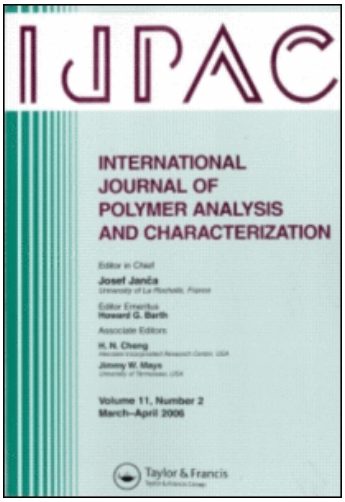

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Chemical Groups and Structural Characterization of Brown-Rotted *Pinus massoniana* Lignin

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Abstract: Milled wood lignins (MWLs) were isolated from sound and naturally brown-rotted *Pinus massoniana* (decayed by brown-rot fungus *Poria cocos*) by Björkman and Lundquist techniques. Both lignins were characterized by FT-IR, UV, ^{1}H NMR, GPC, ESR spectroscopy, and chemical analyses methods. The results indicated the brown-rotted lignin was higher in the total acidic group, carbonyl, carboxyl, and phenol hydroxyl contents, but lower in methoxyl content than sound lignin. The C_9 formulas were calculated for sound and brown-rotted lignin as $C_9H_{8.78}O_{2.85}(OCH_3)_{0.83}$ and $C_9H_{8.63}O_{3.01}(OCH_3)_{0.71}$, respectively. Sound lignin had 0.75 aliphatic hydroxyl group and 0.17 phenolic hydroxyl group per C_9-unit, and brown-rotted lignin had 0.62 aliphatic hydroxyl group and 0.30 phenolic hydroxyl group per C_9-unit; lignins were mainly guaiacyl-type lignin, and β-O-4 structures constituted the main intermonomeric connections. Based on the analytical results and proposed reaction mechanism, demethylation, oxidation, and depolymerization were the most important modifications of lignin by brown-rotted fungi.

Keywords: Brown-rot fungi; Demethylation; ESR; ^{1}H NMR; Milled wood lignins (MWLs); Oxidation

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INTRODUCTION

A variety of functional groups (phenolic hydroxyl, aliphatic hydroxyl, carbonyl, carboxyl, and methoxyl groups) and types of bonds between the structural units subject lignin to a vast array of reactions in fundamental and applied studies aimed at defining its structure, separating it from associated polysaccharides, and evaluating its potential as a source of chemicals. Thus, lignin is of interest to the specialist working in various fields of science and industry, such as wood adhesives, molding powders, rubbers, films, and so on.

Besides Kraft lignin reported previously,[1,2] brown-rot fungi could decompose and remove the cellulose and hemicelluloses in wood, leaving a modified, brown-colored lignin residue, which provides a potentially useful material. However, previous studies provide limited information on the chemical structure of brown-rotted lignin, including the increased content of phenolic hydroxyl caused by demethylation of methoxyl groups and hydroxylation[3–5] and the depolymerization, oxidation, and cleavage of lignin side-chain structures.[6] Moreover, most of the work to date has focused on hydroxyl radicals[7–10] produced by brown-rot fungi attacking lignin, but fewer reports have studied the connection between the mechanism of brown-rot decay and the chemical modification of lignin by brown-rot fungi.

The objective of this article is to describe the structure of brown-rotted lignin obtained from the natural environment so as to determine the content of active groups in it: phenolic hydroxyl, carboxyl, and carbonyl. Based on the results obtained from FT-IR, UV, ^{1}H NMR, GPC, ESR spectroscopy, and chemical analyses, the proposed reaction mechanism of lignin by hydroxyl radicals is deduced to explain how brown-rot fungi modifies lignin. In addition, China has large amounts of waste wood decayed by brown-rot fungi because of the use of the fungus *Poria cocos* in traditional Chinese medicine, and this study will also provide information on enhancing the reactive activity of brown-rotted lignin, give insight into the mechanism of brown-rot decay, and pave the way for making full use of this abundant, renewable, naturally brown-rotted wood resource.

EXPERIMENTAL SECTION

Wood Samples

Sound *Pinus massoniana* and *Pinus massoniana* naturally decayed by *Poria cocos* were collected from Huangmei Hill, Taiyang Village, Heping Township, Yuexi County, Anhui Province, China. Two kinds of wood

were selected, brushed and air dried, and then ground to pass a 20-mesh screen.

Preparation of MWL

Two kinds of milled wood lignins (MWLs) were extracted with the standard technique of Björkman[11] and purified with the technique of Landquist[12]: sound *Pinus massoniana* milled wood lignin (S-MWL) and brown-rotted *Pinus massoniana* milled wood lignin (B-MWL).

Elemental, Methoxyl Content, and Functional Group Analysis

Elemental analyses for C, H, N were carried out on a Flash EA 1112 Elemental Analyzer, and O on a Elementar Vario EL Elemental Analyzer. Methoxyl content for two MWLs was performed by ASTM D1166-84 standard technique. The content of functional groups in MWL was determined as follows[13]: the total hydroxyl groups were determined by acetylation; total acidic groups and carboxyl groups by the chemisorption method; carbonyl groups by oximation; the content of phenolic hydroxyl groups by the difference between total acidic groups and the content of carboxyl groups; and the content of aliphatic hydroxyl groups by the difference between the total hydroxyl groups and the content of phenolic hydroxyl groups.

FT-IR Spectroscopy

Fourier transform-infrared (FT-IR) spectra analyses of the two lignins were obtained with KBr pellets (1.0 mg dry lignin in 200 mg KBr) using a Nicolet Avatar-370 FT-IR spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector in the 4000–1000 cm^{-1} range at room temperature. Resolution: 4 cm^{-1}; 64 scans.

UV Spectroscopy

Samples of the two lignins were dissolved in a mixture solvent of dioxane and water (96:4) recorded with a UV-2501 PC spectrophotometer over the range 400–200 nm at room temperature.

Molecular Weight Determination

The number-average molecular weight, the weight-average molecular weight, and viscosity-average molecular weight of both acetylated lignins were estimated by gel permeation chromatography (GPC; Waters 515-2410 system, 515 HPLC Pump, 2410 Refractive Index Detector, 996 Photodiode Array Detector, Styragel HT3-HT5-HT6E) using tetrahydrofurane (THF) as an eluent at 30°C, 1 mL/min flow rate, polystyrene as the calibration standard.

^{1}H NMR (Nuclear Magnetic Resonance) Spectroscopy

Acetylated samples (200 mg) of each lignin were prepared by treatment with acetic anhydride in pyridine as described by Kirk and Chang.[14]

The acetylated samples were dissolved in $CDCl_3$ and the spectra were recorded with a 600 MHz continuous wave instrument (Bruker AV-600). Tetramethylsilane (TMS) served as a chemical shift reference.

ESR Spectroscopy

Electron spin resonance (ESR) measurements of the two lignins were taken at room temperature (298 K) with an X-band ESR spectrometer (Bruker ER200D-SRC). The condition for ESR was as follows: X-band, 100 kHz modulation with 1.0 G amplitude, microwave power 20 mW, microwave frequency 9.53 GHz, central magnetic field 3385 G with scanning 200 G, scanning time 100 s. The intensities of the ESR signals were measured as the height difference between the first maximum and the first minimum of the signal.

RESULTS AND DISCUSSION

Elemental, Methoxyl Content, and Functional Group Determinations

The elemental and methoxyl analyses, the calculated C_9-unit formulae,[13] and C_9-unit weights are summarized in Table I. All data were obtained in duplicate on all samples. B-MWL was higher in oxygen and carbon than S-MWL, but lower in hydrogen, methoxyl, and molecular weight; the insignificant amount of nitrogen might result from pyridine contamination during purification of lignin. From the analysis above, B-MWL was higher in carbon and lower in molecular weight than those reported in Kirk's research,[3] which could result from different wood and

Table I. Element composition, methoxyl contents, and C_9-formula of S-MWL and B-MWL

Samples	Element composition C (%)	H (%)	O (%)	N (%)	Methoxyl group content OCH_3 (%)	C_9-formula	Molecular weight/ C_9-unit
S-MWL	60.38	5.78	30.14	0.43	13.14	$C_9H_{8.78}O_{2.85}(OCH_3)_{0.83}$	Mw = 188.30
B-MWL	60.71	5.62	30.95	0.48	11.43	$C_9H_{8.63}O_{3.01}(OCH_3)_{0.71}$	Mw = 186.98

brown-rot fungi species. However, after decay by brown-rot fungus *Poria cocos*, B–MWL was lower in methoxyl group content, which was the same as those previously reported.[3,4]

The total hydroxyl, phenol hydroxyl, aliphatic hydroxyl, carbonyl, carboxyl, and total acidic group contents of the lignins are listed in Table II. The total acidic group, carbonyl, carboxyl, and phenol hydroxyl contents in B-MWL were about two times higher than those in S-MWL. However, B-MWL was slightly lower in aliphatic hydroxyl and slightly higher in total hydroxyl than S-MWL. As reported by Kirk,[3] this high phenolic hydroxyl content caused the higher total hydroxyl content in B-MWL; the lower aliphatic hydroxyl content was in accord with the higher carbonyl content. The increase of carbonyl and carboxyl contents in B-MWL showed that lignin was oxidized by brown-rot fungi.

Spectroscopic Characterization

FT-IR spectra of S-MWL and B-MWL are presented in Figure 1. Table III summarizes all the absorption bands found in both lignins. Brown-rot fungi just resulted in ring and side-chain modification and modified the aromatic skeleton to a very limited extent,[3,6] so the aromatic band at around 1510–1504 cm^{-1} served as the reference band,[15] and all transmittance values of both lignins were normalized

Table II. Contents of functional groups in S-MWL and B-MWL

Samples	Total hydroxyl (%)	Phenol hydroxyl (%)	Aliphatic hydroxyl (%)	Carbonyl (%)	Carboxyl (%)	Total acidic group (%)
S-MWL	8.02	1.25	6.77	0.77	1.24	2.49
B-MWL	8.72	2.36	6.36	1.37	2.33	4.69

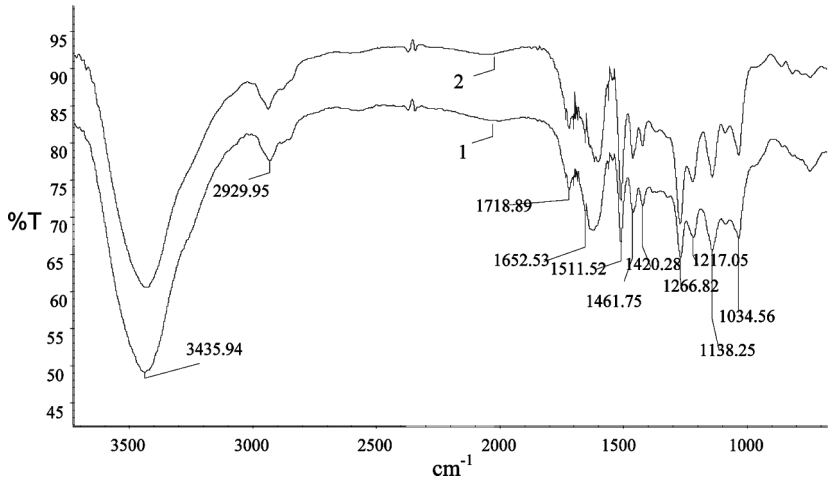

Figure 1. FT-IR spectra of S-MWL and B-MWL: 1, S-MWL; 2, B-MWL.

to the aromatic skeletal vibration at 1507 cm^{-1}.[16] The peak patterns in the FT-IR spectra for both ligins were similar, and the changes in functional group contents were also demonstrated in the FT-IR spectra. The band at 1369–1268 cm^{-1} showed that *Pinus massoniana* S-MWL and B-MWL (softwood lignins) were guaiacyl lignin (G-lignin) polymers. The band intensities at 3435–3424 cm^{-1} and 1653 cm^{-1} increased in B-MWL, which indicated the increase of hydroxyl and conjugated carbonyl contents. The increased band intensities at 1141–1139 cm^{-1}, 1034–1032 cm^{-1} in B-MWL showed that the carbon-hydrogen bonds in aromatic skeleton increased, namely by the partial depolymerization of lignin.

The UV spectra of both lignins are presented in Figure 2. Both lignins had a peak at about 288 nm, and a shoulder at 305–350 nm. In general, softwood lignin showed a maximum at 280–285 nm because of guaiacyl compounds.[17,18] The specific location of a maximum around 280 nm depended on the position and nature of the substitutes in the benzene ring, so the ionized hydroxyl and aldehyde groups in S-MWL and B-MWL caused a marked bathochromic spectral shift. The increased absorption in B-MWL at 305–350 nm indicates that reactions within the side chains of lignin took place. Although the conjugated carbonyl groups and the appearance of the conjugated α-β-double bonds were possible candidates for causing this effect, based on the above analysis, the conjugated carbonyl groups had the best likelihood. The higher degree of conjugation stabilizes π-π^* transitions and thus results in absorption bands at longer wavelengths.[19,20]

Table III. FT-IR absorption peak locations and assignments of S-MWL and B-MWL

S-MWL			B-MWL			
Peak range (cm^{-1})	Transmittance (%)	Relative value	Peak range (cm^{-1})	Transmittance (%)	Relative value	Assignment
3435.94	48.501	0.728	3424.37	60.439	0.850	Stretching in hydroxyl group
2929.95	77.381	1.161	2938.25	84.265	1.185	C–H stretching in methyl and methyene groups
1718.89	73.580	1.104	1718.53	80.589	1.133	C=O stretching in unconjugated ketone and carbonyl and in ester groups
1652.53	71.918	1.079	1652.53	80.287	1.129	C=O stretching in conjugated carbonyl groups
1511.52	66.641	1	1507.37	71.129	1	Aromatic skeletal vibration; G > S
1461.75	70.553	1.059	1465.65	77.326	1.087	C–H deformation in methyl and methyene groups
1420.28	71.662	1.075	1419.82	78.982	1.110	Aromatic skeletal vibration plus C–H in-plane deformation
1266.82	64.346	0.966	1269.84	68.857	0.968	G rings plus C=O stretching
1217.05	67.136	1.007	1217.00	74.669	1.050	C–C plus C–O plus C=O stretching
1138.25	65.088	0.977	1141.15	75.055	1.055	Aromatic C–H in-plane deformation, typical for G units, $G_{condensed} > G_{etherified}$
1034.56	66.865	1.003	1032.89	77.999	1.097	Aromatic C–H in-plane deformation plus C–O deformation in primary alcohols plus C=O stretching

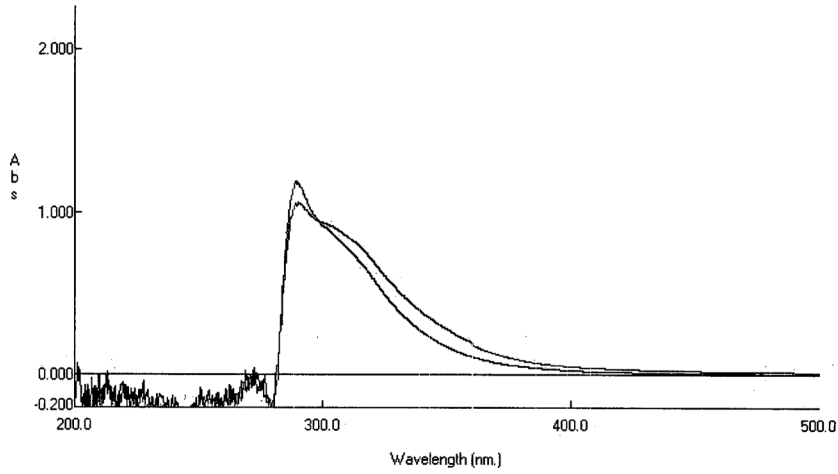

Figure 2. Ultraviolet spectra of S-MWL and B-MWL: 1, S-MWL; 2, B-MWL.

Molecular Weights

Several kinds of average molecular weights of both lignins are summarized in Table IV. Except for the polydispersity, the Mn, Mw, Mp, and Mv in B-MWL were lower than those in S-MWL, which indicated the lignin was depolymerized by brown-rot fungi to a limited extent.[6] Presumably, the depolymerization could cleave the side chain to the formation of more aromatic carboxylic acids and cleave the aryl-ether bonds to form more phenolic hydroxyl (Table II).

^{1}H-NMR Spectrum

^{1}H-NMR spectra of the acetylated S-MWL and B-MWL are presented in Figure 3. Table V summarizes the distribution of protons in the ^{1}H-NMR

Table IV. Average molecular weights of S-MWL and B-MWL

Samples	Number-average molecular weight (Mn)	Weight-average molecular weight (Mw)	Peak molecular weight (Mp)	Viscosity-average molecular weight (Mv)	Polydispersity
S-MWL	4166	7506	5492	6953	1.801754
B-MWL	3599	5997	5095	5630	1.666597

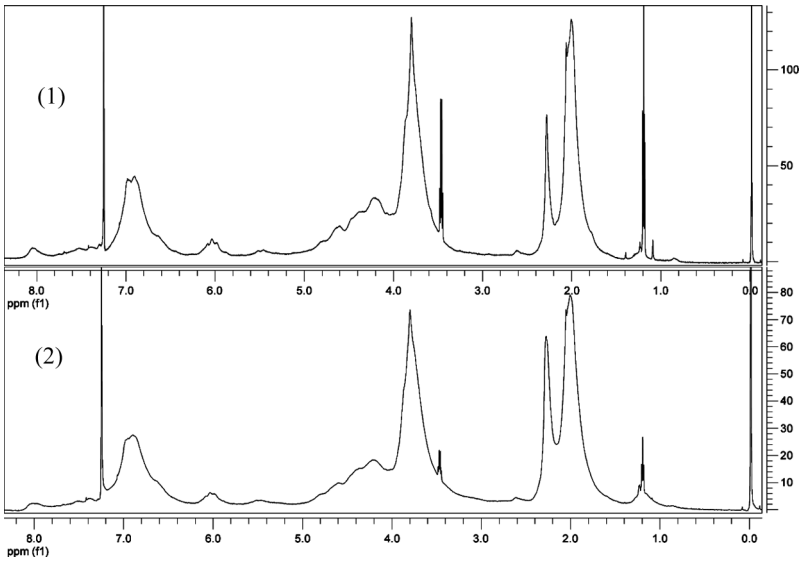

Figure 3. ^{1}H NMR spectra of acetylated S-MWL and B-MWL: 1, acetylated S-MWL; 2, acetylated B-MWL.

spectra of acetylated derivatives of S-MWL and B-MWL, which was determined by previous studies.[18,21]

For both acetylated lignins, the number of hydroxyl groups was estimated from the acetate signals by considering C_9-unit formulae (Table I). The detailed procedures followed previous studies.[18] From the empirical formula of S-MWL, the total OCH_3 protons per C_9-unit were calculated to be $0.83 \times 3 = 2.49$, and the number of protons per unit integral was $2.49/19.64 = 0.127$; other protons were measured and are listed in Table V. From Table V, we could obtain the aliphatic and aromatic hydroxyls of S-MWL per C_9-unit, the mole ratio $OAc/OCH_3 = (0.51 + 2.25)/2.49 = 1.11$. Thus the total OCH_3/C_9 ratio $= (0.83\ OCH_3/C_9) \times (1.11\ OAc/1\ OCH_3) = 0.92$; the number of aliphatic $OCH_3/C_9 = (0.83\ OCH_3/C_9) \times (2.25\ OAc/2.49\ OCH_3) = 0.75$, and the number of aromatic $OCH_3/C_9 = (0.83\ OCH_3/C_9) \times (0.51\ OAc/2.49\ OCH_3) = 0.17$. Therefore, the numbers of aliphatic and aromatic hydroxyls in S-MWL were estimated to be 0.75 and 0.17 per C_9-unit, and the numbers of aliphatic and aromatic hydroxyls in B-MWL were estimated to be 0.62 and 0.30 per C_9-unit. The number of aromatic hydroxyl per C_9-unit in B-MWL was about two times higher than those in S-MWL, and B-MWL was slightly lower in the number of aliphatic hydroxyl per C_9-unit than S-MWL, which had some similarities with the results obtained from UV technique[3] and was consistent with the results from Table II.

Table V. Assignments of signals and protons per C_9 structural unit in the ^1H NMR spectra of acetylated S-MWL and B-MWL

Range no.	Range (10^{-6})	Maximum value (10^{-6})		Main assignments	Protons per C_9-unit	
		S-MWL	B-MWL		S-MWL	B-MWL
1	7.30	7.25	7.25	$CDCL_3$		
2	7.25–6.80	6.90	6.90	Aromatic protons in guaiacyl units	1.37	1.15
3	6.25–5.75	6.03	6.03	H_α of β-O-4 (H_α in β-1 structures)	0.13	0.07
4	5.75–5.24	5.50	5.51	H_α of β-5 structures	0.02	0.02
5	4.90–4.00	4.20 4.50	4.21	H_γ in several structures, H_α and H_β of β-O-4 structures, H_α of β-β structures, and H of xylan residue	1.40	1.04
6	4.00–3.48	3.80	3.80	H of methoxyl groups	2.49	2.13
7	3.48–3.45	3.47		$H_{\alpha'}H_\beta$ and H_γ except ranges 3 and 4	0.19	
8	2.50–2.22	2.28	2.28	H of aromatic acetates	0.51	0.89
9	2.22–1.60	2.06	2.05	H of aliphatic acetates	2.25	1.87
10	1.40–1.10	1.19	1.19	Hydrocarbon contaminant	0.16	0.13

Based on the ^{1}H-NMR spectra and Table II, we could separately calculate the content of aliphatic hydroxyl (phenol hydroxyl) and aliphatic hydroxyl in total hydroxyl. In the ^{1}H-NMR spectra, the contents of aliphatic hydroxyl and aliphatic hydroxyl of S-MWL in total hydroxyl were $0.75/0.92 = 81.5\%$ and 18.5%; the contents of aliphatic hydroxyl and aliphatic hydroxyl of B-MWL in total hydroxyl were 67.4% and 32.6%. From Table II, the content of aliphatic hydroxyl and aliphatic hydroxyl of S-MWL in total hydroxyl were 84.4% and 15.6%, and the contents of aliphatic hydroxyl and aliphatic hydroxyl of B-MWL in total hydroxyl were 72.9% and 27.1%. The results of two techniques were similar; the difference between the results was in the range of $3\% \sim 5\%$. However, in ^{1}H-NMR spectra, the aromatic and aliphatic OAc signals were overlapping, consequently, the results of hydroxyl determination can be only approximate.[16]

From the ^{1}H-NMR spectra of both acetylated lignins, *Pinus massoniana* S-MWL and B-MWL were guaiacyl lignin (G-lignin) polymers, in agreement with the FT-IR and UV spectra results. The aryl glycerol-β-O-4 aryl ether linkages constituted the main intermonomeric connections in both lignins. There were also β-5, β-β, and β-1 structures, but the proton signals in these structures were insignificant, for example, the protons of β-5 in both lignins were the same 0.02 per C_9-unit. Moreover, B-MWL was lower in proton numbers in ranges 2, 3, 5, and 6 (Table V) than S-MWL, which indicated that various structures in S-MWL had been modified by brown-rot fungi to some extent, such as aryl ether interunit bond cleavage and the cleavage of the lignin side-chain structures.

ESR Analysis

The ESR spectra of S-MWL and B-MWL are shown in Figure 4. For S-MWL, the principal singlet signal had a $g = 2.0026$ and a Gaussian line width of 13.3 G, and the overall concentration of stable free radicals was 9.52×10^{17} spins/g; for B-MWL, the principal singlet signal had a $g = 2.0026$ and a Gaussian line width of 11.5 G, and the overall concentration of stable free radicals was 7.94×10^{17} spins/g. The observed spectral parameters in both lignins indicated that the spectrum could be assigned to the phenoxy radicals, which was previously observed for solid pristine lignin.[22] Phenoxy radicals in lignin could be generated readily with many forms of energy, as were carbon radicals. Thus, radicals had been detected in lignin after oxidation, mechanical treatments, and high energy irradiation. After oxidation and partial depolymerization, B-MWL should generate more radicals, but the overall concentration of stable free radicals in B-MWL was lower than that in S-MWL, which

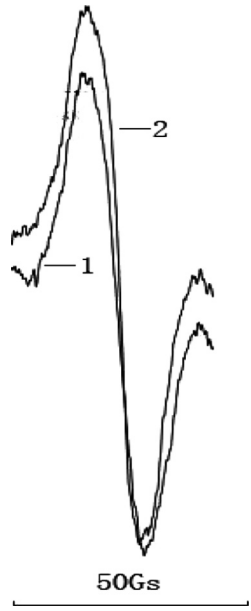

Figure 4. ESR spectra of S-MWL and B-MWL: 1, S-MWL; 2, B-MWL.

may be a result of intramolecular or intermolecular rearrangement reactions caused by some unstable radicals.

Although the mechanism of brown-rot decay has not been determined, some studies showed that brown-rot fungi produced ·OH, and the initial stages of decay were thought to involve the action of Fenton chemistry ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + OH$) for the production of hydroxyl anions and radicals,[7] then ·OH resulted in diverse reactions, some of which were expected to degrade the polymer.[8,23,24] So, based on the results of the above analysis and the previous studies, we concluded that the proposed reactions in *Pinus massoniana* lignin during the decay are caused by brown-rot fungus *Poria cocos* (Figure 5). The main structures (guaiacyl lignins A and B) of *Pinus massoniana* lignin are shown in Figure 5. There are five proposed reactions. (I), (II), and (IV) resulted in higher content of phenolic hydroxyl in B-MWL (Table II and the numbers of aromatic hydroxyls resulting from ^{1}H-NMR spectra analysis) and the stable phenoxy radicals generated from guaiacyl derivatives; (I) and (IV) resulted in the decrease of methoxyl content (Table I); (II) was expected to degrade the polymer and could be consistent with the results of ^{1}H-NMR spectra analysis; (III) could be ascribed to the formation of α-carbonyl (i.e., conjugated carbonyl) groups, which was indicated by the FT-IR and UV spectra; (IV) and (V) might result in the formation of catechol moieties and

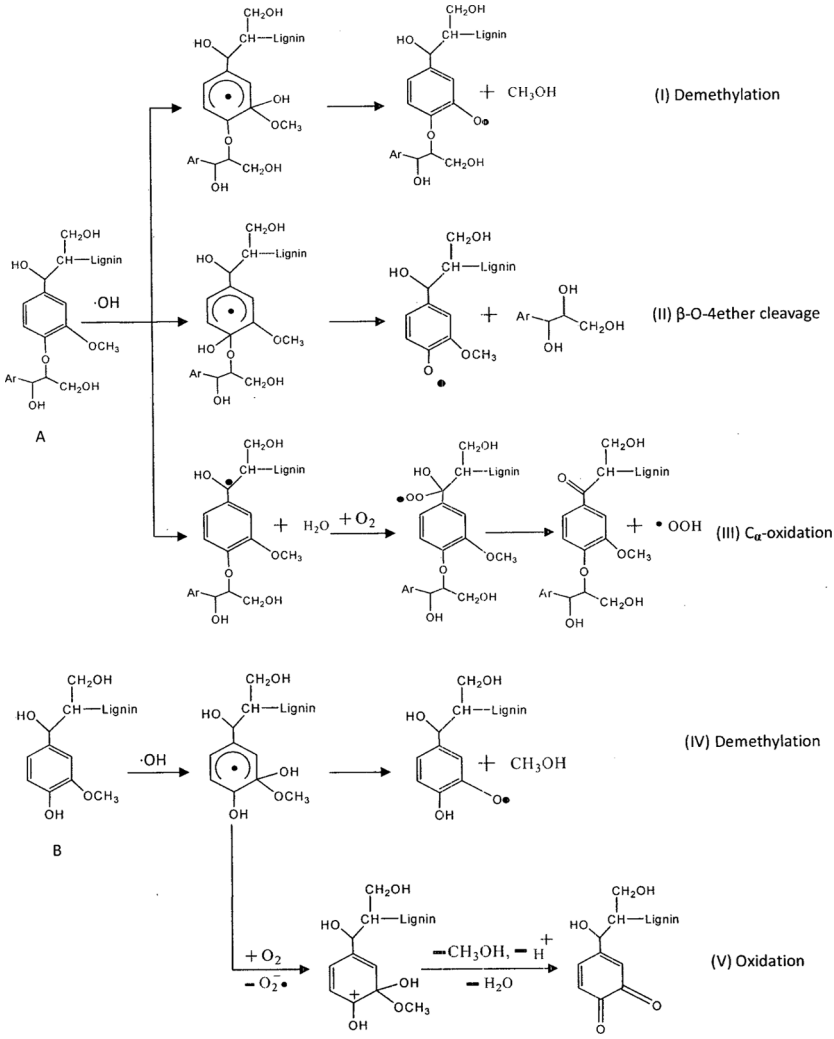

Figure 5. Proposed reactions of hydroxyl radicals with *Pinus massoniana* lignin.

ortho-quinone, although there were no direct results to prove them besides the higher phenolic hydroxyl content and carbonyl content in B-MWL. Aromatic hydroxylation did not take place in softwood guaiacyl-type lignins when the wood was under brown-rot fungal attack,[6] so hydroxylation was not taken into account. The main modification of *Pinus massoniana* lignin by brown-rot fungus *Poria cocos* could be illustrated from the analysis discussed above.

CONCLUSION

In conclusion, the results presented in this study clearly confirmed that the main modifications of *Pinus massoniana* lignin by brown-rot fungus *Poria cocos* were oxidation, demethylation, and partial depolymerization. All these modifications of lignin were well evidenced by functional groups analyses and various spectroscopic techniques. Moreover, based on the proposed reactions in lignin and the corresponding results, during the decay process, the hydroxyl radical (·OH) resulted in diverse reactions, demethylation, β-O-4 aryl ether cleavage, and C_α-oxidation, which made the softwood guaiacyl-type lignin produce more content of phenolic hydroxyl and degrade the lignin polymer. Eventually, all the proposed reactions made the natural brown-rotted lignin chemically more reactive.

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