Comparative Study of Corn Stover Pretreated by Dilute Acid and Cellulose Solvent-Based Lignocellulose Fractionation: Enzymatic Hydrolysis, Supramolecular Structure, and Substrate Accessibility

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ABSTRACT: Liberation of fermentable sugars from recalcitrant biomass is among the most costly steps for emerging cellulosic ethanol production. Here we compared two pretreatment methods (dilute acid, DA, and cellulose solvent and organic solvent lignocellulose fractionation, COSLIF) for corn stover. At a high cellulase loading [15 filter paper units (FPUs) or 12.3 mg cellulase per gram of glucan], glucan digestibilities of the corn stover pretreated by DA and COSLIF were 84% at hour 72 and 97% at hour 24, respectively. At a low cellulase loading (5 FPUs per gram of glucan), digestibility remained as high as 93% at hour 24 for the COSLIF-pretreated corn stover but reached only ~60% for the DA-pretreated biomass. Quantitative determinations of total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC) based on adsorption of a non-hydrolytic recombinant protein TGC were measured for the first time. The COSLIF-pretreated corn stover had a CAC of 11.57 m²/g, nearly twice that of the DA-pretreated biomass (5.89 m²/g). These results, along with scanning electron microscopy images showing dramatic structural differences between the DA- and COSLIF-pretreated samples, suggest that COSLIF treatment disrupts microfibrillar structures within biomass while DA treatment mainly removes hemicellulose. Under the tested conditions COSLIF treatment breaks down lignocellulose structure more extensively than DA treatment, producing a more enzymatically reactive material with a higher CAC accompanied by faster hydrolysis rates and higher enzymatic digestibility.

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KEYWORDS: biofuels; biomass; cellulose accessibility to cellulase; cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF); dilute acid pretreatment; substrate accessibility

Introduction

Production of second generation biofuels such as cellulosic ethanol from renewable lignocellulosic biomass will lead the biindustrial revolution necessary to the transition from a fossil fuel-based economy to a sustainable carbohydrate economy (Lynd et al., 2008; Zhang, 2008). Use of biofuels will offer several benefits, including reduced greenhouse gas emissions, decreased competition with tightening food supplies, enhanced rural economic development, and increased national energy security (Demain et al., 2005; Himmel et al., 2007; Lynd et al., 2002; Zhang et al., 2006b).

Lignocellulosic biomass, such as agricultural and forestry residues, municipal and industrial solid wastes, and herbaceous and woody bioenergy plants, is a natural complex composite primarily consisting of three biopolymers: cellulose, hemicelluloses, and lignin (Fengel and Wegener,
Efficient, cost-competitive production of fermentable sugars from recalcitrant biomass remains the largest obstacle to emerging cellulosic ethanol biorefineries (Lynd et al., 2008; Wyman, 2007; Zhang, 2008). Biomass saccharification via biological conversion involves two steps—lignocellulose pretreatment or fractionation followed by enzymatic cellulose (and perhaps hemicellulose) hydrolysis. Dilute acid pretreatment (DA), typically using sulfuric acid, is the most investigated pretreatment method (Bernardez et al., 1993; Grethlein, 1985; Grethlein and Converse, 1991; Lloyd and Wyman, 2005; Ooshima et al., 1990; Schell et al., 2003; Thompson et al., 1992). Conducted at relatively high temperatures (150–200°C) and pressures (120–200 psia), DA pretreatment solubilizes acid-labile hemicellulose and thereby disrupts the lignocellulosic composite linked by covalent bonds, hydrogen bonds and van der Waals forces (Burns et al., 1989; Lloyd and Wyman, 2005; Ooshima et al., 1990). As a result, the condensed lignin remains on the surface of crystalline cellulose following DA, potentially hindering subsequent enzymatic hydrolysis (Bernardez et al., 1993; Jeoh et al., 2007; Liu and Wyman, 2003; Lloyd and Wyman, 2005). In cellulose solvent and organic solvent lignocellulose fractionation (COSLIF), a cellulose solvent (e.g., concentrated phosphoric acid or ionic liquid) enables the crystalline structure of cellulose to be disrupted. This type of pretreatment can also be carried out at low temperatures (e.g., ~50°C) and the atmospheric pressure where sugar degradation is minimized (Moxley et al., 2008; Zhang et al., 2007a). Subsequent washing steps are used to fractionate biomass; a first washing with an organic solvent to remove lignin; and a second washing with water to remove fragments of partially hydrolyzed hemicellulose. The COSLIF approach produces highly reactive amorphous cellulose, which can be enzymatically hydrolyzed quickly with high glucan digestibility yield (Moxley et al., 2008; Zhang et al., 2007a).

The root causes of biomass recalcitrance could be attributed to a number of factors, such as substrate accessibility to cellulase, cellulose degree of polymerization (DP), cellulose crystallinity, lignin content and structure, and hemicellulose content (Chandra et al., 2007; Himmel et al., 2007; Kim and Holtzapple, 2005; Zhang and Lynd, 2004; Zhang et al., 2006b). A functionally based mathematical model of fungal enzyme-based enzymatic cellulose hydrolysis has been developed, accounting for cellulose characteristics (DP and substrate accessibility) and different modes of action for endoglucanase and cellobiohydrolase enzyme system components (Zhang and Lynd, 2006). This model not only correlates disparate phenomena reported in the literature but also clearly suggests that low cellulose accessibility is the most important substrate characteristic limiting enzymatic hydrolysis rates (Zhang and Lynd, 2006). More recently, a quantitative assay for determining cellulose accessibility to cellulase (CAC) has been established based on adsorption of a non-hydrolytic fusion protein (TGC) containing a cellulose-binding module and a green fluorescence protein (Hong et al., 2007). This new approach more accurately assesses substrate characteristic related to enzymatic cellulose hydrolysis than traditional methods such as nitrogen adsorption-based Brunauer–Emmett–Teller (BET), size exclusion, and small angle X-ray scattering (Hong et al., 2007; Zhang and Lynd, 2004). Regenerated amorphous cellulose (RAC) that is prepared from microcrystalline cellulose (Avicel) has ~20-fold higher CAC (Hong et al., 2007, 2008b) and exhibits much faster enzymatic hydrolysis rates than microcrystalline cellulose (Zhang et al., 2006a), which is in agreement with the model prediction that increasing CAC is more important for increasing hydrolysis rates than decreasing DP (Zhang and Lynd, 2006). The CAC value of Avicel (m² per gram of Avicel) based on the TGC adsorption was only one-tenth of that based on the BET method (Marshall and Sixsmith, 1974), implying that about 90% gross surface area measure based on nitrogen adsorption cannot be accessible to large-size cellulase protein molecules, at least initially. Traditional size exclusion techniques are labor intensive and cannot distinguish the real cellulase binding area (110 face) or ignore the external surface area (Hong et al., 2007; Zhang and Lynd, 2004).

Enzymatic hydrolysis of pretreated lignocellulose is more challenging than enzymatic hydrolysis of pure cellulose because any remaining lignin and residual hemicellulose could adsorb cellulase components and thereby block or impede cellulose hydrolysis (Berlin et al., 2005; Bernardez et al., 1993; Converse et al., 1990; Grethlein and Converse, 1991; Kurabi et al., 2005; Ooshima et al., 1990; Wyman, 2007). The total substrate accessibility has been measured previously by using cellulase-size molecule exclusion (Burns et al., 1989; Esteghlalian et al., 2001; Grethlein, 1985; Thompson et al., 1992), low-temperature cellulase adsorption (Gerber et al., 1997; Kumar and Wyman, 2008; Lee et al., 1994; Lu et al., 2002; Mooney et al., 1998) or labeled cellulase (Jeoh et al., 2007; Palonen et al., 2004). However, in pretreated lignocellulose materials it remains relatively challenging to quantitatively differentiate accessibilities for cellulose and non-cellulose fractions.

In this study, we compared the enzymatic hydrolysis behaviors (enzymatic cellulose hydrolysis rates and yields) of corn stover pretreated by DA and COSLIF approaches. We also used scanning electron microscopy (SEM) to examine the supramolecular structures of DA- and COSLIF-pretreated corn stover samples. Additionally, we developed and applied new quantitative assays for substrate accessibility by distinguishing cellulose and non-cellulose fractions of pretreated lignocellulose.

**Materials and Methods**

**Chemicals and Microorganism**

All chemicals were reagent grade, purchased from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Fungal cellulase Spezyme CP was prepared...
gifted from Genencor (Palo Alto, CA). Novozymes 188 \( \beta \)-glucosidase was purchased from Sigma. Microcrystalline cellulose (Avicel PH105) was purchased from FMC (Philadelphia, PA). RAC was prepared through Avicel dissolution in concentrated phosphoric acid followed by regeneration in water (Zhang et al., 2006a). Corn stover was obtained from the National Renewable Energy Laboratory (NREL, Golden, CO). Corn was grown from biomass AgriProducts (Harlan, IA). The tub-ground materials were approximately 9 months old (harvested in fall 2005). The recombinant thioredoxin–green fluorescent protein–cellulose binding module (TGC) fusion protein was produced in *Escherichia coli* BL21 (pNT02) (Hong et al., 2007) and purified by affinity adsorption on RAC followed by modest desorption using ethyl glycol (EG) (Hong et al., 2008b). The EG was removed by using membrane dialysis in a 50 mM sodium citrate buffer (pH 6.0). The TGC protein solution was re-concentrated using a 10,000 Da molecular weight cut-off centrifugal ultrafilter columns (Millipore Co., Billerica, MA).

### Corn Stover Pretreatments

Dilute sulfuric acid pretreated corn stover was produced in a pilot-scale continuous vertical reactor at 190°C, 0.048 g acid/g dry biomass, 1 min residence time, and a 30% (w/w) total solid loading by using procedures discussed elsewhere (Schell et al., 2003). The acidic slurry was stored at 4°C prior to use. Before experiments, the slurry was dewatered and the pretreated solids were washed with deionized water until the pH of the washed water reached pH \( \sim 6 \).

COSLIF was conducted as described previously (Moxley et al., 2008; Zhang et al., 2007a). One gram of dry corn stover (particle size >60 mesh and <40 mesh screen, i.e., 0.25–0.42 mm in diameter) in a 50-mL plastic centrifuge tube was mixed with 8 mL of 84% phosphoric acid using a glass rod, and the mixed slurry was then incubated at a 50°C water bath for 20 or 45 min. The reaction was then stopped by an ice-water bath. Forty milliliters of acetone was then added to precipitate dissolved cellulose and hemicellulose. The slurry was spun down for 20 min at room temperature using a swinging bucket centrifuge at 3,600 rpm. The pellets were re-suspended and washed in 40 mL of acetone twice more. After three acetone washes, the pellets were washed two more times with water. The residual amorphous solid pellet was neutralized to pH 5–7 using 2 M sodium carbonate.

The amounts (dry weight percentages) of sugars and lignins in the DA- and COSLIF-pretreated corn stover were measured using a modified quantitative saccharification method, which can determine acid-labile hemicellulose composition more accurately (Moxley and Zhang, 2007). The compositions of ashes and extractives in the intact and pretreated biomass samples were measured according to the protocol from NREL LAP-002 (Sluiter et al., 2006). The protein contents in the biomass samples were measured by the ninhydrin assay (Starcher, 2001).

### Enzymatic Hydrolysis of Pretreated Corn Stover

The pretreated corn stover samples were diluted to 10 g glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) supplemented with 0.5 g/L sodium azide for enzymatic hydrolysis. All hydrolysis experiments were carried out in a rotary shaker at 250 rpm and 50°C. Two enzyme loadings were used: (1) 15 FPUs cellulase and 30 units Novozyme 188 \( \beta \)-glucosidase per gram of glucan (12.3 mg cellulase and 9.4 mg \( \beta \)-glucosidase per gram of glucan) as well as (2) 5 FPUs cellulase and 30 units \( \beta \)-glucosidase per gram of glucan (4.1 mg cellulase and 9.4 mg \( \beta \)-glucosidase per gram of glucan). Eight hundred microliters of evenly-mixing slurry were taken at different time and centrifuged at 13,000 rpm for 5 min. Exactly 500 \( \mu \)L of the supernatant was transferred to another microtube and held at room temperature for 30 min, ensuring conversion of nearly all cellubiose to glucose by free \( \beta \)-glucosidase. The supernatant was acidified by adding 30 \( \mu \)L of 10% (w/w) sulfuric acid and then frozen overnight. The freshly thawed liquid samples were mixed well and then centrifuged at 13,000 rpm for 5 min to remove any solid sediments. Glucose concentration in the clear supernatants was measured by HPLC using a Bio-Rad HPX-87H column operating at 65°C with a mobile phase of 0.005 M sulfuric acid at a flow rate of 0.6 mL/min (Zhang and Lynd, 2003a, 2005a). After 72-h hydrolysis, the remaining hydrolysis slurries were transferred to 50-mL centrifuge tubes and centrifuged at 3,600 rpm for 20 min. After decanting the supernatant, the pellets were re-suspended in 20 mL of water and then centrifuged to remove soluble sugars. After centrifugation, the remaining sugars and lignin in the lyophilized pellets were measured by quantitative saccharification. The soluble glucose and xylose was measured by HPLC as described above.

Glucan digestibility (\( X_G \)) at the end of hydrolysis (hour 72) was calculated using the ratio of soluble glucose (\( G_{solv} \)) in the supernatant to the sum of \( G_{solv} \) and the residual glucan expressed in terms of glucose equivalents (\( G_{res} \)) in the solid phase (Eq. 1) (Moxley et al., 2008; Zhang et al., 2007b).

\[
X_G = \frac{G_{solv}}{G_{solv} + G_{res}} \times 100\%
\]  

(1)

### Scanning Electron Microscopy

Supramolecular structures of the intact and pretreated corn stover samples were examined by scanning electron microscopes, as described elsewhere (Selig et al., 2007; Zhang et al., 2006a).
Protein Mass Concentration Assays

The mass concentrations of the non-adsorbed proteins—bovine serum albumin (BSA), β-glucosidase, and cellulase—were measured by the BioRad Bradford protein kit (Richmond, CA) with BSA as the protein standard. Mass concentration of the non-adsorbed TGC protein was measured based on fluorescence reading using a BioTek multi-detection microplate reader, as described elsewhere (Hong et al., 2007, 2008a).

Protein Adsorption

Adsorption of cellulase, β-glucosidase, and BSA on pure cellulose samples or birch xylan was conducted at room temperature for 1 h in a 50 mM sodium citrate buffer at various concentrations (0.5–100 g Avicel/L, 0.1–10 g RAC/L or 0.5–50 g birch xylan/L). After centrifugation at 13,000g for 5 min, the protein concentrations in the supernatant were measured by the Bradford method, as described previously (Zhang and Lynd, 2003b, 2005b).

The maximum TGC adsorption capacity was calculated based on the Langmuir isotherm (i.e., a fixed amount of adsorbent in terms of various concentrations of TGC). Eight hundred microliters of pretreated corn stover slurry solutions containing 1 g glucan/L and a final TGC concentration from 0.05 to 0.3 g/L was well mixed in a 50 mM sodium citrate buffer (pH 6.0) at room temperature for 1 h. After centrifugation, the free TGC concentrations were measured by the BioTek multidetection microplate reader. A_{max,TGC} was calculated based on the maximum TGC adsorption capacity of the pretreated samples. A_{max, BSA/TGC} was calculated based on the maximum TGC adsorption capacity of the pretreated samples that had been blocked by adding an excess of BSA (5 g/L, final) for 1 h before adding TGC. The BAS blocking was conducted at a pH 4.80. After BSA blocking, the pH was adjusted back to 6.00 by adding sodium carbonate, and TGC adsorption was assessed by fluorescence measurement as described above.

Quantitative Substrate Accessibility Determination

Cellulase adsorption on the surface of cellulose can be described by the Langmuir equation:

\[ E_a = \frac{W_{max}K_pE_t}{1 + K_pE_t} \]  

(2)

in which \( E_a \) is adsorbed protein (μmol/L), \( W_{max} \) the maximum protein adsorption per L (μmol/L), \( E_t \) the free cellulase (μmol/L), and \( K_p \) the dissociation constant (\( K_p = E_t/E_fS \)) in terms of L/g cellulose. The \( W_{max} \) and \( K_p \) values in Equation (2) can be calculated by a number of mathematical data fitting methods.

Results

For COSLIF pretreatment, we have previously found that (1) phosphoric acid at concentrations beyond the critical value (≥ 83%) acts as a cellulose solvent, (2) reaction time should be sufficient to dissolve biomass but be short enough to prevent complete hydrolysis, and (3) reaction temperature is set below 60°C for no detectable xylose degradation (Moxley et al., 2008). The optimal reaction condition for corn stover is 84% phosphoric acid, 50°C, and 45 min. Although the DA pretreatment conditions used in this study are known to produce enzymatically digestible material, they have not been optimized. Table I shows glucan, hemicellulose, and lignin contents of the COSLIF-pretreated and DA-pretreated corn stover. COSLIF pretreatment
removes more lignin than DA pretreatment, producing a material with lower levels of residual lignin (19.7% vs. 30.3%, respectively). On the other hand, COSLIF pretreatment removes less hemicellulose than DA pretreatment; residual levels of hemicellulose are 6.2% and 3.4%, respectively.

**Enzymatic Hydrolysis**

Figure 1 shows the glucan digestibility profiles for the corn stover pretreated by DA and COSLIF at two different enzyme loadings (A, 15 FPU cellulase per gram of glucan; and B, 5 FPU cellulase per gram of glucan). At the high enzyme loading, glucan digestibility of the COSLIF-pretreated corn stover (45 min) was greater than 90% at hour 12 and reached 97% at hour 24. When COSLIF pretreatment time was decreased to 20 min, hydrolysis rates were slower and digestibility was lower. But a long COSLIF reaction time was not recommended because it resulted in low solid glucan retention. If concentrated phosphoric acid completely hydrolyzed cellulose to soluble sugars, cost-effective separation of soluble sugars and soluble acid would be challenging, similar to what occurs using concentrated sulfuric acid for cellulose saccharification (Fengel and Wegener, 1984; Zhang et al., 2007a). In contrast, DA-pretreated corn stover exhibited considerably slower enzymatic hydrolysis rates, with glucan digestibility reaching 84% at hour 72. At a low enzyme loading (5 FPU per gram of glucan), final glucan digestibility of the COSLIF-pretreated biomass was 93% within 24 h, while digestibility of DA-pretreated biomass only reached 60% at hour 72. The significant difference in observed enzymatic hydrolysis behaviors between the COSLIF-pretreated and DA-pretreated biomass samples motivated additional studies to develop a better understanding of the causes.

**Supramolecular Structures**

The supramolecular structure changes in corn stover before and after the different pretreatments are shown by using SEM (Fig. 2). The intact plant cell wall structure of corn stover shows evidence of plant cell wall vascular bundles and a highly fibrillar structure (A). Dilute acid pretreatment disrupts the lignocellulosic structure by mainly dissolving hemicellulose. As a result, major microfibrous cellulose structures remain (Fig. 2B) and some lignin or lignin-carbohydrate complexes may be condensed on the surface of the cellulose fibers. Treatment with concentrated H₃PO₄ significantly alters the fibrillar structure. A well-pretreated lignocellulose (corn stover) COSLIF sample (84.0% H₃PO₄, 50°C and 45 min) shows no clear fibrous structure (Fig. 2C). These qualitative images are consistent with the observations that faster hydrolysis rates and higher glucan digestibilities are obtained for COSLIF-pretreated biomass than for DA-pretreated biomass.

**Protein Adsorption on Pure Cellulosic Substrates**

Adsorption of three proteins (cellulase, β-glucosidase, and BSA) was conducted on pure crystalline cellulose—Avicel.
The free cellulase concentration decreased with increasing cellulose concentrations. In contrast, there was no adsorption of β-glucosidase and BSA because they do not contain cellulose-binding modules. Similarly, cellulase was adsorbed by RAC but neither were β-glucosidase and BSA (data not shown). Because significant cellulose hydrolysis occurs (especially for amorphous cellulose fraction) during the active cellulase adsorption process, accompanied by a change in substrate accessibility (Steiner et al., 1988), we have proposed to determine CAC based on adsorption of a non-hydrolysis fusion protein, TGC, containing a cellulose-binding module (CBM) and a green fluorescent protein (GFP) (Hong et al., 2007).

Figure 4 shows the adsorption equilibrium curves of the TGC protein on Avicel and RAC. The maximum protein adsorption capabilities ($A_{max}$) after data fitting were determined to be $7.38 \pm 0.13 \mu$mol TGC per gram of RAC and $0.32 \pm 0.01 \mu$mol TGC per gram of Avicel. The CAC value of RAC ($51.94 \pm 0.91 \text{ m}^2 \text{ per gram of RAC}$) was greater than 20-fold higher than that of Avicel ($2.25 \pm 0.07 \text{ m}^2 \text{ per gram of Avicel}$). The effects of adsorption temperature and substrate concentration on maximum adsorption capacity were also investigated. No significant change was observed in $A_{max}$ over ranges of 1–5 g RAC/L and 2–50 g Avicel/L at both room temperature and 50°C (data not shown). In addition, the TGC maximum binding capacity on hemicellulose was determined to be $0.17 \mu$mol TGC per gram of birch xylan.

**Lignocellulosic Substrate Accessibilities**

In order to quantitatively determine pretreated lignocellulosic substrate accessibility to cellulase in the presence of...
residual lignin and hemicellulose (Table I), it is important to distinguish substrate accessibility for cellulose and non-cellulose (lignin-rich) fractions. Here we have applied a new scheme for quantitatively determining CAC and NCAC for pretreated lignocellulosic substrates (Fig. 5), based on the facts that (i) BSA can irreversibly bind with the accessible lignin fraction of lignocellulosic biomass (Berlin et al., 2005; Yang and Wyman, 2006) and (ii) BSA cannot bind with cellulose (Fig. 3). Similarly to several substrate accessibility assays, TSAC can be determined based on one-protein adsorption, where in this case TGC is used rather than a hydrolytic cellulase. For CAC measurement, a high concentration of BSA (5 g/L, final) was mixed with the pretreated biomass for blocking accessible lignin, where 5 g BSA/L was much higher than $A_{\text{max,lignin}} \times$ non-cellulose content/1 g biomass/L prior to the TGC adsorption. Consequently, the TGC protein was then added to assess the maximum adsorption capacity of the blocked pretreated biomass, and the maximum TGC adsorption capacity of the BSA-blocked biomass was used to represent CAC. The difference between TSAC and CAC was NCAC that represented the accessibility of the non-cellulose (lignin-rich) fraction. Figure 6 shows TGC adsorption equilibrium curves obtained using corn stover pretreated by either DA or COSLIF with or without BSA blocking. The $A_{\text{max,TGC}}$ and $A_{\text{max,BSA/TGC}}$ are 2.05 ± 0.15 and 1.64 ± 0.13 μmol per gram of COSLIF-pretreated biomass and 1.09 ± 0.08 and 0.84 ± 0.05 μmol per gram of DA-pretreated biomass, respectively. The impact of remaining hemicellulose on CAC was very low because of low hemicellulose contents in the COSLIF- and DA-pretreated samples relative to cellulose contents and low TGC binding capacity of hemicellulose compared to the $A_{\text{max}}$ of pretreated cellulose.

Table II shows the TSAC, CAC and NCAC values of the pretreated corn stover. For COSLIF-pretreated sample, TSAC was 14.44 ± 1.09 m² per gram of biomass, where CAC and NCAC were 11.57 ± 0.90 and 2.88 ± 0.20 m² per gram of biomass, respectively. The TSAC, CAC, and NCAC of the DA-pretreated sample were 7.66 ± 0.55, 5.89 ± 0.34, and 1.78 ± 0.09 m² per gram of biomass, respectively. The normalized CAC of the COSLIF-pretreated and DA-pretreated biomass were 19.94 ± 1.53 and 10.90 ± 0.63 m² per gram of cellulose, respectively. The normalized NCAC of the COSLIF-pretreated and DA-pretreated biomass were 6.89 ± 0.45 and 3.48 ± 0.19 m² per gram of non-cellulose, respectively, suggesting that non-cellulose fraction of the COSLIF-pretreated biomass had higher substrate accessibility based on mass weight than that of the dilute acid-pretreated biomass.

**Discussion**

Hydrolysis results clearly showed that soluble sugars were released faster and to a greater extent in the COSLIF-pretreated corn stover than in the DA-pretreated corn stover studied here. Such differences (93% digestibility for COSLIF samples achieved within 24 h vs. 60% for DA samples within 72 h) were more significant at a low enzyme loading of 5 FPUs. Although intensive efforts have been made to increase specific cellulase activity and decrease cellulase production costs (Himmel et al., 2007; Lynd et al., 2008; Zhang et al., 2006b), additional reductions in enzyme usage costs are important to promote the economy of biorefineries.

Beyond different enzymatic hydrolysis characteristics, these substrates exhibited significant differences in their supramolecular structures and substrate accessibilities. Qualitative SEM images clearly indicate that the cellulose solvent (concentrated phosphoric acid) treatment conducted
at a low temperature substantially disrupts the biomass fibrillar structure (Fig. 2C), whereas dilute acid pretreatment at a higher temperature does not (Fig. 2B). The CAC of the COSLIF-pretreated corn stover was nearly double that of DA-pretreated biomass, consistent with the hypothesis that CAC was one of the most important (rate-limiting) factors influencing enzymatic hydrolysis (Zhang and Lynd, 2006). However, while changes in CAC appear to be a major causative factor for the changes in hydrolysis rates (especially initial rates), additional factors involving feedstock structure and composition undoubtedly contribute to the increased glucan digestibility (enzymatic hydrolysis yields) observed in COSLIF-pretreated as compared to DA-pretreated samples.

Quantitative determination of lignocellulosic substrate accessibility to enzymes (or chemicals) is important to understand the mechanism of cellulose hydrolysis and modeling hydrolysis kinetics (Zhang and Lynd, 2004, 2006). Such information may help improve the methods for evaluating pretreatment efficiency. As discussed in the introduction, total substrate accessibility has been measured previously using several approaches. The TGC-based CAC method used here has been previously applied to determine the CAC of pure cellulosic samples (Hong et al., 2007). This work found that a transition from substrate excess to substrate limitation occurred over the course of the process of enzymatically hydrolyzing crystalline cellulose (Hong et al., 2007).

Although it is well-known that cellulase adsorption on cellulose often can be empirically described using a Langmuir isotherm model (Lynd et al., 2002; Zhang and Lynd, 2004), Jeoh et al. (2007) attempted to estimate total substrate accessibility based on the linear range of cellulase adsorption. This estimation can be good only when a very low concentration cellulase is used for adsorption experiments because any small variations in free protein measurement may result in large deviations in calculated A_{max} values in the first order equation for approximation of the Langmuir equation. Another deviation could be large especially for the easily hydrolyzed pretreated biomass studied here (COSLIF samples) because some hydrolysis occurs during the active stage of cellulase adsorption, even when experiments are carried out at a decreased temperature (Beldman et al., 1987; Ooshima et al., 1983; Steiner et al., 1988).

But not all of the accessible surface in pretreated lignocellulosic biomass can be hydrolyzed by cellulase, as reflected here by the measurement of NCAC. Previous efforts reported in the literature to assay lignin accessibility to enzymes (lignin being the dominant component in the non-cellulose fraction) can be divided into two classes: (1) extracting lignin from lignocellulosic materials using chemicals (Bernardez et al., 1993; Converse et al., 1990; Gerber et al., 1997; Lee et al., 1994; Mooney et al., 1998; Palonen et al., 2004) and (2) leaving primarily only lignin remaining by hydrolyzing (dissolving away) the hemicellulose and cellulose fractions (Bernardez et al., 1993; Converse et al., 1990; Ooshima et al., 1990; Palonen et al., 2004). Both classes of methods may suffer from the large changes in lignin (or non-cellulose) substrate properties that occur during extraction or hydrolysis.

Here we attempted to distinguish cellulose accessibility and non-cellulose (lignin-rich) fraction accessibility without substrate hydrolysis or lignin extraction. We blocked accessible lignin by using 5 g BSA/L prior to the TGC adsorption because BSA can non-specifically irreversibly bind with lignin (Berlin et al., 2005) and cannot bind with cellulose (Fig. 3). The results shown in Table II suggest that TSAC overestimated CAC by 24.8% for the COSLIF-pretreated biomass (14.44 vs. 11.57 m^2/g) and by 30.0% for the DA-pretreated biomass (7.66 vs. 5.89 m^2/g).

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<th>TSAC</th>
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<td>μmol/g biomass</td>
<td>m^2/g biomass</td>
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<tr>
<td>Non-pretreated</td>
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<td>1.13 ± 0.006</td>
<td>0.06 ± 0.001</td>
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<tr>
<td>COSLIF</td>
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<td>DA</td>
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Table II. Total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC) values of corn stover samples before pretreatment and after COSLIF and DA pretreatment.
COSLIF-pretreated biomass (2.88 m²/g biomass) is higher than that of the DA-pretreated biomass (1.78 m²/g biomass).

In conclusion, these results improved the understanding of how DA- and COSLIF-pretreatments have different mechanisms of reducing biomass recalcitrance to enzymatic hydrolysis. DA pretreatment substantially removes hemicellulose and thereby breaks recalcitrance of biomass. COSLIF pretreatment partially removes lignin and hemicellulose but also substantially disrupts the fibrillar structure of biomass. The resulting faster hydrolysis rates and higher glucaen enzymatic digestibility of COSLIF-pretreated corn stover as compared to DA pretreated corn stover are in a good agreement with (i) more efficient biomass structure destruction qualitatively shown by SEM images and (ii) the almost twofold higher CAE levels measured by quantitative TGC adsorption. COSLIF pretreatment produces more highly digestible material than DA pretreatment, but similar to DA pretreatment it is not yet commercially proven and its economic viability for use in large scale biorefining remains to be demonstrated. The COSLIF technology remains at an earlier stage of development than DA pretreatment technology and more detailed economic analysis based on rigorous Aspen-plus models are needed to understand its potential for practical applications.

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