Improving Cellulose Hydrolysis with New Cellulase Compositions

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Abstract

Cellulose is the most abundant organic molecule on the earth, and a renewable and seemingly inexhaustible feedstock for the production of fuels and chemicals. Evolved as a structural polymer, cellulose is remarkably recalcitrant to breakdown into its monomeric form, glucose. Bacteria and fungi have evolved complex enzymatic systems enabling their growth on plant material rich in cellulose, but these organisms typically require weeks, months, or even years to decompose a fallen log or a tilled corn stalk. For chemical or fuel production from these same materials, industry requires affordable chemical or enzymatic systems that can do the job in hours or in days. Under a research subcontract from the National Renewable Energy Lab (NREL), with funds from the U.S. Department of Energy, Novozymes has conducted a four year project focused on improving the performance and reducing the cost of fungal cellulases for the conversion of dilute acid pretreated corn stover to fermentable sugars. One aspect of this project has been the identification of novel proteins that, when added to the cellulases produced by a widely used soft rot fungus *(Trichoderma reesei)*, increase the specific performance of the enzyme mixture. Applying state of the art tools of biotechnology to identify, clone, and express these boosting proteins, we have successfully transferred a number of genes from other organisms to *T. reesei*, creating a super-cellulase producing fungus capable of meeting industrial needs.

Introduction

Increasing concerns over the geopolitical, environmental, and economic cost of utilizing finite petroleum resources for the production of fuels and chemicals have provoked significant governmental policy changes supporting research and development of means for the economic utilization of biomass as a source of biobased products. Moving from petroleum towards renewable carbohydrate-based economy has the potential to decentralize energy and chemical feedstock production, while at the same time spurring rural economic development and dramatically reducing greenhouse gas emissions. The primary barrier to realizing these benefits is economic: petroleum remains a relatively inexpensive feedstock with a well-established, fully depreciated system for extraction, purification, and delivery. Over the next 50 years, the cost of petroleum is expected to rise precipitously as increasing demand from developing countries collides with dwindling supplies of easily-extractable petroleum. In addition, global concern over the environmental impacts of petroleum use have left the world searching for alternatives. Biomass, in the form of crop residues like straw and corn stover, forest residues, municipal solid wastes, and dedicated energy crops like switchgrass and yellow poplar, has the potential to offer a geographically widespread supply of sugars that can be converted to fuels and chemicals in so-called biorefineries.

The biorefinery concept is based on the large scale conversion of sugars from sugar-rich crops or biomass using the large-scale fermentation of microorganisms. While sugarcane, sugar beets, and grains can supply the necessary sugars, such use of these crops will ultimately compete with food uses. The long-term success of the biorefinery concept rests on our capability to efficiently and cheaply convert biomass to fermentable sugars. In 2001, the US Department of Energy recognized the importance of recruiting technology leaders in

industry to focus R & D on improving the enzymatic conversion of biomass carbohydrates into sugars.

Novozymes, the world leader in industrial biotechnology, was awarded subcontracts through the National Renewable Energy Laboratory (NREL), with total funding of 17.8 million USD over four years. Corn stover, the leaves and stalk residue from corn crops, was selected as the single most abundant and accessible feedstock in the US and a reasonable starting point for the contract work. The project was a collaboration with NREL, who contributed expertise in biomass pretreatment and enzyme evaluation. The project yielded a highly efficient enzyme system for the saccharification of lignocellulosic biomass. Biotechnological and pretreatment improvements resulted in a more than 30-fold reduction in the cost required for biological breakdown of cellulose to sugars for the world's developing biorefineries. Herein we provide an overview of the technological advances that have led to the significant reduction in cellulase enzyme cost. Prior to this research initiative, the cost of producing a kilogram of fermentable sugar from corn stover was dominated by the high cost of the enzymes required for cellulose hydrolysis. Novozymes and NREL have effectively removed enzyme cost as the major economic barrier to realizing the biorefinery.

Enzymatic hydrolysis of cellulose

Cellulases have been the target of active research for over five decades, and are an important class of industrial enzymes for various industries outside the biorefinery area. The classification of these enzymes into glycosyl hydrolase (GH) families is based on degree of sequence identity, with cellulases falling into the following families: 1, 3, 5-9, 12, 44, 45, 48, 61 and 74 [1]. Based on mechanism, cellulases can be grouped into exo-1,4-β-D-glucanases or cellobiohydrolases (CBHs, EC 3.2.1.91), endo-1,4-β-D-glucanases (EGs, EC 3.2.1.4) and β-glucosidases (βGs, EC 3.2.1.21). Cellulases are often modular, containing a catalytic core, a linker and a carbohydrate-binding module. EGs cleave glycosidic bonds within cellulose microfibrils, acting preferentially at amorphous cellulose regions. EGs fragment cellulose chains to generate reactive ends for CBHs, which act "processively" to degrade cellulose, including crystalline cellulose, from either the reducing (CBH I) or non-reducing (CBH II) ends, to generate mainly cellobiose. These fundamentally different catalytic mechanisms allow different types of cellulases to interact synergistically. At high concentrations, cellobiose inhibits CBH activity. Thus βG, which converts cellobiose into glucose, is often required for optimal cellulose performance in conditions where cellobiose accumulates, as it relieves end-product inhibition.

Cellulases are essential for survival of many organisms, including bacteria, fungi, plants, protozoa, insects and herbivores (through symbiotic microbes). Cellulases used for current industrial applications are mainly fungal in origin, primarily due to efficiencies in fungal enzyme secretion. A renowned degrader of cellulose, *Trichoderma reesei* is one of the best-studied fungal cellulolytic systems [2]. In the presence of available cellulose, *T. reesei* secretes CBH I (~60%), CBH II (~15%), and a number of EGs (~20%), in addition to over 30 other minor proteins. Commercially available *T. reesei* cellulase preparations, such as Novozymes' Celluclast 1.5L and Genencor International's Spezyme, are widely used in various applications. Available cellulase products, developed for detergent, textile and other industries, are too expensive for a viable refinery. The economic considerations of protein production led us to choose Celluclast 1.5L, produced by large scale batch fermentation of *T. reesei*, as a starting point for developing the next generation cellulase for use in a biorefinery

with a biomass feedstock. We focused on improving the activity of the *T. reesei* cellulase system while maintaining its already high protein productivity during fermentation. All studies were performed using acid pretreated corn stover (PCS) as a substrate (~57% cellulose, 28% lignin, 5% hemicellulose and 4% protein; prepared by the National Renewable Energy Laboratory) [3, 4]. Improvements were benchmarked with regard to Celluclast 1.5L supplemented with 1% (v/v) Novozym 188, Novozymes' *Aspergillus niger* βG (~404 IU/mL) product [5].

Cellulase Development for Biomass Conversion

Fundamental biochemical properties of the cellulase enzyme mix were optimized with the goal of improving the total enzyme activity on PCS per gram of total protein. The improvements had direct impact on final cost. A number of different strategies were employed, to increase k_{cat}, and to improve enzyme thermostability, so that an increased reaction temperature could be used to leverage a faster rate of reaction. Enzymatic changes were made to minimize end-product inhibition, and we also considered improvements to alleviate non-specific binding of enzymes, to reduce non-specific enzyme inhibition. Optimizing cellulase component mixtures was important in achieving optimal enzyme synergy throughout the reaction.

Novozymes' technology platform was fully utilized toward improving activity of enzymes and toward reducing enzyme production costs. In the area of enzyme improvement, bioinformatics, proteomics, microarray technology, and directed molecular evolution were employed to probe cellulolytic machineries of *T. reesei* and mine nature's cellulase repertoire, and to improve upon naturally occurring systems. Improvements identified by protein chemistry were adapted into a final enzyme mix by heterologously expressing and secreting proteins at optimal levels in a single fungal host. Fermentation engineering tools, as well as classical and directed fungal molecular biology tools were used to optimize enzyme production and minimize fermentation costs. All the approaches were coordinated in an interdependent, dynamic and productive loop with the ultimate goal of reducing the cellulase cost.

Optimization of the CBH-EG-βG System

Fundamental studies of synergies among cellulase enzymes have identified a "complete" cellulase system as that containing two CBHs with different cellulose chain-end affinity, an EG and a βG. Synergy is achieved as the EG cleaves a cellulose chain to generate reactive ends, allowing access by CBHs which degrade from both the non-reducing and reducing ends. Cellobiose generated by CBH action is hydrolyzed to glucose by βG, relieving inhibition of CBHs, and providing a fermentable sugar. Balancing the ratio of these enzymes could lead to improved cellulose hydrolysis.

βG supplementation. T. reesei secretes at least two enzymes with cellobiase activity at very low levels during normal cellulose-induced growth. We observed that Celluclast hydrolyzed cellulose with improved performance when assayed in a diafiltration-saccharification device which enables continuous removal of small sugars by filtration, compared with a closed vessel. Accumulation of CBH-inhibiting cellobiose in a closed vessel resulted in inhibition of the overall reaction [6]. We supplemented Celluclast with an *Aspergillus oryzae* βG (belonging to GH3). Adding a small amount of the enzyme, present as a few percent of total protein, enabled us to achieve equivalent conversion of cellulose in PCS

with half the enzyme dosage of the unsupplemented Cellulase mix. We found that the optimal βG content, expressed as a percentage of total protein, increased as the substrate loading increased. In general, the economy of biomass conversion is improved as higher substrate loadings are achieved due to reduced capital costs for smaller reaction vessels, decreased water usage, and the higher sugar concentration after saccharification. We optimized *Aspergillus oryzae* βG expression in *T. reesei* for optimal hydrolysis of PCS in saccharification of 13.67% (w/w) PCS, the benchmark conditions for hydrolysis of the model biomass material. Expression of the cellobiase in the *T. reesei* strain eliminated the need to ferment a BG product separately, further reducing enzyme production costs.

Improvement of enzyme thermal properties. Toward improving performance of the classical CBH-EG-βG mix, we focused on engineering and discovery or enzymes with improved thermal stability. Thermally stable enzymes could enable cellulose hydrolysis at elevated temperature, which in theory should result in thermal enhancement of enzymatic rate. We implemented a screen for *T. reesei* CBH I variants with improved rates of hydrolysis at temperatures higher than 60^{o} C, a temperature where the native enzyme is unstable during the long time periods required for cellulose hydrolysis. Random and directed mutagenic approaches were coupled with an automated high throughput screen for improved enzymatic hydrolysis of methyl-umbelliferyl lactoside at high temperatures (>60^{o} C), relative to moderate temperature (50^{o} C). *In vitro* evolution of variants was performed to recombine mutations that conferred improved thermal properties, and to reduce the presence of mutations deleterious to enzyme activity. Using this approach, a more than seven-fold improvement in "thermal activity" of the enzyme, measured as the ratio of the rate of reaction at a high temperature relative to the rate of activity at 50^{o} C on the fluorescent substrate. Resulting variants were shown to have improved performance in long term (5-7 day) hydrolysis of PCS at 60^{o} C and higher, when combined with thermotolerant CBH II, EG and βG.

A directed evolution strategy was also employed to improve the thermal properties of *A. oryzae* βG. Random mutagenesis was employed, and a high throughput assay where enzymes were subjected to short thermal challenges followed by measurements of enzyme residual activity was used to identify thermostable variants. Mutants with >10x improved thermal stability were identified, where the residual activity of enzymes following a ten minute heat treatment at 68^{o} C was compared.

Bioinformatic tools were also exploited toward discovery of novel cellulase-encoding genes with improved thermal properties. We concentrated our efforts on thermotolerant and thermophilic fungi known to have a high capacity for cellulose degradation. Expression of recombinant cellulases in a filamentous fungal host, and, ultimately in combination with other partner cellulases in *T. reesei*, was used. We assessed thermal properties, as well as ability to hydrolyze the selected biomass. Over eighty novel cellulases genes were cloned, expressed, and characterized for performance in the degradation of PCS. We identified several enzymes with superior thermal stability relative to the *T. reesei* counterparts.

Exploring Cellulase Natural Diversity to Improve Classical Cellulase Mix

The two CBHs, six EGs, and one BG known to be secreted by *T. reesei* belong to eight GH families, representing approximately half of the GH families known to include cellulases. It is possible that other cellulases from other GH families could be beneficial to the *T. reesei*

cellulolytic system, by providing complementary specificities, stronger synergism, reduced inhibition, enhanced reactivity, or increased stability. In addition to focusing discovery efforts to clone, express and characterize enzymes within the "canonical" CBH-EG-βG system, we investigated the full range of enzymes secreted by fungi such as *T. reesei*, assessing their ability to work alone or in concert with other glycosyl hydrolases toward improved hydrolysis of PCS.

DNA microarrays were employed to assess gene expression during growth of filamentous fungi on cellulosic carbon sources, in comparison to growth on glucose. Various cellulases, hemicellulases and non-hydrolytic proteins were identified as upregulated genes in *T. reesei* grown on PCS. Expected genes include cellulases (*e.g*. GH 7, 6, 5, 12, 45), hemicellulases (*e.g*. Xyn11, Man5, AXE3/5, Chi18), and other glycoside hydrolases. Other non-hydrolytic proteins were also found. Twenty-four genes with no significant homology to previously cloned genes were detected.

Proteomic techniques were also employed in a targeted discovery of proteins secreted by fungi that actively degrade cellulose. To catalog the secreted proteins (secretome), we applied a proteomic technique coupling two-dimensional (2D) electrophoresis and mass spectrometry, and targeted a number of fungal broths obtained from growth of diverse organisms in the presence of cellulose. Proteins were excised and analyzed by either tandem LC-MS/MS or MALDI-TOF mass spectrometry. The major identified proteins included CBH I (Cel7A), CBH II (Cel6A), and the EGs Cel7B and Cel5A, as well as Xyn11A and swollenin. We also analyzed the secretomes of other cellulolytic fungi, including many with strikingly different secretomes, compared with that of *T. reesei.*

We explored whether crude fungal broths could improve the *T. reesei* cellulolytic mixture screening for enhancement of PCS hydrolysis activity. An example of this is seen in Figure 1, where a fungal cellulolytic system has been combined with Celluclast in a 1:1 mixture. This mixture performed as well as the individual cellulase systems dosed at *twice* as much enzyme as the *T. reesei* cellulolytic system alone, indicating a significant synergism between the two systems.

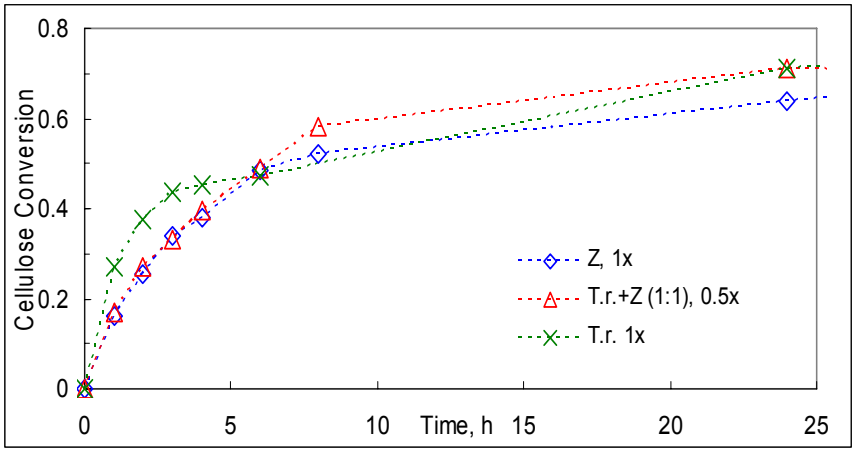

Fig. 1. Effect of adding fungus Z proteins to *T. reesei* broth in hydrolyzing PCS at 50°C.

A biochemical approach was used to fractionate the proteins in the crude fungal broth assessed in Figure 1. PCS hydrolysis assays were used to identify fractions of proteins that were capable of improving activity of a Celluclast-based mixture. Upon identification of fractions that enhanced cellulase performance, mass spectrometry was used to identify proteins in the various fractions with enhancing activity. Some of the fractions with enhancing activity contained protein(s) with sequence identity to proteins classified as family GH61 glycosyl hydrolases. We followed this lead, cloning and expressing proteins from the GH61 family from this fungus, as well as from other cellulolytic fungi. Characterization of recombinantly expressed GH61 proteins showed that a number of these polypeptides can enhance the cellulase performance Celluclast 1.5L in hydrolysis of PCS.

Identifying limiting enzyme activities for late stage hydrolysis

Late-stage hydrolysis, defined herein as the stage of the reaction where 50% or more of the cellulose has been saccharified, is characterized by a drastic slow down in the rate of cellulose conversion. To understand what enzyme component(s) might be limiting in the later stage of hydrolysis, we tested monocomponents in “restart” experiments, testing for the ability of enzymes to synergize with residual cellulases in stalled hydrolysis reactions. We identified enzyme components that could be added during late-stage hydrolysis to significantly enhance cellulose degradation. These components were shown to act in synergy with residual cellulases that were still active after the initial part of the reaction, during which 50% of cellulose was converted. Identifying the rate-limiting components in the enzyme mix allowed us to amend the *T. reesei* enzyme system to include higher amounts of these limiting components, thereby improving the effectiveness of the enzyme system in achieving complete cellulose saccharification.

High-Yield Expression of Cellulases

Many individual enzymes acting synergistically form an effective cellulase mix for conversion of lignocellulosics to sugars. Production of cellulase components individually is not economically feasible; instead all the proteins necessary should be expressed and secreted by a single host. Protein composition should be well balanced to take advantage of the optimal mix for synergy. At the same time, the overall total protein yield must be high.

The use of genetic engineering to introduce and manipulate gene expression was indispensable to our cellulase research program. We used several selective genetic markers to follow gene integration, and developed a variety of promoter elements to enable variable levels of gene expression. Transformation procedures for high efficiency simultaneous co-transformation of different transgenes were developed.

Conclusion: Enhanced cellulolytic mixture for lignocellulosic feedstocks

This extended abstract outlines the concerted research approach used at Novozymes to reduce cellulase cost for conversion of lignocellulose to raw sugars. Figure 2 demonstrates the relative increases in enzyme specific performance that have been achieved by Novozymes, including implementation of some of the discoveries outlined here. By reducing the cost of production in addition to increasing the effectiveness of the cellulase, a 30-fold decrease in cost has been achieved. The overall enzyme cost for the process of converting corn stover to ethanol is now estimated to be 0.10-0.18 USD per gallon in laboratory trials mimicking an NREL process model system. The enzyme cost is no longer a dominating economic barrier in producing sugars from corn stover.

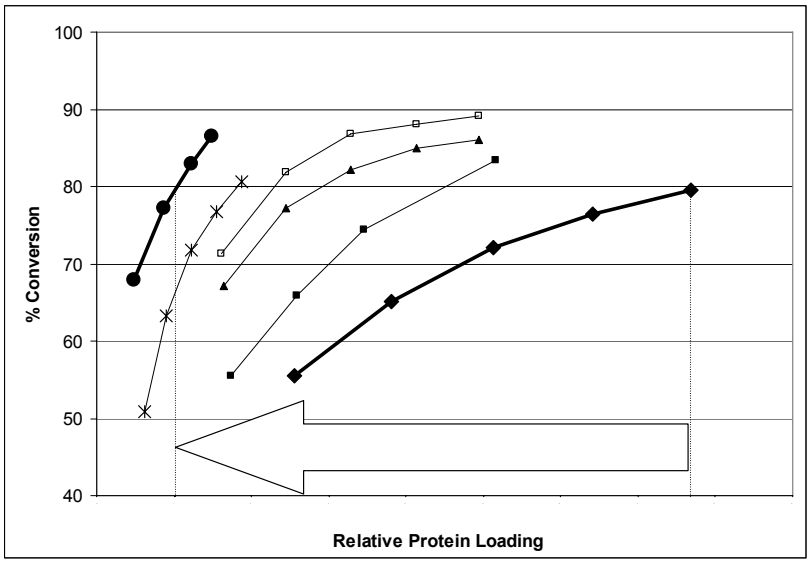

Fig. 2. Stepwise improvements in enzyme performance in hydrolysis of PCS. Relative protein loading is plotted vs. percent cellulose conversion. The large arrowhead shows reduction in enzyme loading required to achieve 80% conversion from the benchmark cellulase under original, benchmark conditions (diamonds) to the final improved cellulase (circles).

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