CHARACTERIZATION OF EXTRACTED LIGNIN OF BAMBOO (*NEOSINOCALAMUS AFFINIS*) PRETREATED WITH SODIUM HYDROXIDE/UREA SOLUTION AT LOW TEMPERATURE

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Ball-milled bamboo (*Neosinocalamus affinis)* was first treated under ultrasound at 20 °C in 95% ethanol solution for 0 to 50 min, dissolved in sodium hydroxide/urea solution (7% NaOH/12% urea) at –12 °C, and then extracted with ethanol and dioxane to isolate lignin. The structure of the isolated lignin was characterized with a set of wet chemical and spectroscopic methods, including UV, FT-IR, ^{13}C NMR, and HSQC spectroscopies. The results showed that the lignin extracted from bamboo consisted of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) type lignins with minor cinnamate units. The predominate lignin inter-units were β-*O*-4´ ether linkages, followed by phenylcoumaran and a lower proportion of resinol and spirodienone. It was also found that the ester groups of lignin were cleaved during the pretreatment process with cold alkaline solution.

Keywords: ^{13}C NMR; Bamboo; Heteronuclear single quantum correlation (HSQC); Lignin structure

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INTRODUCTION

Lignocellulosic biomass is mainly composed of cellulose, hemicelluloses, and lignin. The predominant component of lignocellulosic biomass is cellulose, a β (1-4)-linked chain of glucose molecules. Hemicelluloses are composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose, and xylose. Lignin, accounting for about one fourth of the lignocellulosic biomass, is the third most abundant biopolymer after cellulose and hemicelluloses. Lignin is polymerized of three phenyl-propanoid units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). Hardwood lignins consist principally of G and S units, and trace amount of H units, while softwood lignins are composed mostly of G units and low levels of H units. Lignins from grasses incorporate G and S units at comparative levels, and they have more H units than wood. It is speculated that the carbohydrate fraction (hemicelluloses and cellulose) of lingo-cellulosic biomass is covalently linked to lignin through lignin-carbohydrate bonds.

The major challenge of lignocellulosic biomass as a feedstock is the development of cost-effective methods to refine and transform it into chemicals and fuels. To increase the economic viability of the biomass conversion, production of multiple products is necessary for the process, which is consistent with the concept of a biorefinery–integrating conversion of biomass to fuels, power, and chemicals (National Renewable

Energy Laboratory 2008). Examples of chemicals produced from a biorefinery include ethanol, methanol, furfural, paper, lignin, vanillin, lactic acid, DMSO, xylitol, etc. In most of the process, using biomass as a feedstock for chemical production requires an initial step to separate or fractionate the main components to obtain usable fractions, aiming to maximize the usage (Ragauskas et al. 2006).

Traditionally, the extraction of lignin is mainly achieved through chemical pulping processes. In these processes, except for organic acid pulping, most of the lignin is liberated under high temperature and pressure, with a high energy cost. For the kraft pulping process, which accounts for 80% of the chemical pulp production (Kroschwitz and Howe-Grant 2001), wood or other plant fiber is cooked in a solution of sodium hydroxide and sodium sulfide at a temperature around 165 ^{o}C. Under such a high temperature, lignin undergoes extensive degradation due to the cleavage of α-aryl ether and β-aryl ether linkages (Chakar and Ragauskas 2004). Organosolv pulping processes, including ethanol, methanol pulping, a set of novel technologies developed in the past decades, makes it possible to obtain lignin with only minor changes compared to its native form in the tree. The organic acid pulping process is operated under mild conditions, but the corrosiveness of the acid is a major drawback. In addition, the energy usage and solvent recovery have not been fully investigated (Delmas 2008).

Recently, cold sodium hydroxide solution and sodium hydroxide/urea solution (generally 7% NaOH/12% urea solution at –12 ^{o}C), which are capable of breaking linkages between cellulose, have been used to dissolve cellulose (Łaszkiewicz 1998; Le Moigne and Navard 2010; Qi et al. 2008; Zhou et al. 2004), and to serve as solution for producing regenerated cellulose (Zhou et al. 2002) and homogeneous synthesis cellulose derivatives (Qi et al. 2009; Yan et al. 2009). However, little attention has been paid to their applications in conversion of lignocellulosic biomass into bio-based products. Raw plant fibers (low content of lignin and hemicelluloses), such as flax, abaca, sisal, and ramie were treated in 5% NaOH solution at –5 ^{o}C by Cuissinat and Navard (2008), and the researchers found that the fibers swelled homogeneously but did not become dissolved. In the effect of sodium hydroxide pretreatment on spruce, Zhao et al. (2008) found that the pretreatment chemicals, either NaOH alone or NaOH/urea mixture solution at low temperature, disrupted the connections between hemicelluloses, cellulose, and lignin, removing minor amounts of these components, which made cellulose more accessible to hydrolysis by enzymes.

Bamboo is a perennial woody grass belonging to the *Gramineae* family and Bambuseae subfamily encompassing about 1250 species within 75 genera, distributed mainly in China, India, Thailand, Bangladesh, Indonesia, and South Korea (Scurlock et al. 2000), with a total annual production of 6 to 7 million tons. The culms of bamboo are reproduced asexually every year with a rapid growth rate of sprouts. Because of their high productivity, the development of effective utilization of the plant materials has attracted more and more attention. Traditionally, bamboos have been used in construction and as reinforcement fibers for paper, textiles, and board. Recently, much attention has been paid to energy feedstock (Kobayashi et al. 2004; Zhang et al. 2007) and bio-based materials (He et al. 2009; Shao et al. 2009).

The aim of this research was to investigate lignin extracted from bamboo with little modification under mild conditions. Ball-milled bamboo was pretreated under

ultrasound, then treated with cold sodium hydroxide/urea solution, and then extracted with neutral solvents to isolate bamboo lignin. Moreover, the structure of the isolated lignin was characterized by a set of wet chemistry and spectroscopy methods, including Ultraviolet (UV), Fourier Transform Infrared Spectroscopy (FT IR), ^{13}C Nuclear Magnetic Resonance (^{13}C NMR), and Heteronuclear Single Quantum Correlation (HSQC) spectroscopy.

EXPERIMENTAL

Materials

Bamboo (*Neosinocalamus affinis*) was collected from the experimental farm of North-Western University of Agriculture and Forestry, Yangling, China. The air-dried clums of bamboo were cut to pieces, then ground and sieved to obtain a 40-60 mesh fraction. This fraction was subjected to extraction with methylbenzene/ethanol (2:1, v/v) in a Soxhlet apparatus for 6 h, and then air-dried. The composition of the dewaxed bamboo was glucose 50.82%, xylose 22.94%, arabinose 1.13%, galactose 0.51%, mannose 0.37%, rhamnose 0.02%, glucuronic acid 0.94%, lignin 19.46% (Klason lignin 16.97% and acid-soluble lignin 2.49%), and ash 2.52%, determined according to the sugar analysis method previously described (She et al. 2010) and Tappi standards T 222 om-02 and T211 om-07. All of the chemicals used to isolate lignin were of analytical-reagent grade and purchased from Beijing Chemical Reagent Company, China.

Isolation of Lignin

A scheme for pretreatment and separation of bamboo lignin is shown in Fig 1. The dewaxed sample was ball-milled with a rotary ball mill at room temperature for 48 h. Briefly, the ball milling was performed in a 2 L porcelain jar in the presence of 68 porcelain balls (15mm in diameter), which occupied 20% of the active jar volume. Sample of 10 g was loaded into the jar, creating a porcelain ball to a sample weight ratio of 28.8. The jar was rotated at the speed of 70 rpm. After that, the milled sample of 3.3 g (oven-dried weight) was suspended in 75 mL of ethanol (95%) solution for 5 min, and then the ultrasonic irradiation pretreatment was performed in a ultrasonic cell crusher machine (SCIENT-D, produced by Ningbo Xinzhi Biotechnology Corporation, China) with a sonic power of 100 W at 20 ^{o}C for 0, 5, 15, 25, 35, and 50 min, respectively. Next, the samples were filtered, and the residues were air-dried, respectively. The six residues were submitted to treatment as follows. For each treatment, 65 mL 7% NaOH/12% urea aqueous solution was pre-cooled to –12 ^{o}C, and the sample was dispersed into it and then stirred vigorously at a rotational speed of 1200 rpm for 10 min at –12 ^{o}C. Subsequently, the mixture was neutralized with 10% H_2SO_4 to pH=7. The solid was precipitated from the aqueous mixture, and then it was washed with water for 5 times and filtered. Next, the recovered solid samples were subjected to successive extraction of lignin. Briefly, the extraction process was performed with 75 mL 95% ethanol at 75 ^{o}C for 3 h, and then with 75 mL dioxane at 75 ^{o}C for 3 h. After that, the ethanol solution and dioxane solution were combined and rotator-evaporated to concentrate it to 4–5 mL under reduced pressure at 45 ^{o}C. Then the concentrated solution was dipped into acidic water (pH=2) and

centrifuged to obtain precipitated lignin fractions (Note L0, L1, L2, L3, L4, and L5 corresponding to sample pretreated with ultrasound time of 0, 5, 15, 25, 35, and 50 min). Finally, the fractions were freeze-dried for weight and structural characterization without further purification.

Structural Characterization of Lignin

Structural characterization of lignin was performed referring to previous studies (She et al. 2010; Lin and Dence 1992) with some modifications. The procedures were briefly introduced as follow. The phenolic acids and aldehydes, liberated by alkaline nitrobenzene oxidation of the lignin preparations, were determined by high-performance liquid chromatography (HPLC, Agilent). Identification of the individual compounds was made at 280 nm by computer comparison of the retention times and peak areas with reference to well characterized phenolics.

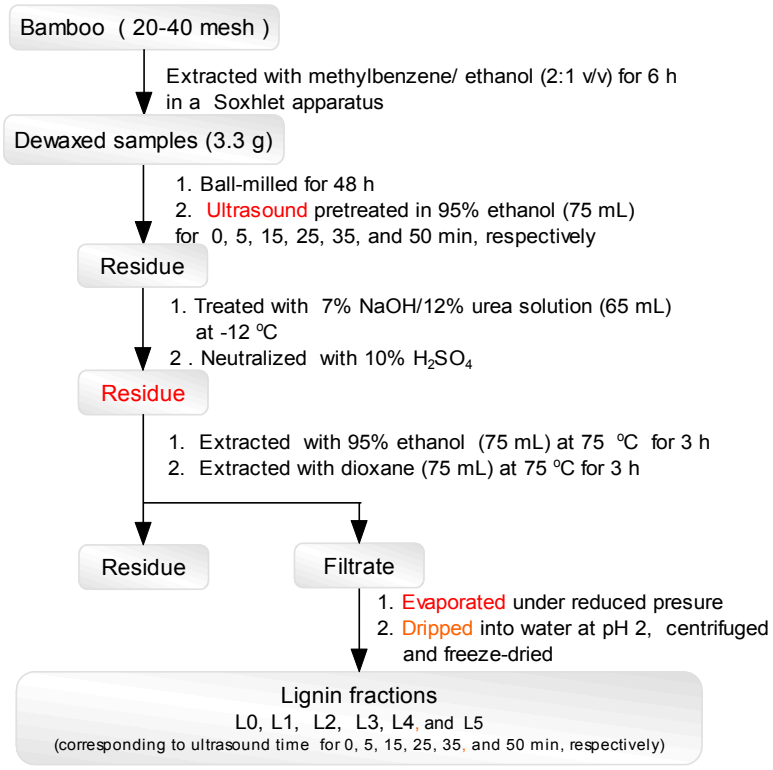

Fig. 1. Scheme for extraction of lignin from bamboo

The molecular-average weight of lignin fractions was determined by Gel Permeation Chromatography (GPC) on a PL-gel 10 mm Mixed-D column. Before the analysis, lignin samples were acetylated with acetic anhydride. Then the samples were dissolved with tetrahydrofuran (THF) with a concentration of 0.2%, and 20 µL solution was injected. The column was operated at 25 ^{o}C and eluted with THF at a flow rate of 1 mL min^{-1}. The column was calibrated using polystyrene standards.

UV spectroscopy was recorded in ethanol solution on UV 2300 (Shanghai Tianmei Science and Technology Corporation, China) using 1 cm cells. The

concentration of lignin sample was 0.001 mg mL^{-1}, and the absorption coefficients were calculated.

FT IR spectra were obtained on a Bruker spectrophotometer using KBr disks containing 1% finely ground samples. Thirty-two scans were taken of each sample recorded from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1} in the transmission mode.

The solution-state ^{13}C NMR spectroscopy was carried out on a Bruker AV III 400 MHz NMR spectrometer. The sample (40 mg) was dissolved in 0.5 mL of dimethyl sulfoxide-d_6 (DMSO, 99.8%), and the spectrum was recorded at 25 ^{o}C after 30 000 scans. A 30 ^{o} pulse flipping angle, a 9.2 μs pulse width, a 1.89 s delay time between scans, an 1.63 s AQ, and a 2 s relaxation time were used.

2D HSQC spectroscopic results were acquired on the same spectrometer in the HSQC GE experiment mode. The spectral widths were 1 800 Hz and 10 000 Hz for the ^{1}H- and ^{13}C-dimensions, respectively. A 128 scanning time (NS), a 2.6 s delay time between transients, and a 1.5 s relaxation time were used. The $^{1}J_{C\text{-}H}$ used was 145 Hz. The central solvent (DMSO) peak was used as an internal chemical shift reference point (δ_C 39.5; δ_H 2.49 ppm). Data processing was performed with standard Bruker Topspin-NMR software.

RESULTS AND DISCUSSION

Yield and Molecular Weight

The ball-milled bamboo was subjected to pretreatment with ultrasound, followed by treatment with cold sodium hydroxide/urea solution and then neutral solution extraction. The yields of extracted lignin (% raw material lignin) obtained were 14.4, 13.4, 16.4, 13.4, 14.9, and 13.9 % for samples under different ultrasound times of 0, 5, 15, 25, 35, and 50 min, respectively. Obviously, the yield of extracted lignin was between 13.4% and 16.4%, and it varied non-significantly with the increment of ultrasound time. To facilitate the separation of the main components of lignocelluloses, three novel assisted separation technologies, including ball-milling, ultrasonic pretreatment, and cold sodium hydroxide/urea pretreatment were utilized. Ball-milling decreased the degree of polymerization (DP) of cellulose and was able to deform the lattice of cellulose to form more amorphous area. Ultrasonic pretreatment disrupted the structure of the wall mainly by the collapse of the bubbles produced by cavitations. In the cold sodium hydroxide/urea pretreatment process, the connection between cellulose, hemicelluloses, and lignin was only partially disrupted to some extent with a minor amount of the main components released. In research reported by Zhao et al. (2008), when spruce was subjected to treatment in NaOH/urea solution at a low temperature, the wood fiber bundles with small and loose lignocellulosic particles were soaked with 7% NaOH/12% urea solution for 24h, resulting in 20% of the original lignin released, and the enzymatic hydrolysis efficiency of the residue was enhanced notably. Usually, the yields of prepared milled wood lignin (MWL) are 20 to 50% (Hu et al. 2006). The low yield in the present study was probably due to the limited effect of ball milling produced from the rotary ball mill. Actually, different types of milling machines produce dissimilar breaking force to the sample, resulting in varied yields of the extracted lignin during the MWL preparation process

(Holtman 2003). It has been reported that when lignocellulosic materials were treated with different scales of machines, the yields of MWL varied widely from 1 to 30% (of Klason lignin) (Sato et al. 1976). In addition, Glasser et al. (1979) reported a low MWL yield of 9.2% when sample was prepared with a rotary ball mill placed with porcelains balls for 13 days. Therefore, to improve the yield of lignin, improvement of the efficiency of ball milling with proper machine is the major concern in a future study.

The molecular weight and polydispersity of extracted lignin of bamboo is shown in Table 1. As can be seen, the weight-average molecular weight M_W was between 5600 and 6400 g mol^{-1}, and number-average molecular weight (M_N) ranged from 3400 to 4000 g mol^{-1}. Obviously, no significant decreases of the molecular weight and DP with increasing of the ultrasound time were found.

Alkaline Nitrobenzene Oxidation Products

Table 2 summarizes the results concerning the analysis of phenolic acids and aldehydes from the lignin fractions, obtained by alkaline nitrobenzene oxidation at 170 ^{o}C for 2.5 h. Obviously, the predominant oxidation products were found to be vanillin and syringic acid, which resulted from the degradation of non-condensed guaiacyl (G) and syringyl (S) units, respectively. The relatively high amount of syringic acid was due to the high temperature of the oxidation in the present study. The presence of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid, suggested the incorporation of H unit in the lignin fractions. The total yield of phenolic acids and aldehydes released from lignin fractions prepared with ultrasonic treatment (L1–L5) were 17.63 to 26.42%, as compared to a low yield of 12.62% from the lignin fraction prepared without ultrasonic treatment (L0). Commonly, the yield of the released products is ~25% (based on Klason lignin) in alkaline nitrobenzene oxidation (Lin and Dence 1992). The lower yield of the released products from L0 was probably due to the higher amounts of bound hemicelluloses in this lignin fraction. The S/G values for all the lignin fractions were 0.74 to 0.83 (varied non-significantly), while H/G values for the fractions prepared with ultrasonic treatment varied from 0.71 to 1.21, higher than that of the fraction prepared without ultrasonic treatment (0.46). As can be seen, the H units accounted for 17 to 30% of the overall lignin units in the lignin fractions. A relatively high proportion of H units has been observed in MWL extracted from *Phyllostachys pubescens*, which accounted for 6~14% of the overall lignin units under varied experiment conditions (Shao et al. 2008; Tai et al. 1990). In addition, minor amounts of *p*-coumaric acid and ferulic acid were detected, which is in agreement with NMR spectra results afterwards.

Table 1. The Molecular Weight and Polydispersity (DP) of Bamboo Lignin

Sample	M_W (g mol^{-1})	M_N (g mol^{-1})	DP
L0	6 360	3 560	1.8
L1	5 950	3 670	1.6
L2	6 650	3 700	1.8
L3	6 480	3 930	1.6
L4	5 650	3 460	1.6
L5	5 610	3 640	1.5

Codes L0, L1, L2, L3, L4, and L5 correspond to samples with ultrasound times of 0, 5, 15, 25, 35, and 50 min.

Table 2. The Yield (% Dry Sample, w/w) of Phenolic Acids and Aldehydes from Alkaline Nitrobenzene Oxidation of Lignin Fractions

Phenolic Acids and Aldehydes	L0	L1	L2	L3	L4	L5
p-Hydroxybenzoic acid	0.36	3.56	3.16	3.14	1.37	4.01
p-Hydroxybenzaldehyde	1.20	1.42	1.73	1.43	1.80	1.52
Vanillic acid	1.18	1.33	2.16	1.62	1.85	1.54
Vanillin	3.58	4.09	7.09	5.35	5.83	4.92
Syringic acid	3.27	3.71	6.22	4.70	5.24	4.35
Syringaldehyde	1.90	1.95	2.94	2.43	2.71	2.75
Acetovanillone	0.52	0.74	0.66	0.71	0.92	0.70
Acetosyringone	0.11	0.22	0.49	0.30	0.21	0.25
p-Coumaric acid	0.12	0.19	0.87	0.22	0.32	0.24
Ferulic acid	0.38	0.42	1.10	0.31	0.49	0.71
Molar ratio (g:s:h)[a]	46:37:17	38:28:34	41:34:24	40:31:29	45:35:20	37:30:33

[a] g represents the sum of total moles of vanillin, vanillic acid, and acetovanillone; s represents the sum of total moles of syringaldehyde, syringic acid, and acetosyringone; and h represents the sum of total moles of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid.

UV Spectra

The UV spectra of bamboo lignin fractions showed similar bands to those of other annual plants (Seca et al. 1998, 2000) (Fig. 2), which are characterized by a sharp maximum at 205 nm, a shoulder at 235 nm, and two low maxima at 282 and 312 nm, respectively. The absorption at 205 nm is assigned to the $\pi \rightarrow \pi^*$ transition in the aromatic ring. In addition, the absorption at 282 nm, corresponding to the $\pi \rightarrow \pi^*$ electronic transition in the aromatic ring of the unconjuated phenolic units, is indicative of free and etherified hydroxyl groups. This result was consistent with the relatively high proportion of guaiacyl units in lignin characterized with other analysis. The absorption at 312 nm, assigned to the $n \rightarrow \pi^*$ transition in lignin units containing C_α=O groups and $\pi \rightarrow \pi^*$ transition in lignin units with C_α=C_β linkages conjugated to the aromatic ring, is indicative of ferulic and *p*-coumaric acids type structures (Sun et al. 2003), which was in agreement with the FT IR spectra. The higher value of extinction coefficients at 282 and 312 nm of 17.7 and 16.6 L g^{-1} cm^{-1} revealed that the lignin had more phenolic groups and C=O and C_α=C_β linkages conjugated with the aromatic ring, which was in agreement with previous research on MWL of bamboo (Tai et al. 1990) and other straws (Oliveira et al. 2009; Seca et al. 2000).

FT IR Spectra

FT IR spectra of lignin are shown in Fig. 3, and the peaks were identified by comparing their wavenumbers with literature data (Faix 1991). In the FT IR spectra, the peaks and the absorption intensity of the six samples were rather similar, indicating an analogous structure of the lignin samples. The absorption at 3400 cm^{-1} is attributed to OH stretch, and 2926, 2853 cm^{-1} is assigned to CH stretch in CH_2 and CH_3 groups, respectively. Absorption of 1460 cm^{-1} is due to methoxyl C-H deformations and bending of lignin. The signal at 1385 cm^{-1} is assigned to aliphatic in CH stretch in CH_3, and aromatic skeleton vibration occurs at 1595, 1510 and 1423 cm^{-1}.

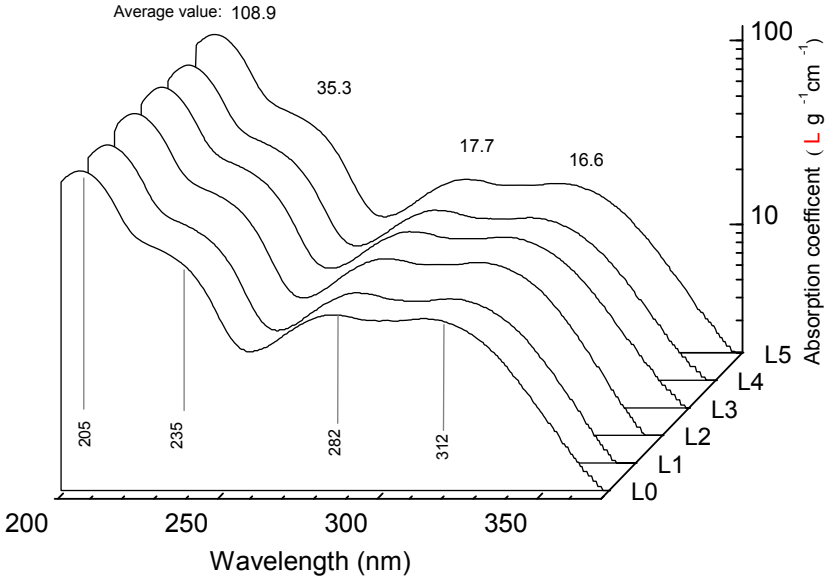

Fig. 2. UV spectra of bamboo lignins

The bands at 1125 cm^{-1} and 832 cm^{-1} and shoulder at 1158 cm^{-1} in lignin indicate a typical structure of lignin with *p*-hydroxy phenylpropane (H), guaiacyl (G), and syringyl (S) units. The shoulder at 1158 cm^{-1}, corresponding to C=O in an ester group (conjugated), gives signals for typical H G S lignins; while 833 cm^{-1} is assigned to C-H out of plane in positions of 2 and 6 of S and in all position of H units. Furthermore, syringyl ring signals occur at 1328 , 1224, and 1127 cm^{-1}, while guaiacyl units exhibit absorption at 1267 cm^{-1} (Fengel and Shao 1985). The absorption of 876 cm^{-1} is attributed to C-H out-of-plane bending in position 2, 5, and 6 of G units.

The weak signal of 1356 cm^{-1}, corresponding to free phenolic OH groups, indicated that a partial cleavage of aryl-ether of lignin in the separation process, and the degree of degradation was lower compared to the MWL of bamboo (Shao et al. 2008). The shoulder peak at 1666 cm^{-1} is attributed to C=O stretch on conjugated *p*- substituted aryl ketones, which is absent in normal bamboo lignin but occurs in wood MWLs. This structure was also found in bamboo extract with an acidified solution of TFA (Fengel and Shao 1985). Compared to the bamboo MWL, the absence of absorption at 1720 cm^{-1}, corresponding to the unconjugated C=O stretch, indicated that the ester groups of lignin was cleaved during the pretreatment with alkaline solution. Similar results was also found in the NaOH extracted lignin from bamboo at room temperature (Fengel and Shao 1985).

^{13}C NMR Spectrum

Structural elucidation for the bamboo lignin (sample L4) was carried out by ^{13}C NMR spectroscopy. The spectral data are presented in Fig 4, and peak assignment based on literature values is summarized in Table 3 by comparing literature data (Capanema et al. 2005; Holtman et al. 2006).

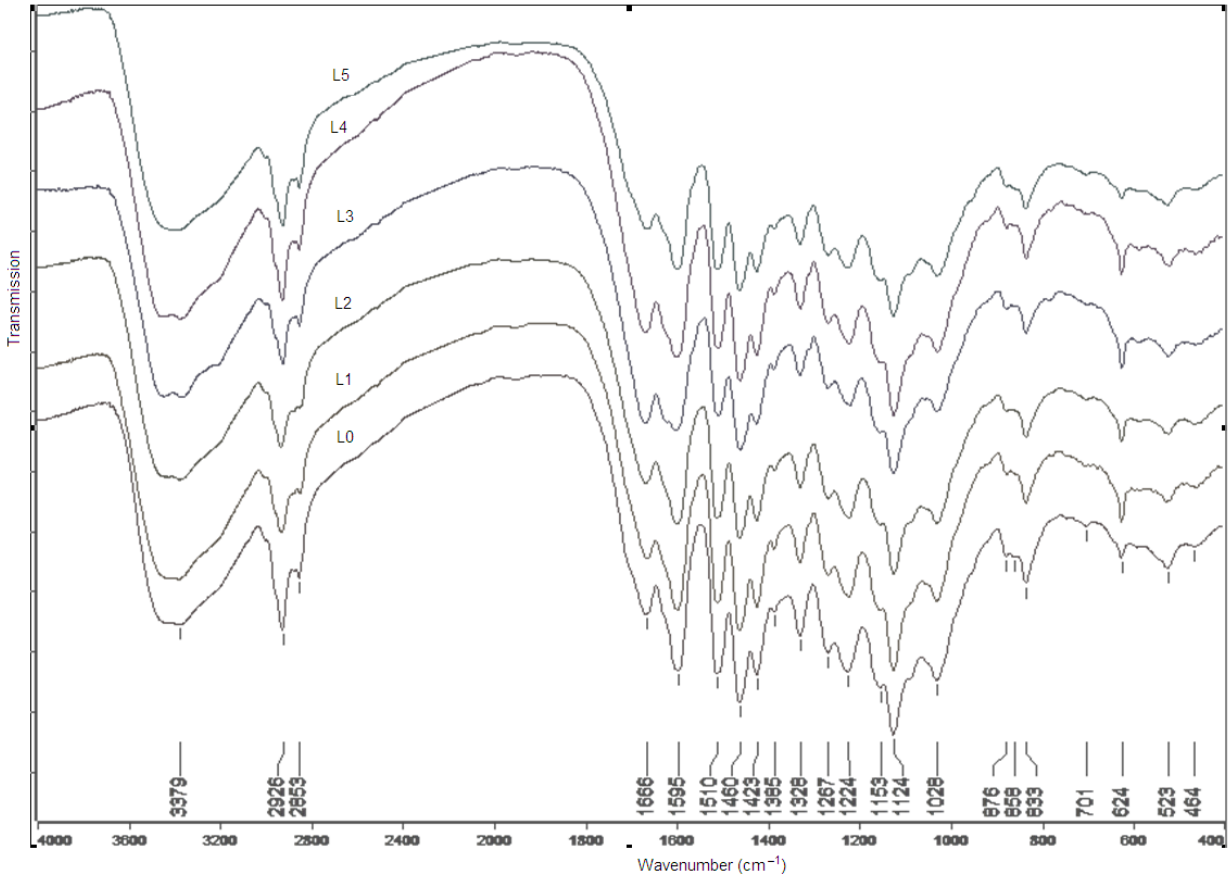

Fig. 3. FT IR spectra of bamboo lignins

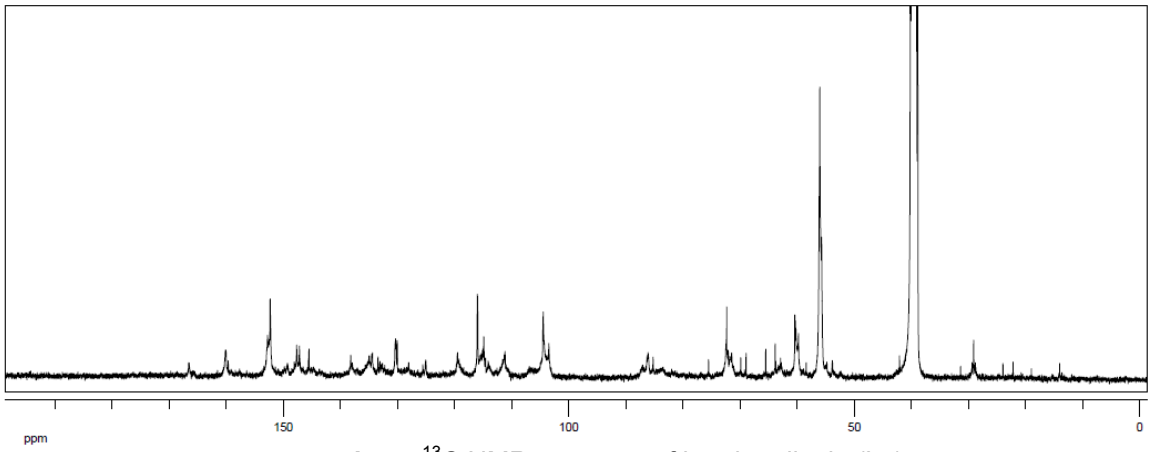

Fig. 4. ^{13}C NMR spectrum of bamboo lignin (L4)

The presence of spirodieneone units is confirmed by the weak signal of peak in the region δ 183–181 ppm. The absence of a signal at δ 173–168 ppm is attributed to the absence of aliphatic COOR, while the weak signal at δ 168–166 ppm is attributed to the structure of conjugated C=O in COOR, resulting from coumaric acid and ferulic acid. It

was confirmed by FT IR that more conjugated C=O structure existed in bamboo lignin. The absence of typical polysaccharide signals between δ 90 and 103 ppm in the ^{13}C NMR spectrum confirmed the low content of associated polysaccharides.

Table 3. Signal Assignment of ^{13}C NMR Spectrum of Bamboo lignin (L4)

Chemical Shift Range (ppm)	Assignments
183–181	C=O in spirodienone unit
168–166	C=O in conjugated COOR
162–158	C_4 in H unit
155–142	C_3/C_4 in G units, C_3/C_5 in S units, C_α in cinnamate
140–123	C_1 in G, S, and H units, C_4 in S, $C_{2/6}$ in H
123–118	C_6 in G units
117–113	C_5 in G, $C_{3/5}$ in H, C_β in cinnamate
113–110	C_2 in G units
109–103	C_2/C_6 in S units
90–78	C_β in β-O-4, C_α in β-5 and β-β
78–67	C_α in β-O-4
65–62	C_γ in β-5 and β-O-4 with C_α=O in G and S units
61–57	C_γ in β-O-4 without C_α=O
56–54	Methoxyl
53–51	C_β in β-β and C_β in β-5 structures

The peak at δ 160 ppm is indicative of *p*-hydroxyphenyl (H) units. The characterization of guaiacyl (G) and syringyl (S) units in the lignin is identified by the peaks in the regions 125–110 ppm and 109–103 ppm, respectively. Tai et al. (1990) reported that the bamboo MWL and Kraft lignin were composed of syringyl, guaiacyl, and *p*-hydroxycinnamic acids, and this was consist with these previous findings. The relatively strong signals at 78 to 76 ppm correspond to β-*O*-4′ linkages, which are the major linkage types in bamboo (Shao et al. 2008; Tai et al. 1990).

HSQC Spectrum

For a further comprehensive structural characterization, bamboo lignin (sample L4) was subjected to 2D NMR analysis. The HSQC NMR spectrum of lignin showed three regions including aliphatic, side chain and aromatic ^{13}C-^{1}H correlations. The side chain (δ_C/δ_H 50–90/2.5–6.0 ppm) and the aromatic (δ_C/δ_H 100–155/5.5–8.5 ppm) regions of the HSQC spectrum, which represent useful information of lignin structure, are shown in Fig 5. The main cross-signals assigned by comparing with the literature data (del Río et al. 2009; Martínez et al. 2008; Rencoret et al. 2009a; Rencoret et al. 2009b) is presented in Table 4, and the main substructures found are depicted in Fig 6.

The side-chain region of the spectrum gives useful information about the different inter-unit linkages present in lignin. The HSQC spectrum showed prominent signals corresponding to methoxyl and β-*O*-4′ substructures (**A**). The C-H correlations in β-*O*-4′ substructures were observed for α- and γ-C positions at δ_C/δ_H 72.0/4.85, 59.8/3.22 ppm, and for β-C positions at δ_C/δ_H 83.7/4.30 ppm in G and H type lignin, and 83.7/4.12 ppm in S type lignin.

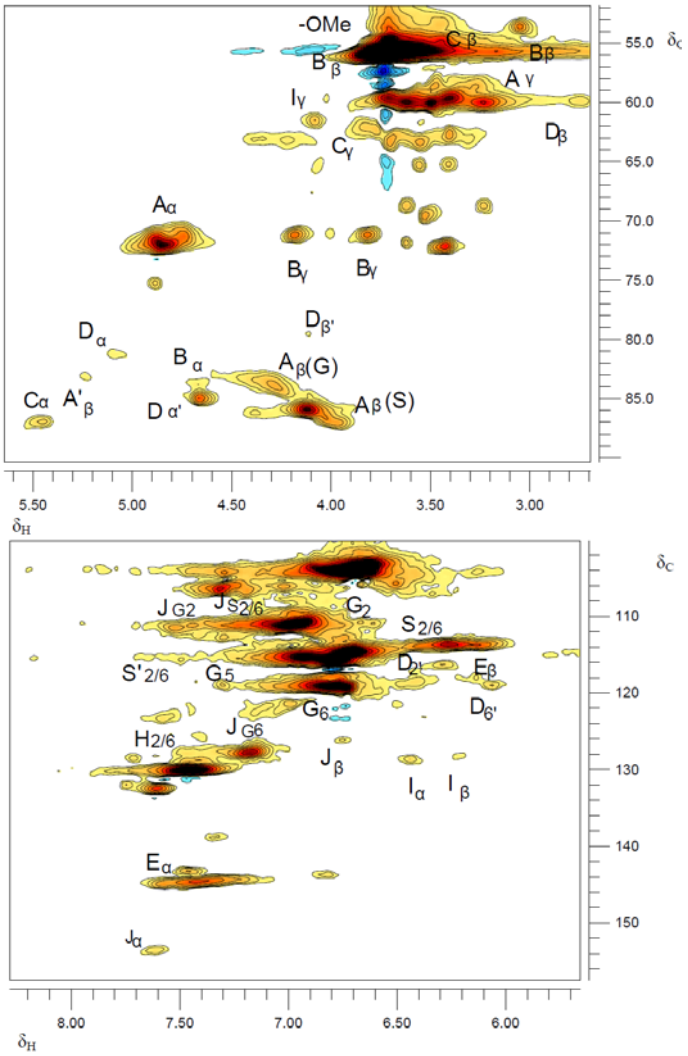

Fig. 5. HSQC spectrum of bamboo lignin (L4)

In addition to β-*O*-4′ aryl ether structures, other linkages were also observed in significant amounts. Strong signals for resinol structures (β-β′/α-*O*-γ′/γ-*O*-α′ linkages, **B**) were observed in the spectrum, with their C-H correlations for α-, β-C positions at δ_C/δ_H 84.9/4.66, 53.6/3.05 ppm, and double γ-C positions at δ_C/δ_H 71.2/4.18 and 80.0/3.81 ppm. The presence of phenyl coumaran substructures (**C**) was observed by C-H correlations for α-, β-, and γ-C positions at δ_C/δ_H 86.8/5.45, 53.2/3.40, and 63.2/3.70 ppm. Traces of spirodienone unit (**D**) were observed by its respective C_α-H_α, $C_{\alpha'}$-$H_{\alpha'}$, C_β-H_β, and C_β'-$H_{\beta'}$ correlations at δ_C/δ_H 81.2/5.09, 83.7/4.66, 59.8/2.74, and 79.6/4.11 ppm, and this was consistent with the results confirmed by ^{13}C NMR. Other small signals observed in the HSQC spectrum were α-, β-, and γ-C positions C-H correlations at δ_C/δ_H 128.7/6.42, 128.1/6.21, and 61.6/4.07 ppm assigned to cinnamyl alcohol end-groups (**I**). In addition, other small signals in the side-chain region of the HSQC spectrum corresponded to C_β-H_β correlations (at δ_C/δ_H 83.1/5.23 ppm) of β-*O*-4 substructures bearing a C_α carbonyl group (**A′**).

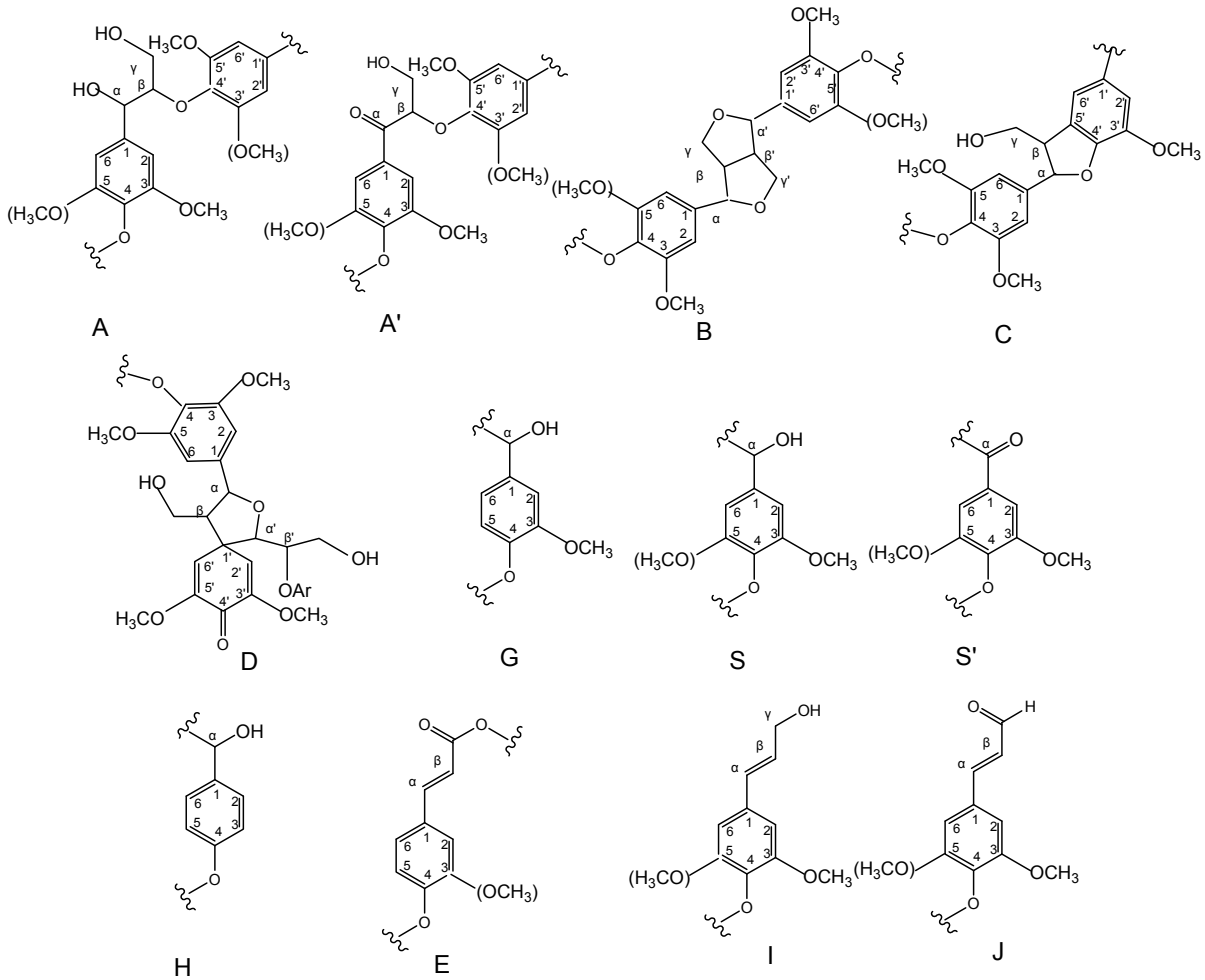

Fig. 6. Main substructures of bamboo lignin (L4) involving different side-chain linkages, and aromatic units identified by HSQC. (A) β-O-4 linkages; (A´) β -O-4 linkages with a carbonyl group at $C_α$; (B) resinol structures formed by β-β´/α-O-γ´/γ-O- α´ linkages; (C) phenylcoumaran structures formed by β-5´/α-O-4´ linkages; (D) spirodienone structures formed by β-1´/α-O-α´ linkages; (E) cinnamate unite; (I) cinnamyl alcohol end-groups; (J) cinnamaldehyde end-groups; (G) guaiacyl unit; (S) syringyl unit; (S') oxidized syringyl unit with a carbonyl group at $C_α$ (phenolic). (H) *p*-hydroxyphenyl unit

The main cross-signals in the aromatic region of the HSQC spectrum corresponded to the substituted phenyl rings of the different lignin units. Signals from guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units were observed. **G** lignin units showed different correlations for C_2-H_2 (δ_C/δ_H 110.9/6.96 ppm), C_5-H_5 (δ_C/δ_H 115.2/6.93, 115.6/6.79, 114.6/6.70 ppm), and C_6-H_6 (δ_C/δ_H 118.7/6.77 ppm). The multiple C_5-H_5 signals revealed some heterogeneity among the G units, especially affecting the C_5-H_5 correlations, probably due to different substituent of phenolic or etherified structure at C_4-H_4 (Rencoret et al. 2009a). Signals corresponding to $C_{2,6}$-$H_{2,6}$ correlations in **S** units were observed at δ_C/δ_H 104.1/6.70 ppm, while structure with $C_α$ carbonyl group (**S´**) gives signals at δ_C/δ_H 106.2/7.31 ppm. Besides, a significant amount of *p*-hydroxyphenyl (**H**) units was observed from $C_{2,6}$-$H_{2,6}$ correlations at δ_C/δ_H 129.9/7.45 ppm.

Table 4. Assignment of Main ^{13}C-^{1}H Correlation Signals in the HSQC Spectrum of Bamboo Lignin (L4)

Labels	C_α/H_α	C_β/H_β	C_γ/H_γ	C_2/H_2	C_5/H_5	C_6/H_6
A-H/G	72.0/4.85	83.7/4.30	59.8/3.22			
A-S	72.0/4.85	83.7/4.12	59.8/3.22			
A´		83.1/5.23				
B	84.9/4.66	53.6/3.05	71.2/4.18 80.0/3.81			
C	86.8/5.45	53.4/3.4	63.2/3.70			
D	81.2/5.09 83.7/4.66	59.8/2.74 79.6/4.11		113.7/6.25		118.9/6.05
E	144.2/7.42	113.5/6.26				
I	128.7/6.42	128.1/6.21	61.6/4.07			
J-G	153.6/7.62	126.0/6.74		111.1/7.45		121.9/7.12
J-S	153.6/7.62	126.0/6.74		106.1/7.02		106.1/7.02
G				110.9/6.96	115.2/6.93 115.6/6.79 114.6/6.70	118.9/6.77
S				104.1/6.70		104.1/6.70
S´				106.2/7.31		106.2/7.31
H				129.9/7.45		129.9/7.45

In addition, the cinnamate unit (**E**) characterized by C_α-H_α correlations at δ_C/δ_H 144.2/7.42 ppm and C_β-H_β correlations at δ_C/δ_H 113.5/6.26 ppm, arose from *p*-coumarate and ferulate, which has been commonly observed in straw plant. Other signals in this HSQC region of the spectrum arise from cinnamyl alcohol end-groups (**I**), with their C_α-H_α and C_β-H_β correlations observed at δ_C/δ_H 128.7/6.42 and 128.1/6.21 ppm, respectively; and cinnamaldehyde end-groups (**J**), with C_α-H_α and C_β-H_β correlations observed at δ_C/δ_H 153.6/7.62 and 126.0/6.74 ppm, respectively. Unlike the aromatic cross-signals overlap of the cinnamyl alcohol end-groups with that of S and G units, some of the cinnamaldehyde aromatic cross signals indicated the presence of (1) sinapaldehyde end-groups ($\mathbf{J_S}$), characterized by a unique $C_{2,6}$-$C_{2,6}$ (δ_C/δ_H 106.1/7.02 ppm), and (2) coniferaldehyde end-groups ($\mathbf{J_G}$), characterized by C_2-H_2 (δ_C/δ_H 111/7.45 ppm) and C_6-H_6 (δ_C/δ_H 121.9/7.12 ppm).

To evaluate the relative ratio of the major lignin side chains, semi-quantitative analysis of the HSQC cross-signal intensities was performed, and the calculation was conducted according to the literature by integration of the signals on inter unit linkages (**A**, **A´**, **B**, **C**, and **D**) (Rencoret et al. 2009a). The results showed that the relative abundances were 76.3, 3.1, 5.3, 9.2, and 6.1% for **A**, **A´**, **B**, **C**, and **D**, respectively. Obviously, the major lignin interunit linkages of the lignin fraction were the β-O-4´ ether linkages (near 80%), followed by phenylcoumaran (**C**). The other condensed linkages (**B**, **D,** e.g. β-β´, β-5´, β-1´,) were present in lower proportions.

Compared to the lignin obtained in the kraft pulping or steam explosion, the structure of bamboo lignin was not significantly degraded under the mild conditions. The kraft lignin of bamboo (Tai et al. 1990), having more phenolic hydroxyl and less non-etherified guaiacyl and syringyl type structure with trace amounts of β-*O*-4´ linkages, indicated the occurrence of serious degradation in the higher temperature and alkaline

solution during the kraft pulping process. In addition, because the steam explosion process operated under higher temperature and acidic medium (resulting from the acetic acid formed in the process), the lignin obtained in the this process also was heavily degraded (Shao et al. 2008).

Due to the relatively high molecular weights and abundance of β-O-4 linkages, the extracted lignin in the present study could be utilized as macromolecular feedstocks to synthesize polyurethanes, polyesters, or epoxy resins (Gandini and Belgacem 2008). Furthermore, aromatic monomers used for fine chemicals can also be obtained through cracking, reduction, or oxidation. The residual fraction rich in cellulose is a feed stock for bio-ethanol production, thus assessment of its enzymatic hydrolysis is being investigated. In addition, as the yield of the extracted lignin was relatively low in the present extraction process, research on improving the effect of pretreatments will be conducted thoroughly.

CONCLUSIONS

1. The above results showed that the lignin obtained from the pretreated bamboo with sodium hydroxide/urea solution at low temperature was an HGS type lignin incorporated with cinnamate units.
2. HSQC analysis indicated that the major lignin side chains were β-*O*-4′ ether linkages followed by phenylcoumaran. In addition, lower proportions of resinol and spirodienone were also presented. The ester groups of lignin were significantly cleaved in the cold alkaline solution. Overall, only minor changes of the lignin structure occurred in such an alkaline solution at –12 ^{o}C.

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