View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Digital.CSIC

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Understanding pulp delignification by laccase-mediator systems through isolation and characterization of lignin-carbohydrate complexes

Xueyu Du[1], Jiebing Li[1*], Göran Gellerstedt[1], Jorge Rencoret[2], José C. Del Río[2], Angel T. Martínez[3] and Ana Gutiérrez[2]

1. Department of Fibre and Polymer Technology, Royal Institute of Technology, KTH, Teknikringen 56-58, SE-10044 Stockholm, Sweden

2. Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, PO Box 1052, E-41080 Seville, Spain

3. Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

* Corresponding author: jbing@kth.se

Abstract

The effects and mechanism of pulp delignification by laccases in the presence of redox mediators have been investigated on unbleached eucalyptus kraft pulp treated with laccases from *Pycnoporus cinnabarinus* (PcL) and *Myceliophthora thermophila* (MtL) and 1-hydroxybenzotriazole (HBT) and methyl syringate (MeS) as mediators, respectively. Determination of corrected kappa number in eucalyptus pulps after the enzymatic treatments revealed that PcL-HBT exhibited more remarkable delignification effect than MtL-MeS. To obtain further insight, lignin-carbohydrate complexes (LCCs) were fractionated and subsequently characterized by NMR, thioacidolysis (followed by GC and SEC) and pyrolysis-GC/MS analyses before and after the enzymatic treatments and their controls. It can be

concluded that the laccase-mediator treatments altered the lignin structures in such a way that more lignin was recovered in the xylan-lignin fractions, as shown by Klason lignin estimation, with lower amounts of both syringyl (S) and guaiacyl (G) uncondensed units, as shown by thioacidolysis -GC, especially after the PcL-HBT treatment. The laccase-mediator treatment produced oxidation at Cα and cleavage of Cα and Cβ bonds in pulp lignin, as shown by pyrolysis-GC/MS. The general mechanism of residual lignin degradation in the pulp by laccase-mediator treatments is discussed in the light of the results obtained.

Keywords: Eucalyptus pulp, Delignification, Laccase-mediator systems, Lignin-carbohydrate complex (LCC), Thioacidolysis, Pyrolysis-GC/MS

Introduction

Lignin removal is a central issue in the paper pulp manufacture, which has been traditionally handled by chemical pulping and bleaching. Recently, there is a general trend worldwide to apply biotechnology for the lignin removal using e.g. ligninolytic enzymes as safer and more environmentally friendly alternatives for the chlorine-containing bleaching chemicals. Among different ligninolytic enzymes, lignin peroxidase (LiP) [1], manganese peroxidase (MnP) [2] and versatile peroxidase (VP) [3] are three well-known enzymes secreted by fungi with high oxidation capacity. LiP can directly oxidize lignin constituents, MnP generates Mn^{3+} that serves as a diffusible oxidizer, and VP combines the LiP and MnP degradative abilities [4]. As a fourth ligninolytic enzyme, laccase belongs to the largest group of blue multicopper oxidases, which exists in many plants, fungi as well as microorganisms. Laccase has outstanding redox ability, by which a wide range of aromatic substrates could be oxidized into phenoxy radicals under the catalysis of its copper ions using oxygen as the electron acceptor and producing water as by-product [5-8].

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Generally speaking, laccase is the most promising ligninolytic enzyme for industrial delignification applications since it is more readily available and easier to manipulate than LiP, MnP and VP. However, unlike the above peroxidases, this green biocatalyst possesses a relatively low redox potential (≤0.8 V). Thus, its direct application for lignin degradation would be restricted to the oxidation of phenolic lignin moiety since non-phenolic structures have a redox potential above 1.3 V. Laccase alone is unsuitable for lignin degradation in practice since there is only a small portion of phenolic groups in the entire lignin structure [9, 10] and, on the other hand, there is a limited ability of the bulk laccase molecule to penetrate into the cell wall. These limitations have however been overcome through mimicking the nature by using redox mediators, known as building a laccase-mediator system (LMS), which can achieve the oxidation of non-phenolic lignin [11]. Mechanistically, the small mediator molecule is firstly oxidized or activated by the laccase, and then it penetrates the compact lignocellulosic structure more easily than the enzyme and thus realizes the oxidation of lignin inside the sample.

The overall delignification efficiency of LMS depends on the laccase and mediator used and their combination. An effective LMS for lignin degradation should have the following features: an effective oxidation of the mediator, a high redox potential of the activated mediator for effectively oxidizing non-phenolic lignin and enough stability of the laccase from being inactivated by the free radical form of the mediator. Among many combinations investigated in the literature, the *Myceliophthora thermophila* laccase (MtL) with methyl syringate (MeS) and *Pycnoporus cinnabarinus* (PcL) laccases with 1-hydroxybenzotriazole (HBT) are successful examples of low and high-redox potential laccases using natural and synthetic mediators, respectively, as outlined below. The lignin degradation capacity by MtL-MeS has been demonstrated in experiments with model lignin compounds [12] and biobleaching of flax [13] and eucalyptus pulps [14, 15]. Additionally, the effectiveness of MtL-MeS on deinking

ACS Paragon Plus Environment

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

[16] and pitch removal [14] has also been shown. Likewise, the efficiency of PcL-HBT has been revealed on redox chemistry research [17], reactions on non-phenolic lignin models [18], biobleaching of flax [19, 20] and eucalyptus pulps [21, 22], pitch removal from different pulps [23-25] as well as on delignification of woody and nonwoody feedstock [26]

In order to know the potential of LMS as industrial biocatalyst it is important to understand the biodegradation mechanism and to evaluate and optimize the efficiency. At a theoretical level, this has commonly been achieved by performing LMS tests on lignin models and synthetic lignin structure (e.g. DHPs). For example, commercial lignosulphonates were used to investigate molecular weight increase effect by laccase catalysis [27]. At the practical level on the lignin macromolecule in the real paper pulps, the LMS tests have conventionally been investigated by measuring e.g. kappa number (a rough estimation of lignin content), brightness and viscosity. Further judgments have been reported by pulp analyses of thermogravimetry [28], microscopy [29], 1D [30] and 2D NMR [31], FTIR [22, 30], XPS [32] and analytical pyrolysis (Py-GC/MS) using lignin markers [33], lignin/carbohydrate ratio [21] or Ph-C1, Ph-C2 and Ph-C3 ratio [33].

It has now been known that the lignin is present in biomass as lignin-carbohydrate complex (LCC) [34]. After chemical pulping, the residual lignin still presents as LCC structures [35]. Therefore, fractionation of LCC structures before and after laccase-mediator treatment followed by structural characterization will supply more profound insights of the lignin biodegradation by LMS. This has been demonstrated by using *Streptomyces ipomoea* laccase together with acetosyringone as mediator where different LCC structures were fractionated following the universally applicable LCC fractionation protocol developed at KTH [34] followed by structural characterizations using thioacidolysis-GC and -SEC and NMR analysis [36]. In order to further deepen the LMS knowledge, the above-mentioned MtL-MeS and PcL-HBT combinations have been applied in this study on unbleached kraft pulp from eucalyptus

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(*Eucalyptus globulus*) followed by a comprehensive structural characterizations of pulp LCCs to reveal the presence, reactivity and biodegradation reaction mechanism of the residual lignin in the kraft pulp by the LMS.

Experimental

Samples

Industrial unbleached *Eucalyptus globulus* kraft pulp was provided by ENCE pulp mill (Pontevedra, Spain). Its kappa number and hexenuronic acid (HexA) content were 13.7 and 57.8 μmol g-1 respectively.

Laccases

A commercial fungal laccase from the ascomycete *M. thermophila* (MtL), provided by Novozymes (Bagsvaerd, Denmark), and a fungal laccase preparation obtained from a laccase-hyperproducing strain of the basidiomycete *P. cinnabarinus* (PcL) provided by Beldem (Andenne, Belgium), were used in this study. Laccase activity was measured as initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical (ε_{436} 29300 $M^{-1}·cm^{-1}$) in 0.1 M sodium acetate (pH 5) at 24ºC. The laccase activity of the two enzyme preparations were 945 U/ml and 45 U/ml, respectively. One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per min. Same loading in terms of laccase activity (20 $U·g^{-1}$ pulp) was used for comparative studies.

Mediators

The mediators used were methyl syringate (MeS; methyl 4-hydroxy-3,5-dimethoxybenzoate) from Alfa Aesar (Karlsruhe, Germany) and 1-hydroxybenzotriazole (HBT) from Sigma-Aldrich (Steinheim, Germany).

Laccase-mediator treatment

Pulp treatments with MtL-MeS were carried out using 10 g (dry weight) of pulp at 3% consistency (w:w) in 50 mM sodium dihydrogen phosphate buffer (pH 6). Laccase loading was 20 $U \cdot g^{-1}$ pulp and concentration of MeS in the reaction was kept at 6.75 mM. The treatments were carried out in 500-mL flasks with O_2 bubbling, placed in a thermostatic shaker at 170 $rev \cdot min^{-1}$ and 50 ºC, for 12 h. Pulp treatments with PcL were performed as described above for MtL, but using 50 mM sodium tartrate (pH 4) as a buffer and HBT (3mM) as mediator. Controls without laccase and mediator and controls including laccase alone (without mediator) were also performed. The different pulps obtained, which are listed in Table 1, were extracted with acetone in Soxhlet extractor for 24 h and further analyzed as extractives-free pulp samples.

LCC fractionation

LCC fractionation was performed as described previously [34, 37]. After Wiley milling to particle sizes smaller than 40 mesh and a subsequent 12 h ball-milling using the miller from SiebTechnik (Mulheim, Germany), around 500 mg of the milled pulp sample was suspended and then dissolved in 10 ml DMSO and 40% tetrabutylammonium hydroxide (TBAH) solution (v:v=1:1) under stirring for 4 h. The sample solution was then dispersed into 100 ml deionized water with stirring and was allowed to stand still overnight for the formation of precipitates. Separation of the precipitates by centrifugation was conducted followed by washing with deionized water until neutral pH. The washed precipitates were obtained after freeze drying as glucan-lignin. From the centrifugation supernatant, xylan-lignin was obtained after ultrafiltration (molecular weight cutoff: 1000 Da) for 48 h and a subsequent freeze drying.

Lignin content and polysaccharide composition

1
2
3 Kappa number (ISO 302) and content of HexA [38] were determined for all pulp samples.
4
5 Klason lignin and carbohydrate composition of LCCs were determined according to TAPPI
6
7 Standards T 222 om-06 and T 249 cm-00, respectively.
8
9
10 *NMR analyses of LCC*
11
12
13 ^{1}H-NMR spectra of the xylan-lignins were recorded after dissolution in DMSO-d_6 on a Bruker
14
15 Advance 400 MHz instrument using the standard Bruker pulse program at room temperature.
16
17 ^{1}H-NMR spectra of the glucan-lignin fractions were conducted after acetylation [39] and
18
19 dissolution in chloroform-*d*.
20
21
22
23 *Thioacidolysis-GC and –SEC of LCCs*
24
25
26 The thioacidolysis reaction was carried out as described[40]. The thioacidolysis product mixture
27
28 (about 6 mg) was silylated for 30 min at room temperature with 100 µl of pyridine and N,O-
29
30 bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (v:v=1:1) before analysis by gas
31
32 chromatography (GC) to quantify the monomeric degradation products, i.e. the uncondensed
33
34 syringyl (S) and guaiacyl (G) units present in the sample. GC analysis was carried out using a
35
36 DB-5MS column with helium as carrier gas. The initial temperature was 150 ^{o}C for 2 min,
37
38
39 and it was then increased by 5 ^{o}C/min to 170 ^{o}C, followed by an increase at 25 ^{o}C/min to 330
40
41 ^{o}C. Injector temperature was 230°C. Another portion of the thioacidolysis product mixture
42
43
44 (about 20 mg) was acetylated and analyzed by SEC to evaluate the size distribution of lignin
45
46 fragments and the extent of lignin condensation. SEC analysis was performed using a Waters
47
48 system with a Waters 2487 UV detector at 280 nm. The analyses were carried out at room
49
50 temperature using THF as the eluent, at a flow rate of 0.8 mL min^{-1}. The samples were
51
52
53 dissolved in THF and injected onto three ultrastyragel columns (HR 2.5, HR2, and HR 4)
54
55 coupled in series. The SEC system was calibrated with polystyrene standards in the molecular
56
57 weight range from 484 to 17900 g mol^{-1}.
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Py-GC/MS of LCC

Py-GC/MS analysis of approximately 100 µg sample were performed with a 3030 microfurnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS system equipped with a DB-1701 fused-silica capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness) and an Agilent 5973 mass selective detector (EI: 70 eV). The pyrolysis was performed at 500 ºC. The GC oven temperature was programmed from 45ºC (4 min) to 280 ºC (10 min) with the heating rate of 4ºC/min. Helium was served as the carrier gas (2 mL/min).

Results and discussion

Lignin content in whole eucalyptus enzymatically-treated pulps

The delignification by LMS was explored using one industrial eucalyptus kraft pulp. The well-established two combinations of different laccases and different mediators were investigated, MtL with MeS and PcL with HBT. The two treatments were conducted at pH 6 and pH 4, which were known as the optimal conditions of each enzyme, respectively [22, 31, 41]. Control experiments were also performed (Table 1).

The biodegradation effects by the LMS treatments were firstly analyzed on the pulp samples by kappa number and hexenuronic acid (HexA) content determination for evaluation of the removal of lignin and HexA, a mimicking lignin structure formed during kraft pulping and consuming MnO_4^- in kappa estimation [42]. To better express the lignin content by kappa number, the corrected kappa number was calculated out by subtraction of the kappa number contribution from HexA from the pulp kappa number by the conversion factor of 0.086 kappa number per 1 $\mu mol \cdot g^{-1}$HexA [43] (Table 1).

1
2
3 As shown in Table 1 the delignification produced by MtL-MeS (1.4 kappa number units reduction) was weaker than with PcL-HBT (kappa number reduction of 2.4 units). This is in agreement with the difference in the redox potential values of the two enzymes, 450 mV and 750 mV for MtL and PcL respectively [18]. Since the MeS used in a laccase-mediator treatment was known being able to graft onto the fibre ([14] and see below) which will increase the kappa number [44], the actual delignification effect of MtL-MeS could be larger than the 1.4 kappa units observed. Interestingly, the control experiment with a buffer solution of pH 4 removed a part of the residual lignin (0.5 unit), while the control experiment at pH 6 did not delignify the pulp (corrected kappa number of 8.6 vs. 8.7 of the initial pulp). The removable part of lignin would be a type of acid-leachable lignin located at the pulp surface. Very obviously, without mediator both enzymes had only a gentle and similar biodegradation capability, 0.6 and 0.4 kappa unit reduction for MtL and PcL respectively. Mechanistically, the lignin removed by the laccases without mediators should be those moieties located at the pulp surface and of phenolic group rich type. As shown in Table 1, no HexA removal was observed for all the pulp samples investigated here.

Characterization of LCC from enzymatically-treated pulps

In this study, we have performed lignin-carbohydrate complexes (LCCs) fractionation and characterization following the universally applicable protocols previously developed [34]. Pulps treated with LMS and their corresponding controls without laccase and mediator were fractionated into two LCCs, one glucan-lignin fraction, whose sugar constitution is mainly glucose (> 90%), and one xylan-lignin fraction, the major sugar fraction of which is xylose (> 88%) (Table 2).

Comparatively, glucan-lignin fractions are the dominating LCC fractions (around 80% of yield) with smaller amounts of lignin (0.4-2.4%) while xylan-lignin fractions (20% yield)

contain higher amounts of residual lignin (2.7-7.3%), (Table 2). Here we would like to point out that due to limited amounts of ball-milled pulps used in the experiments (around 500 mg) there were weighing errors for the LCC Klason lignin values obtained. Nevertheless, it is certain that higher Klason lignin contents were observed in the xylan-lignin fractions after the LMS treatments, 7.3 (MtL-MeS) vs. 4.5% (Control pH 6) and 6.7 (PcL-HBT) vs. 2.7% (Control pH 4). This could be attributable to the lignin biodegradation. When the lignin from the glucan-lignin fractions was degraded, the lignin fragments, free from the glucan part of the original LCC structure, will remain in the solution which contained DMSO, instead of being precipitated by the addition of water, and eventually be a part of the xylan-lignin fractions after freeze-drying (see the experimental part). Due to the above mentioned experimental errors in Klason lignin determination, a decrease of lignin content in glucan-lignin was not observed in the case of MtL-MeS, 1.5 (MtL-MeS) vs. 1.4 % (Control pH 6) while the decrease was evident in the case of PcL-HBT, 0.4% (PcL-HBT) vs. 2.4% (Control pH 4).

^{1}H NMR Analysis

^{1}H NMR was applied to characterize the structures of the fractionated LCCs. Since it was difficult to dissolve glucan-lignin in any common NMR solvent, acetylation was performed to enhance the sample solubility in chloroform-*d*. After the acetylation, all glucan-lignin fractions from the 4 pulp samples (Table 2) gave very similar ^{1}HNMR spectra to each other, the typical one of PcL-HBT treated sample being shown in Fig. 1A, where the six types of proton signals from glucosyl units are clearly observed from 3.53-5.06 ppm. In the region for aromatic protons, no lignin signals could be noticed even after a signal magnification. This is due to the fact that the lignin contents were low (0.4-2.4% Klason lignin).

All the xylan-lignin fractions could be readily dissolved in DMSO-d_6 for ^{1}HNMR analysis. Their ^{1}HNMR spectra were also considerably similar to each other. The example from the PcL-HBT treated pulp is depicted in Fig. 1B; the five types of protons signals from xylosyl units are located from 3.04-4.26 ppm. The aromatic proton signals are barely noticeable compared with the signals of the carbohydrate parts, which is due to the low lignin quantity in these LCC fractions (2.7-7.3% of Klason lignin). After signal magnification of the 6.0-7.0 ppm region as performed for the glucan-lignin NMR spectra, the lignin aromatic signals could then be more clearly seen. The broad-peak shape of the signals confirms the presence of the lignin as a part of the xylan-lignin fraction. The much stronger aromatic signals in the xylan-lignin than glucan-lignin fractions are attributable to the higher lignin contents.

Thioacidolysis-GC and SEC analysis

Since β-O-4′ linkage is the most abundant lignin inter-unit connection type and thioacidolysis is an analytical technique for a complete cleavage of all β-O-4′ bonds into various lignin fragments, thioacidolysis was conducted followed by GC and SEC analysis of the fragments to further evaluate the LCC structures [40]. The results from thioacidolysis-GC analysis showed that only xylan-lignin contained detectable uncondensed monomeric lignin structures, while no uncondensed monomeric lignin structures could be detected in any of the glucan-lignin fractions analyzed (Table 2) that implies that the lignin present in glucan-lignin fractions is more condensed than that in the xylan-lignin ones, since lignin content in glucan-lignin fractions was generally higher than in the xylan-lignin ones. As typically found in eucalyptus pulps, uncondensed S units are more abundant than G units in the xylan-lignin, the S/G ratio being 2.5-3.4. This ratio is lower than the one found in wood lignin from eucalyptus (4.1-4.9) [37] but similar to that reported by [22] from Py-GC/MS of eucalypt pulp, due to the pulping process during which S units would be preferentially removed than G units. In

agreement with the results on kappa number, the lignin modification effect by MtL-MeS was lower than with PcL-HBT. The uncondensed S and G unit contents in the xylan-lignin of treated pulps compared to those from control pulp (pH 6), decreased from 18 to 15 and from 7 to 5 μmol/g, respectively. On the contrary, in xylan-lignin fractions from pulps treated with PcL-HBT a higher delignification effect was observed, and uncondensed S and G units were sharply reduced from 17 to 5 and from 5 to 2 μmol/g, respectively.

From the thioacidolysis-SEC analysis, the molecular weight distribution of all lignin fragments (as detected by UV detection at 280 nm) was studied. Figure 2 shows that a buffer pH 4 treatment (control pH 4) could remove the fraction of condensed lignin structure eluted at 33.6 min (present in control pH 6), which would be responsible for the observation of a slight reduction (0.5 unit) of the kappa number mentioned above. This SEC analysis shows that both laccase-mediator treatments depolymerized the lignin resulting in more fragments eluted at longer times than 35.2 min after the thioacidolysis. They also polymerized the lignin by forming more fragments eluted at the times shorter than 34 min, showing especially a large polymer fraction showed up at around 32.7-33 min at both MtL-MeS and PcL-HBT treated xylan-lignin fractions. Interestingly, more polymeric fragments were found in the case of MtL-MeS treatment by a larger peak area shown in the thioacidolysis-SEC chromatogram at the times shorter than 34 min after the peak normalization to viable the comparison.

Py-GC/MS analysis of LCC

Py-GC/MS is another sensitive technique which was conducted for more detailed investigation of the changes in the lignin structures. The identities and relative abundances of all pyrolysis products, after performing single-ion integration due to low intensity of their original Total Ion Current (TIC) signals (cf. [21]), are listed in Table 3. As a sharp contrast to the thioacidolysis-GC analysis mentioned above, even the lignin structure in glucan-lignin

fractions could now be characterized since various S and G unit fragments were detected from both LCCs. When comparing the fragmentation patterns between MtL-MeS treated pulp with its control (pH 6), big increases of syringol amounts in both LCCs could be noticed, 17.4 vs. 12.4% in glucan-lignin and 30.7 vs. 14.4% in xylan-lignin. An increase of S/G ratio was previously reported as the result of PcL-HBT treatment [21]. However, if we include all the lignin fragments for the S/G ratio calculation, we can conclude that there is actually no noticeable change in the S/G ratio, except for the glucan-lignin fraction in the case of MtL-MeS. For PcL-HBT, the ratio is always around 2.1-2.5 for all LCCs.

From the lignin fragments, obvious lignin oxidation by LMS could directly be noticed. In the case of PcL-HBT, Cα carbon oxidation was observed in the xylan-lignin fraction since the amounts of all Cα carbonyl structures increased, reaching 0.24 compared with 0.13 in the control (Table 3). Such an evaluation method was reported in the literature [45]. Here these carbonyl groups could be attributable solely to the oxidations during the laccase-mediator treatment since the pyrolysis process itself was performed under an inert gas. Interestingly, this Cα carbon oxidation could not be noticed from the glucan-lignin fraction. Neither do this in the case of MtL-MeS. In the case of MtL-MeS, Cα-Cβ bonding cleavage reactions could be noticed from both LCCs. Due to the cleavage that reduced the amounts of longer side chain structures than one carbon already after the LMS treatment, there were obvious declines of both Ph-C2 and Ph-C3 fragments after the pyrolysis. The amounts of Ph-C2 and Ph-C3 fragments decreased from 27.5 to 21.9% and 31.0 to 22.0% in glucan-lignin respectively and 25.5 to 20% and 30.6 to 24.3% in xylan-lignin respectively. Interestingly, this Cα-Cβ bonding cleavage could not be noticed for the case of PcL-HBT. Indeed, the two LMS worked obviously differently from each other.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Based on the observations from this study and the knowledge reported in the literature, we could conclude the lignin biodegradation mechanism by LMS as follows. For phenolic lignin structures, the initial enzymatic reaction would be the oxidation by laccase+O_2 ±mediator leading to the formation of phenoxy radicals [9]. Since non-phenolic units are expectedly being dominating in the residual lignin structures of chemical pulps, two possible routes are important to initiate radical structures in the non-phenolic units, electron transfer (ET) route and hydrogen atom transfer (HAT) route [46]. Both reactions lead to the formation of benzyl carbon-centered radicals of non-phenolic units.

Concerning the treatment with PcL-HBT, it is worth mentioning that the HBT used is known among the most successful mediators found so far and there is actually a radical HAT pathway as revealed by model experiments [46]. The formation of the carbon-centered radicals of non-phenolic lignin units of both S and G units could be drawn as structures **I** as depicted in Fig. 3. Subsequently there is a non-enzymatic reaction of O_2 attacking the carbon-centered radical intermediates, forming structures **IIα** which are not stable and will give rise to a variety of reactions, such as Cα oxidation, Cα-Cβ cleavage and aromatic ring cleavage [47]. The Cα oxidation leads to structures **III**, which explains the observations of more Cα carbonyl fragments obtained after pyrolysis of the LCCs obtained from the PcL-HBT treated pulps. Due to the change at the Cα positions, the thioacidolysis will not any longer lead to formation of “ordinary” G and S uncondensed units which could be detected by the thioacidolysis-GC analysis [40]. That is why reduced amounts of uncondensed G and S units were observed in the analysis after the PcL-HBT treatment. Alternatively, the radical structures **IIα** abstract H-atoms from a substrate, forming non-radical structures **IIβ**. From **IIβ** the cleavage between Cα and Cβ via Baeyer-Villiger reaction takes place, which reduces the molecular sizes of the lignin and generates structures **IV** and **V** with methoxybenzoic acid groups, as reported for LMS treatment of model dimer [48] and lignocellulose fungal treatment

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

[49]. This explains the observations of reduced amounts of PhC2 and PhC3 fragments after the Py-GC/MS analysis of the LMS treated LCCs by MtL-MeS. Interestingly, a significant increase in syringic acid (identified as methylated derivative by Py-GC/MS after using tetramethylammonium hydroxide as derivatization reagent) was observed after both MtL-MeS and PcL-HBT treatments (not shown in Table 3). In the former case this compound could be mainly derived from MeS, but its significant increases in the treatment with PcL-HBT, from 1% to 2.4% (glucan-lignin) and from 2.4% to 6.2% (xylan-lignin) supports formation from S units during LMS treatment. Chemically, the actual route of the LMS oxidation depends on the conversion efficiency of **IIα** to **IIβ** which requests a substrate to supply H-atom. Since MeS has a phenolic structure and may be a better H-atom supplier than the HBT and a higher amount of MeS than HBT was applied (6.75 vs. 3 mM, see the experimental part), more Cα oxidation was noticed in the case of PcL-HBT and more Cα-Cβ cleavage was noticed for MtL-MeS (by Py-GC/MS). In addition, there are also subsequent or separate reactions to the Cα oxidation or Cα-Cβ cleavage, leading to aromatic ring cleavage (not shown in Fig. 3). From the radical structure **I**, two other non-enzymatic reactions will take place: i) eventual radical coupling with mediator, which would result in MeS grafting ; and ii) radical coupling among lignin radicals resulting in lignin condensation, one explanation of the more condensed structures seen by the thioacidolysis-SEC analysis. Moreover, when the entire LCC structure becomes smaller, it will have a higher solubility in the DMSO containing solution and be found in the xylan-lignin fractions. That is why the Klason lignin contents in the xylan-lignin fractions increased after LMS treatments. If the lignin structure is degraded to such an extent that it become soluble in the LMS process solution, the pulp is delignified. This will result in a reduced pulp corrected kappa number, as obtained after HexA content determination, which was more important in the PcL-HBT treatment. Concerning MtL-MeS, previous studies had

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

shown that a sequence including alkaline extraction after LMS is required to obtain significant pulp delignification with this commercial laccase and natural mediator system [14].

Conclusion

In the light of the results obtained by this study, the lignin biodegradation reactions by LMS include Cα carbon oxidation into keto structure, cleavage of Cα and Cβ carbon-carbon bond and aromatic ring cleavage. All reactions will eventually lead to lignin degradation and finally delignification of the chemical pulp. Different combinations of laccase and mediator will lead to differences in the actual oxidation routes and eventually in the total delignification effects. The characterization of trace amounts and of even tracer amounts of the lignin in chemical pulp samples after LMS treatments could be greatly improved by fractionation of lignin-carbohydrate complexes followed by applications of highly sensitive analytical techniques such as thioacidolysis-GC and –SEC and Py-GC/MS analyses so that more comprehensive lignin structural information could be obtained.

Acknowledgement

This study was financially supported by the EU BIORENEW Project (NMP2-CT-2006-026456). Novozymes (Bagsvaerd, Denmark) and Beldem (Andenne, Belgium) are acknowledged for the *M. thermophila* and *P. cinnabarinus* laccases, respectively. J. Romero (ENCE, Spain) is acknowledged for providing the eucalyptus pulp samples and E.D. Babot for performing the enzymatic treatments. J. Rencoret acknowledges the CSIC JAE-Doc contract of the program “Junta para la Ampliación de Estudios” co-financed by the European Social Fund (ESF).

References

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(1) Tien, M.; Kirk, T. K. *Science* **1983,** 221, 661-662.
(2) Kuwahara, M.; Glenn, J. K.; Morgan, M. A.; Gold, M. H. *FEBS Lett.* **1984,** 169, 247-50.
(3) Camarero, S.; Sarkar, S.; Ruiz-Duenas, F. J.; Martinez, M. J.; Martinez, A. T. *J Biol Chem* **1999,** 274, 10324-10330.
(4) Ruiz-Duenas, F. J.; Martinez, A. T. *Microbial Biotechnology* **2009,** 2, 164-177.
(5) Yaropolov, A. I.; Skorobogatko, O. V.; Vartanov, S. S.; Varfolomeyev, S. D. *Appl Biochem Biotech* **1994,** 49, 257-280.
(6) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. *Chem Rev* **1996,** 96, 2563-2605.
(7) Giardina, P.; Faraco, V.; Pezzella, C.; Piscitelli, A.; Vanhulle, S.; Sannia, G. *Cell. Mol. Life Sci.* **2010,** 67, 369-385.
(8) Canas, A. I.; Camarero, S. *Biotechnology Advances* **2010,** 28, 694-705.
(9) Higuchi, T. *Proc. Jpn. Acad., Ser. B* **2004,** 80, 204-214.
(10) Kirk, T.; Cullen, D., Enzymology and molecular genetics of wood degradation by white-rot fungi. In *Environmentally friendly technologies for the pulp and paper industry*, Young, R.; Akhtar, M., Eds. TAPPI: Atlanta, 1998; pp 273-308.
(11) Bourbonnais, R.; Paice, M. G. *FEBS Lett* **1990,** 267, 99-102.
(12) Nousiainen, P.; Maijala, P.; Hatakka, A.; Martinez, A. T.; Sipila, J. *Holzforschung* **2009,** 63, 699-704.
(13) Fillat, A.; Roncero, M. B.; Vidal, T. *J Chem Technol Biot* **2011,** 86, 1501-1507.
(14) Babot, E. D.; Rico, A.; Rencoret, J.; Kalum, L.; Lund, H.; Romero, J.; del Rio, J. C.; Martinez, A. T.; Gutierrez, A. *Bioresour. Technol.* **2011,** 102, 6717-6722.
(15) Valls, C.; Cadena, E. M.; Roncero, M. B. *Carbohyd Polym* **2013,** 92, 276-282.
(16) Fillat, U.; Prieto, A.; Camarero, S.; Martinez, A. T.; Martinez, M. J. *Biochem Eng J* **2012,** 67, 97-103.
(17) Xu, F.; Kulys, J. J.; Duke, K.; Li, K. C.; Krikstopaitis, K.; Deussen, H. J. W.; Abbate, E.; Galinyte, V.; Schneider, P. *Appl Environ Microb* **2000,** 66, 2052-2056.
(18) Li, K. C.; Xu, F.; Eriksson, K. E. L. *Appl Environ Microb* **1999,** 65, 2654-2660.
(19) Camarero, S.; Garcia, O.; Vidal, T.; Colom, J.; del Rio, J. C.; Gutierrez, A.; Gras, J. M.; Monje, R.; Martinez, M. J.; Martinez, A. T. *Enzyme Microb. Technol.* **2004,** 35, 113-120.
(20) Fillat, A.; Colom, J. F.; Vidal, T. *Bioresour Technol* **2010,** 101, 4104-4110.
(21) Camarero, S.; Ibarra, D.; Martinez, A. T.; Romero, J.; Gutierrez, A.; del, R. J. C. *Enzyme Microb. Technol.* **2007,** 40, 1264-1271.
(22) Ibarra, D.; Romero, J.; Martinez, M. J.; Martinez, A. T.; Camarero, S. *Enzyme Microb Tech* **2006,** 39, 1319-1327.
(23) Gutierrez, A.; del Rio, J. C.; Ibarra, D.; Rencoret, J.; Romero, J.; Speranza, M.; Camarero, S.; Martinez, M. J.; Martinez, A. T. *Environ Sci Technol* **2006,** 40, 3416-3422.
(24) Gutierrez, A.; del Rio, J. C.; Rencoret, J.; Ibarra, D.; Martinez, A. T. *Appl Microbiol Biot* **2006,** 72, 845-851.
(25) Valls, C.; Molina, S.; Vidal, T.; del Rio, J. C.; Colom, J. F.; Martinez, A. T.; Gutierrez, A.; Roncero, M. B. *Process Biochem* **2009,** 44, 1032-1038.
(26) Gutierrez, A.; Rencoret, J.; Cadena, E. M.; Rico, A.; Barth, D.; del Rio, J. C.; Martinez, A. T. *Bioresour Technol* **2012,** 119, 114-122.
(27) Areskogh, D.; Li, J.; Gellerstedt, G.; Henriksson, G. *Biomacromolecules* **2010,** 11, 904-10.
(28) Barneto, A. G.; Valls, C.; Ariza, J.; Roncero, M. B. *Bioresour Technol* **2011,** 102, 9033-9039.
(29) Speranza, M.; Ibarra, D.; Romero, J.; Martinez, A. T.; Martinez, M. J.; Camarero, S. *Biocatal Biotransfor* **2007,** 25, 251-259.
(30) Sealey, J.; Ragauskas, A. J. *Enzyme Microb Tech* **1998,** 23, 422-426.
(31) Ibarra, D.; Chavez, M. I.; Rencoret, J.; del Rio, J. C.; Gutierrez, A.; Romero, J.; Camarero, S.; Martinez, M. J.; Jimenez-Barbero, J.; Martinez, A. T. *Holzforschung* **2007,** 61, 634-646.
(32) Liu, N.; Shi, S. L.; Gao, Y.; Qin, M. H. *Enzyme Microb Tech* **2009,** 44, 89-95.
(33) del Rio, J. C.; Gutierrez, A.; Romero, J.; Martinez, M. J.; Martinez, A. T. *J Anal Appl Pyrol* **2001,** 58, 425-439.

(34) Du, X.; Gellerstedt, G.; Li, J. *Plant J* **2013,** 74, 328-38.
(35) Lawoko, M.; Henriksson, G.; Gellerstedt, G. *Biomacromolecules* **2005,** 6, 3467-3473.
(36) Du, X.; Eugenio Martin, M. E.; Li, J. *Holzforschung* **2013,** 10.1515/hf-2012-0140.
(37) Li, J. B.; Martin-Sampedro, R.; Pedrazzi, C.; Gellerstedt, G. *Holzforschung* **2011,** 65, 43-50.
(38) Gellerstedt, G.; Li, J. B. *Carbohyd Res* **1996,** 294, 41-51.
(39) Gellerstedt, G., Gel Permeation Chromatography. In *Methods in lignin chemistry*, Lin, S.; Dence, C., Eds. Springer-Verlag: Heidelberg, 1992; pp 487-497.
(40) Rolando, C.; Monties, B.; Lapierre, C., Thioacidolysis. In *Methods in lignin chemistry*, Lin, S. Y.; Dence, C. W., Eds. Springer-Verlag: Heidelberg, 1992; pp 334-349.
(41) Berka, R. M.; Schneider, P.; Golightly, E. J.; Brown, S. H.; Madden, M.; Brown, K. M.; Halkier, T.; Mondorf, K.; Xu, F. *Appl Environ Microb* **1997,** 63, 3151-3157.
(42) Li, J.; Gellerstedt, G. *Nord Pulp Paper Res* **1998,** 13, 153-158.
(43) Li, J.; Gellerstedt, G. *Carbohyd Res* **1997,** 302, 213-218.
(44) Cadena, E. M.; Du, X.; Gellerstedt, G.; Li, J.; Fillat, A.; Garcia-Ubasart, J.; Vidal, T.; Colom, J. F. *Bioresour Technol* **2011,** 102, 3911-7.
(45) Ohra-aho, T.; Tenkanen, M.; Tamminen, T. *J Anal Appl Pyrol* **2005,** 74, 123-128.
(46) Fabbrini, M.; Galli, C.; Gentili, P. *J Mol Catal B-Enzym* **2002,** 16, 231-240.
(47) Kawai, S.; Umezawa, T.; Higuchi, T. *Arch Biochem Biophys* **1988,** 262, 99-110.
(48) Kawai, S.; Nakagawa, M.; Ohashi, H. *Enzyme Microb Tech* **2002,** 30, 482-489.
(49) Camarero, S.; Galletti, G. C.; Martinez, A. T. *Rapid Commun. Mass Spectrom.* **1997,** 11, 331-334.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Kappa number and hexenuronic acid (HexA) content of eucalyptus unbleached kraft pulps after laccase-mediator treatments with MtL-MeS and PcL-HBT compared with the initial untreated material, controls without laccases (control pH 6 and control pH 4) and controls with laccase alone (MtL and PcL). Corrected Kappa number is also included.

Sample	Kappa number	HexA (μmol/g)	Corrected Kappa number*
Initial pulp	13.7	58	8.7
Control pH 6	13.5	57	8.6
MtL	13.0	57	8.1
MtL-MeS	12.3	57	7.4
Control pH 4	13.0	56	8.2
PcL	13.1	56	8.3
PcL-HBT	11.1	56	6.3

* A kappa number reflecting better the true lignin content after subtraction of the pulp kappa number by the contribution from the HexA using the conversion factor of 0.086 kappa number per 1 $\mu mol \cdot g^{-1}$ HexA [43].

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 2.Yield (percentage of starting pulp), lignin content (Klason lignin, percentage on LCC), lignin composition in terms of uncondensed G and S lignin units (µmol/g) released by thioacidolysis, and carbohydrate composition of glucan-lignin and xylan-lignin fractions after MtL-MeS and PcL-HBT treatments compared with the controls without laccases and mediator (control pH 6 and control pH 4).

Samples		Yield (%)	Lignin			Carbohydrates (relative %)				
			Lignin (%)	G units	S units	Ara	Xyl	Man	Gal	Glu
Control pH 6	Glucan-lignin	~80	1.4	0	0	1.9	5.4	0.8	0	91.9
	Xylan-lignin	~20	4.5	7	18	4.3	88.4	0	2.3	5.0
MtL-MeS	Glucan-lignin	~80	1.5	0	0	2.8	4.9	0.7	0	91.5
	Xylan-lignin	~20	7.3	5	15	2.0	92.0	0	1.9	4.1
Control pH 4	Glucan-lignin	~80	2.4	0	0	3.1	4.9	0.7	0	91.3
	Xylan-lignin	~20	2.7	5	17	4.1	90.9	0	2.0	3.0
PcL-HBT	Glucan-lignin	~80	0.4	0	0	2.6	5.3	0.6	0	91.5
	Xylan-lignin	~20	6.7	2	5	2.6	93.6	0	1.6	2.2

Table 3.The different pyrolyzed lignin fragments and their relative intensities (%) from blank experiments (buffer pH 6 and pH 4) and LMS treatments

No	Name	Abbreviation	Ion for integration	Buffer 6		MtL-MeS		Buffer 4		PcL-HBT	
				Glucan-lignin	Xylan-lignin	Glucan-lignin	Xylan-lignin	Glucan-lignin	Xylan-lignin	Glucan-lignin	Xylan-lignin
1	Guaiacol	G	124	3.4	5.0	11.7	4.8	3.5	5.4	3.6	5.2
2	4-methylguaiacol	G-Me	138	5.1	4.4	8.6	3.3	4.7	4.5	4.8	3.1
3	4-ethylguaiacol	G-Et	152	1.1	0.8	0.8	0.7	0.9	0.9	0.8	0.6
4	4-vinylguaiacol	G-Vinyl	150	5.3	5.3	11.6	3.2	3.6	4.7	4.1	3.9
5	Eugenol	G-C-C=C	164	1.3	1.3	0.5	0.7	1.1	1.2	1.2	1.0
6	4-propylguaiacol	G-C-C-C	166	0.3	0.2	1.0	0.2	0.4	0.1	0.0	0.1
7	Isoeugenol (cis)	G-C=C-C (cis)	164	0.7	0.8	0.8	0.6	0.6	0.8	0.7	0.7
8	Isoeugenol (trans)	G-C=C-C (trans)	164	4.4	5.4	6.6	3.0	3.8	4.6	4.3	3.8
9	Vanillin	G-CHO	152	5.8	4.3	12.6	3.6	8.1	2.8	3.7	7.3
10	Homovanillin	G-C-CHO	162	2.7	0.8	0.0	0.5	2.5	1.0	1.7	0.4
11	Acetoguaiacone	G-CO-C	162	1.7	1.2	0.0	1.2	1.9	1.4	1.9	3.1
12	Guaiacyl acetone	G-C-CO-C	166	0.8	1.0	0.0	1.3	0.9	1.1	1.5	2.1
13	Propioguaiacone	G-CO-C-C	166	0.0	0.1	0.0	0.0	0.0	0.1	0.2	0.1
14	1-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one	G-CO-C=C	180	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	Dihydroconiferyl alcohol	G-C-C-C-OH	180	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
18	Coniferaldehyde	G-C=C-CHO	180	0.0	0.2	0.0	0.0	0.5	0.0	0.0	0.0
19	Syringol	S	154	12.4	14.4	17.4	30.7	12.7	16.8	14.4	14.5
20	4-methylsyringol	S-Me	168	10.5	8.4	6.0	7.0	12.0	8.9	10.2	6.9
21	4-ethylsyringol	S-Et	182	2.0	1.2	0.0	1.2	2.7	1.8	2.2	1.3
22	4-vinylsyringol	S-Vinyl	180	10.5	12.6	9.5	9.8	10.3	12.4	9.1	10.9
23	4-allylsyringol	S-C-C=C	194	3.7	3.4	0.0	2.4	4.1	3.4	4.4	2.6
24	4-propylsyringol	S-C-C-C	196	0.7	0.4	0.0	0.4	0.8	0.5	0.8	0.4
25	4-propenylsyringol (cis)	S-C=C-C (cis)	194	2.5	1.8	0.0	1.5	2.5	1.9	2.1	1.5
26	4-propenylsyringol (trans)	S-C=C-C (trans)	194	13.5	12.9	9.7	8.8	14.5	13.0	13.2	10.0
27	Syringaldehyde	S-CHO	182	4.3	7.5	0.0	6.3	2.0	6.3	5.0	9.3
28	Homosyringaldehyde	S-C-CHO	196	1.9	1.1	0.0	0.6	1.2	1.4	1.4	1.2
29	Acetosyringone	S-CO-C	196	2.3	2.5	0.0	2.8	2.2	2.2	3.2	3.5
30	Syringyl acetone	S-C-CO-C	210	2.6	2.5	3.4	4.7	2.2	2.3	4.7	6.0
31	Propiosyringone	S-CO-C-C	210	0.4	0.5	0.0	0.7	0.6	0.5	0.8	0.6
	S/G ratio			2.1	2.2	0.8	3.3	2.1	2.5	2.5	2.2
	Total amount of Cα carbonyl structures			0.15	0.16	0.13	0.15	0.15	0.13	0.15	0.24
	Ph-C2			27.5	25.5	21.9	20.0	25.3	25.8	24.4	24.9
	Ph-C3			31.0	30.6	22.0	24.3	32.0	29.5	33.9	28.9

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure legends

Fig. 1. ^{1}HNMR spectra of LCCs from PcL-HBT treated eucalyptus unbleached kraft pulp. (A) Acetylated glucan-lignin dissolved in $CDCl_3$. (B) Xylan-lignin dissolved in DMSO-d_6. In the spectra, G1 (4.40 ppm), G2 (4.79 ppm), G3 (5.06 ppm), G4 (3.71 ppm), G5 (3.53 ppm), and G6 (4.05 and 4.40 ppm) are the characteristic proton signals from glucosyl units. X1 (4.26 ppm), X2 (3.04 ppm), X3 (3.25 ppm), X4 (3.50 ppm), and X5 (3.16 and 3.87 ppm) are the characteristic proton signals from xylosyl units. X-OH (5.13 and 5.23 ppm) originates from protons of –OH groups of xylosyl units.

Fig. 2. Thioacidolysis-SEC chromatograms of xylan-lignin fractions from blank experiments (buffer pH 6 and pH 4) and LMS treatments

Fig. 3. Biodegradation mechanism of non-phenolic lignin structure by LMS treatment (e.g. PcL-HBT)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

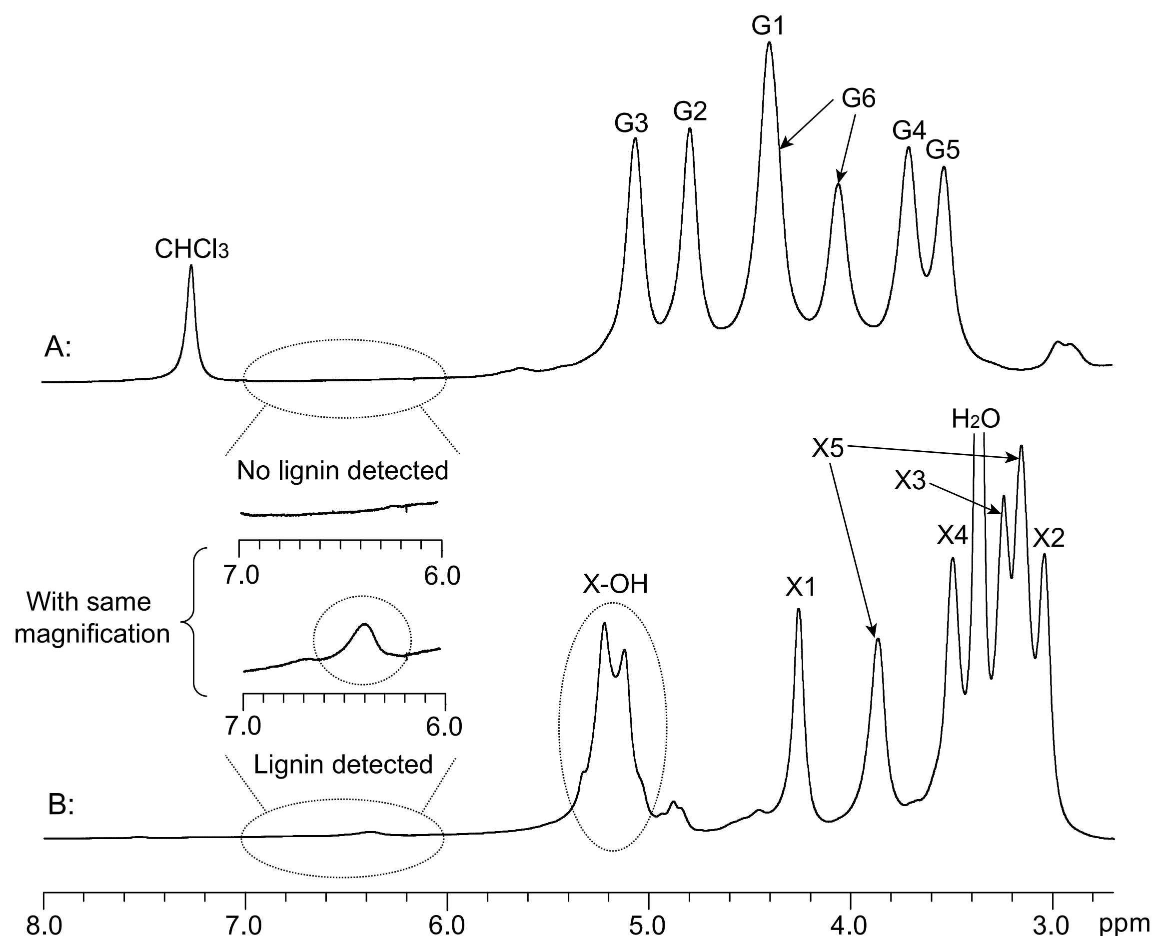

Fig. 1. ^{1}HNMR spectra of LCCs from PcL-HBT treated eucalyptus unbleached kraft pulp.

(A) Acetylated glucan-lignin dissolved in $CDCl_3$. (B) Xylan-lignin dissolved in DMSO-d_6. In the spectra, G1 (4.40 ppm), G2 (4.79 ppm), G3 (5.06 ppm), G4 (3.71 ppm), G5 (3.53 ppm), and G6 (4.05 and 4.40 ppm) are the characteristic proton signals from glucosyl units. X1 (4.26 ppm), X2 (3.04 ppm), X3 (3.25 ppm), X4 (3.50 ppm), and X5 (3.16 and 3.87 ppm) are the characteristic proton signals from xylosyl units. X-OH (5.13 and 5.23 ppm) originates from protons of –OH groups of xylosyl units.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

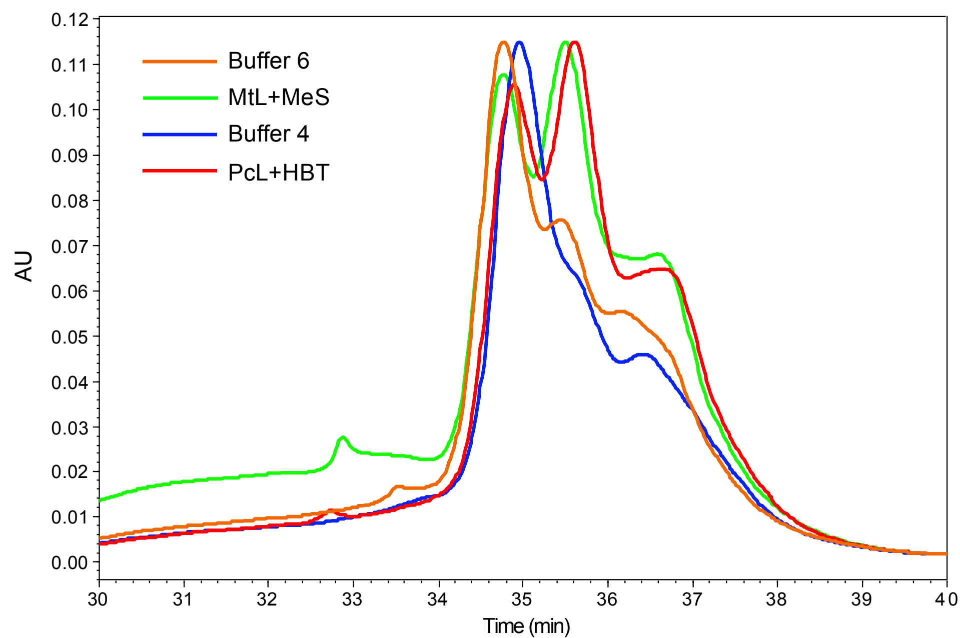

Fig. 2. Thioacidolysis-SEC chromatograms of xylan-lignin fractions from blank experiments (buffer pH 6 and pH 4) and LMS treatments

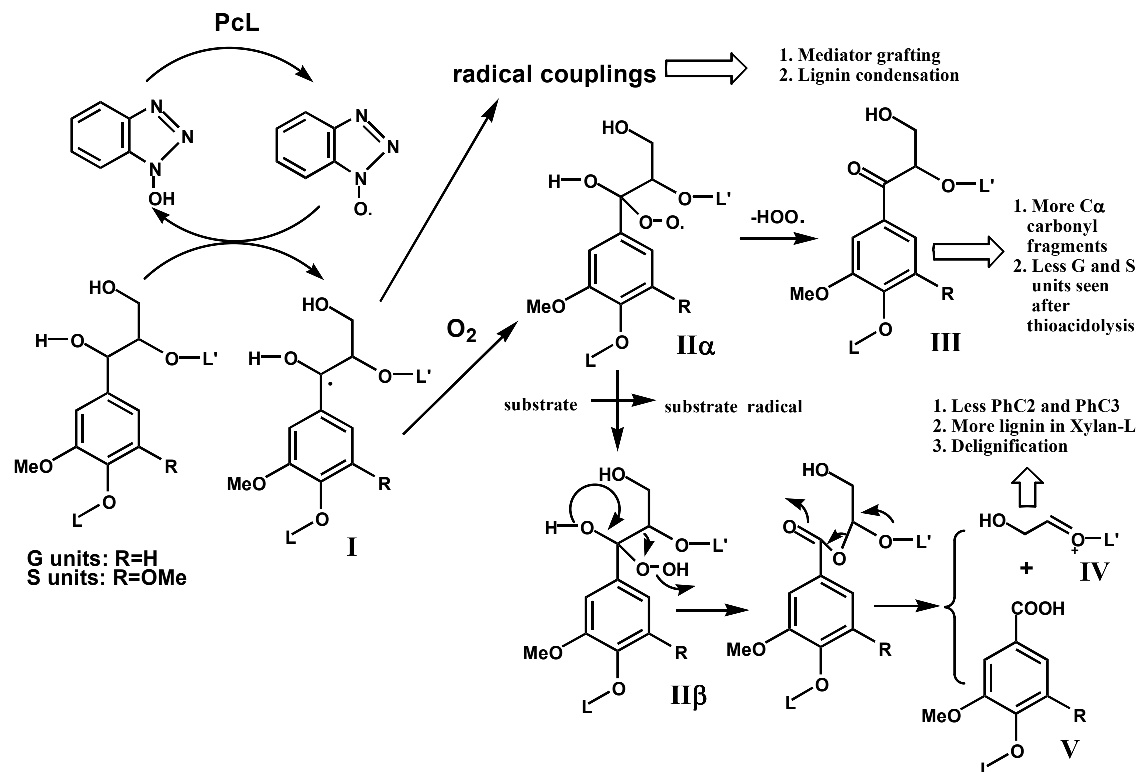

Fig. 3. Biodegradation mechanism of non-phenolic lignin structure by LMS treatment (e.g. PcL-HBT)