

EVALUATION OF THE STRUCTURAL AND MOLECULAR WEIGHT CHANGES OF LIGNIN DURING THE TREATMENT OF HARDWOOD ALKALINE PEROXIDE MECHANICAL PULP WITH LACCASE AND A LACCASE-MEDIATOR-SYSTEM

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Alkaline Peroxide Mechanical Pulp (APMP) of triploid of *Populus tomentosa* was modified by laccase and a Laccase-Mediator-System (LMS). The influence of the following main variables on the pulp physical properties were studied: enzyme dosage, reaction time, treatment temperature, and pH. Under the optimum conditions of laccase treatment – pH 5, temperature 50°C, pulp consistency 4%, and a reaction time of 60 min – the optimum charge of laccase was 2u/g. It was also found that the tensile strength and tear indices of the pulps treated with laccase increased significantly. The two-stage method of enzyme-mild acidic hydrolysis was adopted to isolate lignin from the APMP pulps both before and after enzymatic treatments. The functional groups in all lignin samples were qualitatively and quantitatively analyzed with ³¹P-NMR spectra. The molecular weight distributions of all the lignin samples were obtained through Gel Permeation Chromatography (GPC) after the lignin samples were benzoylated.

Keywords: Laccase; Alkaline peroxide mechanical pulp; Lignin structure; ³¹P-NMR spectra; Molecular Weight distribution; GPC

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INTRODUCTION

Compared to chemical pulps, high yield pulps possess many advantages such as a higher yield, higher bulk, better stiffness, excellent optical performance, and environmentally friendly character. But lignin-rich mechanical pulp fibers display poor flexibility, low fiber strength, low brightness, and rapid photoyellowing. Therefore, mechanical pulps cannot completely replace chemical pulps for the production of high-quality paper. Modification of the chemistry of mechanical pulps is therefore necessary to extend their application (Lachenal *et al.* 1994; Henriksson and Gatenholm 2002). Biotechnology, perhaps the most promising new tool in the arsenal of modern pulping technology, potentially offers a way to circumvent the deficits cited above for mechanical pulp fibers. Enzyme-modified fibers provide desirable properties depending on the enzyme used and fiber response (Poppius 1999; Gruber 2002). Firstly, cellulases can improve the drainability and papermaking performance (Maximino *et al.* 2011; Wong *et al.* 1999; Wong *et al.* 2000; Richardson *et al.* 1998); secondly, hemicellulases can reduce

the beating energy; lastly, lignin-degrading-based enzymes can improve the physical performance of mechanical pulps (Petit-Connil *et al.* 2005).

Nuclear magnetic resonance (NMR) is one of the most powerful ways to investigate how the chemistry of pulp fibers changes after enzymatic treatment by examining the molecular structure of specific fiber components. NMR was first applied to study wood structure in 1964 (Ludwig *et al.* 1964; Jiang *et al.* 1995). The ^1H -NMR technique was used more often in the early days, and then ^{13}C -NMR was more used widely, which greatly enhanced the understanding of lignin structures. In recent years, ^{31}P -NMR, two-dimensional, and three-dimensional NMR techniques have been applied to investigate the structure and understand the resultant function of lignin. ^{31}P -NMR has been most widely used to quantitatively determine hydroxyl groups in lignin (Koda *et al.* 2005; Argyropoulos *et al.* 2002; Faix *et al.* 1994).

The current work provides a detailed analysis of the chemical response of the lignin in Alkaline Peroxide Mechanical Pulps (APMP) of triploid of *Populus tomentosa* to laccase and a Laccase-Mediator-System (LMS) (Mansfield 2002; Li *et al.* 1997). A two-stage method of enzyme-mild acidic hydrolysis was adopted to isolate the lignin from the APMP and the modified pulp samples. The lignin samples were analyzed by ^{31}P -NMR, FT-IR, and Gel Permeation Chromatography (GPC). The differences of the two enzymes systems were investigated and compared to allow for an understanding of the mechanisms of laccase and LMS activity on the lignin in the APMP pulps.

EXPERIMENTAL

Materials

The first-stage APMP was provided by Shandong Zhongmaoshengyuan Group, Lingxian, Shandong. The brightness of this pulp was 53.5% ISO, which was low because it was taken before the stage of final bleaching with alkali H_2O_2 .

The commercial laccase code was NS 51003 offered by Novozymes A/S. Enzyme activity was 5600 u/g at the pH value range from 4.5 to 6.5 and at the optimal temperature of 50°C . The mediator was 1-hydroxy benzotriazole (HBT) of analytical grade, generously provided also by Novozymes A/S.

Modification with Laccase

Based on the previous research, it was determined under the constraints necessary for final pulp production, that the optimum conditions of laccase treatment were: pH 7, temperature 50°C , pulp consistency 4%, reaction time 60 min, and a charge of laccase of 2u/g (Wang and Liu 2010).

Modification with Laccase/Mediator System

1-hydroxy benzotriazole (HBT) was used at the dosage of 0.5% under the above optimum conditions. (Wang and Liu 2010).

Determination of Lignin Contents

Klason lignin (acid-insoluble lignin) and acid-soluble lignin contents of wood and pulps were determined according to TAPPI Test and Useful Methods (T222 om-8 and UM 250, respectively).

Lignin Isolation

In this research, all the residual lignin samples were isolated and purified by the enzymatic-mild acidic hydrolysis method to obtain lignin samples that meet the high standards of lignin isolation criteria that include a high yield, a high purity, and minimal structural changes (Argyropoulos *et al.* 2002; Wu and Argyropoulos 2003).

The pulp was extracted with acetone for 8 hours in a Soxhlet extractor to remove the extractives. The extracted pulp was washed twice by deionized water and air-dried for usage in subsequent steps.

Enzymatic hydrolysis was the first stage. In this stage, the pulp was subjected to cellulase treatment at 40°C for 48 hours in a 0.1 mol/L acetate buffer (pH 4.5) with 5% consistency. The cellulase dosage was 360 IU/g to the pulp. After enzyme treatment, it was centrifuged. The precipitate was collected and washed with acidified water (pH = 2) and then washed with deionized water. It was freeze-dried to obtain the crude lignin for mild acid hydrolysis.

In the mild acid hydrolysis stage, the pulp was subjected to a mixture of dioxane and water (dioxane:water = 85:15, v/v, containing 0.05mol/L HCl) at the consistency of 0.5% and refluxed for two hours at the azeotrope boiling point of 86°C under nitrogen. The resulting solution was filtered, and the solid residue was washed with fresh dioxane-water (dioxane:water = 85:15, v/v) and finally with dioxane. The filtrates were neutralized with sodium bicarbonate. The neutralized solution was concentrated by rotary evaporation. The concentrated solution was added drop-wise to a large amount of acidified deionized water (pH = 2). The precipitated lignin was isolated by centrifugation, washed, and freeze-dried. Then it was washed with dichloromethane to remove any extractives.

Quantitative ³¹P-NMR Spectroscopic Analysis

The ³¹P-NMR spectra of the lignin samples were obtained on a Bruker 400 MHz spectrometer. Approximately 40 mg of oven-dried lignin sample was placed into pyridine/chloroform (1.6:1, V/V) in a small vial and stirred continuously for several hours to fully dissolve it. N-hydroxyl naphthalimide was used as the internal standard, and chromium acetylacetonate as the relaxation reagent. Lastly, 2-chloro-1,4,4,5,5-tetramethyl-1,3,2-dioxaphospholane was used for phosphorylation of all of the lignin hydroxyl groups.

Benzoylation in Ionic Liquid

An ionic liquid ([amim]Cl – 1-allyl-3-methylimidazolium chloride) was added to a specified amount of lignin sample in a 8 mL sample bottle. It was vortexed until all solid particles had dispersed. And then it was heated at 80°C until the solution was clear. A small amount of pyridine was added to the solution and vortexed until it became homogenous, after which it was cooled to room temperature. A specified amount of

benzoyl chloride was added in one portion, and the mixture was vortexed until a homogeneous white paste was formed. The sample was left at room temperature for 3 hours. Deionized water and ethanol with a volume ratio of 1:3 was added, and the mixture was vigorously shaken and vortexed for 5 min. The solid was filtered off through a sintered funnel (grade 3), and then washed with ethanol and purified with methanol (stirred overnight without heat). The solid was then filtered off to give a white powder. The weight percent gain (WPG) was calculated for all the samples. The theoretical WPG was calculated using the value of 18.5 mmol/g of –OH group for cellulosic material, and 6.0 mmol/g of –OH group for lignin.

Lignin Molecular Weight Distribution

The molecular weight distributions of all the benzoylated lignin samples were obtained through Gel Permeation Chromatography (GPC). Approximately 40 mg oven-dried lignin sample was fully dissolved in ([amim]Cl), and then pyridine was added. Each mixture was stirred until it became a homogeneous solution. Benzoyl chloride was added, the mixtures were filtered, and the benzyolated lignin samples were vacuum-dried. The benzoylated derivatives showed good solubility in tetrahydrofuran (THF).

RESULTS AND DISCUSSION

Modification with Laccase and Laccase/Mediator System

It was determined under the constraints necessary for final pulp production that the optimum conditions of laccase treatment were: pH 7, temperature 50°C, pulp consistency 4%, reaction time 60 min, and a charge of laccase of 2u/g. The results of the pulp physical properties are shown in Table 1.

Table 1. Results of the Pulp Physical Properties

Dosage of Laccase /u/g	Dosage of Mediator /%	Brightness /%ISO	Tensile Strength Indexes /N·m/g	Tear Indexes /mN·m ² /g
0	0	53.5	8.56	—
2	0	49.8	9.06	1.80
2	0.5	49.0	10.60	2.25

As can be seen from Table 1, the brightness of the modified pulps with laccase and LMS decreased slightly in comparison to the control pulp sample. The tensile strength indices increased from 8.56 N·m/g to 9.06 N·m/g during the laccase treatment process, which represented a 5.8% increase. During the LMS treatment process, the tensile strength indexes increased from 8.56 N·m/g to 10.60 N·m/g, *i.e.*, an increase of 17.5%. These results indicated that the laccase and LMS modifying process could increase the pulp strength while incurring only a slight brightness decrease penalty. Clearly, the accessibility of the laccase was hindered without the mediator present; the mediator, as shown from previous work, is able to provide a convenient and efficient diffusible electron shuttle system that is free from the constraints of accessibility.

Lignin Contents Before and After the Modifications

From the data in the Table 2, it was clear that the pulp lignin contents were both decreased in the laccase modification and LMS modification processes, and the total lignin contents were decreased by about 9.6% in both cases.

Table 2. Results of the Pulp Lignin Contents

Pulp Samples	Klason Lignin (%)	Acid Soluble Lignin (%)	Total Lignin (%)
Untreated pulp	26.19	0.32	26.51
Laccase modified pulp	23.65	0.32	23.97
LMS modified pulp	23.55	0.31	23.86

Quantitative ^{31}P -NMR Spectroscopic Analysis

The ^{31}P -NMR Spectrum of phosphorous-derived lignin sample is shown in Fig. 1.

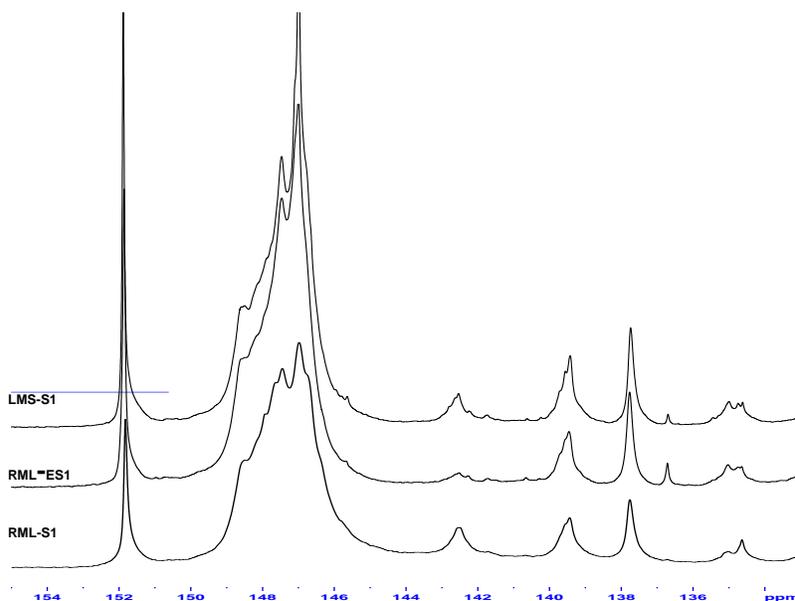


Fig. 1 ^{31}P -NMR spectra of Lignin samples from the APMP Pulps before and after enzymatic modifications

In Fig. 1, there were three lignin samples, RML-S1, RML-ES1, and LMS-S1. RML-S1 represented the lignin sample isolated from the first-stage APMP by the enzymatic-mild acidic hydrolysis method. RML-ES1 was from the modified APMP pulp with laccase. LMS-S1 was from the modified APMP pulp with laccase/mediator system. These codes were uniformly applied throughout this report.

The Lignin Functional Groups Analysis

The spectra demonstrated changes in the aliphatic, phenolic, and carboxylic acid groups. The integration fields corresponding to the various hydroxylic-based groups of lignin that were used in this study are listed in Table 3 (Liu *et al.* 2006).

The lignin structural changes observed are shown in Fig. 2. The A-OH label in the Figure represents aliphatic OH, and Tp represents total phenolic OH. COOH represents the OH in the carboxyl groups. It can be observed that the content of aliphatic hydroxyl increased from 7.32 mmol/g to 7.83 mmol/g in the laccase modifying process, an increase of 7.0%. However, in the LMS treatment process the content of aliphatic hydroxyl decreased from 7.32 mmol/g to 5.57 mmol/g, which represents a 23.9% decrease. In the laccase treatment process, the content of total phenolic hydroxyl decreased from 2.42 mmol/g to 1.48 mmol/g, which amounts to a 38.8% decrease. Similarly, the content of total phenolic hydroxyl decreased by 49.6%. Not surprisingly, it was found that the higher the concentration of carboxylic acid, a hydrophilic group in the lignin structure, the faster the delignification took place. It could be seen from Fig. 2 that the content of carboxylic acid decreased from 0.40 mmol/g to 0.33 mmol/g during the laccase treatment process, which represents a decrease of 17.5%. During the LMS modification process, the content of carboxylic acid decreased by 37.5%. These decreases in acid concentration indicate that the lignin was *not* as degraded in the laccase modification system; hence, less delignification occurred.

Table 3. Signals of Functional Groups of Lignin from the Hardwood and its Pulps

Chemical Shift (ppm)	Functional Groups
150.0~145.0	Aliphatic OH
144.6~143.6 & 142.4~140.2	Condensed phenolic OH
143.6~142.4	Guaiacyl and demethylate OH
140.2~138.6	Syringyl OH
138.6~137.0	<i>p</i> -hydroxy-phenyl OH
136.0~134.0	COOH

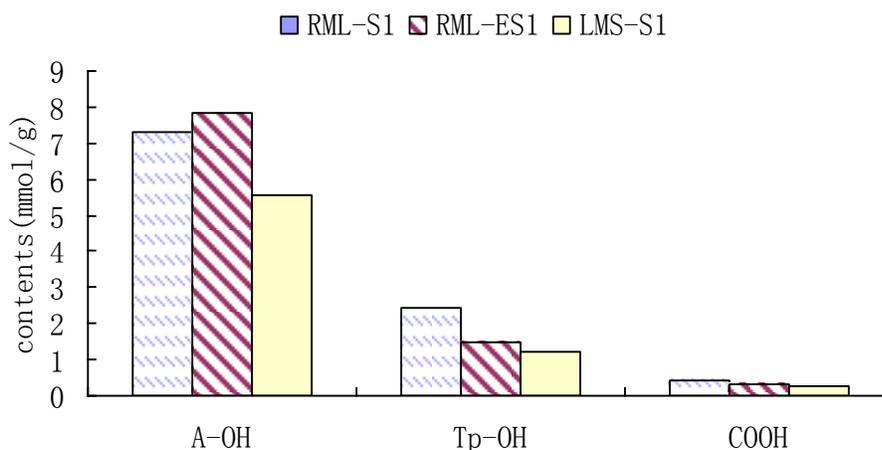


Fig. 2. Contents and comparison of OH and COOH in residual lignin samples

The contents of the phenolic OHs in the residual lignin samples are shown in Fig. 3. In the figure, C-OH represents condensed phenolic OH, S-OH represents Syringyl OH, G-OH represents Guaiacyl and demethylate OH, and *p*-OH represents *p*-hydroxy-phenyl OH, respectively.

The changes of all the phenolic OH of lignin during the modifications are shown in Fig. 3. After the laccase and LMS modifications, the condensed phenolic OH in the lignin decreased from 0.69 mmol/g to 0.43 mmol/g and 0.32 mmol/g with a decrease of 37.7% and 53.6%, respectively. Meanwhile, the Syringyl OH decreased from 0.52 mmol/g to 0.09 mmol/g and 0.16 mmol/g with a decrease of 82.7% and 30.8%, respectively. The contents of Guaiacyl and demethylate OH, *p*-hydroxy-phenyl also decreased after the two modification processes.

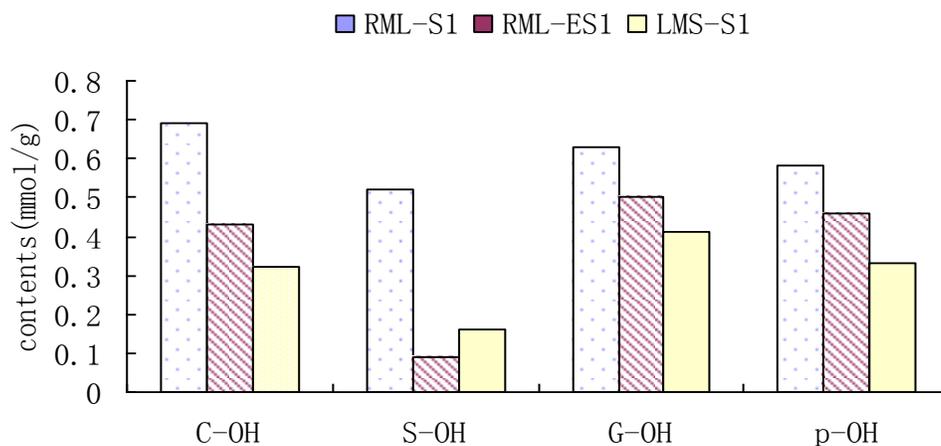


Fig. 3. Contents and comparison of phenolic OHs in residual lignin samples

Average Molecular Weight Changes of Lignin During Modifications

The molecular weight distribution curves of lignin samples obtained from gel permeation chromatography analysis system are shown in Fig. 4. Weight and number-average molecular weights (M_w and M_n) and polydispersity of lignin samples are shown in Table 4.

Table 4. Average Molecular Weights and Polydispersity of Lignin Samples

Code	M_n	M_w	M_z	M_w/M_n
RML-S1	2509	7526	4312	3.00
RML-ES1	267	24036	17220	90.2
LMS-S1	217	8869	5075	40.9

It was found that the average molecular weight of lignin had been greatly influenced by laccase and LMS. During the modification with laccase, the number-average molecular weight of the residual lignin of the first-stage APMP pulp decreased by 89.4%. During the modification with laccase/mediator system process, the number-average molecular weight similarly decreased by 91.4%. The weight of the residual lignin of the first-stage pulp increased by 2.2 times over that of the control pulp during the treatment with the laccase process, while it increased 17.8% during the treatment with the

laccase/mediator system process. During the modification with laccase, the number-average molecular weights of the residual lignin from the second-stage pulp decreased by 93.8%, while the number-average molecular weights of the residual lignin from the second-stage pulp decreased by 94.6% in the modified with laccase/mediator system process. Clearly, the lignin was being severely hydrolyzed by the enzymatic treatments, but the LMS system appeared to have a much more pronounced influence on the oxidation of the residual lignin and hence delignification. This result is not surprising in light of what is already known about LMS systems (Shleeva *et al.* 2006; Bourbonnais and Paice 1996, *etc.*)

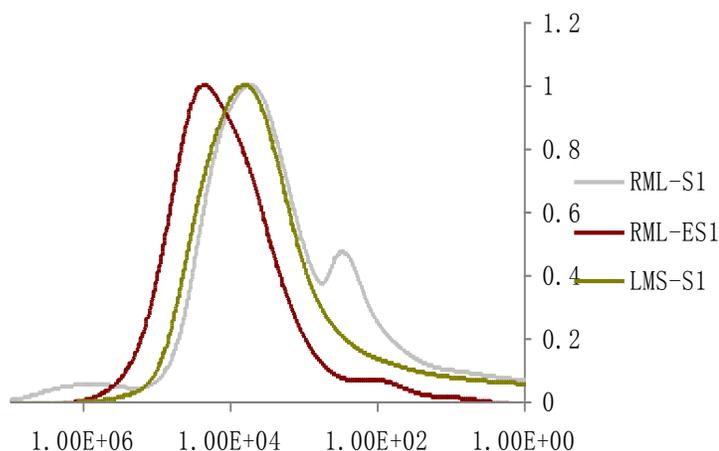


Fig. 4. Molecular weight distribution curves of lignin samples for APMP pulps before and after enzymatic modifications

Lignin polydispersity of the modified pulp with laccase and laccase/mediator system significantly increased during the modification of both the laccase and laccase/mediator system, whereas laccase/mediator system had a greater influence on residual lignin because it likely was based on a more easily mobile shuttle (mediator) to facilitate oxidative cleavage relative to the enzyme (Shleeva *et al.* 2006; Bourbonnais and Paice 1996).

CONCLUSIONS

1. The content of aliphatic hydroxyl groups (A-OH) of residual lignin from the APMP pulp was 7.32 mmol/g, and the content of total phenolic hydroxyl groups (T-OH) was 2.42 mmol/g. The content of carboxylic acid groups (COOH) was 0.40 mmol/g.
2. The content of aliphatic hydroxyl groups of residual lignin of the laccase modified pulp was 7.83 mmol/g, which had slightly increased. Compared to the control pulp there was an increase of 7%. The content of total phenolic hydroxyl groups was 1.48 mmol/g, representing a decrease of 38.8%. The content of carboxylic acid groups was 0.33 mmol/g.

3. The content of aliphatic hydroxyl groups of residual lignin of the LMS-modified pulp was 5.57mmol/g, and the content of total phenolic hydroxyl was 1.22 mmol/g. The content of carboxylic acid was 0.25 mmol/g, which was decreased about 49.6% in the laccase-modified pulp, while in the process of LMS modification its contents decreased by about 37.5%.
4. The results indicated that the treatment influence on residual lignin during the LMS modification process were slightly greater than that in the laccase modification process.

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