Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda

A Research Roadmap Resulting from the Biomass to Biofuels Workshop Sponsored by the U.S. Department of Energy

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Sugar Fermentation to Ethanol

Fermentation of sugars by microbes is the most common method for converting sugars inherent within biomass feedstocks into liquid fuels such as ethanol. Bioconversion or biocatalysis is the use of microbes or enzymes to transform one material into another. The process is well established for some sugars, such as glucose from cornstarch, now a mature industry. Production of fuel ethanol from the mixture of sugars present in lignocellulosic biomass, however, remains challenging with many opportunities for improvement. More robust microorganisms are needed with higher rates of conversion and yield to allow process simplification through consolidating process steps. This development would reduce both capital and operating costs, which remain high by comparison with those of corn.

The growing U.S. industry that produces fuel ethanol from cornstarch has opportunities for incremental improvement and expansion. Processes for the bioconversion of lignocellulosic biomass must be developed to match the success in starch conversion (see sidebar, Starch: A Recent History of Bioconversion Success, this page). Technologies for converting cellulosic biomass into fuel ethanol already have been demonstrated at small scale and can be deployed immediately in pilot and demonstration plants. The challenge, with limiting factors of process complexity, nature of the feedstock, and limitations of current biocatalysts, remains the higher cost (see Fig. 1. The Goal of Biomass Conversion, p. 120). The discussion in this chapter will focus on process improvements that will reduce risk, capital investment, and operating costs. This emphasis is driven by the goal to integrate and mutually enhance the programs in DOE's Office of the Biomass Program (OBP) and Office of Biological and Environmental Research (OBER) related to achieving the president's goal of a viable cellulosic ethanol industry.

Bioconversion must build on its historic potential strengths of high yield and specificity while carrying out multistep reactions at scales comparable to those of chemical conversions. Biology can be manipulated to produce many possible stoichiometric and thermodynamically favorable products (see Fig. 2. Examples of Possible Pathways to Convert Biomass to Biofuels, p. 121), but bioconversion must overcome the limitations of dilute products, slow reactions, and often-limited reaction conditions. For commodity products such as fuels, biologically mediated conversion represents a large fraction

Starch: A Recent History of Bioconversion Success

Biotechnology has a track record of displacing thermochemical processing in the biomass starch industry. In the 1960s, virtually all starch (a sugar polymer in granules) was processed by acid and high temperatures. Inhibitory by-products and lower conversion rates resulted in a soluble starch solution that was lower in quality and yield when further fermented to ethanol. Development of specific thermostable high-productivity enzymes (e.g., alpha-amylase and glucoamylase) produced a higher-quality soluble starch, completely displacing the acid process by 1980. This new process has allowed technologies for producing ethanol from starch to continuously improve to the high yield and rate levels seen today in wet and dry corn mills. Other starch-conversion enzymes (e.g., glucose isomerase) have made possible another commodity product, high-fructose corn syrup, which is used in virtually all domestic sweetened beverages and many other products (www.genencor.com/wt/gcor/grain).

References: p. 154

Fig. 1. The Goal of Biomass Conversion. Securing cost-effective biofuels from biomass feedstocks requires moving biological technology from the laboratory (a. Microbial Cultures at Oak Ridge National Laboratory) through the pilot plant (b. National Renewable Energy Laboratory's Process Development Unit) to the full industrial biorefinery [c. Industrial Biorefinery in York County, Nebraska (Abengoa Bioenergy Corporation)].

of costs and selling prices (unlike the pharmaceutical industry, where bioconversion costs are small) (Lynd, Wyman, and Gerngross 1999). Ultimately, goals in this roadmap seek to define and overcome the biological limitations for key conversion parameters of metabolic flux and product, thermal, and pH tolerances to develop a robust bioconversion process. Several chapters articulate practical advantages and some challenges of biocatalysis and biomass conversion. While most biological research has focused on systems relevant to basic knowledge or medical applications, it has provided a wide base of tools and knowledge for application to the bioconversion of biobased feedstocks.

This discussion focuses on defining and prioritizing requirements for science and technology pathways that reach the maximal potential of biomass bioconversion. Results build on approaches developed in prior workshops (Scouten and Petersen 1999; Roadmap for Biomass 2002). This chapter expands that focus in light of new biological research tools and understanding. The new biology will use such emerging technologies as proteomics, genomics, metabolomics, protein-complex characterization, imaging, modeling and simulation, and bioinformatics. This joint effort will further guide the development of new high-throughput (HTP) biological tools (e.g., screening, functional assays, and resequencing).

Some common themes arose during the workshop. (1) At present, we reaffirm recalcitrance of lignocellulosic biomass as a core issue, but portions of both the science and the conversion solution clearly are within the microbial world. (2) Understanding microorganisms will enable us to manipulate them so they can reach their maximal potential in human-designed processes. (3) The first thrust is to develop biocatalysts that will allow design and deployment of conversion processes that are less costly in operation and capital than current lignocellulose-to-ethanol conversion processes. (4) Another major thrust is to eliminate or combine separate processing steps by developing a "multitalented" robust microorganism. Research and development are addressing both strategies. (5) Even with molecular biology approaches, scientists create alterations (usually a single change) and observe the result. While experimental validation always is needed, new global genomics methods offer the potential for intelligently predicting the impact of multiple simultaneous changes.

The new omic tools enable a deeper and more complete understanding of the microbial "state" and its physiology in its environment—enabling the probing of dynamics, regulation, flux, and function. Combining this understanding with the goal of improving microbial traits by manipulation will
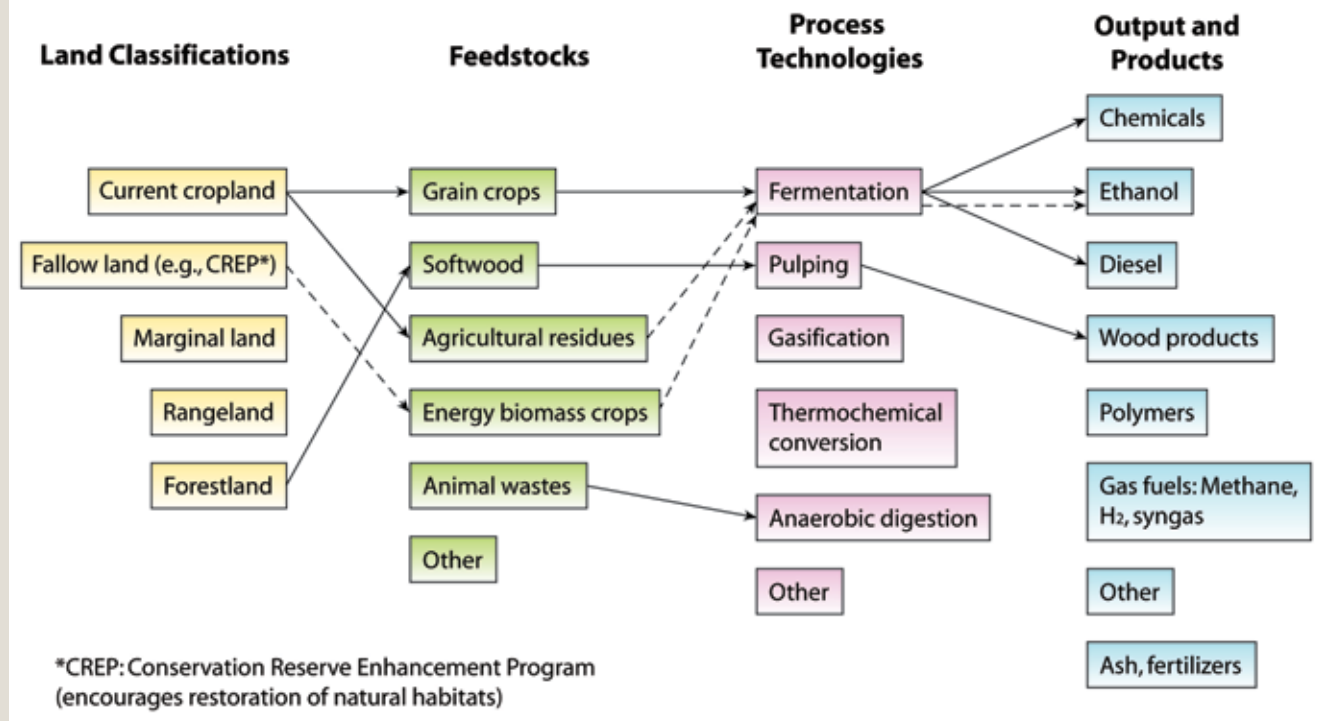
Fig. 2. Examples of Possible Pathways to Convert Biomass to Biofuels. The dotted lines show examples of factors this roadmap can accelerate; solid lines indicate existing paths. [Source: B. Davison, Oak Ridge National Laboratory]

allow regulation of the microbe to achieve desired outcomes. Many traits or phenotypes, such as overall glycolysis rate or ethanol tolerance, will be multigenic. To identify further potential improvements, we especially need rapid methods to assess the state of microorganisms that have been engineered with new properties—either new process traits or industrial robustness. A first step is to analyze of how current industrial microbes have evolved through human selection from their progenitors to be better adapted to their process environments.

As stated, we need to achieve rapid analysis, modification, and understanding of the biocatalytic system to accelerate implementation of organisms for efficient bioconversion of sugars into ethanol. An array of basic microbial requirements includes full microbial system regulation and control, tools for rapid manipulation of novel microbes, and new microbial platforms. More practical requirements for biocatalysts include utilization of all sugars and a robust microorganism. The first may require deeper metabolic and regulatory understanding. The second requires an understanding of stress response and inhibition. It can be implemented by inserting all capabilities into one host or by using multiple microbial species with unique, complementary capabilities in a controlled, stable mixed culture. To enable this research and development, certain microbial-specific enabling tools are discussed. Through a deeper understanding of the microbial system, new biocatalysts can be developed to reduce process cost and risk in developing a truly sustainable industry.

Three core biological barriers have been identified as high-priority research areas for improving current bioconversion processes: Optimizing microbial strains for ethanol production, developing advanced microorganisms for process simplification, and creating tools and technologies to enhance the analysis, understanding, and use of microbial systems. We also consider several speculative, breakthrough opportunities offering novel approaches to biofuel production that could further reduce cost and risk in the more-distant future. These breakthrough, high-payoff opportunities include use of microbial communities rather than pure cultures for robust energy production, model-driven design of cellular biocatalytic systems, direct production of more energy rich fuels such as alkanes or long-chain alcohols, microbial production of up to 40% ethanol from biomass, and microbial conversion of biomass-derived syngas to ethanol and other products. Although such ideas as a pure in vitro multienzymatic system were considered, they seemed unlikely to compete with advantages microbes offer in producing, regulating, and using complex multistep carbon and energy metabolic pathways as commodities in the next 20 years.

Optimizing Microbial Strains for Ethanol Production: Pushing the Limits of Biology

A major barrier in the efficient use of biomass-derived sugars is the lack of microbial biocatalysts that can grow and function optimally in challenging environments created by both biomass hydrolysis and cellular metabolism. The new tools of biology will facilitate the development of these advanced biocatalysts. Problems include inhibition by deleterious products formed during biomass hydrolysis, yields limited by accumulation of alternative products, unnecessary microbial growth, and suboptimal specific productivity resulting from various limitations in the ethanol biosynthetic pathway and a mismatch in conditions with the hydrolysis enzymes. Another challenge is that inhibition by the main fermentation product (ethanol) results in low alcohol concentration (titer). These problems contribute to the cost of lignocellulosic ethanol by increasing capital expenditure, reducing product yields, and increasing water volumes that must be handled as part of relatively dilute product streams. The research objective is to mitigate these limitations through concerted application of emerging tools for systems biology, working with principles from metabolic engineering and synthetic biology, and using evolutionary approaches combined with quantitative evaluation of candidate high-producing strains.

To foster an industry based on biomass sugars, process parameters must be comparable to those of the cornstarch ethanol industry. Ultimately, the overall cellulosic process can compete with petroleum, whereas cornstarch processes alone cannot achieve the needed quantities. Current technology is based on cornstarch conversion to ethanol utilizing yeast. This process uses glucose as the carbon source and converts it at high yields (90%), high titers (10 to 14 wt %), and reasonable rates (1.5 to 2.5 g/L/h). Recombinant ethanologenic organisms (i.e., yeast, *E. coli*, and *Z. mobilis*) have been created to ferment both glucose and xylose,
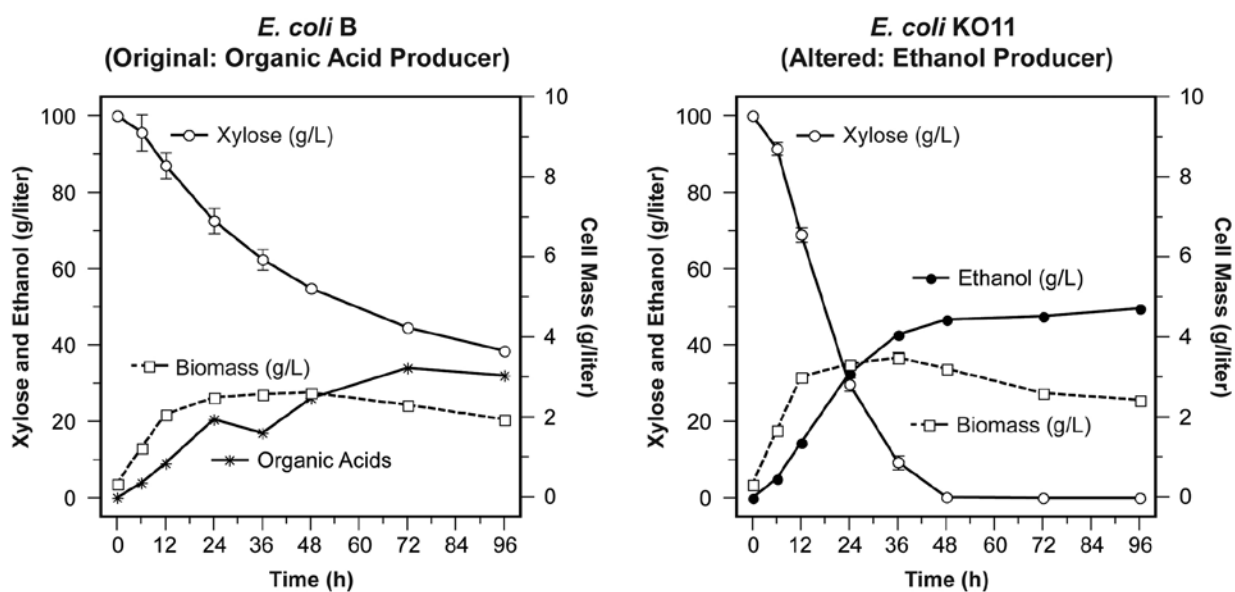
Fig. 3. Changes in Metabolism Brought About by Genetic Engineering. The *E. coli* B strain, an organic acid producer, was altered to the *E. coli* strain KO11, an ethanol producer (ethanologen). The altered KO11 yielded 0.50 g ethanol per g xylose (10% xylose, pH 6.5, 35°C). In the graphs, biomass refers to the cell mass of *E. coli*. [Source: L. Ingram, University of Florida. Based on data reported in H. Tao et al., "Engineering a Homo-Ethanol Pathway in *Escherichia coli*: Increased Glycolytic Flux and Levels of Expression of Glycolytic Genes During Xylose Fermentation," *J. Bacteriol.* **183**, 2979–88 (2001) .]

but they currently produce lower ethanol titers (5 to 6 wt % ethanol). Improvements in ethanol yields and tolerance are needed to increase rates of production (>1.0 g/L/h) from all sugar constituents of lignocellulosic biomass. One successful strategy for utilization of both hexose and pentose sugars takes known ethanologens like yeast and adds abilities to utilize pentose sugars. Another strategy takes mixed-sugar consumers like *E. coli* and replaces native fermentation pathways with those for ethanol production. Figure 3. Changes in Metabolism Brought about by Genetic Engineering, this page, shows an example of how the output of a microbe can be changed. As titers are increased, rates slow down and eventually cease at ~6 wt % ethanol, the upper limit for wild-type *E. coli*. By comparison, wild-type yeast and *Z. mobilis* can reach titers of >15% ethanol from cornstarch glucose but have failed to achieve these levels on pentose sugars (see section, Optimal Strains: Fermentative Production of 40% Ethanol from Biomass Sugars, p. 149).

Most methods of biomass pretreatment to produce hydrolysates also produce side products (e.g., acetate, furfural, and lignin) that are inhibitory to microorganisms. These inhibitory side products often significantly reduce the growth of biocatalysts, rates of sugar metabolism, and final ethanol titers. In all cases, the impact of hydrolysates on xylose metabolism is much greater than that of glucose. Research described here offers the potential to increase the robustness of ethanologenic biocatalysts that utilize all sugars (hexoses and pentoses) produced from biomass saccharification at rates and titers that match or exceed current glucose fermentations with yeast.

From the above analysis of present and target states regarding use of biomass hydrolysates for biofuel production, critical parameters needed for a cost-competitive process are clearly evident:

1. High yield with complete sugar utilization, minimal by-product formation, and minimal loss of carbon into cell mass.

2. Higher final ethanol titer.

3. Higher overall volumetric productivity, especially under high-solid conditions.

4. Tolerance to inhibitors present in hydrolysates.

Specifically, the following figures of merit are suggested for a biomass-to-ethanol process that will be cost-competitive relative to current cornstarch ethanol operations:

• Use of both hexoses and pentoses to produce ethanol at a yield greater than 95% of theoretical yield.

• Final ethanol titers in the range of 10 to 15 wt %.

• Overall volumetric productivity of 2 to 5 grams of ethanol per liter per hour.

• Ability to grow and metabolize effectively in minimal media or on actual hydrolysates (with only minerals as added nutrients).

To achieve the above targets, we must improve our ability to grow organisms in an inhibitory environment of high concentrations of sugars and other compounds, including ethanol. In addition, significant increases in flux through the sugar-to-ethanol metabolic pathway are needed. We present a roadmap below for meeting these objectives.

## Science Challenges and Strategy

Key questions include:

• What are the implications of simultaneous vs sequential consumption of 5-carbon and 6-carbon sugars on cellular metabolism, flux, and regulation, especially when xylose metabolism has been engineered into ethanologens?

• What can allow more rapid and controlled alteration of microbes, especially regulatory controls and "adaptation" to novel inserted genes or deleted genes? This consideration applies also to known industrial microbes.

• What mechanisms control glycolytic flux, and what are their implications for cellular metabolism? For example, could the glycolytic pathway efficiently handle an excess of carbon flux in an organism engineered to rapidly consume a mixture of 5- and 6-C sugars (see Fig. 4. Recombinant Yeast, *S. cerevisiae*, with Xylose Metabolism Genes Added, p. 125)? A systems biology approach will allow insights into the molecular basis for these processes and development of predictive models to refine their design.

- What molecular mechanisms are used by cells to cope with such environmental challenges as high concentrations of sugars and ethanol and the presence of inhibitors from biomass hydrolysis?

- What genetic and physiological characteristics mediate evolution of wild-type organisms into robust laboratory or industrial strains, and which ones control their functional state in the process environment (see sidebar, Proteomic and Genomic Studies of Industrial Yeast Strains and Their Ethanol-Process Traits, p. 126)?

Utilizing a combination of metabolic engineering and systems biology techniques, two broad methods for developing more capable and more tolerant microbes and microbial communities are the recombinant industrial and native approaches.

- Recombinant industrial host approach: Insert key novel genes into known robust industrial hosts with established recombinant tools.

- Native host approach: Manipulate new microbes with some complex desirable capabilities to develop traits needed for a robust industrial organism and to eliminate unneeded pathways.

These methods require genetic understanding of the trait we wish to be added or preserved and robust tools for genetic manipulation. The subset of biochemical pathways potentially involved in glycolysis is complex (Fig. 5. Some Metabolic Pathways that Impact Glucose Fermentation to Ethanol, p. 128). Our goal is to pare this down to just what is essential for xylose and glucose use (Fig. 6. Desired Metabolic Pathways for a Glucose-Xylose Fermenting Ethanologen, p. 129). Both methods can have value; for example, either eliminate unecessary pathways in *E. coli*, which has yielded strains that efficiently metabolize both xylose and glucose (and all other sugar constituents of biomass) to ethanol, or add xylose-fermenting pathways (and others) to ethanol-producing yeast. A number of methods and approaches support the two broad strategies. These and other goals will require certain enabling microbiological tools (see section, Enabling Microbiological Tools and Technologies That Must be Developed, p. 138).
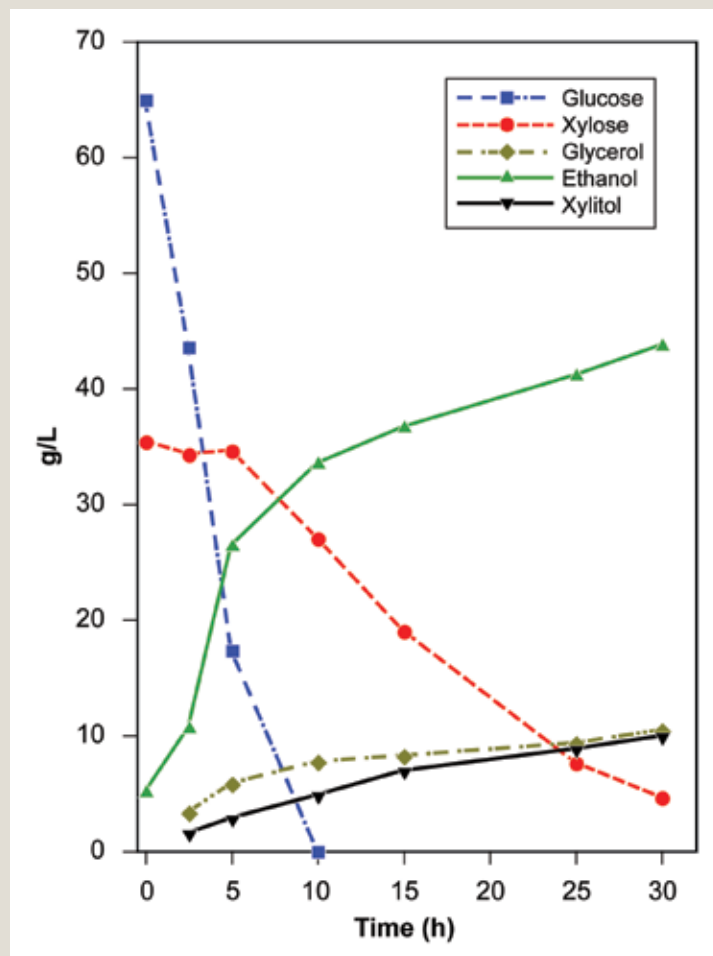
Fig. 4. Recombinant Yeast, *S. cerevisiae*, with Xylose Metabolism Genes Added. Following rapid consumption of glucose (within 10 h), xylose is metabolized more slowly and less completely. Ideally, xylose should be used simultaneously with glucose and at the same rate, but the xylose is not totally consumed even after 30 h. Also note that yield is not optimal. Although ethanol is the most abundant product from glucose and xylose metabolism, small amounts of the metabolic by-products glycerol and xylitol also are produced. [Source: M. Sedlak, H. J. Edenberg, and N. Ho, "DNA Microarray Analysis of the Expression of the Genes Encoding the Major Enzymes in Ethanol Production During Glucose and Xylose Cofermentation by Metabolically Engineered *Saccharomyces* Yeast," *Enzyme Microb. Technol.* **33,** 19–28 (2003). Reprinted with permission from Elsevier.]

Proteomic and Genomic Studies of Industrial Yeast Strains and Their Ethanol-Process Traits: Rapidly Finding the Genetic and Functional Bases

Current industrial yeast strains have been isolated from wild yeast populations for many decades, selected for their capacity to produce ethanol under industrial settings. Understanding how these selected genotypes and phenotypes differ from undomesticated strains of yeast would help us to understand the type of changes needed to develop a robust ethanol producer. Understanding how cells cope with high-ethanol media concentrations is essential to improve fermentation yield and titer. Similar studies would be beneficial for other industrial organisms, such as *Escherichia coli*. Gaining insight about an organism's process of adaptation to an industrial biorefinery environment can help us intentionally replicate these changes (see Fig. A. Importance of Adaptation for Robust Initial Strains, below). The strategy for studying industrial strains follows.

- Compare proteomic and genomic sequences of the most common yeast strains manufactured and sold for ethanol production with those of their ancestral parent strains.

- Compare proteomic and genomic sequences of evolved strains produced through metabolic engineering and metabolic evolution with those of their parental strains. Proteomic studies will be performed on samples taken from industrial fermentations. Genomic studies of strains from the same processes should reveal differences between industrial and laboratory strains that will provide fundamental information regarding multigenic traits essential for high metabolic activity, product tolerance, and adjustments to engineered changes in metabolism.

Studying genomes of industrial yeast strains will help us understand common traits of effective ethanol-producing strains. Proteomic studies will reveal proteins generated under actual industrial production conditions. Complete mapping and reconstruction of the strain's networks will be needed for proper comparisons. Available modeling tools are being improved continuously. Proteomic analysis of membrane proteins is still a challenge and needs to be developed further to guarantee a more complete and meaningful analysis of samples.

Data generated through this effort will require full use of all tools available for systems biology and will stimulate hypothesis generation and testing by the academic community. The effect will be similar to those from metagenomic studies and community proteomics, in which huge amounts of data were made available and are being analyzed by many different groups around the world.
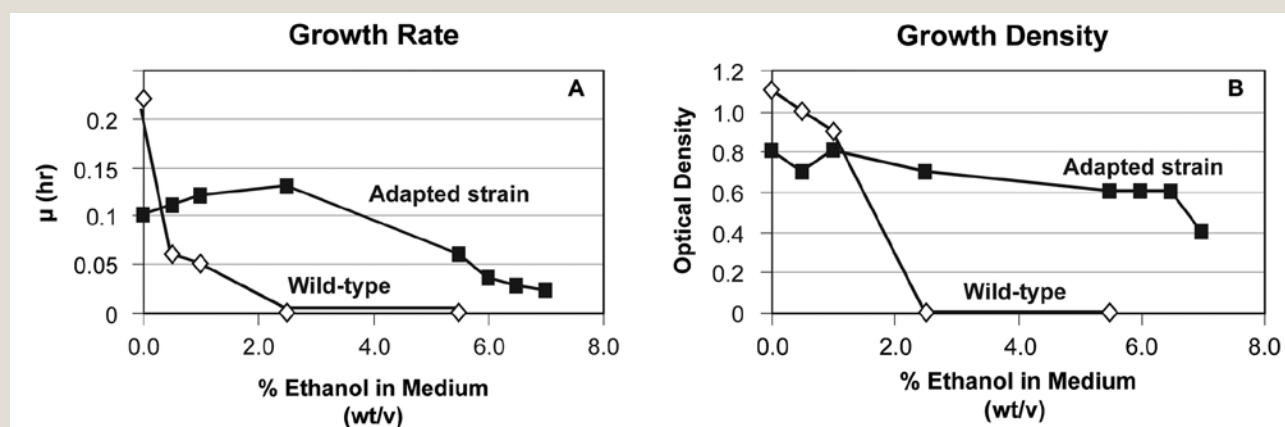

Fig. A. Importance of Adaptation for Robust Industrial Strains. [Source: H. J. Strobel and B. Lynn. 2004. "Proteomic Analysis of Ethanol Sensitivity in *Clostridium thermocellum*," presented in general meeting, American Society for Microbiology, New Orleans, La., May 23–27, 2004.]

Metabolic Engineering

Yield and productivity enhancement will be accomplished by applying *metabolic engineering* concepts and methods. *Yield maximization* is tantamount to by-product minimization, which is achieved by eliminating branches of competing pathways that lead to unwanted products. This usually is done by deleting genes encoding enzymes that catalyze competing reaction pathways. If such pathways are responsible for synthesis of metabolites essential for cell growth and function, downregulation of these genes may be preferable to complete gene knockout. In all cases, optimal balancing of enzymatic activities is critical for satisfactory function of the resulting engineered strain. Current molecular biological methods can be deployed successfully to this end, including specific gene knockout, gene amplification through promoter libraries or regulated (induced) promoters, and other methods combining gene knockout or downregulation and gene amplification. The ability to measure detailed cell behaviors and develop predictive models to refine their design will be critical to speed up and enhance these engineering efforts.

A related part of this work is analysis and regulation of cellular energetics. Careful alteration of growth, energy, and redox often is needed. Frequently, decoupling growth from production will increase yield.

Productivity maximization has been demonstrated in many applications of metabolic engineering with *E. coli* and yeast strains. Examples include 1,3 propanediol, amino acids such as lysine and threonine, biopolymer biosynthesis, precursors of pharmaceutical compounds, ethanol, and many others (see Fig. 7. A 3G Titer from Glucose, p. 130). These examples illustrate the feasibility of significant specific productivity enhancements by applying genetic controls, sometimes in combination with bioreactor controls. Improvements suggest that projected enhancements in specific cell productivity are entirely feasible and that the new technologies of systems biology can dramatically increase and accelerate results.

The first generation of specific productivity improvement will target enzymes important for the sugar-to-ethanol pathway. Stable isotopes will be used as tracers to map the metabolic fluxes of ethanol, including related pathways producing or consuming energy or redox metabolites (e.g., ATP or NADPH), and other key precursors for ethanol biosynthesis. Flux maps, together with transcriptional profiles, will be generated for control and mutant strains to identify enzymes controlling overall pathway flux. Gene amplification of rate-limiting steps will be used to overcome flux limitations. This is anticipated to be an iterative process, as new limitations are likely to arise as soon as one is removed by gene modulation. The goal will be to amplify flux of the *entire pathway* without adverse regulatory effects on the organism's growth or physiology. Again, balancing enzymatic activities, removing limiting steps, and pruning unwanted reactions—all supported by comprehensive analysis and modeling—will be deployed for this purpose.

In addition to specific pathway steps, remote genes with regulatory and other (often-unknown) functions impact pathway flux. Modulation of

such genes has been found to influence significantly the biosynthesis rate of many products. Such genes will be found through inverse metabolic engineering, whereby libraries of endogenous and exogenous genes are expressed in the host strain and recombinants are selected on the basis of drastic improvements in the desirable phenotype (e.g., ethanol production and tolerance). Genes conferring these phenotypes can be sequenced and identified for expression in clean genetic backgrounds.

Recombinant Approach

Tolerance to inhibitors is a multigenic property. In the example systems given above, this trait is founded primarily on membrane fluidity and other membrane properties and functions. In general, efforts to improve microorganism tolerance by recombinant gene manipulation have been confounded by the limited ability to introduce *multiple* gene changes *simultaneously* in an organism. Development and use of a systems approach that allows multiple-gene or whole-pathway cell transformation are important milestones.

Evolutionary Engineering

A strategy for increasing ethanol tolerance or other traits could use *evolutionary engineering* concepts and methods. This strategy would allow the microbial process to evolve under the proper selective pressure (in this case, higher ethanol concentrations) to increasingly higher ethanol tolerances.
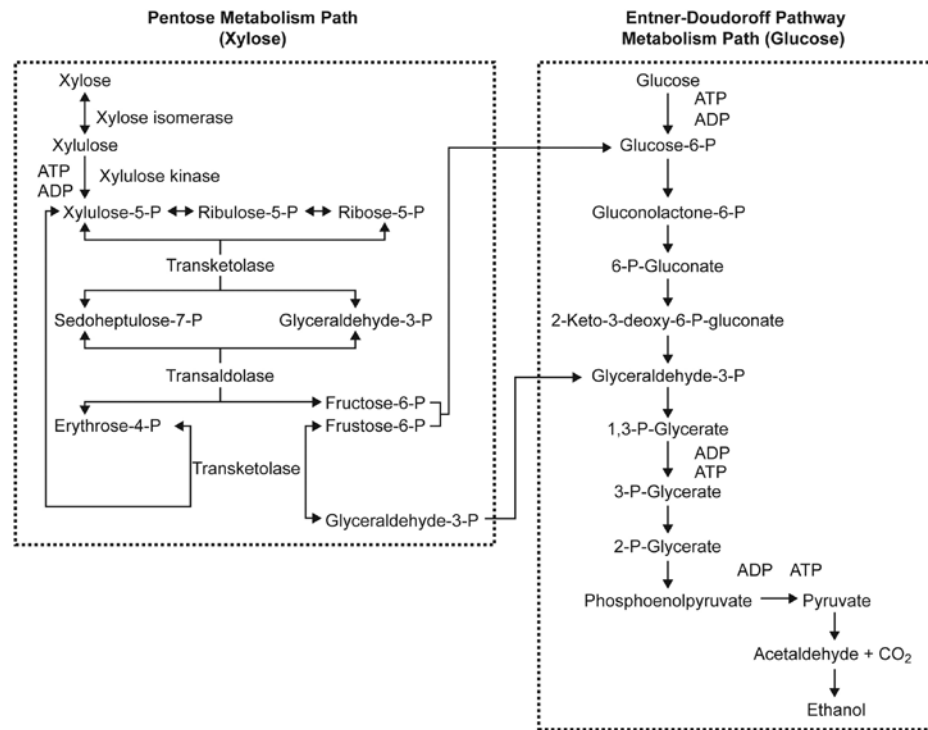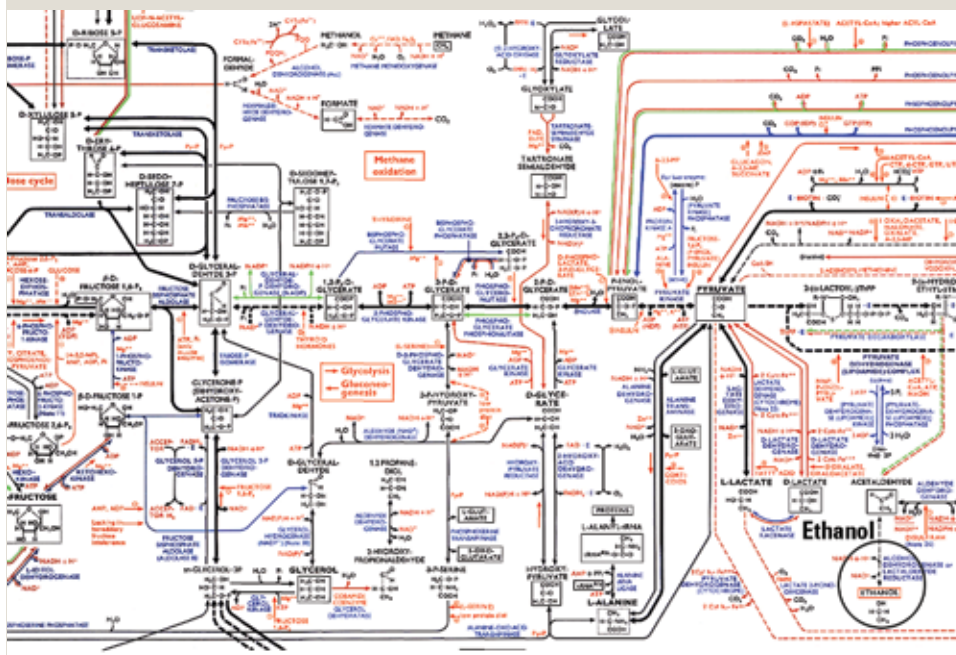
Fig. 5. Some Metabolic Pathways that Impact Glucose Fermentation to Ethanol. This pathway map demonstrates the complexity of even a simple, widely utilized, and relatively well-understood process such as glucose fermentation to ethanol. Glucose
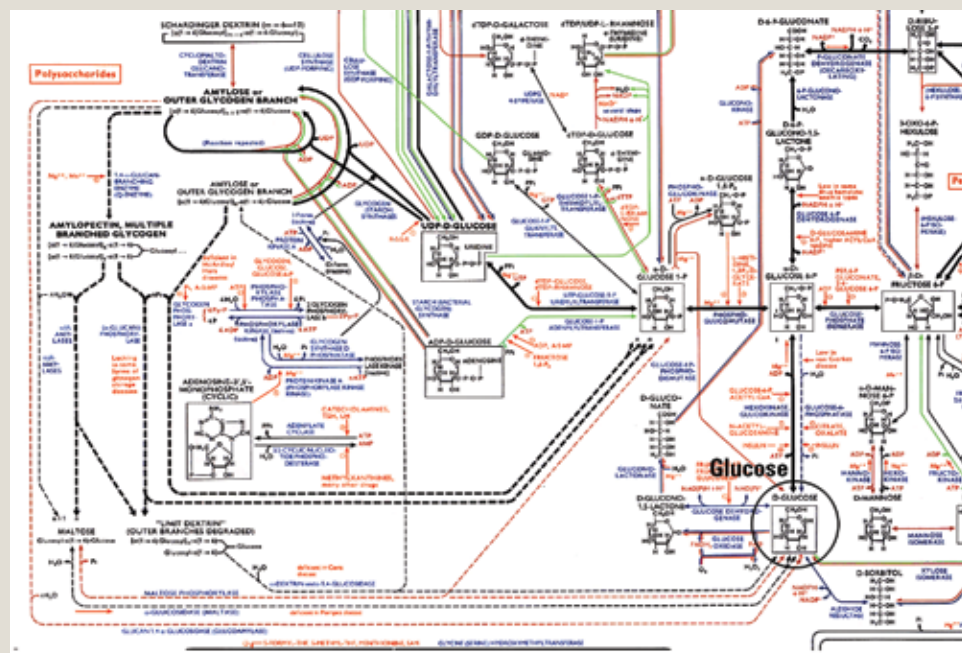
Evolutionary engineering can be applied to the ethanol-producing organism as a whole or to specific proteins, in particular those with regulatory functions. In the latter case, evolutionary engineering emulates the methods of *directed evolution,* which has proven very successful in engineering protein mutants with specific desirable pharmaceutical, regulatory, or kinetic properties.

Accurate characterization of cell and protein mutants will be needed to allow an understanding of principles for improving and rationally carrying out the designs. This task will require sequencing, large-scale binding experiments,

Fig. 6. Desired Metabolic Pathways for a Glucose-Xylose Fermenting Ethanologen. The goal is to genetically engineer an industrial organism that can metabolize both sugars. Pathways indicate the many involved genes that would have to be functional in such an organism.

and ethanol are identified. [E. Gasteiger et al., "Expasy: The Proteomics Server for In-Depth Protein Knowledge and Analysis," *Nucleic Acids Res.* **31**, 3784–88 (2003). Screenshot source: http://ca.expasy.org/cgi-bin/show_thumbnails.pl.]
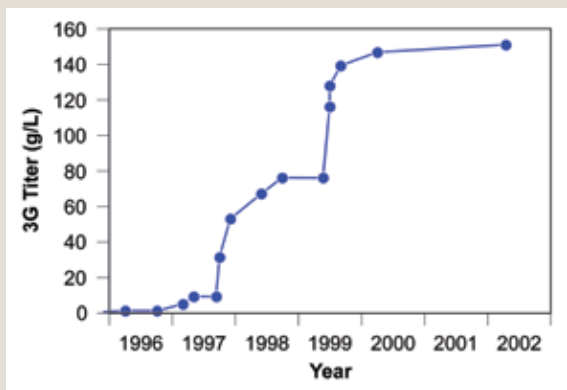
Fig. 7. A 3G Titer from Glucose. The graph shows DuPont-Genencor success in altering *E. coli* to maximize yield and titer of 3G (1,3, propanediol). Projects such as this could greatly benefit from the deeper systems biology understanding that GTL seeks. [Source: Adapted from C. E. Nakamura and P. Soucaille, "Engineering *E. coli* for the Production of 1,3-Propanediol," presented at Metabolic Engineering IV, Tuscany, Italy, October 2002.]

transcriptional studies, proteomics, metabolomics, and physiological functional evaluation, all well suited for GTL capabilities described in more detail below.

Evidence is growing that methods of evolutionary engineering and directed evolution of regulatory proteins have the potential to achieve the targets of tolerance to ethanol and other inhibitory compounds. Recent studies with *E. coli* have increased that organism's ethanol tolerance by more than 50% while comparable increases also have been obtained for yeast at high ethanol (6%) and glucose (100g/L) concentrations. Applying systems biology methods and HTP technologies and computing will accelerate the process by revealing the genetic, molecular, and mechanistic impacts of evolutionary methods.

These methods also will be used to isolate fast-growing organisms. High growth rates and final biomass concentrations are imperative for achieving high-volumetric productivities, since the latter depend on fermentor biomass concentrations. More detailed investigation is needed on the effects of various biomass-hydrolysate compounds on cell *growth*, especially factors responsible for gradual reduction in specific ethanol productivity during fermentation as sugars are depleted and products, particularly ethanol and other inhibitors, accumulate.

Technical Milestones

Within 5 years

- Mesophilic microbes demonstrated at scale that are capable of full utilization of all lignocellulosic sugars for reduced commercialization risk. This requires optimization of developed and partially developed strains.

- Increased strain tolerance to inhibitory hydrolysates and ethanol, with the ability to use all sugars, including mesophile and thermophile strains.

- Understanding of multigenic causes of industrial robustness.

- Candidate microbes such as thermophilic ethanologens compatible with desired cellulase enzyme optima. This allows process simplification to single-vessel fermentation with efficient use of all biomass-derived sugars (see section, Advanced Microorganisms for Process Simplification, p. 132).

- Development of coproducts.

Within 10 years

- Rapid tool adaptation and regulation of genetically engineered strains, including use of minimal media.

- Ability to engineer ethanol tolerance and robustness into new strains such as thermophiles.

- Higher-yield microbes via control of growth and energetics.

- Increased product titer to simplify product recovery and reduce water use.
- Full predictive metabolic pathway systems model for common industrial microbes, including regulation and identification of unknown genes (see section, Model-Driven Design of Cellular Biocatalytic Systems Using System Biology, p. 142).

Within 15 years

- Thermophillic microbes demonstrated at scale to enable simultaneous saccharification and fermentation.
- Further refinement of biofuel process and operation.

The Role of GTL Capabilities

As discussed, achieving these objectives will require the use of rational-combinatorial and evolutionary approaches to improve the properties of individual enzymes and organisms. To inform, enhance, and accelerate manipulation of new microbes, systems biology analyses (e.g., omic measurements, knockouts, tagging of proteins and complexes, visualization, and a bioinformatic core structure for data) will be applied. Once the novelties (e.g., pathways, proteins, products, traits, and complexes) are identified, additional genetic tools will move desired genes and traits into a known industrial host or further manipulate novel microbes into an industrial organism by adding gene traits. There are no consistent and rapid tools for these manipulations at present.

The capabilities listed below will play an important role in both cases.

Protein Production

A wide range of proteins (regulatory, catalytic, and structural) will be produced and characterized, and appropriate affinity reagents will be generated. Modified proteins also will be used to understand functional principles and for redesign. Examples include glycolytic proteins and alcohol dehydrogenases from other organisms or those evolved in the lab, structural proteins from high-tolerance organisms, or regulatory proteins with altered properties.

Molecular Machines

HTP methods to identify binding sites of global regulatory proteins and other aspects of membranes and membrane formation will be required. Specific protein complexes of interest are sugar transporters, solvent pumps, or other porins. These measurements will inform our understanding of, for example, the interaction or association of enzymes along the glycolytic pathway. The membrane could be studied as a machine to control inhibitory stress.

Proteomics

Although rational and evolutionary approaches are envisioned, a common component of both is the use of tools that allow quantitative cellular characterization at the systems level, including existing tools for global

transcript, protein, and metabolite profiling. Additional HTP tools not currently available will be required to monitor key players that define the redox and cell energy state [e.g., ATP, GTP, NAD(P)H, NAD(P)]. Capabilities could include metabolic flux mapping, a major activity in understanding and manipulating cellular metabolism. The most efficient way to estimate in vivo metabolic fluxes is through labeling experiments. Specific needs include appropriate nuclear magnetic resonance (NMR) and mass spectroscopy (MS) instrumentation and stable isotopes for visualizing pentoses, hexoses, and cellulose. Intensive mathemathical and computational power is required to achieve the final goal of flux estimation. HTP technologies for global identification of genes that impact ethanol biosynthetic pathways are required to select cells capable of high ethanol production and other desired functions.

Cellular Systems

The ability to track key molecular species as they carry out their functions and create predictive models for systems processes will be critical for developing or enhancing cell properties.

DOE Joint Genome Institute

Sequencing and screening of metagenomic libraries for novel genes and processes and analyzing novel organisms will be carried out at DOE JGI. Exploiting microbial diversity by mining for novel pathways or organisms that make a step change in ethanol production could spur the production of other chemicals through fermentation.

## Advanced Microorganisms for Process Simplification

Methods and technologies discussed above will be applied to consolidating process steps, which is widely recognized as a signature feature of mature technologies and has well-documented potential to provide leap-forward advances in low-cost processing technology. In light of the complexity of underlying cellular processes upon which such consolidation depends, fundamentally oriented work will be a highly valuable complement to mission-focused studies and can be expected to accelerate substantially the achievement of applied objectives.

Realizing the benefits of targeted consolidation opportunities requires understanding and manipulating many cellular traits, an approach much more fruitful at a systems level than at the individual gene level. As discussed previously, examples of such traits include transporters, control mechanisms, and pathways relevant to use of non-native substrates (e.g., 5-C sugars and cellulose), microbial inhibition (e.g., by pretreatment-generated inhibitors or ethanol), and the ability to function well in simple and inexpensive growth media. Investigation of these traits provides an important way to apply and extend new systems biology tools to nonconventional host organisms such as thermophiles.

The current process has undergone many improvements in the last decade. In Fig. 4 of the Introduction, p. 14, the process cartoon illustrates pretreatment (probably dilute acid hydrolysis), followed by a detoxification and neutralization step, then separate fermentation of the soluble pentose sugars. Some biomass solids are used to make the cellulases, which then are added to the biomass solids to convert cellulose to glucose, followed by a separate glucose fermentation. This section discusses recent and ongoing developments to make a single microbe for cofermentation of hexose and pentose sugars (e.g., glucose and xylose).

Eliminating process steps may reduce capital and operating costs and allow other synergistic benefits. Some of these simplification steps are under limited active research. We focus here on three immediate consolidation opportunities:

1. Elimination of a dedicated step to detoxify pretreatment hydrolysates before fermentation. These inhibitors can be by-products of the hydrolysis process and include acetate, furfurals, and other undetermined substances. Figure 8. Recombinant Yeast Cofermentation of Glucose and Xylose from Corn Stover Hydrolysate Without Detoxification (this page) shows the impact of these inhibitors. In process configurations under consideration (e.g., acid hydrolysis), such detoxification requires equipment (e.g., solid-liquid separation and tanks), added materials (e.g., base for overliming followed by acid for neutralization before fermentation), and added complexity. Obvious savings can be realized by developing improved biocatalysts not requiring the detoxification step. For detoxification elimination, research will support development of organisms having a high tolerance to pretreatment-generated inhibitors or those that detoxify these inhibitors (e.g., by consuming them) while preserving other desired fermentation properties. Some inhibitors have been identified, such as furfurals and acetate, but not all are known.

2. Simultaneous saccharification and cofermentation (SSCF), in which hydrolysis is integrated with fermentation of both hexose and pentose
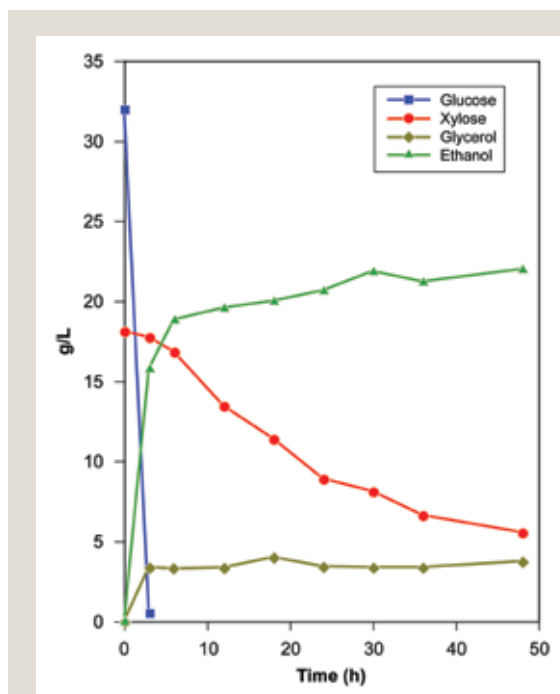
Fig. 8. Recombinant Yeast Cofermentation of Glucose and Xylose from Corn Stover Hydrolysate Without Detoxification. Note slower xylose use and lower ethanol titer and yield than in Fig. 4. Corn stover hydrolysate was prepared by aqueous pretreatment followed by enzyme hydrolysis. [Source: M. Sedlak and N. Ho, "Production of Ethanol from Cellulosic Biomass Hydrolysates Using Genetically Engineered *Saccharomyces* Yeast Capable of Co-Fermenting Glucose and Xylose," *Appl. Biochem. and Biotechnol.* 114, 403–16 (2004) (also see Mosier et al. (2005)].

sugars but with cellulase produced in a separate step. For example, development of thermophilic ethanol-producing organisms for use in SSCF could allow the consolidated process to run at higher temperatures, thus realizing significant savings by reducing cellulase requirements. Previous analyses (Svenson et al. 2001) have shown that a midterm strategy to produce ethanol from biomass would be to develop new strains capable of yielding ethanol at 50°C pH 6.0, the optimal conditions for saccharification enzymes generated today by the industry.

3. Combining cellulase production, cellulose hydrolysis, and cofermentation of C-5 and C-6 sugars in a single step termed "consolidated bioprocessing" (CBP). Widely considered the ultimate low-cost configuration for cellulose hydrolysis and fermentation, CBP has been shown to offer large cost benefits relative to other process configurations in both near-term (Lynd, Elander, and Wyman 1996) and futuristic contexts (Lynd et al. 2005).

The goal is a process more like Fig. 5 of the Introduction, p. 15. Further simplifications can be envisioned beyond these examples. Unique challenges are the expression of multiple enzymes for cellulose and hemicellulose hydrolysis or the engineering of native cellulose-hydrolyzing organisms to produce ethanol. Selecting optimal enzyme targets for expression will require extensive screening and characterization of heterologous genes. Developing a unique enzyme suite capable of complete cellulose and hemicellulose hydrolysis will require insights into the plant cell-wall assembly and structure as well as new tools for cell-wall investigations.

Research must determine which aromatic hydrocarbon degradative pathways can solubilize lignin and how they can be integrated into a productive host for additional ethanol production. Fortunately, aromatic hydrocarbon-biodegradation pathways have been studied extensively over the past two decades, and many are known. Integrating necessary components into single hosts and channeling carbon to ethanol will be major challenges.

It will be a challenging goal to optimally achieve all the traits at one time. Expression, regulation, tolerance, growth, and metabolism must be designed and synchronized to function in the process. At present for this approach, we appear to be limited to anaerobic bacteria and not the aerobic fungi used to make current cellulases. We have limited knowledge and less ability to manipulate most of these bacteria. The cellulolytic bacteria also have some interesting differences, such as the cellulosome discussed in the biomass deconstruction chapter.

For all these consolidation opportunities, native or recombinant industrial strategies will be employed:

1. The recombinant industrial strategy, engineering industrial organisms with high product yield and titer so cellulose or pentose sugars are used by virtue of heterologous enzyme expression.

2. The native host strategy, engineering organisms with the native ability to use cellulose or pentose sugars to improve product-related properties

(e.g., yield, titer) and process-related properties (e.g., resistance to toxic compounds).

However, a third combined strategy is possible.

3. Mixed culture conversion strategy to separately modify microbes to work on different parts of the substrates or pathways. This has been suggested but not well tested for cofermentation of pentoses and hexoses (see section, Microbial Communities for Robust Energy Production, p. 140).

As described before, the key difference is how challenging the complex trait is. Some cases like the elimination of lactic acid as a by-product might involve the deletion of a single gene; in others, the production of a complex extracellular cellulase or cellulosome may appear impossible at present. Delineating the genetic changes needed to confer resistance to toxic compounds generated in biomass processes is even more challenging. Researchers must balance the ease of manipulation against the trait's complexity in pursuing improvements of biocatalysts for industrial ethanol from lignocellulose.

For both SSCF and CBP, causes of cellulosic-biomass recalcitrance need to be understood not only with respect to enzymatic hydrolysis, in which enzymes act independently of cells (cellulose-enzyme complexes), but also to microbial hydrolysis, in which hydrolysis is mediated by cellulose-enzyme-microbe complexes. Growing evidence shows that free-enzymatic and microbial hydrolyzes differ in substantial ways. Studies of recalcitrance in microbial cellulose hydrolysis will build on and complement, but not duplicate, investigation of enzymatic hydrolysis.

Further process simplifications can be considered. For example, development of robust, intrinsically stable pure or mixed microbial cultures could eliminate the need for costly sterilization. Alternative routes for process simplification also should be considered—such as gasifaction of the entire biomass followed by catalytic or biological conversion into fuels like ethanol (see section, An Alternative Route for Biomass to Ethanol, p. 152).

Science Challenges and Strategies for Process Simplification

The physiology or microbial state of modified organisms within the conversion process needs to be understood to help determine when simplification is helpful and what conditions must be achieved to make it effective. Part of this is regulation of native and modified pathways and traits, many of which appear to be multigenic, complex, poorly understood, and difficult to control. For all three consolidation opportunities, understanding the sensitivity of organism performance to growth-medium formulation would benefit from use of systems biology tools. Although separate processes are more cost-effective at times, simplification tends to win historically.

A. Elimination of Detoxification

• Fundamental mechanisms of toxicity and resistance.

• Evaluation of tolerance among a diversity of species and strains with and without opportunity for adaptation and evolution.

- Characterization and evaluation of detoxification mechanisms.

B. Simultaneous Saccharification and Cofermentation

- Fundamentals of fermentation in the presence of high solid concentration. The microbe-enzyme-solid interface should be analyzed.

- Understanding and reconciling factors responsible for differences in optimum conditions for cellulase function and fermentation of sugars to ethanol.

C. Consolidated Bioprocessing

- A key question is, How do microorganisms break down cellulose? How is breakdown in microbially attached cellulosome complexes different from enzymatic hydrolysis with added fungal enzymes? A significant number of fundamental issues needing to be addressed are over and above questions implicit in seeking to understand how enzymes hydrolyze cellulose. They include:

  - Bioenergetics, substrate uptake, and metabolic control (including regulatory circuits) related to cellulose hydrolysis.

  - Relative effectiveness of cellulose-enzyme-microbe complexes as compared to cellulose-enzyme complexes and the mechanistic basis for such differences and possible synergies.

  - Extent to which products of microbial cellulose hydrolysis equilibrate or do not equilibrate with the bulk solution and the fraction of hydrolysis products that proceed from the cellulose surface directly to adherent cells.

  - Features of cellulolytic microorganisms favored by natural selection and how selection can be harnessed for biotechnology (especially for the recombinant strategy).

  - Documentation and understanding of the diversity of cellulose-utilizing organisms and strategies present in nature.

- How do microorganisms respond to cellular manipulations undertaken in the course of developing CBP-enabling microorganisms? Specific issues include:

  - For the native strategy, how cells respond to changes in end-product profiles in terms of the cell's state (transcriptome, proteome, and metabolite profiles) as well as key properties of industrial interest (product tolerance and growth rate).

  - For the recombinant strategy, understanding gained from recombinant cellulolytic microorganisms developed one feature at a time, including those in addition to hydrolytic enzymes (e.g., for substrate adhesion, substrate uptake, and metabolism). Such step-wise organism development provides an outstanding opportunity to advance applied goals and gain fundamental insights simultaneously.

D. Other Simplification Opportunities

- The development of intrinsically stable cultures could involve contamination-resistant thermophiles or acidophiles or techniques to control mixed microbial cultures (see section, Microbial Communities for Robust Energy Production, p. 140).

- The development of microbial growth-independent processes will reduce waste-treatment volumes of biosolids and allow better return of nutrients to the land as sustainable fertilizers.

Technical Milestones

Of the targeted consolidation opportunities, CBP is the most ambitious and probably will require the largest effort to achieve. Thus, we may well see substantial progress toward SSCF and detoxification elimination before CBP.

Key milestones associated with targeted consolidation opportunities can be pursued beneficially by complementary mission-oriented and fundamentals-focused research activities. These milestones include:

Within 5 years

- Improve hydrolysate-tolerant microbes.

- Achieve SSCF under desirable conditions (high rates, yield, and titer; solids concentration and industrial media).

- Functionally express heterologous cellulases in industrial hosts, including secretion at high levels and investigation of cell-surface expression.

- Conduct lab tests of modified initial CBP microbes.

Within 10 years

- Eliminate the detoxification step by developing organisms highly tolerant to inhibitors.

- Have the same response with undefined hydrolysates as with defined hydrolysates.

- Move to pilot demonstration of CBP.

Within 15 years

- Develop intrinsically stable cultures that do not require sterilization.

- Achieve CBP under desirable conditions (high rates, yield, and titer; solids concentration and industrial media), first on easily hydrolyzed model cellulosic substrates, then on pretreated cellulose.

- Develop methods to use or recycle all process streams such as inorganic nutrients, protein, biosolids, or coproduct carbon dioxide (see sidebar, Utilization of the Fermentation By-Product CO_2, p. 138).

The Role of GTL Capabilities

Protein Production

Protein production resources could be very useful in synthesizing enzymes and mixtures of enzymes as controls in experiments comparing enzymatic and microbial hydrolysis. These controls have the potential to be quite complex and thus demanding in terms of protein synthesis capability.

Molecular Machine Analysis

These resources can provide advanced analytical and computational science to study cell and cellulose interaction and particularly to gain insights into what is going on in the gap between an adhered cell and cellulose surface.

Proteomics

Proteomic capabilities can assist researchers seeking to understand system-level responses to metabolic manipulation in the course of developing microorganisms to achieve all three targeted consolidation opportunities as well as diagnosis and alleviation of metabolic bottlenecks and flux analysis.

Cellular Systems

These capabilities also can assist researchers in understanding system-level responses, removing bottlenecks, and conducting flux analysis (e.g., via metabolite analysis).

DOE Joint Genome Institute

DOE JGI can play a key role in sequencing genomes of new microorganisms with relevant features (e.g., ability to use C-5 sugars, resistance to pretreatment-generated inhibitors, and cellulose utilization), thus enabling virtually all lines of inquiry described in this chapter and in Crosscutting 21st Century Science, Technology and Infrastructure for a New Generation of Biofuel Research, p. 155.

Utilization of the Fermentation By-Product CO_2

Carbon dioxide is a major by-product of alcoholic fermentation by both yeast and bacteria. This relatively pure gaseous stream requires no primary separation or enrichment step to concentrate the CO_2, which can be sequestered as part of the national climate-protection program or processed by biological or other means to useful coproducts. Technologies could be developed to produce value-added compounds that might provide income for financing ethanol biorefineries.

The fundamental challenge is how to supply chemical energy to use and reduce CO_2, produce useful compounds, and elucidate factors governing efficient use of CO_2.

Enabling Microbiological Tools and Technologies that Must be Developed

Cellulosic biofuel research will use a broad range of powerful omic tools targeted for fuller development of the plant, enzyme, and microbial arena. However, some specific microbiological tools will need to be created to further understand and exploit microorganisms. These tools include analytical technologies and computational approaches and technologies for revealing the state of a microbial system, permitting assessment of perturbation effects on the system, and providing the information needed to construct useful models to guide engineering efforts.

The chapter, Crosscutting 21st Century Science, Technology and Infrastructure for a New Generation of Biofuel Research, p. 155, discusses in detail barriers in (1) gene-transfer methods and expression of genes in nonconventional host organisms; (2) tools for rapid analysis and modeling of cellular composition and physiological state; and (3) HTP screening methods for novel and evolved genes, enzymes, cells, and communities. Additional required tools include the following:

- Devices and requirements for preparing well-controlled microbial samples for omic analysis.

  – Integrating biological studies into a whole-systems understanding is being made possible by new analytical techniques. Systems biology needs to be driven by an organism's biological context and its physiological state, which is linked tightly to its complete history, including that of culture. For experimentation in all aspects of work with omic tools, high-quality reproducible samples are paramount for subsequent analysis or purification.

  – Controlled cultivation is the method to provide these samples. Cultivation also is that part of the experiment where knowledge of the biology is critical, and the quality of subsequent understanding is driven by the design of microorganism cultivation. The emphatic consensus of workshop attendees was that chemostat or continuous, stirred-tank reactors will provide the highest-quality biological samples for measuring multiple properties (omics) because they maintain environmental conditions at a steady state. For some omic techniques, batch operation will be chosen because of limitations in current cultivation technology and because of sample-number and amount requirements. Investigators must realize, however, that the increased amount or number comes at some cost to quality.

  – Apparatus is needed to characterize mixed microbial populations. Cellulose is degraded in nature by mixed populations needing characterization beyond identification of its members. New tools and approaches are required to understand each population's contribution to cellulose degradation.

- Development of novel techniques and approaches is needed to carry out evolutionary biotechnology, especially for multigenic traits. New and more efficient methods to generate genetic and phenotypic variation in microbes are needed to increase capabilities for obtaining new phenotypes that require multiple simultaneous changes.

- Techniques and approaches are required for studying interactions between cellulolytic microbes and their substrates. A key step in cellulose degradation in nature is adhesion of microbes to the substrate. This dynamic process needs to be characterized with new and more quantitative and spatially, temporally, and chemically sensitive approaches and techniques.

Realization of targeted consolidation opportunities and advancement of relevant fundamentals will be served by a variety of crosscutting technologies and capabilities, including the following.

- Bioreactors—novel configurations, in some cases—to evaluate performance for consolidation opportunities and to test several key hypotheses.

- Evolutionary biotechnology to develop needed strains, including those with new capabilities. Application of these techniques will be advanced by miniature reactors and automated or controlled systems (continuous, semicontinuous, or serial culture) to maximize evolution rates. Some special consideration probably will be required to adapt evolutionary biotechnology to insoluble substrates.

- Improved gene-transfer and -expression technologies for unconventional host organisms and particularly for Gram-positive organisms, which have potential to be profoundly enabling with respect to all three consolidation targets.

- HTP screening for functional abilities and traits. This is needed for selection of the most improved strains, especially for nongrowth-associated functions. It also is needed to identify the function of *unknown* or *hypothetical* genes to allow better models and metabolic engineering.

- Tools to understand microbial mixed cultures in an industrial context.

- Scanning and other microscopic techniques, as well as experimental and computational approaches drawn from biofilm research, to characterize adhered cells.

- Systems biology tools (e.g., transcriptome, proteome, metabolome) to characterize intracellular events associated with targeted consolidation opportunities in both naturally occurring and engineered cells. This includes omic analysis for characterization of existing industrial microbes under production conditions to inform development of new biocatalysts.

- Quantitative modeling at the cellular level to test fundamental understanding and provide guidance for experimental work relevant to all three consolidation opportunities.

- Mesoscale molecular modeling to understand critical events occurring in the gap between cellulose and an adhered cell and its accompanying enzymes (see sidebar, The Cellulosome, p. 102).

- Models to confirm that consolidation and process simplification will be more cost-effective than separate optimized steps (see chapter, Bioprocess Systems Engineering and Economic Analysis, p. 181).

Breakthrough, High-Payoff Opportunities

Microbial Communities for Robust Energy Production

Most industrial bioconversions rely on pure cultures. All environmental bioconversions are based on mixed cultures or communities, with specialists "working" together in an apparently stable fashion. Examples of mixed

communities capable of cellulolytic conversion are ruminant cultures and termite-gut cultures. Are there intrinsic biological reasons why communities could not be used for biofuel production? The fundamental question is, Are there stable self-regulating multiplex solutions for biofuels?

Microbial communities offer flexibility not present in monocultures, because the collective multiple metabolic pathways of microorganisms are activated as conditions demand. For example, microbial communities potentially could produce multiple forms of cellulase enzymes for use in industrial production of ethanol. In fact, multiple cellulases have been shown to be more effective than a single cellulase at processing complex and variable feedstocks. Mixed cultures tend also to be more robust, a characteristic needed for industrial-scale use.

Research Directions

This goal would require the ability to manipulate and use microbial communities to achieve industrial goals—not just the natural microbial goals of reproduction, survival, and net energy utilization. Current applications of mixed microbial cultures primarily are for waste treatment (i.e., anaerobic digestion or biofiltration). However, these technologies are poorly understood and exploit natural selection for survival. There are limited examples of products from mixed cultures in the food industry, but modern biotechnology has used only pure cultures for pharmaceuticals or for bioproducts such as ethanol. The first steps in applying microbial communities to biofuels are (1) characterize and understand existing cellulolytic microbial communities of microbes (e.g., ruminant, termite, and soil) and (2) develop techniques to understand and stabilize intentional mixed cultures. Research can elucidate detailed population interactions (e.g., both trophic and signaling) that stabilize the community. Support also is needed to evaluate robustness and population drift over time, since many mixed-culture operations will be continuous. Gaining a deeper understanding about community evolution will allow the use of selective pressure methods to evolve consortia with increased cellulose-processing efficiency. As an additional benefit, knowledge of mixed-culture dynamics may allow development of new methods to make pure cultures resistant to biological contamination.

Scientific Challenges and Opportunities

The most basic requirements are for mixed-culture identification and enumeration. Major science challenges are analysis and measurement of the mixed-culture "state." Most current omic analyses are predicated on knowledge of the gene sequence. Mixed cultures increase the challenge—for sequence, transcriptomics, and proteomics. The challenge increases geometrically for lower-number (<1%) representatives of the community. Single species existing as a population of clonal variants also adds complexity. Therefore, new techniques need to be developed and tested for sequence-based "metaomic" analysis. Also possible are nonsequence-based, metaomic techniques (e.g., transcript function–based microarrays) (Zhou et al. 2004). The metabolome is the only analysis that will not be

significantly more difficult compared with that analysis in pure culture. One subset of mixed cultures is the many industrial strains that live as a group of clonal variants (a single-species population). Analysis of how these actual strains have adapted to their working environment could be helpful.

After basic analysis, enumeration, and quantitative meta-omics, the goal is to understand community structure. Signaling molecules are known to be important in many communities, so we need to identify and confirm these molecules and determine their importance. Then we can consider how to modify these signals to control the community. Regulatory and metabolic modeling of individual members, as well as the community, will be essential to deciphering the regulatory structure. Understanding the physical structure will require imaging technologies to acquire detailed visualization and specific labeling of individual species. This might require individual-species modification to express tagged marker proteins and then to reassemble the mixed culture. Computational models combining omics and biochemical and spatial variables will be critical to accomplishing this goal.
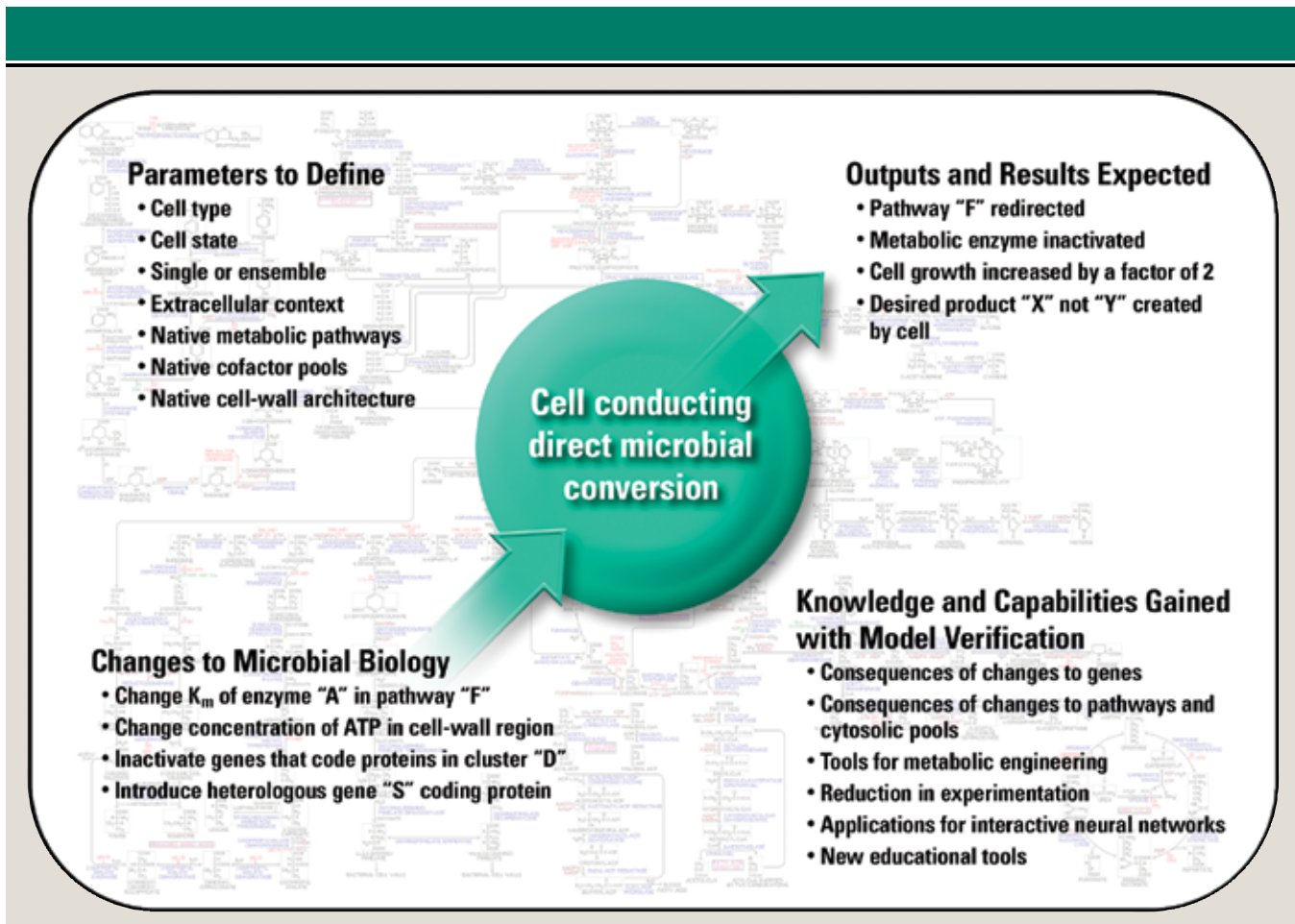
Reproducible samples and improved cultivation techniques in highly instrumented chemostats, for example, also will be required, especially when lignocellulosic solids are introduced. In this case, reproducible samples are especially needed. Mixed cultures may not be deterministic. Some evidence shows that the final state is highly variable. Previous work has shown that parallel enrichments from the same natural source each led to different populations after multiple serial transfers.

GTL Facilities and Capabilities

A major priority, as described in the GTL Roadmap, is to understand microbial communities. Capabilities being developed in the GTL program are ideally suited for developing industrial use of microbial communities. GTL will rely heavily on the DOE Joint Genome Institute (JGI) for sequencing and resulting annotation. Integrated proteomic capabilities will be useful for a wide range of omic analyses. Signaling molecules and tagged proteins and clones would be provided from protein production resources. For advanced community metabolic models, cellular systems capabilities are needed. When studying community interactions with a lignocellulosic medium, the National Renewable Energy Laboratory's Biomass Surface Characterization Laboratory will be valuable.

Model-Driven Design of Cellular Biocatalytic Systems Using Systems Biology

Systems-level modeling and simulation is the modern complement to the classical metabolic engineering approach utilizing all the GTL technologies and computing. Microbial organisms contain thousands of genes within their genomes. These genes code for all protein and enzyme components that operate and interact within the cell, but not all proteins are used under all conditions; rather, an estimated 25% are active under any given

Parameters to Define
- Cell type
- Cell state
- Single or ensemble
- Extracellular context
- Native metabolic pathways
- Native cofactor pools
- Native cell-wall architecture

Cell conducting direct microbial conversion

Outputs and Results Expected
- Pathway "F" redirected
- Metabolic enzyme inactivated
- Cell growth increased by a factor of 2
- Desired product "X" not "Y" created by cell

Changes to Microbial Biology
- Change K_m of enzyme "A" in pathway "F"
- Change concentration of ATP in cell-wall region
- Inactivate genes that code proteins in cluster "D"
- Introduce heterologous gene "S" coding protein

Knowledge and Capabilities Gained with Model Verification
- Consequences of changes to genes
- Consequences of changes to pathways and cytosolic pools
- Tools for metabolic engineering
- Reduction in experimentation
- Applications for interactive neural networks
- New educational tools

condition in a living cell. We currently understand very little about how all these expressed proteins interact and respond to each other to create cellular phenotypes that we can measure or try to establish. With improved understanding of such complex cellular systems, we should gain the ability to design them in an intelligent manner. Figure 9. Microbial Models for Providing New Insights, this page, indicates some of this power and complexity. Two general and complementary methods are taken into consideration: The *synthetic* route, which embraces de novo creation of genes, proteins, and pathways and the *nature-based* route, which uses existing suites of microorganisms and seeks to improve their properties via rational design. This is a qualitative step beyond recombinant and native strategies discussed in the Metabolic Engineering section, p. 127.

The challenge starts with the ability to characterize cellular networks and then moves toward establishing computer models that can be used to design them. These models would capture all aspects of a microbe's metabolic machinery—from primary pathways to their regulation and use— to achieve a cell's growth. Figure 5 illustrates part of this pathway complexity. Through these computational models, we could design and optimize existing organisms or, ultimately, create novel synthetic organisms. Specific to biomass-to-biofuels objectives, organisms engineered using these technologies potentially will consolidate the overall process and reduce unit operations (see Fig. 9. Microbial Models for Providing New Insights, this page).

Fig. 9. Microbial Models for Providing New Insights. Information gained in GTL systems biology research will enable metabolic engineering and modeling to enhance microbial characteristics. Using defined experimental parameters, the biology can be changed to perform desired new tasks. This will allow new biological system outputs, increase knowledge, and, ultimately, improve predictive models. [Source: M. Himmel, National Renewable Energy Laboratory]

Research Directions

This research would provide the ability to control and optimize a microbial transformation from carbohydrates to ethanol and related value-added coproducts in a selected microbe. Gaining complete control over cellular networks implies a capability for engineering and consistently performing transformation with the best available yields, rate, and titers. It also includes the ability to design the microbe intelligently by using computational technologies for determining consequences and optimal approaches to intervene and engineer within cellular networks. Furthermore, this model would enable us to assess the limits of a bioprocess's microbial biotransformation (e.g. the maximal productivity and rates achievable) and potentially to engineer entirely novel biotransformation pathways and systems.

Specific research directions would include the following.

1. Enumeration of Cellular Components, Interactions, and Related Phenotypes. Before any predictive computational models can be built, we need to generate the underlying data sets for relevant process conditions:

- Identification of all proteins and enzymes participating in the metabolic pathways relevant to carbohydrate metabolism for cell growth, ethanol synthesis, and related by-products. This also would include characterization of key enzyme complexes relevant to cellulose degradation, carbohydrate transport, and respiratory mechanisms. Experimental technologies that may be useful include protein tagging, proteomics, and in vivo activity measurements.

- Characterization of novel protein and gene function. About 30% of genes have no understood function, yet some of these unknown genes are thought to be involved in the microbial metabolic systems and stress responses under process conditions.

- Identification and quantification of all metabolites present within the cell. Experimental approaches could involve NMR or MS.

- Characterization of the cell's energetics under various relevant conditions. These would include measurements to characterize the stoichiometry of energy-transducing complexes, parameters such as the P/O and P/H+ ratios, and a cell's maintenance energy associated with cellular functions.

- Characterization of transport mechanisms. In particular, this would focus on determining the components associated with transport of nutrients into and out of the cell as well as those of the mitochondria in eukaryotic organisms. Determining the stoichiometry of transporters and transport processes, as well as their kinetics and differential regulation, is envisioned.

- Characterization of membrane composition for process tolerance (i.e., alcohol and toxin tolerance) and environmental and community interactions.

- Elucidation of regulatory networks enabled by development of experimental approaches to identify protein-protein and protein-DNA interactions.

- Localization data indicating where proteins are operating within the cellular and community space.

2. Knowledgebase to Develop Dynamics and Kinetics Modeling Techniques. One of the most attractive features of a model-driven, rational approach is its predictive capacity, requiring the inclusion of regulatory events at the genetic and metabolic levels. Creation of such models requires data sets, as well as development of HTP tools with capabilities beyond those currently available.

- Acquisition of high-quality and dynamic omic data.
- Development of HTP methods to identify binding sites of global regulatory proteins and other interactions.
- Development of HTP tools to monitor key players that define the cell's redox and energy states [e.g., ATP, GTP, NAD(P)H, and NAD(P)].
- HTP quantification of in vivo enzyme-activity metabolic fluxes.

3. Network Reconstruction. From data sets generated, we can develop the complete mapping and reconstruction of microbial networks and physiology related to the conversion of sugars to ethanol.

- Automated techniques to integrate data sets and rapidly create reconstructed networks.
- Integrated representation of metabolism, regulation, and energetics.
- Approaches to account for the impact of spatial localization of proteins and enzyme complexes within integrated models.

4. Development of In Silico Analysis Tools. Methods are needed to interrogate and simulate the functioning of constructed networks to address key questions about microbial physiology. Any method should develop testable hypotheses that can be integrated with experimental studies. Methods should do the following:

- Assist in network reconstruction, particularly in metabolic pathways and regulatory networks. These methods may involve new approaches that use artificial intelligence.
- Interrogate mechanisms associated with toxic responses and tolerance to product and intermediate levels.
- Assess physicochemical limitations of cellular systems and enzyme components to determine maximum achievable rates (e.g., identify rate-limiting steps, kinetic as well as diffusion limited).
- Generate prospective designs of cellular networks by modifying and testing existing cellular systems.
- Design systems de novo from cellular components.

5. Design of Cellular Systems. Designing engineered and synthetic organisms to convert carbohydrates to ethanol through the use of computational models and methods would include the following:

- Dedicated transforming microbes with focused abilities to perform biotransformation and necessary supporting operations for the conversion of carbohydrates to ethanol.
- Self-replicating synthetic microbes to support biofuel production under optimal conditions.
- Novel pathways for producing biofuels and value-added coproducts from biomass could involve the generation of new enzymes and organisms through the use of evolutionary design concepts (e.g., directed evolution and adaptive evolution).

Scientific Challenges and Opportunities

To address this model-driven design goal, a number of broad scientific and conceptual challenges will need to be overcome, including the ability to make high-quality measurements of cellular components and states to simulate physiology and design networks with models generated from these data.

The Role of GTL Capabilities

Many GTL capabilities, either centralized or distributed, can be leveraged to aid in accomplishing these goals. Particular ones are noted below.

Protein Production

GTL capabilities will be used to characterize proteins by rapid isolation, production, and biochemical characterization in an HTP manner.

Molecular Machines

Molecular machine analysis will enable characterization of large complexes containing many active components of biotransformation networks.

Proteomics

HTP analysis of all proteins present in the cell, their relative abundance, spatial distribution, and interactions will be important to model development.

Cellular Systems

Ultimately, cellular systems analysis is about developing computational models of systems that can be used reliably to engineer microbes. Resources dedicated to the analysis and modeling of cellular systems can be used reliably by technologists to engineer microbes for biofuel production on an industrial scale.

Outcomes and Impacts

The GTL Roadmap describes scientific goals and milestones and the technology and computing needed to meet these research directions. These resources can be focused on the problem of engineering existing or synthetic organisms for biofuel production from biomass. This type of "rational design" and organism engineering has the potential to transform various stages of biomass conversion to biofuels consistent with goals of consolidating the overall process and reducing unit operations. The practical impact

is in reducing the time required to modify a microorganism to perform as desired in an industrial setting.

Although the direct applied benefit will be in biomass-to-biofuel processes, technologies and methods derived from the ability to reliably engineer biological systems will have far-reaching impacts on basic and applied research across many sectors of biotechnology.

Direct Bioproduction of Energy-Rich Fuels

This breakthrough, high-payoff opportunity focuses on microbes for direct production of hydrophobic alternative fuels (i.e., alkanes, longer-chain alcohols, and fatty acids). This would overcome one limitation of nearly all bioconversions—they result in dilute aqueous mixtures. Typical industrial product concentrations are 100 to 150 g/L for ethanol and other such products as organic acids. This limitation imposes separation requirements that increase process and energy costs. New fermentation systems would be highly desirable to allow significant increases in product concentration, new types of products, and new processes for product recovery. Strong increases in efficiency also could be achieved by developing continuous processes.

Research Directions

Microorganisms produce a wide variety of potentially useful compounds but in relatively low amounts. Recently, because of expanded knowledge about the identity of genes for important pathways and mechanisms of pathway regulation, increasing the flux of microbially produced chemicals by up to six orders of magnitude (Martin et al. 2003) has been possible (from trace levels of primary products). A new opportunity is now offered to explore whether or not similar methods can be applied to developing modified microorganisms that secrete nontoxic molecules possibly useful for fuels. Examples may include alkanes, longer-chain alcohols, fatty acids (Voelker and Davies 1994), esters, and other types of molecules with low aqueous solubility that facilitate continuous product removal during fermentation. Advances in understanding how hydrophobic molecules are secreted by specialized cell types (Zaslavskaia et al. 2001) may facilitate the development of radically new production systems. The challenges described here for fermentation into hydrophobic fuels also would apply to potential photosynthetic systems.

Additionally, advances in systems biology and protein engineering may facilitate new approaches to the overall process of fermentation. For instance, developing chemical regulators of cellular processes such as cell division may be possible to allow cultures to be held in highly efficient steady states for prolonged periods. Such process controls may be synergistic with the development of novel product types not normally produced in high concentrations by microorganisms. For example, cocultures may possibly be used for directly combining alcohols and organics into ether or ester production. This would be an advantageous use of acetate

released from biomass hydrolysis——taking it from a harmful by-product to a fuel cosubstrate.

Scientific Challenges and Opportunities

The explosion of sequence information resulting from GTL and other genome sequencing programs has greatly facilitated identification of genes for a wide variety of processes. This information expansion also has allowed the development of systems tools such as whole-genome DNA chips for measuring gene expression. A next-phase challenge is to bring that information and associated tools to bear on identifying entire pathways and cellular processes of relevance to biofuel production. Additionally, understanding how such pathways and processes are regulated is essential. New protein-production and proteomic tools envisioned for GTL will greatly facilitate the elucidation of pathways and their regulation. Important challenges are to understand how the permeability properties of membranes are controlled by composition and how the structure of membrane proteins such as transporters relates to function. Progress has been slow in elucidating membrane protein structure by conventional methods, requiring new approaches that may be addressed by GTL. Identification of microorganisms with high levels of resistance to biofuel compounds (but not necessarily to any production capabilities) could provide useful insights into strategies for improving fermentation efficiency.

The Role of GTL Capabilities

The full suite of GTL resources for genomics and systems tools will be essential in clarifying the underlying mechanisms associated with these and related problems. Examples of the types of contributions envisioned are listed below.

Protein Production

Protein production capabilities will enable elucidation of enzyme function in novel pathways for biofuel production; optimization of enzymes and transporters by protein engineering and evolution; and revelation of components for in vitro pathways. This could lead to development of novel chemical regulators of microbial cellular processes for use in industrial fermentation.

Molecular Machines

These resources will allow nanoscale interrogation of membrane interactions with biofuel compounds (e.g., using patterned membranes); identification of protein complexes; and mechanistic understanding of transporters involved in biofuel secretion. Development of nanoscale materials will facilitate product separations.

Proteomics

The proteomic approach involves biological-state omics for microbes under inhibitory stress; characterization of post-translational modifications of proteins that regulate enzymes or pathways for biofuel production; and analysis of biofuel exposure effects on microbial gene expression.

Cellular Systems

Cellular system capabilities include modeling of cellular carbon flux from uptake of biomass-derived sugars to secretion of finished biofuel compounds, systems engineering of batch and continuous fermentation for biofuel production, and modeling of protein structures in aqueous and nonaqueous environments.

DOE Joint Genome Institute

DOE JGI will characterize organisms with such useful properties as high productivity of or resistance to prospective biofuel compounds and will develop gene-expression interrogation systems.

Other Needs

Other needs (e.g., screening for new pathways and functions) include assessment of maximal redox balances (reduced fuel products yield more CO_2 in fermentation).

Outcomes and Impacts

If alternate fuels were made with higher fuel value (i.e., diesel, alkanes, lipids), both separations and life-cycle costs would be altered because these hydrophobic fuels would separate spontaneously from water. Fuel-density issues of ethanol also would be reduced. Additionally, transportation costs might be lowered because compounds such as alkanes would be significantly less corrosive than ethanol. These biofuels could be used more easily in the nation's current transportation infrastructure. If continuous fermentation with product removal were implemented, higher throughput would result in lower capital expenditures as well as costs associated with product dehydration, as in ethanol production.

Translation to Applications

DOE EERE would lead in pilot-scale tests of strains that produce novel biofuels and in developing fermentation processes based on new strains, products, and product-recovery processes. EERE would analyze the potential market and cost impacts for new and existing biofuels and then take the lead in separation technologies and in integrative separations. Additionally, EERE would carry out testing and possible engine-design modifications for new types of biofuels.

Optimal Strains: Fermentative Production of 40% Ethanol from Biomass Sugars

Current corn-to-ethanol processing plants typically produce titers of 10 to 14% w/w. Because of limits on biomass sugar concentrations, these ethanol levels are at least threefold higher than those produced from lignocellulose using current technology and biocatalysts. Tthe dilute product stream for lignocellulosic ethanol imposes a two- to threefold increase in fermentation volume per annual gallon of ethanol, with corresponding increases in pumps, nutrients, and management. Use of modern molecular tools to harvest the catalytic diversity of nature should facilitate construction of

revolutionary biocatalysts that could increase ethanol titers up to 40% and lower needed investments in capital and operating costs for biomass-to-ethanol plants. This challenge is presented as an example of how systems biology may allow applications to exceed current biological limits. Strategies for obtaining organisms that produce and tolerate high-ethanol concentrations include:

- Engineering current ethanol producers to retain high metabolic activity.
- Engineering naturally ethanol-tolerant organisms to produce ethanol.
- Exploring native diversity of microorganisms to identify those that retain glycolytic and fermentative activity in the presence of high-ethanol titers.

Scientific Challenges and Opportunities

If achieved, production of fermentation broth containing 40% ethanol from biomass or starch sugars would revolutionize process designs. Incremental progress toward this goal would reduce dramatically the size of fermentation plants by decreasing fermentor capacity and associated pumps, nutrient cost, water usage, and waste-water treatment and recovery. For instance, doubling ethanol titers from 5 to 10% would reduce process water volume by 63% for equivalent ethanol production. Further doubling the titer from 10 to 20% would reduce the water needed by an additional 55%. For fermentation broth with a 40% titer, water usage would be only one-tenth the amount currently needed for the biomass-to-ethanol technology that produces 5% titer. At ethanol titers above 40%, viable alternatives to distillation could reduce energy costs associated with purification. However, distillation is a mature technology and its energy costs do not substantially limit the current process because waste heat is reused.

Improving the distillation process also would require developing better upstream processes or conversion technologies to provide highly effective sugar concentrations. A 30% biomass slurry can yield only a 15 to 20% sugar stream that results in just 7 to 10% ethanol. However, reflecting on this challenge illustrates how to further push biology beyond current limits.

Grain ethanol plants produce from 10 to 15% ethanol (Lynd, Wyman, and Gerngross 1999), reaching up to 20% ethanol when provided with very high substrate levels (Scouten and Petersen 1999). Even higher levels of ethanol (25%) are produced very slowly by sake yeasts at a low temperature. The most ethanol-tolerant microorganisms known, *Lactobacillus homohiochii* and *L. heterohiochii*, were isolated as spoilage bacteria in sake (Roadmap for Biomass 2002; Svenson et al. 2001; Lynd et al. 2005). The membrane lipids in these bacteria contain unusually long fatty chains that supposedly are an adaptation to growth in high ethanol. However, no systematic search for a class of extremophiles with resistance to ethanol has been reported.

Cells need not be viable to metabolize sugars to ethanol. At a biochemical level, ethanol production from sugars is a strongly exergonic reaction when coupled to ATP hydrolysis. Individual glycolytic enzymes in yeast

and *Zymomonas mobilis* have been shown to function well in the presence of 20% ethanol (Zhou et al. 2004) and are progressively more inhibited at increasingly higher ethanol concentrations. In vitro disrupted cell preparations of both organisms have been shown to remain active and continue to produce ethanol even in the presence of 20% ethanol. Although organisms continuing to grow at ethanol concentrations above 30% are unlikely to be found, developing microorganisms that remain catalytically active and metabolize sugars to achieve very high levels of ethanol is quite plausible. For instance, over half the ethanol in commercial yeast fermentations is produced after growth has been inhibited by accumulated by-products. Formulating new biocatalysts for biomass presents a challenge and opportunity for engineering improvements to provide concentrated sugar feedstocks (Zhou et al. 2004).

Described below are several approaches for moving toward the goal of producing fermentation broth containing 40% ethanol, including the isolation of native novel microorganisms capable of growing or surviving in the presence of high levels of ethanol. Such organisms can be used as a platform from which to engineer ethanol production, if needed, and as a source of genes and enzymes to improve alcohol production in current ethanologenic biocatalysts. This work is expected to rely heavily on GTL resources for sequencing and transcriptome and proteome investigations, which will identify molecular requirements for ethanol tolerance during growth and for maintenance of active metabolism in the presence of high levels of ethanol.

Retaining the traits of current biocatalysts that do not disturb the metabolisms of all carbohydrate constituents of lignocellulose (hexoses, pentoses, and uronic acids) will be important. Additional genes for using various biomass carbohydrates and other components may be needed to provide high-substrate levels for high ethanol titers. These genes perhaps include hydrolases for cellulose and hemicellulose as well as uptake systems for solubilized products.

Other biomass components offer further opportunities to increase yield. Acetate levels equivalent to 10% of hemicellulose weight represent a potential source of oxidized substrate. Equivalent levels of solubilized lignins represent a source of reduced substrate, a potential electron donor to convert portions of both substrates into additional ethanol. Cometabolizing these substrates by engineering known genes and pathways from soil organisms could increase ethanol yield up to 5%. Acid-stable products represented by 4-O-methyl-glucuronoxylose and 4-O-methyl-glucuronoxylobiose in acid hydrolysates of hemicellulose currently are not metabolized by any ethanologenic biocatalysts. These recalcitrant products typically are not measured by high-performance liquid chromatography analysis and can represent up to 10% of total carbohydrate. Discovery of new genes and organisms to metabolize these saccharides and incorporate them into biocatalysts could provide a further incremental increase in ethanol yield with no increase in capital or operating costs. Together, the more complete use of all solubilized components from lignocellulose and the increase in ethanol

titers would reduce dramatically the size of future ethanol plants and the amount of water use.

Research Directions

The burgeoning amount of sequence information and GTL's current ability to rapidly determine genome sequences for new alcohol-resistant organisms from nature provide an excellent opportunity. We can identify the genes and functions required for growth, survival, and continued metabolism in the presence of high levels of ethanol. Transcriptome and proteomic analyses resources will greatly assist these studies.

Characterizing microbial membrane and wall structures, including lipids, proteins, and carbohydrates, represents a difficult analytical problem and a necessary challenge for future GTL capabilities. Envelope structure is presumed to represent a major determinant for continued metabolic cell activity in high-ethanol and other extreme environments. New tools are needed to facilitate design and modification of biocatalysts for many future processes.

Other yet-to-be-discovered opportunities include potential metabolic pumps for solvents and products, possibly evolved by cells to maintain low intracellular product concentrations. New analytical tools will be needed to investigate properties and functions of these biomachines.

Process improvements also will be needed to make available the high amounts of sugars needed to achieve these ethanol titers, leading to solid-state fermentation in the future.

An Alternative Route for Biomass to Ethanol: Microbial Conversion of Syngas

Biomass can be gasified to produce syngas (mostly a mixture of CO and H_2). Perhaps surprisingly, syngas has been shown to be converted by certain microbes into products including ethanol (Klasson et al. 1990; Gaddy 2000). These microbes are not well understood, but the process has been taken to small pilot scale. The attraction of this alternative approach to bioethanol is that the theoretical yield is quite high since all the biomass potentially is available as syngas for anaerobic fermentation. This gives theoretical yields greater than 130 gal per dry ton of biomass.

Background

Gasification is a combination of pyrolysis and combustion reactions for converting a solid material, such as biomass, to a gasified product (syngas). Gasification is a robust and traditional technology, yet not extensively implemented.

Biopower can use this syngas as a fuel for power production. Once sulfur compounds have been removed, this gas can be converted to other products through catalytic Fisher-Tropsch reactions at high temperatures and pressures. However, these precious-metal catalysts for gas-to-liquid conversion have been explored for over 50 years with incremental improvements. Biocatalysts for some conversion methods are relatively unstudied, operating in

aqueous media with the syngas bubbled past at ambient temperature and pressures and representing a strong alternative to traditional catalysis.

Challenges

How do these biocatalysts carry out transformations that otherwise work only with precious metals at high temperature and pressure? Which enzymes and molecular machines allow these transformations? Can increased understanding of these protein structure-function relationships aid development of either better biocatalysts or insights to improved inorganic metal catalysts?

Trial and error experimentation has shown that process conditions and reactor design will shift the microorganisms to higher product yields. This is the fundamental and unexplored biological question: How does the regulation of the fermentation pathway allow these environmental shifts (e.g., pH, and medium composition) to induce higher yields?

Syngas Status in Industry

Bioengineering Resources, Inc. is a small company developing and soon to be demonstrating its pilot syngas bioethanol process (EERE 2005; BRI Energy 2006). The University of Oklahoma has assembled an integrated gasification and biology program; however, rates remain slow and are limited by the fundamental biology and mass transfer (Klasson et al. 1990).

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