# Report

## Agenda

**8:00** Coffee and refreshments.

**8:30** Welcoming and Introductory Remarks
MARY CLUTTER, Chair, Subcommittee on Biotechnology
FRED HEINEKEN, Chair, Metabolic Engineering Working Group -- Opening Presentation

**9:00** Presentation of NSF Highlights and Introduction of Bernhard Palsson -- Neil Hoffman

Genome-scale Analysis for New Metabolic Engineering Procedures by Bernhard Palsson [Abstract]

**9:40** Presentation of DOD Highlights and Introduction of Jay Keasling -- Harold Bright

Strategies for Metabolic Engineering of Environmental Microorganisms by Jay D. Keasling [Abstract]

**10:20 Break**

**10:40** Presentation of NIH Highlights and Introduction of Claudia Schmidt-Dannert -- Warren Jones

Cell Factory Engineering Using Combinatorial and In Vitro Evolution Strategies by Claudia Schmidt-Dannert [Abstract]

**11:20** Presentation of USDA Highlights and Introduction of Lonnie Ingram -- Chavonda Jacobs-Young

Metabolic Engineering of Escherichia coli W3110 for Redox Neutral and Oxidized Products by L.O. Ingram [Abstract]

**12:00 Lunch**

**1:00** Presentation of NIST Highlights and Introduction of Hal Monbouquette -- Travis Gallagher

Aromatic Biosynthesis in *Archaeoglobus fulgidus* by Hal Monbouquette [Abstract]

**1:40** Presentation of NASA Highlights and Introduction of Vassily Hatzimanikatis -- Steve Davison

Mathematical and Computational Analysis of Central Carbon Pathways for Efficient Metabolic Engineering by Vassily Hatzimanikatis [Abstract]

**2:20 Break**

**2:40** Presentation of DOE Highlights and Introduction of Imran Shah -- John Houghton

Computational Elucidation of Metabolic Pathways by Imran Shah [Abstract]

**3:20** Presentation of EPA Highlights and Introduction of Terry Papoutsakis -- Mark Segal

Stress, Solvent Production and Tolerance by E. Terry Papoutsakis [Abstract]

**4:00** The MetaCyc Database and the Pathway Tools Software: Resources for Metabolic Engineering by Peter D. Karp

**4:15** Future Directions for Metabolic Engineering [discussion notes]

**4:40** Closing Remarks

**4:45** Adjourn

# Background

## Metabolic Engineering

An emerging approach to the understanding and utilization of metabolic processes is Metabolic (or pathway) Engineering (ME). As the name implies, ME is the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular

pathways for chemical transformation, energy transduction, and supramolecular assembly. ME typically involves the redirection of cellular activities by the rearrangement of the enzymatic, transport, and regulatory functions of the cell through the use of recombinant DNA and other techniques. Much of this effort has focused on microbial organisms, but important work is being done in cell cultures derived from plants, insects, and animals. Since the success of ME hinges on the ability to change host metabolism, its continued development will depend critically on a far more sophisticated knowledge of metabolism than currently exists.

This knowledge includes conceptual and technical approaches necessary to understand the integration and control of genetic, catalytic, and transport processes. While this knowledge will be quite valuable as fundamental research, per se, it will also provide the underpinning for many applications of immediate value.

## Scope

The Metabolic Engineering Working Group is concerned with increasing the science and engineering community's level of knowledge and understanding of ME. The Working Group strives to encourage and coordinate research in ME within academia, industry, and government in order to synergize the Federal investment in ME.

## Introduction

In November 1995, Science Advisor John H. Gibbons of the Office of Science and Technology Policy (OSTP) released the report, "Biotechnology for the 21st Century: New Horizons." This report was a product of the Biotechnology Research Subcommittee (BRS) under OSTP, and identifies priorities for federal investment and specific research opportunities in biotechnology. These priorities include agriculture, the environment, manufacturing and bioprocessing, and marine biotechnology and aquaculture. The BRS formed several working groups to facilitate progress on some of these key priorities. The Metabolic Engineering Working Group (MEWG) was created to foster research in Metabolic Engineering, an endeavor that can contribute to all of the key priorities in the aforementioned report. The Working Group is composed of Federal scientists and engineers who participate as part of the activities of OSTP, and represent all of the major agencies involved in Metabolic Engineering research.

In its on-going efforts to promote and enhance the use of Metabolic Engineering (ME), the Working Group sponsored its fifth Inter-Agency Conference on Metabolic Engineering, which was held January 31, 2003 at the National Science Foundation in Arlington, VA. The purpose of the Conference was to: 1. Highlight Metabolic Engineering accomplishments at each of the participating Agencies that have resulted from the Metabolic Engineering Working Group Activities; 2. Educate Federal Agency personnel on emerging Metabolic Engineering issues; and 3. Disucuss Future Directions for Metabolic Engineering.

# Abstracts of Expert Presentations

GENOME-SCALE ANALYSIS FOR NEW METABOLIC ENGINEERING PROCEDURES

Bernhard D. Palsson

University of California, San Diego

With the sequence of E. coli becoming available, genome-scale models of its metabolism have been developed. These models have gone through an iterative model-building procedure and now contain over 900 reactions and over 500 metabolites. These genome-scale models can be used to analyze, interpret and even predict the metabolic genotype-phenotype relationship in E. coli. Most importantly, these models have been able to predict the outcome of adaptive evolution for both wild-type and knock-out strains. This opens up the possibility of designing robust and stable strains for bioprocessing. This goal may now have become reachable in the short term due to the application of optimization methods to determine exhaustively the all such designs based on the genome-scale network. If successful, this procedure would now enable us not only to predict the metabolic genotype-phenotype relationship, but also design it.

## STRATEGIES FOR METABOLIC ENGINEERING OF ENVIRONMENTAL MICROORGANISMS - APPLICATION TO DEGRADATION OF ORGANOPHOSPHATE CONTAMINANTS

Jay D. Keasling
University of California, Berkeley

The goal of this work is to develop the experimental and theoretical methods to introduce multiple, heterologous, biodegradation pathways into a single organism and to optimize the flux through those pathways for the remediation of toxic or recalcitrant organic contaminants. The objectives of this work are: (1) to find and clone a gene that encodes an enzyme capable of degrading diethylphosphate, (2) to clone and express a pathway for complete mineralization of p-nitrophenol phosphate, (3) to clone and express a phosphotriesterase capable of hydrolyzing parathion, (4) to develop a co-culture biofilm capable of degrading parathion (as a proof-of-concept), and (5) to combine all of the genes in a single organism for complete mineralization of parathion or paraoxon.

Parathion (O,O-diethyl-O-p-nitrophenyl phosphorothioate), an organophosphate pesticide which has been widely used and is highly toxic, was chosen as the model compound for this project. Parathion is also structurally and functionally similar to many chemical warfare agents (including VX and soman).

## METABOLIC ENGINEERING OF ISOPRENOID PRODUCTION

Jay D. Keasling
University of California, Berkeley

The objectives of this work are (i) to maximize the production of the isoprenoid precursor isopentenyl diphosphate in E. coli by expressing the genes for either the mevalonate-dependent or the mevalonate-independent synthesis pathway using the metabolic engineering tools developed in this laboratory; (ii) to maximize production of the primary precursors to the terpenoids: geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate; (iii) to introduce into E. coli the genes for specific classes of terpenoids and optimize production of these "natural" terpenoids; and (iv) to use laboratory evolution of terpene cyclases to produce novel terpenoids or to change the distribution of products made by terpenoid biosynthetic enzymes.

To accomplish this work, we are (i) cloning the genes encoding the enzymes in the non-mevalonate IPP biosynthetic pathway and express these genes under the control of inducible promoters on high, medium, and

low-copy plasmids; (ii) cloning the genes for synthesis of DMAPP, GPP, FPP, and GGPP and express these genes under the control of inducible and constitutive promoters on high, medium, and low-copy plasmids; (iii) cloning the genes for various plant and fungal terpenes and express these genes under the control of inducible and constitutive promoters on high, medium, and low-copy plasmids; and (iv) mutating the terpene cyclases genes using mutagenic PCR and gene shuffling. For the maximization of IPP, DMAPP, and GGPP production, we will express the genes for lycopene synthesis and look for deep red colonies (containing large quantities of lycopene).

## CELL FACTORY ENGINEERING USING COMBINATORIAL AND IN VITRO EVOLUTION STRATEGIES

Claudia Schmidt-Dannert
University of Minnesota

We are combining techniques of metabolic engineering with of directed enzyme evolution for the production of useful novel compounds and materials in recombinant cells. To create these cell factories, we make use of the tremendous gene toolbox created in the course of the ongoing (and future) genome sequencing projects. We assemble genes from different organisms into pathways and create new catalytic functions by in vitro evolution. In this project, we apply this approach to the creation of new pathways for the production of a class of complex chemical compounds in E. coli - to the biosynthesis of unnatural porphyrin structures. Porphyrins are highly valuable chemicals with many applications in medicine, chemistry and material sciences. The complexity of many porphyrin structures makes their chemical synthesis often difficult or impossible and typically results in low yields. A biocatalytical approach using metabolically engineered cells, however, could produce specifically functionalized porphyrins in good yields. The biosynthetically produced functionalized porphyrins can then serve as scaffold for chemical modification and/or exhibit specific redox portentials useful for chemical catalysis or incorporation in heme-containing biocatalalysts, such as P450's.

Our first goal was the successful overproduction of porphyrins in E. coli. Presently, no efforts have been undertaken to engineer E. coli cells specifically for porphyrin overproduction. Next, methods for qualitative and quantitative analysis of porphyrin structures synthesized by recombinant E. coli cells needed to be established and methods suitable for high-throughput screening of E. coli libraries developed. To create new porphyrin structures in E. coli, we use combinatorial and in vitro evolution techniques to create in project I expanded porphyrins with more than four pyrrole rings and in project II different unnatural functionalized porpyrins.

During the past year, we have cloned 17 heme biosynthetic genes from different microorganisms. The genes were initially cloned into our constitutive expression vector pUCmod and subsequently several biosynthetic genes were cloned in a modular fashion on two plasmids pACmod and pBBR1, which can be stably propagated together with pUCmod in E. coli, for combinatorial assembly of different porphyrin pathways in E. coli. Because porphyrin overproduction in an engineered microbial host has not yet been described, toxicity of the porphyrins synthesized in E. coli was a major concern. However, after transforming E. coli with our modular heme gene expression plasmids we found that E. coli is capable of synthesizing very large quantities of porphyrins. In fact, the obtained production levels are so high that the produced porphyrins are excreted into the culture medium where they precipitate. HPLC, HPLC-MS and TLC methods for the detection, identification and quantification of

porphyrin compounds have also been established. To produce novel porphyrin structures, we have begun to create libraries of hemF and hemH and transform those libraries into recombinant E. coli that synthesize different porphyrin precursor structures.

Because a first library of randomly mutated hemC did not yield a variant pathway for the synthesis of expanded porphyrins, we changed our strategy towards a semi-rational approach. Reasoning that increased flexibility of the protein structure would be necessary to accommodate an expanded polypyrrole chain, we created random insertions of 2-3 amino acids into loop regions. Surprisingly, the majority of variants of the loop insertion libraries retained their wild type activity. Based on these findings, we physically dissected hemC at these positions such that we express an a and ? fragment of the protein in order to investigate whether these fragments are able to complement each other functionally. In a second series of plasmids we expressed only the a-fragment. To test for functional hemC activity we created a hemC, hemD deletion strain of E. coli into which we transformed these constructs. E. coli cells lacking the ability to produce heme grow only very slow, if at all, under aerobic conditions. Growth is restored in this strain upon complementation with functional genes for hemC and hemD. We also integrated the gene for the heme uptake receptor hasR from Serratia marcescens (kindly provided by C. Wandersmann, Paris) into the E. coli chromosome and thus obtained an E. coli strain without a wild type porphyrin background and capable of aerobic growth when supplemented with hemin in the culture medium. Both properties are desirable when screening libraries for novel porphyrin structures, in particular if the new compounds would inhibit wild type heme biosynthesis. With most of the hemC dissections and surprisingly with most of the hemC truncations as well, heme biosynthesis is restored in E. coli. From these results it appears that domain 1 alone, containing the catalytically active arginine residue, apparently can provide a catalytically active scaffold for the synthesis of sufficient amounts of linear tetrapyrroles to enable growth. We are conducting additional studies (in vitro and in vivo) to further confirm that a minimal hemC protein with only one domain left exhibits catalytic activity and investigate the porphyrin products. If we find this to be the case, we will generate libraries of domain 1 and/or domain 2 to screen for new catalytically active scaffolds that would then allow the synthesis of expanded polypyrrols.

## METABOLIC ENGINEERING OF ESCHERICHIA COLI W3110 FOR REDOX NEUTRAL AND OXIDIZED PRODUCTS

Thomas B. Causey, Shengde Zhou and L. O. Ingram
University of Florida

Microbial processes for commodity chemicals have focused on reduced products and anaerobic conditions where substrate loss to cell mass and CO2 are minimal and product yields are high. To facilitate expansion into more oxidized chemicals, Escherichia coli W3110 was genetically engineered for acetate production using an approach that combines attributes of fermentative and oxidative metabolism (rapid growth, external electron acceptor) into a single biocatalyst. The resulting strain (TC36) converted 333 mM glucose into 572 mM acetate, a product of equivalent oxidation state, in 18 h. With excess glucose, a maximum of 878 mM acetate was produced. Strain TC36 was constructed by sequentially assembling deletions that inactivated oxidative phosphorylation ( atpFH), disrupted the cyclic function of the tricarboxylic acid pathway ( sucA), and eliminated native fermentation pathways ( focA-pflB frdBC ldhA adhE ). These mutations minimized the loss of substrate carbon and the oxygen requirement for redox balance. Although TC36

produces only 4 ATPs per glucose, this strain grows well in mineral salts medium and has no auxotrophic requirement. Glycolytic flux in TC36 (0.5 µmol min-1 mg-1 protein) was 1.5-2.0 fold that of the parent. Higher flux was attributed to a deletion of membrane-coupling subunits in (F1F0)H+-ATP synthase that inactivated ATP synthesis while retaining cytoplasmic F1-ATPase activity. The effectiveness of this deletion in stimulating flux provides further evidence for the importance of ATP supply and demand in the regulation of central metabolism. Derivatives of strain TC36 may prove useful for the commercial production of a variety of commodity chemicals.

## AROMATIC BIOSYNTHESIS IN ARCHAEOGLOBUS FULGIDUS

H.G. Monbouquette, J.C. Liao and I. Schröder
University of California, Los Angeles

The aromatic amino acid synthesis pathway has been engineered successfully for the synthesis of natural and unnatural chiral amino acids, which are important drug intermediates, as well as other industrially important aromatics, such as indigo. Production of aromatics via engineered microbes offers both environmental and economic advantages including exclusive use of aqueous solvent and non-toxic intermediates, and lower raw material cost. Intense interest therefore has developed in the enzymes of these metabolic pathways. A. fulgidus is representative of the third, most primitive domain of life, and the aromatic amino acid synthesis pathways have not been explored in these microorganisms despite the fact that they may offer a far more robust set of biosynthetic enzymes well suited both for in vivo and in vitro synthesis applications. Recently, the entire genome of A. fulgidus was sequenced and a thorough study of open reading frames for sequences homologous to known enzymes was conducted. It is noteworthy that a number of enzymes involved in common aromatic amino acid synthesis routes were not identified on the genome. Our goal is to identify these new enzymes/pathways by a functional proteomics approach made possible by our demonstrated ability to culture A. fulgidus to the 100-liter scale, and to identify, isolate, sequence, clone and express (in E. coli) new enzymes from this microbe. This project focused on the coordinated use of LC/MS-based enzyme assays, DNA microarrays, and gene cloning and expression for screening of enzyme activities and for identification of genes in hypothesized metabolic pathways.

The following has been accomplished in this project to date: (1) the 15 A. fulgidus open reading frames (ORFs) homologous to known genes in the aromatic amino acid synthesis pathways were cloned in E. coli, were sequenced and soluble products were expressed for most, (2) a putative gene for a novel bifunctional phosphoribosyl (PRA) anthranilate transferase/indoleglycerol phosphate (IGP) synthase was found to be two separate genes, (3) a putative trifunctional chorismate mutase/prephenate dehydratase/prephenate dehydrogenase gene was confirmed using LC/MS-based assays to be the first triple activity fusion of its kind in a single polypeptide, (4) over-expressed shikimate dehydrogenase was purified and fully characterized, (5) a method for determining 95% confidence intervals for DNA microarray data was developed, (6) a full-genome DNA microarray for A. fulgidus was created (the first for an archaeon), (7) the presence of a trp operon was confirmed using the DNA microarray.

## MATHEMATICAL AND COMPUTATIONAL ANALYSIS OF CENTRAL CARBON PATHWAYS FOR EFFICIENT METABOLIC ENGINEERING

Vassily Hatzimanikatis
Northwestern University

The availability of the genome sequence for an organism and the application bioinformatics analysis allow the reconstruction of the biochemical networks present in this organism. Based on this knowledge and on information from experimental studies on metabolic fluxes, the intracellular metabolic fluxes can be accurately estimated. However, knowledge about the (steady state) fluxes in a biochemical network does not allow the determination of the responses of the network to changes in its kinetic parameters, such as changes in the activity of the participating enzymes.

In order to overcome this limitation we have developed a bioinformatics framework that employs knowledge about the stoichiometry of biochemical networks and the estimated values of the associated metabolic fluxes, modeling concepts from metabolic control analysis, computational methods, and nonparametric statistics. This framework allows a quantitative ranking of the enzyme manipulations with respect to their probabilities of success in achieving a desired change in metabolic fluxes. Furthermore, we can also characterize and quantify the robustness of a biochemical network in terms of its stability characteristic.

The utility and power of the methodology are illustrated on two examples: a branched biosynthetic pathway and the glycolytic pathway in yeast.

## COMPUTATIONAL ELUCIDATION OF METABOLIC PATHWAYS

Imran Shah
University of Colorado

Elucidating the metabolic network of a living system is an important requirement for modeling its physiological behaviour and for engineering its pathways. With the availability of whole genomes it is theoretically possible to infer the presence of putative enzymes and transporters in an organism. However, piecing this information into a complete picture is still mostly a daunting manual task for at least two reasons. First, we do not have accurate and sufficient annotation of enzymatic function from sequence. Consequently, many proteins in completely sequenced microbes remain functionally uncharacterized. Second, inferring the causal biochemical connections within a metabolic network is not straightforward. We are developing a computational infrastructure to address these challenges. In earlier work we have developed a machine learning (ML) approach to improve the assignment of enzymatic function from sequence. More recently, we have developed an artificial intelligence (AI) approach for the prediction of metabolic pathways and their interactive visualization. In this talk I will present an overview of this work and its relevance to metabolic engineering.

## STRESS, SOLVENT PRODUCTION AND TOLERANCE (in Clostridium acetobutylicum)

E. Terry Papoutsakis, Chris Tomas, Keith Alsaker, Hendrik Bonarius, He Yang, Jeff Beamish and Neil Welker
Northwestern University

Understanding solvent (and other toxic chemical) tolerance of microorganisms is crucial for the production of chemicals, bioremediation, and whole-cell biocatalysis. Past efforts to produce tolerant strains have relied on selection under applied pressure and chemical mutagenesis, with some good results, but not consistently so. We desire to examine if Metabolic Engineering (ME) and genomic approaches can be used to construct more tolerant strains for bioprocessing. The accepted dogma is that toxicity is due to the chaotropic effects of solvents on the cell

membrane. We have found that in C. acetobutylicum, several well-defined genetic modifications not related to membrane function impart solvent tolerance (by 40-70%) without strain selection. This suggests that we need to re-examine the accepted dogma. The objective of this research is to identify genes that contribute to solvent tolerance and to use genetic modifications (involving these genes) to generate solvent tolerant strains. A potential mechanism to overcome solvent toxicity is through the over-expression of heat shock proteins, possibly providing increased protein stability. A C. acetobutylicum strain, 824(pGROE1), over-expressing the molecular chaperone genes groES and groEL, under control of the clostridial thiolase promoter, was created to examine this hypothesis. Final acetone and butanol titers in the over-expressing strain were 66% and 56% higher than in the respective control strain, 824(pSOS95del). Both 824(pGROE1) and 824(pSOS95del) exhibited a sustained solvent production profile (120 hours versus 40 hours for wild type) with increased acetone and butanol formation fluxes. Western analysis of 824(pGROE1) confirmed over-expression of GroEL (3-180 fold) and revealed increased levels of proteins involved in solvent formation. DNA-microarrays suggest that the presence of a control plasmid in C. acetobutylicum results in a generalized stress response with decreased cell motility and chemotaxis. DNA-array analysis of various stresses points to changes to several important cellular programs and can serve as a roadmap for choosing other genes for possible ME intervention.

# Agency Activities in Metabolic Engineering

## U.S Department of Agriculture

The Agricultural Research Service (ARS) and the Forest Service (FS) conduct metabolic engineering research through the Federal laboratory system while the Cooperative State Research, Education, and Extension Service (CSREES) supports metabolic engineering research through competitive research grants (including grants funded through the Interagency Metabolic Engineering Program) and through formula-based programs in cooperation with the states.

USDA research activities encompass animal sciences, plant sciences, commodity conversion and delivery, environmental sciences (air, soil, water), human nutrition, and integration of agricultural systems.

Metabolic engineering technologies are being developed and applied across the above research areas and include the following goals:

- To modify microbial metabolism for the production of commercially useful products, chemicals, biofuels, and biomolecules from agricultural commodities and resources.
- To develop genetic and other techniques for altering metabolic pathways to understand basic processes associated with microbial based natural or newly developed biocontrol agents resulting in elimination, decreased use, or increased environmental bioremediation of both agricultural wastes and agricultural chemicals such as herbicides, insecticides, fungicides, or biocides.

- To improve efficiency of production and decrease losses due to environmental stresses, diseases, pathogens, parasites, or pests by altering host metabolism using genetic or other techniques to apply metabolic engineering at the tissue, organ, or whole organism level of animals or plants, alone or in combination with the microorganisms associated with these hosts.

Ongoing research in USDA labs includes:

- Metabolic engineering for the development of superior fuel ethanol producing microorganisms. Microorganisms that normally use multiple substrates are being engineered for enhanced ethanol production, and microorganisms that normally make ethanol are being engineered to use multiple substrates.
- Metabolic engineering of the castor plant to produce new chemicals that could replace petroleum-derived compounds, and to remove the plant's ability to manufacture its potent toxin, ricin, and allergens that can cause hives and asthma.
- Metabolic engineering of sunflower to produce latex of improved quality and quantity in its stems and leaves.
- Metabolic engineering of toxigenic fungi and host plants. Specifically, the genes involved in aflatoxin biosynthesis have been identified and a master switch gene discovered. By engineering plants to favor production of a metabolite that interferes with this master gene, aflatoxin production may be prevented in the host plant.
- Metabolic engineering of the liquid wax producing capability of jojoba into a metabolic pathway for commercially viable oilseed rape and soybeans.

Recent research funded by CSREES through the Interagency Metabolic Engineering Program includes:

- Metabolic engineering of the model plant Arabidopsis thaliana for higher levels of vitamin C production.
- Study of the metabolic regulation of carbon flow in plant trichome glands into specific diterpene compounds, with the long term goal of introducing coral genes in plant systems to produce pharmaceutically active compounds.
- Study of the response of the E. coli central metabolic pathway to specific genetic manipulations, with the goal of producing flavor compounds in microorganisms.

## National Institute of Standards and Technology

NIST has internal research programs in the Biotechnology Division, and extramural collaboratively funded research and development programs through the Advanced Technology Program that are related to the scientific field known as Metabolic Engineering. Each of these programs have different foci and management structures, but share the overall goal of fostering the commercialization of recent scientific advances in areas related to biotechnology, such as biocatalysis and metabolic engineering.

**Biotechnology Division (Intramural)**

In the intramural programs of the Biotechnology Division ( http://www.cstl.nist.gov/biotech ) , which is one of five Divisions of the Chemical Sciences and Technology Laboratory, the mission is to advance

the commercialization of biotechnology by developing the scientific/engineering technical base, reliable measurement techniques and data to enable U.S. industry to quickly and economically produce biochemical products with appropriate quality control. The mission is carried out in collaboration with industry, other government agencies and the scientific community. The primary research efforts that relate to Metabolic Engineering are in Bioprocess Engineering, Structural Biology, DNA Technologies, and Biomolecular Materials groups.

The Bioprocess Engineering ( http://cstl.nist.gov/div831/bioprocess ) activity includes biophysical property evaluation where thermophysical and thermochemical properties are being obtained, evaluated, codified and modeled for biochemicals, proteins and biosolutions of interest in metabolic pathway engineering. A research program in biocatalysis is underway to solve technical roadblocks in the commercial development of enzymes that build new complex molecules used in advanced drug or food product design. Other investigations include developing DNA-based reference standards for detecting and quantifying biotech crops, and fluorescence standards for interpreting DNA microarrays.

The Structural Biology activity includes x-ray and NMR measurements of atomic structures of prototypical proteins, enzymes, enzyme-substrate complexes and model DNA systems. A research program in biothermodynamics uses state-of-the-art calorimetric methods to study protein-protein and protein-substrate interactions, and computational models are developed that relate structure to function. Physical and biochemical methods are used to characterize protein behavior, including the study of membrane-embedded proteins to understand signal transduction. Computational chemistry and modeling develops methods to model the energetics and dynamics of interactions between substrates and active sites of enzymes. Modeling techniques to understand the relationship between protein sequence and structure are being developed.

The DNA Technologies activity includes development of methods and standards for DNA profiling for forensic and other uses. Research is being conducted to develop the next generation of DNA profiling based on polymerase chain reaction (PCR) technology including new methods development for rapid DNA extraction, amplification, separation, and computer imaging. DNA sequencing develops specific reference materials and technical expertise that are essential for DNA Genomic research in the public and private sector. This activity also provides quality assurance expertise to the developers of technology that proposes to use DNA recognition sites on silicon chips for the diagnosis of human genetic diseases. Research on DNA damage and repair is developing methods to characterize DNA damage on a molecular scale using GC/MS techniques. Studies of both in-vivo and in-vitro systems are underway to understand both damage (as low as one base per million) and repair mechanisms.

The Biomolecular Materials activity develops generic measurement technologies utilizing both optical and electrochemical approaches for applications in clinical diagnostics, bioprocessing, and environmental monitoring. Research on lipid membranes and membrane proteins is being performed to provide an understanding of materials and methods that will enhance the development of this important class of molecules in sensor and other applications. The light-sensitive protein, bacteriorhodopsin is being studied as a potential source for the storage and retrieval of information. Studies are underway to understand and control the mechanism of this optical transition, and to develop methods of

immobilizing this protein to increase its stability.

**Advanced Technology Program (Extramural)**

The Advanced Technology Program within NIST provides funding to support innovative research and development, which are likely to lead to inventive new technologies and products that will have positive economic benefits for the United States. ATP has in the past, and continues to fund projects in Metabolic Engineering. These projects include the modification of enzymatic pathways in microorganisms and improved bioprocessing technologies to produce, in a cost-effective way, monomers used in the synthesis of thermoplastics, essential cofactors for human health, disease-targeted therapeutics and desulfurized crude oil. Support also has been provided to companies seeking to engineer the synthesis of isoprenoids in yeast and biopolymers in the fibers of cotton plants. The production of better goods at lower costs and the utilization of renewable biosystems are potential benefits to be derived from these projects. As documented in more than a dozen White Papers submitted to ATP, industries' future commitments for applications of metabolic engineering are expansive and cover wide areas including immobilized biocatalysis, novel bioreactors, value-added crops, better nutrition and an improved environment

## Department of Defense

The Department of Defense (DoD) currently supports a broad range of research in the area of metabolic engineering through the Army Research Office (ARO) and other Army research activities, the Air Force Office of Scientific Research (AFOSR), the Office of Naval Research (ONR), and the Defense Advanced Research Projects Agency (DARPA). The specific focus of the ARO, AFOSR, ONR, and DARPA efforts will be summarized and future directions in metabolic engineering research and technology development will be addressed.

The broad needs for the DoD that can be served through research efforts in metabolic engineering are summarized below. These science and technology targets will provide enhanced and expanded capabilities for the missions of the services and provide greatly expanded capabilities for the civilian sector.

- Materials
- Processes
- Devices
- Fabrication Schemes
- Information Processing

Current interests in metabolic engineering at ARO are focused on two related topics: the characterization of biochemical pathways and enzymatic mechanisms and the genetic manipulation of protein structure and function. The goal is to develop a detailed understanding of how macromolecules have been tailored to execute their designated functions and how they interact with other macromolecules. With this information, it will be possible to engineer enzymes and metabolic pathways to exhibit a set of specific functions and properties, according to Army needs. ARO currently supports research in several areas, including: how molecular transport, subcellular compartmentalization, and reaction sequences are involved in enzymatic regulation and superstructural formation;

understanding and manipulating aminoacylation of tRNAs to produce, using cellular translation machinery, new polymeric peptide materials containing non-natural amino acids; the role and regulation of "stress" proteins differentially expressed in response to environmental or external stimuli; and the design and implementation of unique enzymatic strategies for the biodegradation of environmental pollutants.

For the AFOSR, space and aerospace materials are often produced by complex sequences of reactions involving toxic solvents and expensive catalysts. Some materials are derived from structures that are difficult to synthesize with traditional chemistry. Because of their remarkable specificity and efficiency, biocatalysts can enable the synthesis of a wide range of materials. They can catalyze de novo synthesis from renewable feedstocks, specific reactions in synthesis of monomers that are difficult to accomplish with conventional chemistry, and modification of polymers or composites at several stages of synthesis and assembly. Biocatalysts have substantial potential for deposition of thin films of organic or inorganic material including silicates. Development of biocatalytic approaches to synthesis will enable the development of materials with novel properties, reduce the cost of the material and eliminate the environmental impact of toxic chemical reagents.

AFOSR-supported work at the Air Force Research Laboratory has also led to the discovery of new catabolic pathways used by bacteria for the biodegradation of synthetic organic compounds. A variety of novel enzymes catalyze key steps in the pathways. The objective of the current work is to characterize the enzymes to determine the reaction mechanisms and then to explore the potential for use of the enzymes as biocatalysts for the synthesis of chemical feedstocks used in the production of space and aerospace polymers. Strategies are also being developed for the biological destruction of chemicals by bacterial enzymes.

One of the metabolic engineering foci at ONR, currently, is the microbial synthesis of energetic materials (EM) and EM precursors for the purposes of cost and environmental impact. Practically all such materials are non-natural products and their biosynthesis therefore requires the re-engineering of existing pathways and/or the assembly of new or hybrid pathways in one or more host organisms. An example of a simple EM precursor now under study is 1,2,4-butanetriol, which as its energetic trinitrate is used as a plasticizer in propellant and explosives formulations. More advanced EM targets, such as RDX, HMX and Cl20, involve high density fused ring cores with multiple nitramino (C-N(NO2)) substituents. While these are very difficult targets, they suggest worthwhile research goals such as the biosynthesis of highly electron withdrawing substituents on carbon (as in C-nitramino) or the assembly of strained heterocyclic rings. Clearly, a theoretical/experimental approach to the prediction of the true scope of enzyme reaction specificity, with energetic boundaries, would be particularly valuable in the design of pathways for EM biosynthesis. Other non-polymeric targets, besides EM, would include novel photonic/electronic/optical materials. Persons interested in metabolic engineering opportunities at ONR are strongly advised to communicate with Dr. Harold J. Bright (703-696-4054, brighth@onr.navy.mil) before submitting a proposal.

DARPA's metabolic engineering programs are driven by an interest in protecting human assets against biological threats and using biology to enhance both human and system performance. The general concept of this thrust is to understand how nature controls the metabolic rate of cells and

organisms (e.g., extremophiles, hibernation) and apply this understanding to problems of interest to DoD. Examples of current investments in metabolic engineering include efforts to develop technologies for engineering cells, tissues and organisms to survive in the battlefield environment so they can be used as sensors. DARPA is also developing technologies that permit the long-term storage of cells including human blood. More complete descriptions of current DARPA programs and solicitations in these areas can be viewed at http://www.darpa.mil/dso.

## U.S. Department of Energy

The Department of Energy is supporting over $25 million in metabolic engineering research, largely through the Offices of Science (SC), Energy Efficiency and Renewable Energy (EE), and Environmental Management (EM). The research falls in two main categories: 1) basic research, which involves the advancement of metabolic engineering fundamental knowledge and capabilities, and 2) applied research, which employs metabolic engineering techniques in development of target products. The basic research efforts of the Department reside within SC, whereas most of the applied research in this area is conducted within EE. In general, these research efforts are conducted by universities, national laboratories, and industry.

The Department's goals related to metabolic engineering research are to:

- To expand the level of knowledge and understanding of metabolic pathways and metabolic regulatory mechanisms related to the development of novel bio-based systems for the production, conservation, and conversion of energy.
- Apply metabolic engineering techniques to enhance and develop plants and microorganisms for use in the production of chemicals and fuels or for environmental remediation of waste sites.

Metabolic engineering research within SC is supported predominantly through the Office of Basic Energy Sciences (BES) and Biological and Environmental Research (OBER). Most of BES's metabolic engineering research resides within the Energy Biosciences program which has the mission to generate the fundamental knowledge required for the development of novel bio-based systems for the production, conservation, and conversion of energy. A significant part of the program has been and continues to be aimed at the development of metabolic engineering capabilities related to plants and fermentative microbes. These activities include defining metabolic pathways, characterization of the catalytic properties of enzymes, determining metabolic regulation mechanisms, development of gene transfer capabilities, kinetic analysis of the flow through a pathway, and in a few instances the actual metabolic engineering of specific pathways. The program focuses on the development of basic scientific knowledge as opposed to the development of specific processes.

The metabolic engineering research within OBER resides in three divisions: Health Effects and Life Sciences Research, Medical Applications and Biophysical Research, and Environmental Sciences. Most of the research is conducted in association with the human genome, microbial genome, structural biology, and environmental remediation programs. OBER's research in this area is directed toward enhancing fundamental knowledge of metabolic pathways and addresses the development of tools and capabilities to elucidate the kinetics and mechanisms of microbial metabolic

pathways; to create useful pathways for biotransformation of metals for biodegradation of toxic organics; and to understand complex relationships between genes, the proteins they encode, and the biological functions of these proteins in the whole organism.

In complement with its core research efforts, SC is conducting joint research with EM in support of their environmental restoration efforts and with EE in support of their fuels and chemicals production efforts. These newly formed partnerships demonstrate the spirit of collaboration and coordination within the Department, which combines science with technology to fulfill DOE's research missions.

Metabolic engineering research within EE is supported through the offices of Transportation Technologies (OTT), Industrial Technologies (OIT), and Utility Technologies (OUT). As applied R&D efforts, the focus is on specific research and market issues within the purview of the respective office. For example, research in OTT focuses on ethanol production using bacteria and yeast that feed on sugars derived from non-agricultural feedstocks. In OIT, the focus is on the development of bioprocesses and new chemical synthesis routes using whole organisms or enzymes in the production of chemicals and materials. Finally, the research in OUT focuses on the use of photosynthetic microorganisms, such as cyanobacterium or alga blue or green algae in the production of hydrogen. In each of these program efforts, the R&D activities address metabolic engineering to increase the production of the product(s) desired by either enhancing existing pathways, constructing new pathways, or designing alternative pathways.

Environmental Management (EM) has a modest biotechnology research effort in support of its mission in waste management related to the clean-up and restoration of the U.S. national laboratory sites. The focus of this research involves bioremediation, including intrinsic, chemical bioaugmentation, and phytological approaches to clean-up chlorinated compounds, heavy metals, and other hazardous organics. Metabolic engineering approaches are being used to improve the effectiveness and efficiency of their environmental clean-up efforts by enhancing, augmenting, or creating new metabolic pathways within target organisms or plants. More recently, EM has teamed with SC to pursue basic research needs in various areas of national laboratory clean-up issues and waste management.

## Environmental Protection Agency

### Developing Metabolic Engineering Strategies

The mission of the Environmental Protection Agency is to protect human health and the environment from adverse effects of anthropogenic activity. Included in this mission are various elements for which metabolic engineering can play a useful role.

One prominent concern is the introduction of chemicals to the environment which may have detrimental effects on humans and other biota. As mandated by statute and implemented by rule, the Agency routinely conducts evaluation of chemicals intended for use, currently in use, or determined to exist at significant levels in the environment. From these evaluations, the Agency may decide to implement management strategies designed to limit the potential for adverse effects.

The application of novel technologies such as the use of biotechnology as a substitute to conventional manufacturing and processing of raw materials into final products is consistent with the mission of the Agency. EPA implements this by supporting development of technologies which 1) use chemical substitutes that are less toxic; 2) produce more efficient activity resulting in decreased requirement for the chemical or; 3) develop engineering procedures which produce little or no toxic end products. Finally, consistent with the pollution prevention ethic is the reevaluation of chemical stewardship from one of "cradle to grave" to a more multigenerational philosophy in which a chemical may be utilized successively in different forms prior to final disposal. Metabolic engineering has a role to play by enabling the development of biological mechanisms for production or use that meet one or more of these criteria.

While it is generally accepted that chemical-based technologies have evolved to provide a higher standard of living for the general population, it is also recognized that the use of some chemicals, either through the chemical characteristics or the handling, synthesis or disposal, have produced negative effects on human health and/or the environment. Advances in technology allow scientists to better predict the potential for adverse effects from exposure to chemicals as well as mechanisms to diminish the negative effects of chemical production such as production of toxic byproducts and disposal of the chemical. The approach, which strives to identify synthetic pathways that are less polluting than existing pathways and that encourages the development of nontoxic chemical products, is referred to as "Green Chemistry". The use of metabolic engineering to evaluate the potential for increased risk from chemicals, by allowing the study of responsible metabolic pathways and by permitting modification of such pathways to reduce risk, is another way in which metabolic engineering firs within the EPA mission.

Finally, basic research, which utilizes methods of metabolic engineering, can provide longer range approaches to assist EPA in its overall mission of protecting human health and the environment. The EPA supports extramural metabolic engineering research through the Technology for a Sustainable Environment (TSE) program, which awards grants in the area of pollution prevention. Since 1995, the TSE program has funded metabolic engineering research related to methanol conversion, solvent tolerance, biopolymer production and pesticide production-all focused on the elimination of pollution at the source

## National Institutes of Health

### National Institute of General Medical Sciences

The National Institute of General Medical Sciences (NIGMS) supports metabolic engineering research, usually in the form of grants to investigators in universities (R01s) or in small businesses (SBIRs). These grants support basic research in two general areas: (1) the development of microbial or plant-based metabolic routes to useful quantities of small molecules such as polyketides; (2) the development of a much better understanding of the control architecture that integrates the genetic and catalytic processes in normal and aberrant cells. During fiscal 2002, the NIGMS is providing $13.6 million (47 grants) for the support of research directly involving metabolic engineering. Examples of funded projects include (1) a study of the pikromycin biosynthetic pathway, and (2) an in silico studies of E. coli growth.

## National Science Foundation

The **Directorate of Engineering** supports several investigators in the area of metabolic engineering. One common feature of these research projects involves purposeful changes in organism behavior for increased product yields and levels for both wild type and recombinant systems. In addition, the improved biodegradation of toxic compounds is also being approached through metabolic engineering. Biological processes of this type have significant industrial potential, but in many cases still require the necessary biochemical engineering to translate them into a scalable process. In order to obtain the highest yields of metabolite products, restructuring of the central pathways for carbon catabolism and dispersal of incoming carbon into synthetic pathways will be necessary. Because of the tight integration among these pathways and the energy-producing pathways, restructuring of this central core of metabolism will require a systems approach, which considers the interactions of the pathways concerned with the other metabolic subsystems in the cell. The system is complicated by regulation at both genetic and enzyme levels of all of these interacting metabolic subsystems. Therefore, an important aspect of the engineering research is the development of the mathematical systems, and control theory needed for a quantitative analysis and understanding of the metabolic changes which are initiated by the manipulation of the enzymatic, transport and regulatory functions of the cell. Examples of metabolic engineering research supported in this Directorate include: (1) the use of linear optimization theory for the network analysis of intermediary metabolism, (2) the development of methods to select the internal fluxes for experimental measurement based on their sensitivity to experimental error, (3) the development of a method to determine flux control coefficients using transient metabolite concentrations, and (4) a study of network rigidity to help overcome the cell control mechanisms that resist flux alterations at branch points in metabolic pathways.

The **Directorate for Biological Sciences** (BIO) supports a broad range of research activities directed at increasing the knowledge base required for metabolic engineering. Examples of several BIO activities with implications for Metabolic Engineering include the following: (1) the ãArabidopsis Genome Research Initiative:ä a multinational research cooperation to sequence the entire genome of the model plant, Arabidopsis thaliana, in order to establish baseline genomic data for plants, and to develop microarrays and other technology that can be used for further applications; (2) the ãPlant Genome Research Programä which supports research on plant genome structure and function. Research supported by this program is characterized by a systems approach to plant genome research that builds upon recent advances in genomics, bioinformatics, and plant biology. This program has already funded over 70 groups of investigators, often consortia of several universities and industries, to carry out sequencing and functional genomics projects. Supported efforts range from sequencing agriculturally important genomes (maize, soybean, tomato), to technology development, to focused applications (stress tolerance, pathogen responses, cotton fibers). (3) The "Microbial Observatories Initiative includes the study of novel microorganisms in soils, marine sediments, and aquatic environments. The tremendous diversity of currently undescribed microorganisms offers potential metabolic engineering spin-offs such as new pathways for biodegradation of environmental toxins and novel pharmaceuticals. (4) The BIO Directorate is in the second year of the ã2010 Projectä that supports research to determine the function of all genes in Arabidopsis thaliana by the year 2010. In the first year, 26 awards were made in support of creative and

innovative research designed to determine the function of networks of genes and to develop new tools for functional genomic approaches.

The **Directorate for Geosciences** supports research related to ME in marine ecological systems. Examples of research areas include: (1) determination of the physico-chemical requirements for the maintenance, growth, and regulation of marine microbes; (2) identification, isolation, and determination of the function of enzymes responsible for useful degradation processes; (3) exploration of marine viruses and how they can be used in genetic engineering; (4) development of molecular assays for harmful species of marine microbes; (5) determination of cellular and biochemical control of trace metal limitation; (6) characterization of enzymes and genes associated with nitrogen fixation in cyanobacteria; and (7) identification and characterization of marine microbes and consortia that degrade, detoxify, or metabolize marine pollutants.

The **Directorate for Mathematical and Physical Sciences** supports a number of projects involving metabolic engineering. Of particular interest is the use of new enzymes to facilitate catalytic processes such as the desymmetrization of achiral molecules and the development of new bacterial strains that will be useful for the conversion of petrochemical and other industrial byproducts into useful or benign derivatives. Theoretical work continues to explore the basis of information encoding which is the foundation of molecular genetics and its associated properties of self-replication and the nonrandom organization of genetic material into specific shapes. Bridges to the experimental realm provide ever more elegant examples of synthetic structures that mimic genetic principles. These experiments are expanding our understanding of the underlying chemistry of genetic and biochemical processes and provide the basis for such functional examples of chemical systems patterned after living systems as enzyme mimics. Additionally, the increasing understanding of the specific ways that drug molecules interact with gene-derived entities is the basis for a new era of chemotherapy.