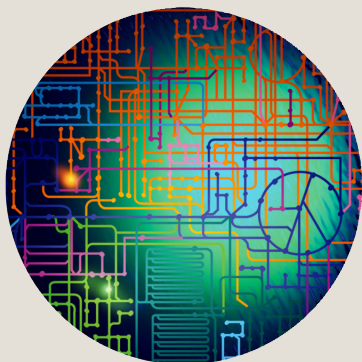


Appendix A. DOE Mission: Energy Security

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The Department of Energy's (DOE) strategic energy goal is to "protect our national and economic security by promoting a diverse supply and delivery of reliable, affordable, and environmentally sound energy" (*DOE Strategic Plan 2003*).

GTL supports this goal by providing the fundamental scientific knowledge needed to develop biological technologies that produce clean, renewable, carbon-free, and carbon-neutral alternatives to fossil fuels (see *Mission Science Goals and Challenges*, below right). These biotechnical advances will expand and improve our current portfolio of options and ultimately help lead to a secure, sustainable energy future for the United States and the world.

DOE Mission: Energy Security

Develop Biofuels as Major Energy Source

A.1.1. The Energy Challenge

Meeting projected increases in energy demand while decreasing dependence on foreign sources of energy defines America's energy challenge. From 2003 to 2025, U.S. energy demand is projected to increase by 35%, much greater than the projected increase in domestic production (*Annual Energy 2005*). Making up this projected shortfall without increasing imports will require investments in science and technologies that will improve conservation and efficiency and expand the domestic energy supply system. A primary goal of the national energy policy is not only to increase domestic supply but also to broaden our range of options in ways that will reduce vulnerabilities to supply disruptions and protect the environment (*Reliable 2001*).

Another key factor in America's energy challenge is rising carbon dioxide (CO₂) emissions. CO₂ is the most abundant greenhouse gas (GHG) in the atmosphere, and, based on projected energy use between 2003 and 2025, U.S. CO₂ emissions could increase almost 40% (*Annual Energy 2005*). With accelerated growth in fossil-fuel consumption projected for developing regions of the world, by 2025 annual global CO₂ emissions could be 55% higher than in 2001 (*International Energy 2004*). In 2002, global energy use emitted about 7 gigatons of CO₂ into the atmosphere. Several long-term projections estimated that CO₂ emissions could be as high as 30 GtC/year by 2100 (*Nakicenovic et al. 2000*). Stabilizing the concentration of CO₂ at any level requires that global CO₂ emissions must peak eventually and begin a long-term decline, ultimately falling to virtually zero.

Mission Science Goals and Challenges

Mission Science Goals: Understand the principles underlying the structural and functional design of microbial and molecular systems, and develop the capability to model, predict, and engineer optimized enzymes and microorganisms for the production of such biofuels as ethanol and hydrogen.

Challenges: Analyze thousands of natural and modified variants of such processes as cellulose degradation, fermentative production of ethanol or other liquid fuels, and biophotolytic hydrogen production.

A variety of breakthrough energy technologies will be needed to significantly reduce CO₂ emissions. To illustrate the scale of the CO₂ emissions challenge, Table 1. How Big is a Gigaton?, this page, provides examples of the types of technological actions required to reduce emissions by 1 GtC per year (Pacala and Socolow 2004).

Strategies for understanding the impacts of energy use on climate change and for developing technologies that will ensure economic prosperity while reducing GHG emissions are provided under the guidance of several government agencies through the Climate Change Science Program (CCSP 2003) and the Climate Change Technology Program (CCTP, www.climatechange.gov) (see Appendix F. Strategic Planning for CCSP and CCTP, p. 249).

A.1.2. The Role of Biology and Biotechnology in America’s Energy Future

Biology played a key role in producing the fossil fuels so critical to meeting today’s world energy demand. Fossil fuels were once living biomaterials synthesized eons ago by photosynthetic and biochemical processes. A series of fortuitous geological events trapped these materials beneath the sediments of ancient seas, and, over millions of years, the right mix of heat, pressure, and other factors transformed the biomaterials into fossil fuels.

With biotechnological innovations, biology once again can play an important role in producing high-energy fuels. Plants and photosynthetic microorganisms are masters at harvesting chemical energy from sunlight—a virtually inexhaustible supply of energy. By harnessing their photosynthetic and other biochemical capabilities, biological systems can be used to satisfy a greater portion of energy demand.

Applying biology to build a new U.S. bioenergy industry can benefit this nation’s energy security, economy, and environment in many different ways. Biofuels, especially ethanol from plant materials (biomass), have the potential to reduce our dependency on foreign oil in the transportation sector and diversify our energy-technology portfolio. As renewable alternatives that can be harvested on a recurring basis, bioenergy crops (e.g., poplar trees and switchgrass) and agricultural residues (e.g., corn stover and wheat straw) can provide American farmers with important new sources of revenue. Consumption of biofuels produces no net CO₂ emissions, releases no sulfur, and has much lower particulate and toxic emissions than fossil fuels (Greene et al. 2004). In addition to ethanol, other biobased energy alternatives include biodiesel, methanol, hydrogen, and methane (see sidebar, Biological Energy Alternatives, p. 200).

Biomass currently is used to meet only 3% of U.S. energy consumption (Annual Energy 2005). In 2004, the U.S. produced 4 billion gallons of ethanol from corn grain, enough to meet about 2% of U.S. gasoline

Table 1. How Big is a Gigaton?

Today’s Technology	Actions that Provide 1 Gt/year of CO ₂ Mitigation
Coal Plants	Replace 1000 conventional 500-MW plants with “zero-emission” power plants
Geologic Sequestration	Install 3700 sequestration sites the size of Norway’s Sleipner Project
Nuclear	Build 500 1-GW plants
Efficiency	Deploy 1 billion cars running at 40 mpg instead of 20 mpg
Wind	Install 150× current U.S. wind generation
Solar photovoltaics	Install 10,000× current U.S. solar PV generation
Biomass fuels from plantations	Globally convert open land >15× the size of Iowa’s farmland to biomass production
Storage in new forests	Reforest open land >40× the size of Iowa’s farmland

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consumption (Homegrown 2005; Mann 2004). Ethanol from biomass has promise for meeting a significantly larger portion of U.S. gasoline demand, but higher production costs, technical difficulties, and inefficiencies in biomass conversion currently prevent ethanol from being cost-competitive with gasoline. Another concern has been the uncertainty in determining how much land must be dedicated to growing bioenergy crops to make a real difference in oil demand and how this would impact current agricultural and forestry practices. A recent report prepared for the U.S. Department of Agriculture and Department of Energy (DOE) has projected that relatively modest changes in the use of farmlands and forests could produce more than 1.3 billion dry tons of biomass per year, enough to reduce current oil demand by about

Biological Energy Alternatives

Most biological processes that produce energy require solar energy either directly or indirectly via photosynthesis, a complex biochemical pathway in which solar energy is used to drive the chemical conversion of low-energy inorganic molecules such as water and carbon dioxide into energy-rich organic molecules. The organic products of photosynthesis are used to build biomass (proteins, fats, carbohydrates, and cellulose) and store chemical energy needed to drive cellular processes. The biomass of photosynthetic organisms can be used directly as a burnable fuel or converted to such other high-value energy sources as ethanol, biodiesel, methanol, hydrogen, or methane.

Liquid Fuels

Ethanol: Currently the most widely consumed biofuel in the United States, used as a substitute or octane booster for gasoline. A gallon of this biofuel has about 2/3 the energy content of gasoline. Some 3 billion gallons of ethanol were produced from cornstarch in 2004, equaling about 2% of U.S. gasoline consumption (Homegrown 2005; Mann 2004). Inefficiencies in the conversion of biomass (e.g., agricultural residues, plant stems and leaves, grasses, trees, and municipal wastes) to ethanol prevent yields that could meet a larger portion of gasoline demand.

Methanol: High-octane liquid fuel that has about half the energy density of gasoline. Engine modifications are required to improve cold starts and prevent corrosion. In the United States, about a billion gallons of methanol are produced each year, primarily from methane, but methanol also can be thermochemically derived from biomass gasification. Methanol could be a future source of hydrogen for fuel cell vehicles.

Biodiesel: Diesel fuel substitute or extender obtained from chemically reacting organically derived oils and fats (e.g., excess soybean oil and restaurant greases) with alcohol to form ethyl or methyl esters. In its pure form, biodiesel reduces fuel economy and power by about 10% when compared with diesel. Biodiesel blends perform similarly to diesel and can be used in unmodified engines. Only about 30 million gallons of biodiesel are produced each year in the United States today—a tiny fraction of the billions of gallons of diesel consumed each year (National Biodiesel Board).

Gaseous Fuels

Hydrogen: Potential energy source that can be released from the breakdown of biomass by microorganisms or produced directly from water and sunlight via photobiological processes that do not require biomass as an intermediate. Much research is needed, however, before we can use these systems for clean, renewable hydrogen production. Currently, most hydrogen is derived from steam reformation of nonrenewable natural gas and used primarily for industrial chemicals production. Only a small fraction is used as an energy carrier. Each year in the United States, about 9 million tons of hydrogen are produced, enough to power 20 to 30 million hydrogen cars or 5 to 8 million homes (National Hydrogen Energy Roadmap 2002).

Methane: Main chemical component of the fossil-fuel natural gas, which currently makes up about 20% of the U.S. energy supply. Microorganisms naturally produce methane during biomass degradation. Extensive infrastructure already is in place for widespread distribution and use. Organic materials in agricultural, municipal, and industrial wastes could be used as feedstock for biomethane production; however, high production costs and incomplete biological conversion (as much as 50% of organic matter is not used) are major limitations.

one-third (Biomass as Feedstock 2005). As research improves efficiencies in both agricultural production and biomass conversion, land and sunlight availability in the United States should be sufficient to produce enough biofuels to meet domestic transportation-related demand without disrupting agricultural land use for food and fiber crops.

In addition to reducing our dependence on oil, biofuels also have great potential for decreasing greenhouse gas emissions associated with fossil-fuel consumption. Figure 1. Potential Role of Biotechnology in the Global Energy System, p. 202, presents the results of an economic analysis exploring conditions under which markets for commercial biofuels could develop (Edmonds et al. 2003). In this figure, two potential scenarios for global energy consumption in the 21st Century are compared: A reference case in which innovations in energy technology take place without constraints on CO₂ emissions and a CO₂-stabilization case in which emissions are limited. In the stabilization case, biomass becomes a major component of the energy-technology portfolio, and by 2100 biomass usage is greater than that of all current fossil fuels (oil, natural gas, and coal) combined. A transition to such large-scale use of biofuels and biotechnologies could create a new bioenergy industry potentially worth trillions of dollars over the 21st Century.

Before biomass and biotechnologies can compete successfully with established energy sources for market share, basic research is needed for a more complete understanding of the biological processes underlying biofuel production. Applying this understanding in innovative ways will enable the development of breakthrough technologies. Since it can take 30 to 50 years for an energy technology to go from research to large-scale commercial deployment, this basic research is needed today.

A.1.3. GTL's Vision for Biological Energy Alternatives

GTL will provide a systems-level understanding of biological processes for developing and deploying large-scale, environmentally sound biotechnologies to produce biofuels and other high-value chemical products that reduce dependence on foreign energy sources and enhance national economic prosperity.

A national vision for bioenergy and biobased products was defined by the Biomass R&D Technical Advisory Committee (BTAC): “By 2030, a well-established, economically viable bioenergy and biobased products industry will create new economic opportunities for rural America, protect and enhance our environment, strengthen U.S. energy independence, provide economic security, and deliver improved products to consumers” (Vision for Bioenergy 2002). BTAC, established as a result of the Biomass Research and Development Act, is responsible for advising the Secretary of Agriculture and the Secretary of Energy on issues relevant to biomass research and development. BTAC also coordinates partnerships among government agencies, industry, researchers, and other groups with interests in biomass R&D (U.S. Congress 2000).

GTL supports this national vision by providing a detailed understanding of the microbial processes that mediate the production of biofuels (see sidebar, Mission Science Goals and Challenges, p. 198). Our limited understanding of many of these processes presents fundamental scientific challenges that must be overcome before we can develop and deploy successful bioenergy technologies. In addition to advancing biofuel production, the capabilities and understanding of microbial systems provided by GTL will be applicable to the biotechnological development of other commercial chemical processes. Techniques used to design microbial systems for biofuel production could be used to develop other microbial systems optimized to convert biomass to biodegradable plastics and other chemical products currently derived from fossil fuels. Insights from GTL research could benefit several research areas supported by DOE's Office of Energy Efficiency and Renewable Energy (EERE) (see sidebar, DOE Activities Complementary to GTL Research, p. 203).

The rest of this chapter will explore current science and technology gaps and research capabilities needed to overcome key challenges in two areas of applied research in bioenergy: Ethanol from biomass and biohydrogen.

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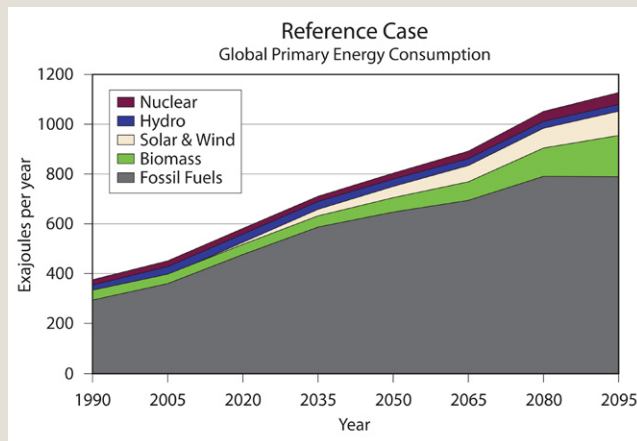


Fig. 1A. Reference Case

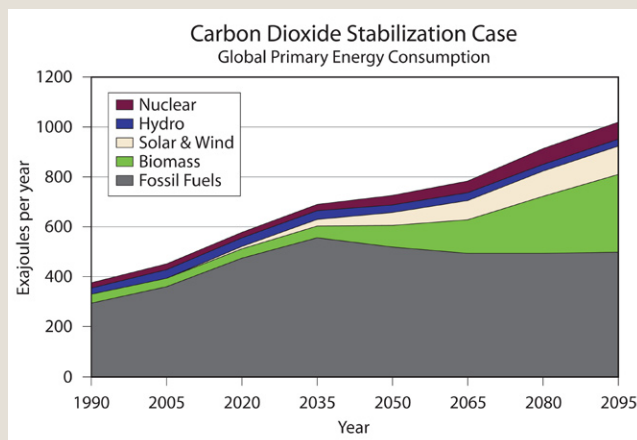


Fig. 1B. Carbon Dioxide Stabilization Case

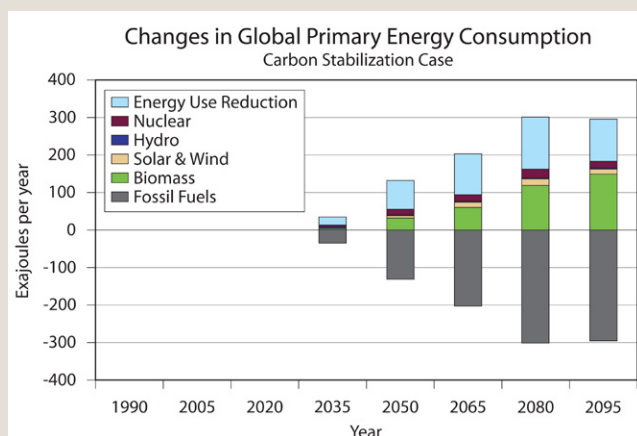


Fig. 1 C. Changes in Global Primary Energy Consumption

*The case of 550 ppmv was chosen to illustrate the types of changes that might occur; currently, no scientific basis exists for preferring any particular CO₂ concentration.

Fig. 1. Potential Role of Biotechnology in the Global Energy System. These diagrams show results of an economic analysis that considered competition among energy technologies in the 21st Century and explored conditions under which biological energy sources could develop (Edmonds et al. 2003). Fig. 1A presents the MiniCAM B2 reference case (Edmonds et al. 2004). In this scenario, the world's economic activity and number of inhabitants continue to grow, with the population reaching 9.4 billion by 2100. Energy technologies continue to improve; however, strategies to address global environmental challenges (such as mitigating greenhouse gas accumulations) are not a priority.

Fig. 1B shows another possible energy-consumption scenario in which a global commitment has been made to stabilize long-term atmospheric CO₂ concentration at 550 ppmv (about double the preindustrial level of 280 ppmv); the current level is around 380 ppmv.* Placing limits on CO₂ emissions provides an incentive for developing noncarbon-emitting energy technologies and reducing energy consumption through conservation and improvements in energy efficiency. Over the century, increased biofuel consumption combined with reductions in energy use would displace hundreds of exajoules of fossil-fuel energy (Fig. 1C), and by 2100 biofuels would equal roughly all fossil-fuel usage today (coal + oil + natural gas). By decreasing fossil-fuel use in the stabilization case, hundreds of gigatons of CO₂ emissions would be avoided (Fig. 1D).

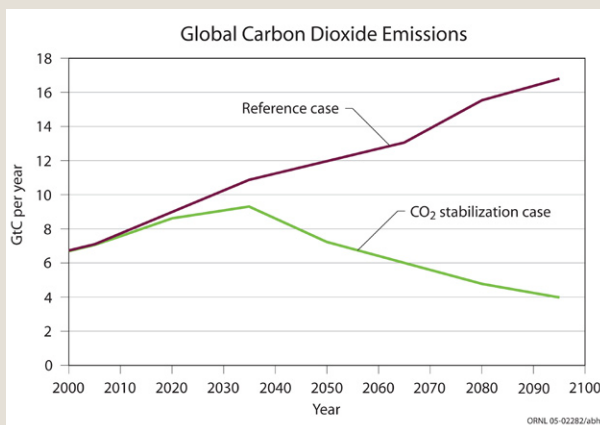


Fig. 1D. Global Carbon Dioxide Emissions

A.1.4. Ethanol from Biomass

A.1.4.1. Cellulose Degradation and Conversion

Understanding the conversion of biomass to ethanol begins with understanding the structural and chemical complexity of the three primary polymers that make up plant cell walls: Cellulose, hemicellulose, and lignin (see Fig. 2. Cellulose Structure and Hydrolysis Challenges, p. 204). Depending on plant species and cell type, the dry weight of a cell wall typically consists of about 35 to 50% cellulose, 20 to 35% hemicellulose, and 10 to 25% lignin (Saha 2004). Cellulose is the most abundant biomaterial on earth. Each cellulose molecule is a linear polymer of glucose residues. Depending on the degree of hydrogen bonding within and between cellulose molecules, this polysaccharide is found in crystalline or paracrystalline (amorphous) forms. Cellulose exists within a matrix of other polymers, primarily hemicellulose and lignin. Hemicellulose is a branched sugar polymer composed of mostly pentoses (five-carbon sugars) and some hexoses (six-carbon sugars). Lignin is a complex, highly cross-linked aromatic polymer that is covalently linked to hemicellulose, thus stabilizing the mature cell wall. These polymers provide plant cell walls with strength and resistance to degradation, which also makes these materials a challenge to use as substrates for biofuel production.

Enzymes such as cellulases, hemicellulases, and other glycosyl hydrolases synthesized by fungi and bacteria work together in a synergistic fashion to degrade the structural polysaccharides in biomass. These enzyme systems, however, are as complex as the plant cell-wall substrates they attack. For example, commercial cellulase preparations are mixtures of several types of glycosyl hydrolases, each with distinctly different functions (exocellulases, endocellulases, exoxylanases, endoxylanases, cellobiases, and many others). Optimization of these enzymes will require a more detailed understanding of their regulation and activity as a tightly controlled, highly organized system.

DOE Activities Complementary to GTL Research

Office of Energy Efficiency and Renewable Energy (EERE)

www.eere.energy.gov

EERE Biomass Program: www.eere.energy.gov/biomass/. The Biomass Program supports the research and development of advanced technologies that transform biomass into biofuels, biopower, and high-value bioproducts. Through partnerships with industry, the Biomass Program is fostering a new domestic bioindustry that will use liquid-based biofuels to reduce U.S. dependence on foreign oil. The program has five core R&D activities: (1) Biomass Feedstocks, which develops technologies to provide biomass feedstock supplies to biorefineries; (2) Sugar Platform, which studies and optimizes the chemical and biological processes that break down biomass into raw sugar components; (3) Thermochemical Platform, which uses gasification, pyrolysis, and hydrothermal processes to convert biomass to intermediate products; (4) Products, which concentrates on chemical and biological processes that convert Sugar Platform and Thermochemical Platform outputs to final products such as fuels and chemicals; and (5) Integrated Biorefineries, which uses technical successes in the other four R&D areas to establish an integrated, market-ready biorefinery capable of employing biomass to make a range of such high-value bioproducts as fuels, chemicals, and biopower. GTL will play an important role in providing a better understanding of current microbial processes and discovering new microbial capabilities relevant to the Sugar Platform and Products research areas.

EERE Hydrogen Production: www.eere.energy.gov/hydrogenandfuelcells/hydrogen_production.html. EERE's Hydrogen, Fuel Cells, and Infrastructure Technologies Program aims to research and develop low-cost, highly efficient hydrogen-production technologies from diverse domestic sources. GTL science could benefit two related research areas: (1) biological and biomass-based production for improving efficiencies of anaerobic fermentation systems and (2) photolytic production of hydrogen by green algae.

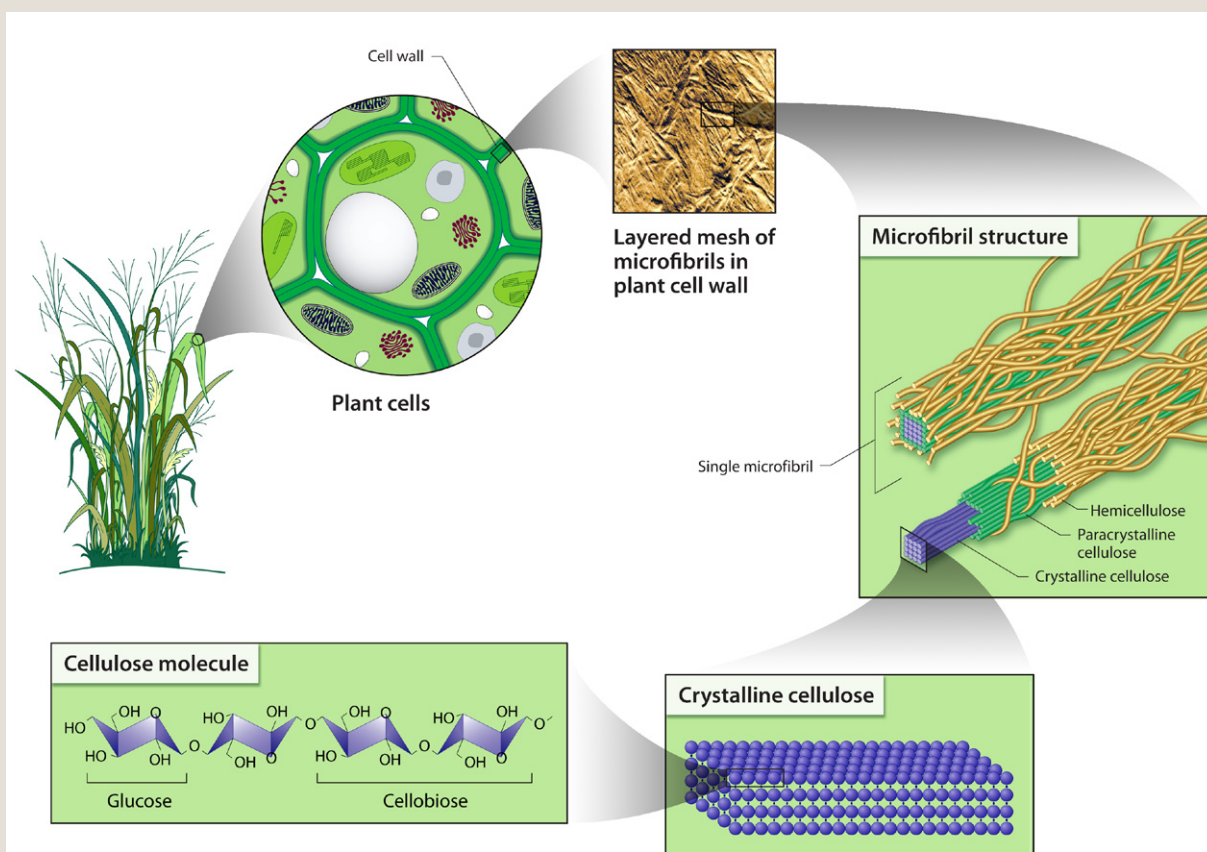
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Fig. 2. Cellulose Structure and Hydrolysis Challenges. Within the plant cell wall, chains of cellulose molecules associate with other polymers to form linear structures of high tensile strength known as microfibrils. Layers upon layers of microfibrils make up the cell wall.

Each microfibril is about 10 to 20 nm in diameter and may consist of up to 40 cellulose chains. A microfibril's crystalline and paracrystalline (amorphous) cellulose core is surrounded by hemicellulose, a branched polymer composed of a mix of primarily pentose sugars (xylose, arabinose), and some hexoses (mannose, galactose, glucose). In addition to cross-linking individual microfibrils, hemicellulose also forms covalent associations with lignin, a rigid aromatic polymer. Lignin is not pictured since its structure and organization within the cell wall are poorly understood. Pretreatment of biomass with enzymes or acids is necessary to remove the surrounding matrix of hemicellulose and lignin from the cellulose core prior to hydrolysis.

The crystallinity of cellulose presents another challenge to efficient hydrolysis. The high degree of hydrogen bonding that occurs among the sugar subunits within and between cellulose chains forms a 3D lattice-like structure. The highly ordered, water-insoluble nature of crystalline cellulose makes access and hydrolysis of the cellulose chains difficult for the aqueous solutions of enzymes. Paracrystalline cellulose lacks this high degree of hydrogen bonding, thus giving it a structure that is less ordered.

Each cellulose molecule is a linear polymer of thousands of glucose residues. Cellobiose, which consists of a pair of glucose residues (one right side up and one upside down) is the repeating unit of cellulose. [Microfibril portion of this figure adapted from J. K. C. Rose and A. B. Bennett, "Cooperative Disassembly of the Cellulose-Xyloglucan Network of Plant Cell Walls: Parallels Between Cell Expansion and Fruit Ripening," *Trends Plant Sci.* 4, 176–83 (1999).]



The biochemical conversion of biomass to ethanol currently involves three basic steps: (1) thermochemical treatments of raw lignocellulosic biomass to make the complex polymers more accessible to enzymatic breakdown; (2) production and application of special enzyme preparations (cellulases and hemicellulases) that hydrolyze plant cell-wall polysaccharides to a mixture of simple sugars; and (3) fermentation, mediated by bacteria or yeast, to convert these sugars to ethanol. A more complete understanding of enzymes and microbes involved in biomass conversion to ethanol is needed to overcome many current inefficiencies in the production process (see Table 2. Cellulosic Ethanol Goals and Impacts, this page; and Table 3. Cellulosic Ethanol Challenges, Scale, and Complexity, p. 206).

A.1.4.2. Bioethanol Research Targets for GTL

Improving Cellulase Systems. GTL will accelerate the development of optimal cellulase systems by providing resources for screening thousands of natural and modified enzyme variants, enabling the high-throughput production and functional analysis of these enzymes, elucidating regulatory controls and essential molecular interactions, and developing models for analyzing the structure and activity of natural and engineered enzyme systems.

Enabling the Development of Integrated Bioprocessing. A long-term target for GTL research is integrated bioprocessing, the conversion of biomass to ethanol in a single step. Accomplishing this requires the development of a genetically modified, multifunctional organism or a stable mixed culture capable of carrying out all biologically mediated transformations needed for the complete conversion of biomass to ethanol.

A.1.4.2.1. Gaps in Scientific Understanding

Without improving our understanding of microbial processes essential to bioethanol production, developing and improving technologies based on this understanding will be difficult. Biotechnology innovation requires basic research that explores a greater variety of enzymes and microorganisms, analyzes enzymes as systems,

Table 2. Cellulosic Ethanol Goals and Impacts

Factors	Today	Interim	Long-Term*
Billion gallons Fossil fuel displaced** CO ₂ reduced	4 2% 1.8%	20 10% 9%	30 to 200 15 to 100%*** 14 to 90%
Feedstock****	Starch (14% energy yield)	Waste cellulose	Cellulosic energy crops (>37% energy yield)
Process	Starch fermentation Little cellulose processing	Acid decrystallization: Transition to enzymes Cellulases Single-sugar metabolism Multiple microbes Some energy crops	Enzyme decrystallization and depolymerization Cellulase and other glycosyl hydrolases Sugar transporters High-temperature functioning Multisugar metabolism Integrated processing Designer cellulosic energy crops Carbon sequestration through plant partitioning
Deployment	Large, central processing	Large, central processing	Distributed or centralized, efficient processing plants
Other impacts: Energy dollars spent at home, third crop for agriculture, land revitalization and stabilization, habitat, soil carbon sequestration, yield per acre roughly tripled (cellulose over corn starch).			

*Enabled by GTL.

**Current U.S. consumption of gasoline is about 137 billion gallons per year, which corresponds to about 200 billion gallons of ethanol (Greene et al. 2004) because a gallon of ethanol has 2/3 the energy content of a gallon of gasoline.

*** Assumes improvements in feedstocks, processes, and vehicle fuel efficiency.

**** Adapted from Smith et al. 2004.

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and determines how certain factors influence biomass degradation or ethanol production. Several fundamental scientific questions in need of further investigation include:

- **What is the extent of natural diversity among biomass-degrading and ethanologenic organisms?** Over the last 30 years, most research devoted to ethanol production from cellulose has focused on fungal systems (primarily *Trichoderma reesei*) for the breakdown of cellulose into sugars coupled with the sugar-fermentation processes of yeast (*Saccharomyces cerevisiae*) (Demain et al. 2005). A deeper understanding of a greater variety of cellulolytic and ethanologenic systems is needed. Bacterial species in diverse physiological groups (e.g., bacteria with various tolerance levels for oxygen, temperature, and salt concentrations) are known to hydrolyze cellulose; thus a wide range of natural habitats could be explored for novel cellulolytic activities in bacteria.
- **How do soluble enzymes act on an insoluble crystalline substrate?** The hydrolysis of crystalline cellulose is the rate-limiting step in biomass conversion to ethanol because aqueous solutions of enzymes have difficulty acting on this insoluble, highly ordered structure. Cellulose molecules in their crystalline form are packed so tightly that enzymes and even small molecules such as water are unable to permeate the structure.
- **How do different biomass-degrading enzymes work together as a synergistic system?** Cellulases and hemicellulases are secreted from cells as free enzymes or as large, extracellular complexes known as cellulosomes. The collective activity of these enzyme systems is much more efficient than the individual activity of any isolated enzyme; therefore, to truly understand how these enzymes function, they must be studied as systems rather than individually or a few at a time. In addition, these systems eventually must be analyzed under laboratory conditions more representative of real-world environments. For example, laboratories often use purified cellulose as the substrate for enzyme analysis rather than more heterogeneous, natural lignocellulosic materials, and this can provide erroneous conclusions about natural enzyme activity.
- **Why are ethanologenic organisms less efficient at using certain sugar substrates?** A varied mix of hexoses (e.g., glucose, mannose), pentoses (e.g., xylose, arabinose), and oligosaccharides are released from the hydrolysis of lignocellulosic materials, and no microorganism is capable of fermenting all these sugars. The most widely studied ethanologenic microbes (e.g., yeast) prefer to use glucose as a substrate. Even when yeast cells are modified genetically to use xylose, they ferment all glucose before switching to the much slower xylose fermentation. Conversion rates can vary greatly depending on such factors as the type of sugar substrate being fermented, environmental conditions (e.g., pH, temperature), and the concentrations of certain products from other metabolic pathways.
- **How effective are sugar transporters at translocating different sugars across the cell membrane?** Sugar transporters are membrane-bound proteins that take up sugars from the environment and deliver them to

Table 3. Cellulosic Ethanol Challenges, Scale, and Complexity

Research and Analytical Challenges	Scale and Complexity
<ul style="list-style-type: none"> • Screening of databases for natural variants of cellulases (generally glycosyl hydrolases) and other enzymes or molecular machines in metabolic networks and characterization of variants • Analysis of modified variants to establish design principles and functional optimization • Modeling and simulation of cellulase, sugar transport, and multiple sugar-fermentation processes and systems • Integration of processing steps into single microbes or stable cultures 	<ul style="list-style-type: none"> • Thousands of variants of all enzymes; screening of millions of genes, thousands of unique species and functions • Production and functional analysis of potentially thousands of modified enzymes, hundreds of regulatory processes and interactions • Models at the molecular, cellular, and community levels incorporating signaling, sensing, regulation, metabolism, transport, biofilm, and other phenomenology and using massive databases in GTL Knowledgebase • Incorporation of complete cellulose-degradation and sugar-fermentation processes into microbes or consortia—hundreds of metabolic, regulatory, and other interconnected pathways

the metabolic pathways inside cells. The inefficient transport of different sugar substrates by microbes can result in low product yield and is a major obstacle to the efficient conversion of biomass to ethanol. Our limited understanding of sugar transporters is due to a lack of adequate techniques for producing membrane proteins and studying their structure and function. Questions in need of investigation include: Can a glucose transporter transport other sugars, and, if so, how efficiently? Are some transporters better than others? Can transporters be modified for improved function?

- **Why do different enzymatic and microbial processes operate optimally at different temperatures?** Cellulases operate optimally at temperatures (>40°C) higher than those tolerated by ethanologenic organisms, so these two processes currently cannot be consolidated into a single process step. Thermophily (tolerance of high temperatures) improves the robustness of enzymes or microbes needed for industrial-scale processes and reduces the likelihood of culture contamination. The basis by which enzymes, pathways, and entire microbes are made thermophilic is understood poorly, and methods for inserting cellulolytic or fermentative pathways into thermophilic organisms are not well developed.
- **What are the requirements for producing and maintaining stable mixed cultures?** At a minimum, cultures used in bioethanol-production systems will need to be resistant or stable despite contamination by “outside” microbes or other potentially toxic materials or life forms. We currently do not understand in sufficient detail the dynamics of microbial consortia that carry out stable mixed processes such as aerobic and anaerobic digestion. Without this understanding, we will not be able to “design” or “engineer” such systems.
- **How can we improve systems for genetically engineering microorganisms involved in bioethanol production?** While many studies have expressed genes from cellulolytic organisms in *Escherichia coli* or other mesophilic organisms, systems for expressing foreign genes in cellulolytic or thermophilic organisms are in need of further development. Our current limited understanding of microbial regulation prevents the successful engineering of a microbe capable of versatile expression of lignocellulolytic enzymes, utilization of multiple sugars, and glycolysis.

A.1.4.2.2. Scientific and Technological Capabilities Required to Achieve Goals

Improving current understanding of bioethanol production will require a variety of new capabilities including techniques for surveying enzyme diversity; visualizing enzyme systems; efficiently producing enzyme systems and membrane proteins; cultivating microbial consortia; integrating transcriptomics, proteomics, and metabolomics; and genetically engineering microorganisms for integrated bioprocessing (see Table 3, p. 206). Specific needs include the following:

- **Ecogenomic approaches to explore the natural diversity of cellulases.** High-throughput sequencing and computational analysis of DNA from environments in which cellulose is widely available will lead to the discovery of genes for novel cellulase systems that could be used as templates for protein production.
- **Techniques to visualize cellulase systems in motion.** Advanced imaging techniques will provide new insights into how cellulases interact with crystalline cellulose and overcome current barriers to efficient cellulose hydrolysis (e.g., substrate accessibility, product or substrate inhibition, low product yield). Structural information and imaging from X-ray, nuclear magnetic resonance spectroscopy, scanning transmission electron microscopy, and other techniques will be needed to identify additional interactions between cellulases and other molecules needed for efficient function.
- **Large-scale production of cellulase enzyme systems, sugar transporters, and other proteins.** This will require improved methods for protein production and characterization. Currently, synthesis of sugar transporters and other membrane proteins is difficult, so analyzing the structure and activity of these proteins is challenging, if not impossible. High-throughput techniques and expression systems for efficiently producing membrane proteins, sets of different enzymes that work together, and enzyme complexes such as cellulosomes are in need of development. Access to validated expression systems for microorganisms with mission-relevant capabilities, including thermophilic, cellulolytic, and ethanologenic organisms, would help researchers spend less time on developing expression methods and more time on characterizing and improving proteins.

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- **Methods to grow stable mixed cultures.** Improved experimental and modeling tools are needed to develop methods for producing a mixed microbial culture. The goal is to enable each population carrying out one part of the overall ethanol production process to perform stably.
- **Methods to integrate transcriptomic, proteomic, and metabolomic information.** Techniques that integrate information gathered from these global molecular measurements are essential to determining which genes are expressed and functionally active during cellulose utilization or ethanol fermentation and which metabolites influence the activity of enzymes involved in these pathways. As an insoluble substrate, cellulose cannot enter cells and induce the expression of genes involved in cellulose hydrolysis. Metabolic profiling could be used to identify which substrates or metabolites at what quantities activate or repress expression of key cellulolytic genes. In addition to illuminating regulatory strategies for cellulases and other coexpressed enzymes such as ligninases, these integrated omics approaches could be used to build regulatory and metabolic maps to guide genetic engineering. For example, these maps could be used to identify the best potential gene knockouts that redirect carbon flux from a particular sugar substrate toward ethanol fermentation and bypass competing pathways that produce other organic end products.
- **Methods to genetically engineer organisms for integrated bioprocessing.** *Clostridium thermocellum* is an anaerobic bacterium capable of both hydrolyzing cellulose and fermenting sugars to ethanol, but its yields are poor and conversion is slow. Improved methods for genetically modifying this and other cellulolytic microbes are needed. In one approach to developing an organism for integrated bioprocessing, a microbe naturally capable of hydrolyzing cellulose, such as *C. thermocellum*, is engineered to provide high product (ethanol) yields. In another approach, noncellulolytic microorganisms known to have high yields of ethanol are engineered to express cassettes of genes encoding cellulase enzyme systems. In either case, to achieve this ambitious goal of developing an organism capable of integrated bioprocessing, the current research paradigm must be altered to focus on understanding how microbial systems function and how their interacting pathways influence one another rather than focusing on only a few genes or enzymes.

A.1.5. Biohydrogen Production

Hydrogen is a promising energy carrier of the future: It can be derived from a variety of energy sources and used in fuel cells with high efficiency; “combustion” of hydrogen produces only water as a by-product, making it a nonpolluting, carbon-free energy alternative. The most common industrial methods for producing hydrogen include steam reformation of natural gas, coal gasification, and splitting water with electricity typically generated from fossil fuels. These energy-intensive industrial processes release carbon dioxide and other greenhouse gases and pollutants as by-products. Some microorganisms produce hydrogen naturally, and biotechnologies based on these microbial systems could lead to the development of clean, renewable sources of hydrogen. In a recent report on the hydrogen economy, however, the National Research Council (NRC) noted that “substantial, fundamental research needs to be undertaken before photobiological methods for large-scale hydrogen production are considered” (Hydrogen Economy 2004).

Several reviews have examined the potential of biological hydrogen production (Madamwar, Garg, and Shah 2000; Ghirardi et al. 2000; Melis and Happe 2001; Tamagnini et al. 2002; Levin, Pitt, and Love 2004; Nath and Das 2004; Prince and Kheshgi 2005). Although microorganisms produce hydrogen by different mechanisms, the step can be represented by the simple chemical reaction $2\text{H}^+ + 2\text{e}^- \leftrightarrow \text{H}_2$. This reaction is known to be catalyzed by either nitrogenase or hydrogenase enzymes. Although alternative biological hydrogen production-pathways exist, each with its own set of advantages and disadvantages, the following discussion on biohydrogen production will focus on the challenges that must be overcome to improve one type of biological hydrogen production known as biophotolysis (see sidebar, Other Mechanisms for Biological Hydrogen Production, p. 209, and Table 4. Biophotolytic Hydrogen, p. 209).

A.1.5.1. Biophotolysis of Water

Under certain conditions, green algae and cyanobacteria can use water-splitting photosynthetic processes to generate molecular hydrogen (H₂) rather than fix carbon, the normal function of oxygenic photosynthesis (see sidebar, Photosynthetic Production of Hydrogen from Water, p. 210). Bidirectional hydrogenases in these organisms use electrons from the photosynthetic electron-transport chain to reduce protons to yield H₂. Biophotolysis holds potential for the scale of hydrogen production necessary to meet future energy demand. This approach to hydrogen production is promising because the source of electrons or reducing power required to generate hydrogen is water—a clean, renewable, carbon-free substrate available in virtually inexhaustible quantities. Another advantage of biophotolysis is the more efficient conversion of solar energy to hydrogen. Reengineering microbial systems for the direct production of hydrogen from water eliminates inefficiencies associated with carbon fixation and biomass formation. Theoretically, the maximal energetic efficiency for direct biophotolysis is about 40% (Prince and Khesghi 2005) compared with a maximum of about 1% for hydrogen production from biomass (Hydrogen Economy 2004). Recognizing the important potential of biophotolysis, NRC has recommended that DOE “refocus its biobased program on more fundamental research on photosynthetic microbial systems to produce hydrogen from water at high rate and efficiency” (Hydrogen Economy 2004).

A.1.5.2. Biohydrogen Research Targets for GTL

Engineering Oxygen-Tolerant, Efficient Hydrogenases. Hydrogenases known to tolerate oxygen generally are not very efficient hydrogen producers. During biophotolytic hydrogen production, oxygen is released from the water-splitting reaction, thus engineering hydrogenases with sufficient activity and oxygen tolerance will be needed. Engineered hydrogenases then could be used in bioinspired nanostructures that maintain optimal conditions for hydrogen production.

Other Mechanisms for Biological Hydrogen Production

Nitrogenase-Mediated Hydrogen Production. In the absence of oxygen and presence of light, purple nonsulfur (PNS) photosynthetic bacteria such as *Rhodospseudomonas palustris* and *Rhodobacter sphaeroides* contain nitrogenase enzymes that can generate hydrogen under nitrogen-limited conditions. These microbes obtain the electrons they need to reduce protons to molecular hydrogen (H₂) from the breakdown of organic compounds. Certain species of cyanobacteria also contain nitrogenase enzymes capable of producing hydrogen as a by-product of nitrogen fixation.

Fermentative Hydrogen Production. A variety of bacteria such as *E. coli*, *Enterobacter aerogenes*, and *Clostridium butyricum* are known to ferment sugars and produce hydrogen using multienzyme systems. These “dark fermentation” reactions do not require light energy, so they are capable of constantly producing hydrogen from organic compounds throughout the day and night. Compared with other biological hydrogen-production processes, fermentative bacteria have high evolution rates of hydrogen. However, sugars are relatively expensive substrates that are not available in sufficient quantities to support hydrogen production at a scale required to meet energy demand.

Table 4. Biophotolytic Hydrogen: Goals and Impacts

- Sunlight and seawater, two resources in virtually limitless supply, can be used to produce the ultimate fuel and energy carrier, hydrogen. High-efficiency use of hydrogen in fuel cells can produce electricity directly with water as the by-product.
- This energy cycle is carbon free and can be developed as the complement to the electric grid for all energy applications— industrial, transportation, and residential.
- Development of biological photolytic processes to produce hydrogen at high rates and efficiency will enable the establishment of a hydrogen-economy strategy based on a renewable source.

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Designing Microorganisms Optimized for Hydrogen Production. Photosynthetic microbes that have been genetically modified to produce hydrogen at high rates and efficiency from the biophotolysis of water could be grown in extensive farms of sealed enclosures (photobioreactors). Hydrogen would be harvested for use in energy applications, with oxygen released as a by-product.

A.1.5.2.1. Gaps in Scientific Understanding

Understanding biophotolysis well enough to model hydrogenase structure and function, regulatory and metabolic networks, and eventually entire organisms will stimulate the kind of biotechnological innovation needed to engineer the ideal organism to use in hydrogen bioreactors or the ideal enzyme-catalyst to use in bioinspired nanostructures for hydrogen production. But achieving this level of understanding will require basic research that investigates a greater range of hydrogen-producing enzymes and organisms, mechanisms of hydrogenase assembly, oxygen sensitivity of hydrogenase, electron-transfer rate limitations, and regulatory and metabolic processes that influence hydrogen production. Some specific issues relevant to these basic research needs follow.

- What is the extent of natural diversity among hydrogenases and hydrogen-producing organisms? A vast majority of organisms that contain hydrogenases have not been identified and probably cannot be cultured

Photosynthetic Production of Hydrogen from Water

Although microorganisms are capable of carrying out different types of photosynthesis, that found in plants, algae, and cyanobacteria is best understood. Photosynthesis in these organisms is a complex series of reactions that use light energy to drive electron transfer from water to carbon dioxide to yield carbohydrates.

Instead of using electrons harvested from water to synthesize carbohydrates from CO_2 , under certain conditions green algae and cyanobacteria can use them to reduce protons and produce hydrogen gas (H_2). Molecular complexes involved in mediating electron flow from water to carbon-fixing or hydrogen-production reactions make up the photosynthetic electron-transport chain found in the thylakoid membranes of cyanobacteria and green algae. In eukaryotic green algae, thylakoid membranes are housed within a cellular organelle known as the chloroplast; in prokaryotic cyanobacteria, thylakoids are found in the cytoplasm as an intracellular membrane system (see Fig. A).

An overview of steps involved in using light energy to produce carbohydrates or hydrogen is depicted in Fig. B and described below.

1. **Light Absorption by Photosystem II (PSII) Initiates the Photosynthetic Pathway.** PSII is a large molecular complex that contains several proteins and light-absorbing pigment molecules. The primary pigment molecules are chlorophylls and carotenoids, but cyanobacteria also have other pigments called phycobilins that absorb light at different wavelengths. The pigments are bound to proteins to form antenna complexes that absorb photons and transfer the resultant excitation energy to the reaction center of PSII, where energized electrons move to a small electron-carrier molecule. This molecule shuttles the excited electrons to the next complex in the photosynthetic electron-transport chain. To replace electrons lost in the transfer, the reaction center strips low-energy electrons from two water molecules, releasing four protons and an oxygen (O_2) molecule into the thylakoid space.

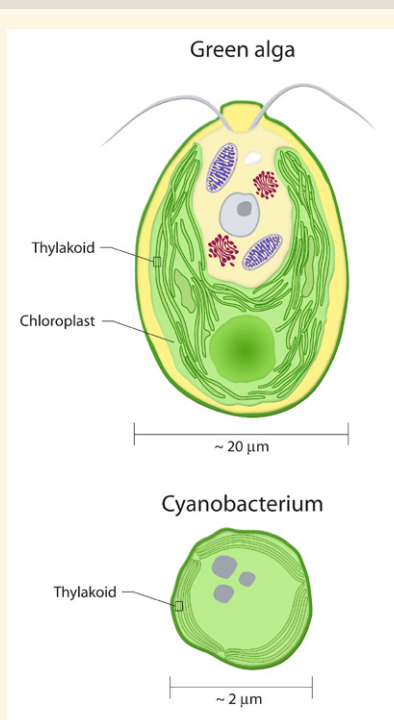


Fig. A. Thylakoids in Green Algae and Cyanobacteria.

- Electron Transport Through the Cytochrome Complex Generates a Proton Gradient.** The electron carrier from PSII passes through the thylakoid membrane and transfers its electrons to the cytochrome complex, which consists of several subunits including cytochrome *f* and cytochrome *b₆*. A series of redox reactions within the complex ultimately transfer the electrons to a second electron carrier that acts as a shuttle to photosystem I (PSI). As electrons are transported through the complex, protons (H^+) outside the thylakoid are carried to the inner thylakoid space. The increase in proton concentration inside the thylakoid space creates a proton gradient across the thylakoid membrane.
- Light Absorption by PSI Excites Electrons and Facilitates Electron Transfer to an Electron Acceptor Outside the Thylakoid Membrane.** PSI is another large protein-pigment complex that contains light-absorbing antenna molecules and a reaction center. Light absorbed by the PSI reaction center energizes an electron that is transferred to ferredoxin (Fd), a molecule that carries electrons to other reaction pathways outside the thylakoid. The reaction center replaces the electron transferred to ferredoxin by accepting an electron from the electron-carrier molecule that moves between the cytochrome complex and PSI.
- Under Certain Conditions, Ferredoxin can Carry Electrons to Hydrogenase.** Normally, ferredoxin shuttles electrons to an enzyme that reduces $NADP^+$ to $NADPH$, an important source of electrons needed to convert CO_2 to carbohydrates in the carbon-fixing reactions. Under anaerobic conditions, hydrogenase can accept electrons from reduced ferredoxin molecules and use them to reduce protons to molecular hydrogen (H_2).
- Dissipation of Proton Gradient is Used to Synthesize Adenosine Triphosphate (ATP).** ATP synthase couples the dissipation of the proton gradient generated in step 2 to the synthesis of ATP. Translocation of protons from a region of high concentration (thylakoid space) to a region of low concentration (outside thylakoid) releases energy that can be used to drive the synthesis of ATP from adenosine diphosphate (ADP) and phosphate (P). ATP is a high-energy molecule used to convert CO_2 to carbohydrates in the carbon-fixing reactions.

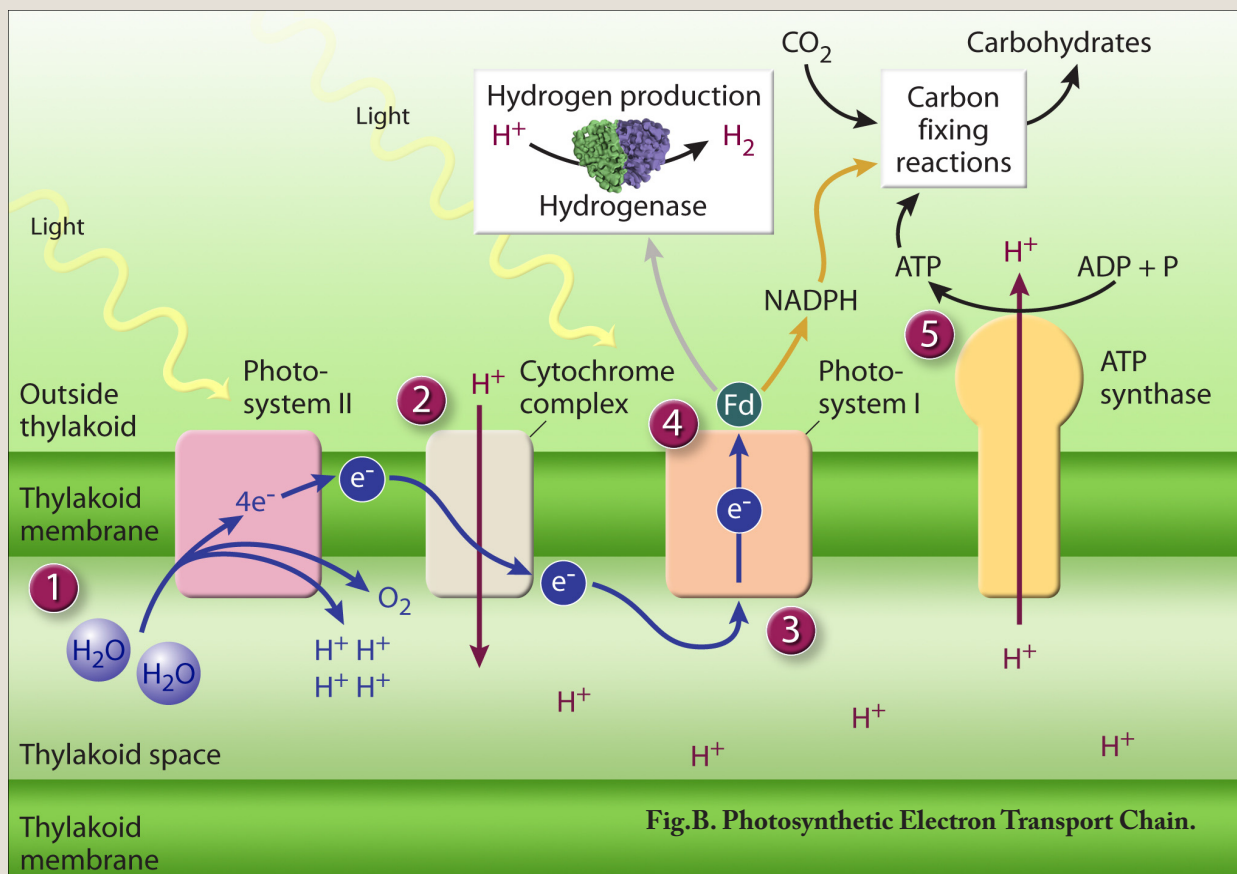


Fig.B. Photosynthetic Electron Transport Chain.

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in the laboratory using current procedures. Studying hydrogenase enzymes involved in nonbiophotolytic pathways could provide structural or functional insights to guide the engineering of biophotolytic systems.

- **How are hydrogenases assembled, and how are metals incorporated into the active site?** Two major types of hydrogenases are defined by their biologically unique metallocenters: Nickel-iron (NiFe) and iron only (Fe). NiFe hydrogenases are found in many bacteria and some cyanobacteria. Fe hydrogenases are found in some bacteria and green algae. In green algae, hydrogenases are bidirectional (capable of catalyzing hydrogen oxidation or proton reduction to produce H_2); in cyanobacteria, hydrogenases are either bidirectional or they uptake enzymes. Although turnover is much higher for Fe hydrogenases, NiFe hydrogenases are more oxygen tolerant. The metallocenters of both NiFe and Fe hydrogenases form complexes with such unusual inorganic cofactors as carbon monoxide (CO) or cyanide (CN). Little is known about the assembly of an active hydrogenase, and several genes may be involved in the synthesis of cofactors required for activity. A better understanding of hydrogenase assembly will enable the engineering of enzymes with improved function.
- **How do we overcome the oxygen-sensitivity problem of hydrogenases?** The bidirectional Fe hydrogenases that catalyze the hydrogen-evolution reaction in biophotolytic systems are highly sensitive to oxygen, a product of the water-splitting reaction in the first step of the photosynthetic pathway. Oxygen sensitivity also makes hydrogenase isolation from cells and its subsequent analysis a challenge that will be met by new technologies.
- **What are the potential electron-transfer rate limitations associated with each step of the biophotolytic hydrogen production pathway?** Key factors that can impact the partitioning of electrons between hydrogenase and competing pathways include the buildup of a pH gradient across the photosynthetic membrane and variations in the concentrations of critical electron-transport carriers. Understanding how electron fluxes in an organism are regulated will aid the development of mechanisms for directing more electrons towards proton reduction and hydrogen production.
- **What are the regulatory and metabolic pathways that influence H_2 production?** A thorough examination of hydrogen metabolism in green algae and several different strains of cyanobacteria from diverse habitats will provide new insights into how hydrogen-production pathways are controlled. By understanding how an organism sustains and regulates hydrogen production, we will be able to determine which metabolic pathways contribute, how eliminating hydrogen-consuming reactions affects hydrogen metabolism and other cellular processes, and how organisms can be adapted to increase hydrogen yields.

A.1.5.2.2. Scientific and Technological Capabilities Required to Achieve Goals

Key capabilities needed to address many of the gaps in current understanding of biophotolytic hydrogen production include developing microbial hosts to produce hydrogenase enzymes, screening large numbers of enzymes for desired functionalities, large-scale molecular profiling to provide a global-view of hydrogen production, in vivo visualization of hydrogenase structure and activity, modeling of regulatory and metabolic networks, and metabolic engineering (see Table 5. Roadmap for Development of Biophotolytic Hydrogen Technologies, p. 213, and Table 6. Biophotolytic Hydrogen Production Challenges, Scale, and Complexity, p. 213). Specific needs include the following:

- **Suites of microbial hosts to produce hydrogenases from many different organisms.** Potentially thousands of enzymes from many different organisms will need to be produced and analyzed. Other requirements include methods for producing eukaryotic enzymes in simpler prokaryotic systems, designing host organisms that can provide the intracellular environment required for proper protein assembly and folding, and screening the proteins produced from these host organisms.
- **Methods to produce large numbers of enzymes to screen for desired hydrogenase properties.** With so much variability among natural hydrogenases and engineered variants, developing high-throughput capabilities for producing large numbers (perhaps hundreds of thousands to millions) of enzymes to screen for O_2 tolerance, H_2 -production activity, spectroscopic examination, and structural analysis could accelerate the discovery of enzymes best suited for biotechnological applications.

- **Molecular profiling to provide a global view of cellular activity during hydrogen production.** Improvements in computational capabilities and large-scale molecular profiling techniques (transcriptomics, proteomics, metabolomics, measurements of metal abundance) are needed to obtain a global view of microbial hydrogen production. Systems-level analyses could guide experimental investigations by defining gene regulatory networks controlling the expression of genes involved in hydrogen production or cofactor synthesis and identify pathways activated or deactivated during hydrogen production for multiple organisms under varying conditions.
- **Methods to perform in vivo visualization and characterization of molecular machines.** Although crystal structures of some hydrogenases have been determined, this information provides only snapshots of enzyme structure. Advanced techniques for visualizing the different stages of hydrogenase assembly or monitoring hydrogenase activity in living cells will be critical to building predictive models that can be used to engineer hydrogenases optimized for biotechnological applications.
- **Support and techniques for systems-level studies to model and simulate regulatory and metabolic networks.** Studying hydrogenase function within the context of a network maintained by living cells is essential to understanding how this process is influenced by different pathways and environmental conditions.

Table 5. Roadmap for Development of Biophotolytic Hydrogen Technologies

Totality of Processes Must Be Optimized with a Number of Challenges for Each

Processes	Challenges	Deployment
Hydrogenases Regulatory pathways Charge transport Partitioning Multiple mechanisms	O ₂ sensitivity Range of hydrogenases Primary and secondary pathways Electron transfer limits Reverse reactions Light capture	Photolytic organisms contained in bioreactors (closed flowing system with hydrogen and oxygen separations) Photosynthetic hydrogen production cassettes deployed in nanostructures
Development Strategy Explore natural range of hydrogenases for variability and design principles Explore mutations and other optimization strategies Understand regulatory and other ancillary pathways for systems optimization (e.g., buildup of protons in cytoplasm, alternative uses of reductants) Capture key functions for cell-free incorporation into nanomembranes		

Table 6. Biophotolytic Hydrogen Production Challenges, Scale, and Complexity

Research and Analytical Challenges	Scale and Complexity
<ul style="list-style-type: none"> • Database screening for and characterizing of natural variants of hydrogenases and other enzymes and molecular machines in the entire set of pathways that underlie this process • Analysis of modified variants to establish design principles for functional optimization of the overall process including oxygen sensitivity, reverse reactions, transport, light capture, and conversion efficiency • Modeling and simulation of photolytic systems to support systems design and optimization 	<ul style="list-style-type: none"> • Screening of millions of genes, thousands of unique species and functions, and thousands of variants of all enzymes • Production and functional analysis of modified enzymes—potentially thousands of each, hundreds of regulatory processes and interactions • Models at the molecular, cellular, and community levels incorporating signaling, sensing, regulation, metabolism, transport, and other phenomenology and using massive databases in GTL Knowledgebase

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Traditional in vitro biochemical methods that study hydrogenase activity one enzyme at a time in the laboratory do not provide sufficient information to understand enzymatic activities in living cells. Tools for monitoring hydrogenase activity in vivo and integrating diverse sets of experiment data are needed to build in silico models of a biophotolytic organism under H₂-producing conditions.

- **Metabolic engineering.** Metabolic engineering involves genetically modifying microorganisms to target and manipulate enzymatic, regulatory, or transport pathways that impact a particular microbial process such as hydrogen production. Models could guide metabolic engineering, for example, by identifying control points for manipulating the flow of electrons to hydrogenase or by predicting how cellular activity and hydrogen yields may be impacted by a variety of conditions. These conditions include the elimination of a particular metabolic pathway or the buildup of a pH gradient across the photosynthetic membrane.

A.1.6. Summary

The broad spectrum of analytical tools in the GTL facilities could be used to rapidly advance our current understanding of fundamental scientific issues that are impeding the development of biofuel production. Both ethanol from biomass and biophotolytic hydrogen represent bioenergy alternatives that could be produced renewably, domestically, and at a scale large enough to reduce our dependence on foreign energy sources. The potential economic and environmental advantages of developing bioenergy options cannot be realized, however, without pursuing answers to key fundamental scientific questions underlying critical R&D breakthroughs. The GTL program seeks to provide answers that can help biotechnology play a more prominent role in our energy future.