Cellulose Accessibility and Zeta potentials of Sugarcane Bagasse Pretreated by Green Liquor and Ethanol for High Hydrolysis Efficiency

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Green liquor (GL) combined with ethanol (GL-ethanol) was selected to pretreat sugarcane bagasse (SCB). The results showed that the maximum lignin removal of 85.2% was achieved at 160 °C and a GL loading of 1.5 mL/g-dry substrate. The glucose yield of pretreated SCB increased with increased pretreatment temperature, and the maximum glucose yield of 97.7% was reached from SCB pretreated at 160 °C. Simons' stain (SS) showed that the glucose yield was affected by cellulose accessibility instead of lignin content when lignin removal was > 70%. The cellulase adsorption isotherm fitted by the Langmuir model showed that the strength of interaction between the cellulase and substrate of GL-ethanol-100/1.5 (100 °C, 1.5 mL GL/g-dry substrate) was declining with increased pH. The adsorption was pH-dependent, and negatively controlled by the pH value. Electrostatic interactions can account for the pH-dependency of cellulase adsorption.

Keywords: Sugarcane bagasse; Green liquor-ethanol pretreatment; Enzymatic hydrolysis; Simons' stain; Adsorption isotherm

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INTRODUCTION

Lignocellulose is the most abundant type of biomass in the biosphere, and its potential for biobased products has attracted increasing attention worldwide. Sugarcane bagasse (SCB) has gained interest from scientists in many countries (Rahikainen *et al.* 2013). In the current production system in China, SCB is burned to supply the energy required in industries, such as the electricity industry, or it remains in the field after mechanical harvest (Silva *et al.* 2010). Due to its abundant availability and high carbohydrate content, SCB could be used to produce value-added products as an ideal substrate during microbial fermentation processes (Pandey *et al.* 2000).

Lignin, an aromatic polymer made up of phenylpropane units (Ma *et al.* 2016), can non-productively adsorb cellulase through hydrophobic interaction (Ko *et al.* 2015), resulting in the reduction of the cellulose conversion rate. A pretreatment is the preferred option to break up the recalcitrant structure of lignocellulose, such as with SCB, to improve the enzymatic hydrolysis. Many pretreatment methods have been investigated, in which alkaline pretreatments coupled with an organic solvent (*e.g.*, ethanol) or sulfite have been regarded as the most effective methods for lignin removal (Bu *et al.* 2012; Yu *et al.* 2015). Green liquor (GL) produced from the pulping process is a type of alkaline

liquid, in which the compositions vary when different pulping methods are used. For example, GL from the soda pulping process is a mixture of sodium carbonate and sodium hydroxide (Yu *et al.* 2015). Green liquor is an alkaline alternative that has been developed as a pretreatment method to improve the enzymatic hydrolysis due to its advantage of high lignin removal efficiency and carbohydrate recovery. Furfural residues (FRs) were pretreated by the GL-ethanol method, and the glucose yield increased from 69.0% to 85.9% compared with raw material (Yu *et al.* 2014). Delignification can increase the accessibility of lignocellulose and decrease the non-productive adsorption of lignin to cellulase. However, relatively high removal of lignin is infeasible because of extensive costs and energy consumption. In the biorefinery process, it is always a question of what extent of delignification is sufficient for promoting the subsequent action by cellulase.

The rate of enzymatic hydrolysis of pretreated lignocelluloses has been related to several factors, among which cellulose accessibility has been reported by many researchers. It was shown that the enzymatic hydrolysis of pretreated furfural residues was not inhibited by reduced Brunauer–Emmett–Teller (BET) surface area, because the specific area of lignin remaining on the surface of lignocellulose could also be measured by the BET method (Yu *et al.* 2014). Solute exclusion as an alternative technology to measure cellulose accessibility was reported to have no advantages of mimicking cellulase adsorption due to the noncellulosic surfaces (Wang *et al.* 2012). Another promising method widely used to determine cellulose accessibility is based on the attraction of a cellulose-binding module (CBM) to cellulose, with the disadvantage of a non-productive adsorption of CBM to lignin (Wang *et al.* 2012). Simons' stain (SS) can estimate the cellulose accessibility of a lignocellulosic substrate in its wet state and can be employed to estimate the pore size of the lignocellulose relative to that of the cellulase enzyme (Chandra *et al.* 2008). In addition to cellulose accessibility, the non-productive adsorption of cellulase to lignin is another aspect that can limit the rate of enzymatic saccharification. The CBM can mediate interaction between cellulase and lignin through the hydrophobic effect (Rahikainen *et al.* 2011). An increase in the carboxylic content of lignin preparation resulted in an increased hydrolysis yield, suggesting that electrostatic interaction was another factor that involved the undesired interaction between lignin and cellulase (Nakagame *et al.* 2011).

To enhance the efficiency of enzymatic hydrolysis, it is necessary to increase the cellulose accessibility of lignocellulosic materials through an appropriate pretreatment, or to reduce the non-productive adsorption of cellulase either by modifying the chemistry structure of lignin or through the addition of some additives such as surfactant. In this experiment, GL-ethanol pretreatment was used to pretreat SCB. The influences of pretreatment conditions on the enzymatic hydrolysis of SCB were compared. The cellulose accessibility was measured by the SS method. The relationships among glucose yield, lignin removal, and cellulose accessibility were the established mechanism of cellulase adsorption to pretreated SCB, which was investigated by the measurement of adsorption isotherms.

EXPERIMENTAL

Materials

Sugarcane bagasse was kindly supplied by the Guitang Corporation (Guangxi, China). The material was immersed in fresh water for 24 h, washed several times until the reduced sugar was removed, and then oven-dried at 50 °C. The dried SCB was ground and screened to 40-mesh. The GL was provided by Chenming Group (Shandong, China). The supernatant for the pretreatment experiment was obtained *via* filtration after precipitation overnight. The main compositions of GL were sodium carbonate (75.2 g/L ± 0.25 g/L) and sodium hydroxide (23.04 g/L ± 0.25 g/L). Other metal elements also were present in the GL, such as iron and calcium. The content of calcium (0.39 g/L ± 0.03 g/L) was lower than that of iron (1.14 g/L ± 0.08 g/L) (Yu *et al.* 2015). Direct Orange 15 (Pontamine Fast Orange 6RN) dyes (DO) were bought from Sigma-Aldrich Corporation (Sigma Co., St. Louis, MO, USA), and the DO dye was ultra-filtered two times to remove the fractions of low molecular weight and intermediate molecular weight (Chandra and Saddler 2012).

Methods

Pretreatment of sugarcane bagasse by GL-ethanol

First, 10 g of dried SCB was placed into a 200-mL polytetrafluoroethylene (PTFE) reactor. Then, 100 mL of a 50:50% ethanol:water mixture (v/v) containing different green liquor loadings (0.5, 0.8, 1, 1.5 mL/g-dry substrate) and 0.1% (w/v) anthraquinone (AQ) (Sigma Co., St. Louis, MO, USA) were mixed well with SCB in the PTFE reactor. After sealing, the PTFE reactor was fixed with a large stainless-steel tank. The system (PTFE reactor and stainless-steel tank) was placed in a chamber equipped with a shaft that could be rotated at different speeds. The system was heated at an average rate of 5 °C/min to reach a desired temperature of 100 °C, 120 °C, 140 °C, and 160 °C. After 3 h of pretreatment, the system was rapidly cooled with tap-water. The insoluble residues were separated by filtration and washed with 200 mL ethanol-water mixture (50:50%, v/v). Next, the solid fraction was washed with distilled water until the pH was neutral. Some of the samples washed to neutral were oven-dried at 105 °C for 6 h to calculate the yield, and the rest was stored at 4 °C for further use. The solid yield was calculated using Eq. 1:

$$\text{Solid yield (\%)} = \frac{\text{Mass of pretreated dry solid (g)}}{\text{Mass of untreated dry solid (g)}} \times 100 \quad (1)$$

Analysis of substrate composition

The Klason lignin and carbohydrate contents of raw and pretreated SCB were calculated according to the National Renewable Energy Laboratory's (NREL) lignin analysis method for biomass (Sluiter *et al.* 2008). The Klason lignin content was defined as the ash free residue after acid hydrolysis. The filtrate was collected to detect the carbohydrate by high performance liquid chromatography (HPLC) (Waters2695e, Waters Corporation, Milford, MA, USA) with an Aminex HPX-87P (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA) at 85 °C and a refractive index detection detector at 35 °C. A 10 μL sample of sugars, such as glucose and xylose, was separated at a flow rate of 0.6 mL/min. Each sample was analyzed in duplicates.

Enzymatic hydrolysis

The enzymatic hydrolysis of untreated and pretreated SCB was performed at 48 °C and a pH of 4.8 with a substrate concentration of 5% (w/v) in a shaking incubator (Huamei Biochemistry Instrument, Taicang in Suzhou province, China) at 200 rpm for 72 h. The filter paper activity of cellulase (Celluclast 1.5 L, Sigma Co., St. Louis, MO, USA) was 130 filter paper unit (FPU)/mL, and the cellobiase activity of Novozyme 188 (A gift from Novozymes China Biotechnology Co., Ltd., Tianjin Shi, China) was 40 CBU/mL. The enzyme loading for the substrate was 12 FPU/g-cellulose for cellulase and 18 CBU/g-cellulose for cellobiase. The hydrolysis of untreated SCB was performed as a control. A glucose yield of 4 h was used to determine the initial rate of enzymatic hydrolysis. The theoretical glucose yield was calculated assuming that 1 g cellulose present in the liquid can generate 1.11 g of glucose.

Substrate characterization

A modified Simons' stain method was employed to calculate the specific area of the untreated and pretreated SCB. Fiber samples (approximately 10 mg dry substrate) were weighed into 2-mL centrifuge tubes, in which 100 uL phosphate buffered saline solution (1M phosphate, pH 6, 2M NaCl) and the required amount of water was added. The tubes containing a series of increasing concentrations of DO dye of high molecular weight (HMW, > 100 KDa) were prepared, which can then be used to measure the dye adsorption isotherm. These tubes were incubated at 70 °C overnight in the same chamber used to perform the pretreatment experiment. After that, the absorbance of the supernatant solution was gained on a UV–vis spectrophotometer (Shanghai Unico Instrument Co., Shanghai, China) at 455 nm, which represents the wavelength of maximum absorbance for DO (Brienzo *et al.* 2017).

Zeta potential measurements were performed to determine the sign of charge of pretreated SCB. Before measuring, the samples were incubated in sodium acetate for 4 h with a solid concentration of 0.5% (w/v) at a pH of 4.8 to 9.0. Six replications were assessed for each sample.

Cellulase adsorption isotherm on pretreated sugarcane bagasse

The protein content was determined based on the bicinchoninic acid (BCA) method described by Walker (1996). Standard working reagent (SWR; Beijing Dingguo Changsheng Biotechnology Co., Beijing, China) was mixed with 100 vol of reagent A (Beijing Dingguo Changsheng Biotechnology Co., Beijing, China) (sodium bicinchoninate 0.1 g, sodium carbonate (Na_2CO_3•H_2O) 2.0 g, sodium tartrate 0.16 g, sodium hydroxide (NaOH) 0.4 g, sodium bicarbonate ($NaHCO_3$) 0.95 g, made up to 100 mL) with 2 vol of reagent B (Beijing Dingguo Changsheng Biotechnology Co., Beijing, China) (copper sulfate ($CuSO_4$•$5H_2O$) 0.4 g in 10 mL of water). The SWR solution was apple green in color and was stable at room temperature for seven days. A 10 uL aqueous protein sample was added to 200 uL SWR solution and incubated at 50 °C for 0.5 h. After the sample was cooled to 25 °C, the absorbance of the supernatant solution was obtained at 562 nm. A calibration curve was constructed using dilutions of a stock 1 mg/mL solution of bovine serum albumin (BSA).

The cellulase adsorption on pretreated SCB was studied with varying pH conditions from 4.8 to 9.0. To determine the adsorption isotherm, different concentrations of cellulase that ranged from 14.2 mg/g-dry substrate to 99.3 mg/g-dry substrate, were mixed with pretreated SCB in sodium acetate and incubated at 4 °C in a shaking

incubator at 200 rpm for 2 h to reach equilibrium. The adsorption was performed in a total volume of 5 mL using a 50-mL tube that contained 100 mg/mL of substrate, enzymes, and 0.05 M citrate buffer (pH 4.8 to 9.0). The protein content in the supernatant was measured using the BCA method mentioned above for the nonadsorbed cellulase. The adsorbed enzyme was calculated from the difference between the total initial protein content and the non-adsorbed protein content. Cellulase adsorption on the pretreated samples was characterized by different isotherm models.

RESULTS AND DISCUSSION

Chemical Component of Pretreated SCB

The raw SCB was composed of 43.8% glucan, 23.3% xylan, and 24.7% Klason lignin. The carbohydrate content (glucan and xylan) of SCB accounted for 67.2% of dry substrate. Due to the high carbohydrate content, the SCB was considered as a potential lignocellulosic material for the production of bioenergy and biochemicals. Lignocellulosic material generally has high heterogeneity, and several factors, in addition to its chemical composition, can influence its recalcitrance (Brienzo *et al.* 2014, 2016; Santos *et al.* 2018). Therefore, in this study GL-ethanol pretreatment was employed to pretreat the raw SCB to reduce its recalcitrance to enzyme.

The SCB was pretreated using the GL-ethanol method at different temperatures and green liquor loading. According to Table 1, at 120 °C, the lignin content decreased from 19.2% to 12.0% in the GL-ethanol pretreatment when the loading of GL was increased from 0.5 mL/g-dry substrate to 1.2 mL/g-dry substrate. The maximal removal of lignin (62.19%) was reached at the 1.2 mL GL/g-dry substrate, and a slight reduction was observed at 1.5 mL GL/g-dry substrate, due to carbohydrate solubilization. Under the same dosage of green liquor (1.5 mL/g-dry substrate), the lignin removal increased with increased temperature. The highest lignin removal of 85.2% was observed at 160 °C. A dramatic increase of lignin removal occurred when the temperature increased from 120 °C (58.3%) to 140 °C (77.0%). The carbohydrate solubilization occurred as GL dosage increased, which caused the lignin content to increase. Lignin was continually removed when the temperature increased from 100 °C to 160 °C. As previously described by Yu *et al.* (2015), 71.8% of lignin was removed when 1.0 mL GL/g-dry substrate was employed during the GL-ethanol pretreatment. The xylan recovery was still keeping above 90% until the temperature of pretreatment reached to 140 °C. Some solubilization of xylan occurred at the pretreatment temperature of 160 °C.

Table 1. Chemical Component of Sugarcane Bagasse Before and After GL-ethanol Pretreatment

Temperature /GL Dosage (°C, mL/g DS)	Glucan (%)	Glucan Recovery (%)	Xylan (%)	Xylan Recovery (%)	Lignin (%)	Lignin Removal (%)	Solid Yield (%)
Untreated	43.2 ± 0.1	100	23.3 ± 0.1	100	24.7 ± 0.1	0	100
100/1.5	51.4 ± 0.2	99.2	26.6 ± 0.2	95.0	14.4 ± 0.2	51.5 ± 0.0	83.3
120/0.5	47.5 ± 1.2	98.7	25.5 ± 0.7	98.3	19.2 ± 0.2	30.5 ± 1.7	89.7
120/0.8	51.5 ± 0.6	99.8	26.3 ± 0.4	94.5	15.3 ± 0.2	48.6 ± 0.7	83.7
120/1.2	53.0 ± 0.8	95.5	27.5 ± 0.3	91.7	12.0 ± 0.0	62.2 ± 0.2	77.8
120/1.5	54.2 ± 0.1	96.5	26.9 ± 0.3	88.9	13.4 ± 0.2	58.3 ± 1.6	76.9
140/1.0	57.5 ± 0.2	99.0	28.8 ± 0.1	91.9	9.4 ± 0.1	71.7 ± 0.6	74.4
140/1.5	58.5 ± 0.5	98.63	29.5 ± 0.4	92.2	7.8 ± 0.2	77.0 ± 0.4	72.9
160/1.5	59.2 ± 0.1	92.75	29.2 ± 0.0	84.9	5.4 ± 0.3	85.2 ± 0.8	67.7

Initial Rate of Enzymatic Hydrolysis

In this study, glucose productivity at 4 h was employed to determine the initial rate of enzymatic hydrolysis. According to Fig.1, the glucose productivity of pretreated SCB increased from 2.34 $g \cdot L^{-1} \cdot h^{-1}$ to 5.40 $g \cdot L^{-1} \cdot h^{-1}$ when the pretreatment temperature was raised from 100 °C to 160 °C with a GL-ethanol pretreatment of 1.5 mL GL/g-dry substrate. Meanwhile, the glucan content increased from 51.4% to 59.2%. During this process, the lignin content declined rapidly from 14.4% to 5.4%. When the GL dosage was varied from 0.5 mL/g-dry substrate to 1.5 mL/g-dry substrate, the glucose productivity increased from 2.10 $g \cdot L^{-1} \cdot h^{-1}$ to 3.51 $g \cdot L^{-1} \cdot h^{-1}$ at the pretreatment temperature of 120 °C, while the lignin content first decreased from 19.2% to 12.0%. It was apparent that the lignin content in the materials was approximately inversely proportional to the initial rate. In fact, the lignin of each pretreated material was different in content, probably, contributing differently to the recalcitrance (Wallace *et al.* 2016).

It has been reported that the rate of enzymatic hydrolysis is essentially constant during the first few hours (Grethlein 1985). The reasoning may be because the adsorption of cellulases onto pretreated lignocellulosic substrates required 30 min to 60 min to reach equilibrium, while it needed a considerably longer time to reach equilibrium onto lignin (Tu *et al.* 2007, 2009). Interaction between cellulose and cellulase occurred most frequently rather than non-productive adsorption of cellulase onto lignin. In addition, the amount of cellulase was considered enough in the cellulosic regions of the substrate during the first few hours.

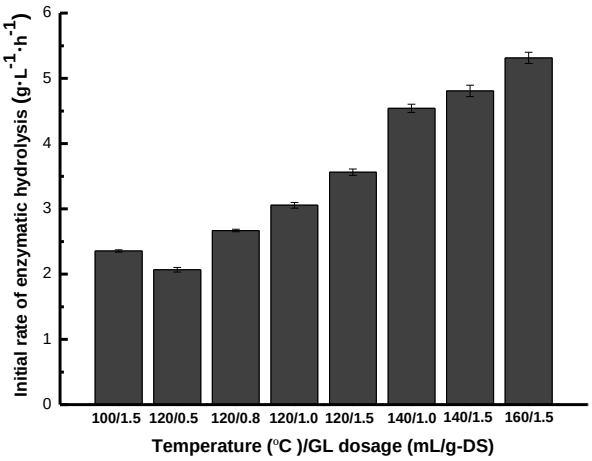

Fig. 1. Initial rate of enzymatic hydrolysis of GL-ethanol pretreated SCB

Relationship between Lignin Removal and Glucose Yield

Lignin is an aromatic cell wall polymer composed of three aromatic precursors that can interact with the action of cellulase, thus decreasing the yield of cellulose hydrolysis (Rahikainen *et al.* 2011). The GL-ethanol pretreatment destroyed the advanced structure of lignocellulose by disrupting the heteropolysaccharide–lignin network surrounding the cellulose fibrils, therefore it has been regarded as an effective way to remove lignin (Yu *et al.* 2014). Lignin removal is a principal factor that is always considered in enzymatic hydrolysis. It is reported that the glucose yield after 24 h can give a measure of the level of lignin removal and final accessibility of the substrate (Grethlein 1985). Thus, it is important to research the relationship between lignin removal and glucose yield.

With respect to the GL-ethanol pretreatment (Fig. 2), the glucose yield rose from 53.9% to 94.6% and then plateaued when the GL concentration was increased from 0.5 mL/g-dry substrate to 1.5 mL/g-dry substrate under the same temperature of 120 °C. In contrast, lignin removal first increased from 30.4% to 62.2% and then decreased to 58.3% with increased GL loading. When the pretreatment temperature increased from 120 °C to 160 °C under the same GL loading of 1.5 mL/g-dry substrate, lignin removal rose from 58.3% to 85.2%, and no remarkable difference was observed in glucose yield at 72 h. The glucose yield seemed no longer affected by lignin when the lignin removal was above 70%.

The non-productive adsorption of cellulase onto lignin has been found to be the limiting factor in the enzymatic hydrolysis of pretreated lignocellulosic substrates (Rahikainen *et al.* 2011). Removing lignin through appropriate pretreatments could reduce or eliminate the lignin inhibition to cellulose enzymatic hydrolysis (Yang and Pan 2016). In previous work it was shown that removing 65% lignin from wheat straw *via* an atmospheric aqueous glycerol autocatalytic organosolv pretreatment could increase the enzymatic hydrolysis yield by 90% after 48 h (Sun and Chen 2008). The add-back of lignin isolated from lodgepole pine and steam pretreated poplar decreased the hydrolysis yields of Avicel, and the nature of the residual lignin obtained after pretreatment significantly influenced hydrolysis (Nakagame *et al.* 2010). In this work, the glucose yield did not increase remarkably with the increase of the delignification degree and the pretreatment intensity of lignin removal being more than 70%; thus some other factors must exist in the enzymatic hydrolysis process.

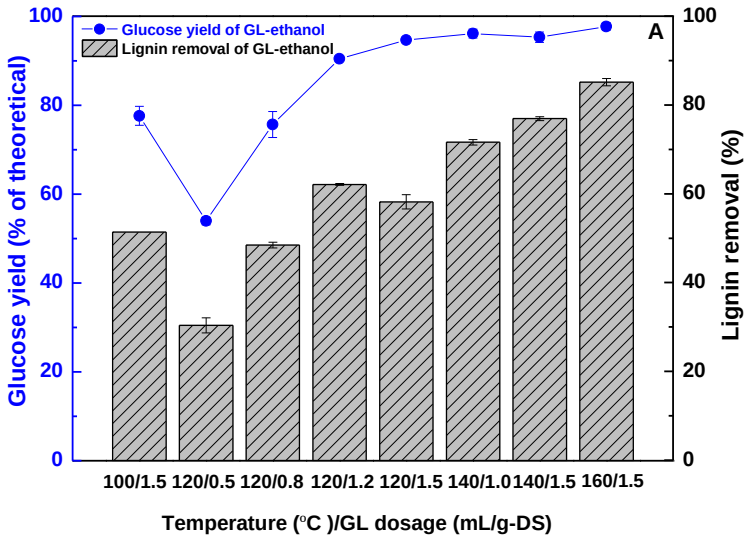

Fig. 2. Glucose yield of enzymatic hydrolysis at 72 h and lignin removal from GL-ethanol pretreated SCB

Relationship between Cellulose Accessibility and Glucose Yield

Based on the specificity of the Simons' staining technique for determining the specific surface area (SSA) of cellulose, the amount of cellulose in a pretreated substrate that is accessible to cellulases (cellulose accessibility), was assessed over a range of cellulosic substrates (Chandra and Saddler 2012). Compared with conventional Simons' Staining, in which two dyes (Direct Orange (DO) 15 and Direct Blue (DB)) were often applied, only DO dye has been employed in modified Simons' Staining methods (Chandra and Saddler 2012). The maximum amount of adsorbed DO dye was calculated by the parameters fitted by the Langmuir adsorption isotherm, which could be the indicator of cellulose accessibility (Esteghlalian *et al.* 2001). In this work, a relationship was established between glucose yield at 72 h and the maximum amount of adsorbed DO dye, which can reflect the external surface area of cellulose of a substrate (Brienzo *et al.* 2017). For the GL-ethanol pretreatment (Fig. 3), the maximum amount of adsorbed DO dye increased from 46.2 mg/g-biomass to 121.0 mg/g-biomass and then plateaued, when the GL concentration increased from 0.5 mL/g-dry substrate to 1.5 mL/g-dry substrate under the same temperature of 120 °C. The maximum amount of adsorbed DO dye did not increase noticeably when the temperature increased from 120 °C to 160 °C. The glucose yield changed correspondingly to the maximum amount of adsorbed DO dye.

The factor that was crucial in the enzymatic hydrolysis of lignocellulosic materials (cellulose accessibility or lignin content) is argued by researchers. In previous work, cellulose accessibility was reported to determine the rate of enzymatic hydrolysis of steam-pretreated spruce, and a clear correlation was observed between the rate of enzymatic hydrolysis and the BET area (Wiman *et al.* 2012). It was reported by Hu *et al.* (2011) that cellulose hydrolysis of steam pretreated corn stover was not improved by blocking non-productive adsorption of cellulase onto lignin at a high enzyme loading, but it was enhanced significantly *via* increased accessibility of the cellulose at low enzyme loading. When two pretreatment methods (dilute acid (DA) and cellulose solvent and organic solvent lignocellulose fractionation (COSLIF)) for corn stover were compared, the COSLIF-pretreated corn stover had a cellulose accessibility to cellulase of 11.6 m^2/g, nearly twice that of the DA-pretreated biomass (5.89 m^2/g). The digestibility remained at

93% at 24 h for the COSLIF-pretreated corn stover but only reached 60% for the DA-pretreated biomass at a low cellulase loading (Zhu *et al.* 2009). The cellulose accessibility was crucial in the enzymatic hydrolysis. In this work, the glucose yield was affected by lignin removal and cellulose accessibility at a low degree of delignification, but it was controlled mainly by cellulose accessibility at a high degree of delignification.

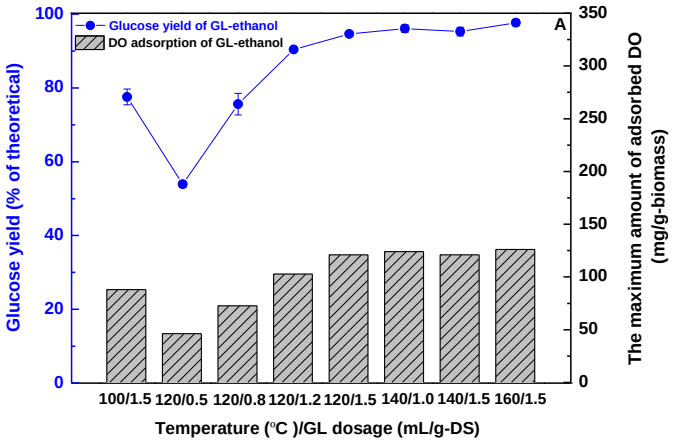

Fig. 3. Glucose yield of enzymatic hydrolysis at 72 h and specific area from GL-ethanol pretreated SCB

Electrostatic Interaction

An evaluation of zeta potential was carried out to measure the net sign of charge on the surface of pretreated SCB at different pH levels ranging from 4.8 to 9.0. As shown in Table 2, with increased pH value, the zeta potential of SCB pretreated at 160 °C and a GL loading of 1.5 mL/g-dry substrate decreased, while that of the SCB pretreated at 100 °C and the same GL loading could not be determined because of the low delignification and less cellulose exposure.

Electrostatic interactions can be expected to contribute to enzyme adsorption because an increase in the carboxylic acid content of the lignin preparation resulted in an increased hydrolysis yield, suggesting that the carboxylic acids within lignin partially alleviate non-productive binding of cellulases to lignin (Nakagame *et al.* 2011). Lignin can be charged under different pH conditions because of phenolic hydroxy and other charged groups, such as carboxyl group and sulfonic acid groups, generated during pretreatment. Cellulase has an isoelectric point (pI) of 3.6 (Wang *et al.* 2013), which implies that its functioning could be sensitive to the prevailing pH during the hydrolysis. Charged cellulase and pretreated lignocellulosic materials, such as SCB, could not only attract but also repel each other under a different pH. Zeta potential has been considered as an effective approach to measure the charged property of pretreated lignocellulosic materials.

Table 2. Zeta Potentials of Sugarcane Bagasse After GL-ethanol Pretreatment

	GL-ethanol-100/1.5 (mV)	GL-ethanol-160/1.5 (mV)
pH 4.8	nd	-5.7
pH 6.0	nd	-6.9
pH 9.0	nd	-10.1
nd: not determined		

Cellulase Adsorption Isotherms on Pretreated Substrates

In this work, two substrates with different delignification degrees were used to establish the adsorption isotherm. For the GL-ethanol pretreatment, the substrate of low delignification (100 °C, 1.5 mL GL/g-dry substrate) was called GL-ethanol-100/1.5, and that of high delignification (160 °C, 1.5 mL GL/g-dry substrate) was named GL-ethanol-160/1.5. The adsorption isotherms of the two substrates were fitted by different equation models. The Langmuir and Freundlich isotherms have been utilized widely to investigate protein adsorption onto various particle surfaces (Tu *et al.* 2009).

The Freundlich isotherm was employed to fit the cellulase adsorption onto both GL-ethanol-100/1.5 and GL-ethanol-160/1.5. Parameter *n* had a value usually in the range of 1 to 10. Another meaning of constant *n* was the heterogeneity factor, which had a larger value for most heterogeneous and multi-layered surfaces (Liu *et al.* 2010). According to Table 3, the cellulase adsorption onto the two substrates was fitted by the Freundlich isotherm when performed at a pH ranging from 4.8 to 9.0. The *n* value of substrate GL-ethanol-160/1.5 was higher than that of GL-ethanol-100/1.5, which indicated that the surface of the GL-ethanol-160/1.5 substrate was more heterogeneous and multi-layered and was confirmed by the results of the SS assay.

Langmuir isotherms are the most often-used isotherm models. They are based on the hypothesis that uptake occurred on a homogeneous surface by monolayer adsorption without interaction between the absorbed substance and that the sites or adsorption are equivalent to each other (Wang *et al.* 2013). Based on Fig. 4, the adsorption isotherm of GL-ethanol-100/1.5 substrate was also fitted well by the Langmuir isotherm. It is proposed that this was because of the low delignification and no modification of lignin (*e.g.*, sulfonation). Cellulase-lignin adsorption could be regarded as the main interaction, and for lignin cellulase adsorption was monolayer and homogeneous. The maximum adsorption capacity (1/k = 61.96 mg/g-biomass) and strength of interaction (1/b = 618.8 mL/g-biomass) were achieved at a pH of 4.8. The strength of interaction declined as the pH increased and cellulase adsorption became impossible when the pH increased to 9, showing that electrostatic repulsion was generated between cellulase and lignin. It was reported by Wang *et al.* (2013) that the pI of cellulase was 3.6, its net charge was influenced by the pH of its surrounding environment, and could get more positively or negatively charged due to the loss or gain of protons. Similarly, the net charge of lignin was also dependent on the pH of the environment. In the present work, the positive charge of cellulase increased at a pH of 4.8, so the strength of interaction reached the highest level. It is worth noting that not only the adsorption capacity (37.7 mg/g-biomass) but also the strength of the interaction (484.0 mL/g-biomass) at pH 6 was lower than of that at pH 4.8, which indicated that the non-productive adsorption of cellulase onto lignin decreased at pH 6. A similar result was described by Lou *et al.* (2013), strongly suggesting that enzymatic saccharification was carried out at an elevated pH of 6 with low capital cost and operating cost. Parameters and correlation coefficients are summarized in Table 3.

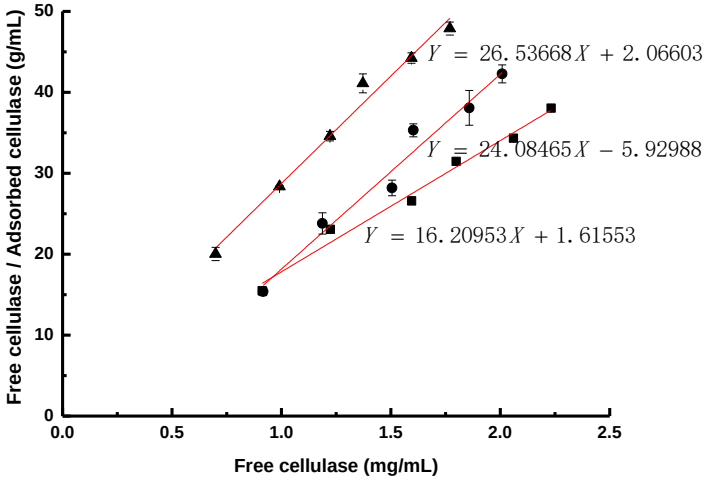

Fig. 4. Cellulase adsorption isotherms of pretreated SCB (GL-ethanol-100/1.5) fitted by Langmuir isotherms,■: pH 4.8, ▲: pH 6.0, ●: pH 9.0.

Table 3. Lists of Parameters of GL-ethanol Substrates

Isotherm Model	Parameters	GL-ethanol-100/1.5			GL-ethanol-160/1.5		
		pH 4.8	pH 6.0	pH9.0	pH 4.8	pH 6.0	pH 9.0
Langmuir isotherms	K ($1/E_{max}$)	16.21	26.537	24.085	-	-	-
	b ($1/E_{max} \times K$)	1.616	2.066	-5.93	-	-	-
	R^2	0.996	0.98	0.965	-	-	-
Freundlich isotherm	K	17.88	29.36	18.82	29.5	36.94	48.13
	n	0.52	0.53	0.46	0.65	0.68	0.75
	R^2	0.996	0.995	0.988	0.996	0.999	0.982
Note: E_{max} represented the maximal adsorbed protein (mg/g of lignin), $E_{max} \times K$ represents the strength of interaction, and R^2 was the coefficient of determination.							

CONCLUSIONS

1. Sugarcane bagasse was pretreated by GL-ethanol. Lignin removal increased from 30.4% to 62.2% and then decreased to 58.3% when GL loading was increased from 0.5 mL/g-dry substrate to 1.5 mL/g-dry substrate at a pretreatment temperature of 120 °C. The maximum lignin removal of 85.2% was achieved at a pretreatment temperature of 160 °C.
2. The glucose yield of pretreated SCB at 72 h rose from 53.9% to 94.6% and then plateaued when the GL concentration increased from 0.5 mL/g-dry substrate to 1.5 mL/g-dry substrate under the same temperature of 120 °C. When the pretreatment temperature increased from 120 °C to 160 °C under the same GL loading of 1.5 mL/g-dry substrate, no remarkable difference was observed in glucose yield at 72 h.

The glucose yield seemed no longer affected by lignin when the lignin removal was above 70%. It was proved by a SS assay that glucose yield at 72 h varied with the maximum amount of adsorbed DO dye.

3. The cellulase adsorption onto substrate of GL-ethanol pretreatment could be fitted by Langmuir and Freundlich isotherm models. Cellulase adsorption onto GL-ethanol-100/1.5 was pH-dependent and negatively controlled by pH value.

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