Microstructure Properties and Cellulase Hydrolysis Efficiency of Hybrid *Pennisetum* with [Amim]Cl Pretreatment

Shengdan Wang,[a] Jiachuan Chen,[b,]* Guihua Yang,[b] Kefu Chen,[a] Rendang Yang,[a] and Jinsong Zeng [a,*]

The complex microstructure of lignocellulosic biomass restricts its conversion into bio-ethanol. In this study, the effects of an ionic liquid (IL) 1-allyl-3-methylimidazolium chloride ([Amim]Cl) pretreatment on the microstructure properties and cellulase hydrolysis efficiency of hybrid *Pennisetum* (*P. americanum* × *P. purpureum,* lignocellulosic biomass) were investigated. After the [Amim]Cl pretreatment, the bonds of lignin-carbohydrate complex (LCC) and C=O in xylan were destroyed and the content of inter-molecular H-bonds O(6)H…O(3’) decreased by 47.2%, while the content of intra-molecular H-bonds of O(2)H…O(6) and O(3)H…O(5) increased by 9.5% and 47.0%, respectively. The crystallinity and the crystallite size decreased by 20.8% and 42.22%, respectively, and the cellulose crystalline structure changed from cellulose crystalline I to cellulose crystalline II. The specific surface area increased from 0.15 to 10.11 m^2/g after the [Amim]Cl pretreatment. The glucose recovery increased by 10.3 times after being pretreated with [Amim]Cl, compared with the unpretreated sample.

Keywords: Hybrid Pennisetum; Microstructure; Pretreatment; [Amim]Cl; Enzymatic hydrolysis

Contact information: a: State Key Laboratory of Pulp and Paper Engineering, Plant micro/nano fiber research center, School of Light Industry and Engineering, South China University of Technology, Guangzhou, 510640, China; b: Key Lab of Pulp & Paper Science and Technology of Education Ministry of China, Qi Lu University of Technology, Jinan, 250353, China;
** Corresponding authors: chenjc@qlu.edu.cn; fezengjs@scut.edu.cn*

INTRODUCTION

Lignocellulosic biomass is a sustainable raw material used for the production of alternative fossil fuels, such as the conversion of cellulose to cellulosic ethanol, furfural, and other high value-added biopolymers (Rose and Palkovitz 2011). As a relatively new feedstock, the energy grass hybrid *Pennisetum*, a perennial herbaceous plant, has attracted growing research because of its advantages, such as its resistance to salinity and drought, suitability for marginal land quality, high productivity, low nutritional requirements, environmental benefits, and its versatility (Chen *et al*. 2014). The grass hybrid is cultivated in north China, the Middle-Lower Yangtze Plain, northeast China, and southwest China, and can grow up to more than 2 m tall in 5 to 6 months, with annual yields as high as 60 to 130 tons per hm^2 (Liu 2009; Zhao and Zhou 2010). Using the cellulose in hybrid *Pennisetum* as a C6 carbon source to produce bio-ethanol would be an effective process for its utilization in China, instead of burning the plant. The hydrolysis of cellulose in the hybrid *Pennisetum* can be carried out to produce bio-ethanol (Tadesse and Luque 2011). However, the complex structure and recalcitrance of its plant cell wall makes it difficult to achieve bio-energy conversion efficiently (Jørgensen *et al*. 2007).

Therefore, a pretreatment is a necessary step to destroy the rigid protective sheath for the improved glucose recovery of cellulose hydrolysis during the production of bio-energy (Zheng *et al.* 2009; Xu *et al.* 2013). Quite a number of pre-processing operations have shown a tendency to improve the hydrolysis efficiency, including physical, biological, and chemical methods (Yang and Wyman 2008). Compared with the above pre-processing methods, ionic liquid (IL) pretreatments have attracted increasing research for the dissolution and subsequent hydrolysis of lignocellulosic biomass. This is due to their desirable properties, such as an environmentally benign nature, low melting point, low volatility and toxicity, and high thermal and chemical stability (Tadesse and Luque 2011). Cellulose samples could be dissolved by many ILs, such as 1-ethyl-3-methylimidazolium acetate ([Emim]Ac), 1-butyl-3-methylimidazolium chloride ([Bmim]Cl), and 1-allyl-3-methylimidazolium chloride ([Amim]Cl). Among the different ILs, [Amim]Cl possesses a high reactivity of allyl chloride and shows better solubility for cellulose samples than the others (Zhang *et al.* 2005; Liu *et al*. 2012).

Recently, the optimization of [Amim]Cl pretreatment conditions for the maximum glucose recovery from hybrid *Pennisetum* has been obtained by response surface methodology (Wang *et al.* 2015). However, the microstructural changes and those effects on the cellulase hydrolysis efficiency of hybrid *Pennisetum* were not elucidated. This study aimed to investigate the microstructure properties of hybrid *Pennisetum* after [Amim]Cl pretreatment. A novel analytical approach of the Gaussian distribution function was used to analyze the H-bond energies (E_H) and bond lengths (R) of different H-bond models, and the crystallite size of the cellulose in the hybrid *Pennisetum*. Furthermore, the effects of the change in microstructure properties of the hybrid *Pennisetum* on the cellulase hydrolysis efficiency were elucidated.

EXPERIMENTAL

Materials

The hybrid *Pennisetum* was collected from an experimental field of the Beijing Academy of Agricultural Sciences (China). It was milled with a FZ120 plant shredder (Truelab, Shanghai, China) and then sieved to 40- to 60-mesh. A comprehensive extraction process with toluene/ethanol (2:1, v/v) was performed in a Blst-250SQ Soxhlet apparatus (Bilon, Shanghai, China). The extractive-free samples were stored at 4 °C in a sealed bag. The ionic liquid ([Amim]Cl) was purchased from the Lanzhou Institute of Chemical Physics (Lanzhou, China). Commercial cellulase (Celluclast 1.5 L, activity 84.5 FPU/g) from *Trichoderma reesei* was purchased from Novozymes (Beijing, China).

Methods

Ionic liquid pretreatment

The pretreatment process was performed according to the authors' previous work (Wang *et al.* 2015). Briefly, 5 g of hybrid *Pennisetum* was placed in 49.95 g of [Amim]Cl in a 150-mL dried screw-capped Synthware tube (Synthware, Beijing, China). The mixture was heated to 139 °C in a preheated oil bath for 178 min. After cooling to 90 °C, deionized water (90 °C) was added to the slurry with an agitation rate of 250 rpm for 1 h.

The mixture was centrifuged to separate the solid (pretreated samples). The precipitate (pretreated sample) was freeze-dried and collected for subsequent analyses.

Enzymatic hydrolysis

The enzymatic hydrolysis of pretreated and unpretreated samples was performed in 25-mL stoppered conical flasks at 50 °C in an air bath shaking incubator (ZWY-2102C, Shanghai, China) at 150 rpm for 72 h. Then, 0.2 g of the pretreated sample was suspended in 10 mL of a 50 mM citrate acid-sodium citrate buffer (pH 4.8) at an enzyme loading of 20 FPU/g substrate. After hydrolysis, the samples were kept in a boiling bath for 5 min to inactivate the enzyme.

The glucose concentrations from the enzymatic hydrolysates were measured by HPLC (Agilent 1200, CA, USA) with a Bio-Rad Aminex HPX-87H analytical column. The column temperature was set at 35 °C (Wang *et al*. 2015).

Component analysis

The chemical composition of the unpretreated and pretreated samples was analyzed using the methods of the Laboratory Analytical Procedure (LAP) provided by the National Renewable Energy Laboratory (NREL) (Sluiter *et al.* 2008).

FTIR analysis

The deconvolution of the spectra (3800 to 3000 cm^{-1}) was carried out using Peak-Fit (Jinan, China) software (4.12.00) in conjunction with the Gaussian distribution function to analyze the H-bond energies (E_H), bond strength, and bond lengths (R) of different H-bond models. After deconvolution, the H-bond characteristic parameters were calculated as follows (Struszczyk and Laine 1986),

$$\mathrm{E_H} = \frac{1}{\mathrm{K}}\frac{v_0 - v}{v_0} \tag{1}$$

where v_0 is the standard free hydroxyl frequency (3650 cm^{-1}), v is the sample hydroxyl frequency, and k is a constant (6.7 × 10^{-2} kJ^{-1}).

$$\Delta v = 4.43 \times 10^3 \cdot (2.83 - \mathrm{R}) \tag{2}$$

In. Eq. 2, $\Delta v = v_0 - v$, v_0 is the stretch vibrational frequencies of hydroxyl (3600 cm^{-1}), v is the sample hydroxyl frequency (cm^{-1}), and R is the bond length (Å).

X-ray diffraction (XRD) analysis

The crystallinity of cellulose I and cellulose II, and the average width of the crystal in the 002/101-lattice plane, were calculated as follows (Langford and Wilson 1978; Struszczyk and Laine 1986),

$$\mathrm{Cr_I} = \frac{\mathrm{I_{002}} - \mathrm{I_{am}}}{\mathrm{I_{002}}} \times 100\% \tag{3}$$

where I_{002} is the diffraction intensity at 2θ = 22.6° and I_{am} is the diffraction intensity at 2θ = 18°.

$$\mathrm{Cr_{II}} = \frac{\mathrm{I_{101}} - \mathrm{I_{am}}}{\mathrm{I_{101}}} \times 100\% \tag{4}$$

In Eq. 4, I_{002} is the diffraction intensity at 2θ = 20.8° and I_{am} is the diffraction intensity at

$2\theta = 16°$.

$$D_{\mathrm{hkl}} = \frac{\mathrm{k}\lambda}{\beta\cos\theta} \quad (5)$$

In Eq. 5, k is the Scherrer constant (0.89), λ is the wavelength of the X-ray source (0.15418 nm), β is the width of the middle height of the 002/101-lattice plane, in RAD, and θ is the maximum of the 002 reflection, in RAD.

The spectra were deconvolved using the Peak-Fit software in conjunction with the Gaussian distribution function to calculate the half-peak width and microcrystalline size.

Scanning electron microscopy (SEM) observation and BET surface area (BET) analysis

The surface characteristics of pretreated and unpretreated samples were observed using a scanning electron microscope (SEM, FEI Quanta-200 ESEM, EIN, Holland). Moreover, the specific surface area of the pretreated and unpretreated samples was analyzed by the nitrogen adsorption method (BET, V-Sorb 2800P, Beijing, China).

RESULTS AND DISCUSSION

Compositional Analysis

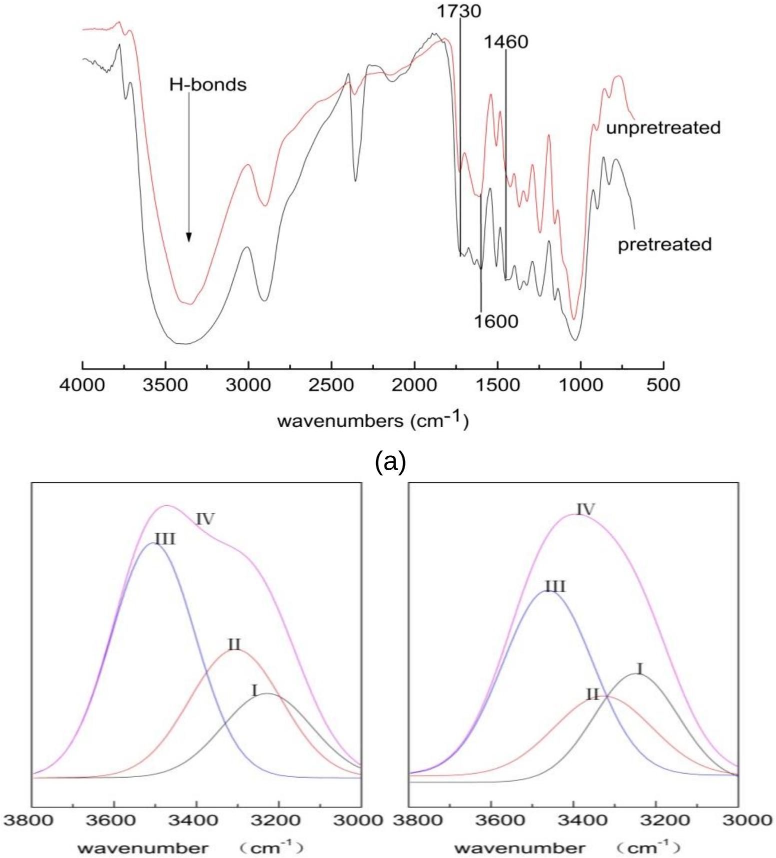
The chemical composition results show that the recovery yield of hybrid *Pennisetum* after [Amim]Cl pretreated was 69.6%. The cellulose, hemicelluloses, and lignin contents in the unpretreated sample were 36.64%, 18.05%, and 11.03%, respectively. The content of cellulose in the pretreated sample rose by 8.39%, to 45.03%, while the hemicelluloses and lignin contents fell by 5.64% and 1.41%, to 12.41% and 9.62%, respectively. This shows that part of the hemicelluloses and lignin in the hybrid *Pennisetum* were removed after the [Amim]Cl pretreatment, especially the hemicelluloses.

Changes in microstructure of cellulose of hybrid Pennisetum

A comparison of the FTIR spectra (Fig. 1a) of the unpretreated and pretreated hybrid *Pennisetum* was used to detect the changes of the structural features. As shown in Fig. 1(a), the spectral intensity of characteristic function groups at 1730 cm^{-1} of C=O in xylan decreased (Kootstra *et al.* 2009), while the spectral intensity at 1460 cm^{-1} of the CH_2 in-plane bending vibration increased for the pretreated sample (Kumar *et al.* 2009). This indicates that the LCC and C=O bonds of xylan in hybrid *Pennisetum* were destroyed after the [Amim]Cl pretreatment (He *et al.* 2008).

The band at 3000 to 3800 cm^{-1} was attributed to the stretching vibration of the intermolecular and intra-molecular H-bonds (Sang *et al.* 2005; Popescu *et al.* 2007). The FTIR spectra in the region of 3000 to 38000 cm^{-1} were fitted into three peaks to analyze the intra-molecular and inter-molecular H-bonds of the pretreated and unpretreated samples. As shown in Fig. 1(b), the intra-molecular H-bonds for O(2)H···O(6) and O(3)H···O(5), and the inter-molecular H-bonds for O(6)H…O(3’) appeared at wave-numbers of 3455 to 3410 cm^{-1}, 3375 to 3340cm^{-1}, and 3310 to 3230 cm^{-1}, respectively (Pimentel and Sederholm 1956). The peak-fitting parameters of different H-bonds are shown in Table 1.

As shown in Table 1, the content of inter-molecular H-bonds of O(6)H···O(3`) in the pretreated sample decreased by 47.25%, while the bond energy of inter-molecular H-bonds O(6)H···O(3') was the maximum, although its bond length was the shortest. This result indicates that the inter-molecular H-bonds are difficult to break and had a more significant positive effect on cellulase hydrolysis efficiency than intra-molecular H-bonds. The decreasing of inter-molecular H-bonds resulted in the loosening of the cellulose chains and helped to expose the free hydroxyl groups of the hybrid *Pennisetum* after the [Amim]Cl pretreatment. Additionally, Table 1 shows that the content of intra-molecular H-bonds O(2)H…O(6) were more than the O(3)H…O(5) in hybrid *Pennisetum*. Moreover, the energy of intra-molecular H-bonds O(2)H···O(6) was higher than O(3)H···O(5). However, the increment of intra-molecular H-bonds O(3) H…O(5) (47.0%) was more than O(2)H…O(6) (9.5%) after the [Amim]Cl pretreatment. This indicated that the intra-molecular H-bonds O(3)H…O(5) were easier to form than O(2)H…O(6) during the [Amim]Cl pretreatment process. Similar results were reported for producing 5-hydroxymethyl furfural *via* waste paper (Wan *et al.* 2015).



(a)

(b)

Fig. 1. The FTIR spectra of the pretreated and unpretreated samples (a), and the fitting of H-bonds stretching (b). (I) inter-molecular H-bonds O(6)H...O(3'); (II) intra-molecular H-bonds O(3)H...O(5); (III) intra-molecular H-bonds O(2)H...O(6); and (IV) raw spectra

The XRD spectra of the unpretreated and pretreated samples are shown in Fig. 2. The diffraction angles of the 002 and 101 lattice planes were changed after the [Amim]Cl pretreatment. This demonstrates that the cellulose crystal type of hybrid *Pennisetum* pretreated by [Amim]Cl changed from cellulose crystalline I to cellulose crystalline II.

Table 1. Characteristics of Different H-bond Models Obtained from FTIR Peak Fitting

Sample	Peak	H-bonds	Wave-number (cm^{-1})	Content (%)	Bond Energy (kJ)	Bond Length (Å)
Unpre-treated	I	O(6)H…O(3’)	3249	27.3	1.6397	2.7698
	II	O(3)H…O(5)	3333	23.2	1.2963	2.7914
	III	O(2)H…O(6)	3462	49.5	0.7689	2.7988
Pretreated	I	O(6)H…O(3’)	3230	14.4	1.7174	2.7465
	II	O(3)H…O(5)	3304	34.1	1.4148	2.7632
	III	O(2)H…O(6)	3505	51.7	0.5929	2.8086

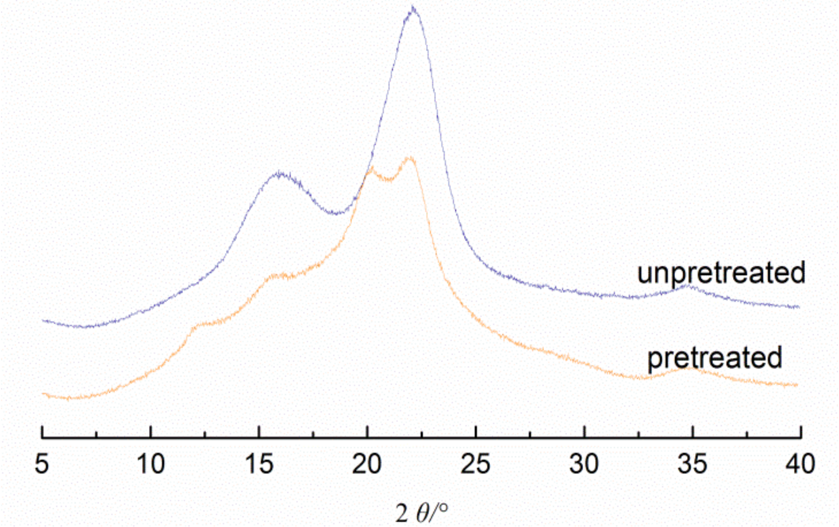


Fig. 2. The XRD spectra of the pretreated and unpretreated samples

Table 2 shows the microcrystalline parameters of unpretreated samples and those pretreated with [Amim]Cl. The relative crystallinities of unpretreated and pretreated samples were 48.1% and 38.1%, respectively. The relative crystallinity of the pretreated sample decreased by 20.8% after the [Amim]Cl pretreatment. Moreover, the I_{002} lattice plane crystallite size of cellulose crystalline I in the unpretreated sample and the I_{101} lattice plane crystallite size of cellulose crystalline II in pretreated sample were 56.16 and 32.45 nm, respectively.

Compared with the unpretreated sample, the crystallite size of the characteristic lattice plane (cellulose crystalline II) in pretreated samples decreased by 42.22%. It is well known that inter-molecular H-bonds contribute to the formation of crystalline portions in cellulose molecules.

From a comparison of Tables 1 and 2, it can be found that the content of inter-molecular H-bonds and the microcrystalline size of cellulose in hybrid *Pennisetum* have similar variation tendencies after [Amim]Cl pretreatment. This result implies that when the content of inter-molecular H-bonds decreased, the adjacent cellulose chains loosened and led to a decrease in the amorphous portions (Vainio and Paulapuro 2007). Therefore, the related microcrystalline size in the crystalline portions decreased.

Table 2. Microcrystalline Parameters of Unpretreated and Pretreated Samples

Sample	Cellulose Crystalline	Diffraction Angle (°)	Lattice Plane	Crystallinity (%)	D_{hkl} (nm)
Unpretreated	I	22.5	002	48.1	56.16
Pretreated	II	21.6	101	38.1	32.45

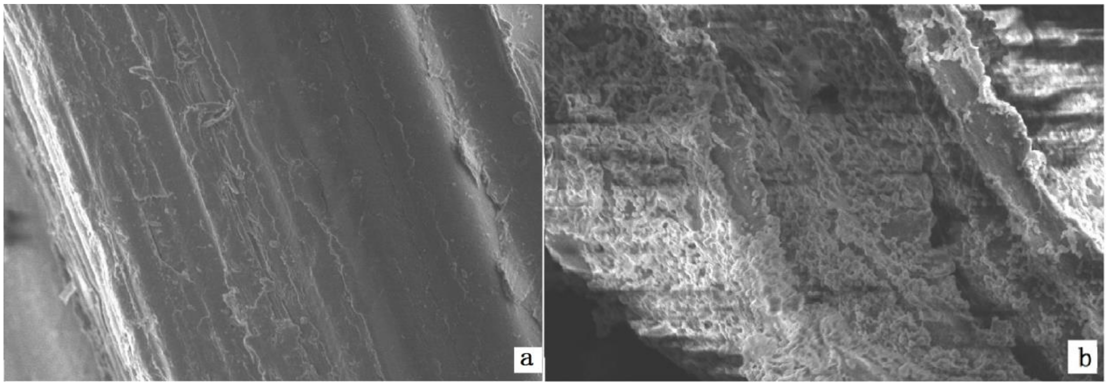


Fig. 3. Scanning electron micrographs of (a) unpretreated and (b) pretreated samples

The SEM images of the unpretreated and pretreated samples are illustrated in Fig. 3. The pretreated samples show a different morphology compared with the unpretreated samples, with rough and porous surfaces because of the removal of lignin and hemicelluloses. Furthermore, the specific surface area of unpretreated samples (11.09 m^2/g) increased by 73 times after the [Amim]Cl pretreatment (0.15 m^2/g). This result implies that the surface structures of hybrid *Pennisetum* were severely damaged by [Amim]Cl pretreatment process.

Cellulase Hydrolysis Efficiency

A comparison of the cellulase hydrolysis efficiency of both the pretreated and unpretreated sample is shown in Fig. 4. Compared with the glucose concentration of the unpretreated sample (1.25 g/L), the glucose concentration of the pretreated sample was markedly increased (14.15 g/L) after 70 h of enzymatic hydrolysis.

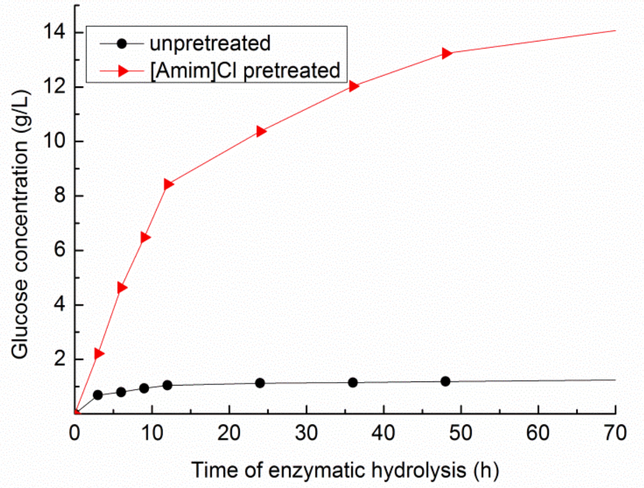


Fig. 4. Comparison of enzymatic hydrolysis before and after [Amim]Cl pretreatment

It can be inferred that there is a direct relationship between the cellulase hydrolysis efficiency and hemicellulose/lignin removal. Similar results have been reported for a dilute sulfuric acid pretreatment of hybrid poplar (Sun *et al.* 2014). The high glucose recovery may be primarily a result of the decrease of inter-molecular H-bonds, resulting in the loosening of the cellulose chains, and the weaker hydrophobic interaction by transforming cellulose I to cellulose II (Wada *et al.* 2010). Moreover, the increasing specific surface area and roughness will accelerate the adsorption of cellulase enzymes and subsequently enhance the enzyme attack.

CONCLUSIONS

1. The highest glucose concentration of 14.15 g/L was obtained after 72 h of enzymatic hydrolysis from the pretreated sample. The glucose recovery increased by 10.3 times after the [Amim]Cl pretreatment compared with the unpretreated sample.
2. A decrease in the relative content of inter-molecular H-bonds O(6)H…O(3'), hemicellulose, and lignin through [Amim]Cl pretreatment can greatly improve the cellulase hydrolysis efficiency of hybrid *Pennisetum*. Similar statements can be made for the decrease in the relative crystallinity and crystallite size.
3. The increasing surface roughness and porosity by [Amim]Cl pretreatment can improve the cellulase hydrolysis efficiency of hybrid *Pennisetum*. Moreover, the cellulose crystalline II of hybrid *Pennisetum* is far easier to hydrolyze than that of cellulose crystalline I.

ACKNOWLEDGMENTS

The authors are grateful for the support of the National Natural Science Foundation of China (Grant No. 31270627, 31370580, 31400511), the Major Science and Technology Projects of Shandong Province (No. 2014ZZCX09101, 2015ZDZX09002), the Taishan Scholars Project Special Funds, 111 plan, and the Guangdong provincial science and technology plan projects (Number: 2015B020241001, Name: Research and Application of Biomass Pretreatment and Ethanol Production Technology).

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Article submitted: October 14, 2016; Peer review completed: November 25, 2016; Revised version received: December 1, 2016; Accepted: December 3, 2016; Published: December 13, 2016.
DOI: 10.15376/biores.12.1.1031-1040