Effects of pH and Sulfonated Lignin on the Enzymatic Saccharification of Acid Bisulfite- and Green Liquor-pretreated Poplar Wood

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The effects of pH and sulfonated lignin (SL) on the saccharification of enzymatic hydrolysis were investigated using acid bisulfite (AS)- and green liquor (GL)-pretreated poplar as substrates. The enzymatic sugar conversions of AS- and GL-pretreated poplar could reach high levels at pH 4.8. The sugar conversions of both AS- and GL-pretreated poplar solids increased when hydrophilic SL was used as an additive in the enzymatic hydrolysis. With SL addition, the optimal pH of AS-pretreated poplar moved to 5.1–5.7, while that of GL-pretreated poplar showed no significant difference. The sugar conversions of AS- and GL-pretreated poplar went up and then leveled off with SL charges from 0.05 to 0.3 g/g-substrate. The highest total sugar conversions increased from 76.4% and 86.9% (pH 4.8, without SL) to 83.5% (pH 5.4, SL 0.3 g/g-substrate) and 90.9% (pH 5.0, SL 0.2 g/g-substrate) for AS- and GL-pretreated poplar, respectively.

Keywords: Poplar; Pretreatment; Enzymatic hydrolysis; pH; Sulfonated lignin

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INTRODUCTION

Ethanol produced from lignocellulosic biomass has great potential to address vital energy security and environmental and economic issues, all of which are a cause for concern for specialists in the chemical and energy field. Lignocelluloses consist primarily of lignin and two polysaccharides, cellulose and hemicellulose, which can be hydrolyzed into sugars by acids or enzymes. However, lignin, a phenolic polymer, restricts the enzymatic hydrolysis of pretreated lignocellulosic feedstocks. Three distinctive mechanisms are thought to contribute to lignin's inhibitions: the lignin as a physical blockage limits the accessibility of cellulase to cellulose (Mooney *et al.* 1998); lignin-derived soluble compounds act as enzyme inhibitors (Jing *et al*. 2010; Ximenes *et al.* 2011); and lignin nonspecifically adsorbs or binds to enzymes (Palonen *et al.* 2004).

Pretreatment is a prerequisite for the efficient enzymatic hydrolysis of lignocelluloses because it breaks down the lignin structure and disrupts the crystalline structure of cellulose, enhancing the accessibility of enzymes to cellulose. An efficient pretreatment minimizes sugar loss during pretreatment and ensures that the subsequent enzymatic hydrolysis productively yields maximal sugar (Jørgensen *et al.* 2007).

Acid bisulfite (AS) pretreatment was firstly developed by Zhu *et al.* (2009) for cellulosic ethanol production from lignocelluloses. With a moderate amount of sulfuric acid and sulfite dosages, AS pretreatment can partially remove the physical blockages *via* the sulfonation of lignin, prevent the degradation of hemicellulose and cellulose, and limit the formation of fermentation inhibitors. Both softwoods (Zhu *et al.* 2009) and hardwoods

with AS pretreatment (Wang *et al.* 2009) had a high final sugar conversion of enzymatic hydrolysis. Green liquor (GL) mainly consists of sodium carbonate and sodium sulfide. In kraft pulp mills, it is completely recoverable by dissolving the smelt from the recovery boiler in the chemical recovery process. GL pretreatment can remove the lignin selectively from the raw materials via the fragmentation and keeps as much polysaccharides as possible in the substrate for enzymatic hydrolysis. Our previous studies confirmed that the enzymatic sugar conversion of GL-pretreated lignocellulosic biomasses could be significantly improved (Jin *et al*. 2010; Gu *et al*. 2012).

However, Bonawitz and Chapple (2013) reported that lignin cannot be simply removed from lignocellulosic biomass without causing undesirable effects. In other words, the residual lignin in the pretreated substrates causes the nonspecific adsorption of cellulase, reducing hydrolysis yield. Therefore, it is important to study the reduction of the nonspecific binding of cellulase to residual lignin rather than further treatments for enhancing enzymatic saccharification.

Three types of interactions between lignin and cellulose have been suggested to explain the nonspecific adsorption of cellulase onto substrate lignin: hydrophobic interaction (Eriksson *et al.* 2002); electrostatic interaction (Nakagame *et al.* 2011); and hydrogen bonding (Berlin *et al.* 2006). Many researchers have reported that some surfactants can enhance the enzyme hydrolysis of cellulosic substrates by altering the substrate structure and making it more accessible to enzymes (Helle *et al.* 1993; Kaar and Holtzapple 1998). Reax 85A is a type of sulfonated softwood kraft lignin (SL) that has good water-solubility and some surface activity. It has been demonstrated that a significant enhancement of enzymatic hydrolysis conversion of pretreated lignocellulosic biomasses can be achieved by adding sulfonated lignin or lignosulfonate during enzymatic hydrolysis (Wang *et al.* 2013, 2015).

In addition, hydrolysis pH is an important control factor for optimal performance of the process. Wood *et al.* (1989) suggested that the activity of enzymes may decrease, or even become lost, when the hydrolysis pH is beyond the optimal range. Many studies attempted to achieve the maximum cellulase productivity by optimizing pH value (Ryu and Mandels 1980; Kansoh *et al.* 1999). Lan *et al*. (2013) reported that the enzymatic hydrolysis of various lignocellulosic substrates with commercial *Trichoderma reesei* cellulases should be carried out at higher pH than what is optimal for enzymes used with pure cellulosic substrates (pH 4.8 to 5.0). Lou *et al.* (2013) indicated that the *Trichoderma reesei* cellulases showed less non-productive binding onto lignin-rich fraction of the lignocellulosic substrates at elevated pH. Non-productive cellulase binding onto lignin decreased with increasing pH, possibly due to increasing electrostatic repulsion between the negatively charged enzymes and lignin (Rahikainen *et al.* 2013). The interactions between lignin and cellulase are lignin structure-dependent in some degree. Therefore, to reduce non-productive cellulase binding without further delignification, an attractive strategy is to modify the lignin in the pretreated substrates.

Poplar has been widely planted in temperate zones because of its rapid growth and ease of stock establishment through stem- or root-cutting (Kang *et al.* 1996). According to incomplete statistics, the planted forest of poplar has an area of roughly 600,000 km^2 (Fang *et al*.1999). In this work, poplar was pretreated by green liquor and acid bisulfite, respectively. The effect of hydrolysis pH on the enzymatic saccharification of pretreated solids, without and with the addition of sulfonated lignin, was investigated to obtain a high enzymatic sugar conversion.

EXPERIMENTAL

Materials

Poplar (*Populus nigra*) was collected from Jiangsu, China. Air-dried poplar (~30 ×15×2 mm) was stored in a refrigerator at 4 °C before use. Cellic® CTec2, generously provided by Novozymes (Bagsværd, Denmark), was used for the enzymatic hydrolysis of the lignocellulosic materials. The cellulase activity of Cellic® CTec2, in terms of "filter paper units" (FPU), was measured by the filter paper method using Whatmann No. 1 filter paper as the standard substrate (Ghose 1987). Reax 85A, kindly provided by Meadwestvaco (Charleston, SC), was used as an additive to the enzymatic hydrolysis. Reax 85A is a chemically modified kraft lignin with a weight-average molecular weight of 10,000. As sulfonate groups are introduced onto the molecule during modification, Reax 85A exhibits good solubility in water. All chemicals were analytical grade and purchased from Nanjing Chemical Reagent Co., Ltd. of China and used as received without further purification.

Pretreatments

AS and GL pretreatments were carried out in a rotary, lab-scale cooking system with an electrically heated oil bath (YRG2–10×1.25, Nanjing Jiezheng, China). Ten 1.25-L stainless-steel bomb reactors with screw caps were contained in the cooking system. First, 100 g of oven-dry (o.d.) materials were impregnated with the pretreatment liquor at 80 °C for 30 min in the cooking pot. Then, the temperature was increased at a rate of 2 °C/min to the target temperature (170 °C) and maintained for 1 h. The AS and GL pretreatment conditions were optimized from a series of experiments to obtain the pretreated solids with a similar lignin content (~ 22%). The detailed conditions of the AS and GL pretreatments are listed in Table 1. All chemicals were added on the weight basis of o.d. materials.

The pretreatment processes were terminated by cooling the bombs to room temperature in cold water. The pretreated solid was collected in cheesecloth and washed with deionized water to remove residual chemicals and dissolved chemical components.

Table 1. Conditions of AS and GL Pretreatment of Poplar

Pretreatment	$NaHSO_3$ (%)	H_2SO_4 (%)	TTA [a] (%)	Sulfidity (%)	L:W [b] (mL/g)	Temp. (°C)	Time at temp. (min)
AS	6	0.92	-	-	6	170	30
GL	-	-	20	25	4	170	60

[a] Total titratable alkali (TTA) charge as Na_2O on the basis of oven-dry (o.d.) material
[b] Liquor to wood ratio

Enzymatic Hydrolysis

Enzymatic hydrolysis of various substrates was carried out at different pH values using sodium acetate buffer at 2% (w/v) consistency and 50 ± 2 °C in a shaking incubator (DHZ-2102, Shanghai Jinghong, China) operating at 180 rpm for 48 h. The activity loading of Cellic® CTec 2 was based on a cellulase charge of 20 FPU/g-cellulose. Varied amounts of Reax 85A (SL) were added to the enzymatic hydrolysis system prior to enzyme addition. Sodium azide was charged at 3 mg/mL of buffer as an antibiotic to inhibit microbial infection. Enzymatic hydrolysis residue and hydrolysate were separated by centrifugation (5000 rpm, 20 min). The hydrolysate was sampled for monomeric sugar (glucose, xylose, arabinose, and mannose) analysis. When it was without SL addition, the enzymatic

hydrolysis pH was the value of sodium acetate buffer solution. It should be noted that the pH was the measured value in enzymatic hydrolysis solution with SL addition.

Analytical Methods

The hydrolysate was diluted 10 times, and the monomeric sugar contents were determined using a high-performance liquid chromatograph (HPLC-Agilent 1200, Santa Clara, CA) with a refractive index detector (RID). A Bio Rad Aminex HPX-87H 20n exclusion column and a Cation-H refill cartridges (Bio-Rad Laboratories, Hercules, CA) were used as the analytical and guard columns, respectively. The column temperature was 55 °C. A 5 mM H_2SO_4 solution was used as the eluent at a flow rate of 0.6 mL/min. Aliquots (10 μL) were injected after passing through a 0.22-μm nylon syringe filter. Monomeric sugars were quantified with reference to standards using the same analytical procedure. The concentration of monosaccharides was corrected by a calibration curve of standard sugars. The glucose, xylose, and arabinose contents were corrected to anhydrous units (*i.e.*, glucan, xylan, and arabinan) for the calculation of the sugar conversion in the enzymatic hydrolysate. The sugar conversion in the enzymatic hydrolysate was reported in weight percent of glucose, xylose, and total sugar in the pretreated substrates. All experiments were replicated twice and data are presented as the average ± error.

The lignin and structural carbohydrates contents of raw and pretreated materials were analyzed following the NREL protocols (Sluiter *et al.* 2008). The hydrolysate from the Klason lignin (KL) content determination was retained for the analysis of monomeric sugars and acid-soluble lignin (ASL). Sugars were determined as described earlier, except that sugar standards were autoclaved at 121 °C for 1 h prior to analysis to compensate for destruction during heating. To obtain acceptable machine pH, 40 μL of 50% (w/w) NaOH solution was injected per mL of hydrolysate sample. Acid-soluble lignin was measured by absorbance at 205 nm in a UV-Vis spectrometer (Puxi TU-1810, Beijing, China).

RESULTS AND DISCUSSION

Chemical Composition of Untreated and Pretreated Poplar

The main chemical components of the untreated, AS-pretreated, and GL-pretreated poplar are listed in Table 2. The carbohydrate content of the poplar was 60.1%, less than that of mixed hardwoods (66.2%) such as maple and oak (Agblevor *et al.* 1996). The carbohydrates of hardwood include cellulose (glucan) and hemicelluloses (xylan and glucomannan). The mannan content was very low (0.4%), meaning that the glucomannan content in poplar was much less than the xylan content. A variety of studies have suggested that the carbohydrate conversion of enzymatic saccharification could be greatly improved when the lignin removal of the substrate reaches approximately 40% (Goyal *et al.* 1991; Chang and Holtzapple 2000; Yang *et al.* 2002). According to previous experiments, the pretreatment conditions (Table 1) of AS and GL were optimized to obtain the highest sugar conversion *via* enzymatic hydrolysis.

With lignin removed, the carbohydrates of the substrates were also degraded to some extent after the AS and GL pretreatments. The lignin removal of the AS (54.2%) was more severe than that of the GL pretreatment (33.4%). The degradation of glucan, xylan, and total sugars of AS-pretreated substrate were 0.5%, 61.7%, and 7.0%, respectively, of which GL-pretreated substrate were 40.3%, 24.5%, and 16.2%, respectively. The degree of carbohydrate degradation of AS pretreatment was higher than that of GL pretreatment.

Compared with cellulose, hemicellulose is easier to degrade in acidic conditions. However, xylan losses had no significant influence on the conversion of total sugars because of the low xylan content.

Table 2. Mass Fractions of the Components of Original and Pretreated Poplar

	Pretreated Solid Recovery (%)	Lignin (%)			Carbohydrates [b] (%)		
		KL	ASL	Total	Glucan	Xylan	Total
Poplar [a]	–	23.3±0.1	2.6±0.0	25.9±0.1	44.1±0.2	15.4±0.2	60.1±0.4
AS-pretreated	57.9±0.0	19.3±0.0	1.2±0.0	20.5±0.1	68.2±0.1	10.2±0.3	78.4±0.4
GL-pretreated	71.3±0.3	22.1±0.2	2.1±0.0	24.2±0.2	57.5±1.1	12.9±0.3	70.6±1.4

[a] Benzene-ethanol extractives content was 1.5±0.1%
[b] Mannan (0.4%) included

Effects of Hydrolysis pH on the Enzymatic Saccharification of AS- and GL-Pretreated Poplar

The effect of pH on the enzyme activity has been extensively studied. The optimal pH widely used for enzymatic hydrolysis is 4.8 to 5.0 to maintain the enzyme activity (Haynes *et al.* 1994). The carbohydrate conversions of the pretreated solids are given in Table 3. Increasing the pH from 4.5 to 4.8 rapidly improved the total sugar conversion for both of GL- and AS-pretreated substrates; when further increasing the pH to 5.7, the total sugar conversion of AS-pretreated substrates leveled off and then exhibited a downward trend. In GL-pretreated substrates, the total sugar conversion was almost invariable compared with that achieved at pH 4.8. Thus, the optimum pH ranges for the enzymatic hydrolysis of AS- and GL-pretreated substrates were 4.8 to 5.4 and 4.8 to 5.7, respectively. The highest total sugar conversions were 76.4% and 86.9%, respectively, achieved at pH 4.8. However, this result contradicts the reported conclusions of Wang *et al.* (2013) that a pH of 5.5 or higher should be used in the enzymatic saccharification of sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL)-pretreated substrates because of electrostatic interactions contributing to non-productive cellulase adsorption at high pH. The enzyme was mostly negatively charged at pH 4.5 to 5.7 in the present work because CTec2 is an enzymatic mixture that contains different components with different p*I* (isoelectric point) values, most of which are less than 5.0 (Chirico and Brown 1987; Medve *et al.* 1988). Lan *et al.* (2013) reported that various functional groups in differently SPORL-pretreated lignocelluloses, especially sulfonic acid groups, can be responsible for pH-induced surface charges affecting the surface hydrophobicity and perhaps the electrostatic interactions between lignin and cellulase. However, according to Wang *et al*. (2015), the acidic groups of AS- and GL-pretreated poplar containing sulfonate and carboxylic acid groups have weak responses to pH changes such that there was no obvious enhancement at elevated pH (4.8 to 5.7). This could be because the structure of the pretreated substrates varied with the pretreatment used.

For both AS and GL pretreatment, the variations in the glucan and xylan conversion with changing pH were similar to the trend exhibited by the total sugar conversion. Compared with that of GL pretreatment, the xylan conversion of AS pretreatment was much lower due to the severe degradation of hemicellulose in the AS pretreatment (Zhu *et al.* 2009). Because the hemicellulose in the AS-pretreated substrates was low, there was no obvious influence of the xylan conversion on the conversion of total sugar during enzymatic hydrolysis.

Table 3. Effect of Enzymatic Hydrolysis pH on the Carbohydrate Conversion of AS- and GL-pretreated Poplar

Pretreatment	pH	Glucan (%)	Xylan (%)	Total Sugar (%)
AS	4.5	58.6±3.2	20.9±2.0	56.9±3.0
	4.8	82.7±2.4	34.2±0.3	76.4±2.1
	5.1	78.3±0.9	26.8±1.9	71.6±1.0
	5.4	80.9±0.4	30.6±0.6	74.3±0.4
	5.7	67.5±3.3	27.6±2.4	62.3±3.2
GL	4.5	52.5±4.0	65.2±2.9	54.6±3.7
	4.8	85.5±0.4	94.8±0.1	86.9±0.3
	5.1	79.5±0.3	79.7±0.7	79.3±0.2
	5.4	81.6±2.5	87.2±4.2	81.1±2.8
	5.7	84.7±0.2	85.3±0.1	84.6±0.2

The Effect of SL on Enzymatic Hydrolysis pH of AS- and GL-Pretreated Poplar

Non-productive adsorption of cellulase onto residual lignin is one of the major inhibitors limiting the efficiency of the enzymatic hydrolysis of lignocellulosic materials (Nakagame *et al.* 2011; Qi *et al.* 2011). Generally, it is believed that the optimal pH is 4.8 to 5.0 for the enzymatic hydrolysis of lignocelluloses using *Trichoderma reesei* cellulase, based on optimization with pure cellulose. In this work, SL was added into the hydrolysis system as an additive to reduce the negative effect of non-productive adsorption of cellulase on the enzymatic hydrolysis of AS- and GL-pretreated poplar at different pH values. The results are illustrated in Fig. 1. With the addition of SL, the total sugar conversions of AS- and GL-pretreated poplar increased when the hydrolysis pH was less than 5.0. It leveled off within the pH range from 5.0 to 5.4, and then decreased.

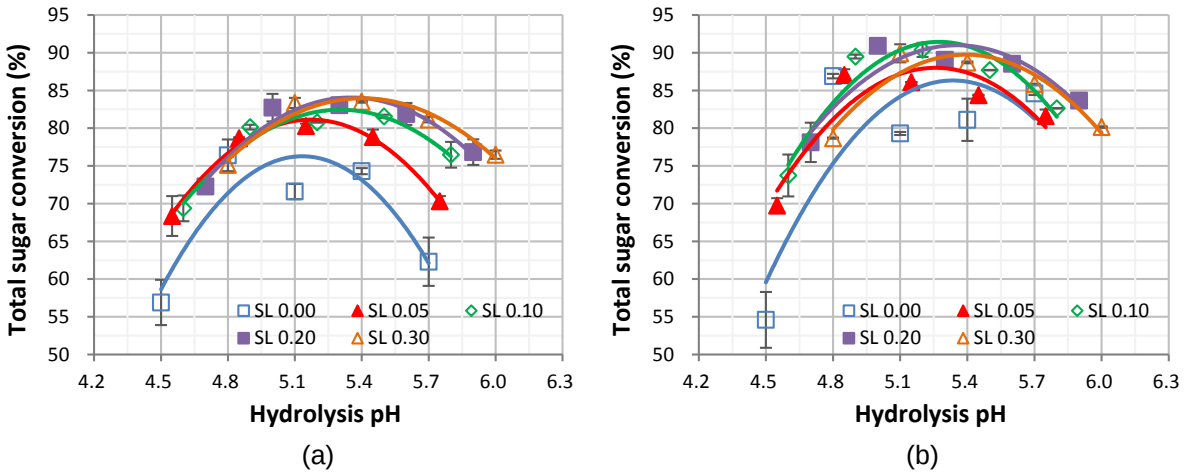

Fig. 1. Effect of enzymatic hydrolysis pH on the total sugar conversion of AS- (a) and GL-pretreated (b) poplar at sulfonated lignin (SL) loadings of 0, 0.05, 0.1, 0.2, and 0.3 g/g-substrate

As shown in Fig. 1a, a high total sugar conversion of AS-pretreated poplar could be obtained within the pH range of 5.1 to 5.7 when the addition of SL was more than 0.1 g/g-substrate. The highest total sugar conversion reached 83.5% at pH 5.4 with an SL addition of 0.3 g/g-substrate. This demonstrates that the application of SL changed the optimal pH for the enzymatic hydrolysis of AS-pretreated poplar. In other words, the elevated pH of 5.4 improved the efficiency of enzymatic saccharification by increasing the

repulsive electrostatic interactions between the residual lignin of the AS-pretreated substrate and the enzyme with SL addition. The optimal pH for AS-pretreated substrate is in accordance with that reported in the cited reference (Lan *et al.* 2013). Lou *et al.* (2012) also suggested that elevated pH values of 5.5 or higher reduced non-productive absorption, and thus improved the saccharification of lignocelluloses. Therefore, the change in the optimal pH was attributed to the interactions of residual lignin of AS pretreated substrate and SL with cellulase. Wang *et al*. (2015) suggested that the application of SL could eliminate nonspecific cellulase binding to lignin in the AS-pretreated poplar through modifying the surface properties of lignin. Therefore, the change in the optimal enzyme hydrolysis pH was likely related to the interactions of the residual lignin and the SL with cellulase.

For GL-pretreated poplar as shown in Fig. 1b, with the addition of SL, the optimal pH value for the highest sugar conversion was about 5.0, which showed no significant difference from that without SL addition. For example, the highest total sugar conversion of GL-pretreated poplar was 90.9% at pH 5.0 and SL loading of s0.2 g/g-substrate.

The structure of the residual lignin in AS-pretreated poplar, which contains sulfonate and carboxyl acid groups (Restolho *et al.* 2009), is rather different from that in GL-pretreated poplar. The role of the elevated pH is to enhance the electronegativity of the cellulose. More negatively charged groups led to stronger electrostatic repulsion between the cellulase and residual lignin of the pretreated substrate. The electrostatic interactions strongly contribute to the non-productive cellulase binding mechanism (Rahikainen *et al.* 2013). Therefore, compared with GL-pretreated poplar, the enzymatic saccharification of AS-pretreated poplar was significantly enhanced by the elevated pH.

Table 4. Effect of Sulfonated Lignin Loading on the Enzymatic Saccharification of AS- and GL-pretreated Poplar

Pretreatment	Sulfonated Lignin (g/g-substrate)	Hydrolysis pH	Carbohydrate Conversion (%)		
			Glucan	Xylan	Total Sugar
AS	0.0	4.8	82.7±2.4	34.2±0.3	76.4±2.1
	0.05	4.8	86.0±0.1	29.8±0.4	78.7±0.1
	0.1	4.9	87.6±0.3	30.2±0.5	80.1±0.3
	0.2	5.0	90.4±2.0	31.7±0.8	82.7±1.8
	0.3	5.1	90.9±1.0	32.9±1.3	83.3±0.7
GL	0.0	4.8	85.5±0.4	94.8±0.1	86.9±0.3
	0.05	4.8	85.7±0.6	94.5±1.7	87.0±0.8
	0.1	4.9	88.4±0.1	95.6±0.9	89.5±0.3
	0.2	5.0	89.9±0.7	97.0±0.3	90.9±0.5
	0.3	5.1	88.8±1.0	96.4±1.9	90.2±1.2

Improving Enzymatic Hydrolysis of AS- and GL-pretreated Poplar by the Addition of SL

As mentioned previously, without the addition of sulfonated lignin, 4.8 was the optimal pH for enzyme hydrolysis. However, both pH value and carbohydrate conversion increased with the addition of SL, as shown in Table 4. With an SL loading of 0.3 g/g-substrate, the highest sugar conversion (83.3%) was achieved in the enzymatic hydrolysis of AS-pretreated poplar. For GL-pretreated poplar, the highest total sugar conversion (90.9%) was achieved with SL loading of 0.2 g/g-substrate. Börjesson *et al*. (2007) reported that the hydrophobic interaction of PEG-lignin contributed to the decrease of nonspecific cellulase binding to the residual lignin of substrate. SL may have a similar effect. Wang *et*

al. (2015) used SL as an additive to improve the enzymatic hydrolysis of AS-pretreated poplar and found that the optimal enhancement in enzymatic digestibility (from 40% to 66%) achieved at SL loading of 0.2 g/g-substrate. The sulfonate and carboxylic acid groups of the residual lignin in AS-pretreated poplar have some effects on the reduction of non-productive cellulase adsorption.

CONCLUSIONS

1. At the optimal pH of 4.8, the highest total sugar conversion rates of acid sodium bisulfite- and green liquor-pretreated poplar were 76.4% and 86.9%, respectively.
2. Because the negatively charged groups of the acid sodium bisulfite-pretreated substrate were more numerous than those in the green liquor-pretreated substrate, the repulsive electrostatic interaction between the negatively charged cellulase and the residual lignin of the AS-pretreated substrate increased with the hydrolysis pH. Thus, the optimal pH of the acid sodium bisulfite-pretreated poplar with SL addition increased to 5.4, whereas that of the green liquor pretreatment did not show much change (pH 5.0).
3. The addition of sulfonated lignin resulted in a noticeable improvement in the enzymatic saccharification of both acid sodium bisulfite- and green liquor-pretreated poplar. The enzymatic saccharification conversion of acid sodium bisulfite- and green liquor-pretreated poplar could reach 83.3% and 90.9%, respectively, at sulfonated lignin loadings of 0.3 and 0.2 g/g-substrate.

ACKNOWLEDGMENTS

The authors are grateful for the support of the National Key Technology Research and Development Program of China (2015BAD15B09), the National Natural Science Foundation of China (31370571), the Specialized Research Fund for the Doctoral Program of Higher Education of China (20133204110006), and the Priority Academic Program Development of Jiangsu Higher Education Institutions of China.

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Article submitted: June 10, 2015; Peer review completed: August 11, 2015; Revised version received: September 6, 2015; Accepted: September 7, 2015; Published: September 16, 2015.
DOI: 10.15376/biores.10.4.7361-7371