Structural Analysis of Fast-Growing Aspen Alkaline Peroxide Mechanical Pulp Lignin: A Post-Enzymatic Treatment

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An enzymatic mild acidic hydrolysis was used to separate and purify residual lignin from alkaline peroxide mechanical pulp (APMP). Using the optimum conditions for the laccase treatment (pH 4.5, temperature 50 °C, lignin consistency of 1%, a reaction time of 60 min, and a laccase dosage of 8 μ/g), oven-dried lignin was treated with laccase and in a laccase mediator system (LMS) to explore the mechanism for laccase and the LMS modification of APMP. The changes of functional groups in lignin were analyzed using nuclear magnetic resonance (^{31}P-NMR and ^{13}C-NMR). The molecular weight distributions of the lignin samples were confirmed by gel permeation chromatography (GPC). The ^{31}P-NMR and ^{13}C-NMR spectra revealed that the lignin structure changed significantly with the laccase and the LMS treatments. Meanwhile, GPC demonstrated that laccase without a mediator could lead to the polymerization of lignin, while the LMS could degrade the lignin. Hence, it was concluded that laccase is an attractive enzyme for lignin modification.

Keywords: Lignin; ^{31}P-NMR; ^{13}C-NMR; GPC; Laccase; LMS

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INTRODUCTION

Currently, high yield pulp (HYP) is a profitable material to produce because of its diversified advantages, such as a higher yield, environmental friendliness, low production costs, higher bulk, and good opacity. However, HYP fibers display poor flexibility, low whiteness, susceptibility to photo-yellowing, thermal reversion, and low contribution to paper strength because of the high lignin content (Liu *et al*. 2012). Therefore, modifications to HYP are needed to improve the quality of the paper and hence broaden its application (Lachenal *et al*. 1995; Henriksson and Gatenhalm 2002). The common modification methods for fiber include mechanical modification, chemical modification (Gruber *et al*. 2002), and physical modification (Goring 1967; Carlsson and Ström 1995). With the development of biotechnology, enzymes are of great interest in studies for the modification of pulp (Sigoillot *et al*. 1997; Richardson *et al*. 1998; Henriksson and Gatenhalm 2002). Enzymes do not generate the pollution that other methods generate. In addition, they provide a low energy consumption method.

Cellulase is an example of an enzyme that has the ability to modify fibers by improving their printability and smoothness (Mansfield *et al*. 1997). Additional examples include hemicellulase, which is capable of reducing the beating energy and

improving the quality of paper (Stork *et al*. 1995), peroxidase, which plays an important role in the degradation process of lignin, and laccase, which is the most promising lignin-degrading and lignin-polymerizing enzyme (Thurston 1994). This is because laccase is an environmentally friendly enzyme, which works in the presence of oxygen and produces water as its only byproduct (Kalia *et al*. 2014). In addition, laccase can be used for many technical processes (Call and Mücke 1997; Couto and Toca-Herrera 2006; Riva 2006; Mikolasch and Schauer 2009), such as bio-bleaching in the pulp and paper industry (Balakshin *et al*. 2001; Zhang *et al*. 2007), decreasing the refining energy (Mansfield 2002), deinking of waste paper pulp (Welt and Dinus 1998; Ibarra *et al*. 2012), lignin degradation (Rico *et al*. 2014), and dyeing in the textile industry (Galante and Formantici 2003).

However, the redox ability of laccase is unfavorable. Moreover, laccase requires a free hydroxyl group on the substrate for the reaction to occur (Euring *et al*. 2012). Bourbonnais and Paice (1990) were the first researchers to discover that laccase can attack a non-phenolic lignin and accelerate the scope of the reaction after adding low molecular weight redox compounds as mediators. Therefore, a laccase mediator system (LMS) plays an important role in indirectly governing and amplifying the low redox ability of laccase (Euring *et al*. 2012). The role of laccase in lignin modification has been explored, and the mechanism of the LMS has been established (Bourbonnais and Paice 1990).

A pulp with a higher lignin content often equates to a more efficient enzymatic process. Therefore, laccase modification with HYP is a potential avenue for improving the quality of HYP and their ultimate usage. Mansfield *et al*. (1997), Wong *et al*. (1999, 2000), and Mansfield (2002) showed that the tensile strength of paper was improved after the laccase treatment of chips processed by steam and a screw extruder. Furthermore, Yamaguchi *et al*. (1994) was successful in finding that after laccase treatment on thermo-mechanical pulp, the tensile strength of paper was greatly improved due to the formation of chemical bonding between pulp lignin and dehydrogenation polymer. When pretreated in a LMS, Jujop (1991) found that the breaking length and tear index of the unbleached and bleached Masson pine ground wood pulp was markedly improved.

This study aims to explore the mechanism of laccase and the LMS for the modification of alkaline peroxide mechanical pulp (APMP). Lignin, separated from the APMP, was treated with laccase and the LMS. Nuclear magnetic resonance (NMR) and gel permeation chromatography were used to analyze the structural changes of lignin and its molecular weight to provide insights into the mechanism of laccase and the LMS on modifying the APMP.

EXPERIMENTAL

Materials

The APMP was provided by Shandong Zhongmaoshengyuan Group, China. The commercial laccase and the 1-hydroxybenzotriazole (HBT; mediator) were supplied by Novozymes, Beijing, China. Enzyme activity was measured at 2400 μ/g at a pH value of 4.5.

Lignin Isolation

The residual lignin in the APMP was isolated and purified by the enzymatic mild acidic hydrolysis method (Argyropoulos *et al*. 2002; Baumberger *et al*. 2002; Wu and Argyropoulos 2003). The isolation process has the ability to produce a higher yield, higher purity, and minimize structural changes (Keiichi *et al*. 2005).

First, extractives were eliminated using an acetone-based 8 h extraction process. The extractive-free pulp was washed repeatedly with deionized water and air dried. Then, the air-dried pulp was milled in a ball mill.

The enzymatic mild acidic hydrolysis was done in two separate stages. In the first stage, cellulase and hemicellulase were added to the milled pulp to remove cellulose and hemicellulose. The slurry was agitated in a Rocking Incubator (HZQ-F100), which was manufactured by Shanghai FUMA Equipment Co., Ltd for 48 h at 40 °C at 5% consistency. After the enzyme treatment, the slurry was centrifuged at 5000 rpm for 15 min in the GL-20G-II High-speed Centrifuge produced by Shanghai Anting Scientific Instrument Factory. The sediment was washed with acidified water (pH 2) prior to freeze-drying to obtain a crude lignin sample.

In the second stage, crude lignin was added to a dioxane-water solution (dioxane: water, 81:15 v/v). Under a nitrogen atmosphere, the mixture was heated and refluxed for 2 h. The resulting mixture was filtered, and the solid component was washed with dioxane-water. The filtrate was neutralized with sodium bicarbonate and concentrated under reduced pressure at 40°C.

The concentrated mixture was added to the acidified, deionized water (pH 2) in order to isolate the lignin by letting it stand for 8 h, centrifuging, and freeze-drying. Lastly, the lignin was washed with hexane to obtain a purified sample.

Lignin Modification

Modification with laccase

The isolated lignin from APMP was directly treated with laccase. The optimum conditions for laccase treatment were: pH 4.5, temperature 50 °C, consistency of lignin 1%, reaction time 1 h, and concentration 8 μ/g (referring to the oven-dried lignin) (Wang and Liu 2010).

Modification with laccase mediator system

1-hydroxy benzotriazole (HBT) was used as mediator for the LMS at a 2% concentration of oven-dried lignin (Wang and Liu 2010).

^{31}P-NMR Spectroscopy

Approximately 40 mg of oven-dried lignin was added to 250 μL of pyridine-d5 and chloroform (1.6:1, v/v) in a small test-tube and stirred to dissolve. Then, 100 μL of N-hydroxy naphlthalimide (internal standard) and 50 μL of chromium acetylacetonate (relaxation regent) were added. Lastly, O-4, 4, 5, 5-tetramethyl-1, 3, 2-dioxaphospholane (TMDP) was added to produce phosphitylation. The sample was thoroughly mixed and analyzed using an Avance II 400 MHz spectrometer (BRUKER, Germany).

Acetylation

Under a nitrogen atmosphere, 15 mL of the acetylated reagents pyridine and acetic anhydride (1:2, v/v) were added to 300 mg of lignin sample in a 50 mL conical

flask for 72 h. After the reaction was run to completion, the mixture was slowly poured into 200 mL of diethyl ether, which produced a white precipitate. The sample was filtered through a glass filter, and then washed with diethyl ether until no pyridine odor was detected; the product formed was acetylated lignin. Finally, the lignin samples were dried in a vacuum drying oven.

^{13}C-NMR Spectroscopy

Approximately 200 mg of oven-dried acetylated lignin and 500 μL of dimethyl sulphoxide-d6 (DMSO-d6) were added and stirred to fully dissolve. The lignin sample was scanned for 16 h by adopting inverse gate decoupling sequence (C13IG sequence) from Bruker Standard Pulse Library.

Lignin Molecular Weight Distribution

The molecular weight distribution of the acetylated lignin samples was obtained using gel permeation chromatography (GPC). Approximately 10 mg of oven-dried acetylated lignin sample was fully dissolved in 10 mL of tetrahydrofuran (THF). The temperature was maintained at 40 °C and the flow velocity was 0.22 mL/min.

RESULTS AND DISCUSSION

^{31}P-NMR Spectroscopy

From the ^{31}P-NMR spectra (Fig. 1), changes in the functional groups of lignin were detected following the treatment with laccase and the LMS.

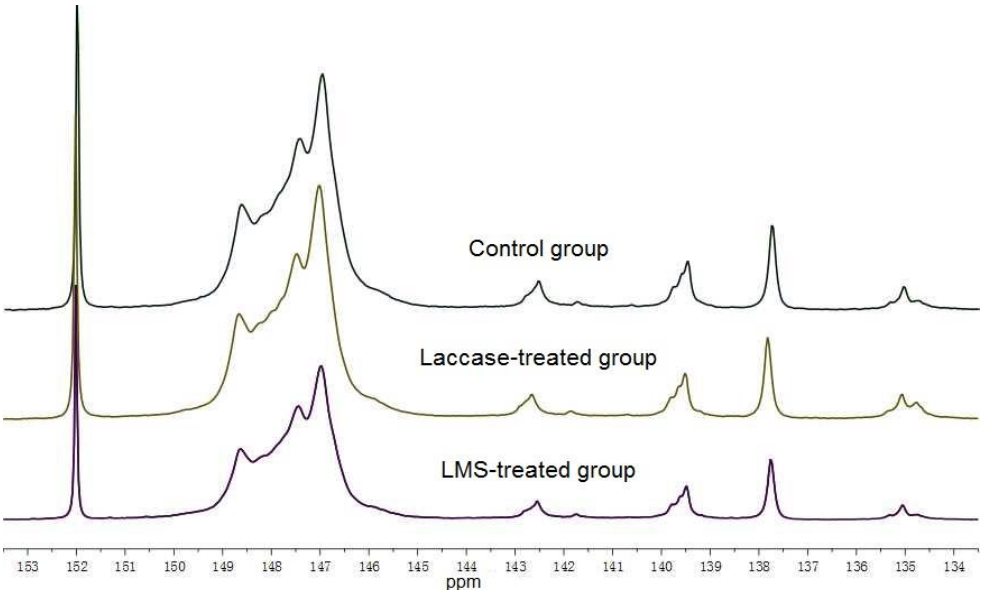

Fig. 1. The ^{31}P-NMR spectrum of lignin samples

As shown in Fig. 1 and Table 1, the concentration of aliphatic OH exhibited reductions of 10.1% and 12.5% after modification of laccase and LMS, respectively. Similarly, the total phenolic hydroxyl content decreased by 15.4% and 22.3%, respectively, demonstrating partial or complete removal of phenolic OH. The content

of condensed phenolic OH was reduced by 19.2% and 30.8%, respectively. Similarly, after the laccase treatment, syringyl OH decreased by 12.5%. However, after the LMS, the change in syringyl OH content was not obvious. Meanwhile, guaiacyl and demethylated OH groups were reduced by 17.9% and 28.2%, respectively. Additionally, the content of p-hydroxyl-phenyl OH also decreased after the laccase and the LMS treatment by12.5% and 22.5%, respectively. Because of the laccase treatment, the total COOH content increased by 10.5%. When the mediator, HBT, was added there was no obvious variation in COOH content, which was in agreement with the result by Crestini *et al*. (2003).

Table 1. Functional Group Frequency and Integration Regions for Quantitative Analysis of ^{31}P-NMR Spectra of Lignin and Relative Absorption Strength (Liu *et. al.* 2012)

Chemical shift (ppm)	Functional groups	Control group	Laccase-treated group	LMS-treated group
		Relative absorption strength (mmol/g)		
150.0-145.0	Aliphatic OH	6.80	6.11	5.95
144.6-143.6 142.4-140.2	Condensed phenolic OH	0.26	0.21	0.18
143.6-142.4	Syringyl OH	0.25	0.22	0.24
140.2-138.6	Guaiacyl and demethylated OH	0.39	0.32	0.28
138.6-137.0	*p*-hydroxy-phenyl OH	0.40	0.35	0.31
136.0-134.0	COOH	0.19	0.21	0.19

^{13}C-NMR Spectroscopy

^{13}C-NMR spectra of each sample are compared in Fig. 2, in which chemical shifts in the range 102.0 to 162.0 are the absorption peaks of the benzene ring carbon.

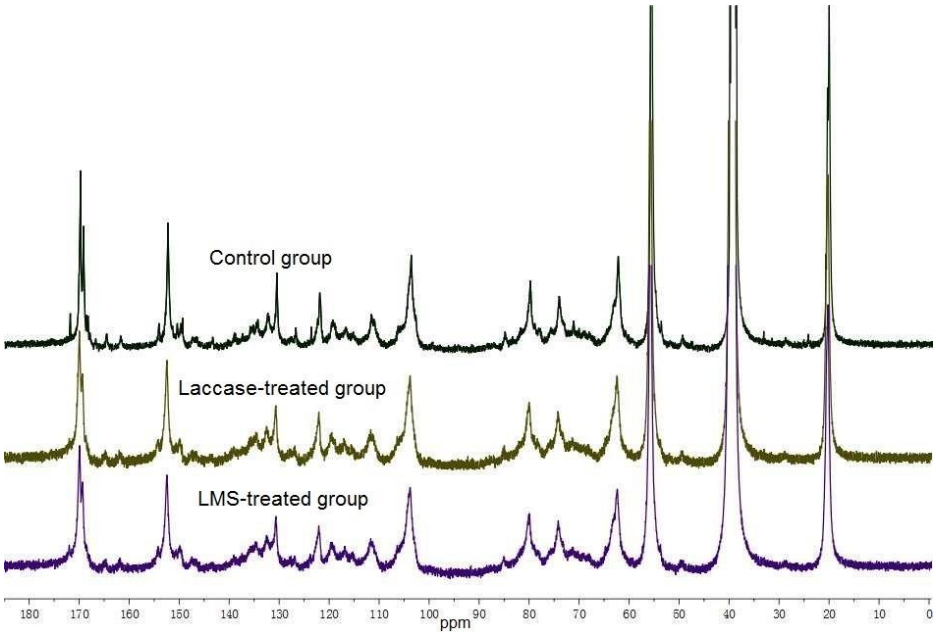

Fig. 2. The ^{13}C-NMR spectra of lignin samples

The integration area of these peaks can be employed as the reference standard to calculate the content of each functional group and connecting structure in lignin structure. However, this integration area is integrated area of 6 benzene rings, so the number of each functional group and connecting structure can be obtained by multiplying by 6. The specific calculation formula is shown as Eq. 1 (Chen 1998),

$$A_{i\text{-}j} = \frac{I_{i\text{-}j}}{I_{102.0\text{-}162.0}} \times 6 \quad (1)$$

where $A_{i\text{-}j}$ is the number of functional groups and connecting structure, of which the chemical shifts are between i to j, $I_{i\text{-}j}$ are the integration areas between i to j, and $I_{102.0\text{-}162.0}$ are the integration areas between 102.0 to162.0 of chemical shifts.

Table 2. Functional Group Frequency and Integration Regions for Qualitative Analysis of ^{13}C-NMR Spectra of Lignin and Relative Absorption Strength

Chemical shift (ppm)	Functional groups	Control group	Laccase-treated group	LMS-treated group
		The number of carbon on each benzene ring		
178.0-167.5	COOH	0.96	1	1
154.0-149.0	C_3-OH	0.63	0.69	0.75
140.0-127.0	C_1-C	0.55	0.84	0.48
123.0-117.0	C_6-H	0.51	0.44	0.42
114.0-106.0	C_2-H	0.3	0.23	0.23
90.0-78.0	C_β in β-O-4	1.24	1.19	1.00
78.0-67.0	C_α in β-O-4	0.91	0.93	0.83
57.0-54.0	OCH_3	2.57	2.42	2.2

Figure 2 and Table 2 show that the laccase and the LMS treatments affected the structure of lignin. After the treatments with laccase and LMS, the content of C_3-OH changed greatly, while the LMS-treated group had the highest content (an increase of 19.1%).

Meanwhile, compared to the control group, the C_1-C content of the laccase-treated group increased by 52.7%, whereas the C_1-C content of the LMS-treated group decreased by 13.7%. In addition, C_β of β-O-4 was reduced by 19.2% after the treatment with LMS. This change was not obvious after the modification with laccase. The changes to the C_α of β-O-4 were inconspicuous; however, the overall content decreased after the treatment with LMS.

These results showed that the LMS could degrade lignin. Moreover, laccase and LMS could diminish the contents of OCH_3 by 5.8% and 14.3%, respectively (Fig. 5).

Average Molecular Weight Changes of Lignin

The molecular weight distribution curves (Fig. 3) of the lignin samples were obtained by GPC. In general, GPC analysis was always done after lignin acetylation.

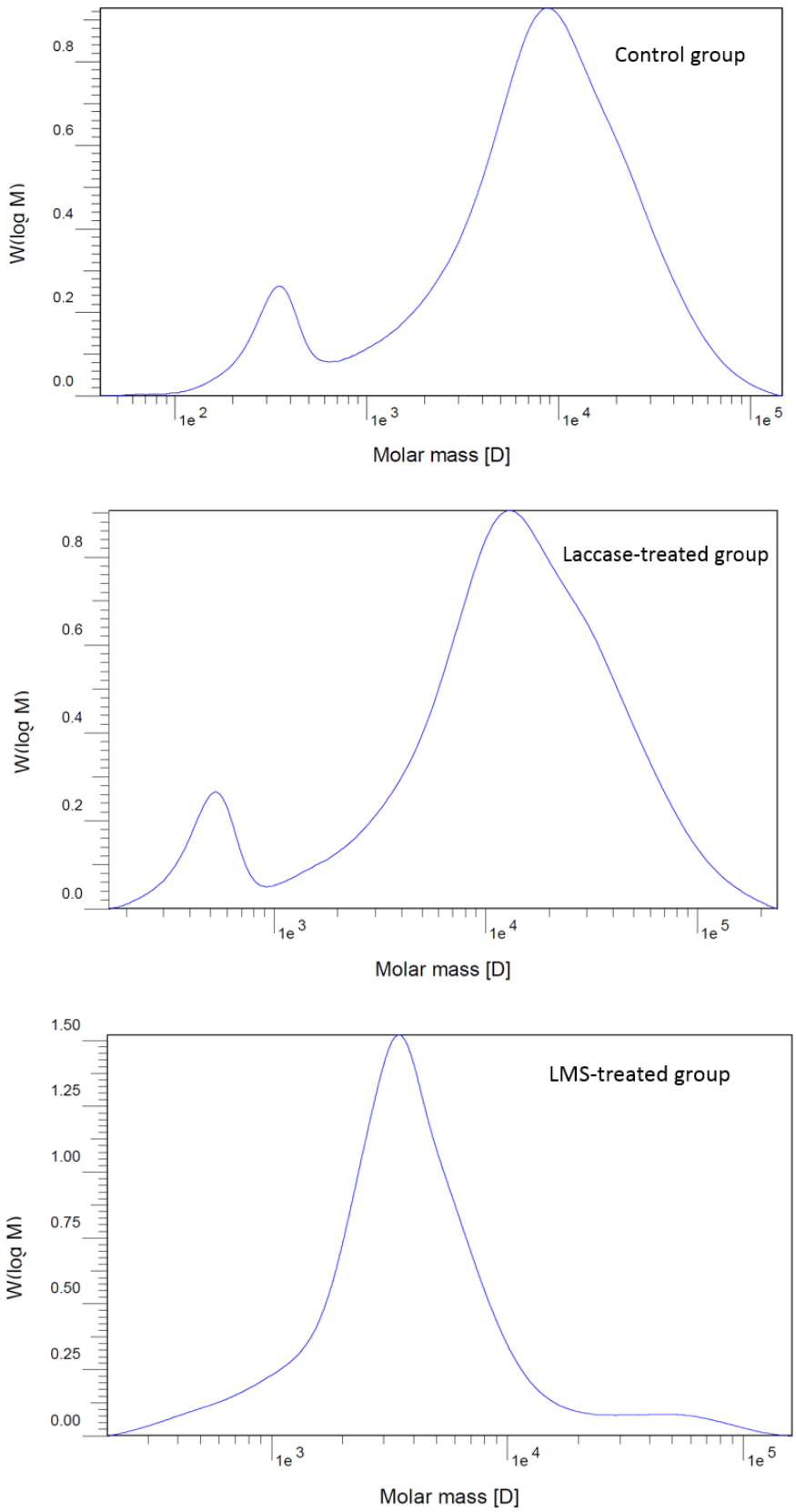

Fig. 3. The average molecular weight distribution curves of the three lignin samples

Table 3. Average Molecular Weight and Polydispersity of Lignin

Lignin	M_n (g/mol)	M_w (g/mol)	M_z (g/mol)	M_w/M_n
Control Group	2017	12624	29163	6.26
Laccase-treated group	3564	21415	50454	6.01
LMS-treated group	2549	6661	28348	2.61

*Values are an average of #samples for each group. M_n: number-average molecular weight, which can be averaged according to the number of molecules; M_w: weight-average molecular weight, which can be averaged according to the weight of molecules; M_z: Z- average molecular weight; M_w/M_n: polydispersity

As shown in Table 3, the average molecular weight of lignin changed after the laccase and the LMS treatments. The M_n of lignin increased for laccase and LMS by 76.7% and 26.4%, respectively. Meanwhile, during the modification reaction with laccase, the M_w of lignin increased 69.6%, while the M_w of lignin decreased after modifying with the LMS (47.2%). These changes in the M_w of lignin can possibly explain why the C_β of β-O-4 was reduced after modifying with the LMS. This also means that the LMS treatment was beneficial for degrading β-O-4 (Kawai *et al.* 2002; Wei *et al.* 2004).

The polydispersities of lignin changed, which may have occurred because the lignin was treated directly with laccase and LMS. During the reaction with laccase, the polydispersities of lignin decreased slightly from 6.26 to 6.01. However, during the reaction with LMS, the polydispersities of lignin decreased from 6.26 to 2.61, with a reduction of 58.3%.

CONCLUSIONS

1. The results of this study revealed that the laccase and LMS treatments could greatly influence the structure of isolated lignin from APMP. After the modification reaction with laccase and LMS, the phenolic OH and aliphatic OH contents decreased. This study also provided a better understanding for the mechanism of laccase and the LMS modification of APMP. The laccase and LMS treatments can improve the quality of fibers through catalytic oxidation of lignin.
2. After the modification by the LMS, the content of the C_β of the β-O-4 functional group decreased, which suggests that the LMS can lead to the degradation of lignin.
3. The ^{31}P-NMR and ^{13}C-NMR spectra analysis indicated that the influence of the LMS modification on lignin was greater than with laccase modification.
4. The GPC analysis provided evidence that the reactions of lignin with laccase and with the LMS were different. For the laccase modification process, the M_w of lignin was greatly influenced and increased, while for the LMS, the M_w decreased dramatically. Meanwhile, after the laccase and the LMS treatments, the M_n of lignin increased.

ACKNOWLEDGMENTS

The authors are grateful for the financial support from the National Science Foundation of China (Grant NO. 31270626) and Taishan Scholars Project Special Funds. In addition, the Life Sciences Laboratory of Shandong University is gratefully acknowledged for the generous use of its laboratory equipment.

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Article submitted: March 18, 2015; Peer review completed: June 20, 2015; Revised version received and accepted: January 11, 2016; Published: February 1, 2016.
DOI: 10.15376/biores.11.1.2723-2733