

Cellulase for commodity products from cellulosic biomass

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A vital objective for second millennium biotechnology will be the enzymatic conversion of renewable cellulosic biomass to inexpensive fermentable sugars; new and more efficient fermentation processes will convert this biological 'currency' to a variety of commodity products. Although early strides will be made using process development and engineering disciplines, mid-term and longer advances must rely heavily on insight gained through protein and metabolic engineering technologies. These challenging goals can be met most effectively by the full integration of academic, federal, and industrial efforts in teams that develop and apply new fundamental knowledge to key cost drivers.

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Abbreviations

CBH	cellobiohydrolase
FPU	filter paper units
NREL	National Renewable Energy Laboratory
SDM	site-directed mutagenesis
SSCF	simultaneous saccharification and co-fermentation

Introduction

Plant biomass, which represents the cellulosic materials that compose the cell walls of all higher plants, is the most abundant source of fermentable carbohydrates in the world. When biologically converted to fuels, such as ethanol and various other low-value high-volume commodity products, this vast resource can provide environmental, economic, and strategic benefits on a large scale, with some, such as reduced release of greenhouse gases, unparalleled by any other sustainable resource [1–3]. As an example, the cost of biomass ethanol production has been reduced dramatically over the past two decades, to the point where the fuel is now competitive for blending with gasoline to reduce greenhouse gas emissions, enhance octane, extend the gasoline supply, and promote more complete combustion [4,5], but further processing cost reduction opportunities have also been identified that would make it competitive as a pure fuel without subsidies [6]. Cellulase enzymes provide a key opportunity for achieving the tremendous benefits of biomass utilization in the long term because of the high glucose yields possible and the opportunity to apply the modern tools of biotechnology to reduce costs. In this review, we present

an estimate of the current cost of making cellulase to produce ethanol and other commodity products from cellulosic biomass on a large scale, review recent developments in cellulase technology in this context, and suggest opportunities to improve cellulases further.

Cellulase production costs

Overall process description

A new analysis of enzymatic hydrolysis of cellulose to glucose for fermentation to ethanol by the National Renewable Energy Laboratory (NREL) provides a useful benchmark of the estimated cost of cellulase enzymes in a large commodity plant [7]. As pictured in Figure 1, the process revolves around dilute acid hydrolysis of hemicellulose in hardwood chips followed by enzymatic hydrolysis of the exposed cellulose to release glucose at high yield. The latter operation is performed in the same vessel used to ferment the sugars from both cellulose and hemicellulose to ethanol to reduce inhibition of enzymes by the sugars released. This combined process step, known as simultaneous saccharification and co-fermentation (SSCF), is carried out in a series of continuous anaerobic fermentors. Before fermentation, the hydrolyzate is conditioned to remove compounds formed (e.g. furfural) or released (e.g. acetic acid) during the hemicellulose pretreatment step that are inhibitory to the fermenting organism and cellulase. After a seven-day residence time, the fermentation broth is transferred to a distillation and dehydration unit for recovery of ethanol, while the residual solids are burned to provide heat and electricity for the process; excess electricity is sold to the grid. A portion of the water from the beer column (distillation) bottoms is recycled, and the methane released during cleanup of the water before recycling or discharge is burned with the residual solids.

Cellulase production

Cellulase is produced by a microorganism fed on a small portion (3–5%) of the conditioned hydrolyzate slurry in eleven 1,000,000 L (264,000 gallon) batch aerobic bioreactors. At any one time, eight bioreactors are in operation, another is being drained, one is being filled, and one is being cleaned and sterilized. Whole corn steep liquor and other trace nutrients are also added to the bioreactors and ammonia is used to control pH and provide fixed nitrogen to the organisms. Three parallel seed trains, of three batch fermentors each, produce the 5% inocula for the process using the carbon source and nutrients described above. All main and inoculum vessels are aerated at 0.577 VVM (volume of gas/volume of reactor/min) without oxygen supplementation, and the temperature is controlled by chilled water running through internal coils. Corn oil prevents excessive foaming in the fermentors.

Cellulase yields above 150 filter paper units (FPU)/g cellulose and productivities above 55 FPU/L-hr were achieved

Table 1**Cellulase production parameters.**

Cellulase requirement for SSCF	15 FPU/g cellulose
Yield	200 FPU/(g cellulose and xylose)
Productivity	75 FPU/L-hr
Initial cellulose concentration	4%

in experiments run in 5 L fermentors using Solka Floc as the substrate [8]. Although producing cellulase on pretreated biomass instead of Solka floc will be more difficult because of the presence of lignin and various inhibitors, it is believed that the yields and productivities can be increased somewhat through improving dissolved oxygen control, and slightly higher performance parameters were used accordingly in this study, as listed in Table 1. The initial substrate concentration is lower because of potential inhibitors in pretreated biomass, and the tabulated entries are based on a specific cellulase activity of 600 FPU/g protein, a yield of 0.33 g protein/(g initial cellulose and xylose), and a productivity of 0.125 g protein/L-hr.

Cost estimate

The purpose of the NREL analysis was to guide the definition of research targets. The cost analysis includes capital and operating costs and is based on mature technology for an n^{th} generation plant using 2000 dry tons of feedstock per day with technology viewed as being currently viable. The ASPEN process simulator was used to determine the flow of the feed to cellulase production and the number of cellulase vessels needed to provide the required amount of

Table 2**Submerged culture cellulase enzyme cost.**

	Feedstock cost		
	\$15/dry ton	\$25/dry ton	\$40/dry ton
\$/gal ethanol	\$0.310	\$0.318	\$0.330
\$/100,000 FPU	\$0.415	\$0.426	\$0.442
\$/kg enzyme	\$2.493	\$2.556	\$2.655

enzyme to SSCF. Operating costs were estimated from previously defined nutrient requirements [4,5] and appropriate labor, utility, and other costs. Equipment costs were estimated from vendor quotes and IcarusTM software, and all capital costs, including working capital, were annualized based on a net present value approach.

The initial results showed that pressure vessels increased the cost of cellulase production over atmospheric vessels. These results also showed that a height to diameter ratio of 2 and an agitation power input of 500 W/m³ (2.54 hp/[1000 gallon]) would be most cost-effective with atmospheric vessels and sparged air. To achieve an oxygen transfer rate (OTR) greater than 80 mmol/L-hr, the air flow was set at 0.577 VVM. The OTR calculated for that set of conditions with the final model is 81 mmol/L-hr.

The enzyme cost per gallon of ethanol in Table 2 was calculated by determining the total cost of ethanol production to achieve a given SSCF performance and then subtracting the cost to achieve the same performance

Figure 1

Process flow diagram of bioethanol production via dilute acid pretreatment followed by SSCF.

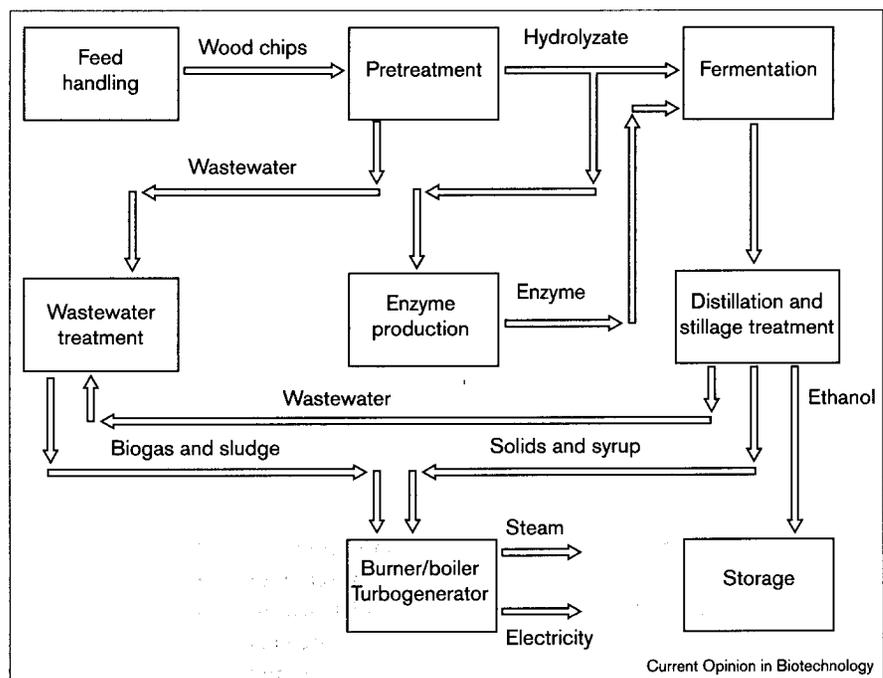
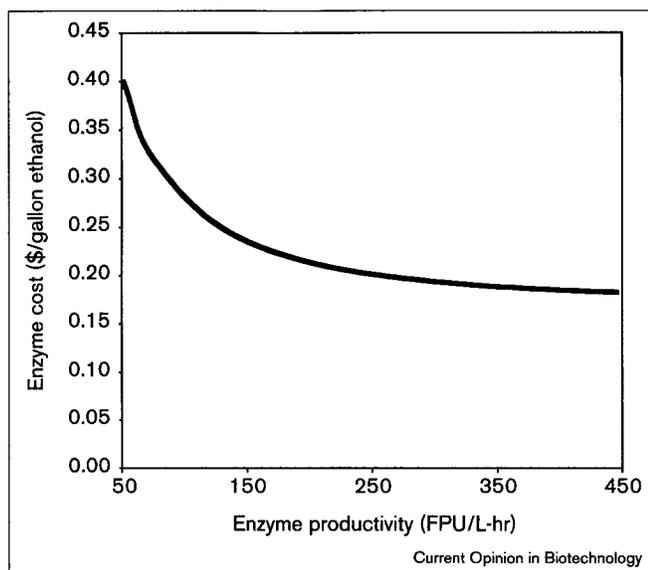
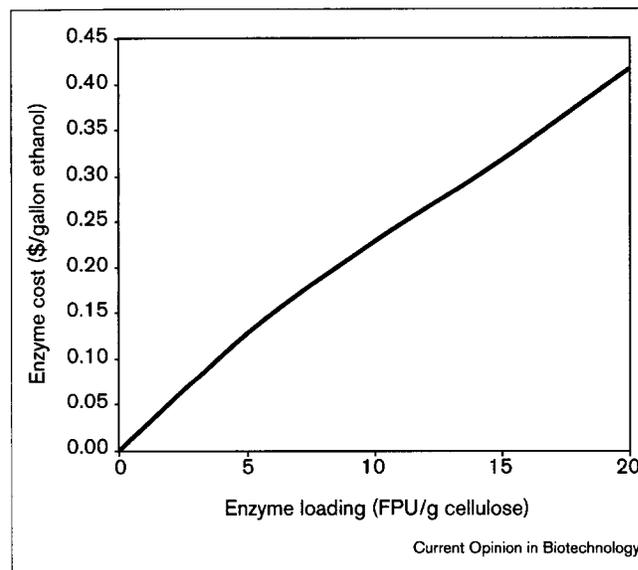


Figure 2



Change in cellulase cost in terms of the quantity of ethanol produced with increasing cellulase productivity measured in filter paper units (FPU) per liter of cellulase production capacity per hour of production time.

Figure 3



Change in cellulase costs with the quantity of cellulase required to achieve the targeted SSCF hydrolysis performance expressed in FPU/g of cellulose in the pretreated solid substrate.

neglecting the enzyme production step. The cost per 100,000 FPU was based on the required enzyme flow in the model and converted to a mass protein basis for a specific activity of 600 FPU/g protein. The primary cost factors for a feedstock case costing \$25/dry ton are capital (\$0.126/gallon of ethanol) and electricity (\$0.107/gallon of ethanol). The remaining \$0.085/gallon of ethanol is made up of fixed costs, feedstock, corn steep liquor, and other raw materials.

Sensitivity studies were performed to determine the impact of improvements in technology on the cost of ethanol production. Increasing cellulase productivity from ~50 to 200 FPU/L-hr, as shown in Figure 2, dropped the cost of enzyme rapidly to about \$0.20/gallon of ethanol, but further increases in productivity had a minor impact on enzyme costs due to the continued need for the extra fermentors for batch cycling and the power law dependence of equipment costs on scale of operation. In Figure 3, we see a nearly direct relationship between cellulase cost and enzyme loading indicating that increases in enzyme activity or improvements in cellulose digestibility through improved biomass pretreatment have major impacts on cellulase costs by reducing cellulase use. Figure 4, however, shows that although increasing the specific activity of cellulase dramatically reduces the cost of enzyme initially, the impact tails off due to the non-linear change in cellulase production costs with scale of operation. Although less expensive feedstocks would have a significant impact on overall ethanol costs, the cost of cellulase only drops by about \$0.08/gallon of ethanol when the feedstock costs drop from a positive \$50/ton to

a negative \$50/ton (due to a tipping fee), showing that capital, aeration, and other costs dominate. Overall, these results suggest advantages to increasing the plant capacity with improvements in cellulase technology to maintain economies of scale in cellulase production and that alternative fermentor configurations should also be considered for improved cellulase technology to minimize nonproductive fermentor volume and costs. Other process configurations may also be needed to ultimately realize low processing costs [6].

Cellulase for biomass conversion to commodity products

The structure of biomass

The predominant polysaccharide in the primary plant cell wall is cellulose, the second most abundant is hemicellulose, and the third is pectin [9,10]. The secondary cell wall, produced after the cell has stopped growing, also contains polysaccharides and is strengthened through polymeric lignin covalently crosslinked to hemicellulose [11]. Cellulose is a crystalline matrix of linear β -(1-4)-D-glucan chains, whereas hemicelluloses include a variety of compounds, such as xylans, xyloglucans, arabinoxylans, and mannans, in complex branched structures with a spectrum of substituents, such as acetyl esters, along its backbone. Hemicelluloses usually hydrogen bond to cellulose, as well as to other hemicelluloses, which helps stabilize the cell-wall matrix and renders the cell wall insoluble in water.

Hemicellulose removal by dilute acid treatment is a classical means of rendering biomass more amenable to cellulase

action [12]. In a hallmark study, Kong *et al.* [13] also showed that biomass with reduced acetylation (produced by treatment with KOH) responded significantly more favorably to cellulase action than native biomass. Although still controversial, there is some indication that biomass with reduced lignin content is more readily hydrolyzed by cellulase action [14,15]. The structural and reactive chemical features of the substrate (primarily defined as acetyl and lignin contents) can be pictured as controlling the accessibility of enzyme to cellulose; the degree of cellulose crystallinity can be visualized as controlling the hydrolytic rate [15].

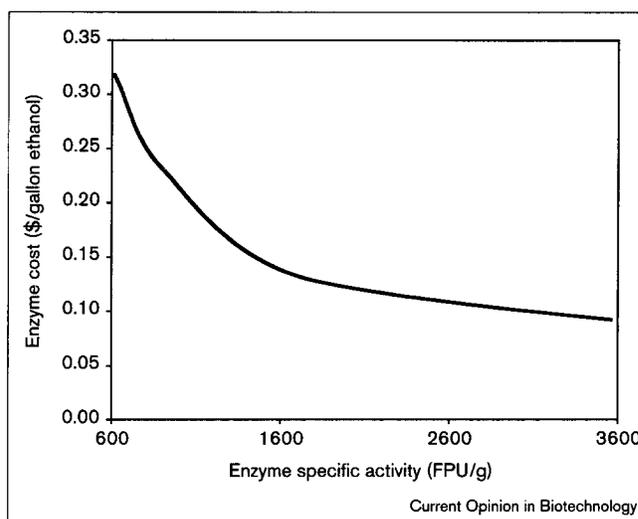
The genesis of cellulase biochemistry

As early as 1886, scientists were aware that enzyme (from fungal extracts) degraded plant cell-wall polysaccharides [16], and Newcombe [17] showed conclusively in 1899 that the cellulose-degrading enzyme (named cytase or cytohydrolyst) in barley malt was distinct from starch-degrading enzymes. From our review of the literature, the first reference to 'cellulases' as enzymes that degrade cellulose was made by Pringsheim in 1912 [18]. During World War II, complex precipitation protocols for segregating blood plasma proteins came into vogue, but it was not until the report of a crosslinked dextran gel (Sephadex) in 1951 that the efficient separation of native enzymes became possible. In many ways, the genesis of cellulase biochemistry dates only four decades, to the time when protein purification became widely available.

It soon became 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 'C₁' decrystallizing cellulose, followed by a consortium of hydrolytic enzymes, known as 'C_x', which break down cellulose to glucose [19]. This early concept of cellulase activity has been augmented, modified, and argued about for the past 40 years [20,21], and the combined action of these enzymes is now described in terms of three major classes of cellulase enzymes: firstly, endoglucanases, which act randomly on soluble and insoluble cellulose chains; secondly, exoglucanases, which include cellobiohydrolases (CBHs) that act processively to preferentially liberate cellobiose (and in some cases glucose) from the ends of the cellulose chain; and finally, β -glucosidases, which liberate D-glucose from cellobiose dimers and exoglucosidases, which preferentially hydrolyze soluble cellodextrins of intermediate chain length.

Although the action of the fungal cellulase system has received extensive and insightful recent review [22,23,24,25] and many models for enzymatic hydrolysis have been proposed [26–30], this process has eluded definition at the molecular level for several reasons. Sinnott [23] provides the classic example comparing k_{cat} (the enzymatic turnover number) values for *Aspergillus* glucoamylase acting on α -glucosyl fluoride and *Trichoderma reesei* CBH II acting on β -cellobiosyl fluoride, which are 730 s^{-1} and 4 s^{-1} ,

Figure 4



Sensitivity of cellulase cost to the specific activity of cellulase enzyme expressed in FPU/g of cellulase protein.

respectively. Why does the cellulase display catalytic efficiencies on this simple substrate that are less than two 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. This argument is further supported by the observation that other non-cellulases (those acting on soluble substrates) have benefited from laboratory-directed [23,31] and, undoubtedly, natural evolution.

The enzymatic depolymerization of insoluble cellulose, whether by exo- or endo-glucanases, necessitates the removal of a cellodextrin chain from the surface of the microcrystallite (at considerable energetic expense), as well as subsequent hydrolysis of this chain. The question of enzymatic decrystallization is thus the second key issue to be resolved before the cellulase system can be fully understood. Some preliminary evidence shows that at least *T. reesei* CBH II does link cellulose crystallite disruption to catalysis [23]; however, the interaction of portions of the cellulase macromolecule with the cellulose surface in ways that direct and enhance decrystallization are already intuitively pleasing. Although direct evidence is lacking, site-directed modifications to the cellulose binding domain of CBH I that could be expected to alter hydrophobic stacking interactions between naturally occurring amino acid residues on its planar interactive surface and glucosyl units of cellulose, have shown a decrease in hydrolytic efficiency on cellulose [32]. Understanding the thermodynamic basis for enzymatic decrystallization, especially the role played by the distribution of water molecules during this process, is thus a key need for the field.

The third impediment to a complete picture of cellulase action is our limited understanding of the changes that occur to the surface of cellulose as enzyme action proceeds [25,33,34**]. Recent attempts by Sild *et al.* [35] to translate kinetic data, aided by Monte Carlo simulation, to models of cellulase action have yielded a 'cellulose erosion' model based on non-productively bound enzymes and changes to the surface topography of cellulose sterically hindering cellobiohydrolase processivity. (Lignin in biomass may also be stimulating non-productive binding of enzymes.) The probable importance of microenvironmental changes in the surface structure, or topography, of cellulose as a function of enzyme action is becoming more widely recognized. As a result of their spatial requirement for action on an insoluble substrate dictated by their molecular size, directional processive enzymes, such as CBH I, create a modified cellulose surface that can no longer accommodate fresh attack by the same enzyme type [35]. Understanding the roles played by various enzyme components on the dynamically eroded cellulose surface should explain the classic 'cellulase slowdown' phenomenon, which becomes evident about midway toward total saccharification and is severe at about 80% hydrolysis.

Opportunities for making better cellulases

To achieve total competitiveness, enzyme costs must be reduced to less than \$0.07/gallon of ethanol or its equivalent for other products, requiring a 10-fold increase in specific activity or production efficiency, or some combination thereof [7]. 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 hydrolases aided by chemical and/or mechanical conditioning for depolymerization. The well-studied *T. reesei* system, for example, produces at least 14 enzymes probably involved in the synergistic hydrolysis of untreated plant biomass [36]. Efforts to reduce the complexity of cellulase mixtures for the hydrolysis of pretreated biomass have been somewhat successful, in that ternary mixtures (90:9:1) of *T. reesei* CBH I/*Acidothermus cellulolyticus* EI/*Aspergillus niger* β -D-glucosidase were shown to hydrolyze cellulose in yellow poplar to the same extent of conversion in 120 hours as a comparable protein loading of the *T. reesei* complex [37]. 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 yellow poplar by 12% relative to wild type EI (tested as a ternary system) [37]. SDM is considered to be an informational approach to protein engineering and relies on high-resolution crystallographic structures of target proteins and some strategy for specific amino acid changes [38,39**]. A 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 [40]. Encouraging results from early SDM work at the NREL certainly demonstrates that classical protein engineering principles can be successfully applied to cellulases; however, rapid advancement 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 generically as 'directed evolution'). Directed evolution, in conjunction with high-throughput screening, allows testing of statistically meaningful variations in protein conformation [41]. Directed evolution technology has undergone significant refinement from initial error-prone PCR methodology and now includes gene shuffling [42,43], site-saturation mutagenesis, and staggered extension process (StEP) technology [44]. In our opinion, the primary challenge in the application of directed evolution technology to cellulase improvement lies almost entirely in adaptation of robotic screening methods to accurately select transformed host cells (clones) producing enzymes displaying enhanced performance on microcrystalline cellulose.

Conclusions

The application of cellulase to the breakdown of cellulosic biomass into sugars for fermentation to ethanol and other commodity products would provide tremendous environmental, economic, and strategic benefits. The key challenge, is to make biomass depolymerization more rapid and less costly, but the key question remains: how can cellulase specific activities, rates and glucose yields be increased by an order of magnitude over the best systems known today? Cellulase performance could be improved in the near term by increasing the catalytic rate constants of cellulase action (i.e. to achieve greater extents of enzymatic conversion with the same protein loading) and by increasing the temperature at which the engineered cellulase system operates [45*]. Longer term objectives include modifying cellulases to decrystallize cellulose more effectively and to act more efficiently on the biomass surface (i.e. become less likely to enter non-productive binding or dead-end binding conformations). Furthermore, improvements in pretreatment technology could increase the accessibility of cellulose to enzymes, create more chain ends, and/or reduce crystallinity [15]. 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 SDM and directed evolution program. The most effective cellulase system documented today displays a specific activity of 600 FPU/g protein and it is possible that pressures for natural selection for systems with 3–10 times this value have not existed. Certainly, no organism in nature has been exposed to biomass similar in composition to that resulting from dilute acid pretreatment at 180°C to 200°C. To be effective, such a program must balance the requirement to reach enzymatic performance goals in a timely manner with a

sufficient understanding of cellulase mechanism and biochemistry to ask the key experimental questions.

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