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Comparative study of lignin characteristic from wheat straw obtained by soda-AQ or kraft pretreatment and effect on the following enzymatic hydrolysis process

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HIGHLIGHTS

- Characteristic of lignin after kraft or soda-AQ pretreatment was compared.
- Quantitative information of different substructures in the lignin was obtained.
- Lignin was more degraded during kraft pretreatment than soda-AQ pretreatment.
- More cellulose was converted to glucose in the wheat straw after kraft pretreatment.
- LCC played a great role in the enzymatic hydrolysis of lignocellulosic material.

GRAPHICAL ABSTRACT

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ABSTRACT

To understand the structural changes of lignin after soda-AQ and kraft pretreatment, milled straw lignin, black liquor lignin and residual lignin extracted from wheat straw were characterized by FT-IR, UV, GPC and NMR. The results showed that the main lignin linkages were β-aryl ether substructures (β-O-4′), followed by phenylcoumaran (β-5′) and resinol (β-β′) substructures, while minor content of spirodienone (β-1′), dibenzodioxocin (5-5′) and α,β-diaryl ether linkages were detected as well. After pretreatment, most lignin inter-units and lignin-carbohydrate complex (LCC) linkages were degraded and dissolved in black liquor, with minor amount left in residual pretreated biomass. In addition, through quantitative ^{13}C and 2D-HSQC NMR spectral analysis, lignin and LCC were found to be more degraded after kraft pretreatment than soda-AQ pretreatment. Furthermore, the subsequent enzymatic hydrolysis results showed that more cellulose in wheat straw was converted to glucose after kraft pretreatment, indicating that LCC linkages were important in the enzymatic hydrolysis process.

1. Introduction

There is an urgent need in utilizing alternative energy sources to supply increasing energy demands for the depletion of low extraction cost fossil fuel reserves and climate change (Agbor et al., 2011). Lignocellulosic biomass has the potential to serve

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as a low cost and renewable feedstock for biofuel production (Hyun Hong et al., 2014). Wheat straw is one of the most plentiful lignocellulosic biomass whose yield is an average of ratio 1.3 kg straw per kg grain, depending on the specific wheat varieties and climatic factors (Kim and Dale, 2004; Talebnia et al., 2010; Ruiz et al., 2011). Hence, wheat straw is considered as one of the greatest potential feedstocks for production of ethanol in 21st century (Talebnia et al., 2010). Cellulose, hemicellulose and lignin are the three main components in wheat straw material (Wörmeyer et al., 2011). In plant cell wall, hemicellulose is cross-linked with lignin and cellulose, which contributes to promote cell wall recalcitrance. Therefore, a prominent conversion route consists of the following key steps: pretreatment, saccharification, fermentation and distillation. In the process, pretreatment is a crucial step to achieve efficient conversion of lignocellulosic biomass to ethanol by breaking down the polymeric matrix of the carbohydrates and lignin in the lignocellulosic biomass, therefore enhancing the accessibility of enzymes to solid substrate during enzymatic hydrolysis step (Ertas et al., 2014; Yao et al., 2010). Typical pretreatments include autohydrolysis or thermochemical methods using alkali, acid or organic solvents which have been widely studied to prepare lignocellulosic substrates from a wide range of raw materials by either remove or redistribute hemicellulose or lignin for subsequent bioconversion (Lee et al., 2009; Oscar et al., 2011).

Alkali pretreatments are much more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose removal than acid or autohydrolysis process (Carvalheiro et al., 2008). The major effects of delignification in the alkaline pretreatment are the cleavage of ester bonds between lignin and hemicellulose and the swelling of the cellulose fibers, resulting in better enzymatic hydrolyzability (Chen et al., 2013). The presence of lignin, which is acting as a shield to prevent the digestible carbohydrates from enzyme attack, limits the rate and extent of enzymatic hydrolysis (Chang and Holtzapple, 2000). In addition, part of cellulase is absorbed onto the solid substrate by binding to lignin, which is less reversible. The non-productive binding of cellulase to lignin physically restrains the enzymes from reaching cellulose surface to reduce cellulose hydrolysis (Pihlajaniemi et al., 2014). Therefore, the removal of lignin in biomass can promote the efficient of enzymatic hydrolysis. In addition, alkali methods can increase the specific surface area for the swelling of cellulose fiber, decrease the degree of polymerization and crystallinity, which makes the cellulose more accessible for enzyme (Kim et al., 2014). In papermaking and bioethanol production industry, the two most important alkali pretreatments are soda-AQ and kraft methods. Kraft process utilizes sulphurous chemicals to break the bonds between lignin and carbohydrate (Wörmeyer et al., 2011), thus the properties of lignin obtained is very different from native lignin (Lora and Glasser, 2002). The soda pretreatment is one of those processes that can produce sulphur-free lignin products from lignocellulosic material which offer potential possibilities for other high quality applications (Wörmeyer et al., 2011).

In this paper, we choose wheat straw as the raw material and two pretreatment methods (soda-AQ and kraft) were used. Different lignin samples were separated from raw materials, black liquor and pretreated materials. Comparative analysis of these lignins properties were done. Lignin substructure and linkages between lignin and carbohydrate were quantitatively studied by combination of ^{13}C NMR and 2D-HSQC. To compare the two different methods, the degradation of lignin and bonds between lignin and carbohydrate were also analyzed. Enzyme hydrolysis of the residue materials after being pretreated by the two methods were designed to explore the effect of the changes of lignin structure on the cellulose conversion.

2. Methods

2.1. Materials

Wheat straw, provided by Hubei Academy of Agricultural Sciences, was size-reduced to the particle size of 3.0–5.0 cm (diameter), air dried and then kept in sealed bags at room temperature prior to pretreatments. The chemical compositions of the ground wheat straw (in % w/w, dry matter) were: cellulose, 35.1; hemicellulose, 9.3; lignin, 26.1 (acid soluble 1.8, acid insoluble 24.3); ash, 4.1; ethanol-extractive, 22.1.

Cellulase (Sino EnzymesR) was purchased from Baiyin Sainuo Technology Ltd. (Gansu province, PR China), and had a filter paper activity of 160 U/g and β-glucosidase activity of 42 U/g.

All the chemical reagents used in this paper were purchased from Sigma–Aldrich.

2.2. Pretreatment of wheat straw

All of the pretreatments were conducted in a one-liter rotary electrothermal pressure digester (ZQS-3, Qinggong Jixie factory of Shanxi University of Science and Technology, Xianyang, Shanxi province, PR China). In soda-AQ pretreatment, milled and air-dried wheat straw was slurried for 5 min with 15% NaOH, 0.05% AQ in a solid:liquid ratio of 1:5 (w/w). The mixture was heated for 90 min to 160 °C and kept for 30 min. In Kraft pretreatment, milled and air-dried wheat straw was slurried for 5 min with 17% NaOH, 25% sulfidity in a solid:liquid ratio of 1:4 (w/w). The mixture was heated for 60 min to 160 °C and kept for 60 min.

After cooling down to room temperature, the residual solid obtained from each pretreatment was separated by filtration, washed to be pH neutral and kept in sealed bags at 4 °C.

2.3. Preparation of lignin samples

2.3.1. Preparation of milled straw lignin (MSL)

Ten grams of wheat straw was milled and homogenized by screening 100 mesh. The well-ground samples were extracted with ethanol/benzene (1:2) and hot water. The dried extractive-free wheat straw powder was ground in a vibratory ball mill for 72 h. The crude lignin was isolated by dioxane/water (96:4, v/v) for 24 h and three times. Then the crude lignin was purified by HAc-H_2O (9:1, v/v) and dichloroethane-ethanol (2:1, v/v) according to the procedure of Björkman (1956).

2.3.2. Preparation of black liquor lignin and residual lignin

The black liquor lignin was isolated directly by acidic precipitation to pH 2.0 with hydrochloric acid under magnetic stirring (Yuan et al., 2009). After filtration, the isolated lignin was washed with diluted hydrochloric acid (pH 2.0) and air-dried under vacuum for 24 h and stored in sealed bags before analysis. Soda lignin (SL) was from black liquor of soda-AQ pretreatment and kraft lignin (KL) was from black liquor of kraft pretreatment.

Residual lignin was prepared and purified according to Kapareju's method (Kaparaju and Felby, 2010). The pretreated material was treated by cellulase at a dry solids concentration of 2% (w/v) in 0.05 M sodium citrate buffer (pH = 4.8), 50 °C, 150 rpm for 72 h in a shaker. Enzyme loading was 100 filter paper unit (FPU) per gram of dry substrate to remove most of carbohydrate from pretreated wheat straw. The crude lignin obtained after cellulase treatment was suspended in acidic dioxane-water (85:15, v/v) mixture. The solution was neutralized with sodium bicarbonate and then rotary evaporated. The concentrated solution was added dropwisely to acidified deionized water (pH = 2.0). The precipitated lignin was isolated and freeze dried. Soda-residue lignin

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(SRL) was from soda-AQ pretreated wheat straw and kraft-residue lignin (KRL) was separated from kraft pretreated wheat straw.

2.4. Analytical methods

2.4.1. Component analysis of isolated lignin samples

Lignin and carbohydrate contents were determined according to the method described by NREL (2006). The procedure uses a two-step acid hydrolysis to fractionate the biomass into two forms that are more easily quantified. The solid obtained after the two-step acid hydrolysis was acid insoluble lignin (AIL). The filtrates were used for sugar and acid soluble lignin (ASL) determination. Sugars hydrolyzed into the monomeric forms in the hydrolysis liquid were determined by high performance liquid chromatography (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. Calibration of polysaccharide content in the isolated lignin samples was performed with a standard solution of D-glucose, D-xylose, D-galactose, D-mannose, and L-arabinose. Measurements were conducted with two parallels.

2.4.2. FT-IR determination

Infrared spectra were determined using an FT-IR 710 infrared spectrophotometer (Nicolet, Madison, WI, USA). Dried samples (1–2 mg) were milled into powder with a diameter less than 1 mm. A certain amount of the powder was dispersed in spectroscopic grade KBr and subsequently pressed into disks using 10 tons of pressure for 1 min. A total of 64 scans with a 2 cm^{-1} resolution were signal averaged and stored; the wave number range scanned was 4000–500 cm^{-1}.

2.4.3. UV determination

UV spectra were recorded on an ultraviolet/visible spectrophotometer (Shimadzu, UV2550). The method is the same as Yuan (Yuan et al., 2009) described before as following: five milligrams grams of lignin sample was dissolved in 10 ml 95% (v/v) dioxane/water under stirring with magnetic force. Then the solution was diluted 10 times with 50% (v/v) dioxane/water. The absorbance between 260 and 400 nm was measured.

2.4.4. Molecular weight determination

The lignin samples were dissolved in N,N-dimethylformamide (DMF, 1 mg/mL), filtered through a 0.45 μm filter and placed in a 2 mL autosampler vial prior to GPC analysis. The molecular weight distributions of the lignin samples were analyzed on an Shimadzu LC-20AD HPLC system equipped with Shim-pack GPC-803D column and a refractive index (RI) detector, using DMF as the mobile phase (1.0 mL/min) with injection volumes of 25 μL at 40 °C. A calibration curve was constructed based on 4 narrow polystyrene standards ranging in molecular weight from 2900 to 19,800 g/mol. Data collection and processing were performed using LC solution software (GPC Postrun). Molecular weights (Mn and Mw) were calculated by the software relative to the universal polystyrene calibration curve.

2.4.5. ^{13}C NMR spectroscopy

All NMR spectra were recorded on a Bruker AVIII 400 MHz spectrometer operated at 25 °C utilizing DMSO-d_6 as the solvent. For quantitative ^{13}C NMR, 125 mg of the lignin was dissolved in 0.5 mL of DMSO-d_6. The quantitative ^{13}C NMR spectra were recorded in the FT mode at 100.6 MHz. The inverse-gated decoupling sequence, which allows quantitative analysis and comparison of the signal intensities, was used with the following parameters: 90° pulse angle; 1.4-s acquisition time; 2-s relaxation delay; 64,000 data points; and 30,000 scans. Chromium (III) acetylacetonate (0.01 M) was added to the lignin solution to provide complete relaxation of all nuclei.

2.4.6. 2D-HSQC spectroscopy

2D-HSQC NMR spectra were recorded in the HSQC experiments using a Bruker AVIII 400 MHz spectrometer. Around 60 mg of lignin was dissolved in 0.5 ml of DMSO-d_6. The spectral widths were 5000 and 20,000 Hz for the ^1H and ^{13}C dimensions, respectively. The number of collected complex points was 1024 for the ^1H dimension with a recycle delay of 1.5 s. The number of transients was 64, and 256 time increments were recorded in the ^{13}C dimension. The $^1J_{CH}$ used was 145 Hz. Prior to fourier transformation, the data matrices were zero filled to 1024 points in the ^{13}C dimension. Integration calculations of the 2D contours in all spectra was performed using MestReNova software (trial version).

2.5. Enzymatic hydrolysis

Pretreated wheat straw was hydrolyzed in 100-ml flasks at a consistency of 2% (w/v) in 0.05 M sodium citrate buffer (pH = 4.8) at 50 °C under continuous agitation at 150 rpm for 72 h in a shaking incubator. Cellulase loading was 25 filter paper unit (FPU) per gram of dry substrate. Liquid samples were taken at different intervals, quenched by submersion in a vigorously boiling water bath, and centrifuged at 10,000 rpm for 10 min, and the supernatant was used for glucose content analysis.

Glucose was determined by high performance liquid chromatography (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.

3. Results and discussion

3.1. Composition analysis of isolated lignin samples

The compositions of the five lignin samples (MSL, SL, KL, SRL and KRL) were determined by the method of NREL (2006). The solid obtained after the two-step acid hydrolysis was acid insoluble lignin (AIL). The filtrates were used for sugar and acid soluble lignin (ASL) determination. The results (as shown in Table 1) showed that the AIL in all lignin samples were more than 92%, with ASL less than 1%. All the five lignins contained 3–5 kinds of sugar contamination. But the total content was less than 5%. The data implied that the lignin samples were pure enough to compare and the characterization difference was due to the structural changes in lignin.

3.2. FT-IR determination

FT-IR spectroscopy has several advantages including high sensitivity and selectivity, high signal-to-noise ratio, accuracy, short time and small amount of sample required for the analysis (Ghaffar and Fan, 2013). FT-IR spectra of lignin samples showed strong absorbance at around 3410 cm^{-1} from –OH stretching, as well as signal at 2920 cm^{-1} of –CH stretching vibration. It was obvious to see the strong signal from aromatic ring at 1600 cm^{-1}, 1510 cm^{-1} and 1420 cm^{-1} (Yang et al., 2013), indicating that the basic structure of lignin was not changed appreciably during pretreatment.

On the other hand, there were also several significant differences observed. Signals with greater intensity from C–H of methyl and methylene units at 2920 cm^{-1} and 2850 cm^{-1} were identified in the residual lignin compared with MSL (Yang et al., 2014), indicating the breakdown and rearrangement of lignin monomer units

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Table 1
Lignin and neutral sugar contents (%) of the isolated lignin samples.

	Acid insoluble lignin	Acid soluble lignin	Glucose	Xylose	Galactose	Arabinose	Mannose
MSL	95.92 ± 1.84	0.34 ± 0.11	0.12 ± 0.05	1.45 ± 0.07	0.48 ± 0.01	0.40 ± 0.02	0.16 ± 0.01
SL	92.35 ± 0.07	0.93 ± 0.21	0.82 ± 0.12	1.86 ± 0.06	0.52 ± 0.02	0.11 ± 0.01	0.05 ± 0.00
KL	92.10 ± 1.54	0.67 ± 0.13	0.60 ± 0.01	1.79 ± 0.01	0	0.49 ± 0.02	0.04 ± 0.00
SRL	92.59 ± 1.87	0.86 ± 0.10	2.10 ± 0.25	1.61 ± 0.01	0.68 ± 0.04	0	0
KRL	93.50 ± 2.40	0.50 ± 0.08	1.61 ± 0.08	2.53 ± 0.02	1.38 ± 0.05	0	0.09 ± 0.00

Note: MSL, milled straw lignin; SL, soda lignin; KL, kraft lignin; SRL, soda-residual lignin; KRL, kraft-residual lignin.

during pretreatment. In addition, intentions of signal from C–H of *p*-hydroxyphenylpropane at 837 cm^{-1} were decreased in both residual lignin and black liquor lignin, compared with milled straw lignin (Faix, 1991), which indicated that part of H-type lignin was probably degraded and dissolved during pretreatment. Combined with signal at 1325 cm^{-1} assigned to syringylpropane ring and guaiacylpropane ring condensed (Faix, 1991), it could be concluded that the main substructures of lignin in wheat straw were H type, G type and S type.

3.3. UV determination

UV spectroscopy has been used to determine the content of lignin, since lignin absorbs much more strongly in the UV and visible regions than cellulose or hemicellulose (Yuan et al., 2009). Five lignin samples had the basic character of lignin UV spectrum. The maximum absorbance wavelengths were at 279.3 nm, 274.5 nm, 273.4 nm, 275.1 nm and 277.5 nm for MSL, SL, KL, SRL and KRL, respectively, which were from non-condensed phenolic groups. If there were more S-type subunits in lignin sample, the maximum absorbance wavelength would be decreased (Pei and Yang, 2014). Comparing with MSL, the maximum absorbance wavelength of all other lignin samples was decreased, suggesting that G-type lignin or H-type lignin was probably degraded to some extent during pretreatment. Black liquor lignin (SL and KL) contained more amount of S-type lignin than residual lignin (SRL and KRL), in which the kraft lignin contained more than soda lignin, indicating that it was easy for S-type lignin to be dissolved during kraft pretreatment than soda-AQ pretreatment. The result was verified later in the quantitative analysis of NMR.

The absorbance between 300 and 320 nm was from phenolic type lignin (Pei and Yang, 2014), which could be only seen in MSL, indicating that phenolic type lignin was degraded during pretreatment.

3.4. Molecular weight determination

The results of molecular weights and polydispersity of lignin samples were shown in Table 2. It could be seen that all five lignin samples did not have wide polydispersity, as shown by Mw/Mn < 1.5. The molecular weights of lignin samples were between 60,000 and 140,000 g/mol. MSL had the lowest molecular weights, followed by black liquor lignin, while residual lignin had the highest molecular weights.

MSL from wheat straw had the widest polydispersity, while residual lignin had the narrowest polydispersity, indicating that there were most lignin fractions with different molecular weight co-existing in native lignin. Compared with black liquor lignin, residual lignin had higher molecular weight, suggesting that lignin with low molecular weight was probably dissolved during pretreatment firstly, followed by lignin fraction with higher molecular weight, which caused lignin fractions with highest molecular weight being left in residual lignin.

Table 2
Results of weight-average (Mw), number-average (Mn) molecular weights and polydispersity indexes (Mw/Mn) of the isolated lignin samples.

Samples	Mw	Mn	Mw/Mn
MSL	97,898 ± 488	66,513 ± 176	1.47 ± 0.01
SL	102,375 ± 672	85,856 ± 489	1.19 ± 0.00
KL	99,275 ± 329	82,067 ± 541	1.21 ± 0.00
SRL	133,716 ± 590	124,472 ± 364	1.07 ± 0.00
KRL	137,472 ± 862	129,442 ± 155	1.06 ± 0.01

Comparing the two pretreatment methods, it was found that Mn, Mw and polydispersity of soda lignin was higher than that of kraft lignin, but residual lignin from soda pretreatment had lower Mn and Mw and wider polydispersity, indicating that the dissolved lignin was more degraded during kraft pretreatment.

3.5. ^{13}C NMR determination

^{13}C NMR is widely applied in lignin characterization as a powerful analytical technique of revealing a large amount of lignin structures including aryl ethers, condensed and uncondensed aromatic and aliphatic carbons. To further study characteristic of lignin samples and compare the two different pretreatment methods, ^{13}C NMR and 2D-HSQC were employed. ^{13}C NMR spectra of lignin samples were shown that the weak signal from 90 to 102 ppm indicated the low content of carbohydrate in lignins (Yang et al., 2014).

In the aromatic region (153–104 ppm), the main signals were from guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) units. Signals assigned to the three substructures were at 148.1 ppm (C_3 in G), 119.9 ppm (C_6 in G), 115.3 ppm (C_5 in G), 152.8 ppm (C_3/C_5 in S), 133.6 ppm (C_1 in S), 128.6 ppm (C_2/C_6 in H) (Kim and Ralph, 2010).

The strong signal at 56.5 ppm was from $-OCH_3$. In addition, in the region of 50–90 ppm, it was easy to find signals assigned to β-O-4′, β-β′, β-5′, β-1′ and 5-5′ at 85.7 ppm (C_β in S type β-O-4′ units), 72.8 ppm (C_α, β-O-4′), 60.7 ppm (C_γ, β-O-4′), 84.3 ppm (C_α, β-β′), 71.1 ppm (C_γ, β-β′), 86.7 ppm (C_α, β-5′), 81.8 ppm (C_α, β-1′), 83.3 ppm (C_α, 5-5′) (Nimz et al., 1981; Yang et al., 2014).

The intensities of some signals, such as C_α, C_β, C_γ in β-O-4′, C_α in β-β′, β-5′, β-1′ and 5-5′ from black liquor lignin and residual lignin, were decreased compared with MSL, indicating that these substructures were degraded during pretreatment. The intensities of the signals between 10 and 34 ppm from the aliphatic side chain of the phenylpropane units were the strongest signals in residual lignin, indicating the possible breakdown and rearrangement of lignin monomer units during pretreatment, which was in accordance with the results from FT-IR.

3.6. 2D-HSQC determination

One of the most applied 2D NMR techniques is heteronuclear single-quantum-coherence (HSQC). Overlapping protons attached to carbons which have different shifts could be separated by their

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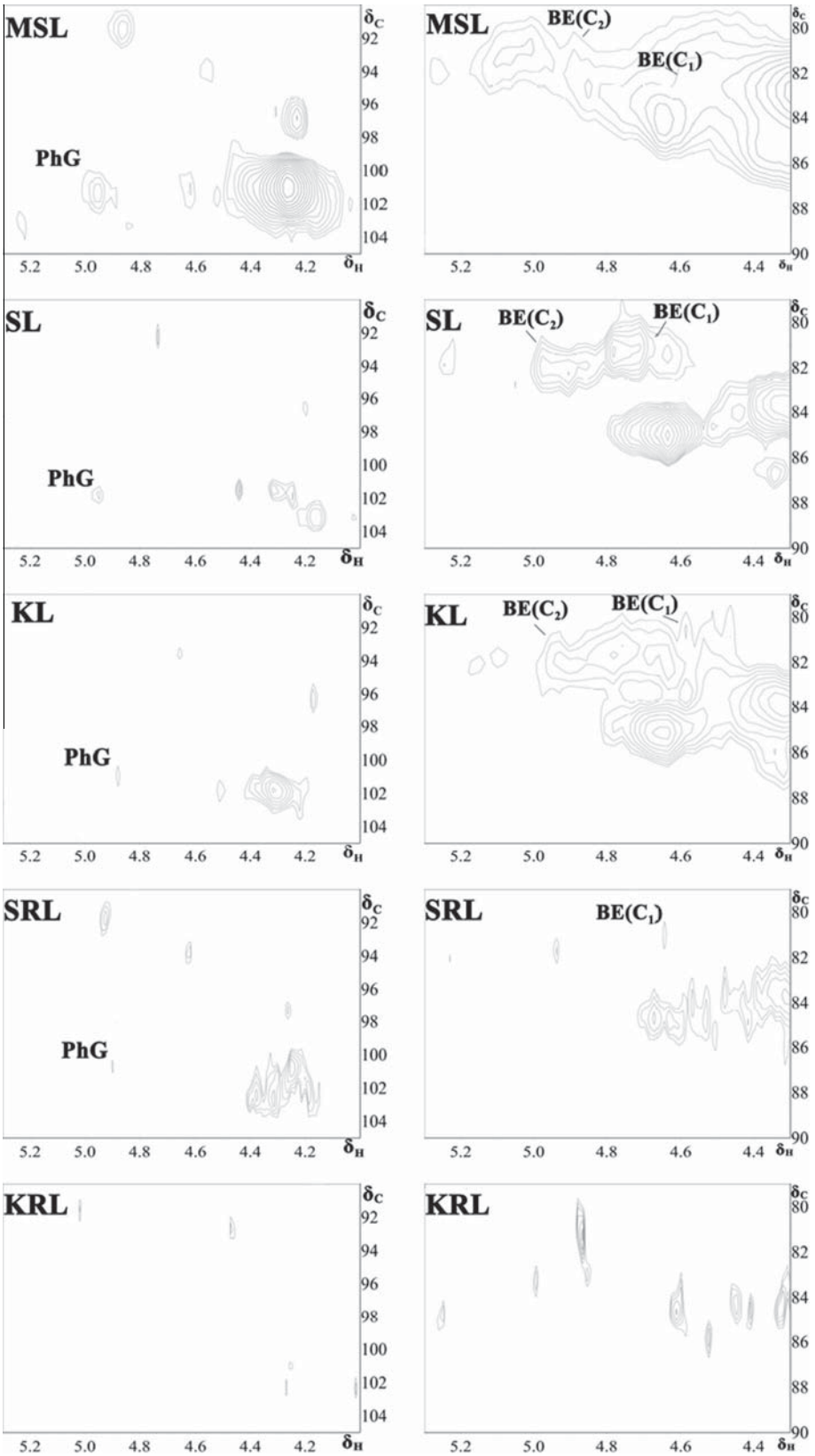

Fig. 1. LCC linkages region. PhG: phenyl glycoside (left side, δ_C/δ_H 103–99.5/5.2–4.8); BE(C_2): benzyl ethers C_2 (right side, δ_C/δ_H 81/5.0); BE(C_1): benzyl ethers C_1 (right side, δ_C/δ_H 81/4.6).

different chemical shifts. While overlapping carbons could also be separated by their attachment to protons with different shifts. Therefore, the apparent resolution of 2D spectra is much better than anything that can be achieved in 1D spectrum (Balakshin et al., 2011). HSQC spectra of aromatic and aliphatic regions of lignin samples were shown in Fig. 2.

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Table 3
Quantification analysis of aromatic moieties and aliphatic region by combination analysis of ^{13}C and HSQC NMR spectra of wheat straw isolated lignin samples.

Aromatic moiety	MSL		SL		KL		SRL		KRL	
	Ar[a]	%[b]	Ar[a]	%[b]	Ar[a]	%[b]	Ar[a]	%[b]	Ar[a]	%[b]
Syringyl (S,S′)	28.10 ± 1.00	38.91 ± 1.09	10.69 ± 0.09	35.24 ± 0.26	21.72 ± 0.58	41.76 ± 1.02	10.91 ± 0.49	46.30 ± 1.32	4.13 ± 0.18	52.79 ± 1.33
Guaiacyl (G)	36.83 ± 0.08	51.01 ± 0.50	15.21 ± 0.10	50.14 ± 0.48	24.76 ± 0.26	47.60 ± 0.59	12.39 ± 0.13	52.62 ± 1.39	3.68 ± 0.04	47.13 ± 1.40
p-Hydroxyphenyl (H)	7.28 ± 0.37	10.08 ± 0.59	4.44 ± 0.31	14.61 ± 0.77	5.53 ± 0.21	10.63 ± 0.43	0.25 ± 0.02	1.06 ± 0.07	0.01 ± 0.00	0.08 ± 0.02
S/G ratio	0.76 ± 0.03		0.70 ± 0.00		0.88 ± 0.03		0.88 ± 0.05		1.12 ± 0.06	
p-Benzonates (PB)	0.04 ± 0.01	0.06 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
p-Coumarate (PCA)	5.37 ± 0.46	7.43 ± 0.59	0.15 ± 0.02	0.50 ± 0.03	0.12 ± 0.03	0.24 ± 0.05	0.02 ± 0.00	0.09 ± 0.02	0.00	0.00
Ferulate (FA)	3.18 ± 0.05	4.41 ± 0.03	3.31 ± 0.04	10.92 ± 0.04	4.15 ± 0.11	7.98 ± 0.20	0.23 ± 0.02	0.97 ± 0.07	0.08 ± 0.02	1.05 ± 0.17
p-Coumarate (PCA)/ ferulate (FA) ratio	1.69 ± 0.12		0.05 ± 0.00		0.03 ± 0.01		0.10 ± 0.02		0.00	
Cinnamates/lignin ratio	0.12 ± 0.01		0.11 ± 0.00		0.08 ± 0.00		0.01 ± 0.00		0.01 ± 0.00	
Cinnamyl aldehyde (J)	0.10 ± 0.02	0.14 ± 0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cinnamyl alcohol (I)	0.07 ± 0.02	0.10 ± 0.02	0.08 ± 0.01	0.25 ± 0.04	1.13 ± 0.10	2.18 ± 0.18	0.00	0.00	0.00	0.00
Tricin (T)	5.56 ± 0.10	7.69 ± 0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aliphatic region	MSL		SL		KL		SRL		KRL	
	Ar[a]	%[c]	Ar[a]	%[c]	Ar[a]	%[c]	Ar[a]	%[c]	Ar[a]	%[c]
α-OH/β-O-4′(A,A′)	30.21 ± 0.67	74.92 ± 1.63	12.78 ± 0.25	67.77 ± 0.65	9.38 ± 0.16	73.90 ± 0.76	10.41 ± 0.36	89.52 ± 1.00	6.33 ± 0.31	63.51 ± 0.38
α-keto/β-O-4′(Aox)	0.18 ± 0.03	0.45 ± 0.07	0.57 ± 0.04	3.01 ± 0.28	0.19 ± 0.01	1.49 ± 0.13	0.04 ± 0.01	0.31 ± 0.06	0.51 ± 0.07	5.08 ± 0.40
Total β-O-4′	30.39 ± 0.64	75.37 ± 1.56	13.34 ± 0.21	70.78 ± 0.93	9.57 ± 0.15	75.38 ± 0.89	10.45 ± 0.37	89.84 ± 0.94	6.84 ± 0.38	68.59 ± 0.02
Phenylcoumaran (B)	4.28 ± 0.26	10.63 ± 0.64	2.44 ± 0.09	12.97 ± 0.12	0.26 ± 0.01	2.07 ± 0.01	0.21 ± 0.05	1.77 ± 0.37	1.01 ± 0.07	10.11 ± 0.13
Resinols (C)	4.07 ± 0.23	10.09 ± 0.57	2.39 ± 0.16	12.66 ± 0.46	2.13 ± 0.14	16.77 ± 0.64	0.87 ± 0.10	7.47 ± 0.52	1.62 ± 0.08	16.31 ± 0.12
Dibenzodioxocin (D)	0.25 ± 0.03	0.63 ± 0.08	0.13 ± 0.02	0.68 ± 0.06	0.14 ± 0.01	1.13 ± 0.05	0.00	0.00	0.00	0.00
Spirodienones (E)	1.28 ± 0.11	3.18 ± 0.27	0.48 ± 0.06	2.51 ± 0.26	0.59 ± 0.04	4.67 ± 0.17	0.11 ± 0.01	0.94 ± 0.07	0.50 ± 0.02	4.97 ± 0.06
α,β-Diaryl ether (F)	0.04 ± 0.00	0.09 ± 0.01	0.08 ± 0.01	0.40 ± 0.03	0.00	0.00	0.00	0.00	0.00	0.00
Total side chains	40.32 ± 0.02	100.00	18.86 ± 0.54	100.00	12.70 ± 0.35	100.00	11.64 ± 0.53	100.00	9.96 ± 0.54	100.00
PhG: phenyl glycoside	3.92 ± 0.38		1.21 ± 0.20		0.75 ± 0.04		0.77 ± 0.01		0.00	
BE(C2): benzyl ethers C2	1.64 ± 0.23		0.19 ± 0.01		0.08 ± ± 0.01		0.00		0.00	
BE(C1): benzyl ethers C1	0.30 ± 0.04		0.15 ± 0.04		0.08 ± 0.02		0.13 ± 0.00		0.00	

[a] The amount of specific functional group was expressed as number per 100 Ar.
[b] The amount of specific interunit linkage was expressed as percentage of S + G + H.
[c] The amount of specific interunit linkage was expressed as percentage of total side chain.

3.6.1. Analysis of lignin aromatic region

Signals assigned to G, S and H units were mainly in the region of δ_C/δ_H 100–135/5.5–8.5. The ^{13}C–^{1}H correlations for $S_{2,6}$ and $S'_{2,6}$ were at δ_C/δ_H 103.9/6.69 and δ_C/δ_H 106.3/7.40. The signals from G_2, G_5 and G_6 were found at δ_C/δ_H 110.3/6.93, δ_C/δ_H 114.8/6.90 and δ_C/δ_H 118.2/6.80, respectively in the HSQC spectra. The resonance from $H_{2,6}$ was observed at δ_C/δ_H 127.5/7.16. There was signal from C_2–H_2 of ferulate (FA) at δ_C/δ_H 110.4/7.32 in MSL and black liquor lignin, indicating dissolving of FA during both pretreatments. Because FA is an important component for forming lignin-carbohydrate complex (Zeng et al., 2013), the disappearance of the signal in residual lignin showed that part of LCC linkages were broken during pretreatments. In addition, it was also easy to find signals from *p*-coumaric acid ($PCA_{2,6}$, $PCA_{3,5}$ and PCA_α), end groups of cinnamyl alcohol and end groups of cinnamaldehyde (I_α, I_β, J_α and J_β) were existed in the spectra of MSL and part of signals from *p*-coumaric acid and end groups of cinnamyl alcohol were also existed in the spectra of SL, KL, SRL and KRL (Fig. 2) (Yang et al., 2014). PCA and FA were two predecessors to *p*-hydroxyphenyl units, and the ratio of PCA/FA in MSL was 1.60, indicating the predominance of PCA in wheat straw. The ratio of PCA/FA was shown in Table 3 were decreased dramatically after both pretreatments, which suggested that PCA was destroyed easier than FA.

Several ^{13}C–^{1}H correlations of tricin were identified only in MSL at δ_C/δ_H 94.1/6.38 (T_8), 99.1/6.14 (T_6), 104.0/7.24 ($T_{2',6'}$) and 105.0/7.10 (T_3). Function as antioxidants, tricin is found in most herbaceous plants in both free and conjugated forms (Li et al., 2003). A recent study suggested that tricin may be an important substructure in the herbaceous lignin complex (del Rio et al., 2012). The disappearance of the signals from tricin in lignin samples except MSL indicated that tricin was unstable, therefore was probably dissolved and destroyed completely during both pretreatments. In addition, a minor amount of signal from *p*-benzonates ($PB_{2,6}$) was detected in MSL. The disappearance of this signal in SL, KL, SRL and KRL suggested that PB was also destroyed completely during both pretreatments.

To obtain quantitative information of different substructures in the lignin, quantitative ^{13}C NMR calculation was also applied. The quantification results showed that the absolute contents of H-type lignin were 7.28, 4.44, 5.53, 0.25, 0.01 per 100 aromatic ring for MSL, SL, KL, SRL and KRL, respectively, indicating the degradation of H-type lignin during pretreatments. Cinnamates/lignin ratio represents the ratio of *p*-hydroxyphenyl units in the lignin, which was 0.12 in MSL and decreased slightly in the black liquor lignin. That ratio was decreased to only 0.01 for the two residual lignins. It indicated that most H-type lignin was dissolved, which were in accordance with FT-IR analytical results.

S/G of lignin is an important parameter to evaluate the degree of delignification (Wen et al., 2013). S/G for the five lignin samples were 0.76 (MSL, milled straw lignin), 0.70 (SL, soda lignin), 0.88 (KL, kraft lignin), 0.88 (SRL, soda-residual lignin) and 1.12 (KRL, kraft-residual lignin). It was found that the content of S and G in KL and KRL were 21.72, 24.76, 4.13 and 3.68 per 100 aromatic ring, respectively, indicating that most S and G substructures were dissolved during kraft pretreatment. While the contents of the two units were 10.69, 15.21, 10.91 and 12.39 per 100 aromatic rings for SL and SRL, respectively, which suggested that only half of S and G subunits were dissolved during soda-AQ pretreatment. Similarly, more PCA and FA were degraded during kraft pretreatment. It could be concluded again that lignin was easier to be degraded during kraft pretreatment.

3.6.2. Analysis of lignin aliphatic region

The main signals in the aliphatic region of lignin δ_C/δ_H 50–90/2.5–6.0 were from $-OCH_3$ (δ_C/δ_H 55.7/3.69), β-O-4′, β-β′, β-5′, β-1′ and 5-5′.

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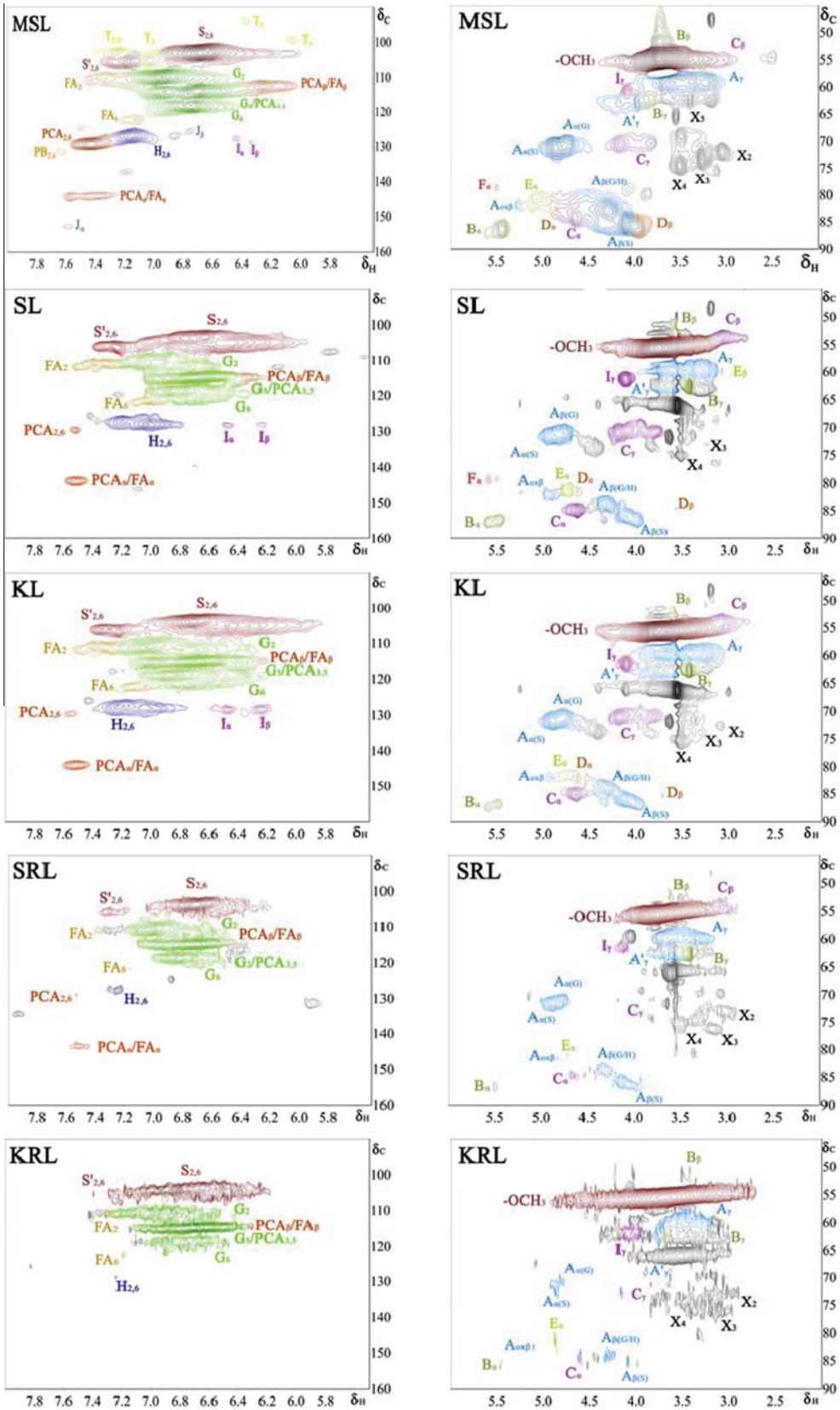

Fig. 2. Aromatic regions (left side, δ_C/δ_H 160–90/8.0–5.5) and aliphatic regions (right side, δ_C/δ_H 90–45/6.0–2.0) in HSQC spectra of the isolated lignin samples from wheat straw: (A) β-O-4′ alkyl-aryl ethers; (A′) β-O-4′ alkyl-aryl ethers with acylated γ-OH; (Aox) β-O-4′ alkyl-aryl ethers with α oxidization structure; (B) phenylcoumarane; (C) resinols; (D) dibenzodioxocins; (E) spirodienones; (F) α,β-diaryl ether; (T) tricin; (I) cinnamyl alcohol end-groups; (J) cinnamyl aldehyde end-groups; (PB) p-hydroxybenzoate substructures; (PCA)p-coumarates; (FA) ferulates; (H) *p*-hydroxyphenyl units; (G) guaiacyl units; (S) syringyl unit; (S′) oxidized syringyl units with a Cα ketone.

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The signal of δ_C/δ_H 84.3/4.29 and δ_C/δ_H 86.1/4.08 were assigned to C_β-H_β in β-O-4′ substructures linked to G and S, while the signal from α-position was shifted to δ_C/δ_H 70.7/4.75 (Aα(G)) and δ_C/δ_H 71.2/4.85 (Aα(S)). The signals of β-O-4′ from γ-position were in the region of δ_C/δ_H 60–64/3.4–4.2. Phenylcoumaran substructures (β-5′) were the second most abundant interunit in wheat straw and the crosspeaks at δ_C/δ_H 86.3/5.43 and 53.1/3.4 were ascribed to α and β-position. Along with these interunits, signals from α-position of resinols (β-β′), dibenzodioxocin (5-5′) and spirodienone (β-1′) could also be observed at δ_C/δ_H 84.2/4.65, 82.8/4.85 and 81.2/5.07, respectively. Besides, minor amount of α,β-diaryl ether substructures (Fα) could be found at δ_C/δ_H 79.2/5.5.

The absolute content of β-O-4′, β-β′, β-5′, β-1′, 5-5′ and α,β-diaryl ether could be calculated by combination of ^{13}C NMR with 2D-HSQC. The results were shown in Table 3. From the table, it was found that the main substructure in wheat straw lignin was β-O-4′ substructures, then β-5′ and β-β′ substructures, with minor content of β-1′, 5-5′ and α,β-diaryl ether substructures. Furthermore, after soda-AQ pretreatment, 34.4% of β-O-4′, 21.4% of β-β′, 4.9% of β-5, 8.6% of β-1′, none of 5-5′ and α,β-diaryl ether were left in residual lignin. While after kraft pretreatment, 22.5% of β-O-4′, 39.8% of β-β′, 23.6% of β-5′, 39.1% of β-1′, none of 5-5′ and α,β-diaryl ether were left in residual lignin. From the results, it was found that during both pretreatment, most part of β-O-4′, β-β′ and β-5′ substructures were degraded. 5-5′ and α,β-diaryl ether substructures were destroyed completely during both pretreatments. In terms of all the interunits in the aliphatic region, 28.9% and 24.7% of total side chains were left in residual lignin after soda-AQ and kraft pretreatment. It could be concluded that interunits in lignin were more degraded during kraft pretreatment.

3.6.3. Analysis of lignin carbohydrate complex

It was obvious to see signals from carbohydrate in the spectra. As signals at δ_C/δ_H 101.9/4.28, δ_C/δ_H 72.8/3.06, δ_C/δ_H 74.2/3.27, δ_C/δ_H 75.3/3.52, δ_C/δ_H 62.7/3.41 from C_1,C_2,C_3,C_4,C_5 of xylose (Cheng, 2011; Yang et al., 2014). Special attention has been paid to the study of lignin carbohydrate complex, as it might be correlated with the recalcitrance of wheat straw, which has great influence on the subsequent enzymatic hydrolysis process (Min et al., 2014a,b). Signals found in the range of δ_C/δ_H 103−99.5/5.2−4.8 were ascribed to phenyl glycoside structure (PhG), as shown in Fig. 1. The crosspeaks from benzyl ethers C_1 (BE(C_1)) and C_2 (BE(C_2)) were found at δ_C/δ_H 81/4.6 and 81/5.0 in HSQC spectra of MSL. BE(C_1) and BE(C_2) referred to linkages between the α-position of lignin and primary or secondary OH groups of carbohydrate, respectively (Balakshin et al., 2011). The obvious existence of BE (C_2) at δ_C/δ_H 80.6/5.08 demonstrated that xylan was the major carbohydrate attached to lignin side chains. The linkages between lignin and carbohydrate could also be determined by combination of ^{13}C NMR with HSQC. The phenyl glycoside (PhG) and benzyl ethers (BE(C_1) and BE(C_2)) in MSL were 3.92, 1.64 and 0.30 per 100 aromatic rings. They were all decreased after both pretreatments. Comparing the two pretreatment methods, it was found that 19.6% of PhG, 43.3% of BE(C_1) and none of BE(C_2) were left in residual lignin after soda pretreatment, while none of PhG, BE (C_1) and BE(C_2) were left after kraft pretreatment. It was suggested that all the LCC linkages were removed after kraft pretreatment.

3.7. Enzymatic hydrolysis

The aim of pretreatment applied on lignocellulosic material was to increase the extent and rate of cellulose conversion, therefore to get more glucose during enzymatic hydrolysis process. Changes of glucose content and cellulose conversion with time from wheat straw after soda-AQ or kraft pretreatment were shown in Fig. 3. As shown in the figure, glucose contents and cellulose conversion were increased rapidly within the first 24 h. At the end of enzymatic hydrolysis process, almost the same content of glucose was got from wheat straw after soda-AQ or kraft pretreatment. Compared with control, 6–7 times more glucose was got from wheat straw after pretreatment, indicating the effectiveness of soda-AQ and kraft pretreatment in enhancing the conversion of cellulose to glucose.

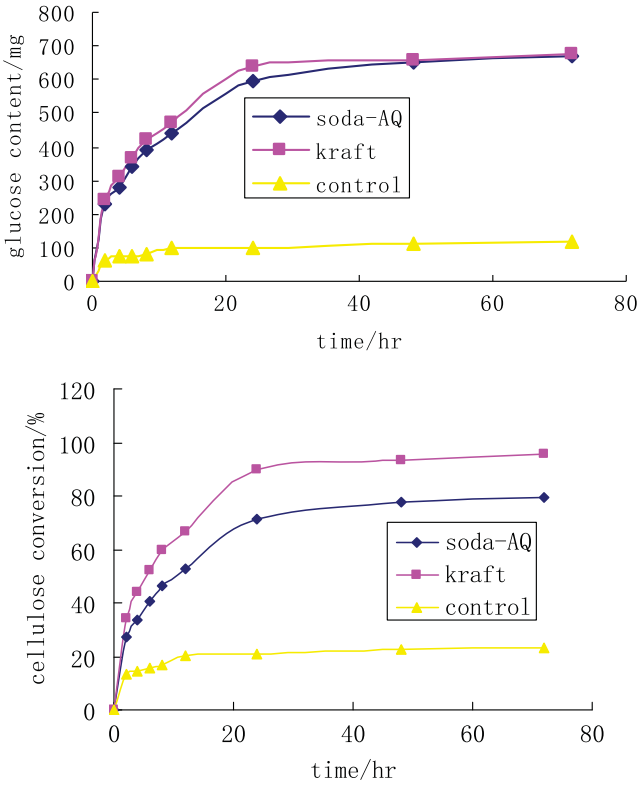

Fig. 3. Changes of glucose contents and cellulose conversion with time from wheat straw after soda-AQ or kraft pretreatment.

Although almost the same content of glucose was got from wheat straw after soda-AQ or kraft pretreatment, percentage of cellulose conversion was quite different. That was because the cellulose content in wheat straw after soda-AQ or kraft pretreatment was different. Based on the results shown before, less lignin carbohydrate complex linkages were present in wheat straw after kraft pretreatment, resulting in less cellulose content. So, cellulose conversion of wheat straw after kraft pretreatment was higher than that after soda-AQ pretreatment. At 72 h, cellulose conversions of wheat straw after soda-AQ and kraft pretreatment were 79.5% and 95.8%, respectively, compared with control which was only around 20%. The enzymatic hydrolysis results also proved that kraft pretreatment was better than soda-AQ pretreatment in terms of reducing biomass recalcitrance. Although the lignin content was all about 5% in wheat straw after both pretreatments, characteristic of lignin was different. Recent studies revealed that lignin carbohydrate complex (LCC) had effect on glucose recovery (Min et al., 2014a,b). They found that there was correlation between LCCs and enzymatic saccharification, LCCs accounting for the recalcitrance of lignocellulosic biodegradation. In the present study, less substructure and LCC linkages was left in residual lignin after kraft pretreatment, resulting in more cellulose to be reacted with cellulase.

4. Conclusion

Characteristics of lignin from wheat straw after soda-AQ and kraft pretreatment were analyzed in this project. It was found that

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after pretreatment, most lignin was degraded and dissolved in black liquor, with a little left in residual lignin. Lignin and LCC were more degraded after kraft pretreatment than soda pretreatment. Wheat straws after soda-AQ and kraft pretreatments were hydrolyzed by cellulase to get glucose. More cellulose was converted to glucose after kraft pretreatment, indicating that LCC linkages had strong negative effect on enzymatic hydrolysis of wheat straw.

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