ABSTRACT: While many pretreatments attempt to improve the enzymatic digestibility of biomass by removing lignin, this study shows that improving the surface area accessible to cellulose is a more important factor for achieving a high sugar yield. Here we compared the pretreatment of switchgrass by two methods, cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) and soaking in aqueous ammonia (SAA). Following pretreatment, enzymatic hydrolysis was conducted at two cellulase loadings, 15 filter paper units (FPU)/g glucan and 3 FPU/g glucan, with and without BSA blocking of lignin absorption sites. The hydrolysis results showed that the lignin remaining after SAA had a significant negative effect on cellulase performance, despite the high level of delignification achieved with this pretreatment. No negative effect due to lignin was detected for COSLIF-treated substrate. SEM micrographs, XRD crystallinity measurements, and cellulose accessibility to cellulase (CAC) determinations confirmed that COSLIF fully disrupted the cell wall structure, resulting in a 16-fold increase in CAC, while SAA caused a 1.4-fold CAC increase. A surface plot relating the lignin removal, CAC, and digestibility of numerous samples (both pure cellulosic substrates and lignocellulosic materials pretreated by several methods) was also developed to better understand the relative impacts of delignification and CAC on glucan digestibility.

Introduction

Biomass-derived fuels offer the only renewable liquid alternative to petroleum-based transportation fuels (Lynd et al., 2009; Zhang, 2008). Biomass resources, such as crop residues, dedicated bioenergy crops grown on marginal land, and timber industry waste, are cheap and abundant renewable feedstocks. A recent report on the technical feasibility of a billion-ton annual biomass supply estimated that enough biomass could be sustainably produced to replace more than a third of the current US transportation fuel demand, with only modest land-use change (Perlack et al., 2005).

The largest obstacle to economical production of cellulosic biofuels is cost-effectively releasing sugars from recalcitrant lignocellulose (Lynd et al., 2008; Zhang, 2008). Saccharification of biomass normally consists of pretreatment or fractionation, which generates more reactive biomass, followed by enzymatic hydrolysis, which produces soluble fermentable sugars. Increasing overall sugar yields and decreasing processing costs can be accomplished by (1) improving pretreatment (Sathitsuksanoh et al., 2009; Wyman, 2007), (2) enhancing cellulase performance.
biomass saccharification (Zhang and Lynd, 2006). Hydrolysis is vital for decreasing costs associated with pretreatment and their relationship with enzymatic cellulose understanding of substrate characteristics after biomass bioenergy plants (Chen and Dixon, 2007). In-depth (Himmel et al., 2007), and (5) producing less recalcitrant et al., 2009b), (4) decreasing enzyme production costs (Heinzelman et al., 2009; Liu et al., 2009; Zhang et al., 2006), (3) recycling cellulase (Lee et al., 1994; Tu et al., 2007; Zhu et al., 2009b), (4) decreasing enzyme production costs (Himmel et al., 2007), and (5) producing less recalcitrant 

Substrate accessibility has long been recognized as an important factor in the enzymatic hydrolysis of lignocellulose (Arantes and Saddler, 2010; Grethlein, 1985; Lee et al., 1994; Zhang and Lynd, 2004). Accessibility of pretreated biomass substrate to cellulase is usually measured based on the adsorption of active cellulase at decreased temperatures (Kumar and Wyman, 2009; Lu et al., 2002), but this method cannot distinguish cellulose and non-cellulose fractions (Zhu et al., 2009a). Recently, a technology has been developed to determine the cellulose accessibility to cellulase (CAC) and non-cellulose accessibility to cellulase (NCAC) based on adsorption of a non-hyrdolytic fusion protein containing a green fluorescent protein and a family 3 cellulose-binding module (Hong et al., 2007; Zhu et al., 2009a). This technique provides a useful metric for pretreatment comparison. Due to the difficulty of accurately measuring CAC previously, many pretreatments have been compared on the basis of lignin or hemicellulose removal and glucan digestibility (Converse, 1993; Mosier et al., 2005). The importance of removing lignin has been frequently suggested, as many studies have shown a correlation between lignin removal and increased sugar release (Chang and Holtzapple, 2000; Fan et al., 1981; Ishizawa et al., 2009; Liu and Wyman, 2003; Yang et al., 2002).

Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) and soaking in aqueous ammonia (SAA) represent two different biomass pretreatment goals (Fig. 1). COSLIF is mainly focused on breaking the linkages among lignocellulose components and disrupting the orderly hydrogen bonds in cellulose fibers, increasing cellulose accessibility greatly (Sathitsuksanoh et al., 2009; Zhang et al., 2007a; Zhu et al., 2009a). The COSLIF pretreatment accomplishes this disruption of biomass under mild conditions by using the successive actions of cellulose, organic, and aqueous solvents to fractionate lignocellulosic biomass into lignin, hemicellulose oligomers, and highly reactive cellulose (Moxley et al., 2008; Zhang et al., 2007a). Using alkaline conditions, SAA aims to remove a large amount of lignin and dissolve some hemicellulose (Kim and Lee, 2005; Sousa et al., 2009). SAA is typically conducted at a moderate temperature (~60°C), but harsher conditions can be employed for more recalcitrant feedstocks (Gupta et al., 2008).

In order to better understand the root causes of biomass recalcitrance and the substrate characteristics impacting enzymatic cellulose hydrolysis, this study compared COSLIF- and SAA-pretreated switchgrass, using compositional analysis, enzymatic hydrolysis, scanning electron microscopy (SEM), X-ray diffraction (XRD), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC).

**Materials and Methods**

**Chemicals and Materials**

All chemicals were reagent grade, purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise noted. Aqueous ammonia (28–30%), 85% phosphoric acid, and 95% ethanol were purchased from Fisher Scientific (Houston, TX). *Trichoderma reesei* cellulase (Novozyme 50013, 84 filter paper units (FPU)/mL) and β-glucosidase (Novozyme 50010, 270 β-glucosidase U/mL) were gifts from Novozymes North American (Franklinton, NC). Alamo switchgrass (*Panicum virgatum*) was gifted by the National Renewable Energy Lab (Golden, CO). The biomass was harvested in November 2007, baled and shipped a week after harvest, hammer-milled to less than one inch, then processed with a Wiley mill to 1 mm. The resulting biomass was then sieved to less than 20 mesh (0.85 mm particle size) and greater than 80 mesh (0.18 mm particle size).

**COSLIF Procedure**

COSLIF pretreatment of switchgrass was conducted as described elsewhere (Sathitsuksanoh et al., 2009, 2010), using 95% (v/v) ethanol as the organic solvent. In brief, one gram of air-dry switchgrass (~5% moisture) was mixed with 8 mL of 85% phosphoric acid at 60°C for 45 min in 50 mL plastic centrifuge tubes. Biomass dissolution was stopped by...
adding 20 mL ethanol and mixing well. Solid/liquid separation was then conducted in a swinging bucket centrifuge at 4,500 rpm at room temperature for 15 min. The supernatant was decanted, and an additional 40 mL of ethanol was mixed with the cellulose- and hemicellulose-containing slurry. Solid/liquid separation was again conducted by centrifugation. After the supernatant was decanted, the pellets were re-suspended and washed twice with 40 mL of deionized water. The remaining pellet (primarily amorphous cellulose) was neutralized to pH ~7 with 2 M sodium carbonate.

Soaking in Aqueous Ammonia (SAA)
A range of SAA conditions were attempted, where the ammonia concentration, pretreatment temperature, pretreatment time, and liquids to solids loading ratio were adjusted. The pretreatment that was most effective for removing lignin as well as a significant hemicellulose fraction was 10% (w/w) ammonia, 140°C, and a 1:20 solid to liquid ratio, for 14 h. One gram of switchgrass was added to a 200-mL serum bottle, followed by 21 mL of 10% (w/w) ammonia. The tube was capped with a rubber stopper and placed into a preheated 140°C furnace. After 14 h, the bottles were allowed to cool to room temperature. Liquid–solid separation was conducted by filtration using glass filters purchased from Fisher Scientific, and the remaining solids were washed with deionized water until a pH of 7 was achieved (~500 mL). It should be noted that heating ammonia to this temperature may cause potential explosive hazards. Caution should be employed during any repetition of this experiment.

Carbohydrate and Lignin Assays
The structural carbohydrate composition of the biomass was determined with a modified quantitative saccharification (QS) procedure (Moxley and Zhang, 2007). In modified QS, secondary hydrolysis was conducted in the presence of 1% (w/w) sulfuric acid at 121°C, for 1 h to more accurately determine the quantities of sugars susceptible to acid degradation (e.g., xylan). The standard NREL biomass protocol was used to measure lignin and ash (Sluiter et al., 2008). In brief, solids remaining after two-stage acid hydrolysis were held at 105°C overnight. The weight of the dried solids corresponds to the amount of acid-insoluble lignin and ash in the sample. The weight of the ash only fraction was then determined by heating the solids to 575°C for 24 h. Percent acid-soluble lignin in the sample was determined by measuring the UV absorption of the acid hydrolysis supernatant at 320 nm. Monomeric sugars were measured with a Shimadzu HPLC, with a Bio-Rad Aminex HPX-87H column (Richmond, CA), at 60°C with 50 mM sulfuric acid as the mobile phase, operated at a rate of 0.6 mL/min (Zhang et al., 2007a). All carbohydrate and lignin assays were conducted in triplicate.

Enzymatic Hydrolysis
Enzymatic hydrolysis of substrate produced by each pretreatment was conducted at two enzyme loadings (15 and 3 FPU/g glucan, each supplemented with 10 U of β-glucosidase), with and without lignin blocking. This series of experiments was conducted in triplicate and repeated in duplicate. To prevent lignin from competitively binding cellulose, blocking was achieved by adding BSA (10 g/L) for 30 min at room temperature prior to hydrolysis. BSA has previously been shown to adsorb irreversibly to lignin binding sites, without interfering with the sites important for cellulose hydrolysis (Berlin et al., 2005; Zhu et al., 2009a). Pretreated switchgrass samples were diluted to 10 g glucan/L in a 50 mM sodium citrate buffer (pH 4.8) and 0.1% (w/v) NaN₃. Hydrolysis experiments were conducted in a shaking water bath at 250 rpm and 50°C. Eight hundred microliters of well-mixed hydrolysate were removed, followed by immediate centrifugation at 13,000 rpm for 5 min. Then exactly 500 μL of the supernatant was transferred to another microcentrifuge tube and incubated at room temperature for 30 min, to allow the conversion of any cellobiose present to glucose, by the action of β-glucosidase remaining in the supernatant. The supernatant was then acidified by adding 30 μL of 10% (w/w) sulfuric acid, after which it was placed in a ~4°C freezer. After being frozen overnight, samples were thawed, mixed well and then centrifuged at 13,000 rpm for 5 min, to remove any precipitated solid sediment. The soluble glucose and xylose in the enzymatic hydrolyzate were measured by HPLC using a Bio-Rad HPX-87H column. Galactose and mannose co-eluted with xylose. After completion of 72 h hydrolysis, the remaining hydrolyzate was transferred to a 50 mL centrifuge tube, centrifuged at 4,500 rpm for 15 min, and soluble sugar content was determined using the same procedure as other hydrolyzate samples, as described above. After all remaining hydrolyzate was decanted, and the pellet was resuspended in 20 mL of water and centrifuged to remove residual soluble sugars from the pellet. The sugar content of the washed pellet was determined by QS. Enzymatic glucan digestibility after 72 h was calculated using the ratio of dissolved glucose in the supernatant to the sum of this dissolved glucose and the glucose equivalent of the residual glucan (Zhang et al., 2007b).

Scanning Electron Microscopy (SEM)
Micrographs were taken of untreated, COSLIF-treated, and SAA-treated switchgrass samples using a Zeiss-DSM940 (Carl Zeiss, Okerkochen, Germany). All samples were sputter-coated with gold and imaged by SEM, as described elsewhere (Moxley et al., 2008; Sathitsuksanoh et al., 2009).

X-Ray Diffraction (XRD)
X-ray diffractograms of all samples were measured using a Bruker D8 Discover X-ray diffractometer (Madison, WI).
with Cu Kα radiation (λ = 1.54178 Å) with the scanning rate of 4°/min, ranging from 10° to 60°.

**Substrate Accessibility Assays**

Total substrate accessibility to cellulase (TSAC) was determined on the basis of the maximum adsorption capacity of the TGC protein (Zhu et al., 2009a). The TGC protein is a non-hydrolytic fusion protein containing a green fluorescence protein and cellulose-binding module (Hong et al., 2007). Recombinant thioredoxin-green fluorescent protein-cellulose binding module (TGC) fusion protein was produced in *Escherichia coli* BL21 (pNT02) (Hong et al., 2007), purified by adsorption onto regenerated amorphous cellulose, and desorbed with ethylene glycol (EG) (Hong et al., 2008). EG was then removed using membrane dialysis in a 50 mM sodium citrate buffer (pH 6.0), and the TGC solution was concentrated using 10,000 Da molecular weight cut-off centrifugal ultrafilter columns (Millipore Co., Billerica, MA). CAC assays were conducted in duplicate. Mass concentration of TGC protein in the liquid phase (the fraction that had not adsorbed) was measured by sample fluorescence using a BioTek multi-detection microplate reader. Cellulose accessibility to cellulase (CAC, m²/g biomass) was measured based on the maximum TGC adsorption capacity after the sample’s lignin was blocked with 5 g/L BSA. Non-cellulose accessibility to cellulase (NCAC, m²/g biomass) was calculated from the equation NCAC = TSAC – CAC (Zhu et al., 2009a).

**Surface Graphing Software**

Lignin removal percentages, normalized CAC values, and 72 h glucan digestibility (15 FPU/g glucan) data were imported to TableCurve 3D v4.0.01 (Systat Software, Inc., San Jose, CA). A conservative smoothing method, Smooth Non-Uniform Rational B-Splines (NURBS), was applied to these data to obtain the surface presented.

**Results**

**COSLIF and SAA**

Untreated switchgrass contained approximately 35 wt% cellulose, 22 wt% hemicellulose, and 20 wt% acid-insoluble lignin. COSLIF conditions were 85% phosphoric acid at 140°C for 45 min, with 95% ethanol used as the organic solvent (Sathitsuksanoh et al., 2009, 2010). The COSLIF pretreatment resulted in decreased hemicellulose (67% removal) and slightly reduced acid-insoluble lignin (34% removal). SAA was conducted with a 120 solids to liquids loading in 10 wt% aqueous ammonia at 140°C for 14 h. Under these conditions, SAA resulted in a substrate with reduced hemicellulose (42% removal) and greatly reduced acid-insoluble lignin (74% removal). As shown in Table I, COSLIF and SAA pretreatments resulted in similar cellulose contents (approximately 50 wt%), but there were large differences in hemicellulose and lignin contents. Comparing the compositions of COSLIF- and SAA-pretreated switchgrass, the largest difference is in lignin content, a result of the different mechanisms of these pretreatments.

**Supramolecular Structures**

A qualitative assessment of the substrates was conducted using SEM microscopy. Figure 3 shows clear cell wall structures in the untreated switchgrass. Vascular bundles and parenchyma are observed in cross-section (A), and closer magnification reveals a complex ordered structure

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cellulose (wt%)</th>
<th>% Cellulose removal</th>
<th>XMG (wt%)</th>
<th>% XMG removal</th>
<th>AIL (wt%)</th>
<th>% AIL removal</th>
<th>% Mass loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>34.9 ± 0.3</td>
<td>—</td>
<td>21.6 ± 0.8</td>
<td>—</td>
<td>20.2 ± 0.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>COSLIF</td>
<td>47.3 ± 1.1</td>
<td>26.9 ± 0.4</td>
<td>12.4 ± 0.6</td>
<td>66.6 ± 2.1</td>
<td>24.6 ± 1.0</td>
<td>34.2 ± 3.9</td>
<td>46.0 ± 1.9</td>
</tr>
<tr>
<td>SAA</td>
<td>50.5 ± 1.0</td>
<td>24.6 ± 1.6</td>
<td>23.9 ± 0.7</td>
<td>42.4 ± 1.1</td>
<td>10.0 ± 0.3</td>
<td>74.4 ± 0.9</td>
<td>49.1 ± 3.0</td>
</tr>
</tbody>
</table>

XMG, xylan, mannans, and galactan; AIL, acid-insoluble lignin.
that has been cut open by the milling process (B). This surface is broken significantly by SAA (Fig. 3C and D), resulting in a delignified structure with a different supramolecular structure than the untreated material. Much different pictures are observed after COSLIF. In Figure 3E and F, the fibril structure is completely disrupted. The difference in ordered structures apparent in these micrographs is in good agreement with the expected results of these pretreatments (Fig. 1).

Quantitative Cellulose Accessibility Assay

A quantitative measure of the total substrate accessibility to cellulase (TSAC) and the cellulose accessibility to cellulase (CAC) were determined with the TGC fusion protein adsorption assay (Hong et al., 2007; Zhu et al., 2009a). Figure 4 shows schemes of TGC protein components (A), the TSAC assay (B), and the CAC assay (C). For both TSAC and CAC, COSLIF caused a much greater increase than SAA (Table II). SAA increased CAC by 1.4-fold compared to untreated switchgrass (0.49 ± 0.049 m²/g biomass), whereas COSLIF increased CAC by 16-fold. Non-cellulose accessibility to cellulase (NCAC) also increased to a much greater extent for COSLIF-pretreated substrate (twofold increase) than for SAA-pretreated material (8% increase). Since NCAC primarily represented lignin binding sites, this surprising result means that although lignin accessibility was much higher after COSLIF, the relatively small amount of lignin accessible after SAA was responsible for the large negative effect seen in the hydrolysis results. Furthermore, although SAA under the conditions tested removed a large fraction of the lignin portion, the lignin accessibility (NCAC) increased slightly compared to the untreated case, suggesting that the lignin remaining after SAA was more
evenly distributed, with a much higher surface area to volume ratio than the untreated case.

X-Ray Diffraction (XRD) Crystallinity Comparison

The XRD spectrum of untreated switchgrass reveals three peaks, corresponding to (1 0 1), (0 0 2), and (0 4 0) lattice planes (Fig. 5). SAA-pretreated materials retained the same three peaks as untreated switchgrass, while COSLIF-pretreated switchgrass effectively eliminated (1 0 1) and (0 4 0) peaks, while broadening and greatly reducing the (0 0 2) peak. Several procedures are available for calculating the crystallinity index, CrI, from XRD spectra, and these methods can give significantly differing, sometimes conflicting results (Thygesen et al., 2005). Two commonly used methods were employed. The Segal method, which compares peak height (Segal et al., 1959), is the most commonly used method, although it gives CrI indices higher than other methods (Park et al., 2009). In the peak deconvolution method, the XRD spectrum is first computationally separated into component peaks. CrI is then determined by taking the ratio of crystalline peak area to total area (Park et al., 2009). In the peak deconvolution method, the XRD spectrum is first computationally separated into component peaks. CrI is then determined by taking the ratio of crystalline peak area to total area (Park et al., 2009). Qualitatively from the XRD diffractograms, SAA had a much less drastic effect on crystallinity than COSLIF. However, after SAA it was unclear whether biomass crystallinity increased or decreased, due to conflicting results provided by the two methods employed. COSLIF resulted in a clear and drastic decrease in CrI, as expected due to the complete dissolution of biomass during this pretreatment.

Discussion

Despite retaining a large lignin fraction, COSLIF-treated substrate was found to have greatly increased cellulose accessibility, resulting in rapid hydrolysis rates. In contrast, SAA removed large amounts of lignin, while causing a mild increase in cellulose accessibility and slower hydrolysis rates. Addition of BSA prior to hydrolysis caused a large increase in the final digestibility of SAA-treated substrate, but did not impact COSLIF-treated material.

Based on the larger lignin content and increased NCAC observed after COSLIF pretreatment, a corresponding increase in digestibility following pre-hydrolysis BSA blocking might be expected, but such an increase was not observed. On the contrary, SAA-treated material exhibited strong negative effects due to lignin, despite drastically reduced lignin content and a very modest NCAC increase. Several factors may explain this apparently contradictory connection between lower lignin accessibility and higher lignin inhibition. One explanation may be that ammonia or the high level of delignification affected the cellulose structure. Zhu et al. (2008) suggested that delignification beyond about 50% might result in cellulose pore collapse, causing a decrease in cellulose accessibility. Another explanation may be different lignin properties after different pretreatments. Kumar and Wyman (2009) showed that lignin from acidic pretreatments had a significantly lower

Table II. TGC adsorption-based substrate accessibility to cellulase.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>TSAC (m²/g biomass)</th>
<th>CAC (m²/g biomass)</th>
<th>NCAC (m²/g biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.27 ± 0.08</td>
<td>0.49 ± 0.05</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>COSLIF</td>
<td>9.6 ± 0.6</td>
<td>8.0 ± 1.1</td>
<td>1.6 ± 1.3</td>
</tr>
<tr>
<td>SAA</td>
<td>1.51 ± 0.04</td>
<td>0.68 ± 0.04</td>
<td>0.83 ± 0.06</td>
</tr>
</tbody>
</table>
inhibitory effect than lignin resulting from basic pretreatments. The basic conditions used for higher lignin removal caused a change of lignin chemistry, resulting in a lignin surface that was more prone to adsorb protein. In addition, biomass dissolution achieved by COSLIF may result in lignin clustering, possibly in a manner similar to its occurrence in dilute acid pretreatment (Donohoe et al., 2008), which would further limit any inhibitory effect observed, despite the relatively large lignin content. This speculation is supported by the fact that while COSLIF caused a CAC increase of 16-fold, the NCAC increase was only 2-fold, much lower than expected based on compositional ratio alone.

In addition to the COSLIF-SAA comparison presented here, an empirical correlation between lignin removal and cellulose accessibility for 72 h enzymatic glucan digestibility was developed by combining quantitative accessibility and delignification data from this study, various other COSLIF studies (Sathitsuksanoh et al., 2009; Zhu et al., 2009a), and several publications of the Consortium for Applied Fundamentals and Innovation (CAFI) (Kumar et al., 2009; Kumar and Wyman, 2009; Wyman et al., 2009). In order to normalize CAC values for different accessibility assay conditions (temperature, protein detection method, etc.), the CAC reading from Avicel was used as a reference for this study and CAFI’s data. The CAFI-reported values based on cellulase adsorption were adjusted to the TGC-based CAC values by a factor of the cellulase adsorption on the pretreated biomass samples relative to that on Avicel (Kumar and Wyman, 2009). TableCurve 3D was used to determine a best fit for these data points ($R^2 = 0.99$), resulting in a surface representation of a variety of pretreatments, presenting glucan digestibility as a function of delignification and CAC (Fig. 6).

The substrates used to create this surface include ammonia recycle percolation (ARP) hybrid poplar, dilute acid (DA) hybrid poplar, lime hybrid poplar, untreated Avicel, regenerated amorphous cellulose (RAC), COSLIF corn stover, DA corn stover, untreated corn stover, COSLIF switchgrass, SAA switchgrass, untreated switchgrass, COSLIF bamboo, untreated bamboo, COSLIF common reed, and untreated common reed. While it was impossible to logically relate hemicellulose removal, CAC, and glucan digestibility with this data set (figure not shown), the effect of lignin removal and CAC on digestibility displayed a clear trend. Analyzing the effect of lignin removal at low CAC, Figure 6 shows an increase from $\sim$10% to 20% digestibility (untreated substrates) to $\sim$60% digestibility (SAA switchgrass, Avicel). When CAC is increased, however, even when little lignin is removed (DA corn stover, COSLIF substrates), much greater glucan digestibility is enabled, up to 97% (COSLIF corn stover). This correlation suggests that increasing the accessibility of the substrate is critical for achieving high lignocellulose digestibility. Lignin removal is important for increasing the digestibility of low-CAC materials, but once high CAC is achieved, removing lignin is a much less impactful pretreatment objective.

In conclusion, increasing cellulose accessibility was a more important pretreatment consideration than delignification for effectively releasing sugars from recalcitrant lignocellulose at high yield. High levels of delignification without a significant increase in CAC did not result in a correspondingly large increase in glucan digestibility. The COSLIF pretreatment was capable of greatly increasing the cellulose accessibility of switchgrass, resulting in rapid digestibility and high final sugar yield, even at low enzyme loadings. Further pretreatment development efforts are recommended to focus on increasing substrate accessibility, the most important factor in enzymatic biomass saccharification, using low-cost processes.

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