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Progress and Challenges in Enzyme Development for Biomass Utilization

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Abstract Enzymes play a critical role in the conversion of lignocellulosic waste into fuels and chemicals, but the high cost of these enzymes presents a significant barrier to commercialization. In the simplest terms, the cost is a function of the large amount of enzyme protein required to break down polymeric sugars in cellulose and hemicellulose to fermentable monomers. In the past 6 years, significant effort has been expended to reduce the cost by focusing on improving the efficiency of known enzymes, identification of new, more active enzymes, creating enzyme mixes optimized for selected pretreated substrates, and minimization of enzyme production costs. Here we describe advances in enzyme technology for use in the production of biofuels and the challenges that remain.

Keywords Biomass · Enzymes · Hydrolysis

1 Introduction

The utilization of lignocellulose for the production of fuels and chemicals has the potential to change the world economically, socially, and environmentally. Today roughly 87% of the energy used in the world is derived from non-renewable sources such as oil, natural gas, and coal, with total energy consumption increasing at approximately 4% per annum. About 28% of that energy consumption is in the form of liquid transportation fuels, derived almost entirely from petroleum [1]. The long-term cost of continued use of these finite fuel sources can already be seen in increased conflict over their control and distribution, climate change linked to increased greenhouse gas emissions, and increasing prices, all of which negatively impact people around the world every day. Lignocellulosic biomass, in the form of plant materials such as grasses, woods, and crop residues, offers a renewable, geographically distributed, greenhouse-gas neutral source of sugars that can be converted to ethanol or other liquid fuels via microbial fermentation.

In the past 30 years, ethanol produced from corn starch and sugarcane has been established as an economically viable supplement to gasoline. In the USA over the past 5 years, production has increased from 175 million gallons per year to nearly 4.5 billion gallons last year, and is growing at more than 25% per year. In the near future, the use of sugar and starch as feedstocks for fuel production is expected to face increasing competition with their direct use as food and animal feed, impacting both availability and price. Current estimates suggest that in the USA, starch-based ethanol output will reach a maximum of between 12 and 15 billion gallons per year [2]. To significantly impact the use of petroleum in the USA, which uses approximately 140 billion gallons of gasoline per year, additional sources of fermentable sugar for ethanol production will be required.

Lignocellulosic biomass has the potential to become a major source of these fermentable sugars in the future. It is estimated that in the USA alone, more than one billion tons per year of biomass could be sustainably harvested in the form of crop and forestry residues, replacing as much as 30% of the total US gasoline consumption [3].

To turn the prospect of replacing a significant proportion of the current liquid fuels into reality, the conversion of lignocellulose to ethanol must become less expensive in both operating cost and capital investment. Current estimates suggest that the cost of producing cellulosic ethanol is $1.80/gallon or higher, or almost twice as high as the cost of producing ethanol from starch [4]. Part of this high cost results from a significantly higher estimated capital investment for the construction of cellulosic plants compared to starch-based production facilities. Cellulose-to-ethanol plants in current design scenarios require more unit operations, must be larger to accommodate more dilute sugar streams, and in some cases require acid-resistant

construction materials, which in sum are projected to increase the investment more than fourfold relative to current dry milling starch-based ethanol plants (from $1.10/gallon installed capacity to $4.70/gal) [4]. On the operating cost side, equipment replacement may be more frequent due to processing materials that are more abrasive than seed, enzyme cost will be significantly higher due to the increased complexity of the substrate and higher enzyme dosage required to release the sugars, and higher water consumption may be required to remove compounds that interfere with the hydrolysis and fermentation processes.

Starch is present in plants as an energy source for growing seeds, while lignocellulose is present as a structural cell wall component to give the plant rigidity; therefore it should be no surprise that the latter is much more resistant to enzymatic attack. On a protein weight basis, it takes anywhere from 40–100 times more enzyme to break down cellulose than starch, yet the cost of enzyme production is not substantially different (Novozymes, unpublished data).

In 2001, Novozymes was awarded a research subcontract by the US Department of Energy with the goal of reducing the cost of cellulases for ethanol production from biomass. This effort, called the Cellulase Cost Reduction Project, was administered by the National Renewable Energy Laboratory (NREL), with Novozymes providing expertise for enzyme improvement and production, and NREL contributing expertise in biomass pretreatment and enzyme evaluation. The stated goal of the project was to achieve a tenfold reduction in the cost of enzymes for the conversion of acid pretreated corn stover to ethanol in laboratory-scale testing. At the beginning of this work, the cost of providing a commercial cellulase preparation for the conversion of 80% of the cellulose in acid pretreated corn stover to fermentable glucose was estimated to be $5.40/gallon ethanol produced. During the course of the contract, significant advances were made in improving the efficiency of the cellulases, increasing the yield in production, and reducing the cost of production. In addition, work focusing on other enzyme activities required for effective enzymatic hydrolysis of lignocellulosic substrates other than acid pretreated corn stover was successfully conducted. In this manuscript, we highlight some of those efforts that have contributed to making enzymes for lignocellulose hydrolysis more affordable.

2
Lignocellulosic Biomass to Ethanol Process Overview

While possible variations in the process of converting lignocellulosic biomass to ethanol are virtually endless, it can most simply be described as the integration of five unit operations: (1) desizing, (2) thermochemical pretreatment, (3) enzymatic hydrolysis, (4) fermentation, and (5) ethanol recovery

(Fig. 1). In the first step of the process, the delivered biomass must be made uniform in size to facilitate handling and transport via conveyor or screw drive and to provide a more consistent surface-to-mass ratio for thermochemical pretreatment. The pretreatment step is typically a short- (minutes) to long-term (hours) exposure to extremes of temperature (150–200 C), pH (<2 or >10) and pressure (2–5 atm) and may additionally involve a rapid pressure release that facilitates chemical infiltration and fiber explosion. Ideally, pretreatment produces a disrupted, hydrated substrate that is accessible to enzymatic attack, but avoids both the production of sugar degradation products and fermentation inhibitors. As discussed below, some pretreatments solubilize hemicellulose to oligomeric and/or monomeric sugars comprised largely of pentoses that can be fermented independently or together with the glucose released from the cellulose fraction. In the next step, the pH is adjusted and enzymes are added to initiate cellulose hydrolysis to fermentable sugars. With pretreatments that do not solubilize the hemicellulose fraction, additional enzymes may be required to hydrolyze the hemicellulose

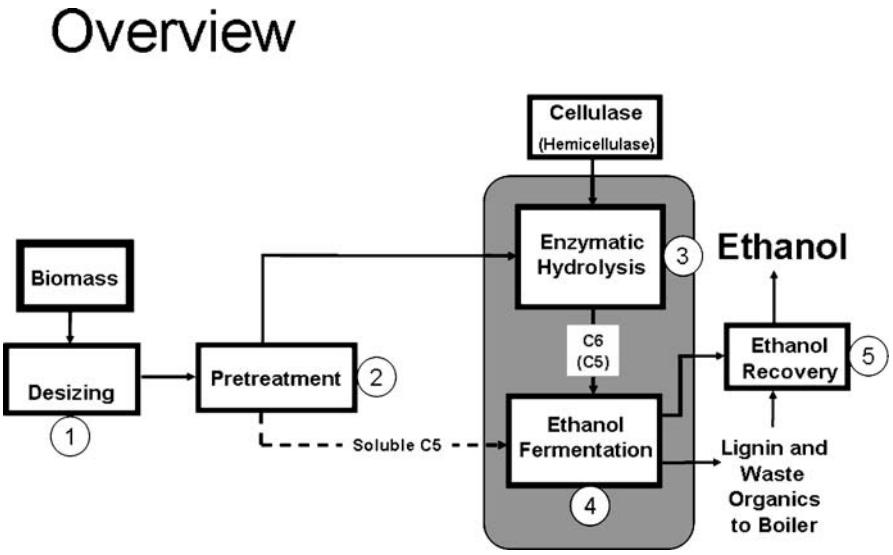

Fig. 1 Five-step process for the conversion of biomass to ethanol. *Step 1* The biomass is physically reduced in size by milling or chopping to increase surface area and uniformity. *Step 2* Some form of thermochemical pretreatment consisting of exposure to high pressure, temperature and extremes of pH is conducted to destroy the plant cell wall and expose the sugar polymers to the liquid phase. *Step 3* Enzymatic hydrolysis using a complex mix of glycosyl hydrolases to convert sugar polymers to monomeric sugars. *Step 4* Fermentation of the monomeric sugars to ethanol by addition of a fermentation organism. *Step 5* Ethanol recovery from the fermentation using distillation or some other separation technology. *C6* refers to glucose derived from cellulose hydrolysis, while *C5* refers to pentose sugars (mainly xylose) derived from hemicellulose

polymer. Hydrolysis typically is performed at pH 5 and 50 °C for 24–120 h, followed by addition of a fermentation organism to begin production of ethanol. In many cases (as described below) fermentation is initiated long before hydrolysis has completed, since both the extent and speed of ethanol production can often be increased by combining the hydrolysis and fermentation steps. In the final step, ethanol is recovered via distillation, and remaining organic waste is burned for production of heat and/or power.

2.1 Minimizing Yield Loss and Cost

The key to developing an economically viable biorefinery is to employ a holistic approach that integrates the unit steps, maximizing the yield at each, while minimizing both capital and operating costs. At each step of the process, from pretreatment to fermentation, effort must be made to minimize any loss in potential ethanol production. In the example in Fig. 2, the production of degraded sugars during pretreatment, incomplete cellulose or hemicellulose

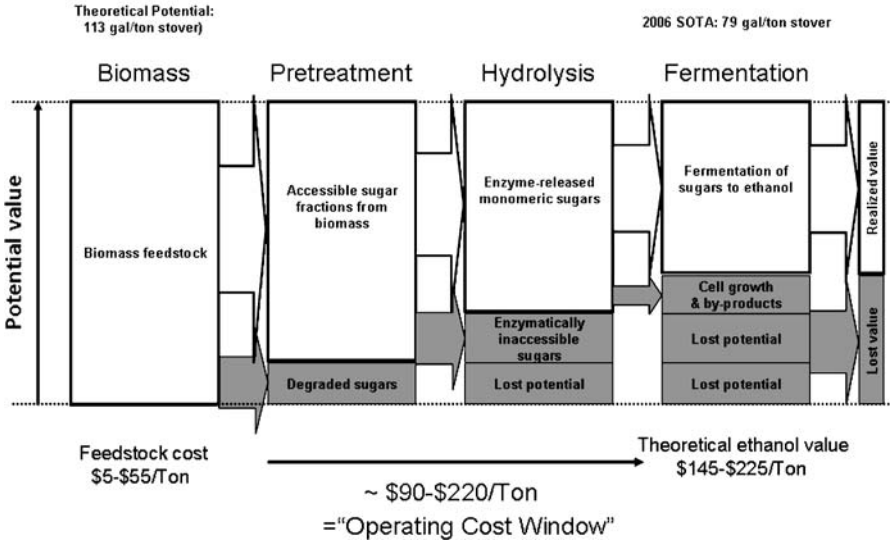

Fig. 2 Defining the operating cost window. These calculations utilized bone-dry corn stover and assumed the only sugar polymers used to produce ethanol are cellulose (40%) and xylan (25%). Ethanol yield was calculated according to the yield calculator from the US Department of Energy [5]. The theoretical ethanol value is based on $2/gallon selling price. 2006 SOTA is a current state-of-the-art scenario for conversion of cellulose (74% of theoretical) and xylan (64% of theoretical) to ethanol to yield 79 gallons of ethanol per bone-dry ton of corn stover. The value of any products other than ethanol, such as excess heat or power, is not included. For reference, corn grain at 72% starch has a theoretical yield of 124 gallons/ton

conversion to fermentable sugars during hydrolysis, and fermentation losses due to sugar consumption by the yeast all contribute to lost value in the conversion. If biomass feedstock such as corn stover, purchased at $5/ton, could be converted with perfect efficiency to its theoretical potential of 113 gallons of ethanol per ton of stover with an ethanol selling price of $2/gallon, the value of the ethanol would be ~$225/ton, creating an "operating cost window" for depreciation of capital, operation, and profit of ~$220/ton [5]. Losses in any unit step that reduces the overall yield will reduce the value per ton, whether the losses result from reduced enzyme hydrolysis, poor fermentability of the hydrolyzate sugars, or reduced fermentation yield. It is also important to note that maximizing the conversion of the two most abundant sugars, glucose and xylose, is important to viable economics. If only cellulose is utilized with no conversion of hemicellulose, the theoretical yield drops 39% to 69 gallons/ton, reducing the cost window to ~ $135/ton. Unless the xylose is utilized to produce something of equal or higher value, it is unlikely that such a process could be viable. Similarly, a pretreatment selected on the basis of a reduced capital cost for installed equipment, but increasing the required enzyme dosage, may reduce the operating cost window significantly.

3 Impact of Process Steps on Enzyme Dosage and Cost

The amount and types of enzymes required for the saccharification of cellulose and hemicellulose are strongly dependent on the biomass being hydrolyzed and the type and severity of pretreatment. Ultimately the selection of biomass feedstock will be based on local availability and economy of supply. In the early stages of commercial development, feedstocks with the greatest potential for demonstrating economic viability of an integrated process are likely to be developed first. These likely will include processing residues that are already available at processing plants such as corn fiber, soybean hulls, sugarcane bagasse, wood waste, and paper mill waste. The selection of both desizing and pretreatment processes may also be strongly influenced by local economics, especially with regard to co-location with existing wood, coal, or municipal solid waste-burning power plants, where inexpensive power and steam are available. With a diversity of potential substrates, different thermochemical pretreatments will be utilized to balance accessibility to enzymatic attack with destruction of valuable sugars. Variations in the severity of the pretreatment (pretreatment severity is defined as the combined effect of temperature, acidity, and duration of treatment) must also be varied to maximize both sugar and fermentation compatibility. For example, an acid pretreatment, run at high temperature, high pressure and for long periods of time is considered more severe than a neutral pH water pretreatment run under the same temperature and pressure conditions. A low severity pretreat-

ment will solubilize less of the hemicellulose fraction, increasing the amount of hemicellulase enzymes required, but may also reduce the production of by-products toxic to the fermentation, increasing the ethanol yield from the fermentation.

3.1 Impact of Substrate Selection on Enzyme Cost

The principal components of biomass are: cellulose (~ 30–50%), hemicellulose (~ 20–30%) and lignin (~ 20–30%); with minor components of starch, protein and oils. The exact composition of each biomass varies depending both on the plant species and the plant tissue utilized. Table 1 shows a variety of substrates in an effort to illustrate the variability of the composition of different substrates. In addition to the variability seen between plant species, work at the US National Renewable Energy Laboratory has demonstrated that even within a single plant species there is considerable variability in composition [6]. Using near infrared spectroscopy, they showed that the total sugar content contributed by cellulose and hemicellulose varied from 45 to 68% of dry mass between 1061 samples of corn stover. Lignin content, which has a direct impact on enzymatic digestibility, varied between 12 and 20%. These differences can be attributed to the genetic background of the corn variety, environmental factors such as weather, location, and pest invasion, and differences in farming practices.

The substrate characteristics that have been shown to impact the rate of hydrolysis include accessibility, degree of cellulose crystallinity, and the type and distribution of lignin [8]. The presence of lignin in a cellulose–cellulase

Table 1 Composition of representative biomass samples

Samples	Variety	% Mass		
		Total lignin	Cellulose	Hemicellulose
Monterey Pine	*Pinus radiata*	25.9	41.7	20.5
Hybrid Poplar	DN-34	24	40	22
Sugarcane bagasse	*Gramineae saccharum* var. 65-7052	24	43	25
Corn stover	*Zea mays*	18	35	22
Switchgrass	Alamo	18	31	24
Wheat straw	Thunderbird	17	33	23
Barley straw	*Hordeum vulgare* sp.	14	40	19
Rice straw	*Oryza sativa* sp.	10	39	15

Source: [7]

reaction is hypothesized to decrease the quantity of the enzyme associated with the cellulose due to nonspecific adsorption of the enzyme to lignin [9] and steric hindrance [10]. Steric hindrance occurs when lignin encapsulates the cellulose and makes it less accessible to enzyme attack [11]. Each of the factors summarized above are known to effect enzyme action and no single parameter correlates absolutely with enzymatic digestibility. The variation in composition of a given biomass requires some tailoring in the conversion method.

3.2 Impact of Pretreatment Selection

For an industrial process to be economically viable, enzymatic breakdown of lignocellulose to fermentable sugars must occur as quickly as possible, preferably in hours or days. No known enzyme or mix of enzymes are able to accomplish this feat on crude biomass. To make lignocellulose more amenable to breakdown, a wide array of thermochemical pretreatments have been devised [12]. Pretreatment has been described as the second most expensive unit cost in the conversion of lignocellulose to ethanol using enzymatic hydrolysis [13], after feedstock cost. A wide variety of pretreatments have been extensively described, including comminution [14], steam explosion [15], ammonia fiber explosion [16], and acid [17] or alkaline treatments [18] with different chemicals [19]. It is not our intent to review these sundry pretreatments, but only to indicate how they differ in terms of their impact on downstream enzymatic hydrolysis.

Pretreatments vary from extremely acidic to quite alkaline, modifying the composition of the biomass and making it more accessible to the enzymes. For example, acidic pretreatments will hydrolyze the majority of the hemicellulose while largely leaving the cellulose and lignin intact [20, 21]. Dilute acid (0.5–1.0% sulfuric) at moderate temperatures (140–190 °C) hydrolyzes most of the hemicellulose to soluble pentose sugars (both monomers and oligomers), with a concomitant increase in the efficiency of enzymatic cellulose digestion [22]. Although very little lignin is solubilized, the lignin is disrupted or redistributed in such a way that enzymatic digestion is enhanced [23]. Alkaline pretreatments typically solubilize less of the hemicellulose and lignin than acidic pretreatments, but modify or redistribute the lignin [24, 25]. Alkaline pretreatments therefore require enzymes that hydrolyze both cellulose and hemicellulose. Pretreatments differ not only in the degree of hemicellulose depolymerization, but also in the formation of compounds such as furfurals, acetate, and other chemicals that may be deleterious to the fermentative organism [26]. The effect of inhibitors released during pretreatment can also inhibit enzyme activity [27, 28]. An ideal pretreatment would be inexpensive both in capital and operating costs, create cellulose and hemicellulose substrates that require low enzyme dosages to release the

monomeric sugars, generate no hazardous waste, and produce a sugar stream that is fermentable without detoxification. Alterations in the type and severity of pretreatment can have a profound impact on enzyme dosage required for hydrolysis, and therefore on the cost of enzymatic hydrolysis.

As an example, a comparison of enzyme digestibility was made at Novozymes between alkaline pretreated and acid pretreated corn stover using the same enzyme mix composed of *T. reesei* cellulase (Novozymes' Celluclast 1.5 L) supplemented with cellobiase (Novozym 188) (Fig. 3). For the alkaline pretreatment, two conditions of severity were supplied for analysis, while for the acid pretreated material, one sample was washed exhaustively with water to remove solubles, while the other was simply adjusted to pH 5 with base. Although the samples contained the same cellulose content, large differences were seen in the enzyme loading required to hydrolyze the cellulose, with only the cellulose in the washed acid pretreated material being hydrolyzed to completion. The unwashed acid pretreated material was more

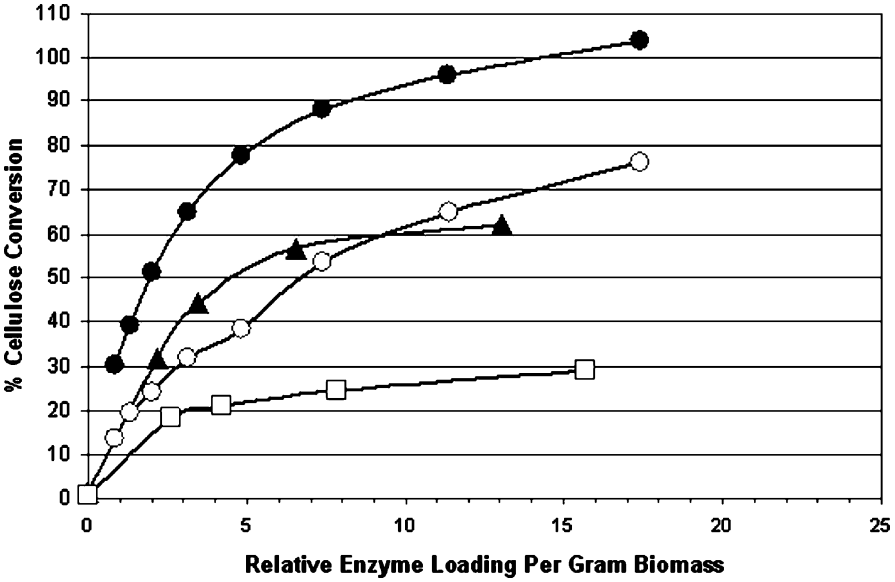

Fig. 3 Enzymatic digestibility of acid and alkaline pretreated corn stover, washed and unwashed. Comparison of the enzymatic digestion of washed (●) and unwashed (○) acid pretreated corn stover, and two (▲, □) severities of an alkaline pretreated corn stover using a mixture of Celluclast 1.5 L and Novozym 188 at various enzyme loadings. Pretreated corn stover was supplied by NREL and others. Acid pretreated corn stover was washed with water until the supernatant reached pH 5. Cellulose content was estimated from compositions provided by biomass suppliers. Enzymatic hydrolysis was conducted with the same enzyme mix in 50 g assays containing 13.5% dry solids at 50 °C for 168 h. Calculation of approximate conversion was based on the amount of glucose released as a percentage of the theoretical yield from cellulose

resistant to hydrolysis, likely due to the presence of inhibitors that block enzyme action. While the cellulose in the unwashed stover was hydrolyzed to a greater extent with increasing enzyme dose, both of the alkaline pretreatments show a plateau in cellulose hydrolysis, likely due to steric hindrance by unremoved hemicellulose or lignin. Addition of hemicellulase activities can improve the cellulose digestion in these cases, but at an increased cost for the additional activities. Our enzyme mix was optimized for an acid pretreatment, and better enzyme mixes for both alkaline and acid pretreatments could and should be possible.

3.3 The Impact of Process Integration on Enzyme Requirements

The process steps of pretreatment, hydrolysis, and fermentation need to be viewed holistically to maximize ethanol yield and overall process cost. As discussed previously, different pretreatments produce different substrates for enzyme action, impacting both the required mix of enzymes, the dosage of those enzymes, and the cost of the hydrolysis step. Similarly, the selection of the fermentation organism determines the pH and temperature optima of the fermentation step, which can affect enzyme performance and loading since hydrolysis and fermentation are often combined at some stage of the hydrolysis in a single reactor.

The enzymatic hydrolysis can either be done separately from the fermentation (SHF, separate hydrolysis and fermentation) or in combination with the fermentation (SSF, simultaneous saccharification and fermentation). In SHF, hydrolysis is allowed to proceed to a point of completion at reaction conditions optimal for enzyme action, which today for *T. reesei* enzymes is 50 °C and pH 5, then the temperature is lowered to allow survival of the fermentation organism (typically 30–40 °C) and the pH is adjusted upwards to pH 5.5–7. The primary drawback to the SHF process is a reduced rate of hydrolysis caused by product inhibition of the enzymes by the released monomeric and oligomeric sugars. The SSF process for producing ethanol is capable of improved hydrolysis rates, yields, and product concentrations compared to SHF because of the continuous removal of the reaction end products (the sugars) by the yeast, preventing competitive inhibition of some of the component enzymes, provided the temperature and pH required for fermentation does not drastically slow enzyme action. Ideally we will see organisms and enzymes developed that have similar growth and reaction optima, allowing optimal growth and enzyme action to occur in a single vessel. Currently, compromises in either or both must be made in the process design since there is a 10–20 °C gap in temperature optima and a 0.5–2 pH unit gap in pH optima.

In hybrid hydrolysis and fermentation (HHF), the hydrolysis and fermentation are temporally separated to optimize the combined rate of hydrolysis and fermentation. Hydrolysis is allowed to proceed to a point at which glucose

release slows significantly, then the temperature is dropped, the pH increased, and fermentation is initiated by addition of the organism. The development of an economically viable process depends on optimizing the timing of the shift from hydrolysis to fermentation, and is dependent on the enzymes, the organism, and all the factors that contribute to process cost, such as feedstock cost, hydrolysis/fermentation residence time, solids loading, and capital investment.

It has been established that digestibility of a biomass substrate is highly dependent upon the type of pretreatment, enzyme efficiency, and dosage. Recent results indicate that mixing is also an important parameter in integrating pretreatment and hydrolysis [29]. In Fig. 4, acid pretreated corn stover (PCS) and hot water autolysed wheat straw (HWS) were hydrolyzed at Novozymes with a Celluclast/Novozym 188 mix at the same loadings using two different types of mixing: shake flask orbital mixing versus tumbling (lift and drop). While the PCS, a well-pretreated substrate whose cellulose can be wholly hy-

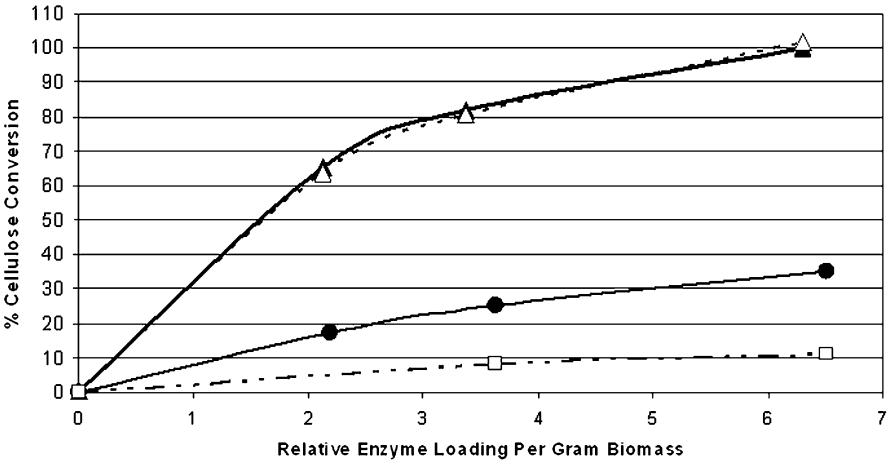

Fig. 4 Comparison of the impact of shake flask orbital mixing versus tumbling on enzymatic hydrolysis. Acid pretreated corn stover (PCS, supplied by the NREL) was washed prior to use. Pretreated wheat straw (HWS) was produced by wet autoclaving at 132 °C for 30 h as estimated by application of the Arrhenius equation to the data of Garrote (1999) so as to produce minimally pretreated biomass. Residual dry weights were determined as per NREL laboratory analytical procedure (LAP) 012 (NREL procedures can be found at [46]). Cellulose content was estimated from published values (HWS) [47], limit enzymatic hydrolysis, and carbohydrate compositional analysis (PCS). Hydrolysis was performed essentially as per NREL protocol LAP 009 (72 h, pH 5, 50 °C), using either flasks in a rotary shaker at 150 rpm (shaker) or in sealed tubes tumbling free in a rolling tub (tumbling). Analysis of resulting hydrolysis sugars was performed according to NREL protocol LAP 13–15. Calculation of approximate conversion was based on the amount of cellobiose and glucose released as a percentage of the theoretical yield from cellulose. PCS tumbling (▲), PCS in a shaker (△), HWS tumbling (●), and HWS in a shaker (□)

drolyzed, shows no difference in either the rate or extent of hydrolysis, the poorly pretreated HWS shows a dramatic improvement in hydrolysis from the more disruptive tumble mixing as compared to orbital mixing. Although conversion is fairly low for the HWS compared to the PCS, we saw both an increase in the rate and endpoint conversion of cellulose to glucose with the tumble mixing. These results indicate that the type and vigor of mixing during hydrolysis may allow less severe pretreatments to be implemented, with the potential to decrease both capital and operating costs during pretreatment. In addition, this type of vigorous mixing may allow higher solids levels during pretreatment and hydrolysis, resulting in a more concentrated sugar stream and higher ethanol titers from the fermentation. This has the potential to reduce operating costs in energy consumption used for ethanol distillation. In addition, utilization of higher solids increases plant throughput, reducing the total capital investment required.

4
Enzyme Discovery: Catalytic Efficiency and Productivity

There are numerous organisms that rely on biomass degradation for their survival, often existing in the natural environment as a complex consortia of fungi, bacteria, and protozoa, working synergistically to decay the plant cell wall. All of these organisms are potential sources of enzyme discovery, but current commercial products for biomass treatment are derived from fungi because these organisms produce a complex mix of enzymes at high productivity and catalytic efficiency, both of which are required for low-cost enzyme supply. Unlike most bacteria, which express complexes of many carbohydrate-degrading activities arrayed on molecular scaffolds physically attached to the bacterial cell wall, fungal cellulases are typically secreted into the growth medium, allowing cost-efficient separation of the active enzymes in a liquid form suitable for delivery to a hydrolysis reactor.

4.1
T. reesei Cellulases: The Current Industry Standard

The most widespread, commercial enzyme products currently available for biomass hydrolysis are produced by submerged fermentation of the saprophytic mesophilic fungus *T. reesei* [30]. This organism, first isolated over 60 years ago from decaying cotton tents during World War II [31] is a prolific producer of secreted cellulases. Since its initial isolation, numerous mutants have been isolated that increase the productivity of the strain by over 20-fold [28, 32, 33]. Three enzymes classes form the core of the *T. reesei* cellulase system: exoglucanases comprised of two primary cellobiohydrolases, a number of endoglucanases, and β-glucosidases (Fig. 5). There are two types of

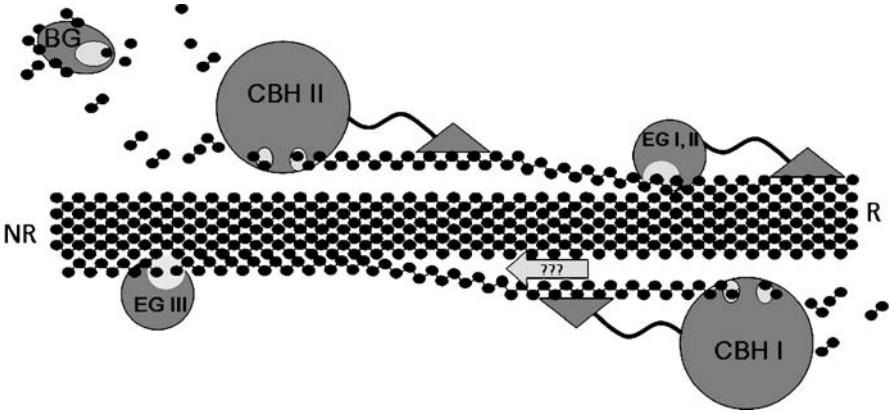

Fig. 5 Schematic of the primary *T. reesei* enzymes involved in hydrolysis of cellulose. Cellulose is represented as stacked chains of *black circles* with reducing (*R*) and non-reducing (*NR*) ends indicated. There are two major cellobiohydrolases that attack the cellulose chain ends processively from the reducing (*CBH I*) and non-reducing (*CBH II*) ends of the chain, releasing the glucose disaccharide cellobiose. In addition, there are three major endoglucanases depicted (*EGI*, *II*, and *III*) that attack the cellulose chain randomly, and two β-glucosidases (*BG*) that hydrolyze cellobiose released by the CBHs to glucose. *Triangles* represent cellulose binding motifs, and the *arrow* represents an additional hypothetical protein components that may assist in cellulase action by disrupting the cellulose crystal structure

cellobiohydrolases, CBH I and CBH II, that constitute roughly 60% and 20% of the secreted protein mix and are critical to the efficient hydrolysis of cellulose [34]. The CBH I and II hydrolyze the cellulose chain processively from the reducing and non-reducing ends of cellulose chains, respectively, releasing the glucose disaccharide cellobiose. Endoglucanases (EG I-IV) constitute roughly 15% of the secreted protein and hydrolyze β-1,4 linkages within the cellulose chains, creating new reducing and non-reducing ends that can then be attacked by the CBHs. β-Glucosidases (BGL I and II), constituting roughly 0.5% of the secreted protein mix, and hydrolyze cellobiose and some other short-chain cellodextrins into glucose.

4.2 Searching for Synergy

The primary factor in the high cost of enzymes for biomass hydrolysis is simply the amount of enzyme that must be used. Compared to starch hydrolysis, 40- to 100-fold more enzyme protein is required to produce an equivalent amount of ethanol (Novozymes data). It was recognized very early on that efficient cellulose hydrolysis requires a complex, interacting collection of enzymes during initial characterization of the *T. reesei* cellulase system [35]. To significantly reduce the amount of these enzymes requires that either more

efficient component enzymes are identified or that additional enzymes can be added that reduce the total enzyme loading. Synergy, the ability of two or more enzymes to work simultaneously more effectively than in succession, was first described in cellulases more than 30 years ago when describing the action of CBH I and EG activities [36]. In this case, the synergy can be mechanistically explained by the production of new cellulose ends by the action of the endoglucanase, creating new sites of exoglucanase attack by the CBH. Similarly, studies of the observed synergism between CBH I and CBH II from *Humicola insolens*, revealed that this CBH II, although capable of acting processively from non-reducing chain ends, does also cleave the cellulose chains in an endo fashion [37]. To drive enzyme loading down, we needed to search for similar synergistic enzyme pairs that could complement the preferred *T. reesei* cellulase system.

4.2.1 β-Glucosidase

An "efficient" cellulase system requires sufficient β-glucosidase (BG) to hydrolyze cellobiose produced by the action of the CBHs to prevent their product inhibition [38]. The addition of BG to a complex cellulase mix such as the Novozymes Celluclast 1.5 L dramatically improves the extent and, during the later stages of hydrolysis, the rate of cellulose saccharification. This is re-

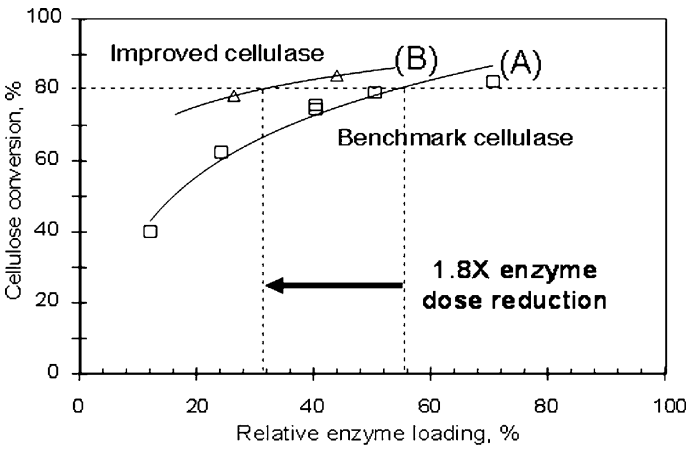

Fig. 6 Improvement of PCS-hydrolyzing cellulases by increasing levels of β-glucosidase (BG) activity. Comparison of *T. reesei* cellulase preparations, with (*B*) and without (*A*) supplementation with purified *A. oryzae* BG, in the hydrolysis of cellulose present in acid pretreated corn stover demonstrates a significant benefit in reducing the amount of enzyme required. Addition of small amounts of BG, present as a few percent of total protein, allowed hydrolysis of 80% of the cellulose to glucose with an enzyme protein dosage 1.8-fold lower that the unsupplemented cellulase

flected in Fig. 6, where the *T. reesei* strain used to produce Celluclast 1.5 L was compared to the same strain expressing *Aspergillus oryzae* BG in hydrolysis assays. Due to relief of the product inhibition at high solids loadings (13.5% w/w in this example), the amount of total enzyme protein required to hydrolyze 80% of the cellulose to glucose was reduced by nearly twofold. At this solids loading, the beneficial effect of BG addition was saturated when it reached ~ 5% of the total enzyme protein, but higher solids would require higher BG levels or a more active BG.

4.2.2 Glycosyl Hydrolase Family 61

In nature, microbes can efficiently degrade biomass by secreting an array of synergistic enzymes, including cellulases, often from numerous microbes intermingled in their growth. In an effort to identify new proteins that could work synergistically with those secreted by *T. reesei*, we conducted mixing experiments by supplementing Celluclast 1.5 L with broth from a wide array of cellulolytic fungi grown under cellulase-inducing conditions. By comparing the saccharification of acid pretreated corn stover using equal protein loadings of either Celluclast alone or mixtures of Celluclast with these broths, fungi secreting components that could work synergistically with the *T. reesei* cellulases could be detected. In Fig. 7, an example of such an experiment shows that a mixture of *T. reesei* broth and *Thielavia terrestris* broth has the same level of hydrolyzing activity as twice as much *T. reesei* or *T. terrestris* broth alone. These results suggested that some activity present in the *T. terrestris* broth was working in synergy with the cellulases present in *T. reesei* broth to more efficiently degrade the cellulose in the corn stover.

In order to identify the protein or proteins responsible for the observed synergism with the *T. reesei* cellulases, the *T. terrestris* broth was fractionated and individual fractions were assayed for synergism similarly. Fractions displaying synergism were separated on one- and two-dimensional polyacrylamide gels, individual proteins were isolated by removal from the gels and subjected to sequencing by tandem mass spectrometry. Several independent chromatographic fractions contained proteins with homology to glycosyl hydrolase family 61A, a protein previously identified in a number of cellulolytic fungi. When purified to homogeneity, a number of these proteins were demonstrated to significantly enhance the activity of the *T. reesei* cellulases in synergism assays. Inclusion of these proteins at less than 5% of the total enzyme dose in some cases could reduce the required cellulase loading by as much as twofold. These results suggested that the GH61 family proteins were the major components responsible for the enhancement of Celluclast 1.5 L activity by crude *T. terrestris* fermentation broth.

We also tested the cellulase-enhancing effect of GH61 proteins on a variety of other lignocellulosic substrates from a variety of pretreatments when

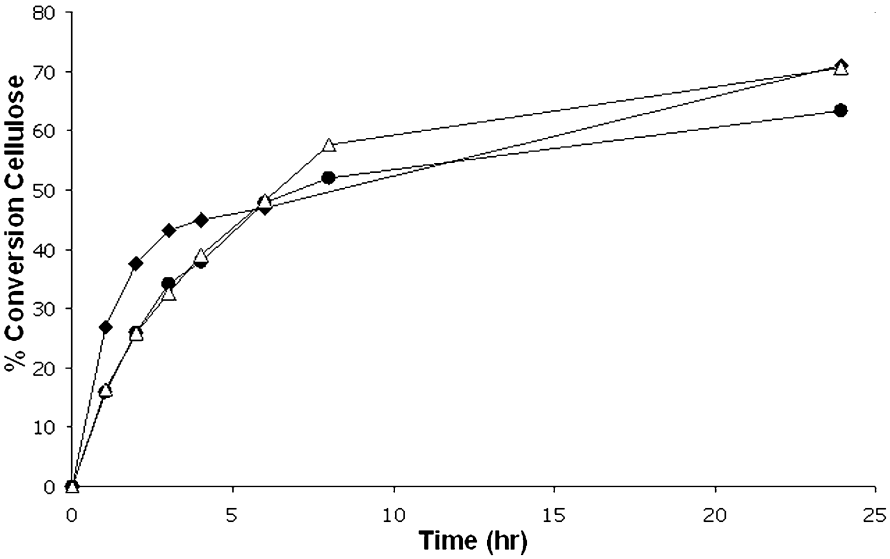

Fig. 7 Synergy between the cellulases of *T. terrestris* and *T. reesei*. Hydrolysis of PCS at 50 °C using cellulase-induced broth samples of *T. reesei* (◆), *T. terrestris* (●), or a 1 : 1 mix of the two broths at one-half the enzyme loading (△). The 1 : 1 mixture of the two cellulase preparations performed as well as the individual system dosed at *twice* as much as the *T. reesei* cellulolytic system alone, indicating a significant synergism between the two systems

combined with *T. reesei* cellulases. Those GH61 proteins capable of enhancing hydrolysis of acid pretreated corn stover also enhanced hydrolysis of other substrates, although they differed in their effectiveness by varying amounts. None of the GH61 proteins were able to enhance the hydrolysis of pure cellulose in the form of filter paper. This lack of enhancement was also shown with other pure cellulose substrates such as Avicel, phosphoric-acid swollen cellulose, and carboxymethyl cellulose.

The GH61 proteins by themselves showed no significant detectable hydrolytic activity on PCS or any other lignocellulosic substrate tested, indicating that the enhancement was not likely to be the result of any intrinsic endo- or exoglucanase activity of the GH61 proteins. The hydrolytic activity of several GH61 proteins was tested on a variety of model cellulose and hemicellulose substrates, but little or no activity was found. These results suggest that the enhancement of cellulolytic activity by GH61 is limited to substrates containing other cell wall-derived material such as lignin or hemicellulose, although there is no clear correlation between the proportions of these materials and the degree of enhancement observed. These findings could be of significant interest for not only the elucidation of the physiological functions of the GH61 protein family, but also the development of a viable enzymatic system to convert biomass to simple platform sugars.

Several of the GH61 genes were transformed into *T. reesei*, resulting in transformants expressing GH61 at various levels, depending on the number of inserts and site of integration. Fermentation broths produced by these transformants were assayed for PCS hydrolysis at various protein loadings to assess their improvement in specific performance relative to control strains not expressing non-native GH61 proteins. The results confirmed that certain GH61 proteins expressed at relatively low levels are capable of significantly enhancing the hydrolysis of cellulose in PCS. For example, expression of *T. terrestris* GH61B in *T. reesei* allows for a reduction in protein loading of 1.4-fold to reach 90% conversion of cellulose to glucose in 120 h. The protein loading reduction made possible by GH61 addition becomes more pronounced at longer incubation times and higher levels of hydrolysis, and higher solids loadings.

4.2.3 Synergistic Hemicellulases

Development of improved enzymes for the hydrolysis of the other major carbohydrate polymer present in lignocellulosic biomass is also of commercial interest, particularly to those utilizing neutral or alkaline pretreatments that leave much of the hemicellulose intact. To develop these enzymes, an industrial residue of the wheat starch industry was used as a model substrate. In Europe, wheat is one of the major substrates for production of fuel ethanol. Processing of wheat starch for glucose results in a by-product stream (vinasse) consisting mainly of the wheat endosperm cell wall material and leftover yeast cells following the fermentation of the starch. The hemicellulose by-product is approximately 33 wt % carbohydrates of which approximately 66 wt % is arabinoxylan. Arabinoxylans consist of a linear backbone of β-1,4-linked D-xylopyranosyl units that are partially substituted with arabinofuranosyls. The major portion of the arabinoxylan in industrial wheat fermentation residues is water-soluble [39], the water-insoluble arabinoxylan is quantitatively more abundant in cell walls isolated directly from unprocessed wheat endosperm [40]. Arabinoxylans are hydrolyzed to monosaccharides by acid treatment or by enzymatic hydrolysis. The enzymatic hydrolysis is usually preferred because it allows for a more specific and controlled modification and fewer undesirable by-products, making it more suitable for microbial fermentation using organisms that can metabolize both xylose and arabinose [41].

The enzymatic degradation of arabinoxylans requires both side-group cleaving and depolymerizing enzymes. Cleavage of the side chains requires the action of several accessory enzyme activities, including α-L-arabinofuranosidases, α-glucuronidases, ferulic acid esterase, and acetylesterases [41, 42]. Depolymerization requires endo-1,4-β-xylanases that result in unbranched xylooligosaccharides, including xylotriose and xylobiose, and

β-xylosidases that cleave xylobiose and attack the non-reducing ends of short chain xylooligosaccharides to liberate xylose [41].

The hydrolysis of arabinoxylan is critical for improved utilization of wheat hemicellulose in the ethanol industry. Three Novozymes cellulolytic and hemicellulolytic enzyme preparations, Celluclast 1.5 L, Ultraflo L, and Viscozyme L were tested in various combinations for their ability to liberate arabinose and xylose from water-soluble wheat arabinoxylan. The substrate was medium viscosity water-soluble wheat arabinoxylan from Megazyme (Bray). The three different enzymes were evaluated individually and also in 50 : 50 combinations to look for possible synergistic effects. Reactions were carried out at pH 5 and 50 °C followed by analysis of arabinose, galactose, glucose, xylose, xylobiose, and xylotriose by high-performance anion exchange chromatography (HPAEC) [43]. The molecular weight and distribution of water-soluble wheat arabinoxylan and hydrolyzates were determined by high-performance size exclusion chromatography (HPSEC).

In those reactions containing the individual enzyme preparations, the levels of arabinose and xylose increased with increasing enzyme dosage and time. Ultraflo L was superior to Celluclast 1.5 L and Viscozyme L in releasing the arabinose from the water-soluble wheat arabinoxylan, meaning that Ultraflo L must contain a significant amount of α-L-arabinofuranosidase. Celluclast 1.5 L was the best enzyme preparation for liberating xylose, resulting in 26 wt % of the available xylose. Ultraflo L released 16 wt % while Viscozyme L released less than 1.5 wt %. In a mixture of 50 : 50 Celluclast 1.5 L and Ultraflo L there was no interaction among the arabinose-releasing side activities since the same amount of arabinose was obtained as when the two individual enzyme preparations were used and then the arabinose total was combined. The Viscozyme L preparation exhibited a weak antagonistic effect with Ultraflo L and Celluclast 1.5 L since the amount of arabinose actually decreased compared to that observed with the individual enzyme preparations. The results indicated that the arabinose-releasing side activities of Viscozyme L had the same activity as those demonstrated by Ultraflo L and Celluclast 1.5 L. Another possibile but less likely explanation is the Viscozyme L contained α-L-arabinofuranosidase inhibitors [43]. The 50 : 50 mixture of Celluclast 1.5 L and Ultraflo L produced an increase in the release of xylose compared with the sum of the individual enzyme preparations (Fig. 8). The mixture released 59 wt % of the available xylose, which was 32 wt % more than the theoretical addition of the individual enzyme preparations alone. Combination of Ultraflo L and Viscozyme L showed no such synergism, but incubation of Celluclast 1.5 L and Viscozyme L showed a weak synergistic effect in liberating some of the xylose from the wheat arabinoxylan.

To further examine the synergistic affect between Celluclast 1.5 L and Ultraflo L the amounts of xylobiose and xylotriose released during enzymatic hydrolysis were quantified using HPAEC for both individual and combined enzyme preparations. During the initial stage of incubation, Celluclast 1.5 L

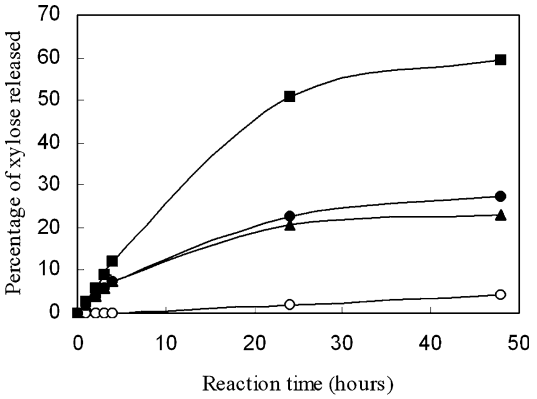

Fig. 8 Synergy between Ultraflo L and Celluclast 1.5 L. Enzyme preparations were from Novozymes (Bagsvæd, Denmark). Weight percent of xylose released from water-soluble wheat arabinoxylan after treatment with: ▲ 5 wt % Celluclast 1.5 L, ○ 5 wt % Ultraflo L, and ■ 10 wt % mix of Ultraflo L and Celluclast 1.5 L (50 : 50 mixture) for 48 h at 50 °C. ● represents the sum of Celluclast 1.5 L and Ultraflo activities, without cooperativity [43].

liberated small amounts of both xylobiose and xylotriose, indicating the presence of endo-1,4-β-xylanase activities. As hydrolysis continued, the released xylobiose and xylotriose was hydrolyzed to xylose, indicating the Celluclast 1.5 L contained one or more β-xylosidase activities.

Ultraflo L treatment resulted in continual liberation of both xylobiose and xylotriose. Ultraflo L showed a low release of free xylose indicating one or more endo-1,4-β-xylanase activities, but little β-xylosidase activity. The synergistic effect between Celluclast 1.5 L and Ultraflo L in releasing xylose is therefore likely to be a result of the action of α-L-arabinofuranosidase and endo-1,4-β-xylanase activities present in Ultraflo L and the β-xylosidase present in Celluclast 1.5 L [43].

Since a strong synergistic effect was observed with a 50 : 50 combination of Celluclast 1.5 L and Ultraflo L for the breakdown of arabinoxylan, a second study was conducted to look for similar effects and viscosity reduction in the fermentation residue, vinasse. The effects of enzyme dosage, optimal temperature, and pH were examined in hydrolysis of whole vinasse, vinasse supernatant, and washed vinasse sediment that was provided by Tate & Lyle, Amylum UK (Greenwich, UK). On whole vinasse, the enzyme-catalyzed release of arabinose and xylose by the 50 : 50 combination of Ultraflo L and Celluclast 1.5 L decreased as the substrate concentration of the vinasse increased. The monosaccharide release also decreased when the substrate concentration of the vinasse increased. Release of arabinose and xylose from the vinasse sediment was very low. The release of arabinose from the whole vinasse varied from 40–50 g arabinose per kilogram vinasse DM while xylose release was between 75–100 g xylose per kilogram vinasse DM after a 24 h hydrolysis. The

Ultraflo L:Celluclast 1.5 L mixture released 53–75 g arabinose and 75–115 g of xylose per kilogram of vinasse DM after a 24 h hydrolysis [44].

Significant viscosity reduction was obtained by enzyme-catalyzed degradation of arabinoxylans present in the fermentation residue stream, vinasse. However, there was limited hydrolysis of the insoluble arabinoxylans in the vinasse sediment. The efficiency of enzymatic degradation of the arabinoxylan in vinasse was dependent on enzyme dosing and substrate dry matter [44].

In an effort to narrow down the specific activities involved in the previous studies, the β-xylosidase from Celluclast 1.5 L was purified and used as a supplement to Ultraflo L enzyme preparation. When dosed at 0.25 g β-xylosidase protein per kilogram of arabinoxylan along with Ultraflo L, this enzyme mix released the same or more xylose as the enzyme mix consisting of 50 : 50 Ultraflo L and Celluclast 1.5 L (Fig. 9).

In order to determine the optimal enzyme mix for the hydrolysis of vinasse arabinoxylan, several recombinant enzymes were made and tested in various combinations. Genes were cloned and expressed in the fungal host *A. oryzae*. Based on our studies the optimal enzyme mix for vinasse hydrolysis consists of α-L-arabinofuranosidase from *Meripilus giganteus*, α-L-arabinofuranosidase II from *Humicola insolens*, and *T. reesei* β-xylosidase. A mixture of 25 : 25 : 50 of α-L-arabinofuranosidase from *M. giganteus*, α-L-arabinofuranosidase from *H. insolens* and β-xylosidase from *T. reesei* was determined to be optimal for maximizing arabinoxylan hydrolysis. The success of this work in identifying and exploiting synergism between hemicellulase component activities is currently being applied to other relevant lignocellulosic substrates that differ significantly in their hemicellulose composition.

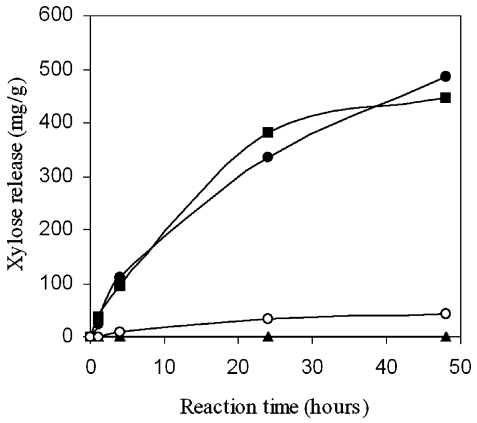

Fig. 9 Xylose released from water-soluble wheat arabinoxylan after treatment with: ▲ 0.25 g β-xylosidase protein kg^{-1} arabinoxylan, ○ 5 wt % Ultraflo L, ● 5 wt % Ultraflo L + 0.25 g β-xylosidase protein kg^{-1} arabinoxylan, and ■ 10 wt % Celluclast 1.5 L/Ultraflo L (50 : 50 mixture) for 48 h at pH 5 and 50 °C [48]. © 2006, with permission from Elsevier

5
Producing Enzymes Economically

There is arguably no other industrial enzyme application that poses a greater challenge to the enzyme producer than supplying cost-effective enzymes for biomass utilization. The high enzyme loading required, combined with the low value of the final product, in the form of ethanol, requires not only that the enzymes be as efficient as possible, but that the cost of producing them be as low as possible. To this end, significant effort has been expended over the past 6 years to increase the productivity of the fungal strains used to produce the enzymes, to reduce the cost of the enzyme fermentation process by reducing the cost of carbon and nitrogen sources for the fermentations, and to reduce the complexity of enzyme recovery and formulation.

Improving the host by classical mutagenesis is one way of developing a host strain with improved total protein production and improved activities. This approach has a long and successful history. Montenecourt and Eveleigh [32] isolated RutC30, one of the best existing *Trichoderma* cellulase mutants, using a combination of ultraviolet irradiation and nitrosomethyl guanidine (NTG). Recently, Toyama, et al. [45] demonstrated a method to screen for increased cellulase production using growth through an overlay of cellulose substrate (Avicel) in Petri plates. In an effort to increase total cellulase productivity, a combination of these methods were utilized on the *T. reesei* strain currently used to produce Celluclast 1.5 L. Chemical mutagenesis was used to generate mutants that were screened using the method of Toyama [45] with minor changes. Briefly, mutagenized spores were suspended in an agar medium, poured into a plate and allowed to harden. The spore-containing layer was then covered with a top layer of agar containing washed, acid pretreated corn stover (PCS) as the sole carbon source. Colonies growing through the PCS layer fastest were isolated and used in a secondary screening. In this, spores from selected fast-growing colonies were inoculated into shake flasks containing cellulase-inducing media. After 5 days of growth, broth samples were tested by robotic assay for production of reducing sugars from hydrolysis of PCS. Total protein assays were then conducted, and mutants expressing elevated cellulase and/or total protein were then re-grown in 2-L fermentors. Broth from the fermentors was then analyzed again in PCS hydrolysis assays and for total protein. Some mutants were identified as having improved PCS hydrolysis and increased total protein secretion compared with the control. Top strains isolated in this manner showed significant increases in protein production and secreted cellulase activity.

Another method of improving a cellulase productivity is through increasing the expression of target proteins using genetic engineering. In many cases the total cost of supplying a heterologous mix of enzymes can be reduced by creating a single expression host expressing not only the native cellulases

and hemicellulases, but expressing additional components, such as the BG and GH61 proteins, without negatively impacting the expression of the native proteins. The introduction of multiple genes into a single host is no easy feat. A significant amount of work was done to identify strong promoters, to identify a number of selectable markers, and to develop a successful transformation technique that allows for co-transformation of multiple transgenes. These technological improvements have allowed us to rapidly and efficiently investigate the effect of introducing various enzymes into the *T. reesei* cellulase mix.

In addition to controlling gene expression transcriptionally, by utilizing promoters of different strengths, we have focused on enhancing individual protein yield by optimizing protein secretion. One example is the replacement of the *A. oryzae* BG signal sequence with a signal peptide from *H. insolens* Cel45A EG, which improved the BG secretion in *T. reesei* by two- to threefold relative to the unfused gene (Fig. 10).

As previously mentioned, several GH61 proteins result in a "boost" in PCS hydrolysis when supplemented to Celluclast 1.5 L. In addition, our studies show that increased levels of β-glucosidase are required in our *Trichoderma* host. Therefore, numerous co-transformations of *T. reesei* with various GH61s, *A. oryzae* β-glucosidase, and other genes of interest were carried out. Those transformants expressing both a GH61 and the β-glucosidase were then screened in PCS hydrolysis assays in order to identify the top strains in true performance assays. Those strains demonstrating the best perform-

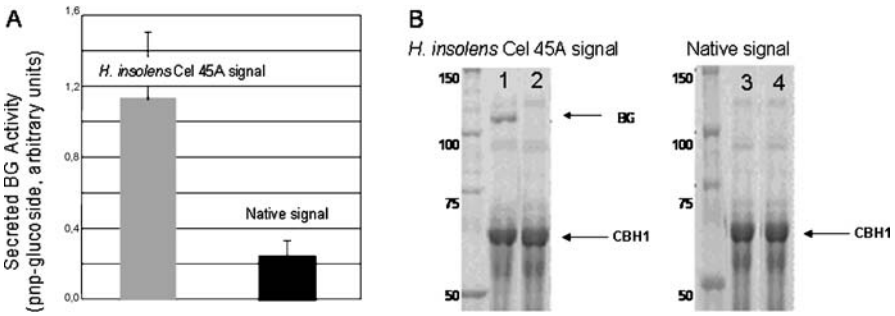

Fig. 10 Signal peptide effect on β-glucosidase (BG) secretion in *T. reesei*. *T. reesei* strains were genetically modified to heterologously express *A. oryzae* BG, using either the native *A. oryzae* signal peptide or the *H. insolens* Cel45A signal peptide. **a** Relative BG activity measured in the secreted fraction, using 4-nitrophenyl β-D-glucopyranoside at pH 5. **b** SDS-PAGE of secreted proteins from the two *T. reesei* strains. *Lane 1* BG expression utilizing the *H. insolens* Cel45A signal sequence. *Lane 2* parent of strain used to generate the strain in lane 1 (untransformed). *Lane 3* BG expression utilizing native signal sequence. *Lane 4* parent of strain used to generate the strain in lane 3. The positions of molecular weight markers are labeled and the positions of *A. oryzae* BG and *T. reesei* CBHI are designated by *arrows*

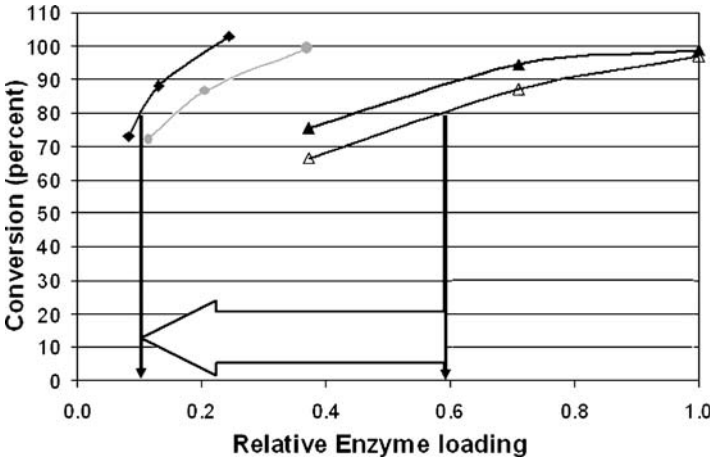

Fig. 11 Stepwise improvements in enzyme performance in hydrolysis of PCS. Relative enzyme protein loading is plotted vs. percent cellulose conversion. Celluclast 1.5 L supplemented with 1% w/w Novozym 188 (Novozymes' BG product) at 38 °C (△) and 50 °C (▲). The Celluclast 1.5 L strain expressing a recombinant BG (●), and the Celluclast 1.5 L strain expressing a recombinant BG, a GH61 protein, and two additional heterologous proteins (◆) were tested to determine the enzyme protein loading required to reach 80% of the theoretical cellulose hydrolysis using acid pretreated corn stover in 168 h. The final *T. reesei* strain produced a cellulase mix roughly sixfold more efficient than the Celluclast 1.5 L supplemented with 1% w/w Novozym 188

ance in PCS hydrolysis were then fermented in 2-L bioreactors and retested in PCS hydrolysis assays. Eventually, a single strain was identified exhibiting improved hydrolysis from our original strains and high total protein production (Fig. 11).

5.1 Reduced Enzyme Recovery

The total production cost for cellulosic ethanol must still be substantially reduced to enable large scale commercialization, and at least a portion of this reduction must come from enzyme cost. Realistically, enzyme cost targets in the range of $0.30/gallon at the commercial scale should be achievable in the near future by avoidance of transportation and formulation costs. In such a scenario, on-site or near-site enzyme production is essential, where enzymes are produced using reduced-cost feedstocks, transported short distances, and not stored for extended periods of time. The least expensive alternative in this situation involves the direct use of whole fermentation broth (including cell mass) to circumvent expensive cell removal and enzyme formulation steps. To investigate this possibility, we compared the use of whole fermentation broth and cell-free broth as catalysts for PCS hydro-

lysis in microtiter-scale reactions at 50 °C, pH 5.0, for up to 120 h. The results of this study strongly suggest that both preparations, dosed at equal volumes, give comparable yields of reducing sugars from PCS, suggesting that costly cell removal may not be required.

6 Conclusions

The development of cost-effective enzymes for the widespread utilization of lignocellulosic biomass will require continued research and development to be successfully deployed. Although great progress has been made in identifying new enzyme mixes with improved catalytic efficiency, improvements in enzyme yield, and improved enzyme production economics, much work remains. There are thousands of organisms involved in the natural decomposition of plant material in our biosphere, and only a fraction of those have been isolated or investigated. Since these organisms work collectively to degrade biomass, better enzymes, with greater synergies, will be uncovered with additional work. Future efforts will also likely require the use of directed evolution techniques to collectively optimize enzymes to perform under conditions more compatible with the fermentation organisms used to produce ethanol and other products. In the short term, there are also great strides to be made in the area of process integration. Here, closely coupling the steps of pretreatment, hydrolysis, and fermentation has the potential to significantly increase overall process efficiency and reduce cost.

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References

1. Energy Information Administration (2005) Official energy statistics from the US Government. Available from http://www.eia.doe.gov/emeu/international/contents.html, last visited: 10 May 2007
2. Jessen H (2006) Ethanol Producer, October, p 44
3. Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes BJ, Erbach DC (2005) Report no ORNL/TM-2005/66, Oak Ridge National Laboratory. Available online at http://www.feedstockreview.ornl.gov/pdf/billion_ton_vision.pdf, last visited: 10 May 2007
4. Gallagher PW (2006) Choices 21(1), Available online at http://www.choicesmagazine.org/2006-1/biofuels/2006-1-04.htm, last visited: 10 May 2007
5. US Department of Energy (2007) Biomass program: theoretical ethanol yield calculator. Available online at http://www1.eere.energy.gov/biomass/ethanol_yield_calculator.html, last visited: 10 May 2007

6. Sluiter AD, Hayward TK, Jurich CK, Newman MM, Templeton DW, Ruth MF, Evans KW, Hames BR, Thomas SR (2003) 25th symposium on biotechnology for fuels and chemicals. National Renewable Energy Laboratory, Golden, CO, p 30. Available online at http://www.nrel.gov/docs/gen/fy03/33925.pdf, last visited: 10 May 2007
7. US Department of Energy (2007) Biomass program: biomass feedstock composition and property database. Available online at http://www1.eere.energy.gov/biomass/feedstock_databases.html, last visited: 10 May 2007
8. Mansfield SH, Mooney C, Saddler JN (1999) Biotechnol Prog 15:804
9. Bernandez TD, Lyford K, Hogsett DA, Lynd LR (1993) Biotechnol Bioeng 42:899
10. Mooney AC, Mansfield SD, Touhy MG, Saddler JN (1998) Bioresour Technol 64:113
11. Fan LT, Lee YH, Beardmond DH (1980) In: Fiechter A (ed) Advances in biochemical engineering. Springer, Berlin Heidelberg New York
12. Mosiera N, Wyman CE, Dale BE, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Bioresour Technol 96:673
13. National Renewable Energy Laboratory (2002) Enzyme sugar-ethanol platform project. NREL, Golden, CO, http://www1.eere.energy.gov/biomass/pdfs/stage2_overview.pdf
14. Cadoche L, Lopez GD (1989) Biol Wastes 30:153
15. Morjanoff PJ, Gray PP (1987) Biotechnol Bioeng 29:733
16. Holtzapple MT, Jun JH, Ashok G, Patibandla SL, Dale BE (1991) Appl Biochem Biotechnol 28/29:59
17. Torget R, Walter P, Himmel ME, Grohmann K (1991) Appl Biochem Biotechnol 28/29:75
18. Bjerre AB, Olesen AB, Fernqvist T, Plöger A, Schmidt AS (1996) Biotechnol Bioeng 49:568
19. Nguyen QA, Tucker MP, Keller FA, Eddy FP (2000) Appl Biochem Biotechnol 84–86:561
20. Lloyd TA, Wyman CE (2005) Bioresour Technol 96:1967
21. Liu C, Wyman CE (2005) Bioresour Technol 96:1978
22. Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY (2005) Bioresour Technol 96:1959
23. Yang B, Wyman CE (2004) Biotechnol Bioeng 86:88
24. Kim TH, Lee YY (2005) Bioresour Technol 96:2007
25. Teymouri F, Laureano-Perez L, Alizadeh H, Dale BE (2005) Bioresour Technol 96:2014
26. Klinke HB, Thomsen AB, Ahring BK (2004) Appl Microbiol Biotechnol 66:10
27. Mes-Hartree M, Saddler JN (1983) Biotechnol Lett 5:531
28. Mandels M, Weber J, Parizek R (1971) Appl Micobiol 21:152
29. Jorgensen H, Vibe-Pedersen J, Larsen J, Felby C (2007) Biotechnol Bioeng 96:862
30. Nidetzky B, Steiner W, Hayn M, Claeyssens M (1994) Biochem J 298:705
31. Reese RT (1976) History of the cellulase program at the U.S. Army Natick Development Center. Biotechnol Bioeng Symp 6:9–20
32. Montenecourt BS, Eveleigh DE (1979) Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. Adv Chem Ser 181:289
33. Mishra S, Gopalkrishnan KS (1984) J Ferment Technol 62:495
34. Suominen PL, Mantyla AL, Karhunen T, Hakola S, Nevalainen KMH (1993) Mol Gen Genet 241:523
35. Reese ET, Siu RGH, Levinson HS (1950) J Bacteriol 59:485
36. Wood TM, McCrae SI (1972) Biochem J 128:1183
37. Boisset C, Fraschini C, Schulein M, Henrissat B, Chanzy H (2000) Appl Envrion Micro 66:1444

38. Sternberg D, Vijayakumar P, Reese E (1977) Canadian J Microbiol 23:139
39. Sorensen H, Pedersen S, Vikso-Nielsen A, Meyer A (2005) Enzyme Microb Technol 36:773
40. Ordaz-Ortiz J, Saulnier L (2005) J Cereal Sci 42:119
41. Saha B (2000) Biotechnol Adv 18:403
42. Biely P (1985) Trends Biotechnol 3:286
43. Sorensen H, Meyer A, Pedersen S (2003) Biotechnol Bioeng 81:726
44. Sorensen H, Pedersen S, Meyer A (2006) Biotechnol Prog 22:505
45. Toyama H, Yamagishi N, Toyama N (2002) J Mol Catal B: Enzymatic 17:175
46. US Department of Energy (2006) Biomass program: standard biomass analytical procedures. Available online at http://www1.eere.energy.gov/biomass/analytical_procedures.html, last visited: 10 May 2007
47. Wiselogel A, Tyson S, Johnson D (1996) In: Wyman CE (ed) Handbook on bioethanol: production and utilization. Taylor & Francis, Washington, DC, p 105
48. Sorensen HR, Pedersen S, Meyer AS (2006) Enzyme Microb Technol (in press) DOI: 10.1016/j.enzmictec.2006.12.009