Study of the Difference between Enzyme Adsorption onto Hydrotropic and Alkali Lignin Separated from Eucalyptus and Bamboo

Hongyan Mou,* Shubin Wu, Mengyun He, Hao Liu, Hongquan Huang, and Chaochao Xu

Enzymatic hydrolysis of lignocellulosic biomass is the key step for controlling the cost of bioethanol production. However, the non-productive adsorption of cellulase onto lignin in biomass severely hampers the enzyme activity and hydrolysis efficiency. Thus, understanding the adsorption mechanism of cellulase onto lignin is critical for the development of enzyme mixtures and enzymatic hydrolysis. In this investigation, cellulase, β-glucosidase (BG), and xylanase adsorption onto lignin from eucalyptus and bamboo, extracted by alkali and hydrotropic techniques, were compared. The physico-chemical properties of the four types of isolated lignin were detected. Langmuir isotherms were used to interpret the cellulase adsorption kinetics of the lignin. The hydrophobicity was found to be the major factor that affected the cellulase adsorption affinity of lignin. The surface charge was important for the adsorption of BG and xylanase onto the lignin. A comparison was made between hydrotropic and alkali lignin, and the hydrotropic lignin from eucalyptus had the highest cellulase adsorption capacity and lowest BG and xylanase adsorption capacities.

Keywords: Alkali lignin; Hydrotropic lignin; Cellulase; Xylanase; β-glucosidase; Adsorption interaction

Contact information: State Key Laboratory of Pulp and Papermaking, School of Light Industry and Engineering, South China University of Technology, Guangzhou, China, PR-510640;
**Corresponding author: fehymou@scut.edu.cn*

INTRODUCTION

Conversion of renewable lignocellulosic biomass into bioethanol is a potential way to alleviate the energy crisis, while also satisfying an increasing demand for industrial bioethanol. Enzymatic hydrolysis of lignocellulosic biomass is crucial for the production of bioethanol that is economically-viable. However, lignin in lignocellulosic biomass is a major contributor to its resistance to enzymatic hydrolysis (Palonen *et al.* 2004; Berlin *et al.* 2006). In addition to the effect of the physical lignin barrier on the enzymatic accessibility of cellulase, the adsorption of cellulase onto lignin could inhibit cellulase activity and impede the recycling of cellulase (Lou *et al.* 2013). Over the years, cellulase adsorption onto lignin that leads to nonproductive binding has frequently been investigated. It has been revealed that the adsorption interaction between cellulase and lignin is affected by the chemical and physical properties of lignin (Lu *et al.* 2016). Pan (2008) claimed that increasing the phenolic hydroxyl content could reinforce the lignin inhibitory effect. However, increasing the carboxylic content in lignin could reduce the cellulase adsorption affinity of lignin (Lou *et al.* 2013). Moreover, lignin is composed of three major

monolignols, which are *p*-coumaryl alcohol (H units), coniferyl alcohol (G units), and sinapyl alcohol (S units). The influences of the basic structural units on the cellulase adsorption capacity of lignin have been verified as well. It has been shown that G-type lignin has a higher cellulase adsorption affinity than S-type lignin (Guo *et al.* 2014). In a previous study, the high polydispersity index (PDI) of softwood lignin was reported to be inversely proportional to the cellulase adsorption capacity of lignin (Berlin *et al.* 2006). Also, the molecular weight correlates with the cellulase adsorption affinity of lignin. Recently, Li *et al.* (2016) reported that alkali lignin with a lower molecular weight had a higher cellulase adsorption affinity. Cellobiohydrolases I (CBH I) and endoglucanase (EG) were two major components of cellulases (*Trichoderma reesei*). Recently, it was demonstrated that aliphatic hydroxyl groups in lignin and basic units (G, S and H) have positive influence on the maximum binding ability of CBH onto lignin samples (Yao *et al.*2017). Langmuir adsorption isotherms can be used to study the adsorption behavior of cellulase onto lignin (Tu *et al.* 2009; Li *et al.* 2016).

Moreover, β-glucosidase (BG) and xylanase are generally used to sufficiently enhance the enzymatic hydrolysis of lignocellulose (Öhgren *et al.* 2007; Petersen *et al.* 2009). Berlin *et al*. (2006) compared the effect of the inhibition of lignin isolated from organosolv-pretreated softwood on cellulase, xylanase, and BG activities. In Berlin *et al*. (2006), it was found that the softwood lignin had the least effect on BG activity. In the case of milled wood lignin (MWL), the effect of the MWL on the adsorption capacity of the BG was lower than on the xylanase (Guo *et al.* 2014). The fractionation process could alter the lignin structure and affect the adsorption behavior of lignin toward enzymes (Lu *et al.* 2016). Native differences in lignin may be the reason for the differences in their enzyme adsorption affinity. It is well known that hydrophobic interactions play a greater role in the adsorption of cellulases and related enzymes onto lignocellulose (Palonen *et al.* 2004; Berlin *et al.* 2005a,b). Hydrogen bonding or electrostatic interaction are also responsible for enzyme adsorption onto lignin (Brash and Horbett 1995; Nakagame *et al.* 2011a, 2011b; Rahikainen *et al.* 2013). Kellock *et al.* (2017) indicated that lignins isolated from steam pretreated spruce (SPS) is more inhibitory to xylanase, β-glucosidase, and individual components of cellulase such as CBH and endoglucanases (EG) than wheat straw (SPWS) lignin. The pH value and temperature could influence the adsorption and desorption behaviors of cellulase on lignin by changing the protein properties (Lu *et al.* 2017). It was shown that enzyme properties, surface charge, and surface hydrophobicity could not alone explain the adsorption behaviour of enzyme onto lignins (Kellock *et al.* 2017). The binding mechanism of enzyme onto lignin is complicated. Despite the extensive research mentioned above, the adsorption interaction mechanism of enzymes onto lignin is not completely understood. Comparison studies on the adsorption capacities of native lignin isolated from diverse types of biomass by different techniques that consider their physico-chemical properties have not been conducted yet.

The present study focuses on the adsorption of enzymes onto lignin separated from eucalyptus and bamboo *via* hydrotropic and alkali methods. The adsorption interactions of different enzyme including cellulase, xylanase, and BG onto the lignin were investigated. The cellulase adsorption kinetics of the lignin were analyzed in detail with Langmuir isotherms. The possible reasons for the change in the adsorption capacity of lignin were considered from the perspective of the lignin properties. The difference in the hydrophobicity, surface charge, molecular weight, and chemical structure of lignin were

determined to interpret the adsorption behavior between the enzyme and lignin. Lastly, the thermal properties of lignin obtained by different isolation processes were determined with thermogravimetric analysis (TGA).

EXPERIMENTAL

Materials

Lignin from eucalyptus and bamboo were extracted by hydrotropic and soda (alkali) processes. The cellulase mixture was obtained from Genencor (Shanghai, China). The xylanase (*Aspergillus oryzae*), BG (Novozym188, *Aspergillus niger*), and all of the other chemicals were commercially purchased (Sigma-Aldrich, Shanghai, China) and applied without further purification.

All of the experiments were conducted in duplicate and the averages of the results were presented.

Methods

Lignin fractionation process

Hydrotropic lignin was extracted from eucalyptus and bamboo in a revolving digester at 160 °C with 30% (w/v) sodium xylenesulfonate for 120 min and 90 min, respectively. The wood to liquid ratio was 1:8. The hydrotropic lignin was recovered from the spent solution by filtration. The precipitation method has been described in detail by Gabov *et al*. (2014).

The alkali lignin was obtained from the black liquor that came from the treatment of eucalyptus and bamboo with 24% alkali at 170 °C for 2 h. The wood to liquid ratio was 1:4. Upon completion, the lignin was precipitated out by adjusting the pH of the black liquor to 2.0 with the addition of 10% sulfuric acid (H_2SO_4) in a water bath at 60 °C. Next, the lignin was separated by centrifugation at 5000 rpm. Then, the obtained lignin was extracted at a dioxan to water ratio of 9:1, and subsequently washed with acidic water (pH = 2.0). Finally, both the alkali and hydrotropic lignin were freeze-dried for chemical analysis.

Elemental analysis

The carbon, hydrogen, nitrogen, and sulfur contents of the isolated lignin were measured with a Vario EL cube elemental analyzer (Frankfurt, Germany). The oxygen amount was calculated by the subtraction method.

Molecular weight of the lignin

The molecular weight of the isolated lignin was analyzed by gel permeation chromatography (GPC). Lignin samples were acetylated and subsequently dissolved in tetrahydrofuran (THF, 1.0 mg/mL) (Kim *et al*. 2013). The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were determined by GPC analysis after acetylation of the lignin.

Prior to the GPC analysis, the acetylated and dissolved lignin samples were filtered through a 0.45-μm filter and placed in 2-mL autosampler vials. The molecular weight distributions of those samples were then analyzed on an Agilent GPC SEC 1200 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Shodex KF-803L column

(Showa Denko, Tokyo, Japan) using THF as the mobile phase (1.0 mL/min) and an injection volume of 100.0 µL.

Surface charge and hydrophobicity measurement of the lignin

The surface charge of the different alkali lignin fractions was determined according to Rahikainen *et al*. (2013). The surface charges (Q, mmol/g) were calculated using the study by Li *et al*. (2016). The lignin hydrophobicity was determined using Rose Bengal solution according to the method described by Gessner *et al*. (2000).

Fourier transform infrared (FTIR) analysis

The functional groups in the lignin structure were determined by FTIR (Nicolet IS50, Thermo Fisher Scientific, Waltham, MA, USA). The same amount of lignin was prepared with KBr pellets before analysis, and the weight ratio of KBr to sample was 100:1. The spectra were collected at a resolution of 4 cm^{-1} in the range of 500 cm^{-1} to 4000 cm^{-1}, and 32 scans per sample were conducted.

Protein analysis

The amount of free protein in solution was determined according to the method reported by Bradford (1976). Samples lacking any enzyme were used as references. The cellulase adsorption capacity was calculated using the following equation:

$$\text{Cellulase adsorption capacity}\,(\%) = \frac{(\text{Total protein - Free protein})}{\text{Total protein}} \times 100 \qquad (1)$$

Enzyme adsorption onto the lignin

To generate the isotherms for the cellulase adsorption onto the isolated lignin, cellulases with different concentrations (0 mg/mL, 0.17 mg/mL, 0.26 mg/mL, 0.55 mg/mL, and 0.90 mg/mL) were incubated with 2% (w/v) lignin in 50 mM citrate buffer (pH 4.8) at room temperature and 150 rpm. After 90 min of incubation, the sample was centrifuged to collect the supernatant for the protein content analysis. The amount of free protein in the solution was determined according to the method reported by Bradford (1976).

The adsorption of cellulase, BG, and xylanase onto the lignin was performed in serum bottles using 165 µL of cellulase in 2.5 mL of sodium citrate buffer (pH = 4.8) at room temperature to avoid changing the surface of the lignin. Samples were taken after 180 min. The supernatant was separated by centrifuge for the protein analysis.

TGA of the thermal properties

Slow pyrolysis of the hydrotropic and alkali lignin was performed for the TGA. The experiments were conducted on a NETZSCH STA 449C thermogravimetric analyzer (Netzsch Group, Selb, Germany). Samples of approximately 30 mg were used during the TGA. Each experiment was conducted in N_2 at a flow rate of 20 mL/min. The sample was held at 30 °C for 1 min and then heated from 30 °C to 900 °C at a heating rate of 20 °C/min. The mass loss (TG) and mass loss rate (DTG) curves were calculated according to the TGA results.

RESULTS AND DISCUSSION

Elemental Analysis of the Lignin

To confirm the chemical structure of the lignin isolated by hydrotropic and alkali processes from eucalyptus and bamboo, an elemental analysis was performed, and the results are shown in Table 1.

Table 1. Elemental Analysis of the Lignin Separated by Hydrotropic and Alkali Processes from Eucalyptus and Bamboo

Lignin	N (%)	C (%)	H (%)	S (%)	O (%)	Ash (%)
Eusxs	0.13	52.89	5.18	8.21	33.59	0.65
EuA	0.13	59.15	6.18	0	34.54	0.05
Bsxs	0.20	55.44	5.88	2.61	35.87	0.87
BA	0.29	59.67	6.46	0	33.58	0.03

Hydrotropic – sxs; Alkali – A; Eucalyptus – Eu; Bamboo – B

Table 1 shows that the contents of the chemical elements in the lignin isolated by alkali and hydrotropic processes varied. In general, the alkali lignin contained more carbon and hydrogen than the hydrotropic lignin. According to the origin of the biomass materials, the hydrotropic and alkali lignin from bamboo seemed to contain more nitrogen than the lignin from eucalyptus. For the eucalyptus lignin, the amount of oxygen in the hydrotropic lignin was slightly lower than that in the alkali lignin, which was the opposite trend seen for the bamboo lignin. Only a small amount of sulfur was detected in the hydrotropic lignin. It probably originated from residual hydrotropic agent (Gabov *et al*. 2014; Mou *et al.* 2013). Therefore, a washing step is important for the hydrotropic lignin recovery operation. However, the ash content of the hydrotropic lignin was much higher than for the alkali lignin. This was probably caused by the different purification treatments used for the alkali and hydrotropic lignin.

Molecular Weight of the Lignin

The hydrotropic and alkali lignin separated from eucalyptus and bamboo were subsequently characterized by GPC analysis. The M_w, M_n, and PDI (M_w/M_n) of the lignin are given in Table 2.

Table 2. Molecular Weight of the Lignin Separated by Hydrotropic and Alkali Processes from Eucalyptus and Bamboo

Lignin	M_n (Da)	M_w (Da)	PDI
Eusxs	2472	5809	2.35
Bsxs	1345	2781	2.07
EuA	1169	2971	2.54
BA	1178	2969	2.52

As shown in Table 2, for both eucalyptus and bamboo, the M_n and M_w of the hydrotropic lignin were higher than that of the alkali lignin, which demonstrated that the

lignin was more severely depolymerized during the alkali separation process. Meanwhile, the PDI value of the hydrotropic lignin was clearly lower than that of the alkali lignin. The M_w of the Eusxs was higher than that of the Bsxs. However, the molecular weight of the alkali lignin from bamboo was similar to that from eucalyptus. In a previous study, a relationship between the molecular weight and PDI and the cellulase adsorption capacity of the lignin was established (Berlin *et al*. 2006). Recently, Lu *et al*. (2016) demonstrated that lignin isolated from corn stover pretreated with hot liquid water had a higher cellulase adsorption affinity when it had a lower PDI value and higher molecular weight. Therefore, the molecular weight and PDI are important factors for determining how cellulase adsorbs onto lignin.

Cellulase Adsorption Isotherms of the Lignin

The enzyme adsorption was investigated using the hydrotropic and alkali lignin incubated with different enzyme loadings at room temperature in an incubator shaker at 160 rpm for 90 min. The adsorption parameters were calculated by fitting the adsorption data to the Langmuir equation (Tu *et al*. 2009).

Table 3 shows the Langmuir adsorption isotherm parameters for cellulase adsorption onto the four types of lignin. The adsorption capacity of cellulase onto the hydrotropic and alkali lignin from eucalyptus was higher than that of the lignin obtained from bamboo. This was most likely because the eucalyptus lignin has a higher molecular weight containing more binding sites than the bamboo lignin (Li *et al*. 2016). In the case of the bamboo lignin, the adsorption cellulase capacity of the Bsxs was similar to that of the BA. The Langmuir constant (K) used to evaluate the cellulase adsorption affinity of the lignin is presented in Table 3 as well. In contrast with the adsorption capacities of the lignin, the Bsxs had the highest K-value of the four samples, which implied a higher cellulase adsorption affinity.

Table 3. Langmuir Adsorption Isotherm Parameters of Enzyme Adsorption onto the Lignin

Lignin	E_{max} (mg/g)	K (mL/mg)	R (L/g)
Eusxs	12.72	0.59	7.54
Bsxs	2.18	0.80	1.74
EuA	11.34	0.44	4.94
BA	2.79	0.96	2.74

Comparing the results in Table 2, it was concluded that the cellulase affinity of the lignin increased with a decreasing lignin molecular weight. This meant that the interaction between the bamboo lignin and cellulase was strong. As interpreted from other works, the distribution coefficient (R), calculated from the Langmuir equation, could be used to estimate the relative affinity of cellulase for the substrates (Li *et al*. 2013). The R value of the Eusxs was higher than that of the other three lignin samples. For the alkali lignin, the relative affinity of the EuA was higher than that of the BA. The E_{max} is the maximum adsorption capacity (mg/g substrate) (Tu *et al.* 2009). According to the Langmuir results, the lowest E_{max} and R values were achieved with the Bsxs, which had the lowest M_w and

PDI values. This was probably caused by the weak adsorption interactions force between the Bsxs and cellulase. Based on the results in Tables 2 and 3, the relationship of the molecular weight to the adsorption affinity of the lignin was consistent with previous literature (Li *et al*. 2016). However, in contrast with the eucalyptus lignin, the PDI of the bamboo lignin was proportional to the cellulase adsorption affinity (K constant).

As a supplementary analysis, the authors studied the change in cellulase adsorption onto the lignin at different reaction times. The results are given in Fig. 1.

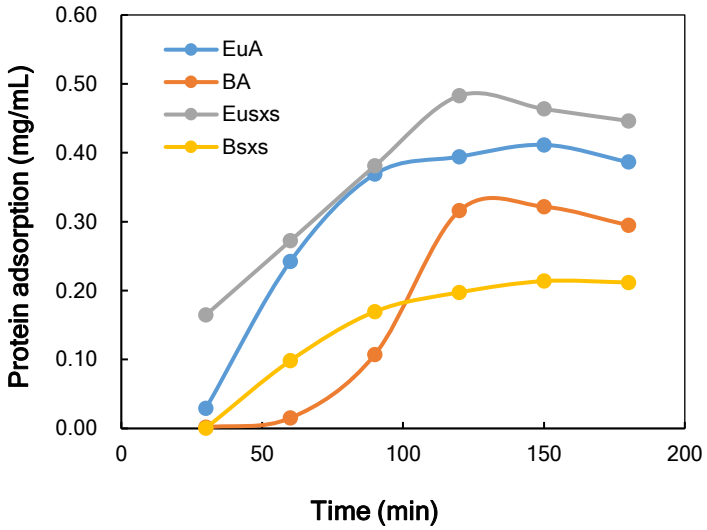

Fig. 1. Cellulase adsorption onto lignins at different reaction times

Figure 1 shows that the protein adsorption amount of the lignin increased remarkably from 30 min to 90 min. The adsorption rate of cellulase onto Eusxs was obviously faster than that onto other three samples. The hydrotropic lignin from eucalyptus adsorbed more cellulase than the other three lignin samples. The alkali lignin from eucalyptus could adsorb more cellulase than the alkali lignin from bamboo. In contrast to the eucalyptus lignin, the cellulase adsorption interaction rate of the BA was faster than that of the Bsxs. As a result, the cellulase adsorption capacity of the alkali bamboo lignin was higher than that of the hydrotropic bamboo lignin. In addition, the maximum adsorption of cellulase onto lignin appeared at 2 hours as shown in Fig. 1. Subsequently, the adsorption of cellulase onto lignins has slightly reduction. This probably because the adsorption of cellulase onto lignin effected by the different adsorption behavior of monocomponent of cellulase onto lignin, which needs further investigation (Li *et al*. 2017; Kellock *et al.* 2017). From the results shown in Fig. 1, it was indicated that the adsorption behavior of cellulase onto lignin was complicated. Moreover, because of the different isolation techniques used and biomass sources, the physical and chemical properties of the lignin were distinct. Therefore, different cellulase adsorption behaviors were observed in the different lignin samples.

Adsorption Capacity of Different Enzymes onto the Lignin

Other than hydrophobicity, the surface charge of lignin, and the stability of cellulase have been found to synergistically influence the cellulase-lignin interaction (Lu *et al.* 2017). Different types of enzyme may differ in their interaction with lignin. Hence, in addition to cellulase, the adsorption behavior of BG and xylanase onto the lignin was studied in this work. After enzyme interaction with the lignin at a pH of 4.8 for 3 h, the amount of free protein in the buffer solution was detected and calculated.

Figure 2 shows that these individual enzymes had different adsorption behaviors. Of the three enzymes, the adsorption of xylanase onto lignin was the highest. After comparison with the Eusxs, Bsxs, and BA lignin samples, the xylanse and BG adsorption amounts were the highest for the EuA. The lowest adsorption amounts for BG and xylanase occurred with the Eusxs lignin, which indicated that the adsorption capacity of the hydrotropic eucalyptus lignin with the xylanase and BG was the lowest. At a pH of 4.8, the adsorption capacity of cellulase onto the EuA and Bsxs lignin was higher than for the BG and xylanase. This result was different from the effects of organosolv-softwood lignin on enzymes reported by Berlin *et al*. (2006) and Kellock *et al.* (2017). Haven and Jørgensen (2013) found that the BG (Novozyme 188) did not adsorb onto the lignin from steam-pretreated wheat straw. The hydrophobic and electrostatic interactions were thought to be important factors that affected the adsorption behaviors between the lignin and proteins (Norde and Haynes 1995; Palonen *et al*. 2004; Lu *et al*. 2016). Therefore, to explain the difference in the enzyme adsorption behavior onto the lignin, the surface charges and hydrophobicity of the lignin were determined.

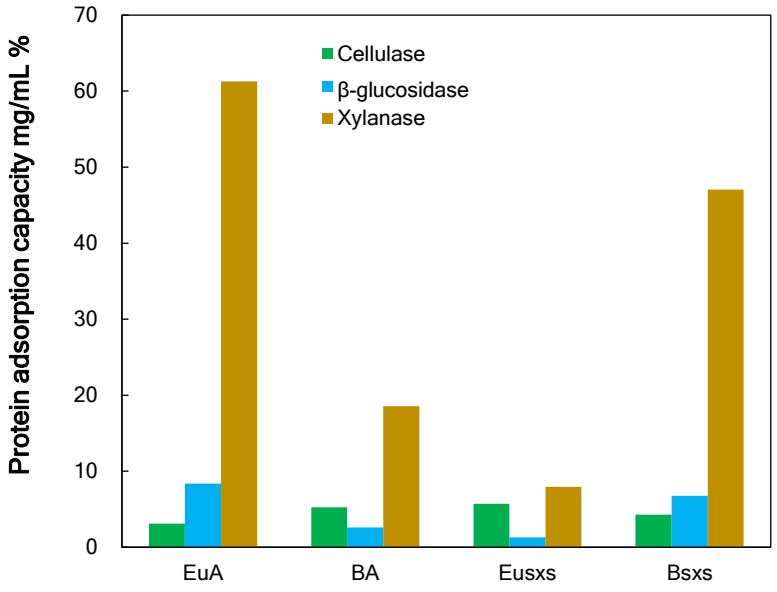

Fig. 2. Cellulase, BG, and xylanase adsorption onto the lignin

Surface Charge and Hydrophobicity of the Lignin

The hydrophobicity and surface charge of the different lignin samples were determined, and the results are summarized in Table 4.

Table 4. Surface Charge and Hydrophobicity of the Lignin

Lignin	Surface Charge (mmol/g)	Hydrophobicity (L/g)
Eusxs	-0.78	1.21
Bsxs	-1.47	1.00
EuA	-1.0	1.15
BA	-0.85	1.13

Table 4 shows that the isolation procedure dramatically changed the surface properties of the isolated lignin. In this study, the hydrophobicity of the Eusxs lignin was the highest. For the bamboo lignin, the hydrophobicity of the Bsxs lignin was lower than that of the BA. Combining the Langmuir isotherm parameters given in Table 3 and adsorption results shown in Fig. 2, the rank of cellulase adsorption capacity for the four lignin types was determined as: Eusxs > EuA > BA > Bsxs. This implied that the hydrophobicity was the main factor that influenced the adsorption of cellulase onto the lignin samples. The same conclusion was reached in previous studies (Gessner *et al*. 2000; Nakagame *et al*. 2010, 2011a; Rahikainen *et al*. 2013). The surface charges of the Eusxs, Bsxs, EuA, and BA were -0.78 mmol/g, -1.47 mmol/g, -1.0 mmol/g, and -0.85 mmol/g, respectively. Studying the results in Tables 2 and 4, it was revealed that all of the lignin samples were hydrophobic and their hydrophobicity increased with an increasing molecular weight. However, the negative surface charge of the lignin increased with a decreasing molecular weight. Table 4 showed that the negative surface charges of the Eusxs and BA were lower than that of the EuA and Bsxs. It has been stated previously that cellulase is negatively charged at a pH of 4.8 (Chen *et al*. 2013). Thus, a higher surface charge for the lignin could produce a relatively stronger electrostatic repulsion of the cellulase, which would result in a decreased adsorption affinity of cellulase. Similar results were reported by Li *et al*. (2016). The EuA and Bsxs lignin had a relatively lower hydrophobicity and higher negative surface charge values than the Eusxs and BA samples. Considering the results in Fig. 2 and Table 4, it seemed that the negative surface charge had a positive influence on the lignin adsorption of the BG and xylanase. As a result, xylanase was adsorbed onto almost all of the lignin and BG was the least affected by the Eusxs and BA lignin (Fig. 2). In contrast with the adsorption behavior of cellulase onto the lignin, the adsorption effect of the EuA and Bsxs on the xylanase and BG was more noticeable than the effect of the Eusxs and BA. This was probably because of the difference in the protein structure (Guo *et al*. 2014). Compared with the Bsxs, the BA had a higher hydrophobicity and lower surface charge, which led to a stronger cellulase adsorption affinity onto the lignin. The dual influence of the hydrophobic interaction and static-electronic interaction resulted in the different cellulase adsorption isotherms for the Bsxs and BA in Fig. 1.

Based on the results above, it was further demonstrated that the hydrophobicity of the lignin mainly affected the cellulase adsorption. In addition, the surface charge of the lignin was a main factor that influenced the adsorption of the BG and xylanase onto the lignin.

Pan (2008) mentioned that the lignin chemical structure played an important role in the lignin inhibition of cellulase. In the following sections, the chemical and thermal properties of the different lignin samples were determined by FTIR and TGA, respectively.

Table 5. Signal Assignments and Relative Intensities of Lignins Separation by Hydrotropic (sxs) and Alkali (A) Process from Eucalyptus (Eu) and Bamboo (B)

Assignment	Peak (cm^{-1})	Eusxs	EuA	Bsxs	BA
O-H stretching in aromatic and aliphatic hydroxyl groups	3440	0.41	0.67	0.50	0.72
C-H vibration in methyl and methylene groups	2935	1.37	0.97	1.10	1.10
	2842	1.85	1.14	1.42	1.36
Nonconjugated carbonyl groups (C=O)	1770	2.08	1.12	1.58	1.34
Aromatic skeletal vibration	1599	0.70	0.86	0.73	0.80
	1515	0.67	0.70	0.61	0.62
	1423	0.81	0.87	0.94	0.88
C-H deformation and aromatic ring vibration (methyl)	1462	0.50	0.69	0.64	0.69
Phenolic hydroxyl groups	1380	1.35	1.14	1.25	1.17
Syringyl (S) C-O stretching	1326	0.78	0.83	0.90	0.90
Guaiacyl (G) C-O units	1268	0.89	0.83	0.71	0.77
Aromatic methyl ether bridges	1225	0.31	0.57	0.39	0.54
C-O stretching in ester groups	1160	0.64	0.90	0.59	0.87
Aromatic C-H deformation in syringyl (S) ring	1123	0.17	0.49	0.33	0.44
Aromatic C-H in-plane deformation G/S	1032	0.66	0.84	0.81	0.73
Aromatic C-H out-of-plane deformation in G/S lignin	915	2.14	1.31	1.90	1.57
Aromatic C-H out-of-plane deformation in position 2 and 6 of S, and in all positions of H	831	1.73	1.24	1.35	1.33

FTIR Analysis of the Lignin

The difference in the chemical structure of the lignin was tested by FTIR. The signal assignment and relative intensities in the FTIR spectra (Fig. 1) of the lignin are given in Table 5.

The peak intensity at 3440 cm^{-1} in the alkali lignin, which was attributed to O-H stretching in the aromatic and aliphatic hydroxyl groups of lignin, was higher than in the hydrotropic lignin. The peaks at 2935 cm^{-1} and 2842 cm^{-1} were assigned to C-H vibration in the methyl and methylene groups of lignin, which had the highest intensities in the Eusxs lignin compared with the other lignin samples. Moreover, in Table 2, the molecular weight of the Eusxs was higher than that of the EuA, BA, and Bsxs, which indicated that the hydrotropic eucalyptus lignin was less degraded during the isolation process. Therefore, the band intensity for C=O stretching (1770 cm^{-1}) in the Eusxs lignin was higher than for the other three lignin samples (EuA, Bsxs, and BA). However, the intensities of the peaks for aromatic skeletal vibration that appeared at 1599 cm^{-1}, 1515 cm^{-1}, and 1443 cm^{-1} in the hydrotropic lignin were close to that of the alkali lignin. The peak at 1462 cm^{-1} in the Eusxs lignin was recognized as C-H deformation and aromatic ring vibration and its intensity was the lowest for all of the lignin samples. The S/G ratio was calculated from the peak intensities for C-O in syringyl and guaiacyl lignin at 1326 cm^{-1} and 1268 cm^{-1}, respectively (Gouveia *et al*. 2012). The Eusxs lignin had a relatively lower S/G ratio value than the EuA, Bsxs, and BA, which was one of the reasons for its higher cellulase adsorption capacity (E_{max} = 12.72 mg/g). Additionally, the low peak intensity at 1123 cm^{-1} (aromatic C-H deformation in S ring) and high peak intensity at 1032 cm^{-1} (aromatic C-H in plane deformation G + S) could have indicated a lower S/G ratio in the lignin samples. The low S/G ratio and high uniform lignin fragment size correlated well with the high adsorption capacity of the lignin that has been reported before by Guo *et al*. (2014). Compared with the alkali lignin, the ether and ester peak intensities at 1225 cm^{-1} and 1160 cm^{-1} in the hydrotropic lignin were lower than in the alkali lignin, which indicated that there was a higher amount of carboxylic acid groups in the lignin. Carboxylic acid groups negatively correlate with the protein adsorption affinity by reducing the hydrophobic interactions (Nakagame *et al*. 2011b; Pareek *et al*. 2013). Furthermore, the amount of phenolic hydroxyl groups in the lignin correlates with the enzyme binding/inhibition capacity (Sewalt *et al*. 1997; Rahikainen *et al*. 2013). Compared with the EuA, Bsxs, and BA, the phenolic (1380 cm^{-1}) content in the Eusxs was obviously too high to contribute to the higher cellulase adsorption capacity of the lignin. This was consistent with the results given in Table 3 and Fig. 1.

Based on the lignin chemical structural properties given in Table 5 and considering the promising influence of the phenolic content and S/G ratio of the lignin on the enzyme adsorption capacity (Pan 2008; Guo *et al*. 2014), it was further revealed that the adsorption behavior between the lignin and enzymes was comprehensively affected by the physical and chemical properties of the lignin.

TGA of the Lignin

The thermal properties of the hydrotropic and alkali lignin obtained from eucalyptus and bamboo were analyzed by TGA. The TG and DTG curves of the isolated lignin at a heating rate of 20 °C/min under N_2 are shown in Figs. 3 and 4.

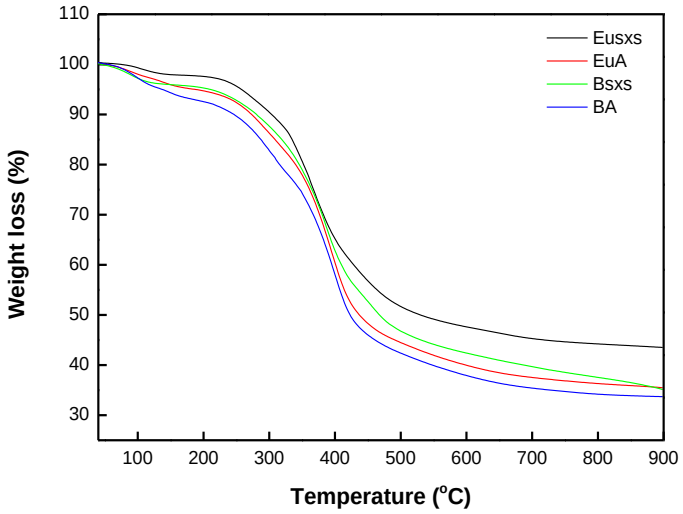

Fig. 3. TG curves of the hydrotropic and alkali lignin from eucalyptus and bamboo

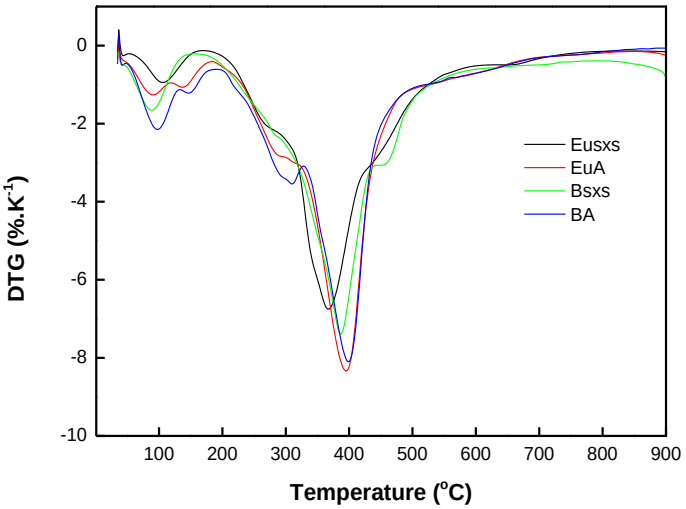

Fig. 4. DTG curves of the hydrotropic and alkali lignin from eucalyptus and bamboo

It was apparent that the thermochemical stabilities of the four lignin samples were quite different. In Fig. 3, the quick weight loss of the four types of lignin mainly occurred from 275 °C to 550 °C. The decomposition of the lignin samples could be divided into three stages: the initial pyrolysis stage before 200 °C, mainly attributed to the release of water; the second pyrolysis stage between 200 °C and 275 °C; and the third pyrolysis stage from 275 °C to 600 °C. The hydrotropic lignin exhibited a better thermochemical stability than the alkali lignin. The weight loss of the hydrotropic lignin was lower than that of the alkali lignin, especially for the Eusxs lignin. The char residue, which was approximately 43.5% for the Eusxs and 35.5% for the EuA, was considered to be the main product from the pyrolysis of the wood lignin. In the case of the bamboo lignin, the amount of char residue from the Bsxs and BA was approximately 35.2% and 33.7%, respectively. Figure 4 shows that the weight loss rate of the hydrotropic lignin (Eusxs, Bsxs) was slower than that of the alkali lignin (EuA and BA). After 700 °C, there was still a great amount of hydrotropic lignin present, which was probably because of its condensation or relocation

during the fractionation process (Sun *et al*. 2000). The condensed reaction took place during the hydrotropic isolation treatment, and has been demonstrated previously (Gabov *et al*. 2014). The distinct chemical structure of lignin definitely influenced the thermal properties.

According to all of the results presented in this study, the Eusxs had the best thermal properties and cellulase adsorption capacity, and so it could be potentially used as an adsorbent or stabilizer for water soluble enzymes.

CONCLUSIONS

1. The results presented in this study showed that the enzyme adsorption interaction for the hydrotropic and alkali lignin isolated from eucalyptus and bamboo obviously differed. The distinct physico-chemical properties of the lignin played important roles in the different adsorption behaviors of the various lignin samples.
2. The hydrophobicity of lignin and its surface charge appeared to be a main factors affecting the adsorption capacity. The adsorption capacity general increased with increasing hydrophobicity and with decreasing absolute value of (negative) charge.
3. From the results of this study, the challenge in enzyme recovery during the production of bioethanol was clearly revealed. How the individual enzymes adsorbed onto the lignin affects the hydrolysis efficiency still needs to be investigated.

ACKNOWLEDGMENTS

This study was financially supported by the Chinese National Natural Science Foundation (31600469) and State Key Laboratory of Pulp and Paper Engineering (201711). The authors would like to thank Dr. Hao Cheng for kindly helping with the preparation of the alkali lignin.

REFERENCES CITED

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., and Saddler, J. (2006). "Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations," *J. Biotechnol.* 125(2), 198-209. DOI: 10.1016/j.jbiotec.2006.02.021

Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Markov, A., Skomarovsky, A., Okunev, O., Gusakov, A., Maximenko, V., Gregg, D., *et al.* (2005a). "Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates — Evidence for the role of accessory enzymes," *Enzyme Microb. Tech.* 37(2), 175–184. DOI: 10.1016/j.enzmictec.2005.01.039

Berlin, A., Gilkes, N., Kurabi, A., Bura, R., Tu, M., Kilburn, D., and Saddler, J. (2005b). "Weak lignin-binding enzymes - A novel approach to improve the activity of cellulases for hydrolysis of lignocellulosics," *Appl. Biochem. Biotech.* 124(1-3), 163-170. DOI: 10.1007/978-1-59259-991-2_14

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem.* 72(1-2), 248-254. DOI: 10.1016/0003-2697(76)90527-3

Brash, J. L., and Horbett, T. A. (1995). "Proteins at interfaces, an overview. Proteins at interfaces, fundamentals and applications," in: *Proteins at Interfaces II: Fundamentals and Applications*, T. A. Horbett and J. L. Brash (eds.), American Chemical Society, Washington, D.C., pp. 1–23.

Chen, G., Song, W., Qi, B., Lu, J., and Wan, Y. (2013). "Recycling cellulase from enzymatic hydrolyzate of acid treated wheat straw by electroultrafiltration," *Bioresource Technol.* 144, 186-193. DOI: 10.1016/j.biortech.2013.06.089

Gabov, K., Gosselink, R., Smeds, A., and Fardim, P. (2014). "Characterization of lignin extracted from birch wood by a modified hydrotropic process," *J. Agr. Food Chem.* 62(44), 10759-10767. DOI: 10.1021/jf5037728

Gessner, A., Waicz, R., Lieske, A., Paulke, B.-R., Mäder, K., and Müller, R. (2000). "Nanoparticles with decreasing surface hydrophobicities: Influence on plasma protein adsorption," *Int. J. Pharm.* 196(2), 245-249. DOI: 10.1016/S0378-5173(99)00432-9

Gouveia, S., Fernández-Costas, C., Sanromán, M. A., and Moldes, D. (2012). "Enzymatic polymerisation and effect of fractionation of dissolved lignin from *Eucalyptus globulus* kraft liquor," *Bioresource Technol.* 121, 131-138. DOI: 10.1016/j.biortech.2012.05.144

Guo, F., Shi, W., Sun, W., Li, X., Wang, F., Zhao, J., and Qu, Y. (2014). "Differences in the adsorption of enzymes onto lignins from diverse types of lignocellulosic biomass and the underlying mechanism," *Biotechnol. Biofuels* 7, 38-47. DOI: 10.1186/1754-6834-7-38

Haven, M. Ø., and Jørgensen, H. (2013). "Adsorption of β-glucosidases in two commercial preparations onto pretreated biomass and lignin," *Biotechnol. Biofuels* 6(1), 165-178. DOI: 10.1186/1754-6834-6-165

Kellock, M., Rahikainen, J., Marjamaa, K., Kruus, K. (2017). "Lignin-derived inhibition of monocomponent cellulases and a xylanase in the hydrolysis of lignocellulosics," *Bioresource Technol.* 232, 183-191. DOI: https://doi.org/10.1016/j.biortech.2017.01.072

Kim, J. Y., Oh, S., Hwang, H., Kim, U.-J., and Choi, J. W. (2013). "Structural features and thermal degradation properties of various lignin macromolecules obtained from poplar wood (*Populus albaglandulosa*)," *Polym. Degrad. Stabil.* 98(9), 1671-1678. DOI: 10.1016/j.polymdegradstab.2013.06.008

Li, M., Tu, M., Cao, D., Bass, P., and Adhikari, S. (2013). "Distinct roles of residual xylan and lignin in limiting enzymatic hydrolysis of organosolv pretreated loblolly pine and sweetgum," *J. Agr. Food Chem.* 61(3), 646-654. DOI: 10.1021/jf304517w

Li, Y., Qi, B., Luo, J., and Wan, Y. (2016). "Effect of alkali lignins with different molecular weights from alkali pretreated rice straw hydrolyzate on enzymatic hydrolysis," *Bioresource Technol.* 200, 272-278. DOI: 10.1016/j.biortech.2015.10.038

Lou, H., Zhu, J. Y., Lan, T. Q., Lai, H., and Qiu, X. (2013). "pH-induced lignin surface modification to reduce nonspecific cellulase binding and enhance enzymatic saccharification of lignocelluloses," *ChemSusChem* 6(5), 919-927. DOI: 10.1002/cssc.201200859

Lu, X., Zheng, X., Li, X., and Zhao, J. (2016). "Adsorption and mechanism of cellulase enzymes onto lignin isolated from corn stover pretreated with liquid hot water," *Biotechnol. Biofuels* 9(1), 118-129. DOI: 10.1186/s13068-016-0531-0

Lu, X., Wang, C., Li, X., and Zhao, J. (2017). "Temperature and pH influence adsorption of cellobiohydrolase onto lignin by changing the protein properties," *Bioresource Technol*. 245(Pt A):819-825. DOI: https://doi.org/10.1016/j.biortech.2017.08.139

Mou, H.-Y., Orblin, E., Kruus, K., and Fardim, P. (2013). "Topochemical pretreatment of wood biomass to enhance enzymatic hydrolysis of polysaccharides to sugars," *Bioresource Technol.* 142, 540-545. DOI: 10.1016/j.biortech.2013.05.046

Nakagame, S., Chandra, R. P., Kadla, J. F., and Saddler, J. N. (2011a). "The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose," *Bioresource Technol*. 102(6), 4507-4517. DOI: 10.1016/j.biortech.2010.12.082

Nakagame, S., Chandra, R. P., Kadla, J. F., and Saddler, J. N. (2011b). "Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin," *Biotechnol. Bioeng*. 108(3), 538-548. DOI: 10.1002/bit.22981

Nakagame, S., Chandra, R. P., and Saddler, J. N. (2010). "The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis," *Biotechnol. Bioeng.* 105(5), 871-879. DOI: 10.1002/bit.22626

Norde, W., and Haynes, C. A. (1995). "Reversibility and mechanism of protein adsorption," in: *Proteins at Interfaces II: Fundamentals and Applications*, T. A. Horbett and J. L. Brash (eds.), American Chemical Society, Washington, D.C., pp. 26-40.

Öhgren, K., Bura, R., Saddler, J., and Zacchi, G. (2007). "Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover," *Bioresource Technol.* 98(13), 2503-2510. DOI: 10.1016/j.biortech.2006.09.003

Palonen, H., Tjerneld, F., Zacchi, G., and Tenkanen, M. (2004). "Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin," *J. Biotechnol.* 107(1), 65-72. DOI: 10.1016/j.jbiotec.2003.09.011

Pan, X. (2008). "Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose," *J. Biobased Mater. Bio.* 2(1), 25-32. DOI: 10.1166/jbmb.2008.005

Pareek, N., Gillgren, T., and Jönsson, L. J. (2013). "Adsorption of proteins involved in hydrolysis of lignocellulose on lignins and hemicelluloses," *Bioresource Technol.* 148, 70-77. DOI: 10.1016/j.biortech.2013.08.121

Petersen, M. Ø., Larsen, J., and Thomsen, M. H. (2009). "Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals," *Biomass Bioenerg.* 33(5), 834-840. DOI: 10.1016/j.biombioe.2009.01.004

Rahikainen, J. L., Martin-Sampedro, R., Heikkinen, H., Rovio, S., Marjamaa, K., Tamminen, T., Rojas, O. J., and Kruus, K. (2013). "Inhibitory effect of lignin during cellulose bioconversion: The effect of lignin chemistry on non-productive enzyme adsorption," *Bioresource Technol.* 133, 270-278. DOI: 10.1016/j.biortech.2013.01.075

Sewalt, V. J. H., Glasser, W. G., and Beauchemin, K. A. (1997). "Lignin impact on fiber degradation. 3. Reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives," *J. Agr. Food Chem.* 45(5), 1823-1828. DOI: 10.1021/jf9608074

Sun, R. C., Tomkinson, J., and Jones, G. L. (2000). "Fractional characterization of ash-AQ lignin by successive extraction with organic solvents from oil palm EFB fibre," *Polymer Degradation & Stability* 68(1), 111-119. DOI: https://doi.org/10.1016/S0141-3910(99)00174-3

Tu, M., Pan, X., and Saddler, J. N. (2009). "Adsorption of cellulase on cellulolytic enzyme lignin from lodgepole pine," *J. Agr. Food Chem.* 57(17), 7771-7778. DOI: 10.1021/jf901031m

Article submitted: September 21, 2017; Peer review completed: December 17, 2017; Revised version received and accepted: January 2, 2018; Published: January 8, 2018.
DOI: 10.15376/biores.13.1.1441-1456