

Effects of Green Liquor Pretreatment on the Chemical Composition and Enzymatic Hydrolysis of Several Lignocellulosic Biomasses

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Pretreatment of biomass is an extremely important step in a commercial biorefinery. For realization of lignocellulosic biomass as an alternative fuel source to occur, a fundamental understanding and critical investigation of the chosen pretreatment are essential. In this work, green liquor (GL) pretreatment of four plant species, namely Masson pine, poplar, moso bamboo, and miscanthus, was investigated to understand its effect on the chemical composition and enzymatic hydrolysis of different lignocellulosic materials. The results indicated that herbaceous materials exhibited better delignification selectivity in GL pretreatment than woody materials according to the order: miscanthus > moso bamboo > poplar > Masson pine. The effect of GL pretreatment on the enzymatic sugar yield was rather different depending on the varieties of lignocellulosic materials. Higher lignin removal with less polysaccharide degradation during GL pretreatment improved the enzymatic sugar yield.

Keywords: Green liquor; Lignocellulosic biomass; Chemical composition; Enzymatic hydrolysis; Delignification; Sugar yield

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INTRODUCTION

Lignocellulosic materials are the most abundant polymeric carbohydrates that can be used for second-generation bioethanol production (Goldemberg 2007; Somerville *et al.* 2010). The efficient bioconversion of lignocellulosic feedstock to bioethanol *via* the sugar platform involves three key steps: pretreatment, enzymatic saccharification, and fermentation or catalytic conversion of sugars (Lynd *et al.* 2008; Conde-Mejía *et al.* 2012; Zhu and Zhuang 2012). Pretreatment is used to break down the lignin structure and disrupt the crystalline structure of cellulose for enhancing accessibility of the cellulose to enzymes during enzymatic hydrolysis (Mosier *et al.* 2005), which has been recognized as a technological bottleneck for the cost-effective development of bioconversion (Alvarado *et al.* 2009). Much research has been conducted to find an effective pretreatment approach to improve bioconversion efficiency, including physical, chemical, biological, or a combination of pretreatments (McMillan 1994; Hsu 1996). Few pretreatment methods have proven to be effective and promising technologies for industrial applications.

Green liquor (GL), composed mostly of Na₂S and Na₂CO₃ in aqueous solution, can be recovered from spent liquor in the recovery furnace of a kraft pulp mill. Under the consideration of capital costs, investment risks, technical feasibility, and the efficiency for bioethanol production, the GL pretreatment process has been developed as an effective method for the bioethanol production from lignocellulosic biomass. The GL process has the following features: (1) removal of a certain amount of lignin, effectively disrupting the

material structure and exposing cellulose to enzyme; (2) keeping a large proportion of the polysaccharides in the substrate under mild alkali condition for enzymatic hydrolysis; (3) recovery of fermentable sugars in one step; and (4) production of no toxic byproducts (*e.g.*, furfural, acetic acid), which affect fermentation and cause equipment corrosion. Integrating GL pretreatment ethanol production with a pulp mill can be an attractive bioconversion by lowering the investment risk and cost with use of mature pulping technology (Jin *et al.* 2010; Wu *et al.* 2010; Gu *et al.* 2012; Meng *et al.* 2014).

Four species of lignocellulosic materials, Masson pine (*Pinus massoniana*), poplar (*Populus deltoides*), moso bamboo (*Phyllostachys edulis*), and miscanthus (*Miscanthus sinensis*), were used to investigate the applicability of GL pretreatment. Masson pine and poplar are typical softwood and hardwood species, respectively. Bamboo plants are giant woody, tree-like, perennial evergreen C4 grasses with more than 70 genera and about 1000 species (Gratani *et al.* 2008). Miscanthus are perennial rhizomatous grasses native to China, Southeast Asia, and the Pacific islands (Xi and Jezowski 2004). For characteristics such as high biomass productivity, strong adversity resistance, extensive adaptability, and relatively low input cost, miscanthus has received considerable attention as a feedstock for bioethanol production (Lewandowski *et al.* 2003). These four materials exhibit many differences in physical properties, chemical composition, and component chemical structure, so were chosen for better understanding of GL pretreatment applicability for bioconversion among softwoods, hardwoods, and herbaceous plants.

EXPERIMENTAL

Materials

Masson pine was provided by a paper mill in Fujian, China. Poplar, moso bamboo, and miscanthus were respectively collected from Jiangsu, Zhejiang, and Hunan provinces of China. Air-dried bark-free Masson pine (~3 cm × 1.5 cm × 0.2 cm), bark-free poplar (~3 cm × 1.5 cm × 0.2 cm), maso bamboo (~3 cm × 1.5 cm × 0.2 cm), and miscanthus (~3 cm in length) were sealed in plastic bags and stored in a refrigerator at 4 °C. The samples were ground in a Wiley mill (FZ102, Tianjin Taisite, China), and the sawdust between 40 and 80 mesh was collected for the analysis of main components. Prior to composition analysis, the sawdust was extracted with benzene-ethanol (2:1, v/v) in a Soxhlet apparatus for 8 h. All of the weight percentages were calculated based on the oven dry (o.d.) samples. All chemicals were of analytical grade and purchased from Nanjing Chemical Reagent Co., Ltd. (China). They were used as received without further purification. Cellic[®] CTec2 used for enzymatic hydrolysis in this study was kindly provided by Novozymes (Novo Nordisk A/S, Demark).

Methods

Green liquor pretreatment

The GL pretreatment was carried out in a rotary lab-scale cooking system with an electrically heated oil bath at a speed of 6 rpm. Ten 1-L stainless steel bomb reactors with screw caps were contained in the cooking system. Green liquor was simulated using Na₂S and Na₂CO₃ in the lab. The detailed conditions of the pretreatment, including total titratable alkali charge as Na₂O on the basis of o.d. material, sulfidity of the liquor, the ratio of liquor to biomass (mL/g), final temperature, and the time at maximum temperature, are listed in Table 1.

Table 1. Detailed Conditions of Green Liquor Pretreatment

Materials	Total titratable alkali (% as Na ₂ O)	Sulfidity (%)	Liquor : Biomass (mL/g)	Temperature (°C)	Time (min)
Masson pine	16 to 28	25	4	170	60
Poplar	4 to 24	25	4	160	60
Moso bamboo	4 to 20	25	4	150	60
Miscanthus	4 to 20	20	6	140	60

The samples were impregnated with the liquor at 80 °C for 30 min, and then the temperature was raised to the designed temperature at a rate of 2 °C/min. The pretreatment was maintained at the designed temperature for an additional 60 min and then terminated immediately by cooling the bombs to room temperature in cold water. The pretreated solid was collected and washed with deionized water to remove the residual chemicals and dissolved wood compounds. Pretreated solid yield, defined as the o.d. weight of the solid fraction divided by that of the starting material, was calculated according to the wet weight and moisture content of the collected solid. Part of the pretreated solid was air dried and ground for chemical composition analysis.

Enzymatic hydrolysis

A laboratory 300-mm disk refiner (KRK; Jilin, China) was used under atmospheric conditions at 3000 rpm to defiberize the pretreated solids to produce substrates. Enzymatic hydrolysis was performed with a consistency of 5% (w/v) in sodium acetate buffer (pH 4.8) using a shaking incubator (DHZ-2102, Shanghai Jinghong, China) at 50 °C and 180 rpm for 48 h. The series enzyme Cellic[®] CTec2 was used for enzymatic hydrolysis. The enzyme loading was 20 filter paper units (FPU) *per* gram of cellulose in the substrate based on cellulase activity. Sodium azide was charged at 30 µg/mL of buffer to prevent microbial growth during the enzymatic hydrolysis. The residues and hydrolysate of enzymatic hydrolysis were separated by centrifugation. The hydrolysate was sampled for monomeric sugar analysis.

Analytical methods

The FPU activity of cellulase was determined following the standard method described by Ghose (1987). The enzymatic hydrolysate was diluted 10 times, and monomeric sugars were determined using high performance liquid chromatography (HPLC, Agilent 1200 Series; Santa Clara, CA, USA) with a differential refractive index detector (RID). The HPLC analysis was carried out using a Biorad Aminex HPX-87H 20n exclusion column (300 × 7.8 mm, Bio-Rad Laboratories, Hercules, CA) with a Cation H Refill Cartridge guard column (30 × 4.6 mm, Bio-Rad Laboratories) at 55 °C. A 5 mM H₂SO₄ solution prepared with degassed super-purified deionized water 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 standards using the same analytical procedure. The concentration of monosaccharides was corrected by a calibration curve of standard sugars. The average of duplicate runs was used in reporting. Data of monomeric sugar contents were corrected to anhydro units for the calculation of sugar yield. Since the peaks of arabinose and mannose of Masson pine obtained by HPLC overlapped and were not clearly separated, the sum of arabinan and mannan was reported in this work.

Lignin and carbohydrate content of the extractive-free and the pretreated substrates were analyzed using the National Renewable Energy Laboratory (NREL) procedure (Sluiter *et al.* 2008). The Klason lignin content of moso bamboo and miscanthus was taken as the ash-free residue after acid hydrolysis. The sugar content was determined by detecting the filtrate captured from the procedure of lignin determination by HPLC as described, except that sugar standards were autoclaved at 121 °C for 1.0 h prior to analysis to compensate for destruction during heating. The ash content was determined by combusting the samples in a furnace at 575 °C.

RESULTS AND DISCUSSION

Characterization of Raw Materials

The main chemical components of Masson pine, poplar, bamboo, and miscanthus are listed in Table 2. As expected, Masson pine had the highest lignin content (29.4%). However, bamboo, as a graminaceous plant, had a high lignin content (28.0%), compared to poplar (25.9%) and miscanthus (20.7%). A high amount of lignin in raw materials is generally not good for enzymatic hydrolysis because of increased blocking of the contact between cellulose and the enzyme. Mild alkali pretreatment mainly relies on delignification to achieve high enzymatic efficiency (Mosier *et al.* 2005). Predominant polysaccharides in Masson pine were glucan and mannan, while they were glucan and xylan in poplar, bamboo, and miscanthus. Total sugars of Masson pine, poplar, and bamboo were 62.6%, 60.1%, and 60.2%, respectively. Miscanthus had a similar amount of polysaccharides (55.7%) as rice straw (Yang *et al.* 2012) and corn stover (Gu *et al.* 2012). The weight percentage of the leaf in miscanthus was 57%. The leaf contains many non-fiber components, such as parenchyma, which have been suggested to be separated from the pulp during pulp preparation to avoid their adverse effect on paper quality (Brown 1958; Kolmer *et al.* 2007). However, the polysaccharides in both the leaf and stem of non-wood fiber materials are expected to be readily hydrolyzed into fermentable sugar, which can be used for bioethanol production (Jin and Chen 2006; Jin *et al.* 2013).

Table 2. Chemical Composition of the Four Untreated Materials (%)

Untreated materials	Extract ^a	Lignin			Polysaccharides				Ash
		KL ^b	ASL ^c	Total	Glucan	Xylan	Ara+man ^d	Total	
Masson pine	2.9±0.1	28.8±0.3	0.6±0.0	29.4±0.2	41.9±0.5	2.2±0.0	18.5±0.3	62.6±0.4	N.D.
Poplar	1.5±0.1	23.2±0.1	2.6±0.0	25.9±0.2	44.1±0.2	15.4±0.2	0.6±0.1	60.1±0.3	N.D.
Moso bamboo	5.8±0.1	26.1±0.1	1.9±0.1	28.0±0.2	37.7±0.3	19.7±0.3	2.8±0.0	60.2±0.6	0.5±0.0
Miscanthus	4.8±0.5	18.1±0.3	2.6±0.0	20.7±0.4	35.2±0.1	17.5±0.2	3.0±0.2	55.7±1.0	6.0±0.1

^a Benzene-ethanol extractives; ^b Klason lignin; ^c Acid soluble lignin; ^d Arabinan and mannan; N.D., Not detectable
Data presented as the average ± standard deviation

Degradation of Polysaccharides and Lignin in GL Pretreatment

The solids recovery and the chemical composition of GL pretreated materials are illustrated in Fig. 1. The GL pretreated materials showed different solids recovery, varying with the pretreatment temperature and TTA charge, which ranged from 56.8% to 87.1%. The solids loss was mostly caused by chemical composition degradation, especially by

hemicellulose and lignin. More than 87% of glucan remained in the pretreated poplar, bamboo, and miscanthus at all pretreatment conditions, while Masson pine had a relatively higher cellulose loss that ranged from 10% to 25% due to alkaline hydrolysis of cellulose under severe pretreatment conditions with a high temperature (170 °C) and a high TTA charge (16 to 28%) (Sjöström 1993).

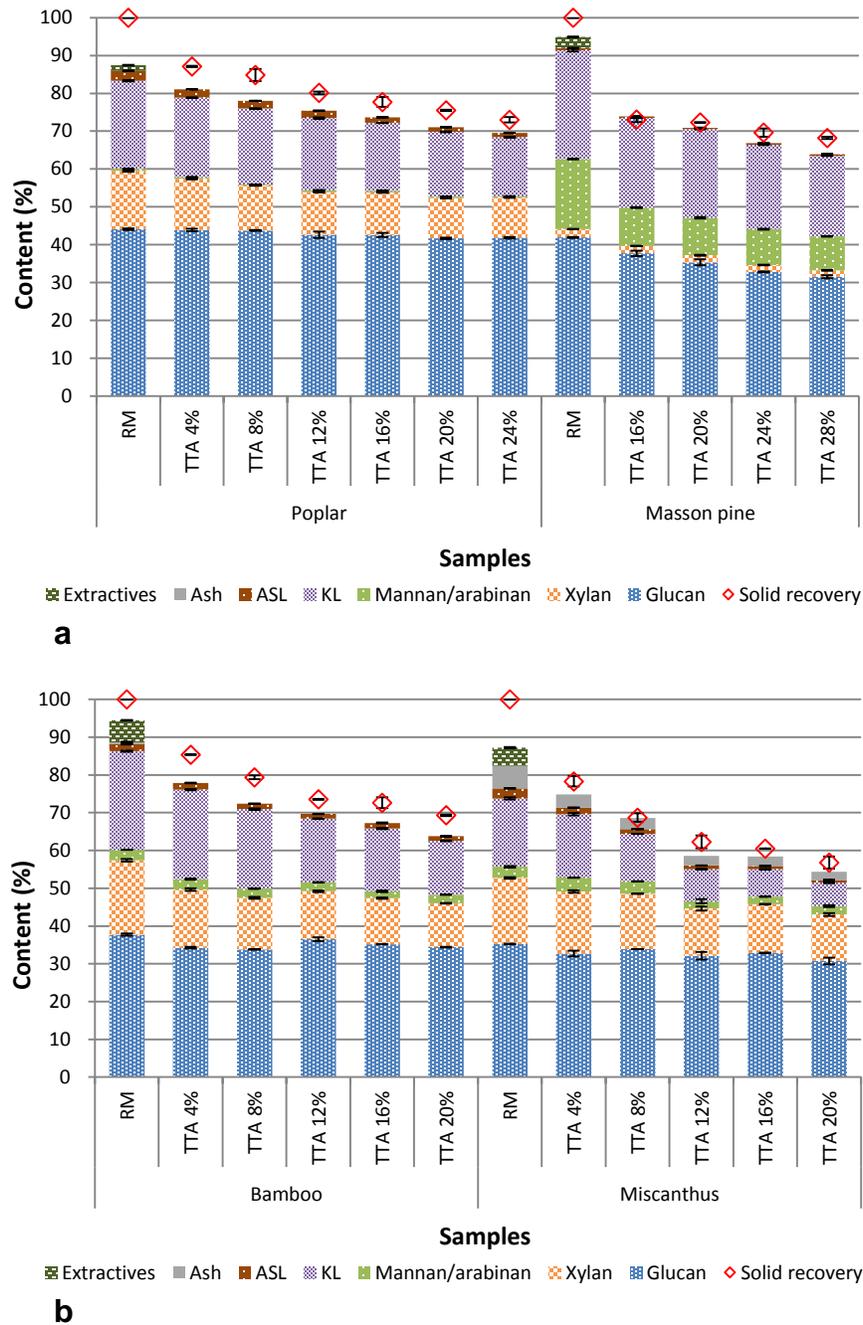


Fig. 1. Solid recovery and retention of various components of (a) GL-pretreated poplar (160 °C) and Masson pine (170 °C), and (b) GL-pretreated moso bamboo (150 °C) and miscanthus (140 °C). Data presented as the average ± standard deviation (RM: Raw Material).

Compared to cellulose, hemicellulose is highly branched and has a low degree of polymerization; therefore it is more easily degraded under alkaline conditions. The hemicellulose degradations at 20% TTA charge were 43.0%, 31.0%, 38.3%, and 29.0%, respectively, for GL-pretreated Masson pine, poplar, moso bamboo, and miscanthus. Leu and Zhu (2013) reported that the removal of hemicelluloses is very important for improving cellulose accessibility because of the direct cross-linkage between cellulose and hemicellulose. However, the recovery of dissolved sugar from pretreated liquor mainly caused by the degradation of hemicellulose needs to be considered for an economical final sugar yield. The pretreated solid containing a large amount of polysaccharides is beneficial to enzymatic hydrolysis for a high sugar yield in one step. The total sugar retention and the lignin removal of GL pretreated materials are depicted in Fig. 2. As shown in Fig. 2a, poplar had a much higher sugar retention than bamboo under the same pretreatment temperature and TTA charge. For example, 87.7% polysaccharides were retained in GL pretreated poplar at 20% TTA and 160 °C, while only 80.2% were retained for bamboo pretreated at the same conditions. Masson pine had relative low sugar retention (< 80%) because of the severe pretreatment conditions. Besides, softwood has a large amount of glucomannan, which is more easily subjected to degradation under alkali pretreatment than glucuronoxylan in hardwood and herbaceous materials (Sjöström 1993).

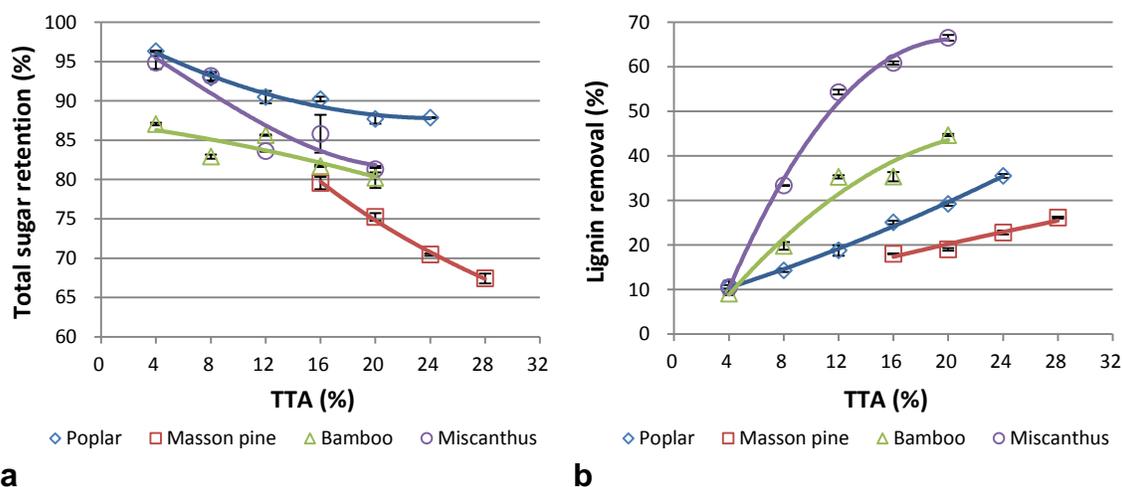


Fig. 2. Effect of TTA charge on (a) total sugar retention and (b) lignin removal in GL pretreatment. Data presented as the average \pm standard deviation

Lignin is recognized as the major barrier for enzymatic hydrolysis either by physical blockage (Mooney *et al.* 1998) or unproductive enzyme binding (Berlin *et al.* 2006), thus reducing the accessibility of cellulase to cellulose. Removing lignin is one of the most effective approaches for increasing accessibility of cellulose towards cellulase by mild alkaline pretreatment. As shown in Fig. 2b, lignin removal increased with the TTA charge, but it was rather different for various raw materials. For example, the lignin removals of Masson pine, poplar, moso bamboo, and miscanthus pretreated at 20% TTA were 19.0%, 29.2%, 44.7%, and 66.5%, respectively. A limited lignin removal was obtained by GL pretreated Masson pine, ranging from 18.0% to 26.2% with 16 to 28% TTA charge at 170 °C. The GL pretreatment was very effective for removing lignin from graminaceous materials, especially for miscanthus. Two-thirds of miscanthus lignin could be removed at 140 °C. In Gramineae, the ester linkages between lignin and hemicellulose

via ferulic and *p*-coumaric acid are quite flexible, especially in alkaline conditions (Buranov and Mazza 2008). Lignin removal of 20 to 65% is sufficient to improve the accessibility of the cellulose to enzymes (Youn *et al.* 1983). A very low lignin removal cannot sufficiently disrupt the structure of lignocellulose for enhancing enzymatic hydrolysis. On the other hand, excess lignin removal usually needs relatively severe pretreatment conditions that result in sugar degradation, which decreases the hydrolysable sugar in the pretreated solid.

Polysaccharide degradation and lignin removal are the primary reasons for solid loss during pretreatment. As shown in Fig. 3, there was a linear relationship between solid recovery and lignin removal. The slope of wood materials was lower than -0.5, while it was higher than -0.5 for graminaceous materials. This implies that GL pretreatment has better delignification selectivity on graminaceous materials than woody materials, with the following order: miscanthus > moso bamboo > poplar > Masson pine. Delignification selectivity is a very important index for alkaline pretreatment. High delignification selectivity means that the pretreated solid is produced with more lignin removal and less sugar degradation. This is very important for improving the subsequent enzymatic hydrolysis effectively.

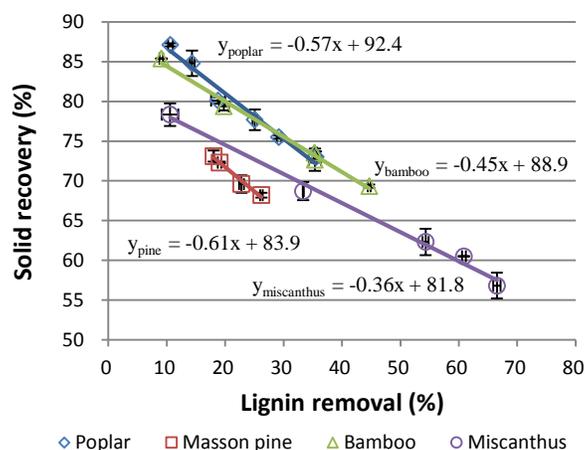


Fig. 3. Solid recovery of GL pretreated materials as a function of lignin removal. Data presented as the average \pm standard deviation

Enzymatic Saccharification of GL Pretreated Materials

Biomass pretreated by GL under the conditions described earlier was hydrolyzed using the series enzyme Cellic[®] CTec2. The hydrolytic efficiency was evaluated by substrate enzymatic digestibility (SED) and sugar yield, which were calculated using Eqs. 1 and 2, respectively.

$$\text{SED (\%)} = \frac{\text{Sugar in enzymatic hydrolysate (g)}}{\text{Sugar in pretreated solid (g)}} \times 100\% \quad (1)$$

$$\text{Sugar yield (\%)} = \frac{\text{Sugar in enzymatic hydrolysate (g)}}{\text{Sugar in raw material (g)}} \times 100\% \quad (2)$$

The sugar yield of GL pretreated materials hydrolyzed at an enzyme loading of 20 FPU/g-cellulose is listed in Table 3. The sugar yield increased rapidly with the TTA charge from 4% to 20% for GL pretreated poplar and bamboo, while miscanthus achieved the

highest sugar yield at 16% TTA charge. The maximum sugar yields of poplar, bamboo, and miscanthus were 61.9%, 48.5%, and 74.8%, respectively. The total sugar yield of GL pretreated Masson pine was extremely low with less than 20% at 20 FPU/g-cellulose and had little change with increased TTA charge from 16% to 28%. Bamboo had a relatively low sugar yield compared with other herbaceous materials pretreated by GL, such as miscanthus, corn stover (Gu *et al.* 2012), and rice straw (Gu *et al.* 2013). The relative density of moso bamboo at different horizontal and vertical locations ranged from 0.553 to 1.006 g/cm³ (Yu *et al.* 2008) which is even higher than poplar (0.41 g/cm³) (Kiaei and Samariha 2011) and Masson pine (0.449 to 0.509 g/cm³) (Zhang *et al.* 2012). The high density of the raw material with low porosity may affect the penetration of pretreatment liquor and enzyme accessibility. In addition, the morphological structure, chemical composition, and its distribution of raw materials (Wu *et al.* 2010) are very important factors for bioconversion. The GL-pretreated miscanthus had relatively high enzymatic digestibility, compared with the rest of the materials. The sugar yield of GL-pretreated (140 °C, 16% TTA, 1 h) miscanthus was 74.8% at 20 FPU/g-cellulose (Table 3). This is presumably because natural low density, loose physical structure, and high delignification selectivity during the pretreatment improved the enzyme accessibility for enzymatic hydrolysis.

Table 3. Substrate Enzymatic Digestibility and Sugar Yield of GL Pretreated Masson Pine, Poplar, Bamboo, and Miscanthus Hydrolyzed at 20 FPU/g-cellulose

Material	TTA (%)	Substrate enzymatic digestibility (%)			Sugar yield (%)		
		Glucan	Xylan	Total ^a	Glucan	Xylan	Total ^a
Masson pine	16	23.3±1.2	19.5±0.7	21.7±1.0	20.8±1.0	10.8±0.0	17.3±0.8
	20	23.3±0.6	19.0±0.7	21.6±0.6	18.9±0.5	9.7±0.0	16.3±0.5
	24	26.3±0.2	19.8±0.0	24.0±0.2	19.8±0.2	9.7±0.0	16.9±0.2
	28	26.6±0.6	19.7±2.3	24.2±1.0	20.0±0.5	9.7±1.1	16.3±0.6
Poplar	4	30.0±0.1	43.7±0.1	33.2±0.1	29.3±0.1	41.1±0.0	32.0±0.1
	8	54.7±0.5	69.0±0.4	57.5±0.5	54.2±0.5	53.2±0.3	53.4±0.5
	12	60.9±0.9	68.1±0.7	61.9±0.9	58.9±0.9	49.9±0.5	56.0±0.8
	16	63.9±0.2	67.1±0.9	64.1±0.3	61.6±0.2	49.1±0.7	57.8±0.3
	20	69.7±0.6	77.0±1.0	70.6±0.7	65.9±0.6	53.1±0.8	61.9±0.6
Bamboo	4	26.2±0.5	39.4±0.6	30.9±0.2	23.9±0.5	30.7±0.4	26.9±0.2
	8	39.5±0.5	54.4±0.4	43.6±0.3	35.4±0.4	37.8±0.3	36.0±2.2
	12	46.3±0.2	62.7±0.1	50.0±0.1	44.8±0.2	40.4±0.1	42.8±0.1
	16	52.0±0.2	66.7±0.4	55.6±0.1	48.5±0.2	41.2±0.2	45.4±0.1
	20	58.2±1.8	71.8±1.3	60.5±1.6	53.1±1.7	42.3±0.8	48.5±1.3
Miscanthus	4	56.5±0.5	45.2±0.8	51.1±0.9	52.4±0.4	42.4±0.7	48.5±0.8
	8	68.0±1.7	64.8±0.2	64.3±0.6	65.3±1.6	54.3±0.2	59.9±0.5
	12	82.1±0.7	79.7±0.3	80.5±0.1	74.8±0.6	57.3±0.2	67.3±0.1
	16	89.2±2.4	85.4±2.6	87.2±2.8	83.3±2.2	62.8±1.9	74.8±2.4
	20	90.9±0.9	85.8±0.4	88.0±0.5	79.3±0.8	60.3±0.3	71.6±0.4

^a Total sugar is the sum of glucan, xylan, arabinan and mannan
Data presented as the average ± standard deviation

Delignification during pretreatment is a crucial factor affecting enzymatic hydrolysis (Yu *et al.* 2011). The relationship between the SED and lignin removal is shown

in Fig. 4. Different materials showed different lignin removal sensitivity. Maximum sugar yield was achieved by 29% lignin removal for poplar, while miscanthus had the maximum sugar yield at 61% lignin removal. Moso bamboo had a similar correlation between lignin removal and total sugar yield with miscanthus. Little enhancement was found in GL pretreated Masson pine with increasing lignin removal from 18% to 26%. Wu *et al.* (2010) also reported that only 41% of the polysaccharides in GL pretreated loblolly pine were converted to monomeric sugars at an enzyme loading of 40 FPU/g-substrate (~70 FPU/g-cellulose). Further treatment such as oxygen delignification or/and mechanical refining is needed to increase the sugar yield. This implies that the delignification selectivity is an important index for high sugar yield by high lignin removal with low polysaccharide degradation during pretreatment.

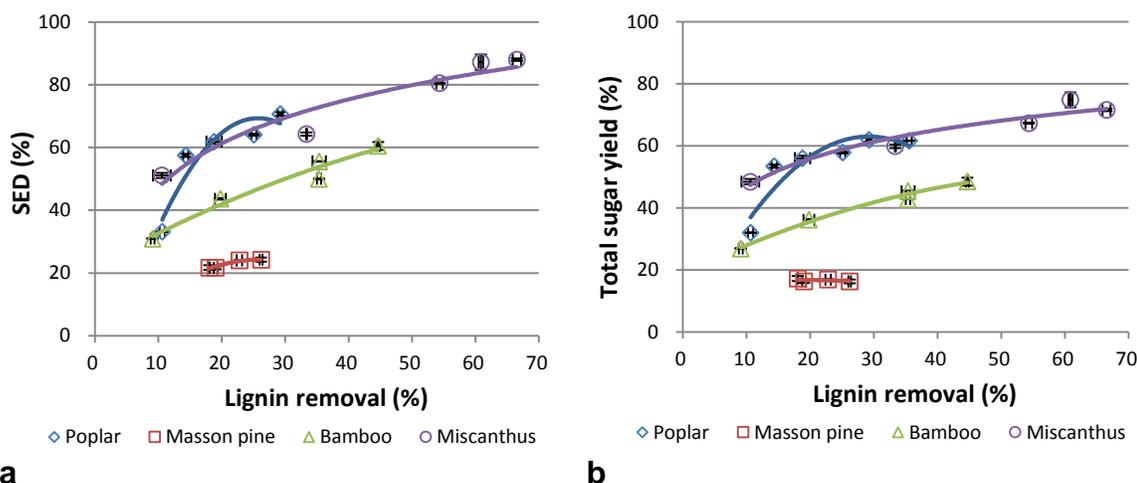


Fig. 4. The effect of lignin removal in GL pretreatment on (a) SED and (b) total sugar yield. The pretreatment temperatures for poplar, Masson pine, moso bamboo, and miscanthus were 160 °C, 170 °C, 150 °C, and 140 °C, respectively. Data presented as the average \pm standard deviation

CONCLUSIONS

1. Green liquor pretreatment improved the enzymatic digestibility of lignocellulosic biomass by partially removing lignin and hemicellulose.
2. The delignification selectivity is an important parameter for obtaining ideal enzymatic digestibility with GL pretreatment. Maximum sugar yields were 17.3%, 61.9%, 48.5%, and 74.8%, respectively achieved at lignin removals of 18%, 29%, 49%, and 61% for Masson pine, poplar, moso bamboo, and miscanthus under given pretreatment conditions.
3. The GL pretreatment had better effects on enzymatic digestibility for miscanthus and poplar than Masson pine and bamboo, which implies that this mild alkali pretreatment may be more suitable for low-density materials, particularly herbaceous materials.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31370571), the Specialized Research Fund for the Doctoral Program of Higher Education, China (20133204110006), and the Priority Academic Program Development of Jiangsu Higher Education Institutions, China. The authors are also grateful for the financial support from the China Scholarship Council (CSC No. 201308320284).

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Article submitted: August 13, 2014; Peer review completed: November 1, 2014; Revised version received: November 15, 2014; Accepted: November 16, 2014; Published: December 3, 2014.