Sulfite (SPORL) pretreatment of switchgrass for enzymatic saccharification

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SPORL pretreatment of switchgrass was optimized by Response Surface Methodology.
SPORL was compared with acid and alkali in pretreating switchgrass.
Hemicellulose removal and lignin sulfonation in SPORL improved hydrolysability.
SPORL pretreated switchgrass had better hydrolysability than DA and NaOH ones.
Removing hemicellulose was more critical than lignin to SPORL substrate hydrolysis.

ABSTRACT

SPORL (Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose) pretreatment was applied to switchgrass and optimized through an experimental design using Response Surface Methodology within the range of temperature (163–197 °C), time (3–37 min), sulfuric acid dosage (0.8–4.2% on switchgrass), and sodium sulfite dosage (0.6–7.4% on switchgrass). Performance of SPORL was compared with that of dilute acid (DA) and alkali (AL) in switchgrass pretreatment. Results indicated that SPORL pretreatment improved the digestibility of switchgrass through sufficiently removing hemicellulose, partially dissolving lignin, and reducing hydrophobicity of lignin by sulfonation. The removal of hemicellulose was more critical to substrate digestibility than the removal of lignin during SPORL pretreatment. SPORL pretreated switchgrass had better enzymatic digestibility than DA and AL pretreated ones. The SPORL pretreated switchgrass could be hydrolyzed by 83% within 48 h with 15 FPU (filter paper unit) cellulase and 30 CBU (cellobiose unit) β-glucosidase/g cellulose.

1. Introduction

Among the available energy crops for cellulosic ethanol production, switchgrass is a promising one because of its high productivity, low inputs, and positive environmental effects (Keshwani and Cheng, 2009). Switchgrass grows throughout North America because it is tolerant to heat, cold, and draught (Casler et al., 2007). Switchgrass provides habitat for grass birds and nesting birds (Roth et al., 2005) and wildlife (Dunn et al., 1993). Switchgrass has an extensive root system, and increases soil carbon storage by carbon sequestration effect (Bransby et al., 1998). It also improves surface water by reducing phosphorous and nitrogen in runoff (Sanderson et al., 2001) and removing herbicides (Mersie et al., 2006).

Physical and chemical pretreatment methods have been applied to switchgrass for cellulosic ethanol conversion. For example, physical pretreatment by grinding and milling (Bridgeman et al., 2007) and mechanical and thermal processing (Rijal et al., 2012) were used to improve enzymatic hydrolysis of switchgrass. AFEX pretreatment of switchgrass was investigated for optimum pretreatment conditions, and resulted in 6-fold improvement in enzymatic hydrolysis of pretreated switchgrass (Alizadeh et al., 2005). Dilute acid pretreatment of switchgrass was studied for optimal acid loading and pretreatment temperature (Chung et al., 2005; Dien et al., 2006). Lime pretreatment of switchgrass was conducted and combined with simultaneous saccharification and fermentation (Chang et al., 1997, 2001). Aqueous ammonium hydroxide pretreatment reduced about half lignin and hemicellulose content in switchgrass (Isci et al., 2008; Pryor et al., 2012). Microwave-alkali pretreatment of switchgrass was investigated for optimum processing condition, and achieves high yield of reducing carbohydrates (Hu and Wen, 2008). Organosolv was also an effective pretreatment for switchgrass by selectively removing lignin and hemicelluloses (Cateto et al., 2011; Cybulska et al., 2012).

SPORL (Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose) is a novel method recently developed for cellulosic ethanol production from lignocelluloses (Zhu et al., 2009). The
SPORL is a two-step pretreatment, a chemical treatment with sulfite followed by a mechanical size reduction. With moderate amount of sulfurous acid and sulfite dosages, SPORL pretreatment worked effectively with softwoods (Shuai et al., 2010; Tian et al., 2010; Zhu et al., 2009, 2010a) and hardwoods (Wang et al., 2009, 2012; Zhu et al., 2011). During the SPORL pretreatment, most of hemicelluloses are removed in the form of fermentable sugars with limited formation of fermentation inhibitors (such as, furfural and hydroxymethylfurfural-HMF). The removal of hemicelluloses and lignin makes the SPORL substrate readily digestible by enzymes. In addition, the energy consumption for size reduction after chemical treatment was significantly reduced (Zhu et al., 2010b). Furthermore, the SPORL pretreatment has a great scalability because the process can adapt existing infrastructure and equipment in paper industry.

With the success of SPORL in woody biomass pretreatment, in the present research we investigated the performance of SPORL in switchgrass pretreatment. Specific objectives of this study included (1) optimizing SPORL pretreatment conditions for switchgrass using Response Surface Methodology; (2) investigating the changes of cell wall components of switchgrass during the pretreatment; (3) evaluating enzymatic digestibility of SPORL-pretreated switchgrass; (4) monitoring the formation of fermentation inhibitors during the pretreatment; and (5) comparing the performance of SPORL, dilute acid, and alkali methods in switchgrass pretreatment.

2. Experimental

2.1. Materials

Switchgrass was harvested from the experimental farm of University of Wisconsin-Madison at Arlington, WI and stored indoors in bales as described previously (Shinn et al., 2010). The switchgrass was air-dried, milled using a Wiley mill to pass through a 1-mm screen, sealed in a zipper-lock bag, and kept in a 4 °C refrigerator until use. Commercial cellulase and β-glucosidase were provided by Novozymes (Franklinton, NC). All chemical reagents were purchased from Fisher Scientific (Pittsburgh, PA) and used as received.

2.2. SPORL pretreatment

Pretreatments were conducted in batch using a MARS microwave reactor, manufactured by CEM Corporation (North Carolina, MA). Because no mechanical stirring was available in the reaction vessels, a high liquor-to-solid ratio 7 (v/w) was used for all experiments to ensure homogeneous pretreatment. Dry switchgrass (15 g per batch) and prepared pretreatment liquor (water containing sodium sulfite and sulfuric acid) were loaded in a 300-ml reaction vessel and evenly mixed. The vessel was then sealed and loaded into the microwave reactor for heating. Heating process was programmed and controlled by a computer. The reactor was heated to the target temperature in 10 min and then held for a preset period. When the pretreatment was complete, a built-in circulation fan cooled down the vessel to room temperature. The spent pretreatment liquor was sampled for analysis of furfural, HMF, soluble lignin, and monosaccharides. Solid residue (pretreated switchgrass) was separated from the spent liquor by filtration, washed thoroughly with water, and kept in a zipper-lock bag for chemical composition analysis and enzymatic hydrolysis.

2.3. Dilute acid (DA) and alkali (AL) pretreatments

Dilute acid and alkali pretreatments were conducted in the same manner as that of SPORL pretreatment as described in Section 2.2. The only difference was the pretreatment chemicals. Instead of sulfite and sulfuric acid, sulfuric and sodium hydroxide were used for dilute acid and alkali pretreatments, respectively.

2.4. Adsorption of enzymes on lignocellulosic substrates

To measure adsorption isotherm, cellulase solutions at varied protein concentrations (0.05, 0.1, 0.2, 0.3, and 0.4 mg/mL) were incubated with 40 mg pretreated substrate sample in 5 mL acetate buffer (50 mM, pH 5.0) for 3 h at 8 °C to reach equilibrium (Yang and Pan, in press). All adsorption experiments were conducted in duplicate. Protein content in supernatant was determined using ninhydrin assay with bovine serum albumin as a standard. Adsorbed protein content on the substrates was calculated from the difference between the initial cellulase loaded and the free cellulase left in the supernatant. Maximal adsorption capacity (σ, mg/g substrate) and affinity constant (A, L/g protein) were estimated by nonlinear regressions of the adsorption data (free protein amount, C, mg/mL and corresponding adsorbed protein amount by substrate, I, mg/g substrate) using Polymath software according to the Langmuir adsorption isotherm: $I = \sigma A C / (1 + AC)$ (Tu et al., 2009). The strength of binding (mL/g substrate) was the combination of maximal adsorption capacity and affinity constant.

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in a shaking incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 50 °C and 200 rpm. Substrate consistency was 1% (1 g cellulose in 100 ml 0.1 M sodium acetate buffer with pH 4.8). Tetracycline (0.004% in the buffer) was used to control microorganisms. Enzyme loadings were 15 FPU (filter paper unit) cellulase and 30 CBU (cellulose unit) β-glucosidase/g cellulose. Periodically, samples of hydrolysate were taken for glucose analysis.

2.6. Analytical methods

Moisture content was determined by drying the materials to constant weight at 105 °C in a convection oven. The chemical compositions of switchgrass were determined according to TAPPI (Technical Association of Pulp and Paper Industry) standard methods: T211 om-93 for ash and T-222 for Klasson (acid-insoluble) lignin, respectively. Water and ethanol extractives were determined according to the ASTM Standard Test Method E1690-01. The hydrolysate from Klasson lignin determination was retained for analysis of monosaccharides and soluble lignin. The soluble lignin was determined using a UV–visible spectrophotometer (CARY 50 BIO, VARIAN Inc., Palo Alto, CA) at the wavelength of 205 nm with 3% H2SO4 as reference using an extinction coefficient of 110 L g⁻¹ cm⁻¹ (Dence, 1992).

Structural monosaccharides (glucose, xylose, mannose, arabinose, and galactose) were determined using a High Performance Ion Chromatography (HPIC, ICS-3000, Dionex, Sunnyvale, CA) equipped with dual pumps, post-column pump, autosampler, and electrochemical (EC, integrated amperometry) detector. The saccharides were quantified with reference to sugar standards. The standards were autoclaved at 121 °C for 1 h prior to analysis to compensate for sugar degradation during heating. HPIC method for determining saccharides is as follows: PA1 analytic column and PA1 guard column (Dionex), column temperature 20 °C, eluent flow rate 0.7 ml/min with gradient (0 → 25 min, 100% water; 25 → 35 min, 40% water and 60% 0.1 M NaOH; 35 → 40 min, 100% water); and post-column eluent (0.5 M NaOH) flow rate 0.3 ml/min for maintaining EC cell pH > 12.5.

Sacharides-derived chemicals (HMF and furfural) were determined also using the Dionex ICS-3000 system with a UV–vis...
2.7. Experimental design

Experiments were designed using Response Surface Methodology (RSM) and small Hartley composite design with the assistance of software SAS 9.1 (Statistical Analysis System, SAS Institute Inc., Cary, NC). The experimental matrix consists of total 21 experimental runs including eight factorial points, eight star points, and five center points with a specified alpha value of 1.7 as an axial scaling, as summarized in Table 1. The investigated parameters were temperature (163–197 °C), time (3–37 min), sulfuric acid dosage (0.8–4.2% on switchgrass), and sodium sulfite dosage (0.6–7.4% on switchgrass) with five levels for each. The conditions were selected according to our preliminary screening experiments and previous results from SPORL pretreatment of woods (Shuai et al., 2010; Wang et al., 2009; Zhu et al., 2009, 2012).

3. Results and discussion

3.1. Chemical composition of the switchgrass

Sugar and lignin composition of the switchgrass was analyzed in detail. The switchgrass had 5.90% ash and 16.58% water–ethanol extractives. The switchgrass had 16.58% acid-insoluble and 2.10% acid-soluble lignin. Majority of the hemicellulosic sugars was xylose (20.42%), accompanied with small amounts of arabinose (2.19%), galactose (0.81%), and mannose (0.38%). Cellulose content was 33.65% (in glucose). Referring to DOE’s (Department of Energy, USA) biomass feedstock composition and properties database (Supplemental Table 1). The switchgrass had 5.90% ash and 16.58% water–ethanol extractives. The switchgrass had 16.58% acid-insoluble and 2.10% acid-soluble lignin. Majority of the hemicellulosic sugars was xylose (20.42%), accompanied with small amounts of arabinose (2.19%), galactose (0.81%), and mannose (0.38%). Cellulose content was 33.65% (in glucose). Referring to DOE’s (Department of Energy, USA) biomass feedstock composition and properties database (DOE, 2004), the switchgrass composition in this study is comparable with the typical values in the database.

Table 1: Experimental matrix and summary of results.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Condition</th>
<th>Solid substrate</th>
<th>Pretreatment spent liquor</th>
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<tbody>
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<td>20</td>
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</table>

Notes: Condition: T – temperature, °C; r – time, min; S – sulfite dosage, % on dry switchgrass; SA – sulfuric acid dosage, % on dry switchgrass.

b Solid substrate: yield – substrate yield, % on dry switchgrass; KL, Glu, and Xyl – klason lignin, glucose, and xylose in the substrate, % on the dry substrate; EHY – enzymatic hydrolysis yield of the substrate at 48 h, % of cellulose-to-glucose, under the conditions of 15 FPU (filter paper unit) cellulase and 30 CBU (cellobiose unit) β-glucosidase/g at 1% substrate consistency (1 g cellulose in 100 ml 0.1 M sodium acetate buffer with pH 4.80); cellulose at 50 °C and 200 rpm with tetracycline (0.004%) in the buffer as microorganisms controller.

c Pretreatment spent liquor: SL, Glu, and Xyl – soluble lignin, glucose, and xylose present in the spent liquor, % on dry switchgrass; furfural and HMF – equivalent pentose and hexose that were converted to furfural and HMF, respectively, in the spent liquor, % on dry switchgrass.

3.2. SPORL pretreatment of switchgrass

3.2.1. Effect of SPORL pretreatment conditions on the yield and composition of solid substrate

The objective of a pretreatment is to remove the recalcitrance of lignocellulose and improve the accessibility of cellulose to cellulases, which can be achieved by removing hemicellulose and/or lignin, grinding/milling the biomass to small particles to increase surface area, destroying cellulose crystalline structure, or prehydrolyzing cellulose to reduce its degree of polymerization. A good pretreatment should be able to generate a readily digestible cellulosic substrate at high yield of substrate and fermentable sugars with limited formation of fermentation inhibitors derived from sugar degradation.

In order to optimize the SPORL pretreatment performance, effect of pretreatment conditions (temperature, time, acid, and sulfite dosage) on the yield and composition of pretreated switchgrass was investigated, and results are summarized in Fig. 1, which was generated with SAS software based on the results from the complete set of the experiments summarized in Table 1. In Fig. 1, when discussing the effect of a factor (such as temperature), other factors (such as time, acid loading, and sulfite loading) were set at the values of center point. The substrate yield varied from below 60% under severe pretreatment conditions (such as high temperature, long pretreatment time, and more acid) to over 80% under mild pretreatment conditions (such as low temperature, short pretreatment time, and less acid). Increasing temperature and/or extending pretreatment time significantly reduced the substrate yield because of enhanced dissolution of hemicelluloses and even cellulose. Higher acid loading (for example more than 2.5%) resulted in a low substrate yield due to accelerated hydrolysis of hemicelluloses and cellulose. Increasing sulfite also decreased the substrate yield, but this was attributed to dissolution of lignin through sulfonation, as will be discussed below. Lignin content in the pretreated substrates was enriched in general at high temperatures, long pretreatment time, and high acid loading because of...
the solubilization of hemicelluloses, but lignin content was re-
duced at high sulfite loading because of promoted delignification
through sulfonation. Glucose content (wt.%) of the pretreated sub-
strates was gradually increased with increased temperature and
prolonged pretreatment because of the dissolution of hemicellu-
loses and probably lignin when sulfite loading was sufficient. Glu-
cose content in substrate increased first with increased acid
loading because of the dissolution of more hemicelluloses, then
versely decreased due to excessive acid-catalyzed hydrolysis of
cellulose. Increasing sulfite loading always improved the retention
of cellulose in the substrate since addition of weak base sulfite ele-
vated pH value of the pretreatment liquor and prevented cellulose
from acidic hydrolysis at low pH. As shown in Fig. 1, dissolution of
hemicelluloses (mainly xylan) was almost linearly increased with
temperature and pretreatment duration. As expected, more acid
promoted the dissolution of xylan, and more sulfite delayed the
dissolution of xylan because of the sulfite-induced pH increase.

In summary, SPORL pretreatment was able to partially delignify
switchgrass through lignin sulfonation by sulfite. In addition, the
addition of sulfite as weak base elevated the pH value of pretreat-
ment liquor, which prevented hemicelluloses and cellulose from
excessive acid-catalyzed hydrolysis and subsequent decomposi-
tion to fermentation inhibitors (furfural and HMF). This is one of
the complete set of the experiments summarized in Table 1.

In Fig. 2, when discussing the effect of a factor (such as tempera-
ture), other factors (such as time, acid loading, and sulfite loading)
were set at the values of center point.

Pretreatment with acid alone typically does not delignify feed-
stock, but with the addition of sulfite, the SPORL pretreatment is
able to partially dissolve lignin through sulfonation. As shown in
Fig. 2, the soluble lignin in spent pretreatment liquor increased lin-
early with the addition of sulfite. High temperature and long pre-
treatment slightly enhanced the solubilization of lignin, but
increasing acid loading retarded the solubilization of lignin due
to the enhanced lignin condensation at lower pH. Increasing tem-
perature resulted in increased cellulose hydrolysis and therefore
glucose in the liquor. However, further increase in temperature re-
sulted in reduced glucose in the liquor due to degradation of glu-
cose to HMF. Prolonging pretreatment and increasing acid
loading increased cellulose hydrolysis, but increasing sulfite load-
ing reduced the cellulose hydrolysis because of increased pH.

Pentoses and hexoses tended to dehydrate and form furfural
and HMF, respectively, at high temperatures and low pH. The fur-
fural and HMF are identified as classic fermentation inhibitors.
Although high temperature, long pretreatment, and high acid load-
ing promoted xylan hydrolysis, as discussed in Fig. 1, the xylene
concentration in spent pretreatment liquor did not increase but de-
creased with temperature, pretreatment time, and acid loading be-
cause of the sugar dehydration and further decomposition to
furfural. The addition of sulfite slowed down the degradation of xy-
lose because of increased pH. As expected, furfural and HMF con-
centrations in the spent pretreatment liquor increased with the
increase in temperature, pretreatment duration, and acid loading
due to the enhanced sugar degradation. Again, addition of sulfite
prevented the sugars from fast and excessive degradation, and
therefore reduced the formation of furfural and HMF.

3.2.2. Effect of SPORL pretreatment conditions on the dissolved sugars,
soluble lignin, and sugar-derived fermentation inhibitors

Effect of SPORL pretreatment conditions on the dissolution of
lignin and carbohydrates and on the formation of fermentation
inhibitors from sugar degradation is summarized in Fig. 2. Simi-
larly, Fig. 2 was generated with SAS software based on the results

Fig. 1. Effect of SPORL pretreatment conditions on the yield and composition (lignin, glucose, and xylose) of solid substrate. Yield: yield of solid substrate (%) on dry
switchgrass; KL: acid-insoluble lignin in substrate (%); GLU_S: glucose content in substrate (%); XYL_S: xylose content in substrate (%); Temp: temperature (°C); Time:
pretreatment time (min); SA: sulfuric acid loading on oven-dry switchgrass (%); and Sulfite: sulfite loading on oven-dry switchgrass (%).
3.2.3. Effect of SPORL pretreatment conditions on the enzymatic digestibility of pretreated substrate

The substrate factors that affect enzymatic hydrolysability include hemicellulose content, lignin structure, content, and distribution, cellulose crystallinity, cellulose degree of polymerization, particle size, accessible surface area and so on. In general, a substrate is expected to have better enzymatic digestibility at low hemicellulose content, low lignin content, less hydrophobic lignin, cellulose with low crystallinity and degree of polymerization, small particle size, and big accessible surface area. The effect of SPORL pretreatment conditions on enzymatic digestibility is shown in Fig. 3. Increasing temperature and prolonging the pretreatment generally improved the digestibility, as shown in Fig. 3(A), because of enhanced removal of hemicelluloses and lignin and prehydrolysis of cellulose (lowering degree of polymerization). As shown in Fig. 3(B), the effect of sulfite and acid loading on digestibility was complex. In general, increasing acid loading in the presence of sulfite would improve digestibility by dissolving more hemicelluloses and increasing lignin sulfonation. Increasing sulfite loading did not always improve digestibility. This is because increasing sulfite loading at constant acid loading can increase lignin removal through sulfonation, but it can also reduce hemicellulose removal. Our previous results indicated that hemicellulose content could be more critical than lignin content to substrate digestibility when lignin was not extensively removed (Li et al., in press; Wang et al., 2012; Zhu et al., 2012).

To compare the effects of lignin and hemicellulose content on the enzymatic hydrolysability of SPORL substrates, the lignin and hemicellulose contents of the 21 SPORL substrates in Table 1 were plotted versus their enzymatic hydrolysability in Fig. 4. It is apparent that the digestibility was nearly inversely proportional to the xylan content in the SPORL substrate with good correlation ($R^2 = 0.83$), indicating that hemicellulose removal facilitated cellulose hydrolysis, which agrees with a previous study using aspen (Zhu et al., 2012). The data also showed that the hydrolysability was proportional to the lignin content. In other words, high lignin content is favorable to cellulose hydrolysis, which was also observed in a previous study (Leu and Zhu, in press). This seems to contradict the common belief that a substrate containing more lignin should have poor hydrolysability. However, it should be noted that the lignin content of SPORL substrates was related to the removal of hemicellulose. Since delignification was not extensive during the SPORL pretreatment, the lignin was actually enriched in the substrate when hemicellulose was removed. In other words, high lignin content was corresponded to low hemicellulose content, which was the real cause why better digestibility was observed at high lignin content in Fig. 4. The results were in agreement with the conclusion by a recent study that the removal of hemicellulose is more critical than lignin content (Leu and Zhu, in press).

The results above suggested that: (1) Temperature in SPORL pretreatment was a key parameter. It significantly affected pretreatment yield, carbohydrates removal, enzymatic hydrolysis of substrate, and formation of fermentation inhibitors. Hemicelluloses removal was sensitive to temperature and could be easily achieved even at a low temperature, while cellulose was partially hydrolyzed only at high temperature. High temperature significantly increased sugar degradation to fermentation inhibitors. (2) To prevent sugars from excessive degradation to fermentation inhibitors, conditions favoring hydrolysability and minimizing sugar degradation should be considered.
inhibitors, prolonging pretreatment at a relatively low temperature was a better strategy to sufficiently remove hemicellulose than a quick pretreatment at a high temperature. (3) Sulfuric acid played a critical role in removing hemicelluloses. Sufficient removal of hemicelluloses is critical to achieve a good digestibility of SPORL substrate. (4) Addition of sulfite partially dissolved lignin during SPORL pretreatment by sulfonation, but too much sulfite could elevate pH to reduce the removal of hemicelluloses. (5) In addition to enhancing delignification through sulfonation reaction, sodium sulfite preserved cellulose and prevents hemicellulose from degradation to fermentation inhibitors.

3.3. Comparison of SPORL, dilute acid and alkali pretreatment of switchgrass

3.3.1. Summary of SPORL, dilute acid, and alkali pretreatments of switchgrass

Dilute acid and alkali pretreatments are the two most investigated methods for pretreating lignocelluloses for enzymatic saccharification. It will be interesting to conduct preliminary comparisons of these two pretreatments with SPORL in terms of performance and the pretreatment fundamentals. The pretreatment conditions are summarized in Table 2. The conditions were selected according to our preliminary results and previous studies (Shuai et al., 2010; Wang et al., 2009; Zhu et al., 2009). As discussed in Section 3.2, the removal of hemicelluloses was critical to the enzymatic hydrolysability of SPORL pretreated switchgrass. Considering the results in Table 1 that the best digestibility achieved was only between 65% and 70% under the pretreatment conditions investigated, to further remove hemicelluloses and thereby improve the digestibility of SPORL substrate, the acid loading was increased to 6% on switchgrass with 6% sulfite on switchgrass. For comparison, the dilute acid pretreatment only used 6% sulfuric acid without any addition of sulfite. Sodium hydroxide loading for the alkali pretreatment was 10% on switchgrass. All the pretreatments were conducted at the same temperature (180 °C) for the same duration (30 min).

3.3.2. Yield and composition of pretreated switchgrass substrates

As shown in Fig. 5, SPORL pretreatment gave the highest substrate yield of 77.2%, while the yields of dilute acid and alkali pretreatments were only 68.1% and 66.6%, respectively. Dilute acid pretreatment removed more carbohydrates than both SPORL and alkali pretreatments, and thereby the dilute acid substrate had lowest glucose and xylose contents (52.9% and 2.4%, respectively). In addition, little delignification occurred in the dilute acid pretreatment. Therefore, lignin in the dilute acid substrate was enriched to 45.3% when hemicelluloses were removed. Compared to dilute acid pretreatment, at the same acid loading, the addition of sulfite elevated the pH of the pretreatment liquor, which reduced the acid-catalyzed hydrolysis of hemicelluloses and cellulose. In addition, lignin was partially sulfonated and dissolved during the SPORL pretreatment. These are the reasons why SPORL pretreatment had more cellulose and hemicelluloses but less lignin in the substrate than dilute acid pretreatment. Alkali pretreatment...
selectively removed lignin and preserved more carbohydrates, in particular hemicelluloses. The alkali-pretreated substrate had the lowest lignin and the highest hemicellulose content, compared with SPORL and dilute acid substrates. It also had comparable cellulose content with the SPORL substrate. The extensive delignification resulted in the low substrate yield of alkali pretreatment.

3.3.3. Sugars and inhibitors in pretreatment liquors

The dissolved sugars and sugar-derived furfural and HMF in the spent liquors from the three pretreatments are summarized and compared in Table 3. Agreeing with the discussion above, more sugars were found in the dilute acid pretreatment liquor than in SPORL one because the addition of sulfite in the latter prevented cellulose in particular hemicelluloses from excessive acidic hydrolysis. This is also the reason why SPORL liquor had slightly lower furfural than dilute acid liquor. Because the alkali pretreatment preserved most of hemicelluloses in the substrate, only small amount of the sugars were detected in alkali pretreatment liquor.

3.3.4. Enzymatic digestibility of pretreated solid substrates

Enzymatic digestibility of the pretreated switchgrass using the three pretreatment methods is compared in Fig. 6(A). It is apparent that SPORL substrate had not only faster initial hydrolysis rate, but also higher digestibility (cellulose-to-glucose hydrolysis yield, 83%) than dilute acid substrate (74%) and alkali substrate (72%). Considering that SPORL substrate had high hemicellulose content than that of dilute acid pretreated substrate (Fig. 5), the higher enzymatic digestibility of the SPORL substrate was likely attributed to its lower lignin content. In addition, the lignin in the SPORL substrate was partially sulfonated, which made the lignin more hydrophilic and thereby reduced the non-productive adsorption of enzymes on lignin through hydrophobic interactions. Comparatively, the alkali substrate had the poorest digestibility, although it had the lowest lignin content (Fig. 5). The poor digestibility of the alkali substrate was presumably attributed to its high hemicellulose content. This is in agreement with the discussion above that the removal of hemicellulose was more critical than that of lignin in the case of grasses and hardwoods that have more hemicellulose, if the lignin was not extensively removed. The observation also agrees with previous studies using aspen (Zhu et al., 2012; Leu and Zhu, in press).

The enzyme adsorption on the substrates pretreated with the three methods was also investigated and compared, as shown in Fig. 6(B). The adsorption parameters of the pretreated switchgrass substrates are listed in Table 4. It is apparent that the alkali substrate had the highest adsorption of cellulases; the SPORL substrate had the lowest adsorption; and the dilute acid substrate fell between the alkali and the SPORL substrates. The binding strength of the three substrates to cellulases followed the same order (Table 4). This was in agreement with the enzymatic digestibility in Fig. 6(A). The observation suggested that more cellulases

![Fig. 5. Comparison of SPORL, dilute acid and alkali pretreatments of switchgrass.](image)

Yield: yield of solid substrates (%); Lignin: lignin content in the substrates (%); Glucose: glucose content in the substrates (%); and Xylose: xylose content in the substrates (%).

<table>
<thead>
<tr>
<th>%</th>
<th>SPORL pretreatment</th>
<th>Dilute acid pretreatment</th>
<th>Alkali pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>0.19</td>
<td>0.96</td>
<td>0.69</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.31</td>
<td>0.55</td>
<td>0.33</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.50</td>
<td>8.30</td>
<td>0.53</td>
</tr>
<tr>
<td>Xylose</td>
<td>2.19</td>
<td>6.01</td>
<td>2.55</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.18</td>
<td>0.00</td>
<td>0.10</td>
</tr>
<tr>
<td>Soluble lignin</td>
<td>5.45</td>
<td>3.46</td>
<td>–</td>
</tr>
<tr>
<td>HMF</td>
<td>0.91</td>
<td>0.85</td>
<td>–</td>
</tr>
<tr>
<td>Furfural</td>
<td>4.83</td>
<td>5.59</td>
<td>–</td>
</tr>
</tbody>
</table>

*Note: % based on dry switchgrass.*

![Fig. 6. Enzymatic digestibility of SPORL, dilute acid, and alkali pretreated switchgrass (A) and Cellulases adsorption on the pretreated switchgrass (B).](image)
adsorbed on substrate do not always mean a faster and better cellulose hydrolysis. If most of the enzymes were adsorbed non-
productively on lignin not productively on cellulose, the adsorption did not benefit the cellulose hydrolysis. The difference of the three substrates in adsorbing enzymes was probably attributed to the affinity of lignin to cellulosates. As discussed above, during the SPORL pretreatment, lignin was partially sulfonated, which made the lignin in SPORL substrate more hydrophilic and therefore reduced the cellulosates adsorption on the lignin through hydrophobic inter-
actions (Lan et al., in press; Nakagame et al., 2011).

4. Conclusions

SPORL pretreatment improved the digestibility through sufficiently removing hemicellulose, partially dissolving lignin, and reducing hydrophobicity of lignin by sulfonation. It was observed that the removal of hemicellulose was more critical to substrate digestibility than the removal of lignin for SPORL pretreatment. SPORL-pretreated switchgrass had better digestibility than DA- and AL-pretreated ones, which was hydrolyzed by 83% within 48 h with 15 FPU cellulase and 30 CBU β-glucosidasase/g cellulose. Compared to DA pretreatment, SPORL pretreatment had higher substrate and sugar recovery yield and generated less fermentation inhibitors. The poor digestibility of AL pretreated switchgrass was presumably attributed to its high hemicellulose content.

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