

Laccase-Mediator Pretreatment of Wheat Straw Degrades Lignin and Improves Saccharification

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Abstract Agricultural by-products such as wheat straw are attractive feedstocks for the production of second-generation bioethanol due to their high abundance. However, the presence of lignin in these lignocellulosic materials hinders the enzymatic hydrolysis of cellulose. The purposes of this work are to study the ability of a laccase-mediator system to remove lignin improving saccharification, as a pretreatment of wheat straw, and to analyze the chemical modifications produced in the remaining lignin moiety. Up to 48 % lignin removal from ground wheat straw was attained by pretreatment with *Pycnoporus cinnabarinus* laccase and 1-hydroxybenzotriazole (HBT) as mediator, followed by alkaline peroxide extraction. The lignin removal directly correlated with increases (~60 %) in glucose yields after enzymatic saccharification. The pretreatment using laccase alone (without mediator) removed up to 18 % of lignin from wheat straw. Substantial lignin removal (37 %) was also produced when the enzyme-mediator pretreatment was not combined with the alkaline peroxide extraction. Two-dimensional nuclear magnetic resonance (2D NMR) analysis of the whole pretreated wheat straw material swollen in dimethylsulfoxide-*d*₆ revealed modifications of the lignin polymer, including the lower number of aliphatic side chains involved in main β -O-4' and β -5' inter-unit linkages per aromatic lignin unit. Simultaneously, the removal of *p*-hydroxyphenyl, guaiacyl,

and syringyl lignin units and of *p*-coumaric and ferulic acids, as well as a moderate decrease of tricin units, was observed without a substantial change in the wood polysaccharide signals. Especially noteworthy was the formation of C α -oxidized lignin units during the enzymatic treatment.

Keywords Enzymatic delignification · Laccase-mediator · Lignin · Wheat straw · 2D NMR · Bioethanol

Introduction

Agricultural and forestry residues represent an enormous source of readily available biomass for biofuel production without the need for additional land cultivation. Among agricultural residues, wheat straw is potentially one of the most favorable feedstocks in terms of the quantity of biomass available [1]. However, in spite of the availability of these residues or other lignocellulosic biomass, their variable composition and recalcitrance represent some technical and economic challenges. Cellulose, hemicelluloses, and lignin are the three main components of lignocellulosic biomass linked into a complex matrix highly resistant to chemical and biological conversion. These components are more resistant to being broken down and fermented than starch and sucrose in the conventional food crops, making the conversion processes more complicated. Biofuel production from lignocellulosic material requires deconstruction of the cell wall into individual polymers and hydrolysis of the carbohydrates into monomeric sugars. One of the major factors causing biomass recalcitrance toward saccharification is correlated with the content and composition of lignin [2–4].

Lignin is a three-dimensional polymer constituted by phenylpropanoid subunits linked together by different ether and carbon-carbon bonds. Lignin is intimately interlaced with

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hemicelluloses in the plant cell wall forming a matrix to cover the largely crystalline cellulose microfibrils. Its aromatic nature and complex structure make lignin degradation very difficult. Lignin has been shown to have a detrimental effect on the hydrolysis of biomass because it physically hinders the access of cellulases and also binds them reducing activity [5]. Therefore, biomass pretreatment to remove lignin is essential for the enzymatic hydrolysis of lignocellulose. Biotechnology can contribute to plant biomass deconstruction by providing biocatalysts being able to degrade or modify lignin [6]. Due to the complex structure of lignin, including heterogeneity of the monomers and linkages, which are not hydrolyzable, these biocatalysts must be oxidative (i.e., oxidoreductases) and also non-specific. Among these, laccases seem to be the most suitable enzymes for industrial application, because they only require dioxygen as oxidant and also because it can be produced on a large scale [7].

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 the phenolic units that only represent a small percentage of the total polymer [8], a fact that limits their biotechnological application. However, the discovery that some synthetic compounds can act as electron carriers between the enzyme and the final substrate [9], 1-hydroxybenzotriazole (HBT) being among the most efficient ones [10], has expanded the utility of laccases. A number of studies have confirmed the potential of laccase-mediator systems for paper pulp delignification [11, 12], pitch control [13], polymer modification [14], and other applications in the forest industry [15] and bioethanol production from pretreated lignocellulose [16–18]. Recently, the ability of fungal laccases to remove lignin (when applied in combination with redox mediators) from whole [19, 20] and ensiled [21] lignocellulosic biomass, making cellulose accessible to hydrolysis, was reported. Additionally, the use of laccases in bioethanol production has also been reported as a biotechnological tool for the removal of phenolic inhibitors generated during steam explosion of lignocellulosic feedstocks [22], although some recent work also shows that some laccase-derived compounds can affect negatively the enzymatic hydrolysis [23].

A previous work on the laccase-mediator treatment of acid-pretreated wheat straw has been reported [17]. The novelty of the pretreatment described here, based on the use of a fungal laccase from the basidiomycete *Pycnoporus cinnabarinus* [24] in combination with HBT as mediator [25], is that it was applied directly in the ground wheat straw feedstock (without a previous chemical pretreatment). Additionally, in the present study, the modification of lignin structure in the pretreated lignocellulosic material was analyzed in depth by two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy of the whole sample at the gel state [26, 27].

Besides lignin modification and removal, the effect of the laccase-mediator on the saccharification yield from the pretreated wheat straw feedstock was also assessed.

Material and Methods

Lignocellulosic Feedstock, Enzyme, and Mediator

Wheat straw (*Triticum durum* var. Carioca) was harvested from an experimental field in Seville (Spain). Wheat straw samples were air-dried and grounded in an IKA MF10 cutting mill to pass through a 100-mesh screen and then finely milled using a Retsch PM100 planetary mill at 400 rev min⁻¹ (with 5-min breaks after every 5 min of milling) using a 500-mL agate jar and agate ball bearings (20 × 20 mm). The total ball-milling time for the samples was 5 h. The chemical composition of wheat straw feedstock (as % dry weight) was as follows: glucose, 39.4 ± 0.7; xylose, 16.0 ± 0.3; arabinose, 3.8 ± 0.2; soluble acid lignin, 1.5 ± 0.1; and Klason lignin, 16.0 ± 0.3.

The laccase was provided by INRA (Marseille, France) and was obtained from a laccase-hyperproducing strain of the fungus *P. cinnabarinus* (Herpoël et al. 2000). Its 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 24 °C. The laccase activity of the enzyme preparation was 102 U/ml (specific activity 156 U/mg). One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per minute. HBT from Sigma-Aldrich (Steinheim, Germany) was used as mediator.

Laccase-Mediator Treatments

The wheat straw samples were treated with the *P. cinnabarinus* laccase in the presence (and absence) of HBT, as mediator. Laccase doses of 13 and 65 U g⁻¹ were assayed, together with 5, 10, and 20 % HBT, all referred to straw 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 4-g (dry weight) samples at 6 % (w/w) solid loading in 50 mM sodium tartrate buffer (pH 4) under O₂ atmosphere (2 bar) for 24 h. Additionally, the treatment with laccase (65 U g⁻¹) and HBT (20 %) was also performed in the presence of 0.05 % Tween 20, to test the effect of adding a surfactant in both the enzymatic delignification and enzymatic hydrolysis of wheat straw [28]. After the treatment, the samples were filtered through a Büchner funnel and washed with 1 L of water. In some cases, a subsequent alkaline peroxide extraction step was performed after the enzymatic pretreatment. In this case, enzymatically treated samples at 6 % (w/w) solid loading were submitted to a peroxide-reinforced

alkaline extraction using 1 % (w/w) NaOH and 3 % (w/w) H₂O₂ (also with respect to sample dry weight) at 80 °C for 90 min, followed by water washing [29]. The solid loading for the latter step was achieved by determining the moisture content of an aliquot. Treatments with laccase (65 U g⁻¹) alone (without mediator) and controls without laccase and mediator were also performed (followed in both cases by the corresponding alkaline peroxide extraction). A control with mediator alone was not included taking into account the results from previous studies. Duplicate experiments of a representative (65 U g⁻¹ laccase and 20 % HBT) laccase-mediator treatment (including control, laccase alone and laccase-HBT) were performed to estimate the variability in biological replicates (as shown in Table 1 footnote). A one-way analysis of variance (ANOVA) was conducted to compare the effects of the different enzymatic treatments on the lignin removal and on the releases of glucose and xylose. Post hoc pairwise comparisons, using the Tukey HSD test, were performed in order to determine which means are significantly different from each other. Klason lignin content was estimated (in triplicate measurements) according to T222 om-88 [30]. The data from both biological and technical replicates were averaged. Weight loss (%) was determined for all the treatments (Table 1) with respect to the control without enzyme-mediator and alkaline extraction: laccase (65 U g⁻¹), 2.3 %; laccase (65 U g⁻¹)-HBT

(20 %), 6.1 %; control/alkaline peroxide, 0.8 %; laccase (65 U g⁻¹)/alkaline extraction, 4.8 %; laccase (13 U g⁻¹)-HBT (10 %)/alkaline extraction, 7.3 %; laccase (65 U g⁻¹)-HBT (5 %)/alkaline extraction, 7.7 %; laccase (65 U g⁻¹)-HBT (20 %)/alkaline extraction, 13.4 %; and laccase (65 U g⁻¹)-HBT (20 %)/T20/alkaline extraction, 13.5 %. The weight loss of control without enzyme-mediator and alkaline extraction with respect to initial wheat straw was 12.7 %.

Saccharification of Treated Wood

The laccase-pretreated samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase (Celluclast 1.5 L; 2 filter paper units (FPU) g⁻¹) and β -glucosidase (Novozyme 188; 6 U g⁻¹) activities, at 1 % solid loading in 3 mL of 100 mM sodium citrate (pH 5) for 72 h at 45 °C, in a thermostatic shaker at 170 rev min⁻¹ (in triplicate experiments). The specific activities of Celluclast 1.5 L and β -glucosidase are 700 EGU/g and 250 CBU/g, respectively.

The different monosaccharides released were determined as alditol acetates [31] by GC. An HP 5890 gas chromatograph (Hewlett-Packard, Hoofddorp, The Netherlands) equipped with a split-splitless injector and a flame ionization detector was used. The injector and detector temperatures were set at 225 and 250 °C, respectively. Samples were injected in the split mode (split ratio 10:1). Helium was used as the carrier gas. The capillary column used was a DB-225 (30 m \times 0.25 mm i.d.,

Table 1 Lignin content and monosaccharide release (% sample weight) by cellulase hydrolysis of wheat straw samples

Wheat straw samples	Lignin	Glucose	Xylose
Initial wheat straw	16.0 \pm 0.3	21.6 \pm 0.2	6.9 \pm 0.1
- Laccase-mediator:			
Control	15.6 a \pm 0.2	24.1 a \pm 0.0	7.3 a \pm 0.4
Laccase (65 U g ⁻¹)	15.0 a \pm 0.1	29.0 b \pm 0.1	9.4 b \pm 0.1
Laccase (65 U g ⁻¹)-HBT (20 %)	9.8 b \pm 0.3	33.6 c \pm 0.2	11.2 c \pm 0.2
- Laccase-mediator/alkaline peroxide:			
Control	14.8 a \pm 0.2	28.4 a \pm 0.4	9.8 a \pm 0.3
Laccase (65 U g ⁻¹)	12.1 b \pm 0.3	31.1 b \pm 0.3	9.8 a \pm 0.2
Laccase (13 U g ⁻¹)-HBT (10 %)	9.4 c \pm 0.1	35.1 c \pm 0.5	11.4 b \pm 0.6
Laccase (65 U g ⁻¹)-HBT (5 %)	9.2 c \pm 0.4	36.5 d \pm 0.3	11.5 b \pm 0.6
Laccase (65 U g ⁻¹)-HBT (20 %)	7.7 d \pm 0.2	41.5 e \pm 0.7	13.3 c \pm 0.2
Laccase (65 U g ⁻¹)-HBT (20 %)/T20	7.5 d \pm 0.2	45.5 f \pm 0.6	13.7 c \pm 0.0

Lignin content (as Klason lignin, corrected for ash) and monosaccharides from cellulase hydrolysis of wheat straw samples treated with the following: (i) *P. cinnabarinus* laccase (13 and 65 U g⁻¹) and HBT mediator (10 and 20 %); (ii) the same previous enzymatic treatment (i) followed by an alkaline peroxide extraction including a treatment in presence of Tween 20 (T20) and (ii) compared with a control without enzyme, a treatment with laccase alone, and the initial wheat straw sample. Biological duplicates for a representative laccase-mediator treatment (including 65 U g⁻¹ laccase and 20 % HBT) were separately analyzed showing that the 95 % confidence intervals of the biological duplicates are smaller than the differences found between the control, laccase alone, and laccase-HBT treatments (data not shown). In the table, means \pm SD from technical triplicates of experiments representative for the different treatments are provided. Letters next to the means, from Tukey test, show whether or not the results from different treatments (shown in each column) are significantly different from each other, at the 0.05 level (means with the same letters are not significantly different, whereas means followed by different letters are significantly different)

0.15- μm film thickness; Agilent J&W). The oven was temperature-programmed from 220 °C (held for 5 min) to 230 °C (held for 5 min) at 2 °C min^{-1} . Peaks were quantified by area, and glucose and xylose were used as standards to elaborate calibration curves. The data from both biological and technical replicates were averaged.

2D NMR Spectroscopy

For gel-state NMR experiments, ~70 mg of finely divided (ball-milled) wheat straw samples was directly transferred into 5-mm NMR tubes and swelled in 1 mL of deuterated dimethylsulfoxide ($\text{DMSO-}d_6$), forming a gel inside the NMR tube [26, 27].

Heteronuclear single-quantum correlation (HSQC) 2D-NMR spectra were acquired at 25 °C on a Bruker AVANCE III 500-MHz spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The 2D ^{13}C - ^1H correlation spectra were carried out using an adiabatic HSQC pulse program (Bruker standard pulse sequence “hsqcetgpsisp2.2”), which enabled a semiquantitative analysis of the different ^{13}C - ^1H correlation signals. Spectra were acquired from 10 to 0 ppm (5000 Hz) in F2 (^1H) using 1000 data points for an acquisition time of 100 ms, an interscan delay of 1 s, and from 200 to 0 ppm (25,168) in F1 (^{13}C) using 256 increments of 32 scans, for a total acquisition time of 2 h 34 min. The $^1J_{\text{CH}}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^1H and a squared cosine bell in ^{13}C . The central solvent peak was used as an internal reference ($\delta_{\text{C}}/\delta_{\text{H}}$ 39.5/2.49). The ^{13}C - ^1H correlation signals from the aromatic region of the spectrum were used to estimate the content of lignin, *p*-coumaric acid, ferulic acid, and triclin (compared with the amorphous polysaccharide content, estimated from the anomeric xylose and glucose signals) and the lignin composition in terms of G, S, and oxidized S (S') units. Correlations in the aliphatic-oxygenated region were used to estimate the inter-unit linkage and end-unit abundances in lignin. The intensity corrections introduced by the adiabatic pulse program permit to refer the side-chain integrals to the previously obtained number of lignin units.

Results and Discussion

Delignification of Wheat Straw by Laccase-HBT

Wheat straw lignin is a guaiacyl-rich lignin [32] that is usually reported to be more resistant to degradation than the syringyl type [33]. For this reason, and also based on results from previous studies, a high-redox potential laccase (from the basidiomycete *P. cinnabarinus*) and mediator (HBT) were selected for wheat straw delignification.

Enzymatic pretreatments using different doses of *P. cinnabarinus* laccase (13 and 65 U g^{-1}) and HBT (5, 10,

and 20 %) were carried out. The selection of these doses was based on previous studies on the pretreatment of another non-woody feedstock (elephant grass) [19] with a lignin having also a high proportion of guaiacyl units although not as high as that of wheat straw [32, 34]. Additionally, the effect of the combination of the enzymatic treatment with a subsequent alkaline peroxide extraction was studied. The lignin content (as Klason lignin) of wheat straw samples after the pretreatments was determined (Table 1). The effect of oxygen (used in the enzymatic reactions) and alkaline peroxide extraction in wheat straw delignification can be observed by comparing the corresponding controls with the initial straw.

The lignin content of wheat straw samples after the laccase-mediator pretreatment using *P. cinnabarinus* laccase (65 U g^{-1}) in the presence of HBT (20 %) decreased about 37 % with respect to the corresponding control (treated under the same conditions except the addition of enzyme and mediator) (Table 1). The pretreatment with laccase alone (in the absence of HBT) only decreased the lignin content by 5 %. Similar values of lignin content decrease have been reported after laccase (alone) treatment of several lignocellulosic feedstocks, as steam-pretreated giant reed and Northern spruce [35].

The laccase alone is not very efficient for delignification because of its low oxidation potential and for steric hindrance reasons. The relatively low oxidation potential of laccases only allows them to oxidize phenolic groups in lignin.

Combination of the enzymatic pretreatment with a subsequent alkaline peroxide extraction step (removing partially degraded lignin) increased wheat straw delignification up to 48 and 18 % (with respect to lignin content of the control sample) in the pretreatments with laccase-HBT and laccase alone, respectively (Table 1). With the aim of exploring if the enzymatic delignification could be enhanced by the presence of a surfactant, as reported for pulp biobleaching [36], addition of Tween 20 was tested, but only a modest effect on the delignification of wheat straw was observed. When the enzymatic pretreatment (followed by alkaline peroxide extraction) was carried out under the same reaction conditions and dose of laccase, but with lower dose of HBT (5 %), 38 % reduction of lignin content (with respect to the control sample) was attained, and a similar value (37 % delignification) was obtained when both the laccase and mediator doses were reduced to 13 U g^{-1} enzyme and 10 % HBT, respectively.

Similar delignification degrees to those above described (with lower laccase-mediator doses) were obtained after four sequential laccase-HBT treatments (including four alkaline peroxide extraction steps) of elephant grass [19]. Interestingly, when the latter pretreatment was applied to eucalypt wood, higher delignification degrees (up to 48 %) were attained [19, 37, 38], showing that some woody feedstocks can be competitive for bioethanol production. The better delignification values obtained in the latter studies can be

related with the predominance of syringyl lignin units in eucalypt wood with respect to elephant grass and wheat straw [32, 34, 39, 40]. On the other hand, no decrease in lignin content was obtained by other authors after treating steam-exploded wheat straw with *P. cinnabarinus* laccase and HBT [41].

Enzymatic Hydrolysis of Pretreated Wheat Straw

The wheat straw samples treated with laccase alone and in the presence of HBT and the corresponding controls (and the initial untreated wheat straw) were hydrolyzed (72 h) using a cellulase and β -glucosidase cocktail [19], and the main monosaccharides released (glucose and xylose) were analyzed by GC.

At low cellulase (2 FPU g^{-1}) and β -glucosidase (6 U g^{-1}) doses, increases in glucose and xylose yields up to 40 and 47 % (with respect to controls), respectively, were attained in the samples pretreated with 65 U g^{-1} of laccase and 20 % mediator (Table 1). Interestingly, in the samples pretreated with laccase alone, 24–25 % increases in glucose and xylose release were produced, although the Klason lignin content was not significantly lowered. The positive effect of laccase (alone) in enzymatic saccharification has been attributed to the binding of laccase to lignin in lignocellulosic substrates that competes with and consequently reduces the non-specific binding of cellulases to lignin improving saccharification [17], in addition to some delignification effect on the phenolic lignin units. Moreover, a surface analysis of lignocellulose substrate after treatment with *Trametes* laccase revealed an increase in carboxylic acid residues after laccase treatment and suggested that this enzymatic modification to lignin may decrease the non-specific adsorption of negatively charged cellulases [16].

On the other hand, increases up to 46 and 9 % in glucose and 35 and 0 % in xylose yields, respectively, were produced when the enzymatic pretreatments of laccase-HBT and laccase alone were combined with an alkaline peroxide extraction step (the highest increases being attained with the highest doses of laccase, 65 U g^{-1} , and HBT, 20 %). Moreover, a similar improvement in saccharification yield was produced after the laccase-mediator treatment alone (without a subsequent alkaline extraction) unlike in a recent study of a laccase-HBT treatment of acid-pretreated wheat straw [17]. In the pretreatment with 13 U g^{-1} of laccase, in combination with 10 % mediator (followed by alkaline extraction), the increases in glucose and xylose yields were 24 and 16 % (with respect to control), respectively (Table 1). Similarly, an increase in glucose and xylose yields up to 29 and 17 %, respectively, was produced using 65 U g^{-1} of laccase, in combination with only 5 % mediator (followed by alkaline extraction). The above glucose release values are higher than those recently reported by other authors in the treatment with laccase-HBT of acid-pretreated wheat straw [17] in which higher laccase doses

were used but also higher solid loading in enzymatic hydrolysis. Other authors have reported no improvement in saccharification yields after different laccase-mediator treatments of steam-exploded wheat straw [41]. On the other hand, the saccharification yields shown here after laccase-mediator pretreatment of wheat straw are higher than those reported for other pretreatments of wheat straw like steam explosion [42]. The sugar degradation and generation of inhibitory compounds during steam explosion that affect the hydrolysis can explain the lower saccharification yields attained in steam-exploded wheat straw. Additionally, it should be mentioned the higher solid loading used by these authors that also may affect enzymatic saccharification.

Finally, noteworthy was the improvement in glucose and xylose yields (up to 60 and 40 %, respectively) obtained in the present study when a surfactant (Tween 20) was added during the laccase-HBT pretreatment (Table 1). Since the addition of Tween 20 did not affect the delignification of wheat straw (as shown above) and, in contrast, enhanced the saccharification, its effect may be related with an increase of cellulase action on cellulose [28].

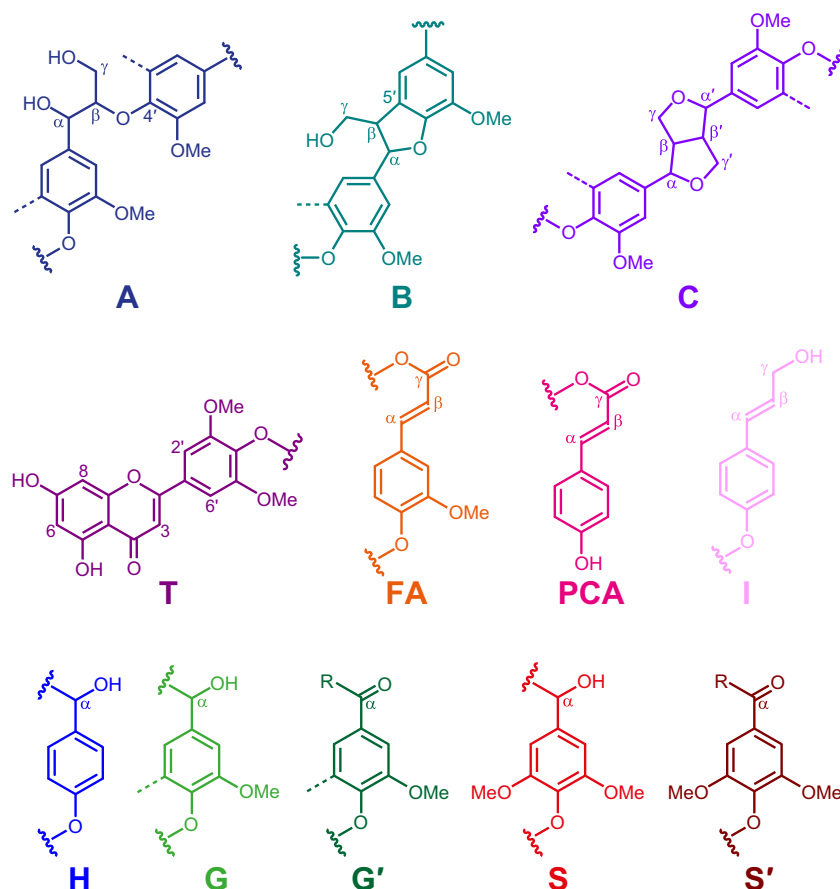
Enzymatic Modification of Wheat Straw Lignin (as Shown by 2D NMR)

The modification of lignin structure produced by the enzymatic pretreatments of wheat straw was studied by 2D NMR. With this purpose, the whole wheat straw samples were swelled in DMSO- d_6 forming a gel and analyzed by HSQC NMR. The main lignin structures identified are shown in Fig. 1, and the different lignin signals assigned on the spectra are listed in Table 2. The HSQC spectra of the different straw samples are provided in Figs. 2, 3, and 4. The composition of lignin, in terms of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, and the *p*-coumaric acid and ferulic acid contents, together with the relative abundance of the main inter-unit linkages in the different samples, estimated from the NMR signal volume integrals, are shown in Tables 3 and 4.

Effect of the Laccase-Mediator Treatment

Figure 2 shows the HSQC spectra of the wheat straw samples after pretreatment with laccase (65 U g^{-1}) in the presence (Fig. 2c, f) and absence (Fig. 2b, e) of HBT (20 %) and the corresponding control (Fig. 2a, d). The aliphatic-oxygenated region of the HSQC spectrum of the control without enzyme (Fig. 2a) shows signals of lignin and carbohydrates, the latter mainly corresponding to amorphous xylan (X), acetylated xylan (X'), and glucan (Gl) units, 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' alkyl-aryl ether lignin substructures (A),

Fig. 1 Main lignin structures identified in the wheat straw samples analyzed by HSQC NMR (Figs. 2, 3, and 4). *A* β -O-4' ether (including a second S or G unit), *B* phenylcoumaran, *C* resinol, *T* triclin, *PCA* *p*-coumaric acid, *FA* ferulic acid (esterified with hemicellulose sugars), *I* cinnamyl alcohol end group, *H* *p*-hydroxyphenyl unit, *G* guaiacyl unit, *S* syringyl unit, *G'* C α -oxidized G unit, and *S'* C α -oxidized S unit (R in *G'* and *S'* can be a hydroxyl in carboxylic acids or a lignin side chain in ketones)



including C α /H α , C β /H β , and C γ /H γ correlations (A α , A β , and A γ , respectively), were observed. The C α /H α and C β /H β correlations gave two different signals corresponding to β -O-4' substructures where the second unit is an S unit or a G unit (A α (S) and A α (G), and A β (S) and A β (G), respectively). The A γ signal overlaps with related signals in lignin and other lignocellulose constituents. Other less prominent signals corresponding to phenylcoumaran (B) and resinol (C) substructures were also observed in the HSQC spectrum. The main signals in the aromatic/unsaturated region of the HSQC spectrum (Fig. 2d) correspond to the benzenic rings and unsaturated side chains of H, G, and S lignin units; *p*-coumaric acid (PCA); and ferulic acid (FA). Additionally, several signals corresponding to the flavone triclin (T) recently reported in wheat-straw lignin [32] were observed. 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 $_6$). From the integrals of the above signals, an S/G ratio around 0.4 and a large predominance of β -O-4' ether linkages, together with some phenylcoumarans and resinols, were estimated for lignin in wheat straw feedstock, in agreement with previous studies [32]. A low-intensity signal corresponding to C $_{2,6}$ /H $_{2,6}$ correlation in H units (H $_{2,6}$) was also observed. On the other hand,

the PCA 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. Additionally, two signals corresponding to C $_2$ /H $_2$ and C $_6$ /H $_6$ correlations in FA (FA $_2$ and FA $_6$, respectively) were also observed, while the other aromatic and olefinic signals of the FA overlapped with similar signals of PCA and lignin G units.

The HSQC spectra of the wheat straw samples after the treatments with the higher laccase-mediator doses showed important differences compared to the control ones. While the carbohydrate signals remained unchanged, most of the lignin (and cinnamic acid) signals were strongly decreased with respect to the initial and control samples (Table 3). The prominent signals of side chains in β -O-4' lignin substructures (A), present in the control spectrum (Fig. 2a), as well as B and C signals were not observed after the enzymatic treatment (Fig. 2c). On the other hand, the signals of lignin units (H, G, and S) present in the spectrum of the control sample (Fig. 2d) also disappeared (Fig. 2f), except the very low-intensity S signal. Moreover, the signals of cinnamates (PCA and FA) also disappeared, whereas, interestingly, the flavonoid structure, triclin, seemed to be more resistant to laccase-mediator treatments (Fig. 2f).

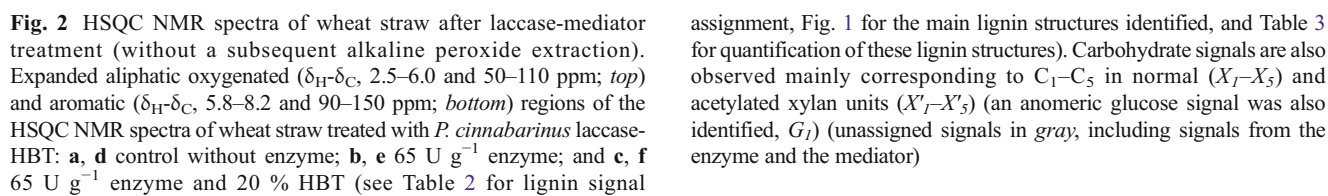
Table 2 Assignments of lignin ^{13}C - ^1H correlation signals in the 2D HSQC spectra of wheat straw samples

Label	$\delta_{\text{C}}/\delta_{\text{H}}$ (ppm)	Assignment
B_{β}	53.1/3.41	$\text{C}_{\beta}/\text{H}_{\beta}$ in phenylcoumaran substructures (B)
C_{β}	53.5/3.06	$\text{C}_{\beta}/\text{H}_{\beta}$ in β - β' resinol substructures (C)
$-\text{OCH}_3$	55.5/3.72	C/H in methoxyls
A_{γ}	59.6/3.37 and 3.71	$\text{C}_{\gamma}/\text{H}_{\gamma}$ in γ -hydroxylated β - $\text{O}-4'$ substructures (A)
I_{γ}	61.5/4.09	$\text{C}_{\gamma}/\text{H}_{\gamma}$ in cinnamyl alcohol end groups (I)
X_5	62.9/3.16 and 3.87	C_5/H_5 in xylopyranose units
$\text{A}_{\alpha(\text{G})}$	71.1/4.71	$\text{C}_{\alpha}/\text{H}_{\alpha}$ in β - $\text{O}-4'$ substructures (A) linked to a G-unit
C_{γ}	71.2/4.17	$\text{C}_{\gamma}/\text{H}_{\gamma}$ in β - β' resinol substructures (C)
$\text{A}_{\alpha(\text{S})}$	71.5/4.81	$\text{C}_{\alpha}/\text{H}_{\alpha}$ in β - $\text{O}-4'$ substructures (A) linked to a S-unit
X_2	72.5/3.03	C_2/H_2 in xylopyranose units
X'_2	73.1/4.49	C_2/H_2 in 2- O -acetylated xylopyranose units
X_3	73.8/3.23	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	75.2/3.50	C_4/H_4 in xylopyranose units
$\text{A}_{\beta(\text{G})}$	83.5/4.35	$\text{C}_{\beta}/\text{H}_{\beta}$ in β - $\text{O}-4'$ substructures linked (A) to a G unit
C_{α}	84.7/4.63	$\text{C}_{\alpha}/\text{H}_{\alpha}$ in β - β' resinol substructures (C)
$\text{A}_{\beta(\text{S})}$	85.9/4.05	$\text{C}_{\beta}/\text{H}_{\beta}$ in β - $\text{O}-4'$ substructures linked (A) to a S unit
B_{α}	86.8/5.42	$\text{C}_{\alpha}/\text{H}_{\alpha}$ in phenylcoumaran substructures (B)
T_8	94.0/6.56	C_8/H_8 in tricin units (T)
T_6	98.8/6.20	C_6/H_6 in tricin units (T)
X'_1	99.3/4.48	C_1/H_1 in 3- O -acetylated xylopyranose units
X_1/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
$\text{S}_{2,6}$	103.8/6.69	C_2/H_2 and C_6/H_6 in etherified syringyl units (S)
$\text{T}_{2',6'}$	103.9/7.28	$\text{C}_{2'}/\text{H}_{2'}$ and $\text{C}_{6'}/\text{H}_{6'}$ in tricin units (T)
T_3	104.6/7.02	C_3/H_3 in tricin units (T)
$\text{S}'_{2,6}$	106.1/7.29, 106.2/7.18	C_2/H_2 and C_6/H_6 in α -oxidized syringyl units (S')
G_2	110.7/6.96	C_2/H_2 in guaiacyl units (G)
G'_2	110.8/7.38	C_2/H_2 in α -oxidized guaiacyl units (G')
FA_2	110.9/7.33	C_2/H_2 in ferulate (FA)
$\text{PCA}_{\beta}/\text{FA}_{\beta}$	113.5/6.29	$\text{C}_{\beta}/\text{H}_{\beta}$ in p -coumarate (PCA) and ferulate (FA)
$\text{H}_{3,5}$	113.6/6.64	C_3/H_3 and C_5/H_5 in p -hydroxyphenyl units (H)
FA_5	114.1/6.77	C_5/H_5 in ferulate (FA)
G_5/G_6	114.9/6.78, 6.94 and 118.8/6.77	C_5/H_5 and C_6/H_6 in guaiacyl units (G)
G'_5	115.0/6.73	C_5/H_5 in α -oxidized guaiacyl units (G')
$\text{PCA}_{3,5}$	115.3/6.76	C_3/H_3 and C_5/H_5 in p -coumarate (PCA)
FA_6	123.2/7.10	C_6/H_6 in ferulate (FA)
$\text{H}_{2,6}$	127.6/7.14	C_2/H_2 and C_6/H_6 in p -hydroxyphenyl units (H)
$\text{PCA}_{2,6}$	130.0/7.48	C_2/H_2 and C_6/H_6 in p -coumarate (PCA)
$\text{PCA}_{\alpha}/\text{FA}_{\alpha}$	145.0/7.58	$\text{C}_{\alpha}/\text{H}_{\alpha}$ in p -coumarate (PCA) and ferulate (FA)

From Figs. 2, 3, and 4 HSQC spectra. See Fig. 1 for chemical structures

A prominent signal corresponding to $\text{C}_{2,6}/\text{H}_{2,6}$ correlations in C_{α} -oxidized S-lignin units ($\text{S}'_{2,6}$) appeared as a consequence of this treatment. Likewise, new signals, tentatively assigned to oxidized G-lignin units (G'), appeared in the spectrum. Generation of oxidized lignin structures is congruent with the nature of the lignin biodegradation process, which has been described as an “enzymatic combustion” where

fungal oxidoreductases play a central role [43]. It is generally accepted that lignin degradation by white rot fungi and their ligninolytic peroxidases starts by aromatic ring oxidation to a cation radical but quickly leads to side-chain C_{α} - C_{β} cleavage causing depolymerization [44]. The same mechanism has been suggested for some laccase reactions mediated by synthetic compounds, e.g., 2,2-azino-bis(3-ethylbenzothiazoline-



after the laccase-mediator treatment of the wheat straw. Concerning C α -oxidized groups, a higher proportion of S and G acid monomers with respect to the aldehyde counterpart has been determined by thermochemolysis in lignins extracted from laccase-mediator-treated wheat straw [17].

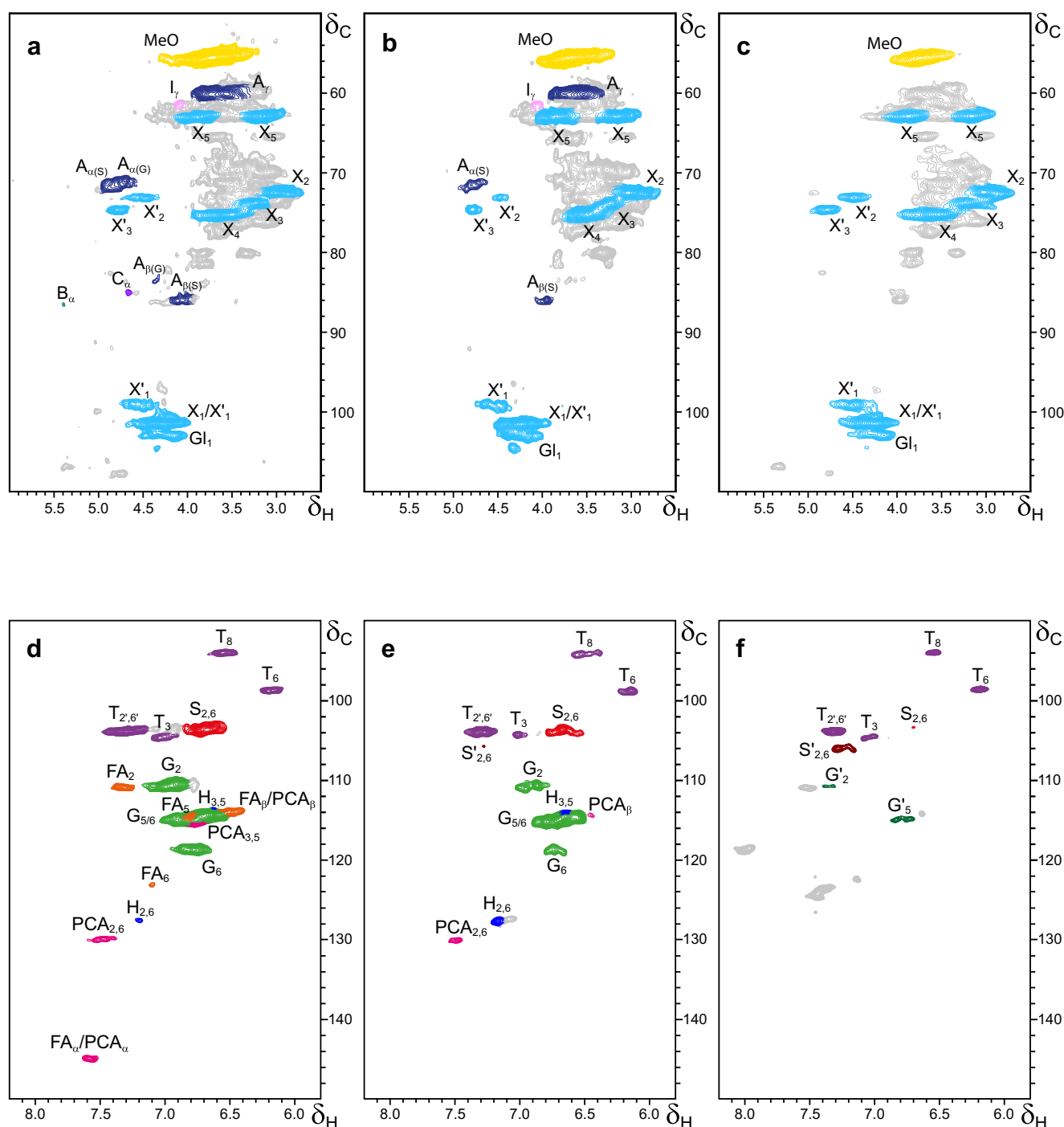


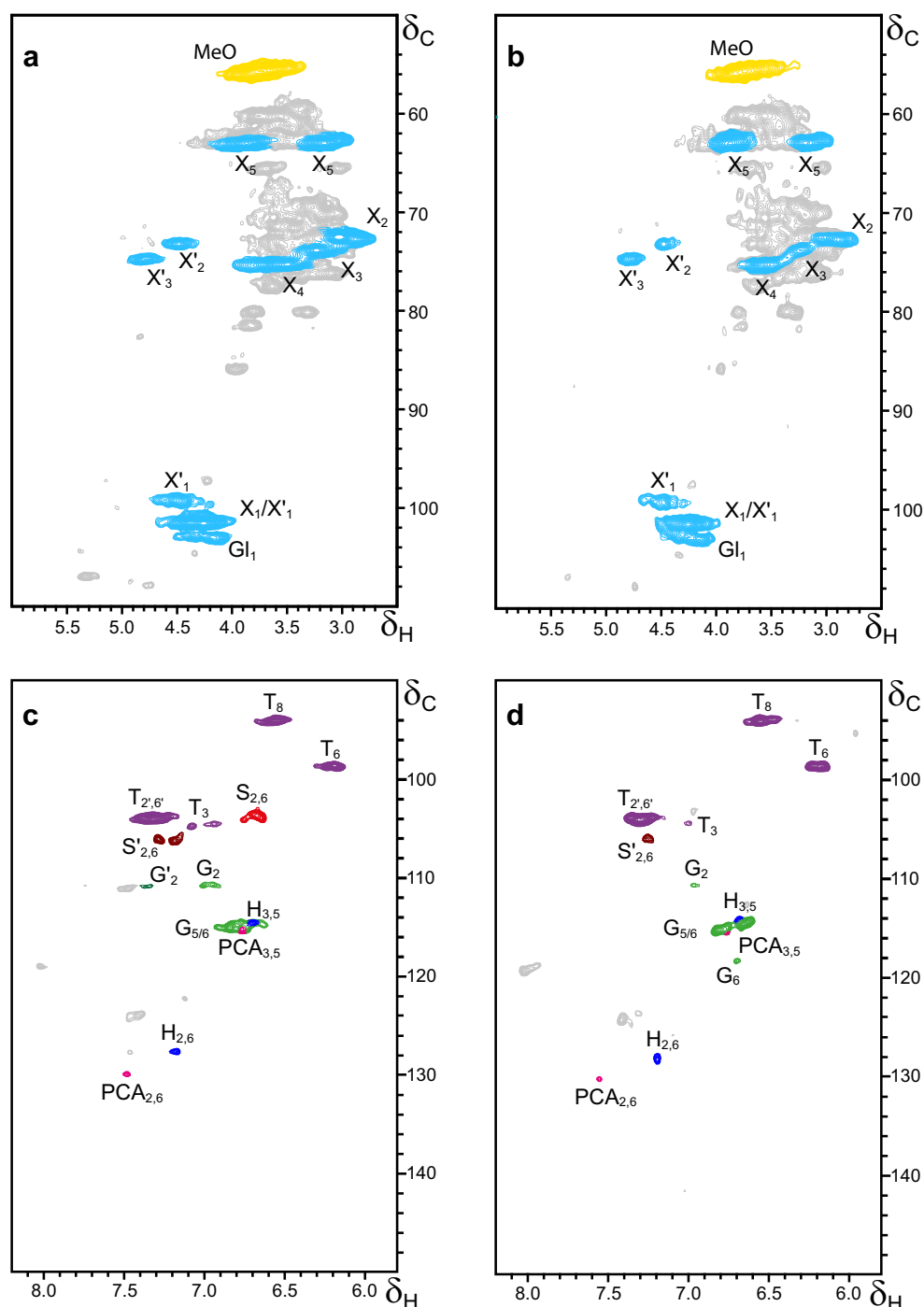
Fig. 3 HSQC NMR spectra of wheat straw after laccase-mediator treatment (higher doses) and alkaline peroxide extraction. Expanded aliphatic oxygenated (*top*) and aromatic (*bottom*) regions of the HSQC NMR spectra of wheat straw samples after treatment with laccase-HBT

followed by an alkaline peroxide extraction: **a, d** Control without enzyme; **b, e** 65 U g⁻¹ enzyme; and **c, f** 65 U g⁻¹ enzyme and 20 % HBT (see Fig. 2 legend for additional information)

Remarkably, some lignin modification and removal were also shown by the NMR spectra of the wheat straw treated with laccase alone (Fig. 2b, e), with a relative decrease of the lignin signals compared to the carbohydrate signals and the initial and control samples (Table 3). Among them, the C signals disappeared, and A_α and A_β and B signals decreased

considerably with respect to the control sample, although the changes were always less intense than those found in the sample treated with laccase and mediator. Concerning the aromatic units, the G lignin signals decreased slightly and C_α-oxidation of S (and G) units was observed to some extent although much less pronounced than in the presence of HBT. The lignin

Fig. 4 HSQC NMR spectra of wheat straw after laccase-mediator treatment (lower doses) and alkaline peroxide extraction. Expanded aliphatic oxygenated (*top*) and aromatic (*bottom*) regions of the HSQC NMR spectra of wheat straw samples after treatment with laccase-HBT followed by an alkaline peroxide extraction: **a, b** 13 U g⁻¹ enzyme and 10 % HBT and **c, d** 65 U g⁻¹ enzyme and 5 % HBT (see Fig. 2 legend for additional information)



modification observed in the pretreatments with laccase alone could be due to the action of laccase catalyzing the oxidation of the phenolic moiety (less than 20 %) of wheat straw lignin since laccase alone is not able to catalyze the oxidation of non-phenolic lignin units. Reactions of high- and low-redox potential laccases with lignin models (monomers) have revealed that any laccase reactions are initiated through oxidation of the phenolic hydroxyl group and that laccase cannot directly oxidize the α -carbon [47]. This side-chain oxidation to an α -

carbonyl leads (in reaction with monomers) to further coupling to the 4- or 5-position (in guaiacyl-type units). However, if the 5-position is occupied (as in syringyl-type groups), the side-chain oxidation to an α -carbonyl serves as a dead-end reaction with higher oxidation potential or to the coupling to the 1-position with subsequent breakage of the bond producing cleavage of the lignin polymer [47]. On the other hand, the cleavage of the $C_{\alpha}-C_{\beta}$ bond catalyzed by laccase has been shown in phenolic lignin model dimers by,

Table 3 NMR analysis of straw treated with laccase-HBT and controls (parentheses refer to total lignin)

	Initial	Control	Laccase	Laccase-HBT
- Sample composition ^a :				
Syringyl lignin units (S)	5.8 (30)	7.7 (31)	5.5 (30)	0.5 (7)
C _α -oxidized S units (S')	0 (0)	0 (0)	0.4 (2)	2.2 (32)
Guaiacyl lignin units (G)	12.6 (63)	15.2 (63)	11.1 (60)	0.4 (6)
C _α -oxidized G units (G')	0 (0)	0 (0)	0.5 (3)	3.4 (51)
<i>p</i> -Hydroxyphenyl units (H)	1.5 (7)	1.5 (6)	0.9 (5)	0.3 (4)
<i>Total lignin</i>	<i>19.9 (100)</i>	<i>24.4 (100)</i>	<i>18.4 (100)</i>	<i>6.7 (100)</i>
Tricin (T)	3.6 (18)	4.7 (19)	3.4 (19)	3.5 (52)
<i>p</i> -Coumaric acid (PCA)	1.3 (6)	1.8 (7)	1.5 (8)	0 (0)
Ferulic acid (FA)	3.4 (17)	3.8 (15)	2.0 (11)	0 (0)
<i>Total aromatics</i>	<i>28.1</i>	<i>34.6</i>	<i>25.3</i>	<i>10.2</i>
Sugar units	71.9	65.4	74.7	89.8
Total	100	100	100	100
Lignin S/G ratio	0.46	0.50	0.51	0.70
- Side chains and end groups ^b :				
β- <i>O</i> -4' ethers (A)	80 (61)	82 (62)	88 (59)	0
Phenylcoumarans (B)	8 (6)	9 (7)	4 (3)	0
Resinols (C)	9 (7)	7 (5)	4 (3)	0
Cinnamyl end groups (I)	3 (3)	3 (2)	4 (3)	0
Total	100 (77)	100 (76)	100 (68)	0

Milled wheat straw was treated with laccase (65 U g⁻¹) in combination with HBT (20 %) and laccase alone and compared with control treatment (without enzyme) and initial material (spectrum not shown) using HSQC NMR (Fig. 2)

^a Sample composition provides the molar amount of normal (S, G, and H) and C_α-oxidized (S' and G') lignin units, S/G ratio, triclin (T), *p*-coumaric acid (PCA), and ferulic acid (FA) from integration of aromatic signals and sugar units from integration of anomeric carbon signals, in the HSQC spectra (parentheses, values referred to total lignin); total lignin (italics)= S+S'+G+G'+H, total aromatics (italics)= total lignin+T+PCA+FA

^b The percentages of lignin side chains forming different substructure (A–C) and cinnamyl alcohol end groups are provided from integration of aliphatic signals in the HSQC spectra (parentheses, values referred to total lignin)

first, oxidizing C_α and by splitting the aryl-alkyl bond [48]. These studies help to explain the lignin degradation observed in the pretreatments with laccase alone. However, it should be taken into account that these reactions occur much easier using model compounds than in polymeric lignin for steric reasons, among others. On the other hand, this lignin modification observed by the action of laccase alone could be also due to the action of natural occurring mediators that can be present in wheat straw.

Noteworthy in the treatment with laccase alone is the preferential decrease of FA signals, with respect to the PCA signals, that would be related to the lower redox potential of FA (due to the presence of an electron-donating substituent on the aromatic ring).

Effect of the Alkaline Peroxide Extraction and Laccase-Mediator Doses

The NMR spectra of the wheat straw treatments followed by an alkaline peroxide extraction are shown in Fig. 3, including

control (Fig. 3a, d), laccase alone (Fig. 3b, e), and laccase-HBT (Fig. 3c, f) treatments, and the results from signal integration are provided in Table 4. The spectra of the latter samples (Fig. 3c, f) were similar to those of samples pretreated under the same conditions but without alkaline peroxide extraction (Fig. 2c, f), although with (slightly) lower-intensity aromatic signals (including C_α-oxidized lignin units) in agreement with lower lignin content (Table 1). The S'/S and G'/G ratios decreased after the alkaline peroxide extraction from 4.4 and 8.5 to 3.3 and 5.5, respectively. During alkaline peroxide extraction, the hydroperoxide anions produced are reported to react with the carbonyl structures existing in lignin resulting in C–C bond cleavage [17], and similar reaction has been reported in alkaline pulping [49].

Differences were also observed between the spectra of laccase-pretreated samples without and with an alkaline peroxide extraction. In the latter spectra, a high decrease in β-*O*-4' side-chain substructures (Fig. 3b) was observed together with the complete disappearance of FA signals and the remarkable decrease of lignin units (Fig. 3e). Finally, some

Table 4 NMR analysis after laccase-HBT (different doses) treatment followed by alkaline extraction (parentheses refer to total lignin)

	Control	Laccase	Lac13-HBT10	Lac65-HBT5	Lac65-HBT20
- Sample composition ^a :					
Syringyl lignin units (S)	4.8 (28)	3.3 (30)	1.3 (24)	0.2 (8)	0.3 (10)
C _α -oxidized S units (S')	0	0.3 (3)	1.0 (19)	0.6 (29)	1.0 (38)
Guaiacyl lignin units (G)	11.6 (68)	6.5 (58)	1.5 (28)	0.7 (37)	0.2 (6)
C _α -oxidized G units (G')	0	0.3 (3)	1.1 (20)	0	1.1 (39)
<i>p</i> -Hydroxyphenyl units (H)	0.8 (5)	0.8 (7)	0.4 (8)	0.5 (26)	0.2 (7)
<i>Total lignin</i>	<i>17.2 (100)</i>	<i>11.1 (100)</i>	<i>5.3 (100)</i>	<i>1.9 (100)</i>	<i>2.7 (100)</i>
Tricin (T)	3.1 (18)	2.7 (25)	2.0 (38)	3.0 (154)	1.4 (50)
<i>p</i> -Coumaric acid (PCA)	1.4 (8)	0.8 (7)	0.3 (6)	0.3 (13)	0
Ferulic acid (FA)	2.6 (15)	0.3 (3)	0	0	0
<i>Total aromatics</i>	<i>24.2</i>	<i>15.0</i>	<i>7.7</i>	<i>5.1</i>	<i>4.1</i>
Sugar units	75.8	85.1	92.3	94.9	96.0
Total	100	100	100	100	100
Lignin S/G ratio	0.41	0.53	0.89	1.02	1.05
- Side chains and end groups ^b :					
β- <i>O</i> -4' ethers (A)	76 (59)	91 (59)	100 (16)	29 (12)	0
Phenylcoumarans (B)	6 (5)	0	0	0	0
Resinols (C)	10 (8)	0	0	0	0
Cinnamyl end groups (I)	8 (6)	9 (6)	0	71 (30)	0
Total	100 (78)	100 (65)	100 (16)	100 (42)	0

Milled wheat straw was treated with 13 or 65 U g⁻¹ of laccase (Lac13 and Lac65) in combination with 5, 10, or 20 % HBT (HBT5, HBT10, and HBT20, respectively) and compared with laccase alone and with a control without enzyme (followed in all cases by an extraction with alkaline peroxide) using HSQC NMR (Figs. 3 and 4)

^a Sample composition provides the molar amount of normal (S, G, and H) and C_α-oxidized (S' and G') lignin units, S/G ratio, triclin (T), *p*-coumaric acid (PCA), and ferulic acid (FA) from integration of aromatic signals and sugar units from integration of anomeric carbon signals, in the HSQC spectra (parentheses, values referred to total lignin); total lignin (italics)= S+S'+G+G'+H, total aromatics (italics)= total lignin+T+PCA+FA

^b The percentages of lignin side chains forming different substructure (A-C) and cinnamyl alcohol end groups are provided from integration of aliphatic signals in the HSQC spectra (parentheses, values referred to total lignin)

effect was also produced by the alkaline peroxide extraction itself. If we compare the NMR spectra of controls with (Fig. 3a, d) and without (Fig. 2a, d) alkaline peroxide extraction, it is evident that B and C signals decreased with the alkaline peroxide extraction. Likewise, the intensities of signals of side chains in β-*O*-4' lignin, especially those where the second unit is a G unit, also decreased. In the aromatic region of the spectrum (Fig. 3d), the most noticeable effect of alkaline peroxide extraction was the decrease in FA. Interestingly, the hemicellulose was not deacetylated (unchanged X₁, X₂, and X₃ signals) in agreement to the mild conditions of the alkaline extraction used.

Finally, interesting results were obtained when lower laccase and HBT doses were assayed, always followed by an alkaline peroxide extraction, including the following: (i) reduced mediator dose (to only 25 % of the initial) (Fig. 4b, d) and (ii) reduced both laccase (to 20 % of the initial) and mediator dose (to 50 % of the initial) (Fig. 4a, c). These two treatments seem to be very efficient in degrading/removing

lignin (including β-*O*-4' side chains and lignin units) similarly to the treatment with higher mediator doses, although the latter showed higher efficacy toward H lignin units, PCA, and triclin. In the three treatments, a preferential degradation of G units with respect to S ones was observed (Table 4). The increase in S/G ratio with the treatment was also revealed in previous studies dealing with laccase-mediator pretreatments of other lignocellulosic feedstocks [19, 37, 38]. On the other hand, in spite of similar lignin removal with both lower doses (Table 1), a higher decrease in S and G lignin units is shown by 2D NMR for the former one that is also correlated with a higher saccharification yield.

Conclusions

Wheat straw can be delignified by a basidiomycete laccase in the presence of HBT, directly on the ground lignocellulosic material (i.e., without a previous chemical pretreatment)

attaining a lignin removal up to 37 %. The delignification can be improved up to 48 % if a subsequent alkaline peroxide extraction step is combined with the enzymatic treatment. Although these lignin removal values were attained with high mediator doses, noteworthy was the lignin reduction reached (up to 38 %) in lower-dose treatments, which would be more industrially attractive. In all cases assayed, the pretreated wheat straw was hydrolyzed with higher efficiency than the untreated material releasing higher yields of glucose and xylose by using relatively low doses of cellulases. The 2D NMR spectra of whole straw samples (at the gel stage) showed the selective action of laccase-mediator on the lignin moiety, while the polysaccharide signals remained unchanged with respect to the controls. This included the breakdown of inter-unit linkages and removal of lignin S and G units, although triclin remained in the residual lignin. The presence of oxidized S and G lignin units in the pretreated wheat straw provides evidence for a C_α-oxidation mechanism (and β-O-4' cleavage) of lignin degradation even in the treatments with laccase alone. It should be pointed out that although promising results have been obtained in the present work with the laccase-mediator pretreatment of wheat straw, further optimization of the treatment including the increase in solid loading and the decrease of the laccase-mediator doses should be necessary to approach industrial conditions.

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