

Physico-chemical Properties of Lignin Fractions from Acid Pretreated Corn Stover and their Effects on Enzymatic Hydrolysis of Microcrystalline Cellulose

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Lignin has been shown to be a recalcitrance factor in many biomass conversion studies. To better understand the effects of lignin on cellulose conversion, different lignin fractions were extracted from the same dilute acid pretreated corn stover by three sequential isolation methods, namely ethanol extraction, dioxane extraction, and enzyme purification. The physicochemical properties of each lignin fraction including molecular weight distribution, surface area, surface charge, and other structural features varied, depending on the isolation methods. All three lignin fractions had negative surface charges, and ethanol-extracted lignin carried the highest surface charges, followed by dioxane-extracted lignin and cellulase-purified residual lignin. These physicochemical properties of lignin fractions also resulted in different extent of inhibitory effects on enzymatic hydrolysis of microcrystalline cellulose (MCC). Dioxane-extracted lignin exhibited the highest inhibitory effect on glucose release from MCC, followed by the cellulase-purified residual lignin fraction and ethanol-extracted lignin. Furthermore, lignin fractions with higher contents of syringyl (S) substructure and β -O-4 aryl ether interunit linkages showed a stronger negative effect on cellulase hydrolysis of MCC.

Keywords: Lignin isolation; physico-chemical properties; Corn stover; Cellulase; Avicel

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INTRODUCTION

Lignocellulosic biomass is an attractive, sustainable feedstock for the production of second-generation bioethanol (Samuel *et al.* 2010). Biomass pretreatment, enzyme hydrolysis, fermentation, and distillation/purification are the four main steps in the bioconversion of lignocellulosics to biofuels (Yang *et al.* 2016). Pretreatment of biomass under elevated pressure and/or temperature with the addition of acid or base reduces the recalcitrance of biomass and increases the accessibility of cellulose to cellulase, helping achieve high glucose yield for the subsequent fermentation process (Pu *et al.* 2008; Meng *et al.* 2013; Ertas *et al.* 2014; Lai *et al.* 2019a).

Among various pretreatment methods, dilute acid (DA) pretreatment is one of the leading and widely studied techniques due to its effectiveness, low energy input, and wide applications for a variety of biomass resources (Chen *et al.* 2011; Teramura *et al.* 2015). The DA pretreatment solubilizes hemicelluloses, and the remaining acid-insoluble residue is mainly composed of cellulose and lignin (Cao *et al.* 2012). Although DA pretreatment increases sugar release of the pretreated biomass (Mosier *et al.* 2005; Hendriks and Zeeman 2009; Yao *et al.* 2010), the lignin in the solid residue still has inhibitory effects on the subsequent enzymatic hydrolysis process (Yao *et al.* 2017).

Lignin, the most abundant non-carbohydrate biopolymer in the plant cell wall, is typically composed of syringyl (S), guaiacyl (G), or *p*-hydroxyphenyl (H) units. The composition of lignin in plants varies depending on its species. For instance, softwood is of primarily G-type, hardwood is of a G-S type, and herbaceous crops are of G-S-H type in its lignin. In recent years, the inhibitory effects of various types of lignin on enzymatic hydrolysis, in particular, non-productive adsorption of cellulase onto lignins, have been reported (Yu *et al.* 2014; Li *et al.* 2016; Sun *et al.* 2016; Yoo *et al.* 2017; Yao *et al.* 2018a,b; Lai *et al.* 2018). Lignin forms droplets on the surface of residual biomass under severe acidic pretreatment conditions (*e.g.*, DA pretreatment at and above 130 °C), thereby hindering the enzymatic digestion of biomass (Selig *et al.* 2007). After incubating with lignin isolated from hot water pretreated hardwoods, 2 to 18% of the initial β -glucosidase and 50 to 60% of cellobiohydrolase and endoglucanase activities were retained (Ko *et al.* 2015). The increase of pretreatment severities results in lignin samples with higher cellulase adsorption ability. Similarly, Lu *et al.* (2016) reported that electrostatic interactions and hydrophobicity were the main cause of cellulase adsorption to hot-water pretreated lignin. Interestingly, certain organosolv lignins or lignosulfonates enhance the performance of enzymatic hydrolysis, and the effect of lignin on cellulase during enzymatic hydrolysis is a function of both hydrophobic interactions and electrostatic repulsions (Huang *et al.* 2017; Lai *et al.* 2019b).

Recently, lignin fractions isolated from dilute acid pretreated *Broussonetia papyrifera* were used to study the impact of the structural properties of lignin on cellobiohydrolases I adsorption (Yao *et al.* 2017). Similar studies were conducted with different lignocellulosic substrates such as switchgrass and poplar (Yao *et al.* 2018a,b). Besides lignin composition, the variety of interunit linkages and hydroxyl group content, surface area and surface charge of lignin affecting cellulase adsorption were studied (Nakagame *et al.* 2011; Lou *et al.* 2013). Despite these recent efforts, the mechanism of lignin inhibition on cellulase hydrolysis is still not fully understood. This is due, in part, to lignin's complexity and heterogeneity. Herein, three different lignin fractions were isolated from the dilute acid pretreated corn stover. Lignin composition, structural features, zeta potential, surface area and molecular weights of each lignin fraction were investigated. The effects of these lignin properties with their inhibitory on enzymatic hydrolysis of MCC were also investigated.

EXPERIMENTAL

Materials

Corn stover was provided by a local farmer from En shi, Hubei province, China. The chemical composition of the corn stover was 17.6% Klason lignin, 32.2% cellulose, 27.6% hemicellulose, 6.58% benzene-ethanol extractives, and 13.9% ash. Cellulase (Sino

EnzymesR) was a kind gift from Baiyin Sainuo Technology Ltd (Baiyin, PR China), with filter paper activity of 160 U/g and β -glucosidase activity of 42 U/g.

Pretreatment

Corn stover was Wiley-milled (screen size of 2 mm), extracted with toluene/ethanol (2:1, v/v) for 8 h, and then air-dried. The extractive-free corn stover was loaded with a 0.5% H_2SO_4 solution (solid-to-liquid ratio of 1:20 (w/w)) to a one-liter rotary electrothermal pressure digester (ZQS-3, Qinggong Jixie factory of Shanxi University of Science and Technology, Xianyang, Shanxi province, PR China) at 170 °C for 60 min (Yao *et al.* 2010). After the pretreatment, the residual solid was separated by filtration and washed with deionized water until the effluent was pH neutral, and the solid residue was stored in a sealed bag at 4 °C prior to further testing. After pretreatment, the chemical composition of corn stover was 60.2% cellulose, 7.23% hemicellulose and 24.9% of lignin.

Methods

Fractionation of lignin from pretreated corn stover

Lignin was isolated from the dilute acid pretreated corn stover by three sequential methods in a previous study with some modifications, as shown in Fig. 1 (Yao *et al.* 2017). The air-dried pretreated corn stover was extracted twice at room temperature with ethanol (10 mL/g biomass) for 24 h (2 x 24h) with a magnetic stirrer. The collected extract was rotary-evaporated and freeze-dried to yield the crude lignin (NO.1). A dioxane-water mixture (96:4, v/v; 10 mL/g biomass) was then used to extract the NO.2 lignin from the residue solid after ethanol extraction. These lignins were further purified according to the purification method of milled wood lignin (MWL) (Björkman 1956).

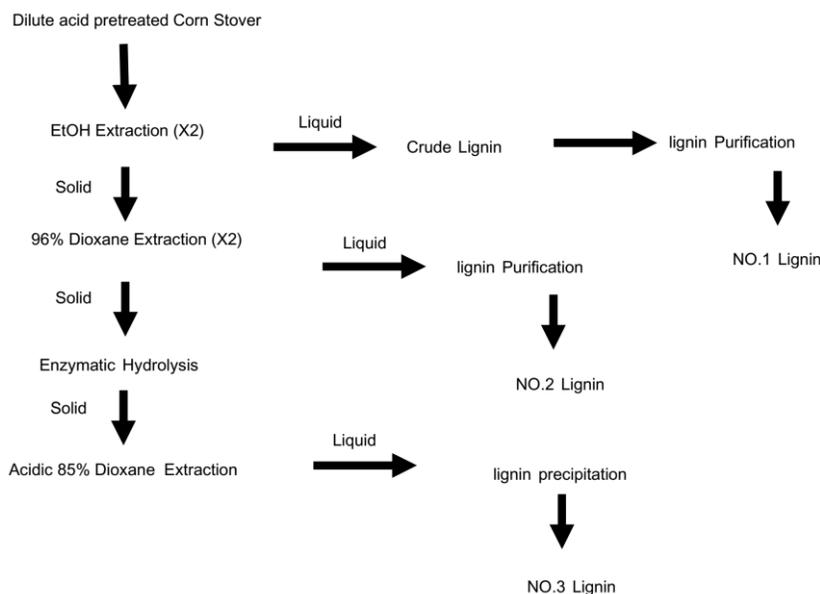


Fig. 1. Isolation process of lignin fractions

The solid residue after the two-step extraction was air-dried and hydrolyzed by overloaded cellulase. The lignin-enriched solid residue after the enzymatic hydrolysis was extracted by acidic 85% dioxane (98.6 mg of 37% hydrochloric acid were mixed with 100 ml of dioxane-water mixture) for 2 h (Wu and Argyropoulos 2003). The solid

was washed with acidic 85% dioxane, and the collected extraction liquid and washings were neutralized with anhydrous sodium carbonate and then rotary-evaporated to reduce the volume. The purified NO.3 lignin was precipitated in acidic water (pH 2), centrifuged, and freeze-dried. The purity of each isolated lignin fractions is shown in Table 1.

Table 1. Purity of the Isolated Lignin Fractions

	Klason lignin (%)	Glucan (%)	Arabinan (%)	Galactan (%)	Total (%)
NO.1 lignin	94.4	1.92	0.13	0.11	96.6
NO.2 lignin	98.7	1.82	0.20	0.05	100.7
NO.3 lignin	98.0	0.51	0.23	0.05	98.8

Lignin characterization

The surface area of lignin fractions was determined by the nitrogen adsorption method. The adsorption/desorption isotherms of nitrogen at 77 K were measured using a BELSORP mini-II surface area and pore size analyzer (MicrotracBEL, Japan; Nakagame *et al.* 2011). The zeta potential of lignin was measured at 25 °C in sodium acetate-acetic acid (NaAc-HAc) buffer (0.05 M, pH 4.8) using a Zeta Potential Analyzer (Zeta plus, Brookhaven, USA) (Lou *et al.* 2013). Lignin was also subjected to Fourier-transform infrared (FTIR) spectroscopy analysis (Spectrum One FTIR system, Perkin Elmer, Wellesley, MA) from 4000 to 500 cm⁻¹.

Lignin and carbohydrate contents were determined by the National Renewable Energy Laboratory (NREL) procedure using high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). Gel permeation chromatography (GPC) analysis was conducted to determine the molecular weights of lignin fractions after acetylation. Tetrahydrofuran (THF) was used as eluent, and the flow rate was 1.0 mL/min. Different sizes of polystyrene standards were used as calibration standards.

Two-dimensional (2D) ¹H-¹³C heteronuclear single quantum coherence (HSQC) NMR experiment was conducted at 298 K using a Bruker Avance III 400-MHz spectroscopy with a 5-mm Broadband Observe probe (5-mm BBO 400MHz with Z-gradient probe, Bruker, Karlsruhe, Germany). The analysis was conducted with a Bruker standard pulse sequence ('hsqcetgpsi2'), spectral width of 11 ppm in F2 (¹H) with 2048 data points and 190 ppm in F1 (¹³C) with 256 data points, 96 scans, and 1-s delay.

Cellulase hydrolysis of microcrystalline cellulose with the addition of different lignin fractions

Prior to the cellulase hydrolysis, microcrystalline cellulose (MCC) having the brand name Avicel was pretreated to increase digestibility by NaOH (Du *et al.* 2018). Enzymatic hydrolysis of pretreated MCC was performed in 500 mL of sodium citrate buffer (50 mM, pH 4.8) with 2% (w/v) substrate loading at 50 °C and 150 rpm for 72 h. The enzyme loading of cellulase was 25 FPU/g glucan. To investigate the effects of each lignin fraction on enzymatic hydrolysis, 500 mg lignin was physically mixed with 500 mg pretreated MCC. Glucose concentration in the hydrolysate was determined by HPLC (Shimadzu, Kyoto, Japan) with a refractive index detector (Shimadzu) on an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) running at a flow rate of 0.6 mL/min at 65 °C, with water as the eluent.

RESULTS AND DISCUSSION

Inhibitory Effect of Lignins on Cellulase Hydrolysis

The inhibitory effects of lignin on enzymatic hydrolysis of cellulose were evaluated with different lignin fractions from dilute acid pretreated corn stover and NaOH pretreated MCC as a standard cellulose resource. As Fig. 2 presents, negative effects were observed from all three lignins (Fig. 2). The hydrolysis yield of pretreated MCC after 72 h decreased from 70.3% (control) to 41.3%, 33.6%, and 39.1% with NO.1, NO.2, and NO.3 lignins, respectively. The results indicated that the NO.2 lignin had the most inhibition impact (52.2% decrease of glucose release). NO.1 and NO.3 lignins had comparable inhibition effects on glucose release from MCC, with a 41 to 44% decrease. This inhibition could be attributed to several factors, such as the non-productive binding of cellulase to lignin that is largely governed by hydrophobic interactions, hydrogen bonding, and electrostatic interactions according to the previous studies (Yoo *et al.* 2017). Structural features of lignin are essential information to understand these inhibitory effects on enzymatic hydrolysis of pretreated MCC.

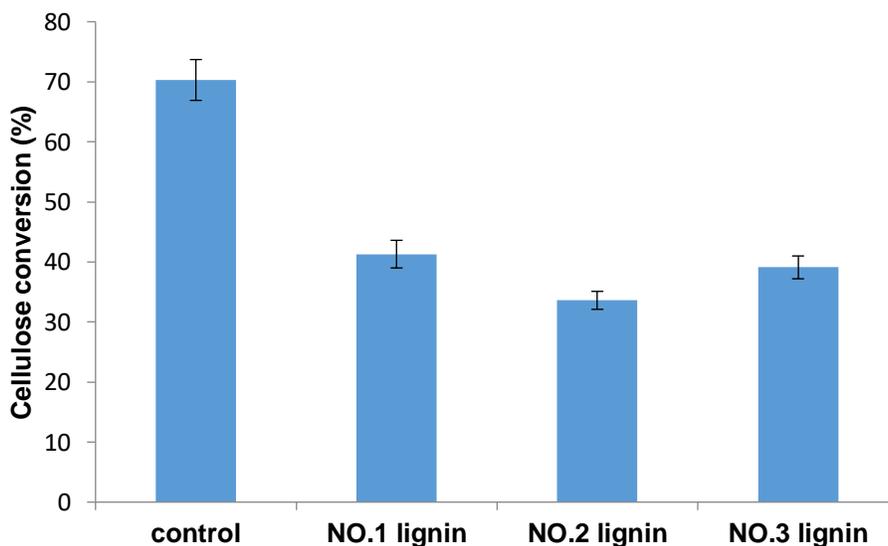


Fig. 2. Cellulose conversion of pretreated MCC with different lignin fractions

Physical Features of Lignin Fractions

Molecular weight of lignin fractions

The molecular weight distribution of each lignin fraction was analyzed by GPC. The molecular weights (M_w : weight-average molecular weight and M_n : number-average molecular weight) and the polydispersity index (*i.e.*, $PDI = M_w/M_n$) of each lignin are summarized in Table 2. The results showed that M_w of NO.1, NO.2, and NO.3 lignins were 1810, 5990, and 14450 g/mol, respectively. The PDI values of these lignin fractions were relatively low (< 1.5), indicating that the distribution of molecular weights in these lignin fractions was relatively narrow. Similar to the previous studies, there was no clear relationship between average molecular weights and inhibitory effect of lignin on cellulase hydrolysis (Pareek *et al.* 2013; Guo *et al.* 2014). Lower polydispersity is favored for the adsorption of cellulase (Berlin *et al.* 2006; Guo *et al.* 2014; Yao *et al.* 2018a); however, this tendency was not observed in the present study.

Table 2. Weight-average (M_w), Number-average (M_n) Molecular Weights and Polydispersity Indexes (M_w/M_n) of Different Lignin Fractions

	M_n	M_w	PDI (M_w/M_n)
NO.1 lignin	1260	1810	1.44
NO.2 lignin	4010	5990	1.49
NO.3 lignin	11880	14450	1.22

Surface area and surface charge

Surface area is one of the factors that influence the non-productive binding of cellulase to lignin. In this study, the surface area of each lignin fraction was determined by a BET analyzer. The results in Table 3 show that the surface area of lignin fractions was 0.17, 0.69, and 2.87 m²/g for NO.1, NO.2 and NO.3 lignins, respectively. However, Fig. 2 shows that NO.2 lignin showed the most inhibitory effect on cellulase hydrolysis of pretreated MCC among the three lignin samples. Other studies have also reported that the maximum protein adsorption capacity was not well correlated with the observed specific surface area and pore size of lignins (Berlin *et al.* 2006; Nakagame *et al.* 2011; Pareek *et al.* 2013).

Zeta potential was used to represent the surface charge of lignin. Table 3 shows that all of the lignin fractions from the pretreated corn stover showed negative zeta potential. NO. 1 lignin exhibited the highest negative surface charge, followed by NO. 2 lignin and NO.3 lignin. As cellulase exhibits a positive charge in the sodium citrate buffer at pH 4.8, cellulase and lignin could interact by electrostatic interactions (Lou *et al.* 2013). At pH 4.8, positively charged enzymes (such as Cel6A and Cel5A) were more strongly adsorbed onto the negatively charged lignins compared to negatively charged enzymes, such as Cel7A and Cel7B due to the electrostatic repulsions (Saini *et al.* 2016; Huang *et al.* 2017). However, Lou *et al.* found that the nonspecific binding of cellulase was not significantly affected by surface charge of lignin at pH 4.8 (Lou *et al.* 2013). In this study, charge of NO.1 lignin was twice the value of NO.3 lignin, but these two lignins showed similar cellulase hydrolysis yields of pretreated MCC.

These results suggested that these structural features of lignin were not significantly involved in the interaction between the cellulases and lignin. Therefore, other chemical characteristics of the lignin fractions were investigated to explore the inactivation mechanism.

Table 3. Surface Area and Surface Charge of Lignin Fractions

	NO.1 lignin	NO.2 lignin	NO.3 lignin
Surface area/ m ² /g	0.17	0.69	2.87
Surface charge/ mv	-30.3	-25.9	-15.1

Chemical Features*FT-IR determination*

FT-IR is a commonly used tool to analyze the structural features of lignin (Ghaffar and Fan 2013). FT-IR spectra of the lignin fractions are shown in Fig. 3. The assignment of signals was based on the published values (Ghaffar and Fan 2013). The peak near 3420 cm⁻¹ represents OH stretching. The peak at 1700 cm⁻¹ was ascribed to the unconjugated carbonyl (Kumar *et al.* 2009). The signals at 1604, 1514, and 1425 cm⁻¹ are assigned to the aromatic ring of each lignin fraction. The shift of band position from 1505

to 1514 cm^{-1} is due to condensation reactions during acid pretreatment and/or lignin extraction process (Esteves *et al.* 2013).

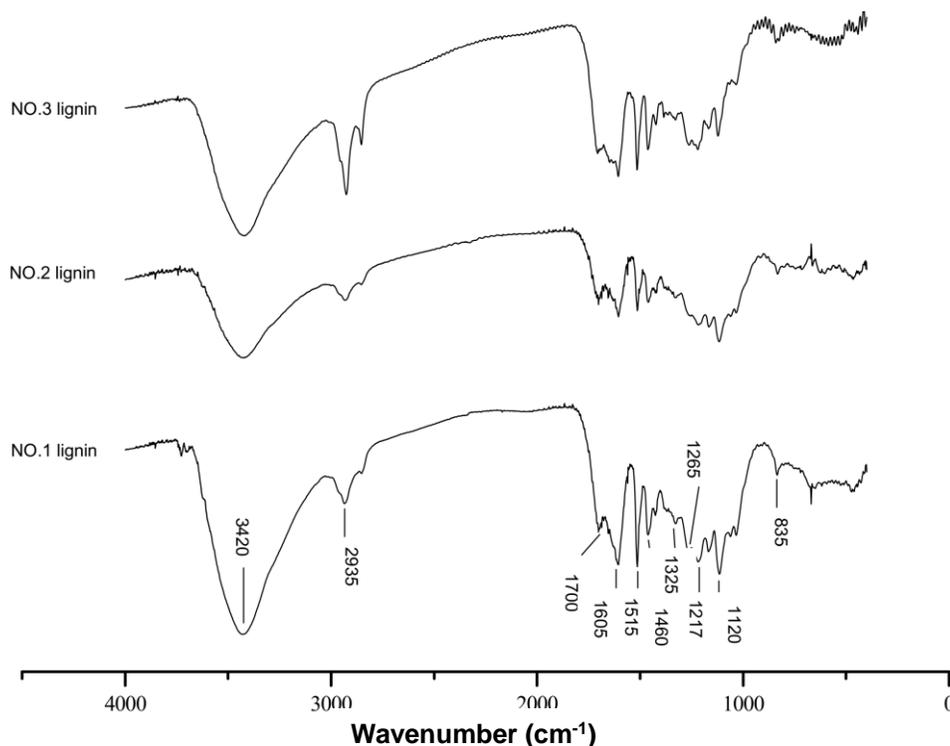


Fig. 3 FT-IR spectra of different lignin fractions

The absorption band position at 2937 cm^{-1} is attributed to stretching of $-\text{CH}$, $-\text{CH}_2$ and $-\text{CH}_3$. Signals at 1325 , 1262 , and 834 cm^{-1} were assigned to C-O bond of syringyl, guaiacyl, and *p*-hydroxyphenyl units (Faix 1991), respectively.

Table 4. Assignment and Relative Intensities of Signals in FT-IR Spectra of Lignin Fraction

Assignment	Wavenumber (cm^{-1})	Relative Intensity		
		NO.1 Lignin	NO.2 lignin	NO.3 Lignin
-OH stretching vibration	3420	1.23	1.10	1.20
$-\text{CH}$, $-\text{CH}_2$ and $-\text{CH}_3$	2937	1.05	1.01	1.12
C=O stretch in unconjugated ketone	1700	0.93	0.94	1.02
Aromatic ring	1604	1.00	0.98	1.03
Aromatic ring	1514	1.00	1.00	1.00
C-H in $-\text{CH}_3$	1460	1.08	1.04	1.05
Aromatic ring	1425	1.11	1.06	1.04
Syringyl	1325	1.05	1.04	0.99
Guaiacyl	1262	0.95	1.01	0.95
C=O in ester group	1168	0.96	1.00	0.94
Aromatic C-H in-plane deformation	1120	1.06	1.03	0.98
C=O stretching	1034	1.14	1.04	0.90
<i>p</i> -hydroxyphenyl units	834	0.75	0.90	0.82

In addition, the band at 1168 cm^{-1} originated from *p*-hydroxyphenyl structures, which indicated that the lignin fractions from dilute acid pretreated corn stover were of the H-S-G type (Sun *et al.* 2003). The relative signal intensities of various functional groups were calculated as the ratio with reference to the signal intensity of the band at 1514 cm^{-1} (Table 4) (Guo *et al.* 2014). The structure of different lignin fractions varied. Syringyl, guaiacyl, and *p*-hydroxyphenyl units were different in the three lignin fractions. As shown in Table 4, stronger intensities of signals at 1262 and 834 cm^{-1} , assigned to guaiacyl and *p*-hydroxyphenyl, were found in NO.2 lignin fraction. To further define the relationship between lignin structure and cellulase interactions, more detailed structural characteristics were needed which was accomplished by NMR.

HSQC determination

HSQC is a commonly applied NMR technique for the structural analysis of lignin. In this study, HSQC was employed to compare the structural features of each lignin fractions extracted from dilute acid pretreated corn stover. The cross peaks were assigned according to the previous studies (Samuel *et al.* 2010; Hu *et al.* 2012; Zeng *et al.* 2013; Yang *et al.* 2016). In the aromatic region ($160\text{-}90/8.0\text{-}5.5\text{ ppm}$), $^{13}\text{C}\text{-}^1\text{H}$ correlations of all three lignin subunits including S, G and H units were observed. This observation is consistent with the FT-IR analysis. The $^{13}\text{C}\text{-}^1\text{H}$ correlation for $\text{S}_{2/6}$ at $\delta_{\text{C}}/\delta_{\text{H}}$ $103.4/6.7$, G_2 at $\delta_{\text{C}}/\delta_{\text{H}}$ $111.5/6.9$, $\text{H}_{2/6}$ at $\delta_{\text{C}}/\delta_{\text{H}}$ $128.0/7.2$, and $\text{pCA}_{2/6}$ at $\delta_{\text{C}}/\delta_{\text{H}}$ $129.9/7.4$ were used to semi-quantitatively measure the S, G, H and *p*CA contents, respectively. The chemical shifts of α -oxidized $\text{S}_{2/6}$ ($\delta_{\text{C}}/\delta_{\text{H}}$ $106.5/7.3$) and FA_2 ($\delta_{\text{C}}/\delta_{\text{H}}$ $110.9/7.3$) were only found in NO.3 lignin. Cross peaks from condensed $\text{S}_{2/6}$ were well resolved at $\delta_{\text{C}}/\delta_{\text{H}}$ $104.7/6.4$, which existed in both NO.1 and NO.2 lignin. Condensed G_2 at $\delta_{\text{C}}/\delta_{\text{H}}$ $112.4/6.7$ was only observed in the aromatic region of NO.1 lignin spectra. The correlations of G_5 , G_6 , pCA_7 and $\text{pCA}_{3/5}$ were also observed. As shown in Table 5, the composition of each lignin fractions was different. Ethanol-extracted lignin fraction (NO.1) was composed of 10.2% H units, 48.6% S (condensed and non-condensed) units and 41.2% G (condensed and non-condensed) units. The S units represented the majority of the other two lignin fractions, which was 88.7% and 84.0% in NO.2 and NO.3 lignin, respectively. The overwhelming percentage of S units was also observed in other studies (Chen *et al.* 2017; Dong *et al.* 2019). That was due, in part, to more degradation of G than S during pretreatment (Chen *et al.* 2019).

Lignin condensation is commonly observed after the dilute acid pretreatment (Yu *et al.* 2014). In the present study, condensed S was found in NO.1 and NO.2 lignin, and it accounted for 18.1% and 13.5% of the total lignin subunits, respectively. The condensed G_2 was only observed in NO. 1 lignin. It is interesting to note that most of the condensed aromatics were isolated by solvent extractions (*i.e.*, ethanol and dioxane), in particular, in NO. 1 lignin fraction. *p*CA is considered to be exclusively acylated at γ -position by ester bonds in various herbaceous crops via lignification of *p*-coumaroylated monolignols (Kim and Ralph 2010). The content of *p*CA increased from 30.8% in NO.1 lignin to 81.5% in NO.3 lignin.

The $^{13}\text{C}\text{-}^1\text{H}$ correlations of methoxyl group ($-\text{OCH}_3$) and various linkages were observed in the aliphatic region $90\text{-}45/6.0\text{-}2.0\text{ ppm}$. The cross peaks corresponding to $\beta\text{-O-4}$ were centered at $\delta_{\text{C}}/\delta_{\text{H}}$ $71.7/4.8$ ($\text{A}_{\alpha\text{-S}}$), $71.1/4.7$ ($\text{A}_{\alpha\text{-G}}$), $83.4/4.3$ ($\text{A}_{\beta\text{-G/H}}$), and $60.5/3.6$ (A_{γ}). The $^{13}\text{C}\text{-}^1\text{H}$ correlations of phenylcoumaran ($\beta\text{-5}$) were found at $\delta_{\text{C}}/\delta_{\text{H}}$ $85.9/5.5$ (B_{α}), $\delta_{\text{C}}/\delta_{\text{H}}$ $52.9/3.5$ (B_{β}) and $62.2/3.8$ (B_{γ}). Signals from $\text{C}_{\alpha}/\text{H}_{\alpha}$ in $\beta\text{-O-4}$ and $\beta\text{-5}$ substructure were used to represent specific total linkages. The results indicated that $\beta\text{-O-4}$ linkages

dominated the interunit bonds in all lignin fractions, followed by β -5. The content of lignin interunit linkages, including β -O-4 and β -5, linkages over total aromatic regions was 4.6%, 24.1%, and 15.3% in NO.1, NO.2, and NO.3 lignin fractions, respectively, indicating that NO.2 lignin fraction contained the most amounts of these lignin inter-unit linkages, followed by NO.3 lignin fraction. Furthermore, lignin extracted by dioxane (NO. 2) had more C-C bond contents than other fractions.

Relationships between structural properties of lignin and its inhibition on cellulase

According to the results of the aforementioned characteristics of lignins and their inhibition on enzymatic hydrolysis, the lignin fractions comprising of more S units resulted in a higher inhibitory impact on cellulase performance ($y = -0.14x + 48.55$, $R^2 = 0.63$), which was consistent with previous study with gramineous crops (Yao *et al.* 2018b). The negative effect of the amount of lignin inter-unit linkages (including β -O-4 and β -5) ($y = -0.39x + 43.69$, $R^2 = 0.91$) or the β -O-4 ($y = -0.40x + 43.58$, $R^2 = 0.88$) in lignin fraction on glucose yield from pretreated MCC was also observed. The results are consistent with other published results (Goujon *et al.* 2003; Xu *et al.* 2012; Jiang *et al.* 2016). These HSQC results indicated that the S/G ratio is a negative factor, whereas the H/G could positively affect saccharification process of herbaceous biomass (Xu *et al.* 2012). The more S units in lignin result in a more linear structure, which could adsorb on the cellulase surface more tightly and decrease the accessibility of cellulose dramatically (Jiang *et al.* 2016). However, some earlier studies showed that low S/G ratio caused higher affinity between lignin and cellulase (Guo *et al.* 2014). It was even pointed out that the phenolic OH group from condensed G had a strong correlation with their inhibition on glucose release (Sun *et al.* 2016). The contradictory results obtained from these studies indicated that there might be other lignin properties that had a more profound influence on lignin interaction with cellulase. It is hypothesized that a higher degree of condensation could result in greater cellulase adsorption via hydrophobic interaction (Ko *et al.* 2015). The degree of condensation (DC) of the isolated three lignin fractions was 0.76, 0.58 and 0.30, respectively, which was calculated from $3.00 - I_{124-102}$ ppm, based on the ^{13}C NMR spectra (Sun *et al.* 2016). These results suggest that DC of lignin did not exhibit significant influences on cellulose conversion in this study.

Table 5. Quantitative Information of Three Lignin Samples in the HSQC Spectra

Lignin Substructure	NO.1 Lignin	NO.2 Lignin	NO.3 Lignin
	% ^a	% ^a	% ^a
Non-condensed S	30.5	75.2	84.0
Condensed S	18.1	13.5	0.0
Total S	48.6	88.7	84.0
Non-condensed G	0.0	3.0	6.7
Condensed G	41.2	0	0.0
Total G	41.2	3.0	6.7
H	10.2	8.3	9.2
pCA	30.8	69.9	81.5
β -O-4	4.1	22.6	15.1
β -5	0.5	1.5	0.2

^aAmount of specific functional group was expressed as percentage of S+G+H

Studies on wheat straw obtained by soda-AQ and kraft pretreatments indicated that residual lignin containing less β -O-4 subunits released more glucose during cellulase

hydrolysis process (Yang *et al.* 2016). A negative correlation was found between the content of β -O-4 bonds in hydrothermal pretreated biomass and its inhibition in pretreated MCC hydrolysis (Kellock *et al.* 2019). In this study, the lignin fraction containing more etherified lignin interunit linkages (NO.2 lignin) also showed more profound inhibitory effect on pretreated MCC saccharification. Earlier studies indicated that chemical modifications produced by pretreatment with laccase and a phenolic mediator resulting in breakdown of the main inter-unit linkages could improve saccharification (Rico *et al.* 2014).

CONCLUSIONS

1. Lignin fractions extracted from dilute acid pretreated corn stover showed different physicochemical properties and inhibitory effects on cellulase hydrolysis of the pretreated microcrystalline cellulose (MCC).
2. The structural features of lignin fractions extracted from dilute acid pretreated corn stover influence the interaction between cellulase and lignin based on the analysis. It is hypothesized that lignin with S-type substructure could affect cellulase activity more. Lignin with more lignin interunit linkages, such as β -O-4 and β -5 linkages, exhibited greater significant inhibitory effect on glucose release from the pretreated MCC.

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