

Coordinated development of leading biomass pretreatment technologies

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Abstract

For the first time, a single source of cellulosic biomass was pretreated by leading technologies using identical analytical methods to provide comparative performance data. In particular, ammonia explosion, aqueous ammonia recycle, controlled pH, dilute acid, flowthrough, and lime approaches were applied to prepare corn stover for subsequent biological conversion to sugars through a Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) among Auburn University, Dartmouth College, Michigan State University, the National Renewable Energy Laboratory, Purdue University, and Texas A&M University. An Agricultural and Industrial Advisory Board provided guidance to the project. Pretreatment conditions were selected based on the extensive experience of the team with each of the technologies, and the resulting fluid and solid streams were characterized using standard methods. The data were used to close material balances, and energy balances were estimated for all processes. The digestibilities of the solids by a controlled supply of cellulase enzyme and the fermentability of the liquids were also assessed and used to guide selection of optimum pretreatment conditions. Economic assessments were applied based on the performance data to estimate each pretreatment cost on a consistent basis. Through this approach, comparative data were developed on sugar recovery from hemicellulose and cellulose by the combined pretreatment and enzymatic hydrolysis operations when applied to corn stover. This paper introduces the project and summarizes the shared methods for papers reporting results of this research in this special edition of *Bioresource Technology*.

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1. Introduction

Biomass, whether as sugar crops, starch crops, or cellulosic materials, provides a unique resource for sustainable production of organic fuels and chemicals that are now primarily made from petroleum. Furthermore, cel-

lulosic materials including agricultural (e.g., corn stover) and forestry (e.g., sawdust) residues and herbaceous (e.g., switchgrass) and woody (e.g., poplar trees) crops can be sufficiently abundant to provide a major resource for making commodity products. They are also inexpensive with cellulosic biomass costing about \$42/dry ton competitive with petroleum at about \$6/barrel based on mass or about \$13/barrel based on energy content (Lynd et al., 1999).

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Petroleum is the largest single energy source in the United States (~40%) and the world (~35%). Although production and consumption of most US energy sources (e.g., coal, natural gas) are almost balanced, almost two thirds of petroleum consumed in the US is imported, creating balance of trade deficits and energy security concerns. Of the total US petroleum consumption, about two thirds is used to power a transportation sector that is almost totally dependent on oil (>96%). Further, transportation is the largest emitter of the greenhouse gas carbon dioxide. Although chemical production consumes much less petroleum than transportation, a large fraction of chemicals are made from petroleum, making this sector vulnerable to price hikes or disruptions. Thus, conversion of cellulosic biomass to transportation fuels and chemicals presents a powerful opportunity to improve energy security, reduce the trade deficit, dramatically reduce greenhouse gas emissions, and improve price stability (Wyman, 1999).

Because cellulose is competitive in price with oil, a key challenge to commercializing production of fuels and chemicals from cellulosic biomass is to reduce processing costs enough to achieve attractive investor returns (Lynd et al., 1999; Wyman, 1999). Biological conversion promises such low costs because it has the potential to achieve nearly theoretical yields and the modern tools of biotechnology can improve key process steps. Overall costs of ethanol production from cellulose have been dropped enough to be competitive with corn processing to ethanol, but the risk of financing first-of-a-kind technology stalls commercial use. Further processing advances are thus needed to improve returns for first projects and eventually compete with fossil-derived products without subsidies (Wyman, 1999).

Cellulosic biomass must be pretreated to realize high yields vital to commercial success in biological conversion (Mosier et al., 2005). Pretreatment is among the most costly steps and has a major influence on the cost of both prior (e.g., size reduction) and subsequent (e.g., enzymatic hydrolysis and fermentation) operations (Wooley et al., 1999; Lynd et al., 1996). For example, better pretreatment can reduce use of expensive enzymes. Thus, more attention must be given to gaining insight into interactions among these operations and applying that insight to advance biomass conversion technologies that reduce costs. In addition, although several pretreatments are promising, their relative attributes differ, but comparisons have been difficult due to differences in research methodology and substrate use. Improving the understanding of differences among pretreatment technologies and the effect of each pretreatment on other operations can facilitate selection, reduce commercialization risk, and suggest opportunities for step-change improvements.

On this basis, a team of researchers from Auburn University, Dartmouth College, Michigan State Univer-

sity, the National Renewable Energy Laboratory (NREL), Purdue University, and Texas A&M University undertook the first coordinated project to develop comparative information on the performance of leading pretreatment options based on the use of a single feedstock, common analytical methods, and a consistent approach to data interpretation. University participation in the project was made possible through funding by the US Department of Agriculture Initiative for Future Agriculture and Food Systems (IFAFS) Program through a competitive solicitation with participation by NREL made possible by the support of the Office of the Biomass Program of the US Department of Energy. This research team is a major part of a somewhat larger Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) that was formed in 2000 to understand and advance biomass conversion technologies and train students in this field. Corn stover was chosen as the single biomass source because of its abundance and potential low cost. This paper gives an overview of this project, the pretreatment technologies considered, and the materials and methods used by all participants. Subsequent papers in this volume and other journals provide more in-depth information on the results for each of the pretreatment systems studied through this coordinated research effort.

2. Overview of biological processing of cellulosic biomass

Cellulosic biomass contains ~40–50% cellulose, a glucose polymer; ~25–35% hemicellulose, a sugar heteropolymer; and ~15–20% lignin, a non-fermentable phenyl-propene unit; plus lesser amounts of minerals, oils, soluble sugars, and other components (Holtzapfle, 1993). Biological routes are built around using enzymes to break down cellulose (cellulase) and perhaps hemicellulose (hemicellulase) to sugars. These sugars are then fermented to ethanol or other products, which are recovered and purified to meet market requirements. A number of references including a recent review paper by this team of authors provide far more detailed information on biological conversion technologies than can be covered here (Mosier et al., 2005).

The key economic driver in biomass conversion is high product yields to enhance revenues and reduce waste treatment costs. Next in importance tends to be the concentration of products formed as concentration strongly influences product recovery costs. Rate is also important, although its impact depends on the process conditions employed with rates being less important for reactions in a mild environment than when more demanding environments, such as those requiring high pressures or extensive mixing, are needed. The cost of materials of construction and chemicals can be

important, and unit costs must be kept minimal for low-value products, such as fuels.

Enzymatic conversion is attractive because nearly theoretical yields of sugars are possible, a key to economic success. However, a pretreatment step is essential to effectively prepare cellulose for enzymatic hydrolysis with these high yields. An effective pretreatment disrupts cell wall physical barriers as well as cellulose crystallinity and association with lignin so that hydrolytic enzymes can access the biomass macrostructure. In addition, pretreatment affects the cost of most other operations including size reduction prior to pretreatment and enzymatic hydrolysis after pretreatment. Pretreatment can also strongly influence downstream costs by determining fermentation toxicity, enzymatic hydrolysis rates, enzyme loadings, mixing power, product concentrations, product purification, waste treatment demands, power generation, and other process variables. And of course, the pretreatment operation itself must be low in cost and avoid high consumption of expensive chemicals, high energy demands, and feedstock degradation. Despite its importance, limited resources have been focused on developing promising pretreatment technologies or comparing how they affect other process steps.

3. Pretreatment technologies evaluated

Although many biological, chemical, and physical methods have been tried over the years, pretreatment advances are still needed for overall costs to become competitive with conventional commodity fuels and chemicals (Wyman, 1999). However, only hemicellulose or lignin removal by adding acids or base, respectively, has been effective at a reasonable cost (Hsu, 1996). In particular, pretreatment by dilute sulfuric acid, with pH control, by ammonia, and with lime appear among the most promising options, with favorable processing conditions summarized for each in Table 1.

Dilute-acid (~0.5–1.0% sulfuric) at moderate temperatures (~140–190 °C) effectively removes and recovers most of the hemicellulose as dissolved sugars, and glucose yields from cellulose increase with hemicellulose removal to almost 100% for complete hemicellulose

hydrolysis (Knappert et al., 1981). Although little lignin is dissolved, data suggest that lignin is disrupted, increasing cellulose susceptibility to enzymes (Yang and Wyman, 2004). Pretreatment has also been performed without adding acid in what is often called auto-hydrolysis (Heitz et al., 1991; Saddler et al., 1993), but hemicellulose sugar yields are lower than when acid is added and hemicellulose sugars are primarily in oligomeric form (Garrote et al., 1999; Parajo et al., 2004). Use of SO₂ enhances yields in a way similar to dilute sulfuric acid (Mackie et al., 1985), but many currently prefer dilute sulfuric acid because it is cheap, up to 90% hemicellulose yields are achieved, and enzymatic hydrolysis yields of glucose can be over 90% (Hsu, 1996). Nonetheless, dilute acid pretreatment results in costly materials of construction, high pressures, neutralization and conditioning of hydrolyzate prior to biological steps, slow cellulose digestion by enzymes, and non-productive binding of enzymes to lignin (Wyman, 1999; Hsu, 1996; Ooshima et al., 1990). Co-current/batch reactors are typical, but percolation reactors reduce times for sugars to degrade. Forcing liquid through a packed biomass bed enhances hemicellulose and lignin removal rates and gives high yields of hemicellulose and cellulose sugars even without acid addition (Bobleter, 1994; Allen et al., 1996; Liu and Wyman, 2003). However, percolation or flowthrough are challenging to implement commercially, and the high amounts of water used result in high energy requirements for pretreatment and product recovery.

An alternative approach is based on maintaining the pH at about 4–7 (Weil et al., 1998). Water under pressure can penetrate the cell structure of biomass, hydrate cellulose, and remove hemicellulose. The pK_a of water is affected by temperature such that the pH of pure water at 200 °C is nearly 5.0. Water also has an unusually high dielectric constant that enables ionic substances to dissociate and dissolve hemicellulose, and one half to two thirds of the lignin dissolves from most cellulosic biomass when they are treated at 220 °C for 2 min. In addition, hot water cleaves hemiacetal linkages and liberates acids that catalyze breakage of ether linkages in biomass (Antal, 1996). The preferred temperature for this approach has been shown to be between 180 and 190 °C for corn stover

Table 1
Technologies and representative reaction conditions for biomass preparation by pretreatment considered in this project

Pretreatment technology	Chemicals used	Temperature, °C	Pressure, atm absolute	Reaction times, min	Concentrations of solids, wt.%
Dilute sulfuric acid—cocurrent	0.5–3.0% sulfuric acid	130–200	3–15	2–30	10–40
Flowthrough pretreatment	0.0–0.1% sulfuric acid	190–200	20–24	12–24	2–4
pH controlled water pretreatment	water or stillage	160–190	6–14	10–30	5–30
AFEX/FIBEX	100% (1:1) anhydrous ammonia	70–90	15–20	<5	60–90
ARP	10–15 wt.% ammonia	150–170	9–17	10–20	15–30
Lime	0.05–0.15 g Ca(OH) ₂ /g biomass	70–130	1–6	1–6 h	5–20
Lime + air	0.05–0.15 g Ca(OH) ₂ /g biomass	25–60	1	2 weeks–2 months	10–20

and 150 and 160 °C for corn fiber. Research results suggest that the pH for corn stover is maintained due to the innate buffering capacity of protein components in fiber and ash (and perhaps hemicellulose), and the benefit of using just water is that extraneous reagents are not needed. Pretreatment using water at a controlled pH minimizes hydrolysis of the oligosaccharides, while causing hydration of the structure by liquid water at a pressure above the saturation vapor pressure of water at the selected temperature. The goal is to minimize degradation by avoiding the formation of monosaccharides that degrade to aldehydes during high temperature pretreatment, and enzymes may be added at lower temperatures for hydrolysis of the resulting cellulose and hemicellulose oligomers (Ladisich et al., 1983; Lynd et al., 1991; Holtzapple, 1993; Mosier et al., 1999). This system is being applied to pretreat corn fiber from a corn wet milling operation, with subsequent enzymatic hydrolysis giving nearly complete conversion of cellulose and hemicellulose, as well as starch (Weil et al., 1998). Data are now being developed on pretreating corn stover as described in another paper in this volume and elsewhere (Kohlmann et al., 1995; Weil et al., 1997, 1998).

Using alkaline chemicals to remove lignin has been known to improve cellulose digestibility for years, but sodium hydroxide and other bases are too expensive and too difficult to recover and recycle to make them viable for producing fuels and chemicals (Hsu, 1996). However, pretreatment with ammonia has more recently been shown to be effective in improving cellulose digestion with the advantage of ammonia being recyclable due to its volatility. Furthermore, rapid release of ammonia following pretreatment in this ammonia fiber explosion (AFEX) process to assist recycle further improves digestibility, probably because the accessible surface area is increased as evidenced by increased water holding capacity of AFEX-treated biomass (Dale and Moreira, 1982). AFEX can be performed in lower cost vessels than are needed for dilute sulfuric acid, the hydrolyzate is compatible with fermentation organisms without conditioning, and residual ammonia in the product enhances fermentations (Dale et al., 1985). Ammonia decrystallizes crystalline cellulose and deacetylates acetyl linkages (Gollapalli et al., 2002; Mitchell et al., 1990). Both of these effects increase the enzymatic hydrolysis of cellulose. AFEX removes or degrades little lignin or hemicellulose, but AFEX treated cellulose can be hydrolyzed to glucose with high yields at low enzyme loadings (Dale et al., 1996; Moniruzzaman et al., 1997). AFEX appears to modify or alter lignin structure in such a way as to diminish the interference of lignin with enzymatic hydrolysis of cellulose (Martinez et al., 1991). AFEX also depolymerizes hemicellulose, probably by general base catalyzed hydrolysis via ammonium hydroxide, further opening up the structure of biomass for enzymatic hydrolysis. However, most AFEX

treated hemicellulose is oligomeric (Ferrer et al., 2000; Sulbaran-de-Ferrer et al., 2003). A primary research challenge is to make this oligomeric pentosan stream fermentable to products. The continuous version of AFEX is called FIBEX (Dale et al., 1999).

A process known as ammonia recycle percolation (ARP) passes aqueous ammonia (10–15 wt.%) through biomass at elevated temperatures (150–170 °C) and then recovers it for recycle (Kim et al., 2003; Iyer et al., 1996; Kim and Lee, 1996). At high temperatures, aqueous ammonia swells biomass, depolymerizes lignin, and breaks lignin–hemicellulose bonds without degrading carbohydrates. ARP can realize high degrees of delignification for hardwood and agricultural residues that can be readily adjusted but is somewhat less effective for pulp mill sludge from softwood (Iyer et al., 1996; Kim et al., 2000). Because lignin is believed to be a major hindrance to enzymatic hydrolysis (Chang and Holtzapple, 2000; Cowling and Kirk, 1976; Dunlap et al., 1976; Yoon et al., 1995; Mooney et al., 1998; Schwald et al., 1988), its removal enhances cellulose digestion and also reduces non-productive binding of cellulose to lignin. For example, the digestibility of ARP treated corn stover is in the vicinity of 90% with 10 FPU/g-glucan of enzyme loading, substantially higher than yields with α -cellulose under similar reaction conditions. Lignin and its derivatives are known to inhibit microbes (Palmqvist and Hahn-Hägerdal, 2000a,b), and lignin removal improves microbial activity. To reduce liquid throughput and energy costs, a simpler process termed soaking in aqueous ammonia (SAA) treats biomass with aqueous ammonia in a batch reactor at moderate temperatures (25–60 °C) under atmospheric pressure and achieves similar results to ARP. SAA retains most of the hemicellulose in the solid, eliminating the need to separately process hemicellulose and cellulose sugars. Commercial cellulases often contain enough xylanase activity to concurrently convert both xylan and glucan in SAA treated solids to ethanol in the simultaneous saccharification and co-fermentation process (Kim and Lee, 2005).

Pretreatment with lime increases pH and provides a low-cost alternative for lignin removal (Chang et al., 1998). Typical lime loadings are 0.1 g $\text{Ca}(\text{OH})_2/\text{g}$ biomass. A minimum of about 5 g $\text{H}_2\text{O}/\text{g}$ biomass is required. Additional water can be added, but it is neither helpful nor harmful. Lime pretreatment can be performed at a variety of temperatures, ranging from 25 to 130 °C, and the corresponding treatment time ranges from weeks (25 °C) to hours (130 °C). An advantage to using temperatures below 100 °C is that a pressure vessel is not required, allowing for the possibility of simply pretreating a pile of biomass without the need for a vessel. Regardless of the temperature, lime treatment removes approximately 33% of lignin and ~100% of acetyl groups. For low-lignin herbaceous materials (e.g., switchgrass), this level of pretreatment

is sufficient to render the biomass digestible (Chang et al., 1997; Gandi et al., 1997; Kaar and Holtzapple, 2000). For high-lignin woody materials (e.g., poplar wood), additional lignin removal is required and can be accomplished by adding either oxygen or air to the lime pretreatment system. The combined action of alkali and oxygen solubilizes significant portions of the lignin (~80%), which renders even recalcitrant biomass digestible. Oxygen can be added at high pressures (~15 atm) and high temperatures (~160 °C), resulting in a relatively rapid reaction (~6 h) (Chang et al., 2001). Alternatively, 1-atm air can be percolated through a 55 °C pile for reaction times of about 1 month. The action of lime is slower than for ammonia or more expensive bases, but its low cost and safe handling make it attractive.

4. Research approach

This research was made possible through a cooperative effort among Auburn University, Dartmouth College, Michigan State University, the National Renewable Energy Laboratory, Purdue University, and Texas A&M University. Most of these CAFI participants have worked on biomass pretreatment and hydrolysis since about the mid 1970s, and all have been involved in developing the front-running pretreatment processes described above. The overall goal of the project was to develop comparative information on these pretreatment operations for production of sugars from the hemicellulose and cellulose fractions of biomass for fermentation or chemical reaction to a wide range of high-volume commodity products. Corn stover was employed as a representative feedstock, and material balances were accurately closed to describe the fate of the key biomass constituents for each pretreatment. Comprehensive analysis was also made of the biomass source and liquid and solid products from pretreatment to support closing of material balances and characterize the key reactions. The cellulose-rich solids from each pretreatment were enzymatically digested to glucose, and the fermentability of the liquid hydrolyzates was determined to provide important data for commercial use and to assess the relationship among pretreatment system, conditions, effects on the biomass substrate, and subsequent downstream processing. The overall approach improved these technologies further, provided valuable comparative data on each technology, and provided new insight into biomass hydrolysis to help define advanced processes that further reduce costs.

The following specific objectives were defined for the project:

1. Apply leading pretreatment technologies to prepare corn stover for cellulose hydrolysis.

2. Monitor the recovery, reactions, and fate of lignin, hemicellulose, cellulose, and protein for each of these pretreatment technologies, and gather comprehensive data to characterize these components.
3. Develop and accurately close material balances for each of the pretreatments and calculate energy balances for each.
4. Hydrolyze pretreated solids with enzymes and acid and evaluate the fermentability of liquid hydrolyzates for the most promising pretreatment conditions for each technology.
5. Compare performance among systems on a consistent basis, publish and otherwise widely disseminate the results, and work with individuals, groups, and firms to apply these results commercially.

An Agricultural and Industrial Advisory Board was vital to the project and particularly to the last objective. First, they served as extension agents for technology transfer to the private sector and helped disseminate the information produced over the course of the project. They also helped identify audiences and contacts for technologies. In addition, they provided diverse perspectives on important needs for application of technology and reviewed data and helped define problems and opportunities. Membership in the Board grew over the project lifetime from initial participants representing four organizations to members from a total of 16 different companies and associations. The representatives and organizations in the Board included Mat Peabody, Applied CarboChemicals; Gary Welch, Aventinerei; Greg Luli, BC International; Paris Tsobanakis, Cargill; Robert Wooley, Cargill Dow; James Hettenhaus, CEA; Kevin Gray, Diversa; Paul Roessler, Dow; Susan M. Hennessey, DuPont; Michael Knauf, Genencor; Don Johnson, GPC (Retired); Dale Monceaux, Katzen Engineers; Mark Stowers, MBI; Rene Shunk, the National Corn Growers Association; Joel Cherry, Novozymes; and Leo Petrus, Shell. Meetings were held with the Board in May and November of each year beginning in late 2000.

The papers in this special volume report many aspects of this coordinated effort and also provide references to other papers documenting additional information on the results of this project. A paper is also included to summarize key data for these pretreatments that we hope will aid the reader in comparing their attributes.

5. Methods

The research groups involved in this project all used the same raw materials, analytical procedures, and data analysis approaches wherever possible to promote easier comparison among pretreatment methods, and these common approaches are described in this section.

Materials and methods unique to a particular pretreatment system are described in the corresponding papers.

5.1. Materials

Feedstock—Approximately 100 kg of corn stover that was originally supplied by BioMass AgriProducts (B/MAP, Harlan, IA) to the National Renewable Energy Laboratory (NREL) was used as a single, consistent feedstock by all research groups in this project. It was washed, air-dried, and milled at NREL in a Mitts and Merrill Model 10 × 12 knife mill (Saginaw, MI) to pass through a 1/4 in. screen and distributed to CAFI member institutions for this research. The initial composition of the corn stover, as determined by NREL, was 36.1 wt.% glucan, 21.4 wt.% xylan, 3.5 wt.% arabinan, 1.8 wt.% mannan, 2.5 wt.% galactan, 17.2 wt.% Klason lignin, 7.1 wt.% ash, 3.2 wt.% acetyl group, 4.0 wt.% protein, and 3.6 wt.% uronic acid. α -cellulose was purchased from Sigma (Cat. No. C-8200, Lot No. 11K0246).

Enzymes—Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was distributed to CAFI members by NREL. The average activity of the enzyme, as determined by NREL, was 31.2 filter paper unit (FPU)/mL. β -glucosidase (Novozyme[®] 188), a supplementary enzyme used with Spezyme CP, was purchased from Sigma Chemical Company (Cat. No. C6150, Lot No. 11K1088).

Microorganisms—The primary microorganism used for SSF was *Saccharomyces cerevisiae* ATCC[®] 200062 (NREL-D₅A). The growth media was YP medium, which contained 1% yeast extract (Sigma Cat. No. Y-0500) and 2% peptone (Sigma Cat. No. P-6588). Recombinant *Escherichia coli* ATCC[®] 55124 (KO11) was employed for SSCF tests where applicable. LB medium (Sigma Cat. No. L-3152) that contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloramphenicol was used for this organism.

5.2. Digestibility test

The enzymatic digestibility of corn stover was determined according to the NREL Chemical Analysis and Testing Standard Procedure No. 009 with a slight modification that the amount of test materials was increased by a factor of 10 (NREL, 2004). The conditions of the enzymatic digestibility tests were 50 °C and pH 4.8 (0.05 M sodium citrate buffer). Screw-capped 250 mL Erlenmeyer flasks were used as reaction vessels and were agitated at 150 rpm in a constant temperature incubator shaker. Two levels of cellulase loading were applied: 15 and 60 FPU of Spezyme CP/g-glucan in treated corn stover. The cellulase enzyme was supplemented with β -glucosidase (Novozyme[®] 188, Sigma Cat. No. C-6150) at a level of 2 CBU/FPU. Biomass samples were loaded into the reactor such that the initial glucan concentra-

tion in the reactor was 1% (w/v) (i.e., 1 g-glucan/100 mL liquid). Pretreated biomass samples were used in wet form for enzymatic digestibility tests. A control was prepared with the identical amount of substrate, water, and buffer, without enzymes. Samples were taken intermittently and analyzed for glucose, xylose, and cellobiose content by HPLC. Total released glucose after 72 h of hydrolysis was used to calculate the enzymatic digestibility. α -cellulose and untreated corn stover were put through the same procedure as reference substrates.

Digestibilities for glucan content were calculated by the following formula

$$\text{Digestibility [\%]} = \frac{\text{Glucose released [g]} + 1.053 \times \text{Cellobiose released [g]}}{1.111 \times \text{Glucan added [g]}} \times 100$$

where necessary, the digestibilities for xylan contents were also determined in a similar manner. For xylan digestibilities, the hydration factors are modified to 1.064 and 1.136 for xylobiose and xylan, respectively, in the above formula.

5.3. Simultaneous saccharification and fermentation (SSF)/co-fermentation (SSCF)

The simultaneous saccharification and fermentation of corn stover was conducted according to the NREL Chemical Analysis and Testing Standard Procedure No. 008 (NREL, 2004). Spezyme CP (Genencor, Lot No. 301-00348-257) was used as cellulase enzyme and supplemented with β -glucosidase, Novozyme 188 (Novo Inc., Sigma Cat. No. C6150, Lot No. 11K1088), at a level of 2 CBU/FPU. Erlenmeyer flasks with a 250 mL volume and shaken in an incubator shaker at 38 °C and 150 rpm were used as bioreactors. Into 100 mL working volume of liquid, treated wet corn stover samples were added such that the glucan content was 3% w/v. The reaction media contained 0.05 M sodium citrate buffer and YP medium, and bubble traps were used to maintain anaerobic conditions. Alpha-cellulose was put through the same procedure as a control. The SSF/SSCF runs were performed without external pH control. The cellulase enzyme loadings were 15 FPU/g-glucan supplemented with 30 CBU of β -glucosidase/g-glucan.

The inoculum preparation procedure involved two growth stages. In the first stage (pre-inoculum stage), a frozen stock culture was inoculated into YPD medium, and the revived culture was then transferred into the second flask, which also contained YPD, for cell growth under aerobic conditions. The inoculum from the second stage was transferred into the bioreactor for the SSF/SSCF tests at an inoculum volume of 10%. Erlenmeyer flasks with a 250 mL volume were used as the SSF reactor, which contained enzymes, 10 mL of × 10 YP medium (10% v/v, 10% yeast extract and 20%

peptone), 10 mL of inoculum (10% v/v), sodium citrate buffer, and water to make 100 mL of total liquid volume.

The ethanol yield was calculated as follows:

Yield based on theoretical maximum

$$= \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial sugar (g) in reactor} \times 0.511} \times 100$$

Note: Sugar is interpreted as glucose in SSF or glucose plus xylose in SSCF.

5.4. Analytical methods

Solid samples were analyzed for sugar and Klason lignin following the two-stage acid-hydrolysis procedures given in NREL Chemical Analysis and Testing Standard Procedures (NREL, 2004). The conditions of the first hydrolysis were 72 wt.% sulfuric acid, 1:10 of solid-to-liquid ratio, and 30 °C for one hour. The conditions for the secondary hydrolysis were 4 wt.% sulfuric acid and 121 °C for one hour. Sugars in the hydrolyzates were determined by HPLC using a Bio-Rad HPX-87P column coupled with a refractive index detector. The sugar values in the hydrolyzates were back-calculated into the amount of sugar polymers in the solids with the extent of sugar decomposition during the acid-hydrolysis factored into the calculation of the original sugar composition.

For the acid insoluble lignin analysis, the autoclaved hydrolysis solution was vacuum filtered, and the captured hydrolyzed solid sample was dried and weighed. The dried samples were then combusted in a furnace at 575 ± 25 °C for 3 h to determine the ash content. The difference of the two weights was taken as the acid insoluble lignin. The absorbance of the hydrolysis liquor in the aliquot obtained from the vacuum filter sample at 320 nm on a UV-Visible spectrophotometer measured the acid soluble lignin.

For the SSF/SSCF tests, HPLC with Aminex-HPX-87H column was used to determine sugar and ethanol concentrations. A glucose/lactate analyzer (YSI Model, 2300/2700, Yellow Springs, OH) was used for rapid analysis of glucose during inoculum preparation.

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