Improved Cellulases for Bioethanol Production

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Abstract
Ethanol is used today as an alternative fuel, a fuel extender, an oxygenate, and an octane enhancer. From just over 10 million gallons of production in 1979, the U.S. fuel ethanol industry has grown to more than 1.8 billion gallons of annual production capacity. These commercial operations use technology that converts corn starch to sugars, which are then fermented to ethanol. Throughout this time, the U.S. Department of Energy has invested in R&D technology that will allow the fuel ethanol industry to expand production using lignocellulosic feedstocks. However, unlike starch, cellulose is highly resistant to enzymatic degradation. It is now clear that cutting-edge biochemical technologies must be used to reduce the cost of cellulase activity delivered to the bioethanol process. We estimate that cellulase usage would contribute around $0.40 to the cost of making one gallon of ethanol using currently available technology. These costs must be reduced ten-fold by 2015. Technically, this objective requires a 10-fold increase in enzyme specific activity or production efficiency or some combination thereof.

Introduction
For well over one hundred years, researchers around the world have pursued ways to make ethanol from biomass such as wood, grasses and waste materials. To distinguish it from ethanol made from starch and sugars in traditional agricultural crops, we refer to ethanol made from biomass as “bioethanol.” The effort to develop bioethanol technology gained significant momentum in the late 1970s as a result of the energy crises that occurred in that decade. This article briefly reviews the broader history of bioethanol technology development. With this as a background, we focus our attention on the remarkable advances in cellulase enzyme research and development, and the role that enzymes will play in the future of bioethanol.

National security still drives much of the interest in bioethanol, since petroleum is a limited resource controlled mostly by other nations. At some point, we must be prepared to deal with future shortages of petroleum. Not everyone shares this view of the future, or sees it as a reason for concern. The American Petroleum Institute does not see foreign imports as a matter of national security [1]. Others have argued that the prediction of increasing Middle East oil dependence worldwide is wrong [2]. The International Energy Agency recently announced that it sees annual petroleum supplies reaching a peak some time between 2010 and 2020. The IEA is one more voice in a growing chorus of concern about the imminent danger of shrinking oil supplies [3]. While many disagree with this pessimistic prediction, concern about our foreign oil addiction is widely held by a broad range of political and commercial perspectives [4]. This concern is appropriate given the almost exclusive dependence our transportation sector has on petroleum.

The Concept of Converting Biomass To Ethanol. At the risk of oversimplifying the Biofuels story, we prefer to view ethanol technology in terms of only four basic steps (see Figure 2). Production of biomass results in the fixing of atmospheric carbon dioxide into organic carbon. Conversion of this biomass to a useable fermentation feedstock (typically some form of sugar) can be achieved using a variety of different process technologies. These processes for sugar production constitute the critical differences among all of the ethanol technology options. Using biocatalysts (microorganisms including yeast and bacteria) to ferment the sugars released from biomass to produce ethanol in a relatively dilute aqueous solution is probably the oldest form of biotechnology developed by humankind. This dilute solution can be processed to yield ethanol that meets fuel-grade specifications. Finally, the economics of biomass utilization demands that any unfermented residual material left over after ethanol production must be used, as well.

Figure 1. Potential biomass feedstock supply @ less than $50 bone dry ton.

Figure 2. General scheme for converting biomass to ethanol.

Keeping this simplistic description of biofuels technology in mind will help provide a clearer, more logical, framework for the various technology development strategies we describe in this plan. In essence, all of the strategies we propose for the program fit into the second or third step shown in Figure 2. Everything that has been done or could be done to improve production of bioethanol from biomass can be categorized in terms of sugar production or fermentation—with one exception. We are currently evaluating a longer term option for ethanol production that involves replacing sugars as the fermentation feedstock with simple single carbon intermediates that can be converted to ethanol. This option, in early stages of development, represents a rather small fraction of our overall effort.

Five Technology Platforms
As indicated earlier, the technology pathways pursued in the Biofuels Program differ primarily in the approach used to produce sugars from biomass (step 2 in Figure 2). Regarding sugar recovery, releasing the sugars from the biopolymers in plant matter involves hydrolysis of the linkages between the sugar moieties. Hydrolysis is a simple chemical reaction in which a water molecule is added across the glycosidic linkages in order to break the bonds. The discovery of sugar production by acid hydrolysis of cellulose dates back to 1819 [5, 6]. By 1898, a German researcher had already attempted to use this chemistry in a commercial process for producing sugars from wood. This early process included fermentation of the sugars to ethanol [7]. In the one hundred years since then, researchers have continued to pursue different approaches to achieving high yields of fermentable sugars from the acid hydrolysis of biomass. It is easy to lose this historical perspective on acid hydrolysis technologies. In identifying priorities for the DOE Biofuels Program, we need to consider the extent of previous research as we assess the directions for R&D that hold the greatest potential for advancement. The Program supports development of five technology platforms for bioethanol production. The first three are based on different approaches to producing sugars. The fourth is a radically different approach to ethanol production.
involving thermal processing of biomass to gaseous hydrogen and carbon monoxide, followed by gas-phase fermentation to ethanol. Finally, we are working on a fifth technology platform that uses a fluidized-bed reactor design as the basis for the fermentation step. The three sugar routes include:

- Low Temperature, Concentrated Acid Hydrolysis
- High Temperature, Dilute Acid Hydrolysis
- Enzymatic Hydrolysis.

Figure 3. Potential for cost savings ($/gal EtOH)

The two acid hydrolysis technology platforms have the longest history of development, while the use of enzymes to produce sugars from biomass is, in the scheme of things, a relatively recent concept and the most promising. Given the long history of development for the acid hydrolysis technologies, it is not surprising that these technologies have less future potential for cost savings, as shown in Figure 3. Enzymes, the relative newcomer in bioethanol technology, offer two to three times the potential for future savings due to R&D. In DOE’s program, the two acid hydrolysis technologies fill the need for near-term pioneer plant start ups, while the enzyme technology occupies the major part of our efforts in research and development.

Enzymatic Hydrolysis of Cellulose in Biomass

It is now clear that the enzyme known as “cellulase” is really a complex of enzymes that work synergistically to attack native cellulose. In 1950, this complex was crudely pictured as an enzyme known as “C₃”, decrystallizing cellulose, followed by a consortium of hydrolytic enzymes, known as “C₄”; which break down cellulose to glucose [8]. This early concept of cellulase activity has been augmented, modified, and argued about for the past 40 years [9, 10], and the combined action of these enzymes is now described in terms of three major classes of cellulase enzymes:

- Endoglucanases, which act randomly on soluble & insoluble chains.
- Exoglucanases, which include cellobiohydrolases that act processively to liberate cellobiose or glucose from the ends of the cellulose chain.
- β-glucosidases, which liberate D-glucose from cellobiose dimers and exoglucosidases that preferentially hydrolyze soluble cellobextrins.

Although the action of the fungal cellulase system has received extensive and insightful recent review [11, 12] and many models for enzymatic hydrolysis have been proposed [13, 14], this process has eluded definition at the molecular level for several reasons. Sinnott [15] provides the classic example comparing $k_{cat}$ (the enzymatic turnover number) values for *Aspergillus* glucoamylase acting on α-glucosyl fluoride and *Trichoderma reesei* cellulohydrolase (CBHII) acting on β-glucosyl fluoride, which are 730 s⁻¹ and 4 x 10⁶ s⁻¹, respectively. Why does the cellulase display catalytic efficiencies on this simple substrate that are less than 2 orders of magnitude that of the intrinsically inefficient glucoamylase? One answer may be that some cellulases use the energy from hydrolysis of the glycosyl bond for functions related to their action on cellulose and not to enhance hydrolysis itself. Thus, cellulases acting on crystalline cellulose may not be under selection pressure to improve catalysis alone.

Opportunities for Making Better Cellulases

To achieve total competitiveness, enzyme costs must be reduced to less than $0.07/gallon ethanol or its equivalent for other products, requiring a ten-fold increase in specific activity or production efficiency or some combination thereof [16]. NREL’s strategy is to reduce the complexity of the cellulase system to a few critical enzymes and, perhaps more importantly, to engineer those enzymes to act more efficiently on pretreated biomass.

Native plant matter requires a suite of glycosyl hydrodolases aided by chemical and/or mechanical conditioning for depolymerization. The well-studied *T. reesei* system, for example, produces at least 14 enzymes likely involved in the synergistic hydrolysis of untreated plant biomass [17]. Efforts to reduce the complexity of cellulase mixtures for the hydrolysis of pretreated biomass have been somewhat successful, in that ternary mixtures (90/8/2) of *T. reesei* CBH II/ Acidothermus cellulolyticus FUS/ *Aspergillus niger* β-D-glucosidase were shown to hydrolyze cellulose in yellow poplar to an extent of 120 h approaching that of a comparable protein loading of the *T. reesei* complex [18]. Initial efforts to improve the performance of this ternary cellulase system have utilized site-directed-mutagenesis (SDM) and show that modifications to the active site of the EI endoglucanase increase the end-point saccharification of pretreated PFP by 12% relative to wild type EI (tested as a ternary system) [19]. Site-directed-mutagenesis is considered an informational approach to protein engineering and relies on high-resolution crystallographic structures of target proteins and some stratagem for specific amino acid changes [20]. Resurgence in SDM technology has appeared following the recent advent of computational methods for identifying these site-specific changes for a variety of protein engineering objectives [21]. Encouraging results from early SDM work at NREL certainly demonstrate that classical protein engineering principles can be successfully applied to cellulases; however, rapid advancement of β-glucosidase to the performance target of a 10-fold increase in specific activity requires efficient access to more protein sequence space than is possible with directed PCR mutation alone. We are thus supporting the full integration of SDM with non-informational mutagenesis techniques (referred to generally as “directed evolution”). Directed evolution, in conjunction with high-throughput screening, allows testing of statistically meaningful variations in protein conformation [22]. Directed evolution technology has undergone significant refinement from initial error-prone PCR methodology and now includes Gene Shuffling [23], site-saturation mutagenesis, staggered extension process (StEP) [24], and DNA synthesis/reassembly [25].

Conclusions

The application of cellulase to the breakdown of cellulolic biomass into sugars for fermentation to ethanol and other commodity products would provide tremendous environmental, economic, and strategic benefits. However, the key challenge is to make biomass depolymerization more rapid and less costly, but the question remains. How can cellulase specific activities be increased by orders of magnitude over the best systems known today? Overall, significant reduction in cellulase cost and thus, “leap-forward” improvements in cellulase specific activity against pretreated biomass, can only arise from the application of an integrated site-directed-mutagenesis and directed evolution program. The DOE Biofuels Program is currently funding research, which seeks to improve both cellulase productivity and enzymatic specific activity using modern biotechnology.
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References


