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A commercial laccase-mediator system to delignify and improve saccharification of the fast-growing *Paulownia fortunei* (Seem.) Hemsl.

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Abstract: It was demonstrated for the first time that a laccase-based enzymatic pretreatment is able to delignify fast-growing paulownia species. The treatment was performed with a commercial low-redox potential laccase isolated from *Myceliophthora thermophila* (Apinis) Oorschot and methyl syringate (MeS) as a natural phenolic mediator. Up to 24% lignin removal was attained by the laccase-MeS treatment (L/MeS), followed by alkaline peroxide extraction in a multistage sequence. The reduction in lignin content was accompanied by a significant improvement in the subsequent enzymatic saccharification, with increases of up to 38% glucose and 34% xylose yields. The structural modifications of the lignin were analyzed *in situ* by two dimensional-nuclear magnetic resonance (2D-NMR) spectroscopy. A considerable removal of guaiacyl and syringyl lignin units with respect to the carbohydrate signals was visible as well as the cleavage of β-*O*-4′, β-5′ and β-β′ linkages leading to elevated amounts of C_α-oxidized guaiacyl and syringyl units. The presence of oxidized lignin compounds in the filtrates of the enzymatic treatments – such as vanillin, vanillic acid, syringaldehyde and syringic acid – conclusively demonstrates the ability of L/MeS treatment to oxidize and depolymerize the lignin in paulownia wood.

Keywords: 2D-NMR, laccase, lignin, paulownia, pretreatment, saccharification

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Introduction

Paulownia (family Paulowniaceae) is a fast-growing woody crop plant with high biomass production (Jiménez et al. 2005). *Paulownia* genus comprises of nine species and a few hybrids native to China and East Asia (Zhu et al. 1986), which are grown commercially for the production of hardwood timber (Bergmann 1998). The physicochemical properties of its wood – including lightweight, strength, insulation, fast drying, high ignition point and rot resistance – make paulownia timber an interesting raw material for house construction and furniture making. Likewise, paulownia wood has also been investigated as raw material for the production of chemical pulp (Jiménez et al. 2005; Caparrós et al. 2007, 2008), being *Paulownia fortunei* (Seem.) Hemsl. the most suitable species for this purpose (Rai et al., 2000). *Paulownia fortunei* shows extraordinarily high growth rates under suitable conditions (Ede et al. 1997), reaching up to 15–20 m high in only 5–7 years, and annual productions as high as 150 t ha^{-1} $year^{-1}$ (Jiménez et al. 2005).

Due to its rapid growth, coppicing property and high carbohydrate content, paulownia also has a potential use as an energy crop for the production of bioethanol (Ye et al. 2016; Domínguez et al. 2017) by enzymatic hydrolysis (Chandra et al. 2007) and subsequent fermentation by yeasts (Park et al. 2013) or bacteria (Ng et al. 1981). For this purpose, the cross-linked macro-molecular assembly of the cell wall must be submitted to a pretreatment (Chen 2014). Lignins especially limit the enzymatic hydrolysis by steric hindrance of the enzymes' access to the polysaccharides and their inactivation (Kumar and Wyman 2009; Rahikainen et al. 2013).

Paulownia wood contains ~24% lignin, which is composed of guaiacyl (G) and syringyl (S) units with an S/G ratio of 0.66 (Rencoret et al. 2009a). Different physical and chemical pretreatments have been proposed for a better saccharification of paulownia wood, such as dilute acid, alkali, ultrasonic-assisted alkali treatments (Ye and Chen 2015, 2016), autohydrolysis (Domínguez et al. 2017) and steam explosion (Radeva et al. 2012). Biological

pretreatments are also possible via ligninolytic fungi or their enzymes, but these methods have not yet been investigated in paulownia.

The aim of the present study is to evaluate the pretreatment of paulownia wood by a commercially available laccase-mediator system to improve the subsequent saccharification. The thermostable laccase from the fungus *Myceliophthora thermophila* (Apinis) Oorschot, which has been cloned and expressed in *Aspergillus oryzae* (Ahlb.) E. Cohn (Xu et al. 1996; Berka et al. 1997), was supplied by Novozymes (Bagsvaerd, Denmark). Methyl syringate (MeS), which can be obtained from syringic acid present in pulp and paper side-streams (Rosado et al. 2012), served as the mediator. MeS is cheaper and less toxic than synthetic mediators such as 1-hydroxybenzotriazole, violuric acid or 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid. The L/MeS treatment was proved to be highly effective on eucalypt wood (Rico et al. 2014), which contains a syringyl-rich lignin (Rencoret et al. 2008) that is easier to degrade under alkaline conditions (González-Vila et al. 1999; Shimizu et al. 2017). The modification of the structure of the lignin polymer in the pretreated paulownia was monitored by two dimensional-nuclear magnetic resonance (2D-NMR) spectroscopy of the whole sample at the gel state (Kim et al. 2008; Rencoret et al. 2009b), and the effect of the L/MeS treatment on the saccharification yield was reported.

Materials and methods

Paulownia wood, enzymes and mediator: Three-year-old *P. fortunei* trees were provided by the University of Huelva (Spain). The wood was manually debarked, chipped, air-dried and ground in a knife mill IKA MF10 (IKA, Staufen, Germany) to pass a 1-mm screen, and then finely milled in a planetary mill Retsch PM100 (Retsch, Haan, Germany) at 400 rpm for 2 h (including pause times to prevent sample heating), using a 500-ml agate jar and agate balls (20×20 mm). The commercial (recombinant) fungal laccase from *M. thermophila* (Novozym 51003) was supplied by Novozymes. The enzymatic activity was measured as the initial velocity during oxidation of 5 mM ABTS from Roche to its cation radical (ε_{436} 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24°C. The laccase activity was 1000 U ml^{-1}. One activity unit (U) is defined as the amount of enzyme transforming 1 µmol of ABTS per min. MeS (methyl 4-hydroxy-3,5-dimethoxybenzoate) from Alfa Aesar (Karlsruhe, Germany) served as the redox mediator.

L/MeS treatments: A sequence of four L/MeS treatments was applied, each one followed by an alkaline peroxide extraction step. Laccase doses of 50 U g^{-1} were assayed, together with 3% MeS (% is b.o. dry wood). Paulownia samples (4 g) at 6% consistency (w:w) in 50-mM NaH_2PO_4 (pH 6.5) were placed into 200-ml pressurized bioreactors (Labomat, Mathis, Switzerland) and treated under an O_2 atmosphere (2 bar), in a thermostatic shaker (adjusted at 50°C and 190 rpm), for 24 h. Then, samples were filtered through a Büchner funnel and washed with 1 l of water. Subsequently, samples at 6% consistency (w:w) were submitted to an alkaline peroxide extraction with 1% (w:w) NaOH and 3% (w:w) H_2O_2 at 80°C for 90 min in a thermostatic shaker at 140 rpm, followed by water washing. Treatments with laccase alone (50 U g^{-1}), without the mediator, and controls without laccase and mediator were also performed. A control treatment with mediator alone was not included in view of the negative results of previous studies. The Klason lignin contents (TAPPI Method T222 om-88, 2004) were corrected for ash.

Saccharification of pretreated samples: After L/MeS treatment, the samples were hydrolyzed in an enzyme cocktail of commercial enzymes (Novozymes) with cellulase (Celluclast 1.5 l; 2 FPU g^{-1}) and β-glucosidase (Novozym 188; 6 U g^{-1}) at 1% solid loading in 3 ml of 100 mM sodium citrate (pH 5) for 72 h at 45°C, in a shaker bath at 140 rpm. The released monosaccharides were determined as alditol acetate derivatives by GC (Selvendran et al. 1979) on an HP 5890 instrument (Hewlett-Packard, Hoofddorp, The Netherlands), as previously described (Rencoret et al. 2017). Chromatographic peaks were quantified by area, and different standards (including glucose and xylose, among others) were used to elaborate calibration curves. Duplicate experiments were performed in terms of L/MeS treatment (including control, laccase and laccase-MeS) and glucose and xylose release, and Klason lignin contents were analyzed in triplicate measurements. At 95% confidence level, the enzymatic experiments are smaller than the differences found between the control, laccase alone and L/MeS treatments. Moreover, analysis of variance (ANOVA) experiments between subjects were performed. Post hoc pairwise comparisons, using the Tukey HSD test, were also performed. The data from both enzymatic and technical replicates were averaged.

2D-NMR analyses: The 2D heteronuclear single quantum coherence (HSQC) NMR experiments were performed at the gel state, which is an *in situ* analysis of the whole cell wall (Kim et al. 2008; Rencoret et al. 2009b). This approach does not require a previous lignin isolation and avoids possible alterations and material losses during the isolation process. These experiments also provided structural information on the hemicelluloses in the cell wall. Seventy milligrams of ball-milled samples (and filtrate samples) were transferred into 5-mm NMR tubes and swelled in 1 ml of DMSO-d_6, forming a gel inside the tube (Kim et al. 2008; Rencoret et al. 2009b). 2D HSQC NMR spectra were recorded at 300 K on a Bruker AVANCE III 500 MHz spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5-mm TCI gradient CryoProbe with inverse geometry. An adiabatic HSQC pulse sequence (Bruker standard "hsqcetgpsisp.2"), which enabled a semiquantitative analysis of the different ^{1}H-^{13}C correlation signals (Kupče and Freeman 2007), was utilized. HSQC spectra were acquired from 10 to 0 ppm (5000 Hz) in F2 (^{1}H) using 1000 data points for an acquisition time (AQ) of 100 ms, an interscan delay (D1) of 1 s, and from 200 to 0 ppm (25 168) in F1 (^{13}C) using 256 increments of 32 scan, for a total AQ of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. The central solvent peak was used as an internal reference (δ_C/δ_H 39.5/2.49). Lignin and carbohydrate correlation signals in the HSQC spectra were assigned by comparison with the literature (Rencoret et al. 2009a,b; Kim and Ralph 2014). The ^{1}H-^{13}C correlation signals from the aromatic region of the spectrum were used to estimate the content of lignin (relative to the content

of amorphous carbohydrates, estimated from the anomeric xylose and glucose signals) and the lignin composition in terms of G, S and oxidized S (S′) and G (G′) units. The C_α-H_α correlation signals in the aliphatic-oxygenated region were used to estimate the abundance of the various lignin inter-unit linkages, whereas the C_γ-H_γ correlation signals were used to estimate the abundance of the cinnamyl alcohol end-units. Likewise, $S_{2,6}$ (and $S'_{2,6}$) and G_2 (and G'_2) signals were used to estimate the relative abundances of the aromatic units – as signals $S_{2,6}$ and $S'_{2,6}$ involve two proton-carbon pairs, their volume integrals were halved. The content of C_α-oxidized S-lignin units in the HSQC spectrum of paulownia treated with laccase and the mediator was corrected for the contribution of MeS to the signal at δ_C/δ_H 106.2/7.20, which was estimated from the integral of its characteristic signal at δ_C/δ_H 52/3.8 ppm.

Analyses of enzymatic filtrates: The filtrates obtained after each enzymatic treatment were combined (from the four cycles), lyophilized and subsequently extracted with chloroform by sonication (20 min). The solution was centrifuged (10 000 rpm for 25 min) and the supernatant, which contains low molecular weight lignin-derived compounds, was then collected by decantation. The extraction process was repeated three times, using fresh chloroform each time and the supernatants were combined. The chloroform was removed by rotary evaporation at 40°C and the compounds were then silylated with bis(trimethylsilyl)trifluoroacetamide (BSTFA)-pyridine (2:1 v/v) and analyzed using gas chromatography-mass spectrometry (GC-MS) on a Varian Saturn 4000 (Varian, CA, USA) equipment. The GC column used was a 15 m × 0.25 mm i.d. and 0.1 µm film thickness (DB5-HT, J&W Scientific). The temperature program was as follows: 50°C → 90°C (30°C min^{-1}), holding time 2 min, → 250°C (8°C min^{-1}), and holding time 2 min. The injector and transfer line temperatures were 250°C and 300°C, respectively. Helium was the carrier gas (2 ml min^{-1}). The chloroform-insoluble fractions of the filtrates, which contain lignin fragments with higher molecular weights, were analyzed using 2D-NMR under the same conditions as described above.

Table 1: Laccase-mediator treatment of paulownia wood (material recovery and final lignin content) and subsequent Celluclast hydrolysis (amount of glucose and xylose per 100 g of sample).[A]

	1) Laccase-mediator treatment[B]		2) Celluclast hydrolysis (2 FPU)	
Samples	**Recovery (%)**	**Lignin (%)**	**Glucose (%)**	**Xylose (%)**
Initial paulownia	100 (4 g)	23.8 ± 0.2	31.5 ± 0.3	6.7 ± 0.1
Control	83 (3.32 g)	22.0 ± 0.1[a]	37.3 ± 0.2[a]	7.3 ± 0.1[a]
Laccase (50 U · g^{-1})	80 (3.21 g)	20.9 ± 0.3[a]	39.4 ± 0.4[b]	7.8 ± 0.1[a]
Laccase (50 U · g^{-1})-MeS (3%)	76 (3.03 g)	16.7 ± 0.2[b]	51.3 ± 0.4[c]	9.8 ± 0.3[b]

[A]Means ± StD presented were obtained from triplicate determination. Letters next to the StD, from the Tukey test, show: no significant differences to the control (a), significant differences (b) and significantly different from both the control and the L-alone results (c), at the 0.05 level. [B]Followed by alkaline-peroxide extraction (four cycles).

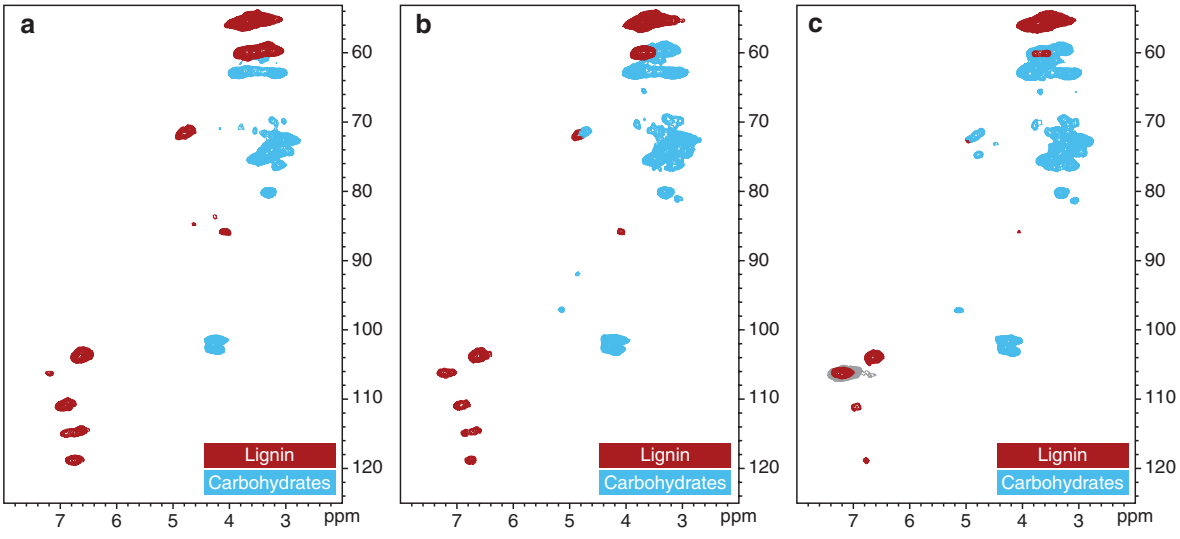

Figure 1: 2D HSQC NMR spectra of paulownia after laccase-mediator treatments followed by alkaline peroxide extractions (four cycles): control without enzyme (a), treated with 50 U g^{-1} laccase (b) and treated with 50 U g^{-1} laccase and 3% MeS (c).
Correlation signals in reddish-brown color largely correspond to lignin, whereas signals in cyan color belong to carbohydrates.

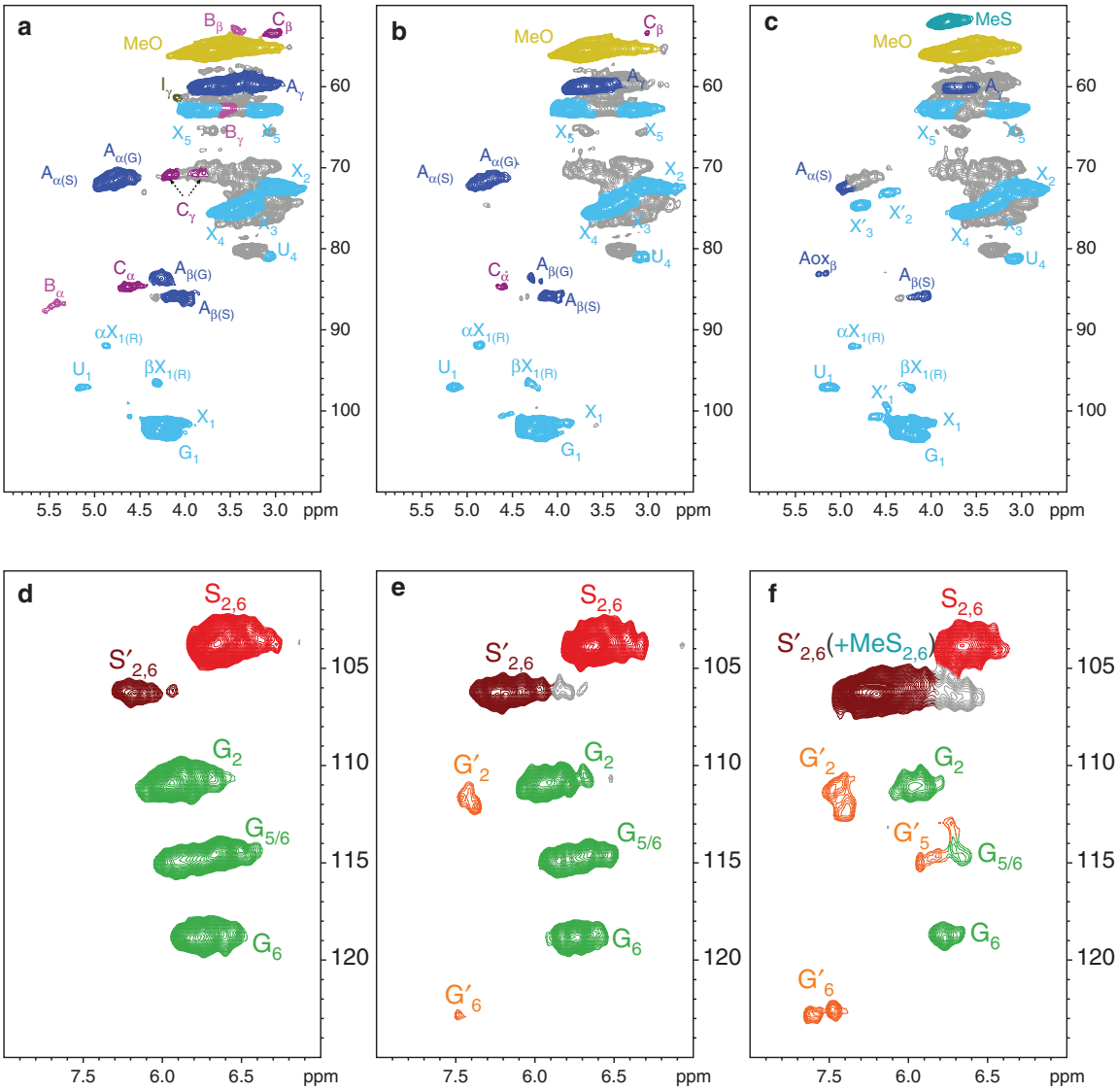

Figure 2: Expanded aliphatic oxygenated (δ_C/δ_H 50–110/2.5–6.0, top) and aromatic (δ_C/δ_H 100–125/6.0–8.0, bottom) regions of the 2D HSQC NMR spectra of paulownia samples: Control without enzyme (a, d), treated with 50 U g^{-1} enzyme (b, e) and treated with 50 U g^{-1} enzyme and 3% MeS (c,f).
The lignin and carbohydrate signal assignments are listed in Table 2 and the lignin structures identified are depicted in Figure 3. Correlation signals of MeS in the HSQC spectrum from laccase-mediator treated paulownia are observed at δ_C/δ_H 52.1/3.82 (C/H in MeS) and δ_C/δ_H 106.4/7.20 (C_2/H_2 and C_6/H_6 in the aromatic ring).

Results and discussion

Delignification of paulownia wood by the enzymatic pretreatment

The Klason lignin content of the paulownia control, processed as the full enzymatic treatment but without laccase and mediator, was only slightly reduced from 23.8 to 22.0% with respect to the initial paulownia lignin content (Table 1). This is the effect of alkaline peroxide extractions. The treatment with laccase alone only resulted in a 5% lignin reduction compared to the control sample. This low efficiency of laccase alone is well known (Lai 1992). The pretreatment with L/MeS, however, resulted in a lignin decrease of up to 24%, with respect to the control, but this delignification degree is lower than that reported about eucalypt wood treatment under the same experimental conditions (47% lignin removal). This difference may be due to the

Table 2: Assignments of the $^{1}H/^{13}C$ correlation signals in the HSQC spectra of the whole cell walls from treated paulownia.

Label	δ_C/δ_H (ppm)	Assignment
Lignin signals		
B_β	53.1/3.43	C_β/H_β in phenylcoumaran substructures (B)
C_β	53.4/3.04	C_β/H_β in resinols substructures (C)
MeO	55.5/3.72	C/H in methoxyls
A_γ	59.6 /3.37 and 3.71	C_γ/H_γ in β-*O*-4′ substructures (A)
I_γ	61.3/4.09	C_γ/H_γ in cinnamyl alcohol end-groups (I)
B_γ	62.6/3.69	C_γ/H_γ in phenylcoumaran substructures (B)
$A_{\alpha(G)}$	70.8/4.73	C_α/H_α in β-*O*-4′ substructures (A) linked to a G-unit
C_γ	70.9/4.17 and 3.81	C_γ/H_γ in resinols substructures (C)
$A_{\alpha(S)}$	71.8/4.83	C_α/H_α in β-*O*-4′ substructures (A) linked to an S-unit
Aox_β	83.0/5.20	C_β/H_β in α-oxidized β-*O*-4′ substructures (Aox)
$A_{\beta(G)}$	83.6/4.26	C_β/H_β in β-*O*-4′ substructures linked (A) to a G unit
C_α	84.7/4.63	C_α/H_α in resinols substructures (C)
$A_{\beta(S)}$	85.8/4.08	C_β/H_β in β-*O*-4′ substructures linked (A) to an S unit
B_α	86.7/5.42	C_α/H_α in phenylcoumaran substructures (B)
$S_{2,6}$	103.8/6.65	C_2/H_2 and C_6/H_6 in etherified syringyl units (S)
$S'_{2,6}$	106.2/7.20	C_2/H_2 and C_6/H_6 in α-oxidized syringyl units (S′)
G_2	110.9/6.93	C_2/H_2 in guaiacyl units (G)
G'_2	111.7/7.42	C_2/H_2 in α-oxidized guaiacyl units (G′)
G_5/G_6	114.9/6.67 and 6.85	C_5/H_5 and C_6/H_6 in guaiacyl units (G)
	118.8/6.76	
G'_5	115.0/6.94	C_5/H_5 in α-oxidized guaiacyl units (G′)
G'_6	122.7/7.54	C_6/H_6 in α-oxidized guaiacyl units (G′)
Carbohydrate signals		
X_5	63.0/3.16 and 3.87	C_5/H_5 in xylopyranose units
X_2	72.5/3.03	C_2/H_2 in xylopyranose units
X'_2	73.0/4.46	C_2/H_2 in 2-*O*-acetylated xylopyranose units
X_3	73.9/3.24	C_3/H_3 in xylopyranose units
X'_3	74.7/4.79	C_3/H_3 in 3-*O*-acetylated xylopyranose units
X_4	75.3/3.49	C_4/H_4 in xylopyranose units
U_4	81.0/3.07	C_4/H_4 in 4-*O*-methyl-α-D-glucuronic acid
$\alpha X_{1(R)}$	91.9/4.88	C_1/H_1 in α-D-xylopyranoside (R) [α-D-glucopyranoside (R)]
$\beta X_{1(R)}$	96.5/4.31	C_1/H_1 in β-D-xylopyranoside (R) [β-D-glucopyranoside (R)]
U_1	97.1/5.15	C_1/H_1 in 4-*O*-methyl-α-D-glucuronic acid
X'_1	99.4/4.48	C_1/H_1 in 2-*O*-acetylated xylopyranose units
X_1	101.5/4.26	C_1/H_1 in xylopyranose units
Gl_1	102.9/4.16	C_1/H_1 in glucopyranose units

high S-unit content of eucalypt wood lignin compared to the G-unit-rich and condensed paulownia lignin.

Saccharification of pretreated paulownia

The percentages of glucose and xylose released by saccharification of treated paulownia samples are listed in Table 1. The saccharification results indicate that there is a direct correlation between the lignin removal and the higher glucose and xylose yields. The pretreatment with laccase alone enhanced the glucose and xylose yields up to 6% and 7%, respectively, but the L/MeS treatment led to glucose and xylose increase up to 38% and 34%, respectively. Rico et al. (2014) found similar glucose (41%) and xylose (37%) yield increments with eucalypt wood after L/MeS under the same experimental conditions.

2D-NMR analysis

The full HSQC NMR spectra of the samples, including those resulting from the treatment with L/MeS, laccase alone and the corresponding control, are shown in Figure 1. A pronounced lignin removal occurred after L/MeS pretreatment, as evidenced by the lower lignin signals (colored in red) after the treatments, in comparison with the signals from carbohydrates (in cyan color) that remained mostly unchanged (Figure 1c), although some lignin degradation can also be seen in the spectrum

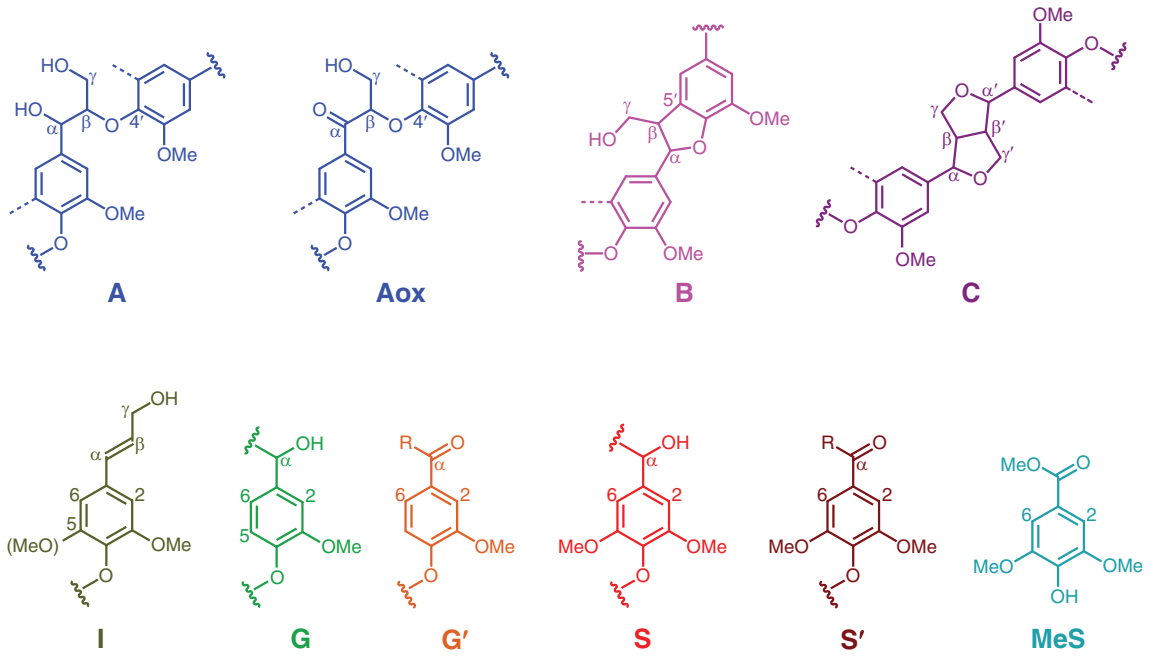

Figure 3: Main lignin units and substructures identified in the 2D-HSQC spectra of treated paulownia samples.
(A) β-*O*-4′ alkyl-aryl ether structures; (Aox) β-*O*-4′ structures with C_α-oxidized; (B) β-5′ phenylcoumaran structures; (C) β-β′ resinol structures; (I) cinnamyl alcohol end-group; (G) guaiacyl unit; (S) syringyl unit; (G′) C_α-oxidized G unit; (S′) C_α-oxidized S unit; (MeS) methyl syringate.

of laccase-treated samples (Figure 1b). More detailed information was gained from these HSQC spectra after increasing their intensities and analyzing the aliphatic oxygenated regions and the aromatic regions individually (Figure 2). The aliphatic oxygenated region (δ_H/δ_C, 2.5–6.0/50–110) of the spectra shows correlation signals from both lignin (side-chain linkages and aromatic methoxy groups) and carbohydrates, which are mainly from hemicelluloses and amorphous cellulose as crystalline cellulose is "invisible" to the HSQC analysis in the gel state. The lignin and carbohydrate cross-signals assigned in the HSQC spectra are listed in Table 2, and the lignin units and substructures identified are depicted in Figure 3. The carbohydrate signals mainly correspond to xylans, both non-acetylated (X) and acetylated (X′), and 4-*O*-methyl-α-D-glucuronic acid, in agreement with previous work that reported an *O*-acetyl-(4-*O*-methylglucurono)xylan as being the main heteroxylan in paulownia (Gonçalves et al. 2008). The lignin signals in this region of the spectra corresponded to MeO groups and different lignin inter-unit linkages such as β-*O*-4′ alkyl-aryl ethers (A), β-5′ phenylcoumarans (B) and β-β′ resinols (C). The aromatic region (δ_H/δ_C, 6.0–8.0/100–125) of the spectra included signals from G and S lignin units, as well as the corresponding C_α-oxidized lignin units (G′ and S′).

The HSQC spectrum of the sample after the enzymatic treatment with laccase alone shows noticeable differences in the lignin correlation signals, in comparison to the control spectrum, whereas the carbohydrate signals remain mostly unchanged. The considerable diminution of signals from the main lignin inter-unit linkages is the most remarkable feature along with the relative signal increment from C_α-oxidized lignin units (G′ and S′). The abundance of β-*O*-4′ alkyl-aryl ether linkages per aromatic units decreased from 51 to 39, whereas the abundance of β-β′ resinol linkages decreased from 4 to 2 per aromatic unit, and the β-5′ phenylcoumaran linkages were not even detectable in the HSQC spectrum of the sample treated with laccase alone, as shown in Table 3, where the integrals of HSQC signals are semi-quantitatively compared. Likewise, the increase of the signals for C_α-oxidized S-lignin units (S′), together with the appearance of signals corresponding to C_α-oxidized G-lignin units (G′), indicates that *M. thermophila* laccase itself was able to oxidize the phenolic substructures to a certain extent. Again, one should emphasize the G-unit-rich character of paulownia lignin (Rencoret et al. 2009a). The G-units have more free OH_{phen} groups than the S-units, which are mainly etherified (Lundquist and Parkås 2011; Pu et al. 2011). This could explain the degradation/modification of the lignin polymer observed during the treatment with laccase alone. However, the lignin content decreased only by 5% after laccase treatment alone.

Table 3: Semiquantitative NMR analysis of paulownia treated with *M. thermophila* laccase (50 U · g^{-1}) and MeS (3%), and laccase alone, compared with the control without enzyme and mediator, including sample composition and linkages/end-groups, from HSQC spectra in Figure 2.

Sample composition[a]	Control	Laccase	L/MeS
Syringyl lignin units (S)	15.6 (37)	13.9 (42)	9.3 (36)
C_α-oxidized S units (S′)	2.6 (6)	5.7 (17)	5.7 (22)[b]
Guaiacyl lignin units (G)	23.5 (56)	11.6 (35)	6.2 (24)
C_α-oxidized G units (G′)	0 (0)	2.0 (6)	4.7 (18)
Total lignin	41.7 (100)	33.2 (100)	25.9 (100)
Sugar units	58.3	66.8	74.1
Total	100	100	100
Lignin S/G ratio	0.8	1.4	1.4
Linkages and end groups[c]			
β-*O*-4′ ethers (A)	51 (81)	39 (95)	22 (69)
β-*O*-4′ ethers (A) C_α-oxidized	0 (0)	0 (0)	9 (30)
Phenylcoumarans (B)	7 (11)	0 (0)	0 (0)
Resinols (C)	4 (6)	2 (5)	1 (4)
Cinnamyl end-groups (I)	1 (2)	0 (0)	0 (0)
Total	64 (100)	41 (100)	31 (100)

[a]Sample composition represents the molar amount of normal (H, G and S) and C_α-oxidized (G′ and S′) lignin units, and sugar units (mainly xylose and glucose) from the integration of anomeric carbon signals (relative percentages of lignin units are shown in parentheses). [b]The contribution of MeS was subtracted. [c]The percentages of lignin linkages involved in substructures A, B and C, and cinnamyl alcohol end-groups (I) are referred to the total aromatic (G + G′ + S + S′) lignin units (% relative to 100 linkages/end-groups are provided in parentheses) obtained from the integration of aliphatic oxygenated signals.

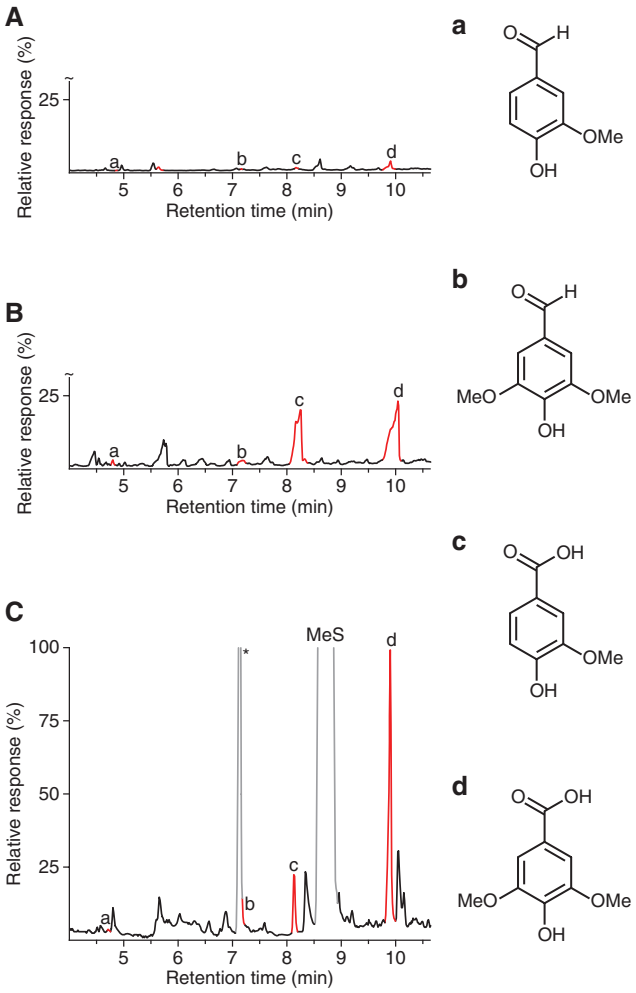

Figure 4: Total ion chromatograms of the chloroform-soluble fraction of filtrates obtained after the enzymatic pretreatment of paulownia with laccase-mediator (C), laccase alone (B) and the corresponding control (A).
The lignin-derived simple compounds identified are depicted (a–e). MeS refers to methyl syringate whereas the peak with asterisk mark (*) refers to contamination peak from methyl syringate.

Interestingly, the HSQC spectrum of the sample treated via L/MeS (Figure 2c, f) revealed a high degree of lignin degradation. The intensity of most of the lignin signals strongly decreased, while the signals from carbohydrates remained mostly unchanged (Figure 2c). Nonetheless, signals from acetylated xylans (X'_2 and X'_3) increased after L/MeS pretreatment, as occurred with other lignocellulosic materials, such as eucalypt wood (Gutiérrez et al. 2012) and sugarcane byproducts (Rencoret et al. 2017), probably due to the better mobility of these groups after lignin removal. This is reflected by the semiquantitative NMR analysis (Table 3) that showed a relative increase of sugar units, with respect to lignin aromatic units, from 58.3% (control sample) to 74.1% (L/MeS pretreatment). The signals of β-*O*-4′ alkyl-aryl ether lignin substructures (A), which were the most intense in the HSQC of the control sample, were hardly visible after the enzymatic pretreatment, whereas the signals from β-5′ phenylcoumarans (B) and β-β′ resinols (C) were not detected at all. These observations confirm the depolymerization of the paulownia lignin. Interestingly, a new correlation signal (Aox_β at 83.0/5.20) corresponding to C_β-H_β correlations in C_α-oxidized β-*O*-4′ alkyl-aryl ether linkages appeared as a result of L/MeS treatment. The formation of this uncleaved ketone is consistent with the changes observed in the aromatic units, described below, and the C_α-oxidation mechanism proposed for the degradation of lignin with the laccase-mediator system (Bourbonnais and Paice 1990; Li et al. 1999). The aromatic region of the spectrum shows a signal decrement from G- and S-units (Figure 2f). A significant part of the lignin is C_α-oxidized, as evidenced from the relative increase in the $S'_{2,6}$ signals (the contribution of $MeS_{2,6}$ correlations was subtracted) and the appearance of G'_2, G'_5 and G'_6 signals. It is obvious that paulownia lignin is degraded by the L/MeS system following the same mechanism

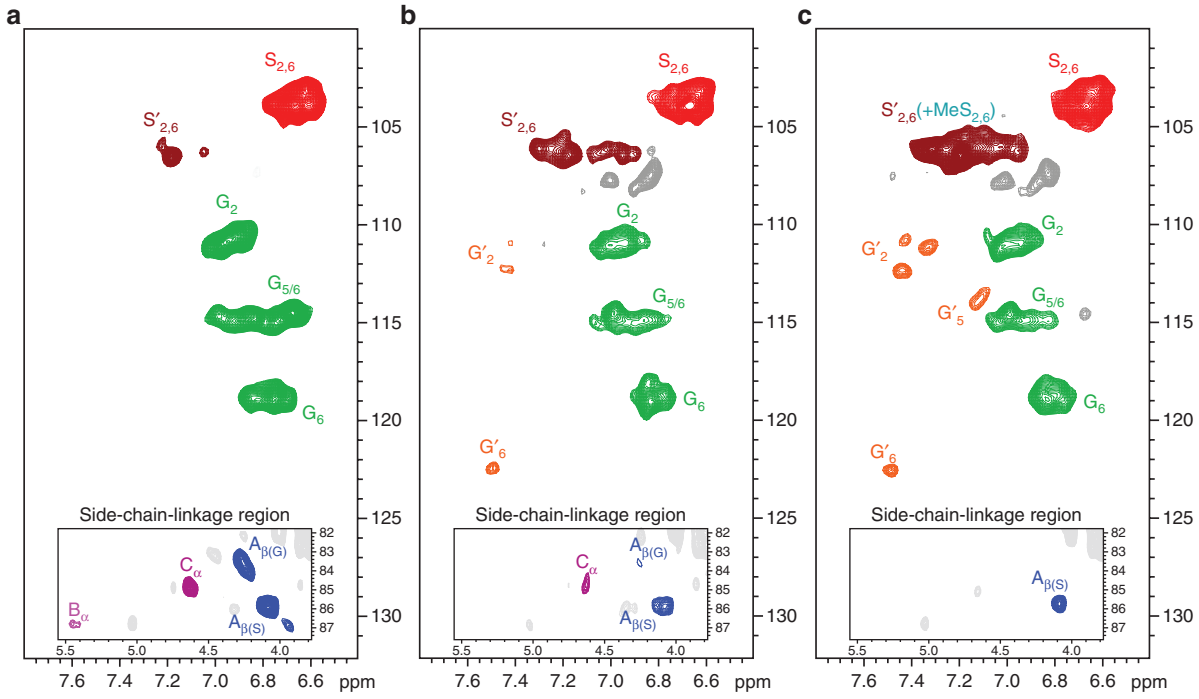

Figure 5: HSQC NMR spectra of lignin compounds in the chloroform-insoluble fraction of filtrates from enzymatic treatment of paulownia with laccase-mediator (c), laccase alone (b) and the corresponding control (a).
See Figure 3 for the main lignin-derived structures identified.

reported for the L/HBT system with non-phenolic lignin model dimers, which occurs via abstraction of a hydrogen atom from the C_α position, forming a ketone group and ending in the C_α-C_β breakdown at a later stage (Fabbrini et al. 2002; Kawai et al. 2002). Clearly, the lignin depolymerization mechanism by L/MeS has an oxidative character.

Analysis of pretreatment filtrates by GC-MS and 2D-NMR

The chloroform-soluble fractions of the filtrates were analyzed by GC-MS to determine the presence of low-molecular weight lignin-derived compounds (Figure 4). The presence of such compounds was negligible in the filtrates of the control wood, whereas they were clearly observed in the filtrates after laccase treatment alone and, especially, after L/MeS treatment. In these samples, the main compounds identified are vanillin, syringaldehyde, vanillic acid and syringic acid, which clearly evidences the oxidative nature of laccase degradation.

The chloroform-insoluble fractions of the filtrates were analyzed by 2D-NMR aiming at the characterization of oligo- and polymers, which are water insoluble at pH 6.5. There are significant differences among these fractions (Figure 5). While the oligomeric lignin in the control filtrate is less oxidized (higher S/S′ ratio) and preserved, the main original inter-unit linkages, the oligomeric lignins in the filtrates after laccase and L/MeS treatments are more oxidized in the low DP fraction. All these data confirm the extensive oxidative depolymerization of paulownia wood during the L/MeS treatment.

Conclusions

The lignin in paulownia wood was modified and partially removed by pretreatment with recombinant laccase of *M. thermophila* in the presence of MeS as the phenolic mediator, despite its high G-unit content. The L/MeS system acts selectively on the lignin polymer, leaving the carbohydrate signals in the HSQC spectra practically unaffected. The alterations produced in the lignin moiety further facilitated the access of the hydrolytic enzymes to cell-wall carbohydrates in the subsequent saccharification step, resulting in higher glucose and xylose yields. The utilization of the commercially available L/MeS system has a high application potential as pretreatment for the saccharification of paulownia wood.

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