - Sensitivity increase of $10^3$.

- New data analysis methods included:
  - Strategies for extracting information on enzyme content per cell based on enzyme kinetics and analysis of product fluorescence distribution
  - Insertion of different autonomous replication sequences into plasmids with marker gene, the expression of which is inducible.

- Also a number of new yeast strains have been constructed to permit future studies of the effect of different plasmid
replication controls on the ability of the yeast cells to retain the plasmid and to synthesize proteins encoded on plasmid genes.

B. MOLECULAR MODELING

Molecular Modeling research activities encompassed two research activities (Table 2-1): (1) Enzyme Reaction Models for Catalytic Processes and (2) Electrocatalysis.

1. Enzyme Reaction Models for Catalytic Processes – California Institute of Technology, Department of Chemistry and Chemical Engineering

   a. Description. This research involves the development of an enzyme catalysis model that would be able to:

   (1) Examine the three-dimensional structure of free and immobilized enzymes.

   (2) Examine the microscopic chemical mechanism involved in the reaction of enzyme with substrate and determine how this is changed upon immobilization.

   (3) Study the role of cofactors in the normal enzymatic reaction.

   (4) Examine strategies of replacing electron transfer and proton transfer cofactor functions by use of appropriate enzyme supports.

   (5) Examine multi-step enzymatic sequences with the goal of finding ways to couple such systems with maximum efficiency and lifetime.

   (6) Study the microscopic details involved in denaturing of thermophilic and normal enzymes at higher temperatures with the goal of learning how to modify enzymatic systems to enhance thermostability.

   In the long term, studies with such enzyme catalysis models might be useful in designing new enzymes that would be suitable in nonaqueous media and in designing new enzymes to be selective for catalyzing nonbiological molecules.

   b. FY 1983 Accomplishments

   • An initial force field model has been developed. This model includes the important molecular interactions of normal protein, such as Van Der Waals interactions, hydrogen bonds, and hydrophobic-hydrophilic interactions. This model has been successful in relating calculated enzyme structural parameters with experimental observations.
The current force field model has been used to examine the dynamics of the enzyme thermolysin. (Thermolysin is the best characterized thermostable enzyme with well established structure and physicochemical properties.)

2. Electrocatalysis - Rockwell International Microelectronics Research and Development Center

   a. Description. The goals of this research were to evaluate the major technological areas in which advances in electrocatalysis could be made. Current key problems of general importance in electrocatalysis include the oxygen electrode whose irreversibility at practical electrodes leads to large energy inefficiencies, and the lack of good catalysts for reducing C\(_1\) compounds and for fixing nitrogen.

   b. FY 1983 Accomplishments

   - A major accomplishment of this research was the development of a new molybdenum-based electrocatalyst for the direct reduction of gaseous nitrogen. This electrocatalyst is based on a molybdenum-phosphine bi-dentate ligand which can be attached to graphite electrodes for heterogeneous catalysis.

   - The potential utility of electrochemical photocapacitance spectroscopy (EPS) was considered for the characterization of electrocatalyst systems. EPS is an in situ method that can provide quantitative information about interface states residing in the electrode/electrolyte region (i.e., where catalysis takes place).

C. PLANNING SUPPORT AND ANALYSIS

Planning support activities for the Project included the Guidance and Evaluation Panel, the Energy and Economic Analysis, Second Law Analysis, and the Research Opportunity Notice (RON) (Table 2-1).

1. Guidance and Evaluation Panel

   During FY 1983, a meeting of the Panel was held during the AIChE Conference at the Biltmore Hotel in Los Angeles, California. During the meeting, the Panel generated a list of key biocatalysis technical issues. These issues provided the starting point for drafting a Research Opportunity Notice.


   a. Description. This research activity has focused on developing and demonstrating an economic assessment methodology for comparing conventional
and biocatalyzed chemical production processes. Initial modeling efforts have used a state-of-the-art fermentation process for acetone/butanol/ethanol (ABE). The objective has been to establish a candidate base case for comparison with new processes that will be developed as a result of research advances in biocatalysis. The defined ABE production facility consists of three major sections: (1) pretreatment and enzymatic hydrolysis of wood, (2) fermentation, and (3) purification.

b. FY 1983 Accomplishments

- Energy and economic analyses of effects on the base case from six potential research advances were completed in FY 1983 as discussed below.

Vacuum Fermentation. Results of this analysis indicated that vacuum fermentation has no advantage for ABE fermentation because volatility (as vapor pressure) is higher for water than n-butanol.\(^8\)

Continuous Fermentation. Because the yield was decreased, the analysis indicated about the same selling price as the base case. However, energy consumption was decreased, and if yield had been constant, the selling price would have been about $0.05/gal less. This result is significant because if the products were removed as they were formed, there would be an increase in yield and substantial further reductions in energy and costs.

Lignol Process. In the base case, lignin was burned as an energy source. In this analysis it was converted to phenol, benzene, and fuel gas and oil. Although capital and energy costs were higher than for the base case, there was a $0.04/gal advantage because the increased costs were more than offset by by-product credits.

Baelene Solvent Extraction. There was a small energy and economic advantage of $0.018/gal; however, most of the apparent potential advantage was not realized because of the need to recover the fluorocarbon solvent from the dilute water waste stream.

Modified Prehydrolysis and Dual Enzyme. Although energy consumption was not decreased, there was a $0.10/gal advantage (primarily a result of decreased capital-related expenses because of the decreased amount of wood being processed).

Increased Butanol Concentration Tolerance. Of the research advances analyzed, this offers the greatest energy and economic advantage: a decrease in selling price from $2.60/gal to $2.09/gal, mainly because of decreased energy consumption and related capital expenses. This price is only about 89% of the selling price estimate for conventional processes to

\(^8\)Analysis omitted the possible effect of the water-butanol azeotrope which may affect the results.
make n-butanol and acetone from alkylenes separated from natural gas. Therefore, with further process development, this advance could result in eventual displacement of conventional processing by a bioproduction process.


a. Description. The objectives of this activity are (1) to develop an analytical method based on the second law of thermodynamics, (2) to provide a general and uniform basis for comparing the energetics of separation processes, (3) to identify the energy-intensive steps of separation processes, and (4) to determine if meaningful and practical energy reductions can be realized for candidate and/or proposed separation techniques and application areas.

b. FY 1983 Accomplishments

- A general analytical methodology was developed based on the second law of thermodynamics to determine the total energy consumption of a separation process. The fundamental thermodynamics upon which the method was developed provides a uniform basis for comparing the energy consumption of competitive or diverse separation processes and for assessing changes in energy consumption resulting from modifications to separation processes.

- The methodology was used to analyze three currently proposed and novel separation processes which are claimed to be energy efficient: membrane separation, supercritical extraction, and condensed-phase (also called moving bed) chromatography. The results indicated that energy efficiencies were not as claimed for two major reasons. First, the claims resulted from focusing attention on one of two steps of the overall process (which were themselves low-energy consuming) but not on other necessary steps in the overall process. Second, for some processes, energy efficiency is a function of product separation, with energy efficiency decreasing as the separation ratio increases.

4. Research Opportunity Notice - Jet Propulsion Laboratory

a. Description. A Research Opportunity Notice (RON) was issued in late April 1983 and solicited expressions of interest for participating in the Activity from petrochemical and chemical companies, bioengineering firms, biochemical engineering consultants, private research laboratories, and universities.

b. FY 1983 Accomplishments. Sixty-seven responses to the RON were received. The results of the RON are summarized below:

- Broad interest exists within the nation's industries, universities, and research institutes for a federally
supported program for investigating potential applications of biotechnology in producing chemicals.

- Some of the country's large chemical companies, including Allied, American Cyanamid, Celanese, Martin Marietta, and UOP, have expressed interest in responding to Request for Proposals (RFPs) released by the ECUT Biocatalysis Research Activity.

- Interest in the Activity has also been expressed by a number of the nation's emerging leaders in biotechnology such as AMGen, Genentech, and Genex.

- Research institutes expressed the broadest interest in the various Research Opportunity Notice (RON) technical issues. In other words, the typical research institute expressed interest in a larger proportion of the technical issues than any other type of respondent.

- The expressions of interest from universities and R&D industry firms (i.e., biotechnology companies and/or firms that perform contracted R&D) most closely paralleled each other. This reflects the close ties that have formed between these two types of respondents.

- Chemical companies expressed levels of interest in the technical issues that were nearly opposite to those expressed by the engineering firms that design and build chemical plants. This result reflects the complementary relationship that exists between these two types of respondents.

- R&D industry firms are primarily interested in the technical issues most relevant to near-term applications of biocatalysis.

- Chemical industry firms also appear to be more interested in the technical issues most relevant to near-term applications; however, their apparent bias is weaker than that exhibited by the R&D industry firms.

- Engineering firms were the only respondents to express strong interest in the issues most relevant to the longer-term, low-value/high-volume applications of biocatalysis.
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SECTION III

FY 1984 PLAN FOR THE BIOCATALYSIS RESEARCH ACTIVITY

The organization of the Activity's planned FY 1984 tasks is shown in Table 3-1.

Table 3-1. Organization of FY 1984 Tasks in the Biocatalysis Research Activity

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Planning Support and Analysis

• Guidance and Evaluation Panel
• Energy and Economic Analysis
• System for Biotechnology Assessment
• RFP

This section briefly describes the FY 1984 content of each of the three Biocatalysis Research Activity work elements: Biocatalysis, Molecular Modeling, and Planning Support and Analysis.

A. BIOCATALYSIS

All of the FY 1983 research activities will continue in FY 1984. Chromosomal Amplification work will isolate derivatives with multiple plasmid copies on the bacterial chromosome from the strains prepared during FY 1984. Attempts will then be made to introduce genes that will block further drift of the number of copies.
Cellulase Hyperproduction work will investigate fungi reproductive and regulatory behavior and cell physiology to better understand growth and cellulase secretion factors. Also, a search will be initiated for mutant fungi that contain favorable cellulase production/secretion characteristics.

Techniques for Plasmid Monitoring work will focus on determining the effects of different plasmid replication controls on the cell's ability to retain and use plasmid-encoded genetic information.

Kinetics for Process Design work will involve (1) calculating the impact of genetic instability on different bioreactor configurations and (2) defining strategies for optimizing the genetic and environmental aspects of biocatalyzed processes.

A competitive procurement is planned for late in the fiscal year. The RFP topics will be chosen based on the interests of the Research Opportunity Notice respondents, results of the Energy and Economic Analysis, and consultations with the Activity's Guidance and Evaluation Panel.

B. MOLECULAR MODELING

The Enzyme Reaction Models for Biocatalysis task will continue in FY 1984.

The Enzyme Reaction Modeling work will focus on refining the current force field model. The new model, based on a combination of theory and experiment, should be more accurate in describing the metal atoms in enzymes, for many of which there is little empirical geometry and energy data. This model will be able to calculate the structure of enzyme substrate and enzyme inhibitor complexes. The information gained from these studies will ultimately lead to the ability to select (design) catalytic systems on the basis of their molecular structure.

C. PLANNING SUPPORT AND ANALYSIS

The Guidance and Evaluation Panel will assist in selecting research topics for competitive solicitations.

Energy and Economic Analysis work will encompass further economic energy analysis of selected combinations of research advances. Vacuum fermentation will be assessed again because the previous study did not account for the real behavior of n-butanol-water mixtures. In addition, base-case economics will be adjusted to reflect the impact of different feedstocks.

A new Planning Support activity -- System for Biotechnology Assessment -- will be initiated during FY 1984. This work will involve modifying the computer program ASPEN (which is currently used for analyzing conventional chemical production processes) so that it can be used to evaluate the economics and energy intensity of biocatalyzed processes. Successful completion of this work will result in an improved capability for conducting sensitivity analyses of the impact of research advances on biocatalyzed processes.
D. RFP

In FY 1984, one or more Request for Proposals (RFPs) will be released by the Activity covering relevant topics identified by the Research Opportunity Notice.


