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PAPER

Strong association of condensed phenolic moieties in isolated lignins with their inhibition of enzymatic hydrolysis

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The residual lignin plays an inhibitory role in the enzymatic hydrolysis of cellulosic biomass. In this study, we examined the structure changes of the isolated lignins from hot water hydrothermal pretreatment of aspen and their inhibitory effects on the enzymatic hydrolysis of Avicel. The functional groups of the isolated lignins were determined by quantitative ^{13}C, 2D HSQC and ^{31}P NMR. The increase of pretreatment severity significantly increased the condensed and non-condensed syringyl and guaiacyl OH group contents in the isolated lignins, but decreased the aliphatic OH, p-hydroxybenzoate OH and carboxylic OH group contents. A compelling adverse association (r^2=0.998) was observed between the condensed syringyl and guaiacyl phenolic OH group contents in lignins and their inhibitory effects on enzymatic hydrolysis. Langmuir adsorption isotherms showed that higher pretreatment severity resulted in higher binding ability between the isolated lignins and the cellulase enzymes, which led to more non-productive binding. It is hypothesized that condensed syringyl and guaiacyl phenolic units are mainly responsible for the inhibitory effect of lignin on enzymatic hydrolysis, in which the condensed aromatic rings enhance the hydrophobic interactions and the phenolic OH group boost the hydrogen bonding. The combination of hydrophobic interactions and hydrogen bonding can further intensify the undesirable non-productive binding.

Introduction

The abundant forest residues and agricultural biomass wastes provide a vast renewable resource for lignocellulosic biorefinery development. The biochemical conversion of plant biomass to biofuels and chemicals typically involves biomass pretreatment, enzymatic hydrolysis and microbial fermentation. The nature of plant cell wall recalcitrance has made the biomass highly resistant to enzymatic hydrolysis.[1, 2] Currently, one of the main challenges in biochemical conversion is the lack of cost-effective approach for releasing fermentable sugars from lignocellulosic biomass due to its recalcitrance.[3] This recalcitrance is related to the structural complexity of lignin and polysaccharides matrix in the cell wall, in which the lignin blocks and inhibits the access of cellulase enzymes and the crystalline cellulose reduces the accessible sites for enzyme to act.[1, 4, 5]

Lignin is a polymer of phenylpropane and can be divided into two major classes based on the structural elements. “Guaiacyl lignin” is a polymerization product of coniferyl alcohol in softwood and “Guaiacyl-syringyl lignin is a copolymer of coniferyl and sinapyl alcohols in hardwood. Lignin typically plays a negative role in enzymatic hydrolysis and can significantly inhibit the enzyme efficiency by non-productive binding.[6-8] The inhibitory effect of residual lignin is believed to be associated to the lignin physicochemical properties and functional groups.[9, 10] Previously, phenolic hydroxyl groups have been demonstrated to be essential for the negative inhibition of lignin model compounds on enzymatic hydrolysis of Avicel.[9] However, the total hydroxyl groups in lignins have been found to be positively associated with their effects on enzymatic hydrolysis reconstructed substrates.[10] Recently, carboxylic acid groups in lignin have been suggested to increase the hydrolysis yield by reducing the non-productive binding through electrostatic repulsion.[11] The aliphatic hydroxyl groups have also been correlated to the adsorption capacity of lignin and enzymes.[12] In addition to residual lignin, soluble lignin degradation compounds have also been observed to inhibit enzymatic hydrolysis considerably.[13-16] Phenolic compounds from hot water pretreatment of maple reduced the hydrolysis yield by 20%.[15] Although the soluble inhibitors can be removed by water washing, the negative effect of residual lignin still presents a significant challenge for enzymatic hydrolysis of lignocellulosic biomass.

Hot water hydrothermal pretreatment is one of the promising pretreatment processes, which can enhance the biomass hydrolyzability by removing the hemicellulose and increasing the porosity of the substrates.[17] Various substrate characteristics (such as particle size, xylan removal, porosity, degree of polymerization (DP) and lignin content) of hot water pretreated hardwood have been correlated to enzymatic hydrolysis,[18] in which a direct association of hydrolysis yield with wet particle and cellulose DP was observed. The study also indicated that the addition of bovine

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serum albumin (BSA) could dramatically reduce the negative effect of residual lignin one enzymatic hydrolysis.[18] The non-productive binding between residual lignin and cellulases could be significant, because the most of lignin remains in the hot water pretreated substrates.[19] It was observed that higher severity pretreatment resulted in more inhibitory lignins. However, how the hydrothermal pretreatment changes the lignin structure is not very clear. Which functional groups are responsible for the inhibition of lignins is not understood yet. We hypothesize that condensed syringyl and guaiacyl phenolic hydroxyl group play a key role in the lignin inhibition due to the hydrophobic interactions and hydrogen bonding. Milled wood lignin (MWL) has been often used as a presentative source of original lignin due to its maximum maintaining the structure of lignin.[20] Both MWL and cellulolytic enzyme lignin (CEL) can be extracted from biomass and used as for plant-cell free lignin to study the cellulosic hydrolysis in the plant cell wall.[21, 22] It is believed that the MWL would has less interference from residual polysaccharides on enzymatic hydrolysis than CEL. Previous study also observed that the addition of isolated lignin back into the delignified substrates reinstated the negative effect of residual lignin in original substrates. [23]

In this study, the functional groups of isolated lignins from the hydrothermal pretreatment of aspen was characterized by quantitative ^{13}C, 2D HSQC and ^{31}P NMR. The functional groups include aliphatic hydroxyls, condensed and uncondensed syringyl and guaiacyl hydroxyls, *p*-hydroxybenzoate phenolic hydroxyls and carboxylic acids hydroxyls. Three milled wood lignins were isolated from the hydrothermal pretreatment of aspen under different temperature (150 -190 °C). The inhibitory effect of isolated lignins on enzymatic hydrolysis of Avicel was correlated to the lignin functional groups. Langmuir adsorption isotherm was used to determine the adsorption affinity of enzyme to isolated lignins. Moreover, zeta potential and molecular weight of the isolated lignins were also characterized. The objective of this work is to elucidate the specific functional groups in lignins that account for their inhibition.

Materials and methods

Biomass and Chemicals

Aspen wood chips were collected by Forest Products Laboratory at Auburn University. The wood chips were ground and sieved to 20-40 mesh size. The ground biomass was dewaxed with toluene/ethanol (2:1, v/v) in a Soxhlet extractor for 6 h to remove extractives (approximate 3.31%) and then dried at 60 °C for 16 h. The composition of dewaxed aspen was 43.62% glucan, 14.85% xylan, 1.67% mannan, and 23.67% lignin (21.20% Klason lignin and 2.47% acid-soluble lignin), which was determined by using National Renewable Energy Laboratory's (NREL) standard analytical procedure, and 0.55% ash, which was measured after heated at 600 °C for 6 h. Microcrystalline cellulose (~50 μm particle size), Avicel® PH-101 was purchased from Sigma Aldrich (St. Louis, MO). A commercial cellulase, Cellic®CTec2, was provided by Novozymes North America, Inc. (Franklinton, NC). Cellulase C2730 was also purchased from Sigma-Aldrich, Co. (St. Louis, MO).

Hydrothermal pretreatment of aspen

The hot water hydrothermal pretreatment was conducted in a 1.0 L stainless steel Par batch reactor with a magnetic stirrer at a solid to liquid ratio of 1:10 (w/w) by a PID controller (Parr 4848, USA). Dewaxed powders (60.0 g) were mixed with deionized water (600 mL) and then heated at 150 °C, 170 °C, and 190 °C for 2.0 h, respectively. The corresponding severity factor (log R_0, ω=14.75) based on temperature and time of pretreatment were 3.55, 4.14, and 4.73, respectively.[24] After the pretreatment, the reactor was cooled to room temperature by tap water. The solid residues were collected with a Buchner funnel, washed thoroughly with distilled water, and further dried in an oven at 60 °C for 16 h.

Milled Wood Lignins (MWL) isolation from pretreated biomass

The 20 g ball-milled aspen (dewaxed raw biomass and hydrothermal pretreated biomass) was directly suspended in dioxane/water (96:4, v/v) with a solid to liquid ratio of 1:20 (g/mL) and extracted at 25 °C for 24 h in a dark condition. After the extraction, the mixtures were filtered and washed with the same solvents until the filtrate was clear. The step was repeated twice. The combined filtrates were concentrated to ~ 50 mL with a rotary evaporator under reduced pressure and then precipitated in acidified water (~ 500 mL, pH 2.0). After washing with acidified water and freeze-drying, the isolated lignins from raw biomass (MWL) and pretreated biomass (150-MWL, 170-MWL and 190-MWL) were obtained. These milled wood lignins were further purified according to a previous method.[10]

Quantitative ^{13}C NMR analysis of the isolated lignins

NMR spectra were conducted on a Bruker AVIII 400 MHz spectrometer. ^{13}C NMR can detects the 1.1% of naturally occurring ^{13}C in lignin. Quantitative ^{13}C NMR employed a 30° pulse angle, a 1.4 s acquisition time and a 2.0 s relaxation delay. The quantitative spectra were recorded in FT mode at 100.6 MHz. The inverse gate decoupling sequence (C13IG) was used and it allowed quantitative analysis and comparison of signal intensities. Chromium (III) acetylacetonate (20 μL, 0.01 M) was added as a relaxation agent to the solution to provide complete relaxation of all nuclei. Non-acetylated isolated lignins (140 mg) were dissolved in 0.5 mL dimethyl sulfoxide (DMSO-d_6). A total of 30,000 scans per sample were collected.

2D HSQC and ^{31}P NMR analysis of the isolated lignins

For 2D-heteronuclear single quantum coherence (HSQC) NMR experiments, 60 mg of lignin samples was dissolved in 0.5 ml of DMSO-d_6. The Bruker pulse program "hsqcetgpsi" was used and the parameters used were listed as below: The number of collected complex points was 1 K for the ^1H dimension with d_1 (2 s), number of scanning is 64, and 256 time increments were always recorded. ^{31}P NMR spectra of the isolated lignins were performed as previously reported, 20 mg lignins were dissolved in 0.5 mL of anhydrous pyridine and deuterated chloroform (1.6:1, v/v) under stirring. This was followed by the addition of 0.1 mL cyclohexanol (10.85 mg/mL in anhydrous pyridine and deuterated chloroform 1.6:1, v/v) as an internal standard (IS), and 0.1 ml chromium (III) acetylacetonate solution (5 mg/mL in anhydrous pyridine and deuterated chloroform 1.6:1, v/v) as relaxation reagent. Finally, the mixture were reacted with 0.1 mL phosphorylating reagent 2-

chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholate (TMDP) and were transferred into a 5 mm NMR tube for analysis.[25, 26]

Chemical composition analysis of isolated lignin and molecular weight and zeta potential determination

Associated polysaccharides of the isolated lignins were determined by HPLC. The neutral sugars in the lignins (5 mg) were liberated by hydrolysis with 6% dilute sulfuric acid (1.475 mL) at 105 °C for 2.5 h. After acid hydrolysis, the hydrolysates were diluted 50-fold, and the filtrate was analyzed by an HPLC system (Dionex ISC 3000) with an amperometric detector, an AS50 autosample, a Carbopac PA-20 column (4 × 250 mm, Dionex), and a PA-20 guard column (3 × 30 mm, Dionex). Molecular weights of the isolated lignins were determined by GPC with an ultraviolet detector (UV) at 240 nm. The column used was a PL-gel 10 mm mixed-B 7.5 mm i.d. column, which was calibrated with PL polystyrene standards. The lignin (4 mg) was dissolved in tetrahydrofuran (THF, 2 mL), and then 20 µL lignin solutions were injected. The column was operated at ambient temperature and eluted with THF at a flow rate of 1.0 mL/min. The zeta potentials of the lignins were varied by mixing 1 mg of the lignin with 1.0 mL of 50 mM citrate buffer solution and dispersing the solution using disperser. The zeta potentials of the lignins were also determined by the Zetasizer (Malvern Instruments Ltd, Worcestershire, UK) measuring response of a small spherical particle in an applied E-field.

Enzymatic hydrolysis of Avicel with the addition of the isolated lignins

Enzymatic hydrolysis of Avicel was performed in 50 mL of 50 mM citrate buffer (pH 4.8) at 2% glucan (w/v) with commercial enzyme (Cellic®CTec2) as previously described.[27] Briefly, the hydrolysis reaction was carried out at 50 °C and 150 rpm for 72 h. The enzyme loading of Cellic®CTec2 was 5.0 FPU/g glucan (2.4 mg protein/ g glucan) in enzymatic hydrolysis of Avicel. To investigate the effects of the isolated lignins on enzymatic hydrolysis, the lignins were added into the enzymatic hydrolysis system prior to the enzyme addition. Specifically, 4 g/L isolated lignins were added to a mixture of the substrates and citrate buffer, and then incubated for 1 h at room temperature. After that, the enzyme was added to initiate the enzymatic hydrolysis. To examine the hydrolysis yield of Avicel, the samples were taken from the hydrolysis solution at various time intervals. The glucose content was determined by HPLC with Aminex HPX-87P column (300 × 7.8 mm id). The mobile phase was nanopure water at a flow rate of 0.6 mL/min. The hydrolysis yield of Avicel was calculated from the released glucose content, as a percentage of the theoretical sugars available in Avicel. Enzymatic

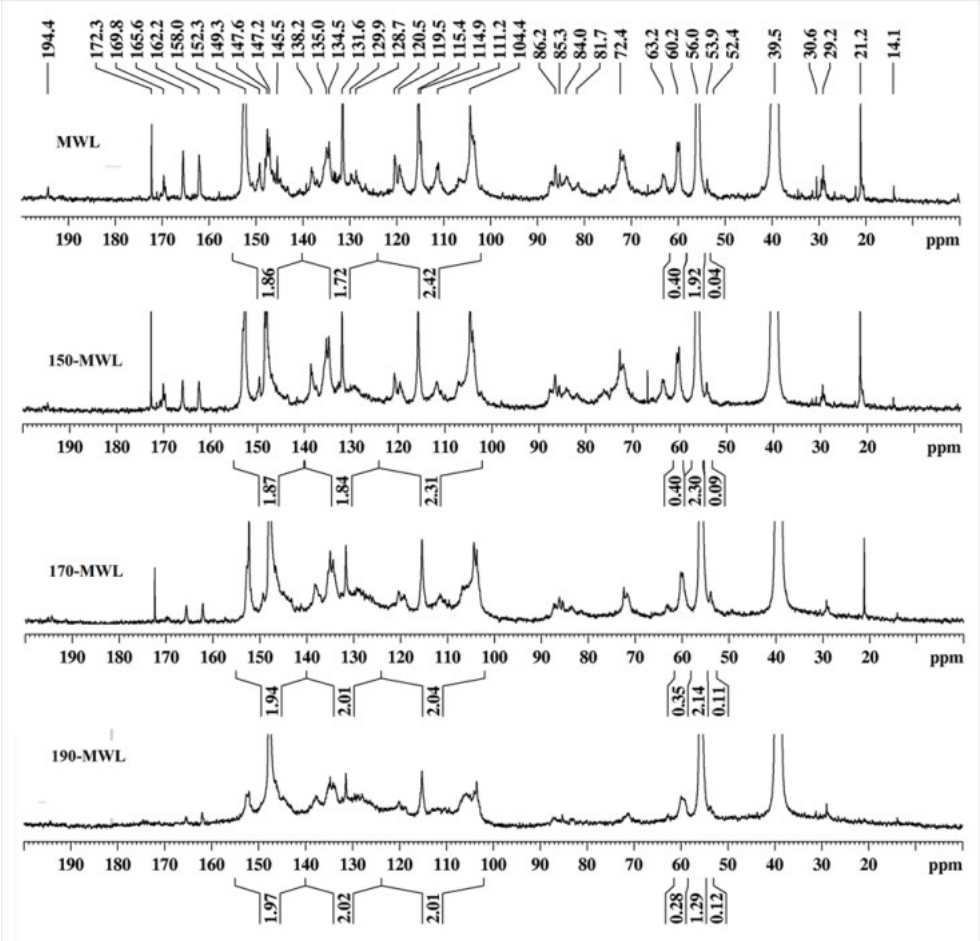

Fig. 1 Quantitative ^{13}C NMR spectra of the isolated lignin from hot water hydrothermal pretreated aspen.

hydrolysis was carried out in duplicate, and each data point was presented as the average of two replicates.

Langmuir adsorption isotherms

To determine the affinity of cellulases on the isolated lignin samples, cellulase C2730 (protein content 41 mg/mL) with low β-glucosidase content was used. Cellulase C2730 was incubated with 2% (w/w) lignin at 4 °C and 150 rpm for 2 h. A range of enzyme concentration was used from 0.01 to 0.40 mg/mL. After reaching equilibrium, the sample was taken to determine the protein content in the supernatant by Bradford assay as free enzyme content in the enzymatic hydrolysis solution. The adsorbed enzyme was calculated from the difference between the initial enzyme content and the free enzyme content. The classical Langmuir adsorption isotherm ($\Gamma=\Gamma_{max}KC/(1+KC)$) was used to fit the cellulase enzyme adsorption on lignins. Where, Γ_{max} is the surface concentration of enzymes at full coverage (mg/g substrate); K is the Langmuir constant (mL/mg); and C is the free enzyme content in solution (mg/mL). The distribution coefficient (R) can be expressed as $R= \Gamma_{max} K$.

Results and discussions

Effect of hydrothermal pretreatment severity on condensed and non-condensed phenolic moieties in lignin determined by quantitative ^{13}C NMR, HSQC NMR and ^{31}P NMR

Quantitative ^{13}C NMR is often used to determine the structure changes in lignin during the biomass pretreatment.[28] The ^{13}C NMR spectra of the isolated lignins were analyzed and compared (**Fig. 1**). It was observed that the amount of β-*O*-4 (based on the signals at 82.5-88.0 ppm) in the isolated lignins decreased significantly when the hydrothermal pretreatment severity increased from 3.55 to 4.73.The integration values of the protonated aromatics (δ 124.0–102.0 ppm), condensed aromatics (δ 140.0–124.0 ppm), and oxygenated aromatics (δ 155.0–140.0 ppm) regions were compared quantitatively (**Table 1**).[29]

The results showed that the condensed aromatics increased with elevating severity from 1.72/Ar (MWL) to 1.84, 2.01 and 2.02/Ar in 150-MWL, 170-MWL and 190-MWL, respectively. The oxygenated aromatics also increased from 1.86/Ar (MWL) to 1.87, 1.94 and 1.97/Ar in 150-MWL, 170-MWL and 190-MWL, respectively. This indicated the condensation and oxidation reactions mainly took place at high severity (4.14 and 4.73). Meanwhile, the amounts of *p*-hydroxybenzoate (PB) decreased with the elevating severity from 0.22/Ar (MWL) to 0.18, 0.17 and 0.15/Ar in 150-MWL, 17-MWL and 190-MWL, respectively. The β-*O*-4 linkages were gradually reduced with the elevating severity, implying that the hydrothermal pretreatment process under higher severity had a significant influence on the amounts of PB and β-*O*-4 linkages. It was also observed that as the pretreatment severity increased, the contents of β-5 and β-β gradually increased, suggesting that more condensed lignin occurred during the pretreatment process. In addition, the amount of OCH_3 was increased from 1.92/Ar in the control MWL to 2.30/Ar in 150-MWL but decreased to 2.14 and 1.29/Ar in 170-MWL and 190-MWL, implying that demethoxylation might occur under the harsh conditions. The 2D-HSQC spectra and structures of the identified lignin sub-units in the isolated lignins are shown in **Fig. 2**.[30, 31] The inter-unit linkages of β-aryl-ether (β-*O*-4, A), resinol (β-β, B), phenylcoumaran (β-5, C), were identified by their cross-peaks at δ_C/δ_H 71.8/4.84 (A_α), 83.4/4.34 ($A_{\beta(G)}$), 85.9/4.11 ($A_{\beta(s)}$), 59.7/3.60 (A_γ) 84.8/4.64 (B_α), 53.4/3.05 (B_β), 71.0/3.81–4.17 (B_γ), 86.8/ 5.45 (C_α), 53.0/3.45 (C_β) and 62.4/3.72 (C_γ) respectively. HSQC NMR spectra showed that β-*O*-4 linkages in lignin decreased significantly from 85% (MWL) to 81%, 69% and 28% in 150-MWL, 170-MWL and 190-MWL, respectively. On the contrary, the β-β linkages increased from 13% (MWL) to 14%, 21% and 51% in 150-MWL, 170-MWL and 190-MWL, respectively. Also, the β-5 linkages increased from 3% (MWL) to 5%, 10% and 21% in 150-MWL, 170-MWL and 190-MWL, respectively. This indicated that condensation probably occurred in the hydrothermal pretreatment and new β-β and β-5 bonds were formed.

Table 1 The assignment and quantification of the signals of the ^{13}C-NMR spectra (results expressed per Ar) of the isolated lignin from hot water hydrothermal pretreated aspen

δ (ppm)	Assignment	MWL	150-MWL	170-MWL	190-MWL
155.0 - 140.0	Aromatic C–O	1.86	1.87	1.94	1.97
140.0 - 124.0	Aromatic C–C	1.72	1,84	2.01	2.02
124.0 - 102.0	Aromatic C–H	2.42	2.31	2.04	2.01
132.7 - 130.8	PB[a]	0.22	0.18	0.17	0.15
61.3 - 58.0	β-*O*-4	0.40	0.40	0.35	0.28
58.0 - 54.0	CH_3O	1.92	2.30	2.14	1.29
54.0 - 53.0	β-β, β-5	0.04	0.09	0.11	0.12
	DC[b]	0.58	0.69	0.96	0.99

[a] PB was calculated from the following formula: PB = 0.5 × $I_{132.7\text{-}130.8\ ppm}$.
[b] DC, degree of condensation. Calculated from 3.00 – $I_{124–102\ ppm}$.

The cross peaks in the aromatic region (**Fig. 2**) showed well separated signals of syringyl (S) and guaiacyl (G) units. The S unit showed a strong signal for $C_{2,6}/H_{2,6}$ correlation at δ_C/δ_H 103.8/6.71 ($S_{2,6}$). The G units showed 3 strong cross correlations at δ_C/δ_H 110.8/6.94 (G_2), 115.0/6.79 (G_5), 119.0/6.78 (G_6). Considerable amount of PB unit was observed at 131.2/7.66 ($PB_{2,6}$) and small amounts of oxidized syringyl (S') unit was shown at δ_C/δ_H 106.4/7.24 ($S'_{2,6}$) for all lignins. The increase of severity in hydrothermal pretreatment increased the S/G ratio from 1.7 in MWL to 3.0, 3.1 and 5.1 in 150-MWL, 170-MWL and 190-MWL, respectively. The indicated that more S unit could be migrated and extracted in pretreated biomass at higher severity. The HSQC spectra also showed the amount of PB (based on $S_{2,6}/2+G_2$) decreased with the elevated severity from 20.6% in MWL to 10.7%, 6.2%, and 4.5% for 150-MWL, 170-MWL, and 190-MWL, respectively. This indicated that some PB units was gradually cleaved and released at higher severity.

^{31}P NMR spectroscopy has been developed to determine various hydroxyl groups in lignin, which allows for quantifying the carboxylic and guaiacyl phenolic hydroxyl groups as well as the primary and secondary hydroxyl groups.[32] It involves phosphitylation of hydroxyl groups in lignin with a ^{31}P reagent such as TMDP, [33] which reacts with different hydroxyl groups (such as aliphatic, phenolic, and carboxylic groups) in lignin. The derived hydroxyl groups can be quantitatively estimated against an internal standard such as cyclohexanol.[34] In this study, to further investigate the effects of pretreatment severity on the major hydroxyl groups in lignin, the lignin samples were derivatized with TMDP and the phosphorylated hydroxyl groups were estimated by ^{31}P NMR based on the integration area of individual peaks (**Fig. 3**). The contents of aliphatic hydroxyls, condensed and uncondensed syringyl and guaiacyl hydroxyls, *p*-hydroxybenzoate phenolic hydroxyls and carboxylic acids hydroxyls were compared in different lignin samples (**Table 2**).[34, 35]

The results showed that the increase of severity in hydrothermal pretreatment decreased the aliphatic OH groups from 3.7 mmol/g in MWL to 3.6, 2.3 and 1.1 mmol/g in 150-MWL, 170-MWL and 190-MWL, respectively. This suggested that the hydroxyl groups in the side-chain of lignin were probably fragmented and eliminated at high severity.[36] On the contrary, the phenolic OH groups in S and G units increased

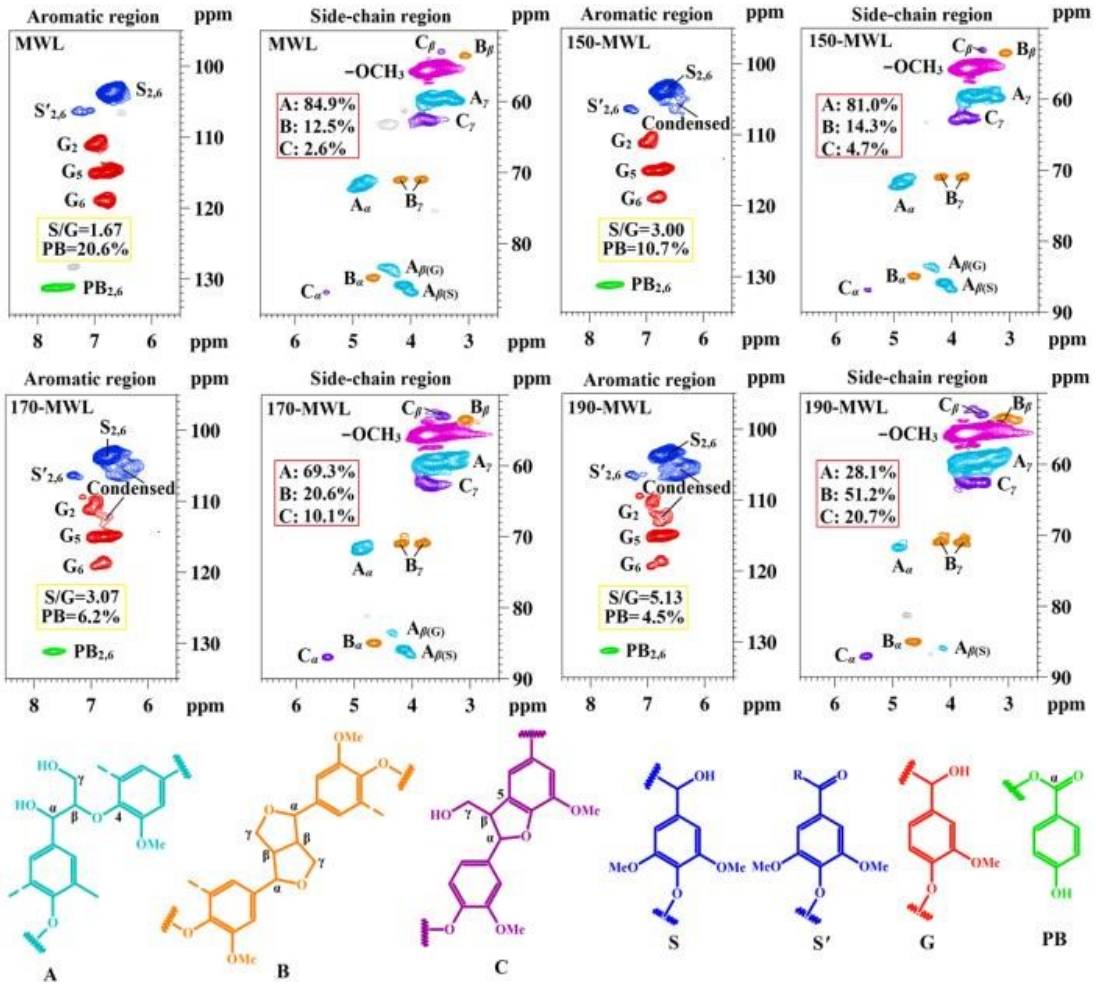

Fig. 2 2D-HSQC spectra and the main structures of the isolated lignins: (A) β-aryl-ether units (β-O-4); (B) resinol substructures (β-β); (C) phenylcoumaran substructures (β-5); (G) guaiacyl units; (S) syringyl units; (S') oxidized syringyl units bearing a carbonyl at C_α; (PB) *p*-Hydroxybenzoate units.

significantly with the increase of pretreatment severity (**Table 2**). Specifically, the condensed syringyl OH groups increased from 0.17 mmol/g in MWL to 0.24, 0.46 and 0.54 mmol/g in 150-MWL, 170-MWL and 190-MWL, respectively. And the non-condensed syringyl OH groups increased from 0.40 mmol/g in MWL to 0.82, 1.29 and 1.42 mmol/g in 150-MWL, 170-MWL and 190-MWL, respectively. The condensed guaiacyl OH groups increased from 0.16 mmol/g in MWL to 0.20, 0.32 and 0.38 mmol/g in 150-MWL, 170-MWL and 190-MWL, respectively. And the non-condensed guaiacyl OH groups increased from 0.66 mmol/g in MWL to 0.73, 0.78 and 0.83 mmol/g in 150-MWL, 170-MWL and 190-MWL, respectively.

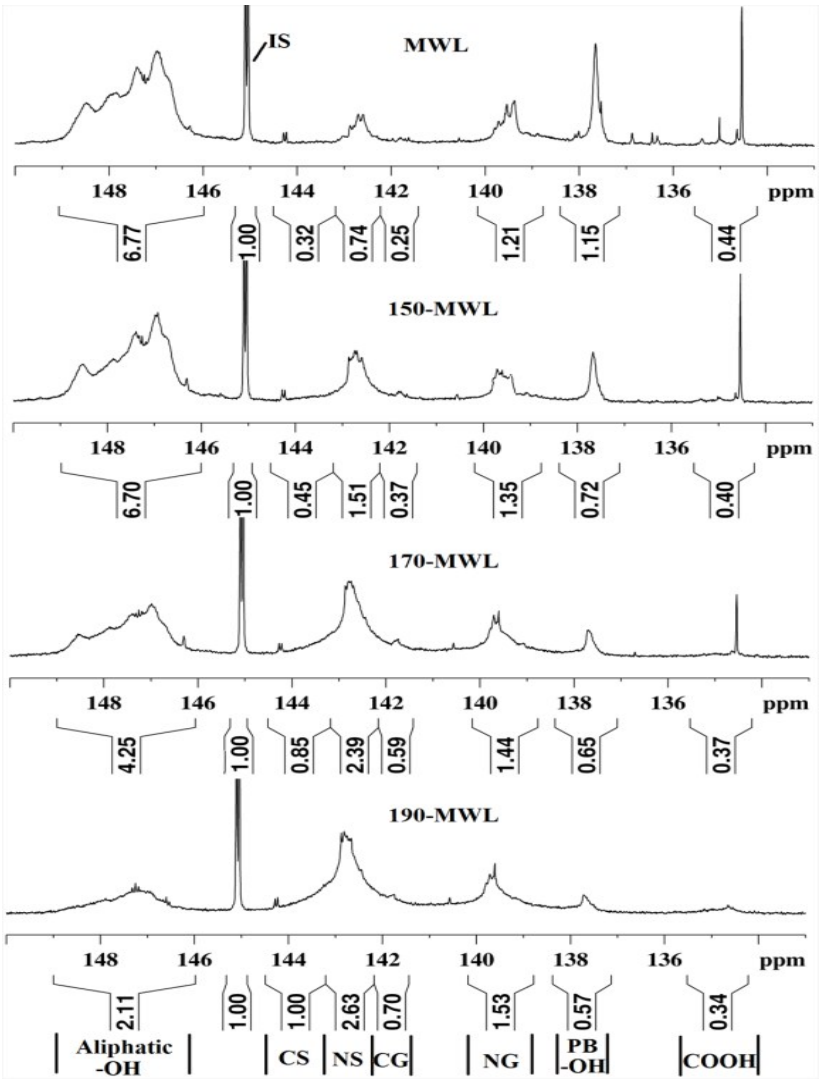

Fig. 3 ^{31}P-NMR spectra of the isolated lignins. Abbreviation: CS, condensed syringyl phenolic hydroxyls; NS, non-condensed syringyl phenolic hydroxyls; CG, condensed guaiacyl phenolic hydroxyls; NG, non-condensed guaiacyl phenolic hydroxyls; PB-OH, *p*-hydroxybenzoate phenolic hydroxyls; COOH, carboxylic groups.

Table 2 Quantification of the functional Groups (mmol/g) in the four MWLs isolated from aspen by quantitative ^{31}P-NMR method

Lignins	Aliphatic OH	Syringyl OH		Guaiacyl OH		PB-OH[a]	Carboxylic group	Total phenolic OH
		C[a]	NC[a]	C	NC			
MWL	3.67	0.17	0.40	0.16	0.66	0.62	0.24	2.01
150-MWL	3.63	0.24	0.82	0.20	0.73	0.39	0.22	2.38
170-MWL	2.30	0.46	1.29	0.32	0.78	0.35	0.20	3.20
190-MWL	1.14	0.54	1.42	0.38	0.83	0.31	0.18	3.48

[a] Abbreviation: C, condensed; NC, non-condensed; PB-OH, *p*-Hydroxybenzoate.

The increased phenolic OH groups were probably due to the cleavage of β-O-4 linkages at high pretreatment severity. Similar observation has been reported in an autohydrolysis pretreatment of poplar.[37] The increased OH groups in condensed S and G units agreed well with the increase of shifted cross correlations of $S_{2,6}$ and G_2 in HSQC spectra. It was also observed that the PB phenolic OH decreased from 0.62 mmol/g in MWL to 0.39, 0.35 and 0.31 mmol/g in 150-MWL, 170-MWL and 190-MWL, respectively. The observation was supported by the decrease of PB cross signals in HSQC spectra. But, previous study also reported that PB-OH groups increased with the increase of pretreatment severity.[37] In addition, it was noticed that higher pretreatment severity resulted in the slight decrease of carboxyl OH groups. This observation was also different from the previous report on enzymatic lignin from autohydrolysis pretreatment of poplar. This discrepancy could be due to the different lignin isolation methods, especially the polysaccharide in enzymatic lignin has been suggested to interfere the value of aliphatic OH in autohydrolysis pretreatment of poplar.[37] Enzymatic lignin has been found to be structurally similar to milled wood lignin.[38] Both of them were suggested to be adequately representative of the total lignin in biomass,[38] although small degradation was expected in MWL. It is believed that the structure changes of the isolated lignins in the hydrothermal pretreatment are relative to what occurred in the lignocellulosic biomass.

Effect of hydrothermal pretreatment severity on lignin inhibitory effect for enzyme hydrolysis of Avicel

To investigate the effects of pretreatment severity on the isolated lignins inhibitory activity, four lignin samples were added in the enzymatic hydrolysis of Avicel. All four lignins showed negative effects on hydrolysis yields (**Fig. 4**). Specifically, the isolated lignins decreased the 72 hydrolysis yield of Avicel by 7-29% from 53.7% (control) to 49.9%, 47.8%, 41.4% and 38.2% with the addition of MWL, 150-MWL, 170-MWL and 190-MWL, respectively. This indicated that higher pretreatment severity resulted in more inhibitory isolated lignins. This inhibition probably was caused by the non-productive binding between cellulase enzymes and isolated lignins.[19] The Isolated lignins can bind with enzymes by hydrophobic interactions, hydrogen bonding and electrostatic interactions. Hydrophobic interactions can occur between the aromatic groups in lignin and aromatic amino acids in enzymes. The hydrogen bonding can take place between the amide groups in enzymes and aromatic phenolic OH groups in lignin. Electrostatic interactions is coming from the surface charges of enzymes and lignin. We believe the phenolic OH groups played an essential in lignin inhibition because the aromatic ring-derived hydrophobic non-productive binding was stabilized by hydrogen bonding. It should be noted that the significant decrease of enzymatic hydrolysis yield with the addition of the isolated lignins was also due to the low enzyme loading (5 FPU, 2.4 mg protein/g glucan), because the lignin inhibition can be mitigated by an excess amount of enzyme that can bind on lignin and leave sufficient enzyme to act on cellulose.[17] Kim et al. (2015) also reported that the negative effect of lignin on cellulase performance intensified at low cellulase loading and higher pretreatment severity.[18]

Effect of hydrothermal pretreatment severity on lignin binding ability to enzyme

The non-productive binding of cellulase enzymes onto residual lignin is known to be detrimental to enzymatic hydrolysis.[39, 40] However, the non-productive binding and inhibitory effect of residual lignin in real cellulosic biomass cannot be quantified without isolating lignin out of the plant cell wall. Although the isolation can potentially change the physicochemical properties of the separated lignin to certain extent, it is believed that the binding ability and the inhibitory effect of isolated lignin can represent those in the biomass residual lignin. Previously, the isolated lignin was added back into the delignified steam pretreated biomass and the inhibitory effect was partially resumed.[23] In this study, to assess the binding ability between isolated lignin and cellulase, Langmuir adsorption isotherms of enzymes onto the isolated lignins were determined (**Fig. 5** and **Table 3**). The results showed that higher pretreatment severity resulted in higher binding ability between isolated lignin and enzymes.

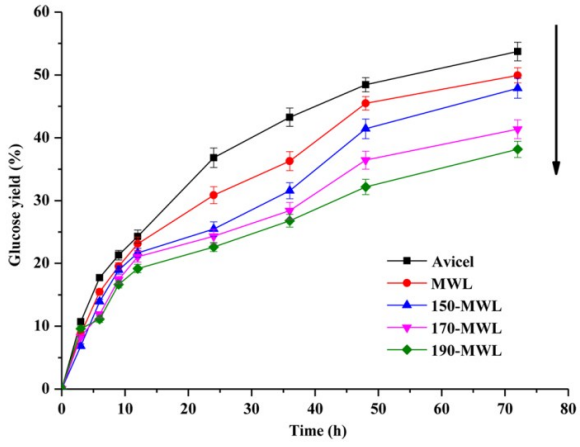

Fig. 4 Effect of the addition of the isolated lignin on enzymatic hydrolysis of Avicel

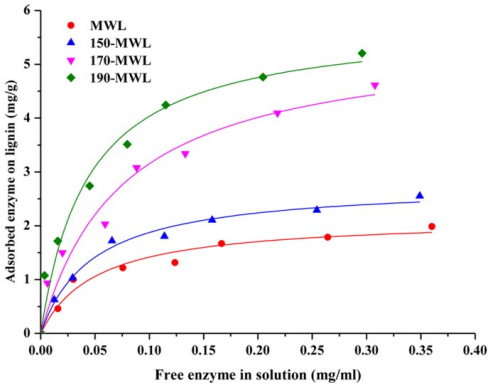

Fig. 5 Cellulase enzymes adsorption on the isolated lignins

Table 3 Langmuir adsorption isotherm parameters from enzyme adsorption on lignins

Cellulases	Γ_{max} (mg/g)	*K* (mL/mg)	*R* (L/g)
Enzyme on MWL	2.142	19.558	0.042
Enzyme on 150-MWL	2.778	20.756	0.058
Enzyme on 170-MWL	5.521	13.305	0.073
Enzyme on 190-MWL	5.793	22.925	0.133

The distribution coefficient (R) increased by 1.4-3.2 fold from 0.042 L/g in MWL to 0.058, 0.073 and 0.133 L/g in 150-MWL, 170-MWL and 190-MWL, respectively. The Langmuir constant (*K*, 13.3-22.9 mL/mg) of isolated lignins was similar to the literature value of ethanol organosolv lignin (EOL-LP) from loblolly pine,[41] but much higher than that of EOL-SG from sweetgum. It should be noticed that previous study showed contrasting effects of EOL-LP (negative) and EOL-SG (positive) on enzymatic hydrolysis of Avicel. Our study showed all four isolated lignins with higher *K* values inhibit the hydrolysis Avicel. This agreed well with the previous report and suggested higher binding affinity of lignin with enzymes resulted in the negative effect. However, to quantitatively estimate the inhibitory effect of lignins, *K* has to be incorporated with Γ_{max} into *R*, because both how much enzymes and how tightly they bind with lignins account for the overall non-productive binding. A good correlation (r^2=0.743) was observed between the distribution coefficient (R value) of the isolated lignins and their 72 hydrolysis yields (ESI, Fig. S2†). The R value has been used as the binding strength between enzymes and lignin, which indicated how much cellulase enzymes will bind onto lignin. The higher R value of lignin typically result in more non-productive binding and higher inhibition. Kumar et al. (2009) also reported that the binding strength was closely related to the degree of inhibition for the isolated lignin.[42]

Effects of hydrothermal pretreatment severity on lignin molecular weight and zeta potential

To further examine the physiochemical changes of isolated lignins from hydrothermal process, the contents of associated polysaccharides, molecular weight and zeta potential were determined (**Table 4**). Small amount of carbohydrates (0.4%-3.9%) was presented in the isolated lignins. GPC analysis showed M_w, M_n and M_w/M_n decreased considerably in isolated as the pretreatment severity increased. Specifically, the M_w of isolated lignin decreased from 3390 in MWL to 2620, 2230 and 1660 in 150-MWL, 170-MWL and 190-MWL, respectively. This suggested that hydrothermal pretreatment cleaved some interunit bonds in lignin and the extent of degradation is severity dependent. Similar results have been reported on the molecular weight of lignin from dilute acid pretreatment of switchgrass.[43] In addition, the degree of condensation (DC) of isolated lignins was calculated from 3.00 – $I_{124–102\ ppm}$ based on the ^{13}C NMR spectra. $I_{124–102}$ is the region of δ=124–102 ppm attributed to aromatic methine carbon atoms (C_{Ar-H}). The theoretical value of C_{Ar-H} in non-condensed units is 3.00, and the difference between theoretical value it and the integral at $I_{124–102\ ppm}$ is considered as the value of DC.[44] Lignin depolymerization and condensation reactions often occur simultaneously in the dilute acid and hydrothermal pretreatment process.[45] It was observed the DC of isolated lignin increased with the increase of pretreatment temperature. It was most likely the cleavage of ether linkages deceased the molecular weight of lignin, and the fragmented lignin also condensed. But, the condensation reaction was not high enough to reverse the decrease of the lignin molecular weight under the given conditions. Higher pretreatment temperature resulted in higher negative zeta potential in isolated lignins. The negative zeta potential of lignin increased from -7.54 mV to -7.68, -8.20 and -8.34 mV in 150-MWL, 170-MWL and 190-MWL, respectively. Typically, higher negative zeta potential generate higher repression between lignin and enzymes and reduced the non-productive binding.[46]. However in this study, the non-productive binding for 190-MWL was the highest (R value), although its negative zeta potential was the largest. This probably indicated that the hydrophobic interactions and hydrogen bonding were more important than electrostatic interactions. It should be noted that the particle size of isolated lignins could also affect the enzymes adsorption. It was observed that the z-average particle

Table 4 Contents of associated polysaccharides, weight-average (Mw), number-average (Mn) molecular weights, polydispersity (M_w/M_n), and zeta potential (mV) of the isolated lignins

	MWL	150-MWL	170-MWL	190-MWL
Arabinose	0.20	0.14	ND	ND
Galactose	0.03	0.15	ND	ND
Glucose	0.32	0.26	0.04	ND
Xylose	3.09	3.40	0.55	0.36
Total sugars	3.64	3.95	0.59	0.36
M_w	3390	2620	2230	1660
M_n	2620	2080	1810	1630
M_w/M_n	1.29	1.26	1.23	1.02
Zeta potential	-7.54	-7.68	-8.20	-8.34

size decreased from 6727 nm in MWL to 5878, 3839 and 2275 nm in in 150-MWL, 170-MWL and 190-MWL, respectively (ESI, Table S1 †). The SEM images of the isolated lignins were provided in ESI (Fig. S1†).

Effect of condensed and non-condensed phenolic moieties in lignin on enzymatic hydrolysis

A key finding was observed that a higher pretreatment severity resulted in a higher binding between the isolated lignins and the enzymes, and that this could be related to the ^{31}P NMR spectra for the different types of lignins (Figs. 3 and 4). To identify which functional groups are responsible for the negative effect of isolated lignin, we estimated the different hydroxyl groups based on ^{31}P NMR and hypothesized that the negative effects of lignin were mainly governed by condensed guaiacyl phenolic OH group. We correlated the content of different hydroxyl groups in lignins with the corresponding 72 hydrolysis yield of Avicel (**Fig. 6**). A compelling adverse association (y=-53.695x+58.559, r^2=0.999) was observed between condensed guaiacyl phenolic OH group and the hydrolysis yield. Higher content of condensed guaiacyl phenolic OH group in isolated lignin resulted in stronger inhibition on hydrolysis. A strong negative correlation (y=-31.261x+55.348, r^2=0.996) was also observed between the content of condensed syringyl OH group and the hydrolysis yield. For the non-condensed guaiacyl and syringyl phenolic OH groups, we also observed good correlations (y=-73.270+99.281, r^2=0.900; y=-11.434x+55.563, r^2=0.907). But, the correlations were not as strong as those from condensed phenolic groups.

This suggested that phenolic hydroxyl groups played more important role in lignin inhibition, especially the condensed phenolic hydroxyl groups. Similar observation has been reported on the correlation of total hydroxyl group content in lignins and the hydrolysis yield of reconstructed lignocellulosic substrates.[10] However, our study showed a weak correlation (r^2=0.561) between the content of total OH group and the hydrolysis yield, but a strong correlation (r^2=0.986) between total phenolic OH group and the hydrolysis yield. This can be explained by the opposite changes of the aliphatic OH group content and phenolic OH group content in lignins with the increase of pretreatment severity. The Aliphatic OH group content in lignins decreased significantly by 69% while the total phenolic OH group increased by 73% at 190 °C pretreatment. This indicated the increase of inhibition from 190-MWL probably was not coming from aliphatic OH group, but from the phenolic OH group. However, the correlation of the total phenolic OH group content and the hydrolysis yield could also be questionable, because it includes the PB OH group. The PB OH group content deceased with the increase of pretreatment severity, not as the syringyl and guaiacyl OH groups. Consequently, we correlated each individual phenolic OH group content with the hydrolysis yield and observed the very strong associations related to both the contents of the condensed syringyl and guaiacyl OH groups (r^2=0.998). The associations with both the contents of the non-condensed syringyl and guaiacyl OH groups were relatively weak (r^2=0.911). This indicated the inhibitory effect of lignin most likely came from the condensed syringyl and guaiacyl OH groups. Previous study has suggested that the increase of phenolic hydroxyls in lignin would increase enzyme non-productive binding and inhibition.[47] Steam explosion pretreatment has been found to increase the lignin hydrophobicity due to the increased lignin condensation.[21] We believe that it is crucial to combine the two properties of condensed phenolic hydroxyls together to evaluate lignin inhibition, because the condensed aromatic rings enhance the hydrophobic interaction and the phenolic hydroxyls bring the enzymes closer to lignin via hydrogen bonding.

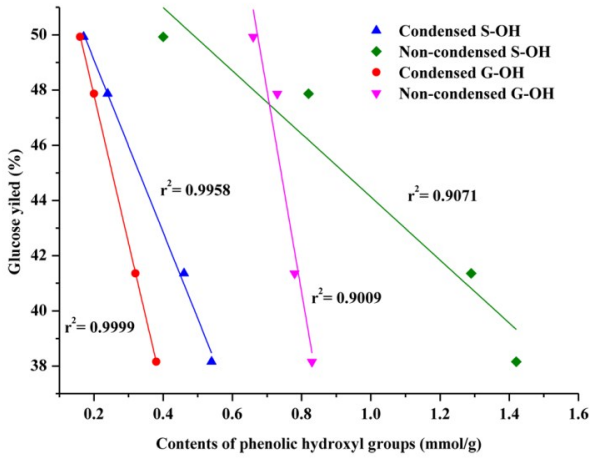

Fig. 6 Correlation between phenolic hydroxyl group content in lignins and the corresponding 72 hydrolysis yield of Avicel.

Higher pretreatment temperature resulted in higher degree of condensation from 0.58 (MWL) to 0.69, 0.96 and 0.99 in 150-MWL, 170-MWL and 190-MWL, respectively. It was observed that the 72 hydrolysis yield decreased with the increase of DC (r^2=0.946). Condensation can bring more aromatic ring close to each other and increase the hydrophobic interactions between the enzymes and lignin, which can lead to more non-productive binding.

As mentioned previously, the carboxylic acid group content decreased from 0.24 mmol/g in MWL to 0.22, 0.20 and 0.18 mmol/g in 150-MWL, 170-MWL and 190-MWL, respectively. A good positive correlation (r^2=0.945) was observed between the carboxylic acid group content and the 72 h hydrolysis yield. This agreed well previous study, which suggested that the presence of carboxylic acids would reduce the negative effects of the isolated lignin on enzymatic hydrolysis.[11] The decrease of carboxylic acid content in lignin will decrease the hydrophilicity of lignin and increase the hydrophobic interactions between the enzymes and lignin. It should be mentioned that the hydrophobic interaction could also be related to the surface area and particle size of the isolated lignins. Especially, the results showed that pretreatment severity changed the size and the shape of the isolated lignins dramatically (ESI, Table S1 and Fig. S1†). Also, the surface properties of the isolated lignins could be different from the residual lignins in biomass. However, the chemical and functional groups identification in isolated lignin will help to improve the understanding of the non-productive

binding between the enzymes and the residual lignin in plant cell wall.

Conclusions

A strong association between the condensed phenolic moieties in the isolated lignins and their inhibition of enzymatic hydrolysis was observed. The functional groups of isolated lignins from hydrothermal pretreatment and their inhibitory effects on enzyme hydrolysis were quantified. Quantitative ^{13}C, HSQC and ^{31}P NMR were used to characterize the effects of pretreatment severity on the functional groups changes in the isolated lignins. It was observed that the increase of pretreatment severity dramatically increased the condensed and non-condensed syringyl and guaiacyl OH groups in isolated lignin. A compelling adverse association was found between the condensed syringyl and guaiacyl phenolic OH group contents and the 72 h hydrolysis yields. The aliphatic hydroxyl, PB hydroxyl and carboxylic hydroxyl group contents decreased as the pretreatment increased, and they were in the positive correlation with the 72 h hydrolysis yield. This suggested that the total OH groups (including aliphatic OH, PB-OH and carboxylic OH) or phenolic OH groups (including PB-OH) probably concealed the true correlation between the condensed syringyl and guaiacyl phenolic groups and the inhibitory effect of lignins. In this study, the hydrophobic interactions and hydrogen bonding most likely played more important role in non-productive binding between the isolated lignins and the enzymes. We believe that the condensed syringyl and or guaiacyl phenolic units can generate synergistic inhibition on enzymatic hydrolysis, in which the condensed aromatic rings enhance the hydrophobic interaction and the syringyl/guaiacyl phenolic OH groups boost the hydrogen bonding.

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