organisms, including plants, has reached the point at which researchers will be able to engineer pathways that take advantage of a variety of conditions with a great degree of confidence. Additionally, genome sequences now provide a straightforward supply of genes to be tested in tentative pathway constructs. Nevertheless, it is important to develop technologies for the synthesis and separation of these alternative fuels, because it is yet unclear what additional requirements such technologies will place in the design of a robust, fast, efficient, commodity-scale process.

In assessing the potential of current and projected technologies to develop cost-efficient B2B processes, it is important to bear in mind that the present state of affairs was reached by minimal investment directly in biofuels research. The major biosciences and bioengineering infrastructure was developed in the course of exploring medical applications of biology and biotechnology. Although this platform is the basis for the present optimism surrounding the use of biosciences for biofuel production from renewable resources, a number of problems still remain in realizing this potential. These problems must be addressed directly and adequately in the immediate future.

References and Notes
9. Data from presentations of K. C. McFarland (Novozymes) and G. Anderl (Genencor International) at the World Congress on Industrial Biotechnology and Bioprocessing, Orlando, FL, July 2006.
18. I wish to thank J. Deutch for valuable discussions on matters related to the cost of cellulose ethanol and for pointing out the need to consider the costs of an ethanol process in its entirety and not at isolated units.
29. I thank H. Alper, J. Nielsen, D. Ramkrishna, and in particular J. McMellen, J. Deutch, and L. Lynd for reading the manuscript and providing many valuable comments.

**Sustainability and Energy**

Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production

Michael E. Himmel,1* Shi-You Ding,1 David K. Johnson,1 William S. Adney,1 Mark R. Nimlos,3 John W. Brady,2 Thomas D. Foust3

Lignocellulosic biomass has long been recognized as a potential sustainable source of mixed sugars for fermentation to biofuels and other biomaterials. Several technologies have been developed during the past 80 years that allow this conversion process to occur, and the clear objective now is to make this process cost-competitive in today’s markets. Here, we consider the natural resistance of plant cell walls to microbial and enzymatic destruction, collectively known as “biomass recalcitrance.” It is this property of plants that is largely responsible for the high cost of lignocellulose conversion. To achieve sustainable energy production, it will be necessary to overcome the chemical and structural properties that have evolved in biomass to prevent its disassembly.

High worldwide demand for energy, unstable and uncertain petroleum sources, and concern over global climate change have led to a resurgence in the development of alternative energy that can displace fossil transportation fuel. In response, many countries have initiated extensive research and development programs in biofuels, a sustainable and renewable energy resource that can provide liquid transportation fuels (1). The U.S. Department of Energy Office of the Biomass Program has developed a scenario for supplying 30% of the 2004 motor gasoline demand with biofuels by the year 2030, which roughly translates to a target of 60 billion gallons per year on a British thermal unit-adjusted basis (2, 3). Similarly, the European Union has developed a vision in which one-fourth of the E.U.’s transportation fuels will be derived from biofuels by 2030 (4). These political timetables result in critical challenges to the scientific community that require cutting-edge tools in the fields of systems and synthetic biology (5).

Starch from corn grain and simple sugars from sugar cane and beets are currently being used directly for ethanol fermentation, but to harness the structural sugars contained in plant fibers, we must first overcome the problems caused by biomass recalcitrance. Cellulose processing cannot commence until we improve (i) the relatively slow kinetics of breaking down pure cellulose into sugars, (ii) the low yields of sugars from other plant polysaccharides, and (iii) the removal of lignin, a relatively intractable polymer of phenylpropanoid subunits. It is clear that technological advances must be realized to make biofuels sustainable and cost effective.

In future biofuineries, biofuels will be produced from biomass resources, including corn grains and lignocellulosic biomass (such as agricultural residues, forestry wastes and thinnings, waste paper, and energy crops). Currently in the United States, approximately 455 million acres are in agricultural production to meet our food, feed, and fiber needs (6). A recent report (7) has suggested that in the near term, more than 1.3 billion tons of biomass could be produced annually in the United States on a sustainable basis, mostly from agricultural and forestry sources. Tilman and co-workers (8) have also described the potential role for low-input, high-diversity grassland perennials for bioconversion. Another study (9) has shown that biomass has the potential to simultaneously meet the nation’s needs for liquid transportation fuel and for food, feed, and fiber, provided that we develop more advanced technologies and make certain land-use changes that would not require more net

---

1Chemical and Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA. 2Department of Food Science, Cornell University, Ithaca, NY 14853, USA. 3National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401, USA.

*To whom correspondence should be addressed. E-mail: mike_himmel@nrel.gov
land. The cost-competitive production of biofuels is currently prevented by the high cost of biomass feedstocks and the processes for converting biomass to sugars—that is, the cost of the thermochemical pretreatment and enzyme hydrolysis unit operations in a biorefinery. Maximizing conversion yield is essential for offsetting feedstock cost.

**Biomass Recalcitrance**

Plant biomass has evolved complex structural and chemical mechanisms for resisting assault on its structural sugars from the microbial and animal kingdoms (Fig. 1). Natural factors believed to contribute to the recalcitrance of lignocellulosic feedstock to chemicals or enzymes include (i) the epidermal tissue of the plant body, particularly the cuticle and epicuticular waxes; (ii) the arrangement and density of the vascular bundles; (iii) the relative amount of sclerenchymatous (thick wall) tissue; (iv) the degree of lignification (10); (v) the structural heterogeneity and complexity of cell-wall constituents such as microfibrils and matrix polymers (11); (vi) the challenges for enzymes acting on an insoluble substrate (12); and (vii) the inhibitors to subsequent fermentations that exist naturally in cell walls or are generated during conversion processes (13). In the context of the biorefinery, these chemical and structural features of biomass affect liquid penetration and/or enzyme accessibility and activity and, thus, conversion costs.

At the molecular level (Fig. 2), the crystalline cellulose core of cell-wall microfibrils (14) is highly resistant to chemical and biological hydrolysis because of its structure, in which chains of cellobiose units are precisely arranged. The chair conformation of the glucose residues in cellulose forces the hydroxyl groups into radial (equatorial) orientation and the aliphatic hydrogen atoms into axial positions. As a result, there is strong interchain hydrogen bonding between adjacent chains and weaker hydrophobic interactions between cellulose sheets. The hydrophobic face of cellulose sheets makes crystalline cellulose resistant to acid hydrolysis because it contributes to the formation of a dense layer of water near the hydrated cellulose surface (15). The strong interchain hydrogen bonding network makes crystalline cellulose resistant to enzymatic hydrolysis (14), whereas hemicellulose and amorphous cellulose are readily digestible. Higher-order structures in plants also contribute to biomass recalcitrance. For example, access to the crystalline cellulose cores of microfibrils is restricted by a coating of amorphous cellulose and hemicellulose (16). At a microscopic and macroscopic scale, the complex heterogeneous nature of biomass creates mass-transport limitations for delivery of chemical or biochemical catalysts.

**Current Biomass Conversion Technology**

The biorefinery is envisioned to comprise four major sections: feedstock harvest and storage, thermochemical pretreatment, enzymatic hydrolysis, and sugar fermentation to ethanol or other fuels. Existing biomass conversion schemes typically rely on a combination of chemical and enzymatic treatments. A pretreatment step is usually conducted to reduce recalcitrance by depolymerizing and solubilizing hemicellulose (approximately 20 to 40% weight by weight of biomass). This step converts hemicellulosic to monosaccharides and oligosaccharides, which can then be hydrolyzed by cellulase enzymes. In addition, pretreatment typically breaks down the macroscopic rigidity of biomass and decreases the physical barriers to mass transport.

**Pretreatment.** thermochemical pretreatment of biomass has long been recognized as a critical technology to produce materials with acceptable enzymatic digestibilities. For example, dilute sulfuric acid pretreatment at 140° to 200°C renders the cellulose in cell walls more accessible to saccharifying enzymes. At moderate severities (17), the hemicelluloses are hydrolyzed and the sugars are solubilized as monomers and oligomers; however, the yields of solubilized sugars are less than quantitative (i.e., 60 to 70%) (18). For the acid treatments, release of mono- and oligomeric sugars from hemicellulose exhibits multimodal kinetics in which a slow component directly relates to the high cost of conversion (19, 20). For example, a number of researchers (20–25) have noted that the solubilization of xylan in hemicellulose appears to be best modeled as a pair of parallel first-order reactions: one that takes place at a fast rate and another that progresses at a much slower rate.

What governs this result is not clear at this time, and it may depend on a number of factors, such as hemicellulose composition; biomass density; the presence of nonsugar components (such as lignin, acid neutralizing ash, and acetyl and other carboxylic acid groups); plant cell structure (including the types of cells or ratios of primary and secondary cell walls); or mass transport. Pretreatment schemes based on alkaline explosive decomposition and organic solvent extractions have been proposed with considerable success (13). The alkaline process, known as ammonia fiber expansion (AFEX), leaves the hemicellulose in place but renders the remaining cell walls considerably more amenable to enzyme hydrolysis (26).

**Fig. 1.** Structural and chemical complexity of cell-wall biomass. (A) Example of high-density bales of corn stover harvested on the eastern plains of northern Colorado. (B) An atomic force micrograph of the maize parenchyma cell-wall surface. The diameter of individual microfibril is only about 3 to 5 nm. Scale bar, 50 nm. (C) A scanning electron micrograph of the cross-section of a maize stem shows vascular bundles and pith tissues, as well as the diverse cell sizes, shapes, and cell-wall thicknesses typical for higher-plant structure. Scale bar, 50 μm.
Enzymatic degradation. In nature, various cellulosic microorganisms produce enzymes that function synergistically and associate with the microorganism [such as the cellulosome (27, 28)] or act independently (such as most fungal and many bacterial cellulases) (29). Although it is not fully known how many enzymes are involved in cell-wall deconstruction, three general categories of enzymes are considered necessary to hydrolyze native cell-wall materials: cellulases, hemicellulases, and the accessory enzymes, which include hemicellulose debranching, phenolic acid esterase, and possibly lignin degrading and modifying enzymes (29). Once the hemicellulose barrier associated with cell-wall microfibrils has been compromised by chemical pre-treatments, cellulase enzymes can be used to hydrolyze the crystalline cellulose cores of these structures.

Crystalline cellulose is hydrolyzed by the synergistic action of endo-acting (with respect to the cellulose chain) enzymes known as endoglucanases, and exo-acting enzymes, known as exoglucanases. The endoglucanases locate surface sites at locations, probably found at random, along the cellulodextrin and insert a water molecule in the β-(1,4) bond, creating a new reducing and non-reducing chain end pair. β-1,4-glucosidases (cellobiases) act to hydrolyze cellobiose, the product of cellulase action, and thus relieve the system from end-product inhibition. Cellulases and other glycosyl hydrolases (30) are known to proceed through a two-step, Kosshland-type mechanism that leaves the terminal C1 carbon hydroxyl in the β configuration (retention of stereochemistry) or a concerted reaction mechanism that leaves the terminal hydroxyl in the α configuration (inversion of stereochemistry) (31). Water molecules could invade the space under the non-reducing chain end and thus prevent it from reannealing into the cellulose crystal. The removal of cellulodextrins from the microfibril core is thought to occur at these new chain ends and this process, considered to be the rate-limiting step in cellulase action, is accomplished by exoglucanases also known as the “processive” cellulases.

Overcoming Biomass Recalcitrance

Current biomass-conversion technologies are primarily developed empirically, based on limited understanding of the biological and chemical properties of biomass. Recent studies of plant development, carbohydrate chemistry, and the ultra-structure of cell walls continue to provide new insights into biomass conversion. To reach the goal of producing cost-competitive biofuels from biomass, these new findings from plant science and carbohydrate chemistry must be translated and integrated into the conversion processes. Further studies will undoubtedly rely on, for example, the development of new techniques for imaging and characterizing the chemical topography of the cell wall at the nanometer scale. The future of research aimed at overcoming biomass recalcitrance will primarily focus on the coengineering of new cell walls to be degraded by newly engineered enzymes designed for this role.

Plants designed for deconstruction. Recent studies of plant cell-wall biosynthesis are beginning to provide new understanding about the structure and chemistry of the plant cell wall (10). Although much of our knowledge is anecdotal, the cell walls of higher plants are viewed as an assembly of biopolymers, in some ways mimicking a “liquid crystal,” synthesized by pathways with as-yet undetermined controls (10, 32). For example, cellulose is synthesized and assembled on plasma membrane, whereas hemicelluloses are synthesized in the Golgi apparatus.

Despite our lack of detailed knowledge regarding cell-wall structure, research during the past 20 years, largely reductionist in approach, has led to a body of information regarding treatments of the cell wall that are effective for enhancing enzyme action. Studies have shown that systematic removal of hemicelluloses, by either acidic or enzymatic processes, results in the marked reduction in cellulase loadings required to convert cellulose.
neous saccharification and fermentation (SSF) scheme used by Gauss in a process developed for Gulf Oil (37). More recently, thoughts about combining SSF with enzyme production have resulted in new approaches to CBP, which could either require engineering an ethanologen (such as *Saccharomyces cerevisiae*) to be cellulosytic or engineering a cellulase producer (such as *Clostridium thermocellum*) to be ethanologenic. For the *c. thermocellum* case, the bioenergetic benefits specific to growth on cellulose result from the efficiency of oligosaccharide uptake combined with intracellular phosphorylative cleavage of β-glucoside bonds, another pathway not known in fungi. Scientists believe that these benefits exceed the bioenergetic cost of cellulase synthesis, supporting the feasibility of anaerobic processing of cellulosic biomass without added saccharolytic enzymes (38).

**Outlook for an Advanced Biorefinery Industry**

Ultimately, biomass conversion processes are attractive because they are in practice today and extension to future scenarios is easy for the public to envision. Although developing the technology for cost-effective motor fuel production by 2030 is challenging, the advances in scientific understanding necessary to achieve this goal appear realizable. The general path forward along the biotechnical fuels production route will generally rely on consolidation of processing steps, both in the engineering and biological sense. Microbial cells will be expected to conduct multiple conversion reactions with high efficiency and to remain robust to process conditions. These improvements require deeper understanding of cellular and metabolic processes. New generations of hydrolytic enzymes will function near their theoretical limits, and energy plants will be modified to serve as improved substrates for these new generation enzymes. Indeed, it is entirely possible that the next generation of energy plants will harbor the genes encoding enzymes necessary for self-deconstruction, activated before harvest or at the normal completion of the growth cycle.

**References and Notes**

17. Pretreatment severity is defined as the combined effect of temperature, acidity, and duration of treatment. Hot-water pretreatments, which actually use acetic liberated from cell-wall hemicellulose, represent the lowest degree of severity.
25. Y. Sun et al., *Bioresource Technol.,* in press.
30. CAZY (Carbohydrate Active Enzymes) is a database for the families of structurally related catalytic and carbohydrate-binding modules of enzymes that degrade, modify, or create glycosidic bonds. The current database is available online (www.cazy.org/CAZY).
42. We acknowledge the support of the U.S. Department of Energy Office of the Biomass Program. We also thank W. Grett for providing the picture shown in Fig. 1A, T. Vinzant for providing the image shown in Fig. 1B, and D. Seely at Pixel Kitchen for helping the image shown in Fig. 3.