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Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks

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HIGHLIGHTS

- A laccase-mediator treatment removed lignin from whole woody and nonwoody feedstocks.
- A high-redox potential laccase and 1-hydroxybenzotriazole (as mediator) were used.
- This laccase-mediator treatment was combined with an alkaline peroxide extraction.
- 2D NMR analyses revealed oxidative removal of lignin aromatic units and side-chains.
- The enzymatic pretreatment increased the sugar and ethanol yields.

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ABSTRACT

The ability of *Trametes villosa* laccase, in conjuction with 1-hydroxybenzotriazole (HBT) as mediator and alkaline extraction, to remove lignin was demonstrated during treatment of wood (*Eucalyptus globulus*) and non-wood (*Pennisetum purpureum*) feedstocks. At 50 U g^{-1} laccase and 2.5% HBT concentration, 48% and 32% of the *Eucalyptus* and *Pennisetum* lignin were removed, respectively. Two-dimensional nuclear magnetic resonance of the feedstocks, swollen in dimethylsulfoxide-d_6, revealed the removal of *p*-hydroxyphenyl, guaiacyl and syringyl lignin units and aliphatic (mainly β-O-4′-linked) side-chains of lignin, and a moderate removal of *p*-coumaric acid (present in *Pennisetum*) without a substantial change in polysaccharide cross-signals. The enzymatic pretreatment (at 25 U g^{-1}) of *Eucalyptus* and *Pennisetum* feedstocks increased the glucose (by 61% and 12% in 72 h) and ethanol (by 4 and 2 g L^{-1} in 17 h) yields from both lignocellulosic materials, respectively, as compared to those without enzyme treatment.

1. Introduction

Lignin removal is an important technical issue for paper manufacturing and a key challenge for the conversion of lignocellulosic feedstock into liquid transportation fuels such as ethanol. Biofuel production from lignocellulosic material requires deconstruction of the cell-wall matrix into individual polymers, and hydrolysis of the carbohydrate polymers into monomeric sugars. Biomass recalcitrance towards enzymatic hydrolysis is correlated with the content and composition of lignin (Studer et al., 2011). Physical, chemical and biological pretreatments, or combinations of these processes, are being studied for deconstructing lignocellulosic biomass and removing lignin (Alvira et al., 2010; Yu et al., 2011). Most biological pretreatments for delignifying lignocellulosic materials employ lignin-degrading fungi, mainly belonging to the group of white-rot basidiomycetes (Kumar et al., 2009; Salvachúa et al., 2011) but such pretreatments require long application periods and consume a fraction of the plant polysaccharides.

Laccases (phenoloxidases, EC 1.10.3.2) are multicopper oxidases that oxidize substituted phenols using molecular oxygen as the final electron acceptor. The direct action of laccases on lignin is, in principle, restricted to phenolic units that only represent a small percentage of the total polymer, a fact that limits their biotechnological application. However, the discovery that some synthetic compounds can act as electron carriers between the enzyme and

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the final substrate (Bourbonnais and Paice, 1990), 1-hydroxybenzotriazole (HBT) being among the most efficient ones (Call, 1994), has expanded the utility of laccases. Studies have confirmed the potential of laccase-mediator systems for pulp delignification (Poppius-Levlin et al., 1999; Ibarra et al., 2006; Babot et al., 2011), pitch control (Gutiérrez et al., 2009), organic synthesis (Kunamneni et al., 2008), polymer modification (Prasetyo et al., 2010), applications in the forest industry (Widsten and Kandelbauer, 2008) and bioethanol production from physically/chemically pretreated lignocellulose (Palonen and Viikari, 2004; Moilanen et al., 2011).

The present study shows the ability of the high redox-potential laccase from the basidiomycete *Trametes villosa* (Li et al., 1999) to remove lignin and make cellulose accessible to hydrolysis for conversion to fuels, when applied on the whole lignocellulosic biomass in combination with HBT as a redox mediator. Eucalypt (*Eucalyptus globulus*) and Elephant grass (*Pennisetum purpureum*) were selected as representative for rapidly growing, high biomass-producing woody and non-woody plant species, respectively. The modification of lignin in the pretreated lignocellulosic materials was analyzed by two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy of the whole sample at the gel state (Kim et al., 2008; Rencoret et al., 2009). In addition to lignin removal, the effect of the enzymatic treatments on sugar and ethanol yield from the two pretreated lignocellulosic materials was also assessed.

2. Methods

2.1. Lignocellulosic samples

Elephant grass (*P. purpureum*) from Viçosa Federal University (Brazil) and eucalypt (*E. globulus*) from ENCE (Pontevedra, Spain), were air-dried and ground in an IKA MF10 cutting mill to pass through a 100-mesh screen, and finely ball-milled in a Retsch PM100 ball mill at 400 rev min^{-1} using an agate jar and balls.

2.2. Fungal laccase and mediators

The laccase preparation from the basidiomycete *T. villosa* was provided by Novozymes (Bagsvaerd, Denmark). Its activity was measured as the oxidation of 5 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, from Roche, Mannheim, Germany) to the cation radical (ε_{436} 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 5) at 24 °C (Li et al., 1999). One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per min. HBT from Sigma–Aldrich (Steinheim, Germany) was used as mediator.

2.3. Laccase-mediator treatments

The eucalypt and Elephant grass samples were treated with the *T. villosa* laccase in the presence (and absence) of HBT, as mediator. Laccase doses of 10, 25 and 50 U g^{-1} were assayed, together with 2.5% HBT (selected after testing several HBT concentrations, from 0.5% to 3%), both with respect to lignocellulosic material dry weight. The treatments were carried out in 200-mL pressurized bioreactors (Labomat, Mathis) placed in a thermostatic shaker at 170 rev min^{-1} and 50 °C, using 2 g (dry weight) samples at 6% consistency (w:w) in 50 mM sodium tartrate buffer (pH 4) under O_2 atmosphere (2 bars) for 24 h. After the treatment, the samples were filtered through a Büchner funnel and washed with 1 L of water. In a subsequent step, samples at 6% consistency (w:w) were submitted to a peroxide-reinforced alkaline extraction using 1% (w:w) NaOH and 3% (w:w) H_2O_2 (also with respect to sample dry weight) at 80 °C for 90 min, followed by water washing (Babot et al., 2011). Cycles of four successive enzyme-extraction treatments were applied. Treatments with laccase alone (without mediator) and controls without laccase and mediator, were also performed (followed in both cases by the corresponding alkaline extractions).

2.4. Enzymatic hydrolysis

The laccase-pretreated samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase (Celluclast 1.5 L; 10 FPU g^{-1}) and β-glucosidase (Novozym 188; 500 nkat g^{-1}) activities, at 1% consistency in 3 mL of 100 mM sodium citrate buffer (pH 5) for 72 h at 45 °C, with magnetic stirring (in triplicate experiments).

The amount of total sugars released during the enzymatic hydrolyses was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). The different monosaccharides present were analyzed in a Waters Alliance 2795 high performance liquid chromatography (HPLC) system with an Aminex HPX-87H column (BioRad) and a Waters 2410 refractive index detector, using 5 mM H_2SO_4 (0.6 ml min^{-1}) as eluent. To improve the separation of the monosaccharides, a HPLC Fast Acid Analysis column (BioRad) was included before the above column, and a Cation-H Refill cartridge (BioRad) was added as a pre-column to remove impurities. Glucose, xylose and arabinose were used as standards.

2.5. Fermentation

Simultaneous saccharification and fermentation was conducted at 10% consistency in a 25-mL volume in Erlenmeyer flasks with airlocks (triplicate experiments). The biomass was pre-hydrolyzed for 6 h at 45 °C as described in Section 2.4, RedStar yeast (from Lesaffre, Marcq-en-Barœul, France) was added with an OD_{600} of 3.5 (about 1 g L^{-1}), and the flasks were incubated at 30 °C with 100 rev min^{-1} shaking for up to 64 h. The progress of the fermentation was monitored by weighing the flasks regularly, and the ethanol production was calculated from the weight loss.

2.6. Klason lignin content and polysaccharide composition

Klason lignin content was estimated according to T222 om-88 (Tappi, 2006). Monosaccharides in the acid hydrolysate were analyzed by high performance anion exchange chromatography using a CarboPac PA-1 column at 30 °C in a Dionex DX 500 series chromatograph equipped with pulse amperometric detection (Dionex ED 40), and expressed in mg of anhydrosugars per 100 mg of sample.

2.6.1. 2D NMR spectroscopy

Fifty to sixty milligram of lignocellulose samples were swollen in dimethylsulfoxide-d_6 for 2D NMR at the gel state (Kim et al., 2008; Rencoret et al., 2009). Heteronuclear single quantum correlation (HSQC) 2D NMR spectra were acquired on a Bruker Biospin (Billerica, MA) AVANCE 500 MHz spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The ^{13}C-^{1}H correlation experiment was an adiabatic HSQC experiment (Bruker standard pulse sequence 'hsqcetgpsisp.2'; phase-sensitive gradient-edited-2D HSQC using adiabatic pulses for inversion and refocusing). Gel HSQC spectra were acquired from 10 to 0 ppm in F2 (^{1}H) with 1000 data points for an acquisition time (AQ) of 100 ms, an interscan delay (D1) of 500 ms, 200–0 ppm in F1 (^{13}C) with 400 increments (F1 acquisition time 8 ms) of 40 scans. 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. Prior to Fourier transformation, the data matrices were zero filled up to 1024 points in the ^{13}C

dimension. The central solvent peak was used as an internal reference (δ_C/δ_H 39.5/2.49). The ^{13}C-^{1}H correlation signals of the different lignin units in the aromatic region were used to estimate the lignin composition in terms of *p*-hydroxyphenyl (H), guaiacyl (G), syringyl (S) and Cα-oxidized syringyl (S′) units, and the *p*-coumaric acid and ferulic acid contents referred to total lignin (H + G + S + S′).

3. Results and discussion

3.1. Elephant grass and eucalypt wood delignification with laccase-mediator

The lignin contents (as Klason lignin) of milled Elephant grass and eucalypt samples after the laccase-mediator sequence were determined and compared with their respective controls (Table 1). The lignin content in both lignocellulosic materials decreased considerably, after the enzymatic sequence, concomitantly with increasing laccase doses. For Elephant grass, the decreases were about 11%, 22% and 32% of the initial lignin content when using laccase doses of 10, 25 and 50 U/g, respectively (the decreases in lignin content did not stabilize in the course of the enzymatic sequence but progressively increased with respect to the previous step in each of the four laccase-mediator/extraction steps). The reduction in eucalypt wood was more pronounced, attaining 32%, 34% and 48% with the above laccase doses. The treatments with laccase alone (without mediator) decreased the lignin content (<5%) in both materials. No significant change in the lignin content, or even a slight increase, have previously been reported after laccase (alone) treatment of steam pretreated giant read (*Arundo donax*) and spruce (*Picea abies*), respectively (Moilanen et al., 2011). Likewise, no substantial variation in the lignin content and composition (discussed below) was reported after laccase-mediator treatment of steam-exploded eucalypt samples (Martin-Sampedro et al., 2011), most probably because of the different enzyme preparation (Novozym 51003 from Novozymes, Bagsvaerd, based on *Myceliophthora thermophila* laccase) (Li et al., 1999) and treatment conditions (note that this enzyme is practically unable to oxidize the HBT mediator used).

The sugar contents after acid hydrolysis were, glucose (44%), xylose (19%) and arabinose (1%) for untreated Elephant grass, and glucose (44%), xylose (12%), galactose (1%) and mannose (1%) for eucalypt wood. These values were basically the same after treatments with laccase alone, and after treating Elephant grass with laccase-HBT; however, an increase in the glucose (up to 49%) and xylose (up to 13%) contents was observed after treating the eucalypt wood with laccase (25 U g^{-1}) in the presence of HBT, due to the removal of lignin.

3.2. Enzymatic modification of Elephant grass lignin (as shown by 2D NMR)

Fig. 1 shows the complete HSQC NMR spectrum of the whole Elephant grass at the gel state, including the aliphatic oxygenated region, with methoxyl, lignin side-chain and carbohydrate cross-signals, and the aromatic region, with the lignin and *p*-coumaric acid signals (the traces of ferulic acid are not visible in the whole spectrum). The detailed assignments of the different signals are shown in Fig. 2, which includes the expanded aliphatic oxygenated (top) and aromatic (bottom) regions of the spectra of the control and laccase-mediator treated samples. The main lignin and cinnamic structures identified are shown in Fig. 3, and the different lignin signals assigned on the spectra are listed in Table 2. The lignin composition and *p*-coumaric and ferulic acids contents in the different Elephant grass samples, estimated from the intensities of the corresponding signals in the aromatic region of the HSQC spectra, are shown in Table 3.

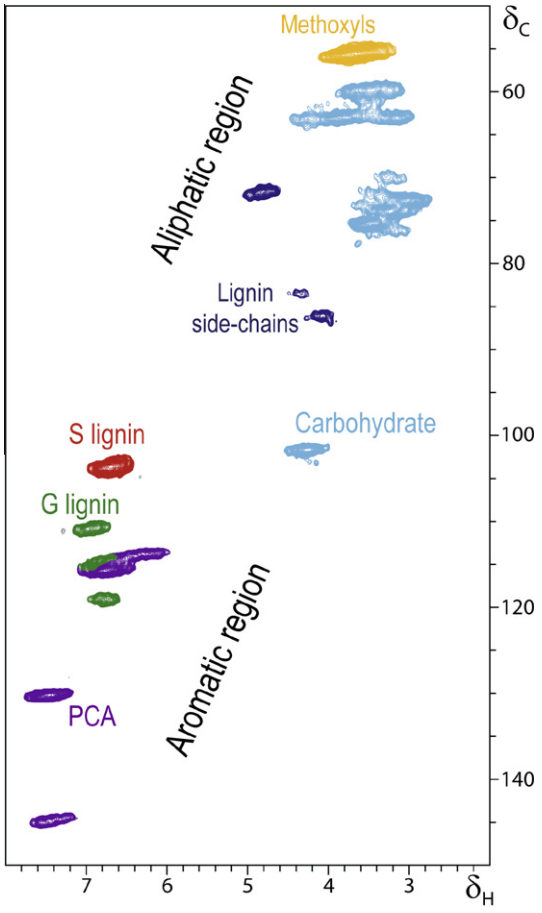

Fig. 1. HSQC NMR spectrum of whole plant biomass (Elephant grass) swollen in dimethylsulfoxide-d_6 showing lignin (G and S units) and *p*-coumaric acid (PCA) signals in the aromatic region, and main lignin inter-unit linkages (β-O-4′) and carbohydrate signals in the aliphatic (oxygenated) region. See Figs. 2 and 5 for detailed signal assignments in Elephant grass and eucalypt samples, respectively, and Fig. 3 for the main lignin structures identified.

Table 1
Lignin content of Elephant grass and eucalypt samples after four enzymatic treatments, each of them followed by an alkaline peroxide extraction, compared with the original untreated material, a control without enzyme and a treatment with laccase alone.

	Elephant grass	Eucalypt
Untreated material	22.1	22.3
Control	21.1	18.0
Laccase (10 U g^{-1})-HBT	18.8	12.2
Laccase (25 U g^{-1})-HBT	16.4	11.9
Laccase (50 U g^{-1})-HBT	14.3	9.4
Laccase (50 U g^{-1})	20.7	17.5

The aliphatic oxygenated region of the spectrum of control Elephant grass (Fig. 2A) showed signals of both lignin and carbohydrates. The latter mainly corresponded to xylan (X), since crystalline cellulose is nearly "silent" in lignocellulose gel spectra under solution NMR conditions. In this region, signals of methoxyls and side-chains in β-*O*-4′ lignin substructures (A), including C_γ–H_γ, C_β–H_β and C_α–H_α correlations (A_γ, A_β and A_α, respectively) were observed. The A_γ signal overlaps with related correlations in lignin and other lignocellulose constituents. The C_β–H_β correlations gave two different signals corresponding to β-*O*-4′ substructures where the second aromatic unit is a G unit or an S unit ($A_{\beta(S)}$ and $A_{\beta(G)}$, respectively).

The main signals in the unsaturated region of the HSQC spectrum of control Elephant grass (Fig. 2D) corresponded to the

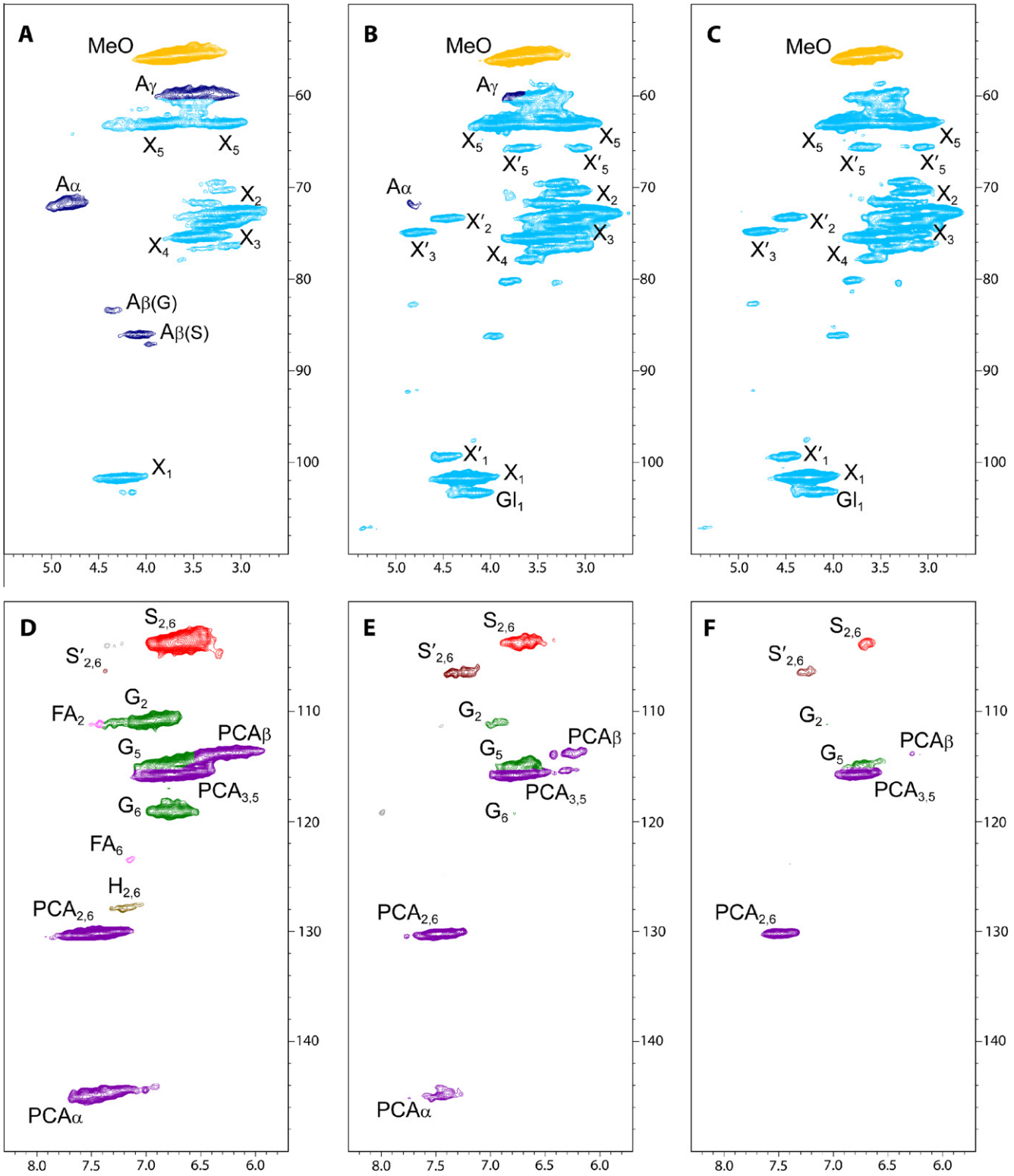

Fig. 2. Expanded aliphatic oxygenated (δ_H-δ_C, 2.5–5.5 and 50–110 ppm; top) and aromatic (δ_H-δ_C, 5.7–8.3 and 100–150 ppm; bottom) regions of the HSQC NMR spectra of Elephant grass treated with low and high doses of *T. villosa* laccase in the presence of HBT: (A and D) Control without enzyme; (B and E) 10 U g^{-1} enzyme; and (C and F) 50 U g^{-1} enzyme. See Table 2 for lignin signal assignment, Fig. 3 for the main lignin structures identified, and Table 3 for quantification of these lignin structures. Carbohydrate signals are also observed mainly corresponding to C_1–C_5 in normal (X_1–X_5) and acetylated xylan units (X'_1–X'_5) (an anomeric glucose signal was also identified, G_1).

benzenic rings of the guaiacyl (G) and syringyl (S) lignin units, and the aromatic and olefinic signals of *p*-coumaric acid. The S-lignin units showed a prominent signal for the $C_{2,6}$–$H_{2,6}$ correlation ($S_{2,6}$), while the G-lignin units showed different correlations for C_2–H_2 (G_2), C_5–H_5 (G_5) and C_6–H_6 (G_6). A low intensity signal corresponding to $C_{2,6}$–$H_{2,6}$ correlation in H units ($H_{2,6}$) was also

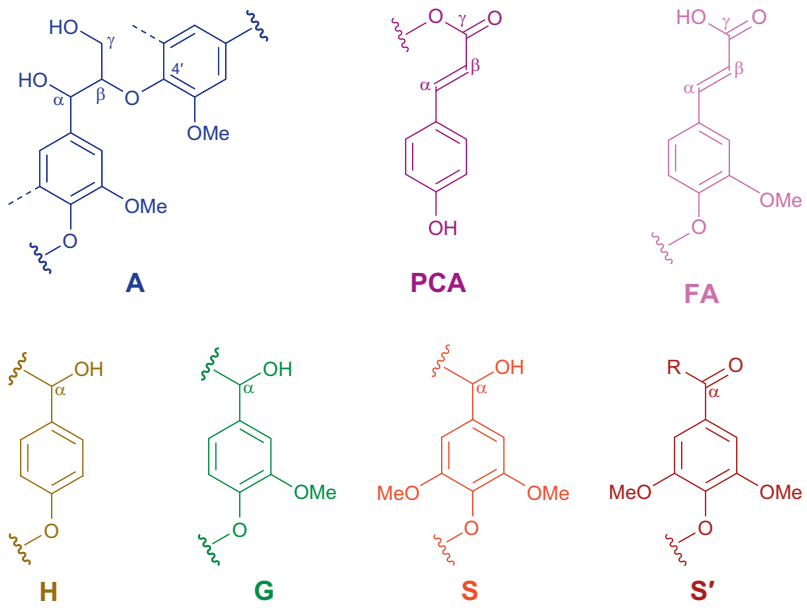

Fig. 3. Main lignin and cinnamic acid structures identified in the Elephant grass and eucalypt samples analyzed by HSQC NMR (Figs. 1, 2 and 5): (A) β-*O*-4′lignin substructures (including a second S or G unit); (PCA) *p*-coumaric acid; (FA) ferulic acid; (H) *p*-hydroxyphenyl units; (G) guaiacyl units; (S) syringyl units; and (S′) Cα-oxidized S units (R can be a hydroxyl in carboxylic acids or a lignin side-chain in ketones).

Table 2
Assignments of lignin and cinnamic acid main ^{13}C–^{1}H correlation signals in the HSQC NMR spectra of the Elephant grass and eucalypt samples swollen in dimethylsulfoxide-d_6. See Fig. 3 for chemical structures.

Label	δ_C/δ_H (ppm)	Assignment
MeO	55.6/3.73	C–H in methoxyls
A_γ	59.4/3.40 and 3.72	$C_\gamma H_\gamma$ in β-*O*-4′ structures (A)
A_α	71.8/4.83	C_α–H_α in β-*O*-4′ structures (A)
$A_{\beta(G)}$	83.4/4.27	C_β–H_β in β-*O*-4′ structures (A) linked to a G-unit
$A_{\beta(S)}$	85.9/4.10	C_β–H_β in β-*O*-4′ structures (A) linked to a S unit
$S_{2,6}$	103.8/6.69	C_2–H_2 and C_6–H_6 in syringyl units (S)
$S'_{2,6}$	106.1/7.32	C_2–H_2 and C_6–H_6 in α-oxidized syringyl units (S′)
FA_2	111.0/7.33	C_2–H_2 in ferulic acid (FA)
G_2	110.9/6.99	C_2–H_2 in guaiacyl units (G)
PCA_β	113.5/6.27	C_β–H_β in *p*-coumaric acid (PCA)
G_5	114.9/6.72 and 6.94	C_5–H_5 in guaiacyl units (G)
G_6	118.7/6.77	C_6–H_6 in guaiacyl units (G)
FA_6	123.2/7.11	C_6–H_6 in ferulic acid (FA)
$H_{2,6}$	127.7/7.20	C_2–H_2 and C_6–H_6 in *p*-hydroxyphenyl units (H)
$PCA_{3,5}$	115.5/6.77	C_3–H_3 and C_5–H_5 in *p*-coumaric acid (PCA)
$PCA_{2,6}$	130.1/7.45	C_2–H_2 and C_6–H_6 in *p*-coumaric acid (PCA)
PCA_α	144.7/7.41	C_α–H_α in *p*-coumaric acid (PCA)

observed. Signals corresponding to $C_{2,6}$–$H_{2,6}$ correlations in C_α-oxidized S-lignin units ($S'_{2,6}$) were hardly observed. On the other hand, the *p*-coumaric acid prominent signals in this region corresponded to the $C_{2,6}$–$H_{2,6}$ ($PCA_{2,6}$) and $C_{3,5}$–$H_{3,5}$ ($PCA_{3,5}$) aromatic correlations, and the C_α–H_α (PCA_α) and C_β–H_β (PCA_β) olefinic correlations. Two low intensity signals corresponding to C_6–H_6 and C_2–H_2 correlations in ferulic acid (FA_6 and FA_2, respectively) were also observed, while other aromatic signals of the ferulic acid traces overlapped with similar signals of *p*-coumaric acid and lignin G units.

The HSQC spectra of the Elephant grass samples after the enzymatic treatments with different laccase doses differed from those of the control (Fig. 2). The signals of side-chains in β-*O*-4′ lignin substructures (A), present in the aliphatic oxygenated region of the control spectrum, decreased and finally disappeared after the laccase-mediator treatment (Fig. 2B and C). Likewise, the signal of S lignin units present in the aromatic region of the spectrum also strongly decreased after the laccase-mediator treatment (Fig. 2E and F), and the signal of Cα-oxidized S-lignin units ($S'_{2,6}$) increased. The enzymatic treatment also enabled detection of new polysaccharide signals corresponding to acetylated xylan (X′), together with a terminal glucose (Gl) signal, that were not detectable in the control samples (probably because of a reduced mobility in the gels due to lignin-hemicellulose linkages). Generation of oxidized lignin structures is congruent with the nature of the lignin biodegradation process, which has been described as an "enzymatic combustion" (Kirk and Farrell, 1987). The action of laccase-HBT on non-phenolic lignin models is produced by hydrogen atom abstraction from the C_α position (Fabbrini et al., 2002). Aromatic ring oxidation, after electron transfer and cation radical formation, has also been reported in model degradation by laccase-HBT, but the C_α attack followed by alkyl–aryl ether breakdown predominates (Kawai et al., 2002). This attack mechanism would result in the increased amount of Cα-oxidized lignin units observed after the laccase-mediator treatment of the Elephant grass, and especially of the eucalypt wood (see below).

In the present study, the decrease in G units observed by 2D NMR, occurred to a greater extent than that of the S ones, and the G units nearly disappeared at the highest laccase dose. Elephant grass lignin which has a similar proportion of S and G units and an S/G ratio around 1.2 in the control sample, became an S-rich lignin after the enzymatic treatments (Table 3). This result was unexpected, since fungal treatment of lignocellulosic biomass often cause a decrease in the lignin S/G ratio (del Río et al., 2002), but the result could have been due to topological reasons favoring the access of laccase mediators and/or alkali to the G-rich lignin present in plant vessels (Musha and Goring, 1975). Interestingly, it has been reported that, under alkaline conditions, the very first lignin removed from hardwood is guaiacyl (Santos et al., 2011). In contrast, the most intense signals of *p*-coumaric acid, corresponding to the double aromatic-ring correlations ($PCA_{2,6}$ and $PCA_{3,5}$) remained in the spectrum at the highest laccase dose, although with lower intensities than the carbohydrate signals.

Table 3
Lignin composition, as molar percentage of H, G, S and Cα-oxidized S units (with respect to total lignin units) and *p*-coumaric acid (PCA) and ferulic acid (FA) content, as cinnamic/lignin (L) molar ratios, from the HSQC NMR spectra of the Elephant grass and eucalypt samples treated with three doses of laccase and HBT (in a sequence including four enzymatic treatments and four alkaline peroxide extractions) compared with a control without enzyme and a treatment with laccase alone.

	H	G	S	S_{ox}	PCA/L	FA/L
Elephant grass						
Control	3	43	54	0	0.47	<0.005
Laccase-HBT (10 U g^{-1})	0	34	43	23	0.68	0
Laccase-HBT (25 U g^{-1})	0	30	53	16	0.82	0
Laccase-HBT (50 U g^{-1})	0	16	49	35	0.86	0
Laccase alone (50 U g^{-1})	0	42	58	0	0.66	0
Eucalypt						
Control	0	23	77	0	0	0
Laccase-HBT (10 U g^{-1})	0	0	56	44	0	0
Laccase-HBT (25 U g^{-1})	0	0	41	59	0	0
Laccase-HBT (50 U g^{-1})	0	0	40	60	0	0
Laccase alone (50 U g^{-1})	0	9	91	0	0	0

The relative molar content of the different lignin units, together with the *p*-coumaric acid content referred to lignin content (PCA/(H + G + S + S′) ratio), are shown in Table 3, revealing a preferential removal of lignin with respect to *p*-coumaric acid.

A general picture on the compositional changes produced by the enzymatic treatments is provided by Fig. 4A, which shows the intensities of the lignin, *p*-coumaric acid, ferulic acid (traces) and carbohydrate signals in the spectra of the Elephant grass treated with laccase doses of 10, 25 and 50 U g^{-1} in the presence of HBT, and with laccase (50 U g^{-1}) alone, compared with the corresponding control. Although the intensities cannot be used for comparison of different C–H couples in different (aliphatic *vs* aromatic) regions of the same spectrum, they permit a comparison between similar C–H couples in the different samples. In this way, the general tendency observed at increasing enzyme doses is the decrease in lignin carbon and an increase in polysaccharide carbon, in agreement with the chemical analyses. In particular, the decrease in the aromatic carbon in lignin H, G and S units and *p*-coumaric acid, and the aliphatic carbon in lignin side-chains and methoxyls (that also include contributions from hemicelluloses) was observed. In addition, an increase in oxidized S units (relatively moderate in the case of treated Elephant grass) and acylated xylan was observed. In the case of laccase alone, the tendency was the same but the changes observed were relatively minor.

3.3. Enzymatic modification of eucalypt lignin (as shown by 2D NMR)

The detailed assignments of aliphatic-oxygenated (top) and aromatic (bottom) signals in the control and laccase-HBT treated eucalypt samples are shown in the spectra expansions included in Fig. 5. The main lignin structures identified are shown in Fig. 3, and the different lignin signals assigned on the spectra are listed in Table 2. Table 3 shows the lignin composition in the eucalypt samples, estimated from the intensities of the main cross-signals present in the aromatic region of the NMR spectra.

The aliphatic oxygenated region of the spectrum of control eucalypt (Fig. 5A) showed signals of both lignin and carbohydrates, the latter mainly corresponding to xylan units (X), as in the Elephant grass spectra. In addition to methoxyl signals, signals of lignin side-chains were observed with lower intensities than those found in Elephant grass, the latter corresponding to C_α–H_α correlations (A_α) in β-*O*-4′ alkyl–aryl ether substructures, and C_β–H_β

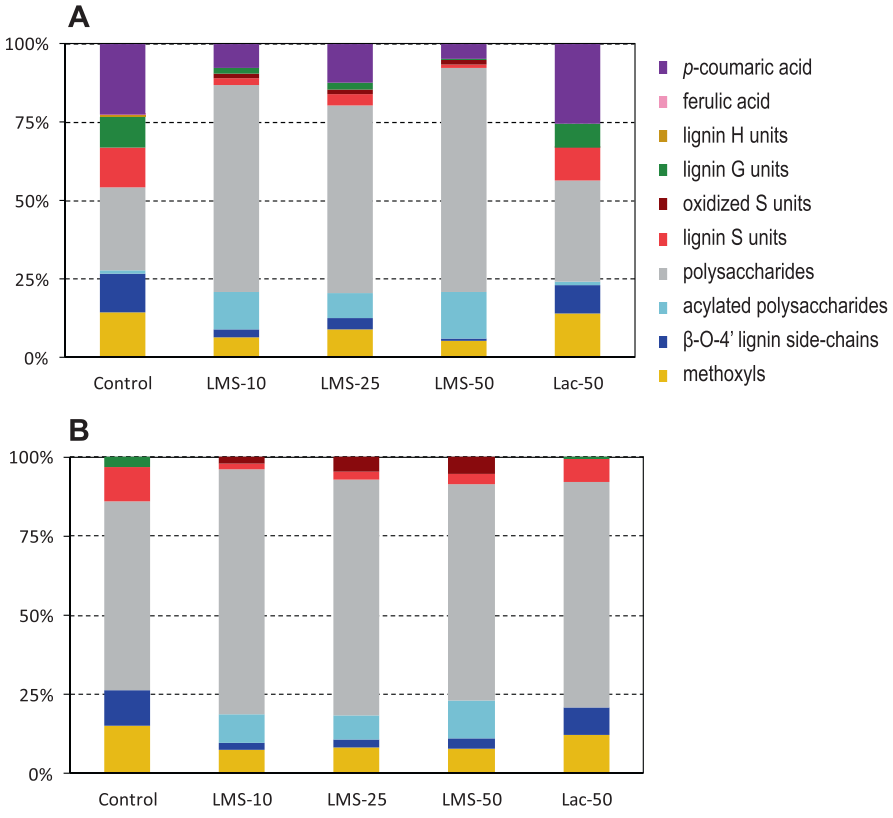

Fig. 4. Comparison of changes in Elephant grass (A) and eucalypt (B) constituents during laccase-mediator treatment with different enzyme doses (10, 25 and 50 U g^{-1}) as revealed by HSQC NMR, compared with a control without enzyme (first bar) and the treatment with laccase alone (50 U g^{-1}; last bar). The presence of *p*-coumaric and ferulic acids, H, G, S and S-oxidized lignin, polysaccharide (xylan), acylated polysaccharide, lignin side-chains (in β-*O*-4′ substructures) and methoxyl groups was, respectively, shown by the $PCA_{2,6}$, $H_{2,6}$, G_2, $S_{2,6}$, $S'_{2,6}$, X_1, X'_3, Aα and MeO signals in Figs. 2 and 5 (shown as percentages of sample carbon corresponding to each structure type).

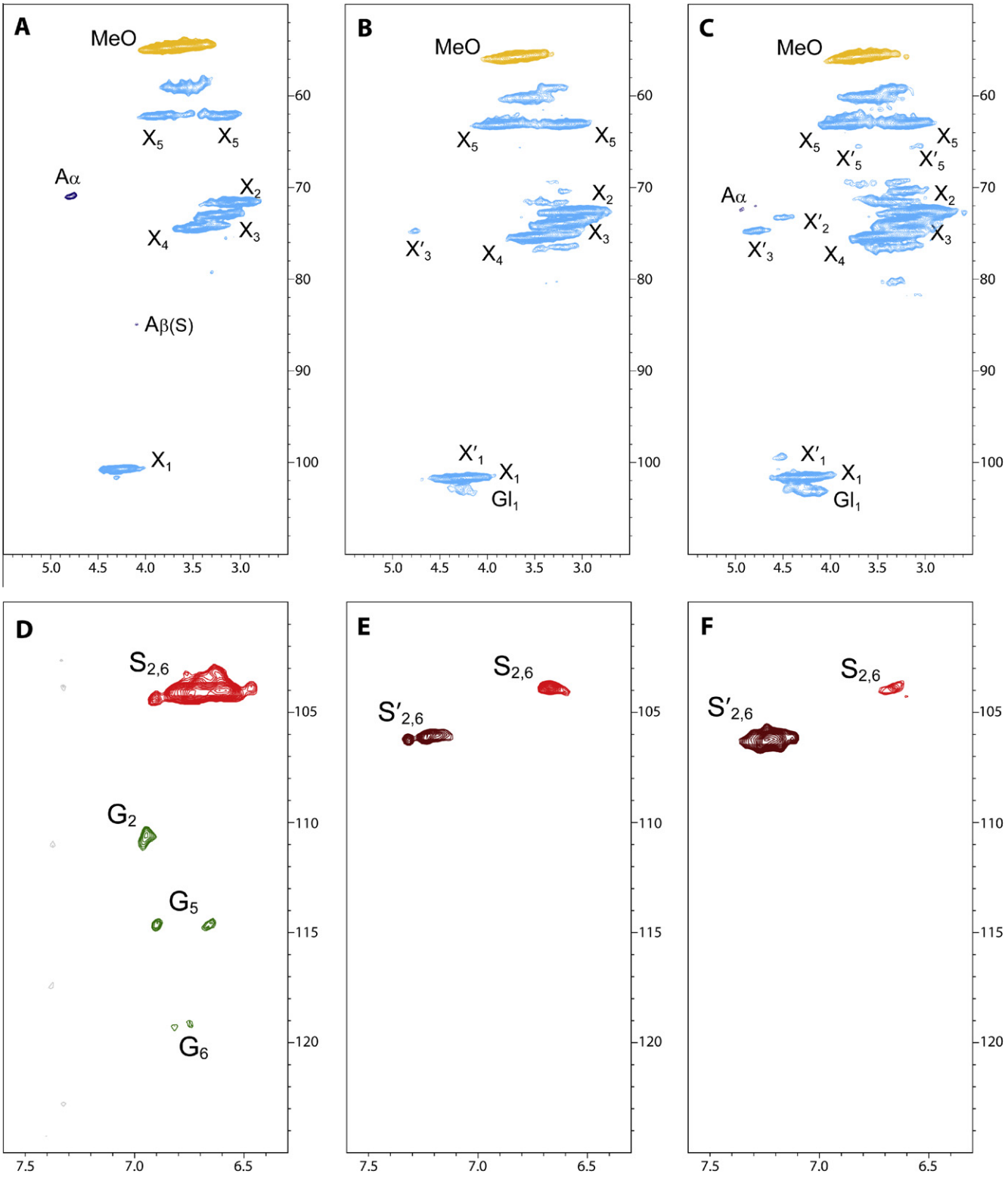

Fig. 5. Expanded aliphatic oxygenated (δ_H-δ_C, 2.5–5.5 and 50–110 ppm; top) and aromatic (δ_H-δ_C, 6.3–7.6 and 100–125 ppm; bottom) regions of the HSQC NMR spectra of eucalypt treated with low and high doses of *T. villosa* laccase in the presence of HBT: (A and D) Control without enzyme; (B and E) 10 U g^{-1} enzyme; and (C and F) 50 U g^{-1} enzyme. See Table 2 for signal assignment, Fig. 3 for the main lignin structures identified, and Table 3 for quantification of these lignin structures.

correlations in β-*O*-4′ alkyl–aryl ether substructures including a second S-unit ($A_{\beta(S)}$). The main signals in the aromatic region of the HSQC spectrum of control eucalypt wood (Fig. 5D) corresponded to the lignin benzene rings, including the G and S correlations described for the Elephant grass. The content in S units of the eucalypt lignin was higher than that in G units, as revealed by the prominent $S_{2,6}$ signal, compared with the G_2, G_5 and G_6 signals, with a S/G ratio around 3.3 (Table 3), in agreement with previous studies (Rencoret et al., 2008, 2011). The higher reduction in lignin content in eucalypt than in Elephant grass samples could have

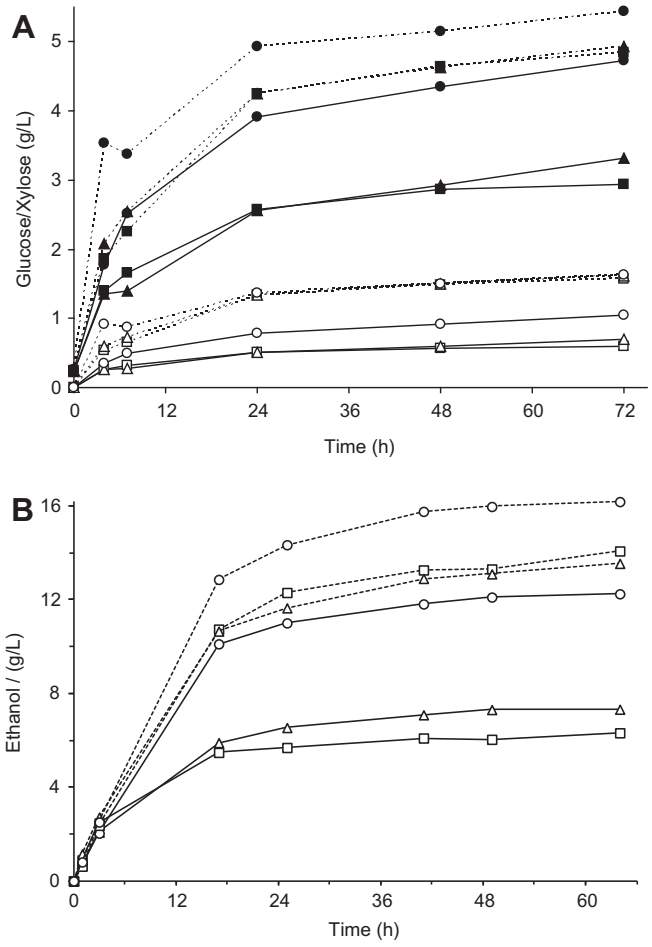

Fig. 6. Effect of enzymatic pretreatment on biomass enzymatic hydrolysis and saccharification: (A) Comparison of glucose (black symbols) and xylose (white symbols) release during 0–72 h hydrolysis of Elephant grass (dashed lines) and eucalypt (continuous lines) pretreated with laccase-HBT (circles) and laccase alone (triangles) and controls without enzyme (squares); (B) Time-course of ethanol production during simultaneous saccharification and fermentation of milled Elephant grass (dashed lines) and eucalypt (continuous lines) pretreated with laccase-HBT (circles) and laccase alone (triangles), in sequences including four enzymatic treatments and four alkaline peroxide extractions, and controls without enzyme (squares). Mean values from triplicate experiments. See Table 4 for final sugar yields and ethanol production.

been due to the higher S/G ratio of eucalypt lignin, which results in a more linear and less condensed polymer.

The HSQC spectra of the eucalypt samples treated with laccase-mediator showed important differences compared to the control ones (Fig. 5). The signal of side-chains in β-*O*-4′ lignin substructures (Aα) present in the aliphatic oxygenated region of the control spectrum completely disappeared even at the lowest enzyme dose (Fig. 5B). Likewise, the G lignin signals, in the aromatic region of the spectrum, also completely disappeared with the lowest enzyme dose, while the S units were Cα-oxidized (and in a significant extent remained as such) as revealed by the strong increase in the $S'_{2,6}$ signal, which became the most prominent signal in this region when the enzyme dose was increased (Fig. 5E and F, and Table 3). Therefore, the results obtained for the eucalypt wood confirmed the Cα-oxidation mechanism for lignin removal by laccase-HBT, and revealed that most of the residual lignin in wood treated with the highest laccase dose corresponds to the oxidized S′ units. Similarly to the Elephant grass enzymatic treatment, the decrease in G units seemed to occurre to a greater extent than in the S ones. The presence of oxidized lignin units was also observed in eucalypt pulp residual lignin after a laccase-mediator treatment, including both Cα ketones and carboxylic acids (Ibarra et al., 2007).

A general picture of the compositional changes revealed by the NMR analyses of the eucalypt samples, enabling comparison of treatments with different laccase doses (in the presence of HBT) and with laccase alone, is shown in Fig. 4B. The general tendency at increasing enzyme doses is a decrease in lignin carbon (in aromatic, side-chain and methoxyl structures), although to a lower extent than in the Elephant grass samples, and a concomitant increase of polysaccharides, including acetylated units. In contrast, the effect of laccase alone was very moderate, being basically reduced to the decrease in lignin G units.

Table 4
Monosaccharide (glucose and xylose) after 72 h (and 4 h, parenthesis) hydrolysis (% of sample weight), and ethanol release 64 h (and 17 h, parenthesis) after adding the yeast to the cellulase reaction (% of sample weight) from the Elephant grass and eucalypt samples treated with laccase-HBT (25 U g^{-1}) in a sequence including four enzymatic treatments (and four alkaline peroxide extractions) compared with a control without enzyme and a treatment with laccase alone. Means ± S.D (from triplicates).

	Hydrolysis		Fermentation
	Glucose (%)	Xylose (%)	Ethanol (%)
Elephant grass			
Control	48.4 ± 1.3 (18.6 ± 1.3)	15.7 ± 0.8 (5.5 ± 0.4)	14.1 ± 0.1 (10.7 ± 0.2)
Laccase-HBT	54.4 ± 1.3 (35.3 ± 1.6)	16.3 ± 0.6 (9.2 ± 0.5)	16.2 ± 0.6 (12.9 ± 0.1)
Laccase alone	49.4 ± 1.2 (20.8 ± 2.7)	16.1 ± 0.4 (5.9 ± 0.6)	13.6 ± 0.6 (10.7 ± 0.2)
Eucalypt			
Control	29.4 ± 5.1 (14.0 ± 2.6)	6.0 ± 1.0 (2.6 ± 0.4)	6.3 ± 0.4 (5.5 ± 0.2)
Laccase-HBT	47.3 ± 1.5 (17.7 ± 1.1)	10.5 ± 0.2 (3.6 ± 0.2)	12.3 ± 0.2 (10.1 ± 0.1)
Laccase alone	33.1 ± 1.1 (13.5 ± 1.6)	7.0 ± 0.3 (2.6 ± 0.4)	7.3 ± 0.9 (5.9 ± 0.1)

3.4. Enzymatic hydrolysis and fermentation

The Elephant grass and eucalypt samples treated with laccase (25 U g^{-1}), alone and in the presence of HBT, were hydrolyzed using a cellulase and β-glucosidase cocktail, and the main monosaccharides released (glucose, xylose and arabinose) were analyzed by HPLC (total reducing sugars from the DNS assay showed similar tendencies). The effect of hydrolysis time was investigated and 72 h hydrolysis was chosen since monosaccharide release already stabilized after this time period, and reached 64–71% of sample weight for Elephant grass and 35–58% for eucalypt (Fig. 6A, Table 4). In the case of eucalypt wood, the effect of the laccase-HBT treatment increased with cellulase hydrolysis times, the highest increases in glucose and xylose releases were obtained after 72 h. However, for Elephant grass the highest increases in sugar releases by the laccase-mediator treatment were observed after only a 4-h hydrolysis. The ability of laccase-mediator treatment to increase polysaccharide hydrolysis by cellulases had been already reported, but only on pretreated (steam-exploded) wood (Palonen and Viikari, 2004). Interestingly, the treatment with laccase alone (without mediator) also slightly increased the hydrolysis yields for eucalypt and Elephant grass, with respect to that of the controls. This agrees with the findings by Moilanen et al. (2011) who reported a 12% hydrolysis increase (after 48 h) by laccase (alone) treatment of steam-pretreated spruce wood, although, surprisingly, the same enzymatic treatment on giant read decreased the hydrolysis yield.

During the subsequent long-term simultaneous saccharification and fermentation (Fig. 6B), the maximal ethanol production rate

(0.32–0.76 g L^{-1} h^{-1}) was achieved during the first 17 h, although further, a slight production was observed during the remaining period (0.02–0.05 g L^{-1} h^{-1}). The latter is explained by the moderate but significant effect caused by milling. In all cases, the highest total ethanol yields were obtained from Elephant grass compared with eucalypt wood. The laccase-mediator pretreatment significantly increased ethanol production after 17 h of saccharification-fermentation. Interestingly, the enzymatic treatment was considerably more efficient improving ethanol production from eucalypt (over 4 g L^{-1} in 17 h) than from Elephant grass (~2 g L^{-1} in 17 h). The presence of the mediator seems necessary to improve ethanol production, since treatment with laccase alone was useless on Elephant grass, and only caused a very moderate increase in ethanol production from eucalypt (0.4 g L^{-1} in 17 h).

4. Conclusions

Woody and nonwoody plant biomass can be significantly delignified by enzymes (30–50% lignin removal) by applying a sequence consisting of successive laccase-mediator and alkaline extraction stages, directly on the ground lignocellulosic material (i.e. without a partial degradation and subsequent deconstruction). The HSQC NMR spectra of the lignocellulosic samples showed a significant decrease of both aromatic and aliphatic lignin signals after the enzymatic treatments, and provide strong evidence for a Cα-oxidation degradation mechanism, with high presence of oxidized S units in the residual lignin. The improved cellulose hydrolysis, and higher ethanol production in enzyme/mediator-treated feedstock demonstrates the potential of this approach in biofuel production.

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