Bioresource Technology 154 (2014) 274–281

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Isolation and structural characterization of sugarcane bagasse lignin after dilute phosphoric acid plus steam explosion pretreatment and its effect on cellulose hydrolysis

Jijiao Zeng [a], Zhaohui Tong [a,*], Letian Wang [a], J.Y. Zhu [b], Lonnie Ingram [c]

[a] Department of Agriculture & Biological Engineering, IFAS, University of Florida, Gainesville, FL 32611, USA
[b] USDA Forest Service, Forest Products Laboratory, One Gifford Pinchot Dr., Madison, WI 53726, USA
[c] Department of Microbiology & Cell Science, University of Florida, PO Box 110700, Gainesville, FL 32606, USA

HIGHLIGHTS

- The structure of lignin after phosphoric pretreatment was elucidated.
- Lignin reserved basic structure and molecular weight after pretreatment.
- Lignin after pretreatment contained relatively lower β-O-4 linkages.
- The removal of ethanol extracted lignin increased ~22% glucose yield.

ARTICLE INFO

Article history:
Received 22 October 2013
Received in revised form 12 December 2013
Accepted 14 December 2013
Available online 23 December 2013

Keywords:
Lignin structure
Soluble lignin
Nuclear magnetic resonance (NMR)
Cellulose hydrolysis

ABSTRACT

The structure of lignin after dilute phosphoric acid plus steam explosion pretreatment process of sugarcane bagasse in a pilot scale and the effect of the lignin extracted by ethanol on subsequent cellulose hydrolysis were investigated. The lignin structural changes caused by pretreatment were identified using advanced nondestructive techniques such as gel permeation chromatography (GPC), quantitative ^{13}C, and 2-D nuclear magnetic resonance (NMR). The structural analysis revealed that ethanol extractable lignin preserved basic lignin structure, but had relatively lower amount of β-O-4 linkages, syringyl/guaiacyl units ratio (S/G), *p*-coumarate/ferulate ratio, and other ending structures. The results also indicated that approximately 8% of mass weight was extracted by pure ethanol. The bagasse after ethanol extraction had an approximate 22% higher glucose yield after enzyme hydrolysis compared to pretreated bagasse without extraction.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Lignocellulosic biomass, including agricultural waste, energy crops, and wood waste, is a renewable resource with potential for the production of fuels and commodity chemicals (Himmel et al., 2007; Ragauskas et al., 2006). However, the presence of lignin, which tightly linked with polysaccharides (cellulose and hemicellulose) in the cell wall, limited the accessibility of chemicals or enzymes during hydrolysis processes (Himmel et al., 2007). A number of thermochemical pretreatment approaches coupled with enzymatic hydrolysis have been developed to effectively remove lignin to hydrolyze polysaccharides into monomer sugars (Chundawat et al., 2011a).

Recently, a promising process has been developed to produce ethanol in a high yield (310–348 L/dry ton) in a pilot scale (80-L fermentor) including a diluted phosphoric acid plus steam explosion pretreatment followed by a liquefaction plus a simultaneous saccharification and co-fermentation process (L plus SScF) (Geddes et al., 2010a,b). A mild pretreatment condition in combination with a hydrolysate-resistant strain *Escherichia coli* (Geddes et al., 2011) could effectively diminish the adverse effect of inhibitors to the following L plus SScF process (Geddes et al., 2011).

Lignin is a cross-linked aromatic polymer composed of hydroxyphenyl, guaiacyl, and syringyl units (Kim et al., 2008). The structure of lignin dramatically affects the efficiency of thermochemical pretreatment process, and hence the knowledge of lignin structure is important to develop an appropriate pretreatment method to remove lignin. Therefore, multiple advanced techniques were used to study structural information of variety lignin from biomass pretreatment, such as acid treated lignin (Samuel et al., 2010), organosolv lignin (Bozell et al., 2011), ionic liquid lignin (Wen et al., 2012), and ammonia treated lignin (Chundawat et al., 2011b). For example, evidences from NMR analysis indicated

* Corresponding author. Tel.: +1 3523921864; fax: +1 3523924902.
E-mail address: ztong@ufl.edu (Z. Tong).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.12.072

that ammonia fiber expansion (AFEX) extensively cleaved *p*-coumarate and ferulate ester to form phenolic amides while preserved lignin polymers (Chundawat et al., 2011b). Hot-water flow through pretreatment severely hydrolyzed β-O-4 inter-unit linkages as well as hydroxycinnamate ester, in conjunction with the preferential removal of S-lignin (Laskar et al., 2013). The effects of ionic liquid pretreatment also cleaved β-O-4 leading to depolymerization of birch wood's lignin (Wen et al., 2012).

The features of lignin after pretreatment can adversely affect enzymatic hydrolysis as well. A comparative analysis of twelve isolated lignin samples confirmed the detrimental effects of residual lignin on the hydrolysis of pretreated lignocellulosic substrates (Nakagame et al., 2010). The hydrophobicity of lignin was identified as a major effect on non-productive binding to cellulase (Tu et al., 2009). Selig et al. (2007) investigated the depolymerization process of lignin during acid pretreatment and suggested that some loosened and modified lignin fragments have been proved to have adverse influence on cellulase digestion which was usually ignored. It was also reported that the increase in pretreatment severity in SO_2-steam explosion pretreatment of Douglas-fir, the higher hydrolysis yield was obtained, which was more related with accessible surface areas of cellulose influenced by lignin but not lignin–enzyme interaction (Nakagame et al., 2011). Recently, lignosulfonate derived from sulfite pulping or sulfite pretreatment (Zhu et al., 2009) was found to have low affinity to cellulase. Furthermore, lignosulfonate also acts as a surfactant to enhance enzymatic hydrolysis (Zhou et al., 2013).

To date, the structure of lignin pretreated by a mild phosphoric acid plus steam explosion process has not been well studied. Therefore, ethanol or ethanol/water extraction step was applied to the pretreated sugarcane bagasse to isolate ethanol soluble lignin after pretreatment and the effect of this extraction step on the subsequent glucose yield was investigated. The coarse ethanol extracted lignin was further purified to ethanol dissolved lignin (EL), water soluble lignin (WSL), and dioxane dissolved lignin (DL). These three lignin and ball milled bagasse lignin (MBL) were characterized by combinatory analysis of quantitative ^{13}C, HSQC NMR, and GPC. The structural information is expected to depict the deconstruction mechanism of this pretreatment method on the cell wall of sugarcane bagasse.

2. Methods

2.1. Materials

Sugarcane bagasse was provided by the Florida Crystals Corporation (Okeelanta, FL) without any further size reduction. Novozymes CTec2 cocktail was provided by Novozymes company. All chemicals were purchased from Sigma–Aldrich.

2.2. Lignin isolation

Lignin was isolated following the procedure described in Fig. 1. The pretreated sugarcane bagasse was collected from the University of Florida cellulosic bioethanol pilot plant. The bagasse was pretreated by soaking in dilute (0.5% v/v) phosphoric acid for 4 h followed by steam explosion at 180 °C for 10 min. Pretreated biomass was extracted twice by 100% ethanol under shaking at room temperature for 4 h with a solid loading of 0.2 g/mL. The supernatant was collected, combined, filtrated and then evaporated in a rotary evaporator in order to obtain crude ethanol-extractable lignin. This lignin was further purified by dissolving in an acetic acid/water solution (9:1 v/v) followed by the precipitation in 10 times volume of cold deionized water. The solid residues were freeze-dried, dissolved in dichloride ethane/ethanol solution (2:1 v/v), precipitated and washed in ethyl ether and freeze-dried again to obtain a purified ethanol dissolved lignin (EL). The soluble part was also freeze-dried and collected as a water soluble lignin (WSL). The solid residues after ethanol extraction were air dried, extracted by dioxane/water solution (96:4 v/v) for 48 h at room temperature and purified using the same procedures described above. The obtained dioxane-dissolved lignin (DL) was used for further characterization. According to the preparation procedure, EL and WSL represents the fragmented lignin. DL theoretically represents the lignin after pretreatment excluding fragmented lignin that could dissolved in ethanol or water. Each purified lignin was carefully weighted to calculate the yield of purified lignin from these two procedures. Ball milled bagasse lignin was prepared according to previous work (Zeng et al., 2013). Prior to lignin extraction, sample was milled (6 h) to 200–500 nm in a Retsch planetary ball mill PM 100 with zirconium dioxide balls at 300 rpm. The ball mill was changed direction every 3 min with a 1-min interval.

The chemical composition of the raw bagasse, the pretreated bagasse, the bagasse residues after ethanol extraction and bagasse residues after dioxane extraction were determined according to a National Renewable Energy Laboratory (NREL) method (Sluiter et al., 2008). The monomer sugar contents after cellulose hydrolysis were measured by a high performance liquid chromatography (Agilent Technologies HPLC 1200 series, Santa Clara, CA, USA) equipped with a BioRad Aminex HPX-87H column (Hercules, CA, USA). The acid soluble lignin was determined by a UV–Vis spectroscopy (Beckman DU800 UV/Vis Spectrophotometer, Brea, CA, USA). Both the acid insoluble lignin and ash contents were obtained by gravimetric analysis.

2.3. Extractive analysis

The extractives of the pretreated bagasse after washing by pure water, pure ethanol, and the water/ethanol solutions with (6.3%, 12.3%, 27.2%, 55.9%, 79.2% mass ratio) were obtained at a 20% w/v solid loading, 25 °C, and 160 rpm. Supernatant was collected and filtrated after 2 h. The extractives were finally dried overnight at 105 °C and the weight of each extractive was measured.

2.4. Enzymatic hydrolysis

To investigate the effects of ethanol extraction steps on enzymatic hydrolysis of the pretreated bagasse, the pretreated bagasse after washed by water and pure ethanol were hydrolyzed using Novozymes CTec2. Citric acid buffer (50 mM; pH 4.8) was used to suspend biomass (2% w/v loading) and CTec2 (0.75 mL/g). Flasks were incubated at 45 °C and 120 rpm in an incubator shaker for up to 108 h. The monomer sugars were analyzed according to previous report (Geddes et al., 2010a).

2.5. NMR analysis

For each sample, 50 mg of extracted lignin was dissolved in 500 μL of 99.9% DMSO-d6 with 0.5% v/v tetramethylsilane as internal standard. For quantitative ^{13}C NMR experiment, 0.01 M chromium (III) acetylacetonate was added to reduce the T_1 relaxation times of carbon signals. The quantitative ^{13}C NMR experiment was carried out in Bruker Avance 600 MHz at 27 °C with the following parameters: 90° pulse width, 0.8 s acquisition time, 2.2 s relaxation delay, and 20,000 scans. The heteronuclear single quantum coherence (HSQC) NMR spectra were recorded on a Bruker Avance II 600 MHz equipped with a 5 mm TXI cryoprobe at 27 °C. The spectral width was 7211.5 Hz for ^{1}H and 31,698 Hz for ^{13}C. The number of scans was 32 with a 512 time increment. The relaxation delay was 0.43 s and the acquisition time was 0.07 s. Interactive integrations of the peaks

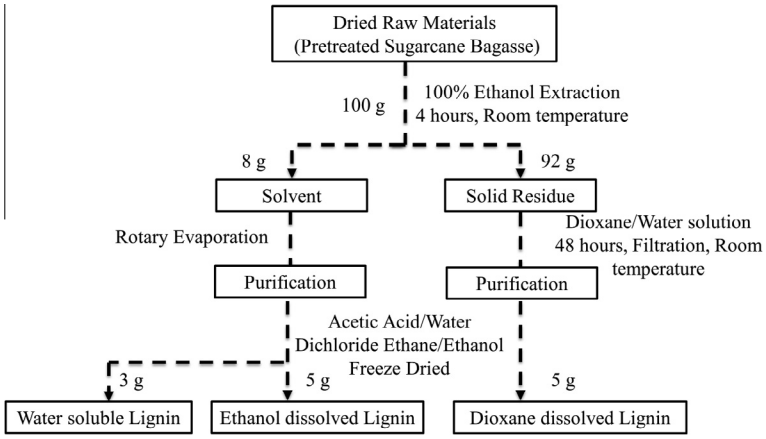

Fig. 1. Lignin isolation procedure and its material balance.

in $^{13}C/^{1}H$ spectrum and contours in 2D HSQC plots were measured using a MestReNova software. To effectively avoid T_2 relaxations, resonance offsets, coupling constant deviations, homonulcear couplings caused by heterogeneous structure of lignin, three independent regions from ^{13}C/HSQC NMR: (1) δ_C/δ_H 113-109/7.6-6.8 ppm, (2) δ_C/δ_H 88-82/5.6-3.9 ppm, and (3) δ_C/δ_H 66-58/4.5-3.0 ppm were selected as internal references to correct quantitative value of lignin units and inter-unit linkages. The detailed calculated method was based on previous report (Zeng et al., 2013).

2.6. Gel permeation chromatography (GPC) analysis

The weight-average (M_w) and number-average (M_n) molecular weights of the lignin were determined by GPC on a 1260 infinity HPLC system (Agilent Technology) with a refractive index detector (RI-G1362A) and a multiple wavelength detector (MWD) (CA, USA). Three columns were connected in series including PLgel mixed B, PLgel mixed E 5 μm with a pore size of 10,000 Å, and PLgel mixed E 5 μm with a pore size of 100 Å. A set of polystyrene standard was used for calibration. Two micrograms of lignin was dissolved in 1 mL of tetrahydrofuran (THF) and 10 μL of filtered solution in each run was injected at 25 °C. All results were processed using the chemstation software package with GPC analysis (Rev.B.04.03).

2.7. Statistical analysis

One way analysis of variance (ANOVA) was used to analyze the extractive yields and the glucose yields after enzyme hydrolysis. All pairwise multiple comparison procedures were based on the Holm-Sidak method and under a 95% confidence interval ($\alpha = 0.05$) in SigmaPlot 12. Error bar represented standard deviation.

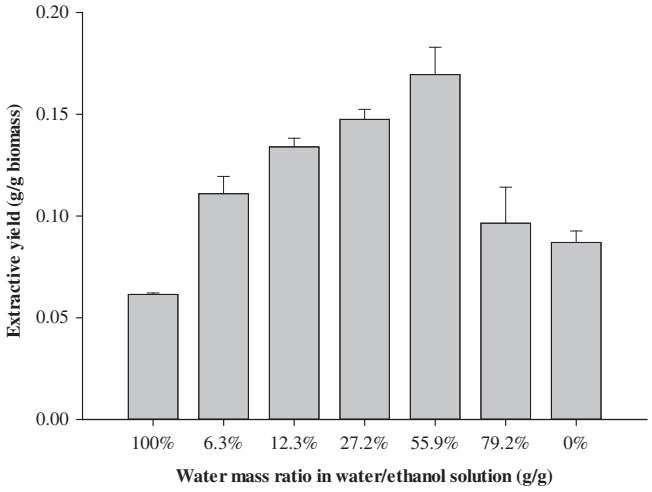

Fig. 2. The yields of the extractives from the pretreated bagasse at different water/ethanol ratios (g/g).

3. Results and discussion

3.1. Composition analysis, and material balance of the extracted lignin fragments isolated from the pretreated sugarcane bagasse

Different types of lignin (EL, DL, and WSL) were isolated from the pretreated sugarcane bagasse as shown in Fig. 1. Approximately 8% (based on the dry weight of the pretreated bagasse) substances were extracted by a pure ethanol solution. The ethanol

Table 1
Chemical compositions of the raw bagasse, the pretreated bagasse, and the pretreated bagasse after ethanol and dioxane extraction.[c]

	Glucan%	Xylan%	Arabinan%	Galactan%	Acetic acid%	Lignin%		Ash%
						AI[a]	ASL[b]	
Raw bagasse	40.57 ± 1.97	16.75 ± 4.31	4.84 ± 0.5	5.92 ± 0.97	1.52 ± 0.22	25.93 ± 0.33	4.69 ± 0.27	2.34 ± 0.17
Pretreated bagasse	51.42 ± 1.40	10.54 ± 0.45	0.84 ± 0.04	4.80 ± 0.34	0	31.44 ± 2.12	3.94 ± 0.57	0.43 ± 0.27
Water extracted bagasse	54.03 ± 0.41	9.60 ± 0.53	0.28 ± 0.20	2.68 ± 0.28	0	31.82 ± 0.40	3.89 ± 0.08	0.37 ± 0.27
Ethanol extracted bagasse	51.72 ± 2.98	10.91 ± 0.79	1.10 ± 0.08	5.14 ± 0.60	0	27.80 ± 1.25	3.44 ± 0.36	0.79 ± 0.13
Dioxane extracted bagasse	65.16 ± 0.76	8.05 ± 0.88	0.79 ± 0.12	4.86 ± 0.40	0	19.92 ± 0.28	3.26 ± 0.27	0.74 ± 0.22

[a] Acid insoluble lignin.
[b] Acid soluble lignin.
[c] The data is based on triplicate experiments.

Table 2
Molecular weight analysis of MBL, EL, WSL, DL, water-only extractive, and ethanol-only extractive (mol/g).

	M_n	M_w	PDI
MBL	1673	3176	1.9
EL	1811	3731	2.1
DL	884	3972	4.5
WSL	207	507	2.4
Water-only extractive	161	302	1.9
Ethanol-only extractive	658	1030	1.6

extractives contained approximately 62.5% of EL and 32.5% WSL. Approximately 5% of DL (based on the dry weight of the pretreated bagasse) was obtained by further isolating the solid residue after ethanol extraction by a dioxane/water extraction. The chemical compositions of the raw bagasse, the pretreated bagasse, and the pretreated bagasse after ethanol and dioxane extraction were listed in Table 1. The contents of xylan and arabinan, main constitution of hemicellulose, were decreased from approximately 22% in the raw bagasse to 11% in the pretreated bagasse. This is because the mild phosphoric acid plus steam explosion pretreatment significantly hydrolyzed hemicellulose to the monomer sugars and furan aldehydes (Geddes et al., 2010a,b). The results also show that pretreatment did not greatly depolymerize cellulose macromolecules. Ethanol extraction removed approximately 12% of Klason lignin from pretreated bagasse and further dioxane/water solution removed 26% of Klason lignin from the ethanol-extracted bagasse residues. As we know, ethanol has been widely used in an organosolv process for the ethanolysis of lignin at high temperature and high pressure condition (Pan et al., 2006; Zhao et al., 2009). However, in this study, ethanol extraction occurred at room temperature and atmospheric pressure. The removal of a large amount of Klason lignin (approximately 12%) by ethanol was attributed to the pretreatment process prior to ethanol extraction. Herbaceous crops form lignin–carbohydrate complex (LCC) through ferulate (FA) which bridges lignin by β-X, 4-O-X or 5-X structure and links hemicellulose by ester bonds (Grabber et al., 1995; Ralph et al., 1995). The detachment of hemicellulose from biomass undoubtedly led to the cleavage of bonds of LCC (Chundawat et al., 2011a). The release of lignin in the bulk liquid phase may re-deposit on the residual surfaces as spherical droplets (Selig et al., 2007). These lignin fragments perhaps are vulnerable to ethanol at ambient conditions.

In order to maximize the extractive yield from the pretreated bagasse, the effects of the ratios of added water in ethanol on the yields of extractives were investigated (Fig. 2). Pure ethanol solution yielded 0.087 ± 0.006 g/g extractive (based on the dry weight of the pretreated bagasse) which was 40% higher than the yield by pure water extraction. However, these two yields exhibit no significant difference statistically ($p > 0.05$). The results also show that the addition of water into ethanol solution (even 5% v/v), significantly improved the extractive yield compared to that by pure ethanol or pure water solution ($p < 0.05$). The maximum extractive

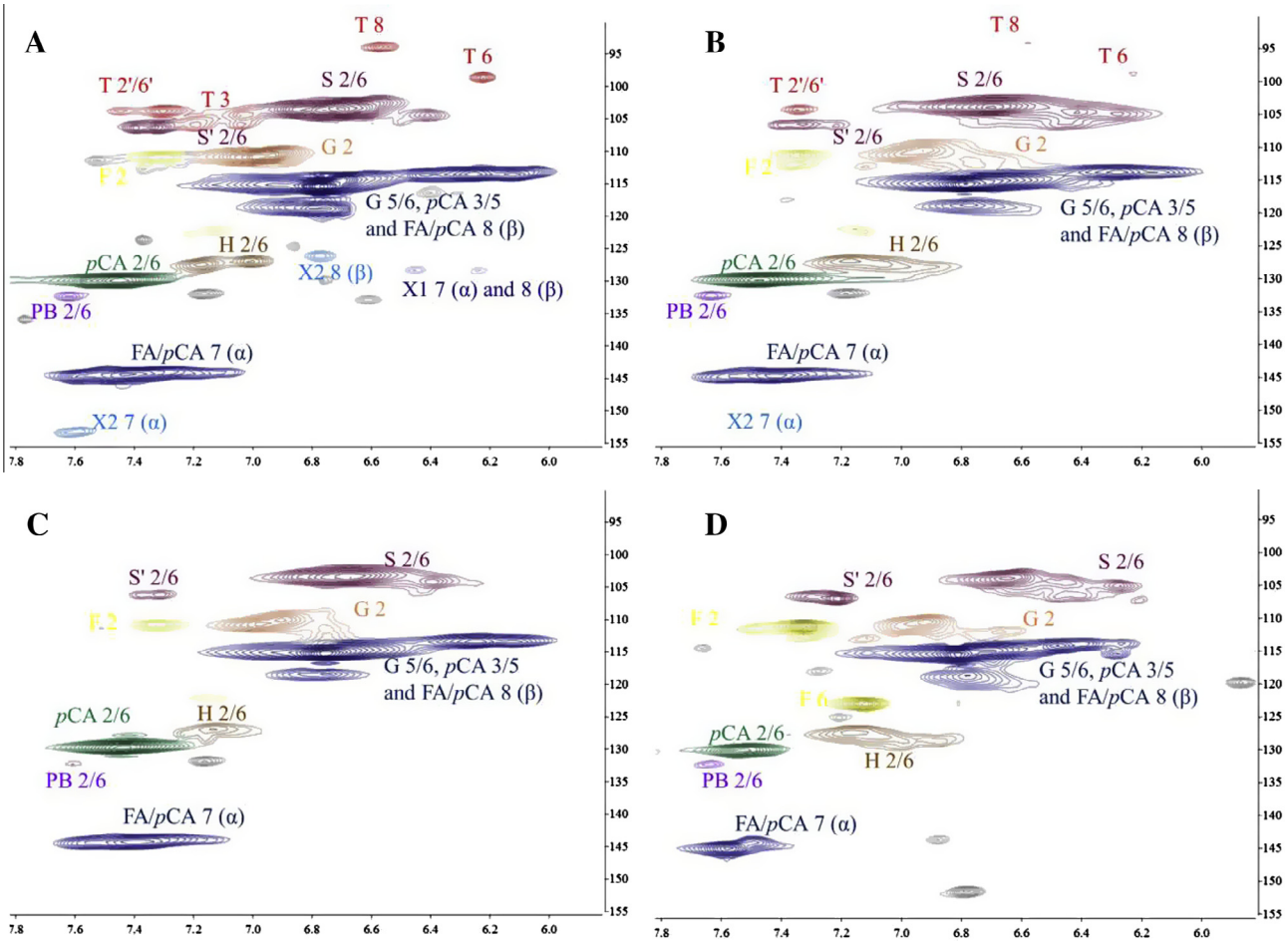

Fig. 3. The aromatic region of extracted lignins (A) MBL, (B) EL, (C) DL and (D) WSL in HSQC spectra. T: Tricin; S: Syringyl units; S′: Syringyl units with α-oxidization. G: Guaiacyl units. H: Hydroxyphenyl units; F: Ferulate; *p*CA: *p*-Coumarate; PB: *p*-Hydroxybenzoate; X1: Cinnamyl alcohol; X2: Cinnamyl aldehyde.

Table 3
Aromatic moieties (lignin, cinnamates, lignin ending groups) by combination analysis of ^{13}C and HSQC NMR spectra of isolated lignin including MBL, EL, WSL, and DL.[a]

Aromatic moieties	σ_C/σ_H (ppm) and assignments	MBL (100 Ar)	EL (100 Ar)	WSL (100 Ar)	DL (100 Ar)
Lignin aromatic units					
Syringyl	103.9/6.7 (S2/6), 106.4/7.4(S′2/6 with α oxidization)	32.3	38.3	29.3	34.2
Guaiacyl	110.8/6.97 (G2), 115.0/6.94 (G5),118.9/6.81 (G6)	22.8	20.0	17.8	23.8
p-Hydroxyphenyl	127.7/7.21 (H2/6)	3.2	7.3	7.6	4.2
S/G ratio		1.4	1.9	1.6	1.4
Hydroxycinnamates					
p-Hydroxybenzoate	132.4/7.63 (PB2/6)	0.5	0.8	0	0
p-Coumarate	130.0/7.47 (*p*CA2/6),115.5/6.79 (*p*CA3/5), 144.74/7.47	29.5	24.7	19.8	30.2
Ferulate	110.9/7.32 (FA2), 122.5/7.15 (FA6)	3.0	5.5	23.4	4.9
p-Coumarate/ferulate		9.8	4.5	1.9	6.2
Cinnamates/lignin and tricin		0.5	0.5	1.3	0.6
Ending group					
Cinnamyl aldehyde	153.5/7.62 (X2α), 126.2/6.78 (X2β)	1.2	ND	ND	ND
Cinnamyl alcohol	128.34/6.44 (X1α), 128.35/6.22 (X1β)	0.5	ND	ND	ND
Lignan					
Tricin	104.04/7.30 (T′2/6), 104.65/7.03 (T3), 94.1/6.56 (T8), 98.8/6.22 (T6)	1.9	0.9	0	0
Inter-unit bondages					
α-OH/*β*-O-4	71.1/4.74(Aα-G), 71.8/4.8 (Aα-S)	30.5	14.1	0	16.7
α-keto/*β*-O-4	82.7/5.1 (Aα-keto)	1.2	3.6	0	4.0
A-H,S,G/*β*-O-4	82–88/4.5-3.9	25.3	6.0	0	12.7
Total *β*-O-4		25.3–31.7	9.6–17.7	0	16.7–20.7
Phenylcoumaran	85.9/5.5(Bα), 53.0/3.4(Bβ)	3.3	4.8	0	2.3
Resinols	84.8/4.7(Cα), 53.5/3.1(Cβ),	1.7	0	0	0
Dibenzodioxocin	83.4/4.9(Dα), 85.5/3.8(Dβ)	2.7	9.2	0	4.0
SD	81.4/5.0 (SDα), 59.5/2.8(SDβ)	1.4	0	0	0
α,β-Diaryl ether	79.6/5.5(Fα)	0.9	0	0	0
Acylation on γ position	60.8-58.8/3.8-3.35	10.6	13.2	11.9	11.7
Functional groups					
CHO and α-CO in *β*-O-4 structure	200-190	3.4	1.3	3.4	5.1
Non-conjugated COOR	175-168	6.4	3.5	12.4	6.5
Conjugated COOR	168-166	24.5	24.5	16.1	26.4
Methoxyl group	57.5-54	108.0	101.3	84.4	100.1

[a] Amount of specific moieties was expressed as number per 100 Ar.

yield (0.169 ± 0.013 g/g) was obtained at 55.9% (g/g) water/ethanol solution.

3.2. Molecular weight analysis

The molecular weights of different lignin including MBL, EL, WSL, DL, and the crude extractives extracted by pure water and pure ethanol solution were investigated by GPC. Ethanol soluble lignin (EL) had a similar molecular weight (M_w: 3731 mol/g) and molecular weight distribution with those of MBL (Table 2). DL had the highest weight average molecular weight (M_w: 3973 mol/g) and a higher polydispersity index (PDI = 4.5). The results approved that lignin fragments dissolved in the ethanol solution as polymers rather monomers. WSL had the lowest molecular weight (M_w: 507 mol/g), which suggested that the water-soluble lignin was mainly composed of monomers or dimers. The molecular weight of the crude extractive from pure ethanol solution extraction (Mw: 1030 mol/g) was between that of EL and WSL, which further indicated that water only dissolved the monomer or dimer fragments.

3.3. NMR analysis

In order to characterize chemical features of lignin, syringyl units (S), guaiacyl units (G), hydroxyphenyl units (H), hydroxycinnamates, tricin (T), various inter-unit structures and main functional groups of isolated MBL, EL, DL and WSL were quantified by combinatory analysis of ^{13}C NMR and 2D HSQC NMR (Zeng et al., 2013). The major aromatic compositions of isolated lignin and their corresponding values were showed in Fig. 3 and Table 3. MBL isolated from untreated sugarcane bagasse was constituted by S, G, H, T units, and other phenolic units including *p*-coumarate (*p*CA), ferulate (FA), *p*-hydroxybenzoate (PB), cinnamyl alcohol (X1), and cinnamyl aldehyde (X2), which was consistent with typical features of most herbaceous lignin (Buranov and Mazza, 2008; Kim et al., 2008). The ^{13}C–^{1}H correlation for S2/6 and α-oxidized S2/6 was found at δ_C/δ_H 104.0/6.70 ppm and δ_C/δ_H 106.4/7.4 ppm, respectively. The peaks of δ_C/δ_H 110.8/6.97 ppm, δ_C/δ_H 110.9/7.32 ppm, δ_C/δ_H 130.0/7.47 ppm, and δ_C/δ_H 132.4/7.63 ppm were assigned to ^{13}C–^{1}H correlations of G2, FA2, *p*CA2/6, and PB2/6. Signals from α and β of cinnamyl alcohol end-groups (X1) and cinnamyl aldehyde end-groups (X2) were separately distributed at δ_C/δ_H 128.3/6.44 ppm (X1α), 128.3/6.22 ppm (X1β), 153.5/7.62 ppm (X2α), and 126.2/6.78 ppm (X2β) in HSQC spectrum of MBL. However, the correlations of α and β of FA and *p*CA strongly overlapped. These two components were not able to be differentiated by NMR analysis. The quantification analysis indicated three main lignin units in sugarcane bagasse: S, G, and H. S is the predominant unit with 32.3 units per 100 aromatic rings of MBL, followed by 22.8 G units and 3.2 H units. The lignin unit distribution was different with that in wheat straw (del Río et al., 2012), but consisted with that in corn stalk (Kim et al., 2008). Approximately 2 units of T were detected in MBL of sugarcane bagasse. As previously reported in wheat straw lignin, tricin was linked with lignin complex by *β*-O-4 linkages and contributed to 10% of total lignin composition (del Río et al., 2012; Zeng et al., 2013). Regarding hydroxycinnamates, the content of *p*CA reached to 29.5 units per 100 aromatic rings compared to a small quantity of FA, PB, X1, and X2 in MBL. The pCA came from the lignification of *p*-coumarolyated monolignols through the formation of ester bonds at γ-OH (Ralph and Landucci, 2010).

Lignin compositions of MBL, EL, WSL, and DL clearly indicated that dilute phosphoric acid plus steam explosion pretreatment significantly degraded and/or fragmented native lignin (Table 3). DL

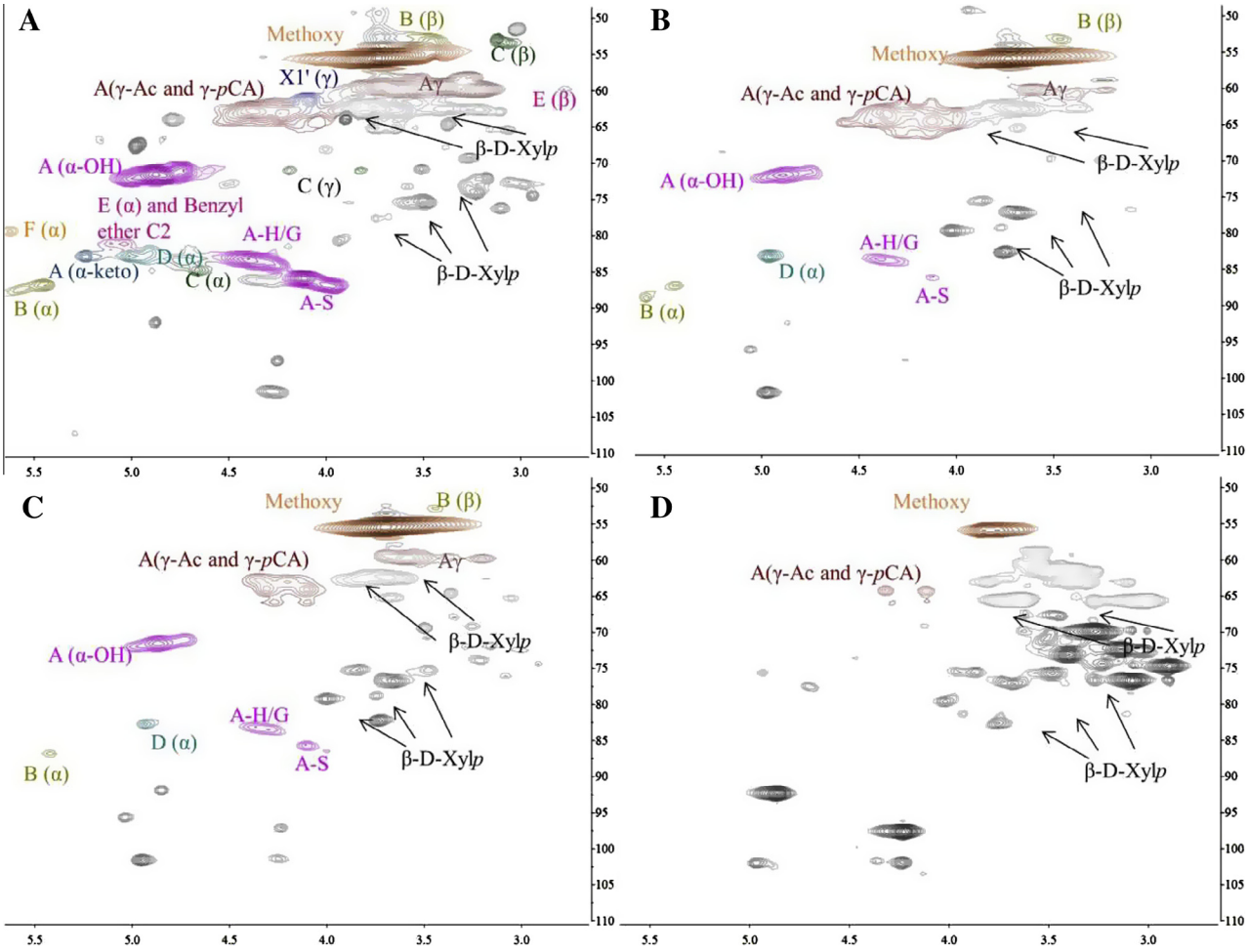

Fig. 4. The aliphatic-oxygenated region of extracted lignin (A) MBL, (B) EL, (C) DL and (D) WSL in HSQC spectra. A (α-OH): G and S units in β-O-4 structure with α-OH; A (α-keto): G and S units in β-O-4 structure with α oxidization; A-H/G and A-S: H, G and S units in β-O-4 structure; A (γ-Ac and γ-*p*CA): Acetate and *p*-coumarate acylated at γ-OH in β-O-4 structure; B (α) and B (β): Phenylcoumaran; C (α) and C (β): Resinols; D: Dibenzodioxocin; E: Spirodienone; F: α,β-diaryl ether.

had a higher amount of S and G units than corresponding values of MBL, which may be due to the loss of T, PB or other unidentified compounds in DL. EL and WSL contained a relatively larger amount of S/G ratio and H units but had a relatively smaller amount of *p*CA/FA ratio. It was known that ethanol could not extract lignin from untreated sugarcane bagasse at ambient conditions, therefore, EL and WSL were the fragmented lignin extracted from pretreatment. The decreased amount of *p*CA of EL and WSL indicated partial cleavage of ester bonds. Special attention was paid to the enhanced S/G ratios of EL and WSL. Zhou et al. (2011) reported that S-unit lignin preferentially located in inner cell wall area. It could be suggested that dilute phosphoric acid plus steam explosion deliginified lignin which was mainly located at secondary cell wall, the middle layer of inner cell wall structure. In fact, preferential removal of S units was not limited to this pretreatment. Many researchers reported that dilute acid pretreatment resulted in the reduction of S-unit lignin content in the pretreated biomass (Cao et al., 2012; Jung et al., 2010). The possible explanation is that the S-unit lignin has linear arrangement linked solely by β-O-4, which is easier to be hydrolyzed compared to other bonds (Studer et al., 2011). WSL was comprised of a significant amount of FA in its HSQC spectrum (Fig. 3), which suggested that the acetic acid–water mixture partially removed lignin–carbohydrate complex(LCC) from crude ethanol lignin (Iiyama et al., 1990). The existing of FA in WSL also indicated that LCC from sugarcane bagasse was mainly linked by FA. This indicated that pretreatment cleaved LCC and released lignin fragment, which was then easily extracted by ethanol.

Inter-units including β-O-4, β-5, β-β′, β-1, 5-5′/4-O-β′ as well as LCC bonds of MBL, EL, WSL, and DL were shown in aliphatic-oxygenated region of HSQC spectrum in Fig. 4. The β-O-4 (A) linkage

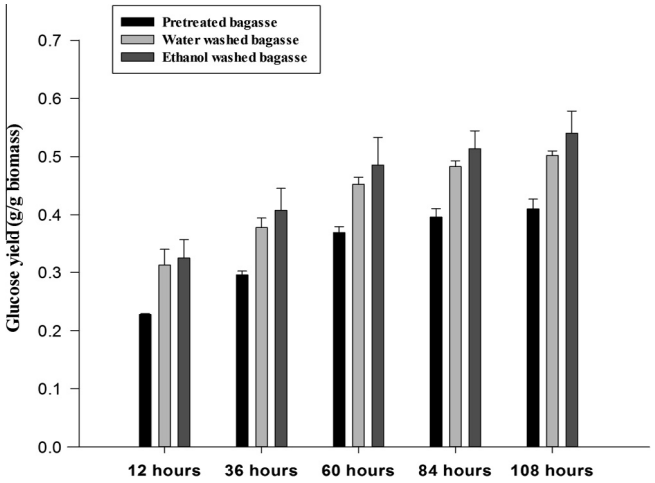

Fig. 5. Effects of different biomass preparation processes on enzymatic glucose yields at 12, 36, 60, 84, and 108 h incubation.

is a good indicator to understand the behavior of lignin complex during the pretreatment process since lignin is mainly linked by β-O-4. The signals of δ_C/δ_H 71.1/4.74 ppm, δ_C/δ_H 71.8/4.8 ppm, δ_C/δ_H 82–88/4.5-3.9 ppm, and δ_C/δ_H 82.7/5.1 ppm were ascribed to the correlation of α position of Aα-OH, β position of A-H/G, S, and α position of Aα-keto (Kim et al., 2008) in β-O-4 structure. Phenylcoumaran (B) was the secondary abundant inter-unit (β-5) formed by coupling available 5 position of lignin units with monolignol' β position. The contours of δ_C/δ_H 85.9/5.5 ppm and δ_C/δ_H 53.0/3.4 ppm were assigned to the correlations of lignin α and β position. In addition to A and B inter-units, resinols (C) with a β-β′ structure was observed at δ_C/δ_H 84.8/4.7 ppm (Cα), δ_C/δ_H 53.5/3.1 ppm (Cβ), δ_C/δ_H 71.5/4.2 ppm(Cγ), and δ_C/δ_H 71.5/3.8 ppm (Cγ). Small quantity of correlation signals from Dibenzodioxocin (D), Spirodienone (E) and α,β-diaryl ether (F) were detected at δ_C/δ_H 83.4/4.9 ppm, δ_C/δ_H 85.5/3.8 ppm, and δ_C/δ_H 79.6/5.5 ppm, respectively. The contours around δ_C/δ_H 65-62/4.5-4 ppm were assigned to the acylation at γ-OH by *p*CA and acetate. The γ-OH in X1 structure was detected at δ_C/δ_H 62-58/3.8-3.0 ppm.

The quantificational results of inter-unit linkages showed that MBL contained 30.5 units of Aα-OH, 1.2 units of Aα-keto, and 25.5 units of A-H/G, S (Table 3). In general, the quantity of A-H/G, S is equal to the quantity of Aα-OH. The difference between A-H/G, S and Aα-OH maybe due to the missing signal of γ-acylated β-O-4, which maybe exist but was not counted in this calculation (del Río et al., 2012). Therefore, total amount of the β-O-4 linkages was represented as the range of A-H/G, S plus Aα-keto (minimal) to Aα-OH plus Aα-keto (maximal) that was 25.3–31.7 units per 100 aromatic rings. As shown in the aliphatic regions of the isolated lignin (Fig. 4 and Table 3), EL and DL contained lower intensities of almost all inter-units except γ-acylation, B, and D. The quantities of β-O-4 were only 9.6–17.7 units for EL and 16.7–20.7 for DL. It is known that acid results in the condensation and cleavage of aryl ether linkages (Yokoyama and Matsumoto, 2010). The reduction of inter-units indicated that phosphoric acid plus steam explosion pretreatment broke down β-O-4 and α,β-diaryl ether. However, the content of B and D structure was stable or even increased in EL and DL. There was no substantial data to support this, but it is possible to conclude that these two structures are resistant to acid or thermal attack. Regarding WSL, there was no any signal derived from β-O-4 or other inter-units in the HSQC spectrum. The absent of inter-units implied that WSL was only a mixture of monomer lignin rather than polymer complex, which is consistent with the GPC results (Table 2).

3.4. Enzymatic hydrolysis of ethanol washed bagasse

The residual lignin associated with cellulose after pretreatment has significant influence on subsequent enzymatic hydrolysis (Selig et al., 2007; Studer et al., 2011). Therefore, it is reasonable to speculate that the removal of EL could enhance the efficiency of enzymatic hydrolysis. The glucose yields after enzyme digestion using the pretreated bagasse, water-washed pretreated bagasse, and ethanol-washed pretreated bagasse as the starting materials were shown in Fig. 5. It was observed that the glucose yields of all samples were generally increased with incubation time. After 108-h incubation, the glucose yield was 0.54 ± 0.038% for the ethanol-washed bagasse, 0.50 ± 0.007% for the water-washed bagasse, and 0.41 ± 0.017% for the pretreated bagasse, respectively. The glucose yield of the ethanol-washed bagasse was enhanced ~22% compared to that of the pretreated bagasse ($p < 0.05$) with the equivalent cellulase loading as they have very similar glucan content (Table 1), while there was only slightly different between water-washed sample and ethanol-washed sample ($p > 0.05$). Although both water- and ethanol- washing steps led to the improvement on enzymatic hydrolysis, their mechanism was different. Ethanol targeted at the delignification of loosened lignin polymers which can be extracted by ethanol (EL) and water (WSL). It is clear that the pretreatment process severely condensed EL by the cleavage of β-O-4 linkages and generated WSL with free phenolic OH by cleavage of LCC bonds (Figs. 3 and 4 and Table 3). These lignin were likely rearranged at the surface of pretreated bagasse (Selig et al., 2007) providing strong ring–stacking interaction, inhibition and/or deactivation effects to cellulase (Rahikainen et al., 2013). The removal of EL and WSL by ethanol wash step not only diminished non-productive binding of lignin to cellulase, but also caused high accessibility of cellulose. In contrast, water solution was proved to dissolve the monomers or oligomers with a very low molecular weight (Table 2). The increase of the glucose yield may be attributed to the removal of various water-soluble degraded products that inhibit subsequent enzyme hydrolysis. However, it is not clear whether there is a correlation between the compositions of the extractives, the extractive yields and the enzymatic conversion rates which will be systematically investigated in the future.

4. Conclusion

The structural features of ethanol soluble lignin after dilute phosphoric acid and steam explosion pretreatment process were analyzed by comprehensive NMR analyses. The results revealed that this pretreatment hydrolyzed the main β-O-4 linkages of lignin resulting in the lignin with riched S units. Meanwhile, during the lignin isolation process, the removal of ethanol-soluble lignin led to significant improvement of glucose yields.

Acknowledgements

We thank the support from National High Magnetic Field Laboratory and McKnight Brain Institute of the University of Florida for the NMR measurement. We also thank UF Bioethanol Pilot Plant to kindly provide the pretreated sugarcane bagasse samples. This project is supported by Biomass Research & Development Initiative Competitive Grant no. 2001-10006-3058 from the USDA National Institute of Food and Agriculture and the US-India Consortium Grant from DOE.

References

Bozell, J.J., O'Lenick, C., Warwick, S., 2011. Biomass fractionation for the biorefinery: heteronuclear multiple quantum coherence–nuclear magnetic resonance investigation of lignin isolated from solvent fractionation of switchgrass. J. Agric. Food Chem. 59, 9232–9242.

Buranov, A.U., Mazza, G., 2008. Lignin in straw of herbaceous crops. Ind. Crops Prod. 28, 237–259.

Cao, S., Pu, Y., Studer, M., Wyman, C., Ragauskas, A.J., 2012. Chemical transformations of *Populus trichocarpa* during dilute acid pretreatment. RSC Adv. 2, 10925–10936.

Chundawat, S.P.S., Beckham, G.T., Himmel, M.E., Dale, B.E., 2011a. Deconstruction of lignocellulosic biomass to fuels and chemicals. Annu. Rev. Chem. Biomol. Eng. 2, 121–145.

Chundawat, S.P.S., Donohoe, B.S., da Costa Sousa, L., Elder, T., Agarwal, U.P., Lu, F., Ralph, J., Himmel, M.E., Balan, V., Dale, B.E., 2011b. Multi-scale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. Energy Environ. Sci. 4, 973–984.

del Río, J.C., Rencoret, J., Prinsen, P., Martínez, A.T., Ralph, J., Gutiérrez, A., 2012. Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. J. Agric. Food Chem. 60, 5922–5935.

Geddes, C., Peterson, J.J., Roslander, C., Zacchi, G., Mullinnix, M.T., Shanmugam, K., Ingram, L.O., 2010a. Optimizing the saccharification of sugar cane bagasse using dilute phosphoric acid followed by fungal cellulases. Bioresour. Technol. 101, 1851–1857.

Geddes, C.C., Peterson, J.J., Mullinnix, M.T., Svoronos, S.A., Shanmugam, K., Ingram, L.O., 2010b. Optimizing cellulase usage for improved mixing and rheological properties of acid-pretreated sugarcane bagasse. Bioresour. Technol. 101, 9128–9136.

Geddes, C.C., Mullinnix, M.T., Nieves, I., Peterson, J., Hoffman, R., York, S., Yomano, L., Miller, E., Shanmugam, K., Ingram, L.O., 2011. Simplified process for ethanol

production from sugarcane bagasse using hydrolysate-resistant *Escherichia coli* strain MM160. Bioresour. Technol. 102, 2702–2711.
Grabber, J.H., Hatfield, R.D., Ralph, J., Zon, J., Amrhein, N., 1995. Ferulate cross-linking in cell walls isolated from maize cell suspensions. Phytochemistry 40, 1077–1082.
Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315, 804–807.
Iiyama, K., Lam, T.B.T., Stone, B.A., 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry 29, 733–737.
Jung, S., Foston, M., Sullards, M.C., Ragauskas, A.J., 2010. Surface characterization of dilute acid pretreated *Populus deltoides* by ToF-SIMS. Energy Fuels 24, 1347–1357.
Kim, H., Ralph, J., Akiyama, T., 2008. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d6. Bioenergy Res. 1, 56–66.
Laskar, D.D., Zeng, J., Yan, L., Chen, S., Yang, B., 2013. Characterization of lignin derived from water-only flowthrough pretreatment of *Miscanthus*. Ind. Crops Prod. 50, 391–399.
Nakagame, S., Chandra, R.P., Saddler, J.N., 2010. The effect of isolated lignins, obtained from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis. Biotechnol. Bioeng. 105, 871–879.
Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N., 2011. The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. Bioresour. Technol. 102, 4507–4517.
Pan, X., Kadla, J.F., Ehara, K., Gilkes, N., Saddler, J.N., 2006. Organosolv ethanol lignin from hybrid poplar as a radical scavenger: relationship between lignin structure, extraction conditions, and antioxidant activity. J. Agric. Food Chem. 54, 5806–5813.
Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., Liotta, C.L., Mielenz, J.R., Murphy, R., Templer, R., Tschaplinski, T., 2006. The path forward for biofuels and biomaterials. Science 311, 484–489.
Rahikainen, J.L., Martin-Sampedro, R., Heikkinen, H., Rovio, S., Marjamaa, K., Tamminen, T., Rojas, O.J., Kruus, K., 2013. Inhibitory effect of lignin during cellulose bioconversion: the effect of lignin chemistry on non-productive enzyme adsorption. Bioresour. Technol. 133, 270–278.
Ralph, J., Landucci, L., 2010. NMR of lignins. In: Lignin and Lignans. CRC Press, Boca Raton, pp. 137–243.
Ralph, J., Grabber, J.H., Hatfield, R.D., 1995. Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. Carbohydr. Res. 275, 167–178.
Samuel, R., Pu, Y., Raman, B., Ragauskas, A.J., 2010. Structural characterization and comparison of switchgrass ball-milled lignin before and after dilute acid pretreatment. Appl. Biochem. Biotechnol. 162, 62–74.
Selig, M.J., Viamajala, S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B., 2007. Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. Biotechnol. Prog. 23, 1333–1339.
Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of Structural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure. National Renewable Energy Laboratory, Golden, CO.
Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E., 2011. Lignin content in natural Populus variants affects sugar release. Proc. Natl. Acad. Sci. USA 108, 6300–6305.
Tu, M., Pan, X., Saddler, J.N., 2009. Adsorption of cellulase on cellulolytic enzyme lignin from lodgepole pine. J. Agric. Food Chem. 57, 7771–7778.
Wen, J.L., Sun, S.L., Xue, B.L., Sun, R.C., 2012. Quantitative structures and thermal properties of birch lignins after ionic liquid pretreatment. J. Agric. Food Chem. 61, 635–645.
Yokoyama, T., Matsumoto, Y., 2010. Revisiting the mechanism of β-O-4 bond cleavage during acidolysis of lignin. Part 2: Detailed reaction mechanism of a non-phenolic C6–C2 type model compound. J. Wood Chem. Technol. 30, 269–282.
Zeng, J., Helms, G.L., Gao, X., Chen, S., 2013. Quantification of wheat straw lignin structure by comprehensive NMR analysis. J. Agric. Food Chem. 61, 10848–10857.
Zhao, X., Cheng, K., Liu, D., 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl. Microbiol. Biotechnol. 82, 815–827.
Zhou, C., Li, Q., Chiang, V.L., Lucia, L.A., Griffis, D.P., 2011. Chemical and spatial differentiation of syringyl and guaiacyl lignins in poplar wood via time-of-flight secondary ion mass spectrometry. Anal. Chem. 83, 7020–7026.
Zhou, H.F., Lou, H., Yang, D., Zhu, J.Y., Qiu, X., 2013. Lignosulfonate to enhance enzymatic saccharification of lignocelluloses: the role of molecular weight and substrate lignin. Ind. Eng. Chem. Res. 52, 8464–8470.
Zhu, J.Y., Pan, X.J., Wang, G.S., Gleisner, R., 2009. Sulfite pretreatment (sporl) for robust enzymatic saccharification of spruce and red pine. Bioresour. Technol. 100, 2411–2418.