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Structural elucidation and antioxidant activity of lignin isolated from rice straw and alkali oxygen black liquor

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ABSTRACT

Alkali oxygen cooking of lignocellulose offers lignin many structural properties and bioactivities for biorefinery. In this work, milled wood lignin (MWL) and alkali oxygen lignin (AOL) were isolated from rice straw and alkali oxygen black liquor, respectively. The lignin structure was characterized by spectroscopy and wet chemistry. Antioxidant activity of lignins was assessed by DPPH·and ABTS scavenging ability assay. Results showed the oxidization and condensation of lignin occurred during alkali oxygen cooking. The *p*-hydroxyphenyl was more easily removed from rice straw than guaiacyl and syringyl units. The ester or ether linkages derived from hydroxycynnamic acids, and the main interunit linkages, i.e. β–O–4′ bonds, were mostly cleaved. Lignin-xylan complex had high reactivity under alkali oxygen condition. Tricin, incorporated into lignin, was detected in MWL but was absent in AOL. Nitrobenzene oxidation showed MWL can well represent the protolignin of rice straw, and the products yield decreased dramatically after alkali oxygen cooking. AOL had higher radical scavenging ability than MWL indicating alkali oxygen cooking was an effective pathway for the enhancement of antioxidant activity of lignin.

1. Introduction

Black liquor, as a bioenergy resource, is the main byproduct of pulp and paper industry. It is the residue of lignin, few polysaccharides and spent chemicals produced during chemical cooking process in digesters [1]. Caustic alkali and kraft sulphate cooking, with the advantage of alkali recovery, are the common methods for wood pulp and bleached-printing, writing papers production [2]. Alkaline cooking induces the separation of carbohydrates and lignin. Simultaneously, this process is also accompanied by the degradation of carbohydrates while the lignocellulose is subjected to direct delignification using alkaline agents [3,4]. For decreasing the carbohydrates degradation and increasing the lignin removal, oxygen is often combined with alkaline cooking, which is considered to be more selective (carbohydrates yield/delignification) than kraft cooking [5]. However, the reactivity of lignocellulose especially the lignin increases in the alkali oxygen system, leading to the formation of various degradation products.

As a major constituent of black liquor and a natural aromatic polymer, lignin is an ideal biopolymer for producing chemicals and functional materials. Lignin is the most abundant aromatic polymer in lignocellulosic biomass, and is derived from three hydroxycinnamyl alcohols (*p*-coumaryl, coniferyl and sinapyl alcohols) [6]. The amorphous lignin is constituted by *p*-hydroxypenyl (H), guaiacyl (G) and syringyl (S) moieties cross-linked via β–O–4′, α–O–4′, β–5′, β–1′, 5–5′, 4–O–5′ and β–β' linkages. These structures give lignin many physicochemical properties, which can be used to produce biofuels or value-added products such as absorbents, dispersants, lignin-based resin, adhesive, gel and so on [7,8].

The structure of lignin is believed to change dramatically after alkali oxygen cooking. These structural changes may give lignin different structural properties and bioactivities for biorefinery. Isolation of lignin from black liquor is a frequently used method for structural analysis and biological activity assay. Many separation methods are reported such as acid precipitation [9], ultrafiltration [10], supercritical fluid extraction and solvent extraction [11], electrolysis [12]. In which, acid precipitation is the most common method to recover lignin because of the simple procedure and low cost.

The structure-bioactivity relationship of lignin plays an important role in the economic viability of lignocellulosic biorefinery or materials production. In order to clarify the effects of the structure of protolignin and chemical lignin on their bioactivities, in this work, the milled wood lignin (MWL) and alkali oxygen lignin (AOL) were isolated from rice

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straw and its alkali oxygen black liquor, respectively. The lignin preparations were structural characterized by spectroscopy and wet chemistry. The effects of structural changes on antioxidant activity of lignin were evaluated via determining the radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) and 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS).

2. Materials and methods

2.1. Materials

Rice straw (*Oryza sativa*) and alkali oxygen black liquor used in this work were obtained from a pulp mill in Jiangsu, China. The alkali oxygen black liquor and air-dried rice straw were stored in a refrigerator at 4 °C before use. The amount of lignin in rice straw was 20.6%, the polysaccharides glucan, xylan and arabinan accounted for 22.4%, 20.6 and 2.7%, respectively. The benzene-ethanol extractives and ash content was 2.5% and 9.3%, respectively. The information of alkali oxygen black liquor was as follows: pH, 10.0; solid content, 0.27 g/mL; density, 1.12 g/cm^3; residual alkali, 1.25 g/L; lignin, 66.7 g/L. The 1,4-dioxane for lignin extraction was purified with NaOH. Other chemicals were all analytical or reagent grade and used as received without further purification.

2.2. Lignin isolation

Lignin in black liquor was precipitated by adjusting pH to 2 with 2 mol/L H_2SO_4, and stirring for 1 h at room temperature. The crude lignin dissolved in 90% (*w*/w) acetic acid, and the soluble fraction was slowly introduced into deionized water. The precipitate was washed with deionized water (pH = 2, adjusted with 2 mol/L H_2SO_4) until the odor of acetic acid disappeared, and then was freeze-dried to obtain AOL.

The rice straw was ground in a Wiley mill. Particles between 40 mesh (0.425 mm) and 80 mesh (0.180 mm) were collected. The meals were extracted with ethanol/benzene (1:2, *v*/v) in a Soxhlet extractor for 8 h. The extractive-free sample was used for component analysis and MWL isolation. The MWL was isolated according to the procedure described by Björkman [13]. Extractive-free materials (4 g in each bowl) were milled in a planetary ball mill (Fritsch GMBH, Pulverisette 7 premium line, Idar-Oberstein, Germany) at a frequency of 10 Hz for 2 h. Two 80 mL zirconium dioxide bowls with 25 zirconium dioxide balls (1 cm diameter) in each bowl were used in the milling. An interval of 5 min was set between every 15 min of milling to prevent overheating. After ball milling, the straw powder was carefully collected and dried under vacuum. Ball-milled samples were suspended in 96% (*v*/v) 1,4-dioxane/water with a solid/liquid ratio of 1/15 (g/mL) at room temperature for 24 h. The extraction procedure was conducted in the dark and under a nitrogen atmosphere. The mixture was centrifuged and washed with 96% 1,4-dioxane/water until the filtrate was clear. Such operations were repeated thrice. The supernatants were combined and the solvent was recycled by vacuum evaporation. The crude lignin was purified by 90% acetic acid identical to the purified procedure of AOL. No further purification was performed for the preservation of the structural features of the lignin preparations.

Table 1
Main components of lignin preparations (%).

Samples	Polysaccharides			Lignin		Ash
	Glucan	Xylan	Arabinan	Klason	Acid-soluble	
MWL	1.3 ± 0.3	1.8 ± 0.4	0.1 ± 0.0	83.6 ± 0.6	3.9 ± 0.4	0.5 ± 0.0
AOL	0.8 ± 0.2	0.2 ± 0.1	0.5 ± 0.2	76.5 ± 0.4	9.1 ± 0.2	2.3 ± 0.0

Table 2
Elemental analysis of MWL and AOL (%).

Samples	C	H	N	S	O [a]
MWL	61.7	6.4	0.9	0.8	31.2
AOL	59.8	6.0	1.0	0.9	32.3

[a] The content of oxygen (O) element was calculated by the difference.

2.3. Analytical methods

The content of lignin and sugars in rice straw, MWL and AOL was analyzed according to the method described by Gu et al. [14]. Elemental analyser (Vario EL III, Elementar, Germany) was used to measure C, H, N and S content of MWL and AOL, and the O content was calculated by difference. The ultraviolet (UV) spectra of lignins were recorded on a UV spectrometer (TU-1810, Puxi, Beijing, China).

The two-dementional heteronuclear singular quantum correlation nuclear magnetic resonance (2D HSQC NMR), Fourier transform infrared spectroscopy (FTIR) spectra of lignins were recorded on an AVANCE III 600 MHz instrument (Bruker, Switzerland) and a VERTEX 80 V FTIR spectrometer (Bruker, Germany), respectively, according to the method described by our previous work [15].

Alkaline nitrobenzene oxidation (NBO) was applied to the extractive-free rice straw (40–80 mesh), MWL and AOL according to the procedure reported by Chen [16].

The DPPH· and ABTS· radical scavenging assay of MWL and AOL was performed using a spectrophotometric method. MWL and AOL was dissolved in 1,4-dioxane/water (9/1, *v*/v). The DPPH· was dissolved in anhydrous ethanol with the concentration of 6×10^{-5} mol/L. ABTS· was generated by reacting 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (7 mM) with 2.45 mM potassium persulfate ($K_2S_2O_8$) in ultrapure water and then letting the solution stand for 15 h in the dark at room temperature. The radical solution was adjusted to obtain an UV absorbance of 0.70 ± 0.02 at 517 nm and 734 nm for DPPH· and ABTS·, respectively. The lignin solution with different volume was diluted to 100 μL using 1,4-dioxane/water and then mixed with 100 μL DPPH· or ABTS·. The absorbance of tested samples was measured using a microplate spectrophotometer (Infinite M200, Kunshan, China). All measurements were performed in duplicate. The radical scavenging ability was calculated using the formula (1):

$$\text{Scavenging ability } (\%) = \left[1-(A_i-A_j)/A_0\right] \times 100\% \quad (1)$$

where A_i is the absorbance of the tested sample; A_j is the absorbance of the blank sample via 100 μL anhydrous ethanol replacing DPPH· or 100

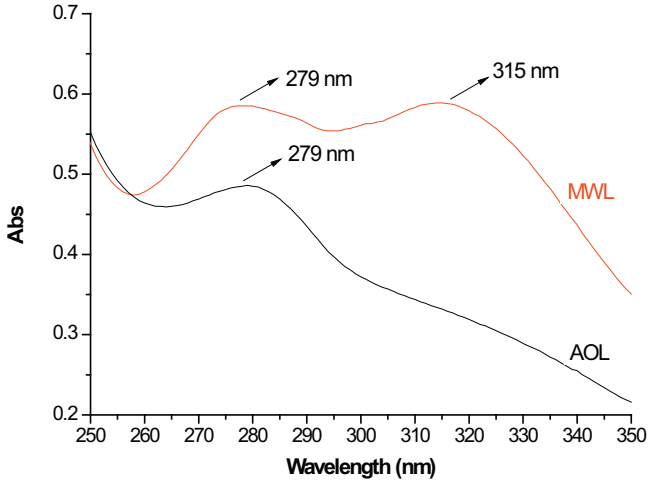

Fig. 1. UV spectra of MWL and AOL.

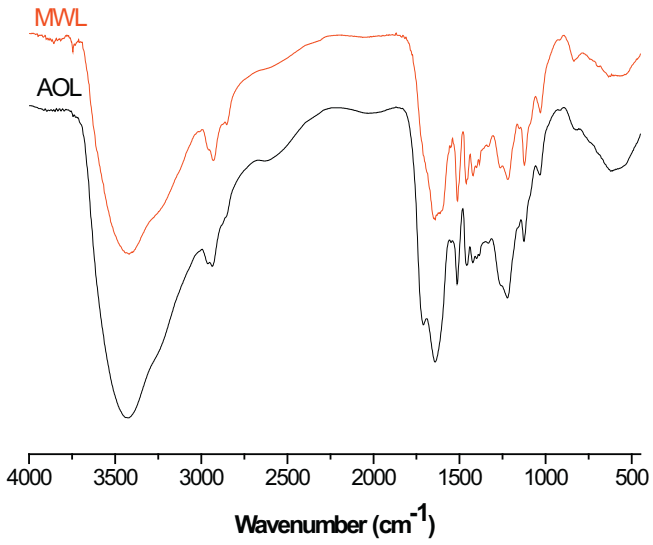

Fig. 2. FTIR spectra of MWL and AOL.

μL ultrapure water replacing ABTS·; A_0 is the absorbance of the blank sample via 100 μL anhydrous ethanol or ultrapure water replacing lignin solution.

3. Results and discussion

3.1. Chemical and elemental composition

The main chemical components of lignins are listed in Table 1. The purity of both lignin preparations was over 85%, but the MWL had higher polysaccharides than AOL. This phenomenon may have two reasons, the alkaline oxygen system has more selective for delignification thus the degradation of carbohydrates is very little. Furthermore, some water-soluble products such as hydroxy acids, volatile acids and methanol from carbohydrates may be produced during alkali oxygen cooking. The content of xylan in MWL was obviously higher than that in AOL. The lignin-xylan is the main LCC linkages especially for gramineous plants [17,18], therefore, it is reasonable to infer that lignin-xylan has high reactivity in alkali oxygen system, causing the cleavage of lignin-xylan linkages.

The carbon (C), hydrogen (H) and oxygen (O) were the primary elements in MWL and AOL, followed by minor nitrogen (N) and sulfur

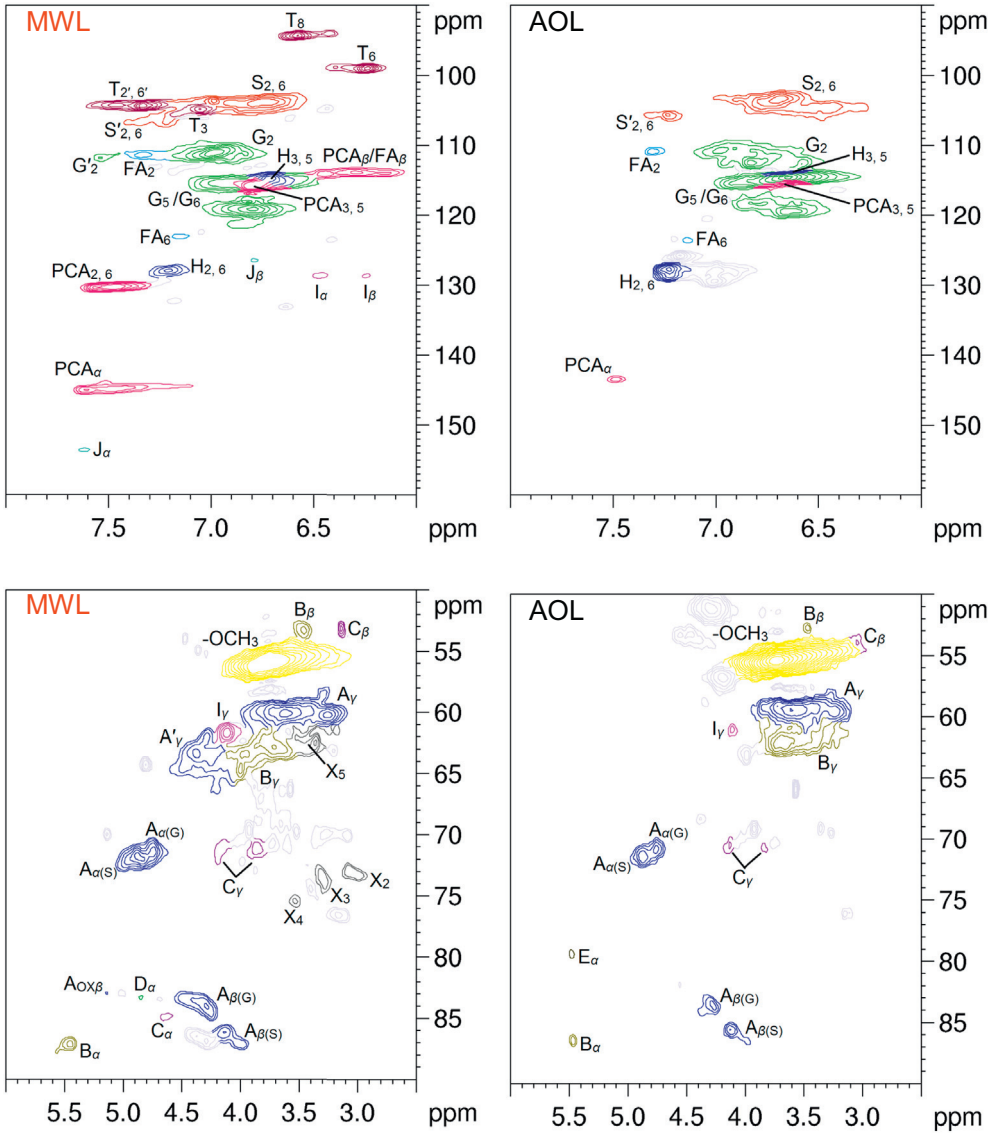

Fig. 3. The aromatic (δ_C/δ_H 90–160/6.0–8.0) and side chain (δ_C/δ_H 50–90/2.5–6.0) regions in the 2D HSQC NMR spetra of MWL and AOL.

(S) elements as shown in Table 2. The raw material was extracted by benzene-ethanol solvent and no specific step was carried out to remove protein. Therefore, the N and S elements in MWL are mainly from protein. After alkali oxygen cooking, the increase of oxygen content in AOL suggests the oxidation reaction occurred or the presence of polysaccharides and fatty acids [19].

3.2. UV and FTIR spectra

As an aromatic compound, lignin has characteristic UV absorption bands while carbohydrate has almost no absorption to UV light. Therefore, the UV spectra of MWL and AOL were acquired to investigate the structure of lignin. Fig. 1 illustrates the UV absorption spectra of MWL and AOL. Both lignins exhibit the typical UV spectra, and display a maximum absorption at about 280 nm, originating from the nonconjugated phenolic groups, which are the characteristics of guaiacyl-syingyl rich lignin [20]. The adsorption at 315 nm of MWL, derived from the conjugated phenolic groups, *p*-coumarates and ferulates, could be observed clearly. However, the signals of conjugated phenolic groups were hardly found in the curve of AOL. This phenomenon reveals that the ester or ether bonds between hydroxycynnamic acids and lignin are mostly cleaved during alkali oxygen cooking. The absorption coefficient can well reflect the conjugate degree of aromatic compounds. As shown in Fig. 1, the maximum absorption of MWL was higher than that of AOL at 280 nm under the same concentration. It is probably because the MWL had higher content of nonconjugated phenolic groups than AOL [21].

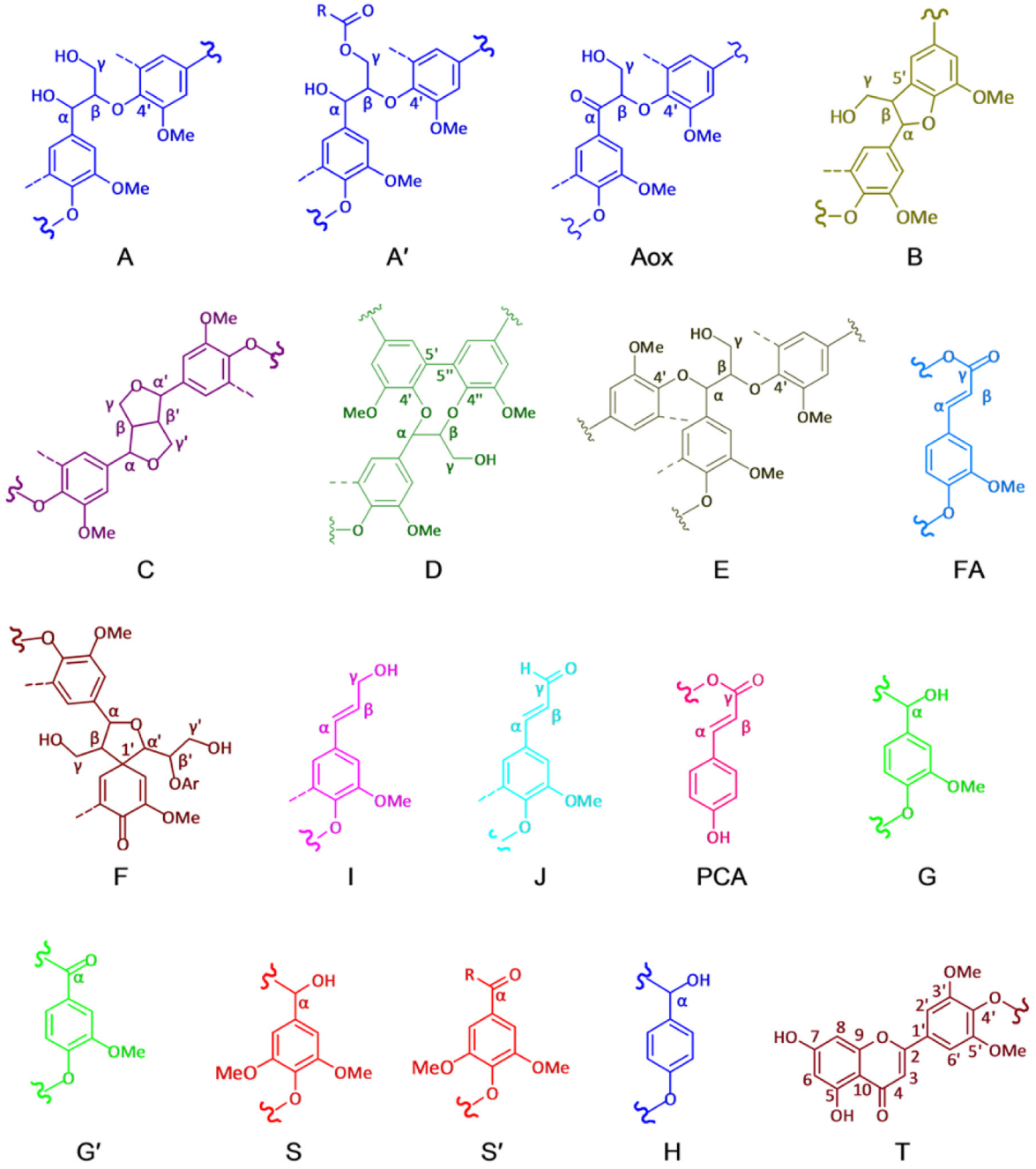

Fig. 4. Main structures present in lignin of rice straw: (A) *β*–O–4′ ethers; (A′) *β*–O–4′ ethers with acylated γ-OH; (A_{OX}) C_{α}-oxidized *β*–O–4′ structures; (B) phenylcoumarans; (C) resinols; (D) dibenzodioxocins; (E) *α,β*–diaryl ethers; (FA) ferulates; (I) cinnamyl alcohol end-groups; (J) cinnamyl aldehyde end-groups; (PCA) *p*-coumarates; (G) guaiacyl units; (G′) C_{α}-oxidized guaiacyl units; (S) syringyl units; (H) *p*-hydroxyphenyl units; (T) tricin units connected with lignin through *β*–O–4′ linkages.

Table 3
Main structural characteristics from integration of C—H correlation peaks in the HSQC spectra of MWL and AOL (%).

	MWL	AOL
Lignin interunit linkages[b]		
β—O—4′ substructures	56	7
β—5′ Phenylcoumaran substructures	2	1
Lignin aromatic units[a]		
p-Hydroxyphenyl (H)	9	27
Guaiacyl (G)	65	50
Syringyl (S)	26	23
S/G ratio	0.4	0.5
p-Hydroxycinnamates [b]		
p-Coumarates (PCA)	32	1
Ferulates (FA)	4	4
PCA/FA ratio	8.0	0.3
Flavonoid[b]		
Tricin (T)	21	0

[a] Molar percentages (H + G + S = 100).
[b] Interunit linkages, *p*-coumarate, ferulate and tricin molar contents as percentages of lignin content (H + G + S).

The FTIR spectra of MWL and AOL are shown in Fig. 2. The assignment of the main absorption bands was assigned according to the published literatures [22,23]. The absorption bands located around 1600 cm^{-1}, 1500 cm^{-1} and 1400 cm^{-1} were related to the vibrations of aromatic rings. The presence of these bands in the spectrum of AOL indicates the presence of intact aromatic rings in it. The intensity of peaks at 1710 cm^{-1} and 1648 cm^{-1} in the spectrum of MWL, attributed to stretching vibration of non-conjugate C═O and conjugate C═O, respectively, was lower than that of AOL. It proves that the oxidization of lignin occurred in the process of alkali oxygen cooking. The absorption band located 1260 cm^{-1} was attributed to stretching vibration of C—O in G units, and the intensity in AOL became higher than that in MWL. The C_5 position of G units is available for branching or some particular interunit linkages such as 5–5′, 4–O–5′, β–5′ linkages. Therefore, it suggests the condensation reaction of lignin occurred under alkali oxygen condition. The stretching vibration of C–O–C at 1160 cm^{-1} was attributed to hydroxycynnamic acids ester or ether linkages, but the intensity became weak in AOL, indicating the cleavage of ester or ether linkages occurred.

3.3. 2D HSQC NMR spectra

^{1}H–^{13}C HSQC NMR is an important tool providing a general picture of the entire lignin and LCC structure. The NMR spectra of MWL and AOL are illustrated in Fig. 3. Fig. 4 depicts the major lignin substructures shown in Fig. 3. The signals related to the structural units and various linkages were assigned according to the published literatures [17,24,25]. A semi-quantitative analysis based on HSQC signals was performed using Bruker's Topspin 2.1 processing software, and the integral method was according to the method described by del Río et al. [17]. The percentage of these structures was calculated by referring these structural signals to the total number of aromatic rings (H + G + S) [15]. The results are given in Table 3.

The main cross-signals in the aromatic region of the 2D HSQC NMR spetra of lignin mainly corresponded to the aromatic rings of H, G and S units. The prominent signals corresponded to *p*-coumarate (PCA) and ferulate (FA) structures were also observed clearly in the aromatic regions of the HSQC NMR spectra [17,24]. The G and S units were the main structures in rice straw, accounted for about 63%, 32%, respectively. The signals derived from tricin (T) were also detected in the HSQC NMR aromatic region of MWL at δ_C/δ_H 94.2/6.56, 99.0/6.21, 104.0/7.32 and 104.7/7.05 assigned to C_8–H_8, C_6–H_6, $C_{2',6'}$–$H_{2',6'}$ and C_3–H_3 correlations, respectively. Other signals in the aromatic region were observed and assigned to the *p*-hydroxycinnamyl alcohol end groups (I) and the cinnamaldehyde end goups (J).

The side chain region (δ_C/δ_H 50–90/2.5–6.0) of the 2D HSQC NMR spectra provides useful information about the interunit linkages present in lignin. As shown in Fig. 3, the correlation peaks from methoxyls and side chain in β–O–4′ substructures (A) were the most prominent in the HSQC spectra of MWL, followed by phenylcoumarans (B) and other substructures such as resinols (C), dibenzodioxocins (D), α,β–diaryl ethers (E). Polysaccharide signals, mainly originated from hemicellulose, were observed in the side chain region of the 2D HSQC spectrum of MWL. The polysaccharide cross-peak signals of X_2 (δ_C/δ_H 73.1/3.08), X_3 (δ_C/δ_H 73.9/3.31), X_4 (δ_C/δ_H 75.9/3.55), X_5 (δ_C/δ_H 62.7/3.34 and 3.95) were assigned to β–D–xylopyranoside [25], indicating that the lignin-xylan is the prominent structural form of LCC.

After alkali oxygen cooking, the structure of lignin was disrupted significantly according to the 2D HSQC NMR spectra of AOL. The correlation signals of G′ units disappeared and the content of G and S units decreased. However, the S/G ratio in AOL (0.5) was higher than that in MWL (0.4). The S units could be dissolved preferentially due to their

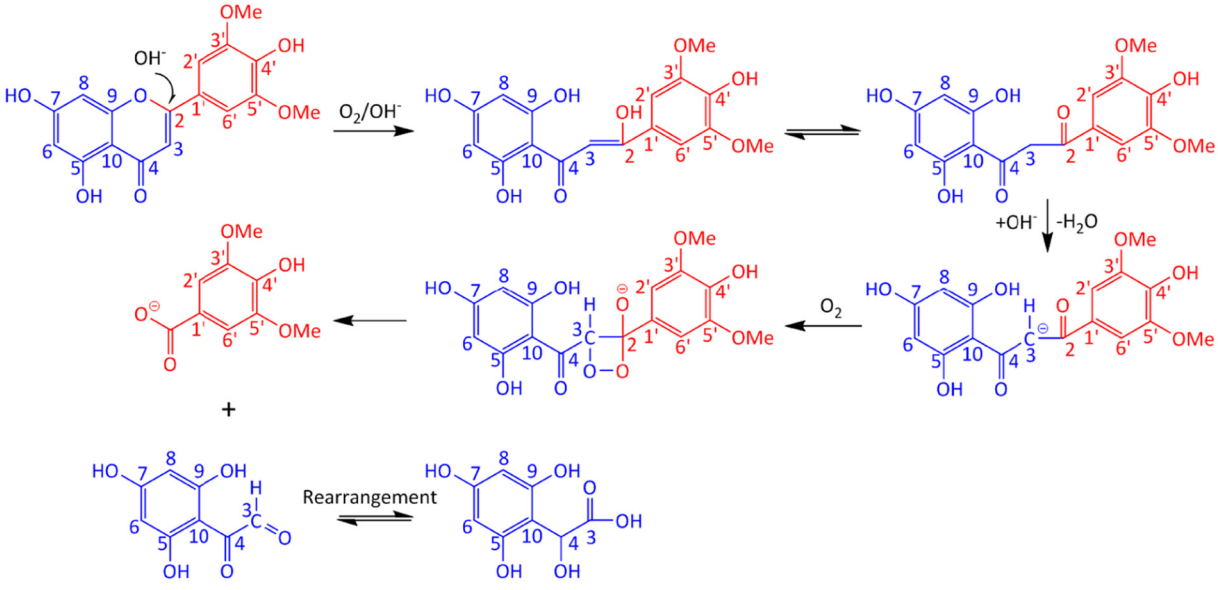

Fig. 5. Possible reaction mechanism of tricin under alkali oxygen condition.

Table 4
The yield and ratio of nitrobenzene oxidation (NBO) products of rice straw, MWL and AOL. Data are the mean of two measurements.

Samples	Yield (mmol/g-lignin)				V/S/H [a]
	V	S	H	Total	
Rice straw	0.60 ± 0.04	0.25 ± 0.04	0.51 ± 0.05	1.36 ± 0.05	44/18/38
MWL	0.59 ± 0.05	0.28 ± 0.07	0.59 ± 0.04	1.46 ± 0.05	41/19/40
AOL	0.34 ± 0.00	0.19 ± 0.02	0.29 ± 0.05	0.82 ± 0.02	41/24/35

[a] V = Vanillin + Vanillic acid; S = Syringaldehyde + Syringic acid; H = *p*-hydroxybenzaldehyde + *p*-hydroxybenzoic acid.

lower degradation temperature and be cleaved with a much faster rate than G units. Therefore, S units could be considered to have high dissolution rate in the alkali oxygen cooking process. The content of H units in AOL was much more than that in MWL, which indicates that H units were more easily dissolved in alkali oxygen system than the G and S units. The C—H correlation signals of PCA changed dramatically but the C_2–H_2 and C_6–H_6 correlations of FA remained unchanged. The C_β–H_β and $C_{2,6}$–$H_{2,6}$ correlations of PCA in MWL were clearly observed at δ_C/δ_H 113.8/6.28 and 130.1/7.47, respectively. The two correlation signals were hard to be detected after alkali oxygen cooking. However, the content of FA in AOL was similar to that in MWL. These results are caused by the different linkages of lignin with PCA and with FA. FA is always present as a bridge to crosslink lignin and carbohydrate, while PCA is just cross-linked to the side chain of lignin [26]. The side chain structures of lignin were destroyed by alkali oxygen system mainly through the cleavage of β–O–4′ linkages. The C_γ-H_γ correlations of β–O–4′ linkages clearly observed in the spectrum of MWL was hardly found in that of AOL, it implies that the ester linkages in γ-acetylated β–O–4′ substructures (A'$_\gamma$) or γ-ester LCC linkages are easily cleaved in alkali oxygen sytem. Furthermore, the correlation signals of β–D–xylopyranoside (X) were not observed in the side chain region of 2D HSQC NMR of AOL, indicating that the covalent bonds of lignin-xylan are cleaved under alkali oxygen condition. It is line with the results of component analysis (Table 1). It is reasonable to deduce that LCC has high reactivity in alkali oxygen system causing the fragmentization of LCC structures.

Interestingly, the intensive signals derived from tricin (T) were detected in MWL but were absent in AOL. Tricin monomer may dissolve in black liquor and cannot be precipitated by H_2SO_4, which gives a new insight into tricin isolation for its further application. However, many researches pointed out tricin was present in alkaline lignin [27,28]. Therefore, the structure of tricin may be disrupted under alkali oxygen condition, and the possible reaction mechanism was illustrated in Fig. 5. The ether linkages between $C_{4'}$ and C_2 positions were attacked by hydroxyl radicals (HO·), and were cleaved through nucleophilic reaction forming enol structure. The enol structure was unstable and the keto-enol tautomerism occurred. The formative structure could be readily attacked by the oxygen under alkaline condition, leading to the cleavage of the C_2–C_3 bonds in etherified structures [29]. The reaction mechanism is similar to the reverse reaction pathway of tricin chemosynthesis [30]. However, it needs to be further investigated through more detection method such as dynamic wet chemistry and spectroscopy.

3.4. Alkaline nitrobenzene oxidation

The yield and ratio of NBO products of rice straw, MWL and AOL are given in Table 4. The NBO products yield and ratio of MWL were similar to that of raw material, indicating that MWL can well represent the protolignin of rice straw. The structure of FA and PCA in rice straw is contributed to V and H units, respectively [31], causing the high content of V and H units in NBO products. After alkali oxygen cooking, the NBO products yield of AOL decreased dramatically, suggesting that AOL has higher condensation degree than MWL. The H and G units, with spare position adjacent to the phenolic hydroxyl group in benzene ring, are easily cross-linked with reactive functional groups. However, it is more difficult for S units to react with other groups since it has two –OCH_3 groups [32]. Therefore, the S/V ratio of AOL was higher than that of MWL. Additionally, the PCA/FA ratio was about 8.0 in MWL (Table 3), and the PCA was more easily removed than FA under alkaline condition. Therefore, the drop of yield of H units (43%) was more severe than that of V units (39%), resulting in the lower H/V ratio in AOL than that in MWL.

3.5. Assessment of radical scavenging ability

The bioactivity especially antioxidant activity of lignin plays an important role in the process of plant growth, which bears outside pressure and has potential application on agriculture [33,34]. The antioxidant activity of lignin is directly related to the structure, thus the structural changes of lignin inevitably cause the changes of the bioactivity. As shown in Fig. 6, the DPPH· and ABTS· scavenging ability of both MWL and AOL increased with lignin concentration. Comparatively, the DPPH· and ABTS· scavenging ability of AOL was obviously higher than that of MWL, which means that alkali oxygen cooking is an effective method for the enhancement of antioxidant activity of lignin. The structural changes of H, G, S units and functional groups such as condensation degree, methoxy, phenolic hydroxyl and carboxy hydroxyl are the potential reasons. Dizhbite et al. [35] pointed out that non-etherified phenolic –OH groups, ortho-methoxy groups, hydroxyl groups and the double bond between the outermost carbon atoms in the side chain contributed the radical scavenging ability. In this work, the increase of non-etherified phenolic –OH groups and carboxy groups content in AOL caused by the cleavage of β–O–4′ linkages and the oxidation under alkali oxygen cooking is the main reason.

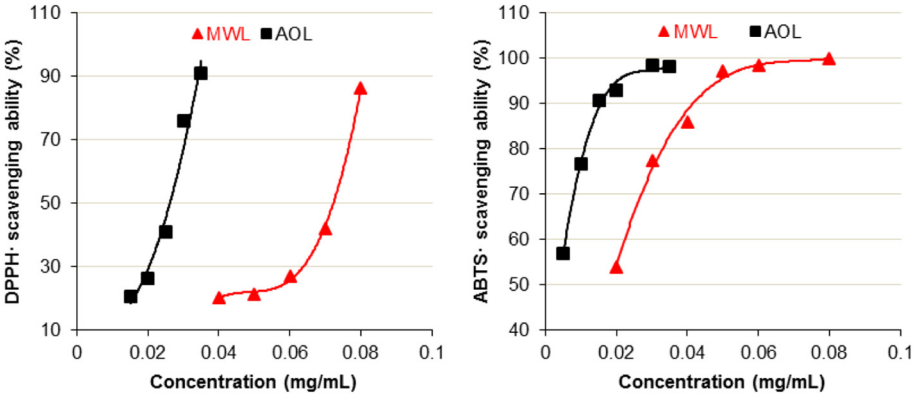

Fig. 6. The DPPH· and ABTS· scavenging ability of MWL and AOL.

4. Conclusions

The alkaline cooking with oxygen represents a combined delignification process but leads to structural and bioactive changes of lignin. The increased oxygen content and high condensation degree of alkali oxygen lignin (AOL) suggest the occurrence of oxidization and condensation, causing a higher syringyl/guaiacyl (S/G) ratio compared with that of milled wood lignin (MWL). The *p*-hydroxyphenyl units are more easily removed from rice straw than G and S units. The signals derived from tricin are found in rice straw MWL but absent in AOL, it implies that tricin is destroyed under alkali oxygen condition. Lignin-xylan complex has high reactivity under alkali oxygen condition. Alkali oxygen cooking is an effective pathway for the enhancement of antioxidant activity of lignin.

Acknowledgments

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