ACS Sustainable Chemistry & Engineering

Research Article

pubs.acs.org/journal/ascecg

Delignification and Saccharification Enhancement of Sugarcane Byproducts by a Laccase-Based Pretreatment

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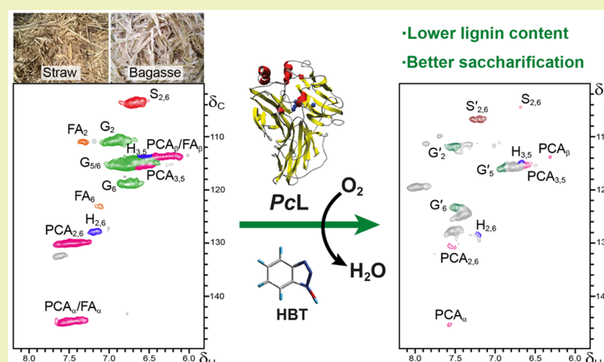

ABSTRACT: Sugarcane bagasse and straw, two major agro-industrial byproducts generated by the sugarcane industry, contain significant amounts of carbohydrates that can be hydrolyzed and then converted into ethanol or other valuable compounds. However, access to them is limited by the presence of lignin, a recalcitrant polymer that protects cell-wall polysaccharides from enzymatic hydrolysis. This work demonstrates the ability of an enzymatic pretreatment, based on the laccase from *Pycnoporus cinnabarinus*, and 1-hydroxybenzotriazole as mediator, to remove and/or modify lignin in sugarcane bagasse and straw residues, improving their subsequent saccharification. Up to 27% and 31% decreases of relative lignin content in ground sugarcane bagasse and straw, respectively, were achieved by the laccase-mediator pretreatment followed by alkaline peroxide extraction. Moreover, the lignin removal directly correlated with improvements in enzymatic saccharification, increasing glucose releases by around 39% and 46% for bagasse and straw, respectively, compared with those of the corresponding controls. Lignin depolymerization and degradation were made evident in the 2D-NMR spectra by a significant reduction in the number of aliphatic side chains involved in the main *β-O*-4′ and *β*-5′ interunit linkages, together with a remarkable removal of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units as well as the associated *p*-coumarates and ferulates, with respect to polysaccharides.

KEYWORDS: *Sugarcane residues, Bioethanol, Lignocellulose, Lignin removal, Oxidoreductases, Hydrolysis, 2D-NMR*

■ INTRODUCTION

In recent years, there has been an emerging effort to increase the production of biofuels from renewable feedstocks as a consequence of growing concerns about the depletion of fossil fuel reserves and global warming from the use of nonrenewable resources. In this context, lignocellulosic biomass represents a sustainable alternative platform to fossil resources since it is the most abundant and renewable biomass on Earth. Second-generation bioethanol, obtained after hydrolysis and fermentation of carbohydrates present in lignocellulosic materials, represents a real alternative to reduce the fossil fuel demand. Among the different lignocellulosic biomasses, agro-industrial lignocellulosic residues, such as sugarcane bagasse and straw, are attractive feedstocks for bioethanol production because of their high carbohydrate contents.[1] Sugarcane bagasse and straw are generated in significant amounts by the sugarcane industry,[2] around 125−140 kg[3] and 85−115 kg (dry weight),[4] respectively, per metric ton of sugarcane, and are readily available at low cost.

Carbohydrates are found in lignocellulosic materials as cellulose and hemicelluloses, two major structural polymers of the plant cell-wall together with lignin. The carbohydrate fraction, especially cellulose, can be converted into fermentable reducing sugars by enzymatic hydrolysis or chemical methods.[5] The hydrolysis is usually carried out by cellulolytic enzymes,[6] and the fermentation is accomplished by using yeasts[7] or bacteria.[8] However, the accessibility of the hydrolytic enzymes to the carbohydrates in the lignocellulosic materials is hampered, to a certain extent, by the presence of lignin. Lignin is an amorphous, cross-linked, and complex aromatic polymer consisting of mainly three different phenyl-propane units linked together by different ether and carbon−carbon bonds.[9] Lignin acts as a biological cement contributing to the formation of a highly recalcitrant lignocellulosic matrix. Lignin has been shown to have a negative effect on the enzymatic hydrolysis of cell-wall polysaccharides because it strongly reduces the access of enzymes[10] and also binds to them thus reducing their activity.[11] Hence, a biomass pretreatment step is needed to partially remove lignin and break down the lignocellulose structure, making cellulose more accessible during subsequent saccharification. Given the potential of sugarcane residues for the production of second-generation bioethanol, many pretreatment processes have been developed to reduce their recalcitrance to enzymatic hydrolysis.[12] These include, among others, steam explosion,[13] organosolv,[14] liquid hot water,[15]

Received: April 28, 2017
Revised: June 8, 2017
Published: July 4, 2017

ACS Publications © 2017 American Chemical Society

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

ammonia fiber explosion,[16] wet oxidation,[17] alkali delignification, and dilute acid hydrolysis.[18]

Biotechnology based on lignin-degrading microbes and their enzymes can contribute to the efficient and ecofriendly use of lignocellulosic feedstocks for the sustainable production of bioethanol.[19] In nature, efficient and selective lignin biodegradation is mediated mainly by white-rot fungi and certain bacteria.[20,21] The ligninolytic enzymes involved in this process are classified as peroxidases (lignin, manganese, and versatile peroxidases) and laccases.[20] Among these, laccases (phenoloxidases, EC 1.10.3.2) seem to be the most suitable enzymes for industrial applications as they can be produced on a large scale,[22] have broad substrate specificity, and utilize atmospheric oxygen as an electron acceptor to produce water. Several laccases have been shown to be capable of degrading different types of lignin, including natural[23−26] and synthetic (dehydrogenation polymer, DHP) lignins.[27] They oxidize either the minor phenolic components of lignin (less than 20%) directly (by themselves) or the phenolic and nonphenolic components, in the presence of a proper redox mediator, indirectly. As a result, radicals are generated in lignin, which can lead to bond cleavage and, consequently, to lignin depolymerization.

However, little is known about the application of biological pretreatments on sugarcane residues, and almost all of them are limited to the use of lignin-degrading fungi.[28−30] These fungal pretreatments present certain advantages over the thermochemical ones, such as mild reaction conditions, higher product yields, and less energy demand; nevertheless, they require a long incubation period. This drawback can be overcome by directly using the oxidoreductase enzymes (laccases and/or peroxidases) secreted by these microorganisms for lignin degradation. The enzymatic treatments, in contrast to those that are fungal-based, require the use of previously milled lignocellulosic materials, to increase the contact surface between enzyme and substrate, although substrate sterilization is not necessary.

Several studies have demonstrated the potential of the laccase-mediator system as an efficient pretreatment to remove lignin from both woody and nonwoody plant feedstocks to enhance their subsequent saccharification.[23−26] In the present study, a laccase-mediator system composed of the high-redox-potential laccase from the basidiomycete *Pycnoporus cinnabarinus* and 1-hydroxybenzotriazole (HBT) as mediator was investigated as a pretreatment to remove and/or modify the lignins in the sugarcane bagasse and straw residues for improved saccharification. *P. cinnabarinus* is one of the fungi selected for the production of high-redox-potential laccases for applications in lignocellulose biorefineries and other industrial processes.[31,32] The secretion rate, over 1.5 g L^{-1} enzyme (in cultures containing 5 g L^{-1} fungal biomass), is among the highest reported for a natural laccase producing fungus, and similar levels have been attained during laccase heterologous expression. Interestingly, under optimized production conditions, laccase is by far the most abundant extracellular oxidoreductase secreted by *P. cinnabarinus*, and therefore, crude culture filtrates could be used for lignocellulose pretreatment, without enzyme purification, as suggested for other applications.[33]

■ EXPERIMENTAL SECTION

Lignocellulosic Feedstocks, Enzyme, and Mediator. Sugarcane bagasse and straw were supplied by a midsized ethanol mill located in Minas Gerais state, Brazil. Sugarcane plants were harvested at the age of 6−8 months from high-performance sugarcane (*Saccharum* sp. hybrids) plantations. The sugarcane plants were manually collected and cleaned in the field where about one third of their weight was removed in the form of tops and leaves; this material is known as sugarcane straw or trash by the sugarcane industry. Bagasse is the solid byproduct that remains after sugarcane stalks are crushed to extract the juice. Sugarcane bagasse and straw samples were air-dried and ground using an IKA knife mill and then finely milled using a Retsch PM100 mill at 400 rpm for 6 h (with 10 min breaks after every 10 min of milling).

A fungal laccase from the basidiomycete *P. cinnabarinus*, provided by INRA (Marseille, France), was used in this study. Its activity, measured as the initial rate during oxidation of 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS (Roche Diagnostics, Mannheim, Germany), to its cation radical (ε_{436} 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate (pH 5) at 24 °C, was 525 U mL^{-1}; the activity unit (U) was defined as the amount of enzyme oxidizing 1 μmol of ABTS per min. HBT from Sigma-Aldrich (Steinheim, Germany) was used as the redox mediator.

Laccase-Mediator Treatments. The enzymatic pretreatment used to delignify the sugarcane bagasse and straw residues consisted of a sequence of four laccase-mediator treatments, using the *P. cinnabarinus* laccase and HBT as redox mediator, each one followed by an alkaline peroxide extraction step. These pretreatment conditions were found to efficiently remove lignin from other woody (eucalyptus) and nonwoody (elephant grass) feedstocks with important improvements in their subsequent saccharification.[23] The treatments were carried out in 200 mL pressurized bioreactors (Labomat, Mathis) placed inside a shaker bath, at 170 rpm and 50 °C, using 2 g (dry weight) of the whole biomass at 6% solid loading (w:w) in 50 mM sodium tartrate buffer (pH 4) under O_2 atmosphere (2 bar) for 24 h. After the enzymatic treatment, the samples were filtered and washed with 1 L of water. In a subsequent step, samples at 6% solid loading (w:w) were submitted to a peroxide alkaline extraction using 1% (w:w) NaOH and 3% (w:w) H_2O_2 (also referred to dry weight) at 80 °C during 90 min, followed by water washing.[25] Treatments with laccase (50 U g^{-1}) alone (without the mediator) and controls without the laccase or mediator, were also performed (followed in both cases by the corresponding alkaline peroxide extractions) for comparison. Duplicate experiments of these treatments (including control, laccase alone, and laccase-HBT) were performed to estimate the variability in biological replicates. A statistical analysis, based on ANOVA (one-way analysis of variance) and the Tukey HSD test, was carried out to compare the effects of the different enzymatic treatments on the lignin removal and on the release of glucose. Lignin content was determined as Klason lignin, in triplicate assays, according to Tappi Method T222 om-88.[34] The data from both biological and technical replicates were averaged.

Saccharification of Treated Sugarcane Bagasse and Straw. The enzymatically treated sugarcane samples were hydrolyzed with a cocktail containing commercial enzymes (from Novozymes, Bagsvaerd) with cellulase [Celluclast 1.5 L; 2 FPU g^{-1} (FPU, filter-paper unit)] and β-glucosidase (Novozym 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 shaker bath at 140 rpm. The released glucose was determined as alditol acetate by gas chromatography (GC).[35] 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), using a capillary column Agilent J&W DB-225 (30 m × 0.25 mm i.d. and 0.15 μm film thickness) and helium as the carrier gas. The oven was temperature-programmed from 220 (held for 5 min) to 230 (held for 5 min) °C at 2 °C min^{-1}. Peaks were quantified by area, and glucose was used as a standard to elaborate calibration curves. The data from three replicates were averaged.

2D-NMR Spectroscopy. For gel-state NMR sample preparation, ~70 mg of ball-milled treated sugarcane bagasse and straw samples were transferred into 5 mm NMR tubes, and swelled in 0.75 mL of deuterated dimethyl sulfoxide (DMSO-d_6), forming a gel inside the

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

NMR tube.[36,37] Heteronuclear single quantum coherence (HSQC) 2D-NMR experiments were run at 298 K on a Bruker AVANCE III 500 MHz spectrometer fitted with a 5 mm TCI (triple cryoprobe inverse) probe. The 2D-HSQC spectra were acquired using an adiabatic pulse sequence (Bruker standard pulse program "hsqcetgpsisp.2"), which enabled a semiquantitative analysis of the different $^{13}C-^{1}H$ correlation signals.[38] Spectra were acquired from 10 to 0 ppm 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 in F1 (^{13}C) using 256 increments of 32 scans, for a total acquisition time of 2 h 34 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typically matched Gaussian apodization in ^{1}H (parameters LB = −0.1 and GB = 0.001) and a squared cosine bell in ^{13}C (LB = 0.3 and GB = 0.1). The central residual DMSO peak (δ_C/δ_H, 39.5/2.49) was used as an internal reference. The $^{13}C-^{1}H$ correlation signals from the aromatic/unsaturated region of the spectrum were used to estimate the content of lignin, *p*-coumarate, and ferulate, and the lignin composition in terms of H, G, S, and oxidized S (S′) and G (G′) units. The amorphous polysaccharide content was estimated by the integration of xylose and glucose anomeric signals, whereas the correlation signals in the aliphatic oxygenated region were used to determine the interunit linkage and end-unit abundances in lignin.[26]

RESULTS AND DISCUSSION

In this work, we evaluate for the first time the ability of a laccase-based enzymatic pretreatment to successfully delignify and improve the saccharification of nonchemically pretreated sugarcane residues (bagasse and straw), which present rather different lignin compositions. Bagasse is enriched in S-lignin units and uncondensed *β*-ether linkages, whereas straw is enriched in G-lignin units and presents more condensed linkages.[1] Furthermore, the structural modifications produced in the lignin polymer of these sugarcane byproducts during the enzymatic treatment were analyzed in situ by two-dimensional nuclear magnetic resonance (2D-NMR) at the gel state,[36,37] which provided useful information regarding the lignin degradation mechanism.

Delignification of Sugarcane Bagasse and Straw with Laccase-HBT. The lignin contents (as Klason lignin) of the sugarcane bagasse and straw samples after the laccase-mediator pretreatment were determined and compared with their corresponding controls (Table 1). The lignin contents in the control samples that were processed as the full enzymatic treatment but without the presence of laccases and mediators were not modified with respect to the initial sugarcane residues. The treatments with laccase alone only decreased the lignin content of bagasse by about 4% (relative to the control), whereas a more pronounced decrease of up to 9% of the lignin content was attained in the case of straw. This low extent of degradation with laccase alone is in agreement with previous works using other lignocellulosic materials.[23−26] It is known that laccases alone are not very efficient for degrading the lignin in lignocellulosic materials because of the steric hindrance caused by their bulky molecular size and also because of their relatively low oxidation potentials, which only allow them to oxidize the minor phenolic hydroxyl groups present in lignin. However, it is important to highlight the higher extent of lignin degradation in the straw (nearly 2-fold higher), although straw only has a slightly lower lignin content than bagasse. This fact seems to suggest that the lignin composition of these materials, which is different in bagasse (S-rich lignin) and straw (G-rich lignin),[1] plays a major role in the lignin degradation with laccase alone. Previous works have shown that G-lignin units are present as phenolic units in a higher extent than the S-lignin units, which are mostly etherified,[39,40] and this would explain

Table 1. Percentage of the Initial Material Recovered, Lignin Content, Glucose Released by Enzymatic Hydrolysis, and in Vitro Digestibility of Sugarcane Residue Samples[a]

sample	recovery (%)	lignin (%)[b]	glucose (%)[b]	digestibility (%)[c]
		Sugarcane Bagasse		
initial sugarcane bagasse		17.8 ± 0.6	35.9 ± 0.7	71.7 ± 1.1
control[d]	88.5	17.5 ± 0.4a	40.1 ± 0.2a	70.9 ± 0.3a
laccase (50 U g^{-1})[d]	80.5	16.8 ± 0.3a	44.2 ± 0.4b	71.3 ± 0.3a
laccase (50 U g^{-1}), HBT (3%)[d]	74.5	12.8 ± 0.3b	55.8 ± 0.4c	82.9 ± 0.6b
		Sugarcane Straw		
initial sugarcane straw		17.0 ± 0.2	34.9 ± 0.2	75.4 ± 0.4
control[d]	90.5	16.6 ± 0.2a	39.2 ± 0.2a	76.5 ± 0.4a
laccase (50 U g^{-1})[d]	82.0	15.1 ± 0.1b	42.3 ± 0.1b	75.0 ± 0.2a
laccase (50 U g^{-1}), HBT (3%)[d]	75.5	11.5 ± 0.3c	57.1 ± 0.3c	93.0 ± 0.5b

[a]Means ± SD (standard deviation) shown were obtained from technical triplicates. Letters next to the SD, from the Tukey test, show results not significantly different from the control (a), significantly different from the control (b) and significantly different from both the control and the laccase-alone results, at the 0.05 level. [b]Values for the lignin content (expressed as Klason lignin) and glucose released after cellulase hydrolysis of samples treated with *P. cinnabarinus* laccase (50 U g^{-1}) and HBT (3%), followed by an alkaline peroxide extraction (Ep), are compared with values for a control without enzyme, a treatment only with laccase, and the initial sugarcane bagasse and straw samples. [c]Digestibility values were calculated on the basis of the total glucan content in the initial sugarcane residues and the percentages of released glucose, with consideration in each case for the percentage of recovery. [d]Enzymatic/Ep pretreated (4 cycles).

their higher degradation extent with laccases alone. However, the lignin content in both lignocellulosic residues significantly decreased after the enzymatic treatment using the laccase-HBT system. For sugarcane bagasse, the lignin reduction was about 27% of the initial lignin content whereas in the case of sugarcane straw up to 31% of lignin removal was achieved. Similar delignification degrees (ca. 32%) were obtained with elephant grass using the same enzymatic conditions, although much higher delignification degrees (nearly 50% lignin removal) could be attained with eucalyptus wood.[23] The differences in the delignification extents of elephant grass and those of eucalyptus wood were attributed to the predominance of syringyl lignin units in the latter.[23] Nevertheless, this seems not to be the case for the sugarcane residues selected for this study since sugarcane bagasse is enriched in S-lignin units[1] but presented a slightly lower delignification extent than the straw, which is enriched in G-lignin units[1] and presented a slightly higher delignification extent. These data indicate that, in the pretreatment of sugarcane residues, other structural features of the lignin polymer beyond its composition (H:G:S ratio) have to be considered to explain the obtained delignification results. In this sense, a possible reason could be related to the presence of *p*-coumarates acylating the *γ*-OH of the lignin side chains, which could hamper in some way the action of laccases. This fact would explain why sugarcane straw, with a percentage of *p*-coumarates of 47% (referred to lignin), is delignified to a greater extent than sugarcane bagasse, which has a higher content of *p*-coumarates (77%). This hypothesis would also

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

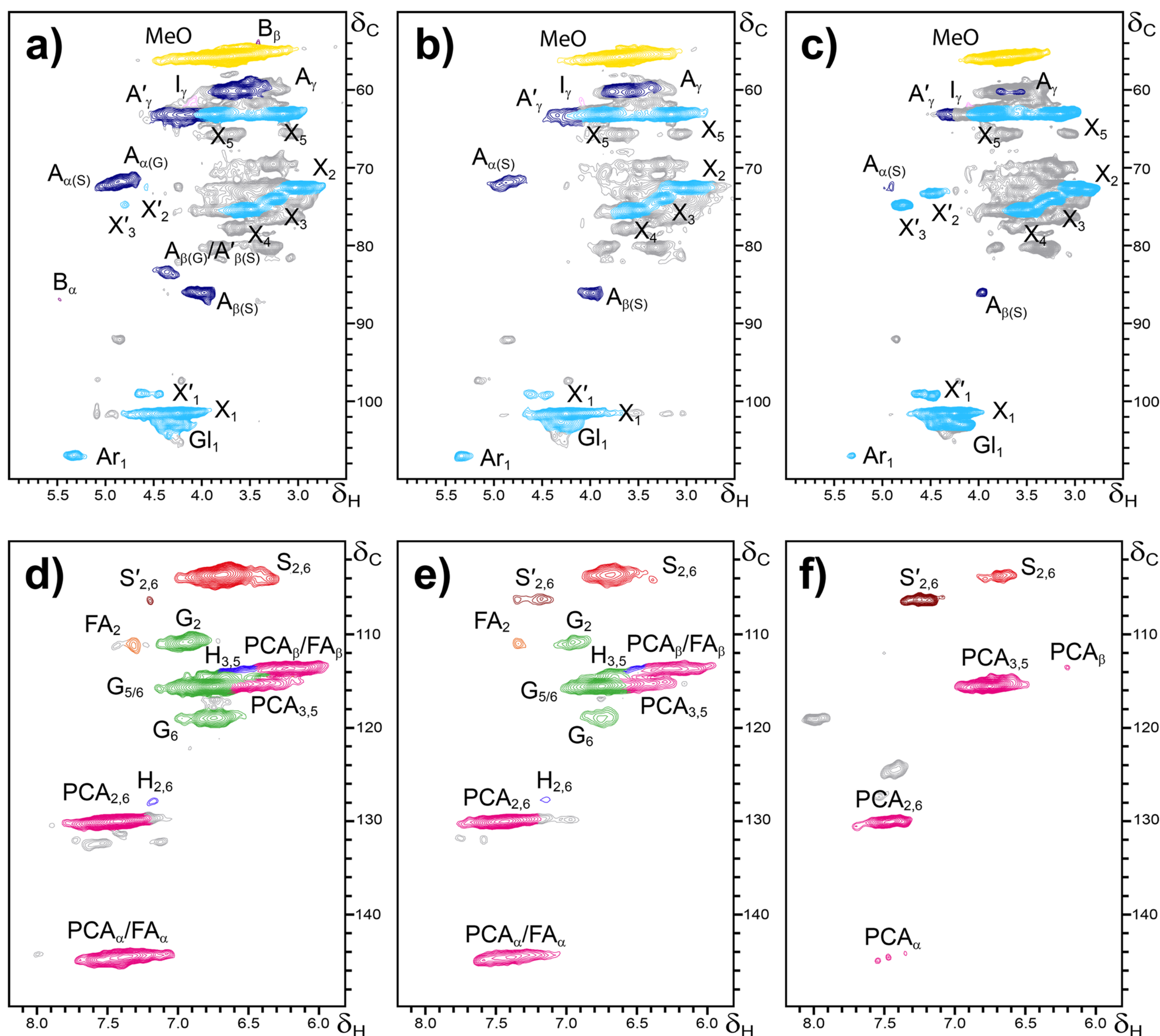

Figure 1. HSQC NMR spectra of sugarcane bagasse after laccase-mediator treatment and a subsequent alkaline peroxide extraction (4 cycles). Expanded aliphatic oxygenated (δ_H/δ_C, 2.5−6.0 and 50−110 ppm; top) and aromatic (δ_H/δ_C, 5.8−8.2 and 90−150 ppm; bottom) regions of the HSQC NMR spectra of sugarcane bagasse treated with *P. cinnabarinus* laccase-HBT: (a, d) control without enzyme, (b, e) 50 U g^{-1} enzyme, and (c, f) 50 U g^{-1} enzyme and 3% HBT. See Table 2 for lignin signal assignments, Figure 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 (X'_1−X'_5) xylan units (anomeric glucose and arabinose signals were also identified: G_1 and Ar_1). Unassigned signals are in gray, including signals from the enzyme and the mediator.

explain that the lignin removal attained after pretreatment of elephant grass (32%)[23] was close to those achieved in the case of sugarcane bagasse and straw pretreatment since elephant grass lignin is also γ-*p*-coumaroylated (∼40%).[41] Finally, in the case of eucalypt wood, whose lignin is not acylated at the γ-OH of the side chains,[42] the pretreatment should be more effective, and this would explain why the attained lignin removal was nearly 50%.

Enzymatic Hydrolysis of Sugarcane Bagasse and Straw Pretreated with Laccase-HBT. The sugarcane bagasse and straw samples treated with laccase-HBT and those treated with laccase alone (without mediator), as well as the control samples, were hydrolyzed (72 h) using low doses of cellulases (2 FPU g^{-1}) and β-glucosidase (6 U g^{-1}),[23] and the released glucose was quantified as alditol acetate by GC. The saccharification results (Table 1) indicate a direct correlation between the lignin removal and the increase in glucose yield obtained by enzymatic hydrolysis. After the treatment with laccase-HBT, relative glucose releases improved up to 39% and 46%, for bagasse and straw, respectively, with respect to the control samples. Interestingly, enzymatic treatments with laccase alone also showed a positive effect in the subsequent enzymatic saccharification, with improvements in glucose release around 10% for both bagasse and straw. This positive effect of the laccase alone has also been observed during the enzymatic pretreatment of wheat straw,[26] and is attributed to both the delignification of the phenolic lignin moiety and the reduction of the nonspecific bindings of cellulases to lignin,

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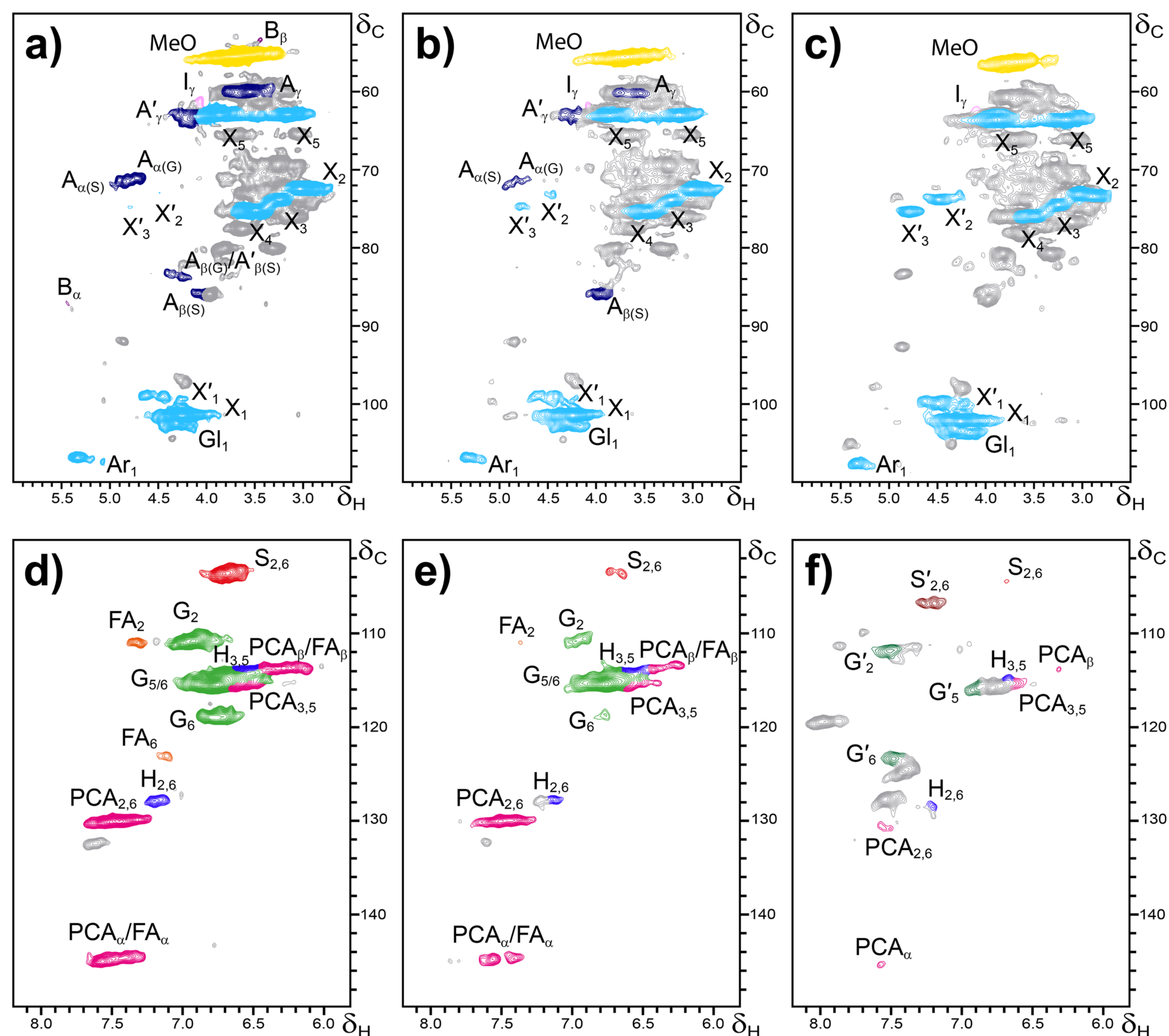

Figure 2. HSQC NMR spectra of sugarcane straw after laccase-mediator treatment and a subsequent alkaline peroxide extraction (4 cycles). Expanded aliphatic oxygenated (top) and aromatic (bottom) regions of the HSQC NMR spectra of sugarcane straw samples after treatment with laccase-HBT followed by an alkaline peroxide extraction: (a, d) control without enzyme, (b, e) 50 U g^{-1} enzyme, and (c, f) 50 U g^{-1} enzyme and 3% HBT. See the Figure 1 legend for additional information.

which make them inactive.[43] Moreover, a treatment with laccase alone could also modify the lignocellulosic substrate surface by increasing the amounts of carboxylic acids, reducing in this way the nonspecific adsorption of negatively charged cellulases.[44] A possibility for the industrial implementation and environmental feasibility of the laccase pretreatments would be the use of natural phenolic mediators instead of synthetic mediators,[45] as already proposed for paper pulp delignification.[46]

2D-NMR Analysis of Sugarcane Bagasse and Straw after Laccase-HBT Pretreatment. The main structural modifications produced in sugarcane bagasse and straw by the laccase-mediator treatment were studied by 2D-NMR of whole cell-walls at the gel state, according to the previously developed methodology.[36,37] This technique allows for the in situ structural characterization of lignins, without the need of prior lignin isolation that sometimes results in alterations of the lignin polymer structure, and carbohydrates in the cell-wall. Finely ball-milled samples were swelled in DMSO-d_6 to form a gel inside the NMR tube and then were analyzed by HSQC 2D-NMR experiments.[36,37] The spectra of enzymatically pretreated (with laccase-HBT and laccase alone) sugarcane bagasse and straw samples (including the respective control samples) are shown in Figures 1 and 2. The aliphatic oxygenated region of the spectra (δ_H/δ_C, 2.5−6.0 and 50−110) shows correlation signals from lignin (side chains and methoxyl groups) and carbohydrates (mainly from hemicelluloses and noncrystalline cellulose), whereas the aromatic/unsaturated region of the spectra (δ_H/δ_C, 5.8−8.2 and 90−150) includes signals from lignin units (H, G, S) as well as signals from the associated *p*-coumarates and ferulates. The lignin and carbohydrate cross-signals assigned in the HSQC spectra are listed in Table 2, and the main lignin units and substructures are depicted in Figure 3. The structural characteristics of the

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

Table 2. Assignments of $^{13}C/^{1}H$ Correlation Signals in the 2D-HSQC Spectra from the Whole Cell-Walls of Treated Sugarcane Bagasse and Straw

label	δ_C/δ_H (ppm)	assignment
	Lignin Signals	
B_β	53.4/3.45	C_β/H_β in phenylcoumaran (B)
OCH_3	55.5/3.72	C/H in methoxyls
A_γ	59.6/3.37 and 3.71	C_γ/H_γ in γ-hydroxylated β-*O*-4′ (A)
I_γ	61.3/4.07	C_γ/H_γ in cinnamyl alcohol end-groups (I)
A'_γ	62.9/4.16 and 4.35	C_γ/H_γ in γ-acylated β-*O*-4′ (A′)
$A_{\alpha(G)}$	71.1/4.71	C_α/H_α in β-*O*-4′ (A) linked to a G unit
$A_{\alpha(S)}$	71.5/4.81	C_α/H_α in β-*O*-4′ (A) linked to an S unit
$A'_{\beta(S)}$	83.0/4.33	C_β/H_β in γ-acylated β-*O*-4′ linked (A′) to an S unit
$A_{\beta(G)}$	83.5/4.38	C_β/H_β in β-*O*-4′ linked (A) to a G unit
$A_{\beta(S)}$	85.9/4.09	C_β/H_β in β-*O*-4′ linked (A) to an S unit
B_α	87.0/5.48	C_α/H_α in phenylcoumaran (B)
$S_{2,6}$	103.8/6.69	C_2/H_2 and C_6/H_6 in etherified S units
$S'_{2,6}$	106.2/7.28 and 106.3/7.16	C_2/H_2 and C_6/H_6 in α-oxidized S′ units
G_2	110.7/6.96	C_2/H_2 in G units
FA_2	110.9/7.33	C_2/H_2 in ferulate
G'_2	111.4/7.50	C_2/H_2 in α-oxidized G′ units
PCA_β and FA_β	113.5/6.29	C_β/H_β in *p*-coumarate and ferulate
$H_{3,5}$	113.6/6.64	C_3/H_3 and C_5/H_5 in H units
G_5/G_6	114.9/6.78 and 6.94 118.8/6.77	C_5/H_5 and C_6/H_6 in G units
G'_5	115.0/6.73	C_5/H_5 in α-oxidized G′ units
$PCA_{3,5}$	115.3/6.76	C_3/H_3 and C_5/H_5 in *p*-coumarate
FA_6	123.2/7.12	C_6/H_6 in ferulate
$H_{2,6}$	127.8/7.19	C_2/H_2 and C_6/H_6 in H units
$PCA_{2,6}$	129.9/7.47	C_2/H_2 and C_6/H_6 in *p*-coumarate
PCA_α and FA_α	144.7/7.53	C_α/H_α in *p*-coumarate and ferulate
	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
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
Ar_1	107.0/5.33	C_1/H_1 in arabinofuranose units

lignins from the enzymatically treated sugarcane bagasse and straw, including the relative abundances of the different interunit linkages and cinnamyl end-groups, and the molar abundances of the lignin units (H, G, S, G′ and S′), *p*-coumarates, and ferulates, estimated from the volume integration of the signals in the HSQC spectra, are indicated in Table 3. In addition, the use of an adiabatic pulse sequence in the 2D-HSQC experiments allowed the estimation of the number of side chains per aromatic lignin unit, a parameter that provides valuable information regarding the lignin depolymerization.

Structural Modification of Sugarcane Bagasse. The expanded aliphatic oxygenated and aromatic/unsaturated regions of the HSQC spectra from the sugarcane bagasse samples are shown in Figure 1. The aliphatic oxygenated region of the spectrum of the control sample (Figure 1a) shows correlation signals of lignin and carbohydrates. Carbohydrate signals corresponded mainly to normal (nonacetylated, X) and acetylated (X′) xylans, whereas the most intense signals of lignin presented in this region corresponded to methoxyl groups (MeO) and β-*O*-4′ alkyl-aryl ether substructures (A), which represent up to 97% of all NMR-measurable (side chain) linkages in the control sample. Other signals corresponded to β-5′ phenylcoumaran substructures (B) and cinnamyl end-groups (I), but the latter were observed with low intensities in this region of the spectrum. The aromatic/unsaturated region of the HSQC spectra of the control sample (Figure 1d) shows that sugarcane bagasse has an S-rich lignin with an H/G/S molar composition of 1:37:61 (S/G ratio of 1.65). In addition, this lignin is associated with important amounts of *p*-coumarates and ferulates (77% and 6%, respectively, referring to the lignin content).

The HSQC spectrum of sugarcane bagasse after the enzymatic treatment with laccase alone (without mediator) is rather similar to that of the control sample, as expected from a lignin removal of only 4%, although the intensity of the signals of β-*O*-4′ alkyl-aryl ether substructures slightly decreased (Figure 1b). Likewise, the signals of the lignin units observed in the aromatic region (Figure 1e) showed a slight increase of Cα-oxidized S-lignin units ($S'_{2,6}$), confirming that laccase alone is able to oxidize the phenolic substructures presented in the lignin moiety, although only to a minor extent.

The HSQC spectrum of sugarcane bagasse treated with laccase and mediator (Figure 1c,f) revealed that the lignin polymer was largely degraded during the enzymatic pretreatment. While the correlation signals of carbohydrates remained mostly unchanged (with the exception of the signals X'_2 and X'_3 from acetylated xylans that increased probably because of the better mobility of these groups after removing lignin–carbohydrate linkages), most of the lignin correlation signals strongly decreased as compared to those of the control sample; hence, the signals for the β-*O*-4′ alkyl-aryl ether substructures (A), which were the most intense side-chain signals in the control sample, were barely detectable, and the β-5′ phenylcoumarans (B) were not detected. This is reflected in the semiquantitative NMR analysis that shows an important relative increase of sugar units from 71.4% (in the control sample) to 94.2% (after laccase-HBT treatment) (see Table 3). In addition, the aromatic region of the spectrum showed the complete removal of signals from H- and G-lignin units, as well as from ferulates, whereas only signals from S-lignin units and those from their associated *p*-coumarates, which are acylating the γ-OH,[1] remain after the laccase-mediator treatment. Similar results were also observed in the lignin of elephant grass after the enzymatic treatment with the same laccase-mediator system.[23] Furthermore, an important fraction of S units were Cα-oxidized as is evident from the relative increase in the $S'_{2,6}$ signals. The formation of oxidized lignin structures is in agreement with the nature of the lignin biodegradation process, described as an "enzymatic combustion".[47,48] Studies with nonphenolic lignin model compounds have reported that the laccase-HBT system first abstracts a hydrogen atom from the Cα position with the Cα-Cβ breakdown at a later stage.[49,50] The decrease of the total side chains (as per aromatic units), together with the increase of the minor cinnamyl end-groups, also confirms the depolymerization of the lignin.

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

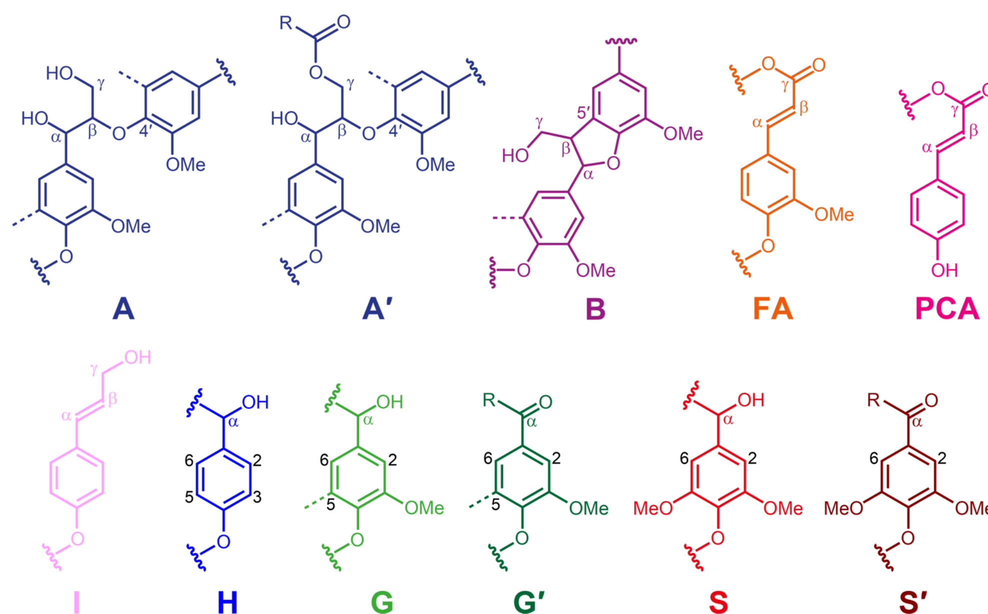

Figure 3. Main lignin units and substructures identified in the 2D-HSQC spectra of sugarcane bagasse and straw samples: (A) β-O-4′ alkyl-aryl ether structures, (A′) β-O-4′ structures with acylated γ-OH, (B) β-5′ phenylcoumaran structures, (FA) ferulate, (PCA) p-coumarate, (I) cinnamyl alcohol end-group, (H) p-hydroxyphenyl unit, (G) guaiacyl unit, (G′) Cα-oxidized G unit, (S) syringyl unit, and (S′) Cα-oxidized S unit. (R in G′ and S′ can be a hydroxyl in carboxylic acids, or a lignin side chain in ketones.)

Table 3. Semiquantitative NMR Analysis[a] of Sugarcane Bagasse and Straw Treated with Laccase (50 U g^{-1}) in Combination with HBT (3%) and Laccase Alone, Compared with Control Treatment (Without Enzyme)

	sugarcane bagasse			sugarcane straw		
	control	laccase	lac-HBT	control	laccase	lac-HBT
Sample Composition[b]						
syringyl lignin units (S)	9.6 (61)	5.0 (59)	1.5 (50)	3.2 (25)	0.9 (17)	0 (1)
Cα-oxidized S units (S′)	0.1 (1)	0.9 (11)	1.5 (50)	0 (0)	0.1 (1)	1.0 (40)
guaiacyl lignin units (G)	5.9 (37)	2.4 (29)	0 (0)	8.3 (66)	3.9 (71)	0 (0)
Cα-oxidized G units (G′)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.4 (52)
p-hydroxyphenyl units (H)	0.1 (1)	0.1 (1)	0 (0)	1.1 (9)	0.6 (10)	0.2 (7)
total lignin	15.7 (100)	8.4 (100)	3.0 (100)	12.6 (100)	5.4 (100)	2.6 (100)
p-coumarates (PCA)	12.0 (77)	8.4 (100)	2.8 (93)	5.9 (47)	4.1 (75)	0.2 (8)
ferulates (FA)	0.9 (6)	0.6 (8)	0 (0)	1.4 (11)	0.4 (8)	0 (0)
total aromatics	28.6	17.4	5.8	19.9	9.9	2.8
sugar units	71.4	82.6	94.2	80.1	90.1	97.2
total	100	100	100	100	100	100
lignin S/G ratio	1.66	2.43		0.38	0.31	0.79
Side Chains and End-Groups[c]						
β-O-4′ ethers (A + A′)	97 (64)	97 (58)	84 (32)	89 (56)	90 (50)	0
phenylcoumarans (B)	1 (1)	0 (0)	0	4 (2)	0 (0)	0
cinnamyl end-groups (I)	2 (1)	3 (2)	16 (6)	7 (5)	10 (6)	100 (10)
total	100 (66)	100 (60)	100 (38)	100 (63)	100 (56)	100 (10)

[a]Values in parentheses refer to total lignin. [b]Sample composition presents the molar amount of normal (H, G, and S) and Cα-oxidized (G′ and S′) lignin units, S/G ratio, p-coumarate (PCA), and ferulates (FA) on the basis of the integration of aromatic signals, and sugar units (mainly xylose and glucose) from the integration of anomeric carbon signals. [c]The percentages of lignin side chains involved in substructures A and B, and cinnamyl alcohol end-groups (I) are obtained from the integration of aliphatic signals in the HSQC spectra. Values in brackets refer to the total lignin units (H + G + G′+ S + S′).

Structural Modification of Sugarcane Straw. Contrary to that in sugarcane bagasse, the lignin in sugarcane straw is enriched in G-lignin units (H:G:S ratio of 9:66:25, S/G ratio of 0.38; for the control sample), and this is reflected in a different relative distribution of the main interunit linkages with respect to bagasse lignin (Table 3). Therefore, a different behavior toward laccase-mediator pretreatment might be expected.

The expanded aliphatic oxygenated and aromatic/unsaturated regions of the HSQC spectra from the sugarcane straw control and treated (with laccase alone and laccase-HBT) samples are shown in Figure 2. The aliphatic oxygenated region of the HSQC spectrum of the control sample showed correlation signals of both lignin and carbohydrates. As for what occurred in the bagasse spectrum, the main signals of carbohydrates corresponded to normal (nonacetylated; X_1, X_2,

DOI: 10.1021/acssuschemeng.7b01332
ACS Sustainable Chem. Eng. 2017, 5, 7145−7154

X_3, X_4, and X_5) and acetylated (X'_1 X'_2, and X'_3) xylans, as well as to arabinose (Ar_1) units. The most prominent signals of lignin present in this region corresponded to methoxyl groups (MeO) and β-*O*-4′ ethers (A), which represent up to 90% of all NMR-measurable linkages. β-5′ phenylcoumaran substructures (B), which require at least one guaiacyl unit to be formed, are also favored in G-rich lignin, and accounted for 4% of total linkages. The aromatic/unsaturated region of the HSQC spectrum of the control sample (Figure 2d) shows that sugarcane straw has a G-rich lignin, as mentioned above. Moreover, this lignin is associated with an important amount of *p*-coumarates and ferulates (47% and 11%, respectively, referring to the lignin content), as what also occurs in the bagasse lignin.

The enzymatic treatment of sugarcane straw with laccase alone produced a higher reduction in the intensity of the lignin signals, when compared to the same treatment on bagasse, in both the aliphatic oxigenated and the aromatic/unsaturated regions. The side-chain region of the spectrum (Figure 2b) showed a decrease of signals from β-*O*-4′ (A) and β-5′ phenylcoumaran (B) substructures, the latest of which completely disappeared from the spectrum. On the contrary, most of the carbohydrate signals remained unchanged, and even signals of acetylated xylan (X'_2 and X'_3) appeared, revealing that the cell-wall structure became altered in a way that some of the xylans increased their mobility. This relative increase of carbohydrates with respect to lignin is also reflected in the NMR semiquantitative analysis (Table 3), where carbohydrate anomeric signals increased from 80% (in the control sample) to 97% (after laccase-mediator treatment). In regards to the aromatic/unsaturated region of the spectrum (Figure 2e), the correlation signals of H-, G-, and S-lignin units, as well as the signals from *p*-coumarates and ferulates, clearly decreased with respect to the control, indicating a modification of the lignin structure by the action of the laccase alone. As mentioned above, laccases are able to degrade phenolic lignin, which is mostly related to G units,[32,33] and this would explain the higher overall lignin removal in the case of sugarcane straw.

The treatment of sugarcane straw with laccase-HBT produced considerable lignin removal (31% compared to the control sample, Table 1) that is reflected in the HSQC spectrum (Figure 2c,f). The aliphatic oxygenated region only showed signals from carbohydrates, which remained largely unchanged, while signals for lignin linkages completely disappeared from the spectrum. In the aromatic/unsaturated region of the spectrum, most of the lignin signals also disappeared, and only small signals from H- and S-lignin units, and from *p*-coumarates (acylating the γ-OH of S units), still remain. Signals for Cα-oxidized G- and S-lignin units (G′ and S′) now appeared in the spectrum, which were generated as a consequence of the oxidation produced by the laccase-mediator treatment. Interestingly, Cα-oxidized G units (G′), which were undetected in the spectrum of the control samples, were now easily observed, indicating the oxidation of the major G-lignin units present in sugarcane straw, as also observed in the laccase-HBT treatment of other G-enriched lignocellulosic materials, like wheat straw.[26]

CONCLUSIONS

Ground sugarcane bagasse and straw residues were partially delignified by the pretreatment with laccase-HBT, thus improving their subsequent enzymatic saccharification. The enzymatic pretreatment produced a rather similar lignin removal from both materials, despite their different lignin composition (H:G:S ratio), suggesting that other lignin structural features, as the presence of *p*-coumarates acylating the γ-OH of the side chains, can also play a role in the lignin removal process. The laccase-mediator treatment selectively acts on the lignin moiety, partially breaking down the interunit linkages and generating Cα-oxidized lignin units, whereas it leaves the carbohydrate fraction mostly unchanged, as indicated by 2D-NMR.

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Author Contributions

J.R. and A.P. contributed equally to this work. A.G. conceived the study and supervised the work. J.R. and A.P. carried out the experimental work. J.R. performed 2D NMR and analyzed the data. J.R., J.C.R., and A.G. wrote the manuscript. A.T.M. critically reviewed the manuscript with substantial contribution to its intellectual content. All authors read and approved the final manuscript.

Funding

This study has been funded by the Spanish projects AGL2014-53730-R and CTQ2014-60764-JIN (cofinanced by FEDER funds) and the EU-project INDOX (KBBE-2013-7-613549).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

A.P. thanks the Spanish MINECO for an FPI fellowship. Dr. Manuel Angulo is acknowledged for running the NMR experiments that were performed on a Bruker Avance III 500 MHz instrument from the NMR facilities of the General Research Services of the University of Seville (SGI CITIUS).

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