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Importance of Mediators for Lignin Degradation by Fungal Laccase

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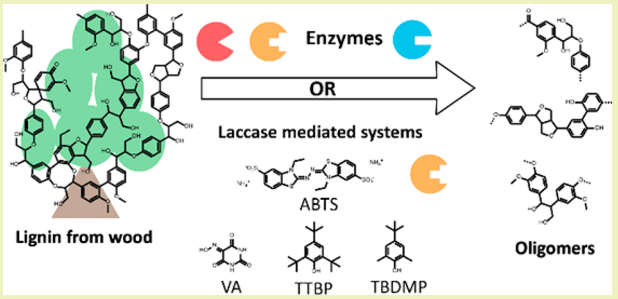
Supporting Information

ABSTRACT: Three of the major ligninolytic enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (LA), as well as the secretome of a white-rot fungi, *Grammothele fuligo*, are tested on three industrial lignins (organosolv, alkali, and Kraft), to investigate and study the differences in biodegradation reactions and mechanism of these three lignins. Strategies involving additives in laccase mediated systems were also considered to produce small phenolic compounds. Three new or underreported additives including 2,4,6-tri-*tert*-butylphenol (TTBP), 4-*tert*-butyl-2,6-dimethylphenol (TBDMP), and 3-hydroxyanthranilic acid (HAA) are compared to three classic laccase mediators violuric acid (VA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 1-hydroxybenzotriazole (1-HBT). Decrease of molecular weight by up to 73% could be obtained on organosolv lignin with LA−VA systems, and by 49%, 43%, and 39% when LA was used with ABTS, TBDMP, and 1-HBT, respectively. In-depth analysis of the degradation products by quantitative 2D HMQC NMR indicated that the oxidation is mediator-dependent and provides new insights on the enzymatic mechanism.



KEYWORDS: *Lignin, Enzymes, Mediator, Biomass, Depolymerization, Lignin peroxidase, Manganese peroxidase, Laccase, Degradation, Biodegradation*

INTRODUCTION

Lignin is the second most abundant renewable biopolymer, which, combined with cellulose, represents over 70% of the worldwide biomass. While cellulose has a well-defined market, lignin remains an industrial waste stream with few value-added applications. As a result, 98% of the world's lignin production is simply burned for energy.[1] As the most important source of phenols and aromatics in the biosphere, lignin has tremendous potential. Its depolymerization in small oligomers or phenolic molecules is critical for the implementation of biorefineries and to deliver new biobased materials. However, lignin recalcitrance has significantly hindered its valorization.

Lignin results from the enzymatic copolymerization of three phenolic monomers (monolignols), leading to a wide variety of chemical bonds and cross-linking either by stable carbon−carbon (*β*−*β*, *β*-1, 5-5, *β*-5) or more labile ether (*β*-*O*-4, 4-*O*-5, spirodienone, dibenzodioxocin) linkages (Figure 1). That heterogeneous complex structure has proved to be a significant obstacle to chemical depolymerization and has prevented upgrading lignin feedstock into fine phenolic chemicals. Some industrial processes exist but involve either extreme conditions of pH, temperature, or pressure or toxic chemicals.[2−4] Greener alternatives are required.

In nature, micro-organisms such as bacteria or wood-rot fungi have developed strategies to degrade and even catabolize the resulting aromatic compounds into carbon and energy.[5−7] These natural depolymerization processes usually combine internal and external enzymes. Different groups of enzymes involved in this process have been identified. The main group includes phenol-oxidases, which comprise lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (LA), and versatile peroxidase. However, it also has been demonstrated that some of these enzymes can oxidize nonphenolic moieties of lignin in certain conditions, a key behavior to drastically increase the biodeterioration of the entire lignin structure.[7−10] It is still unclear whether these ligninolytic enzymes can function independently or if mutual cooperation is required.

Microorganisms can degrade lignin down to small aromatic compounds or even smaller molecules.[11−13] Therefore, a combination of enzymes interacting cooperatively is necessary to cleave the various bonds of lignin. This suggests that the combination of multiple enzymes or their sequential use could improve depolymerization as compared to single enzyme biodegradation, to yield smaller aromatic molecules. A particular enzyme may selectively cleave a specific lignin bond, while another enzyme will cleave another bond. There

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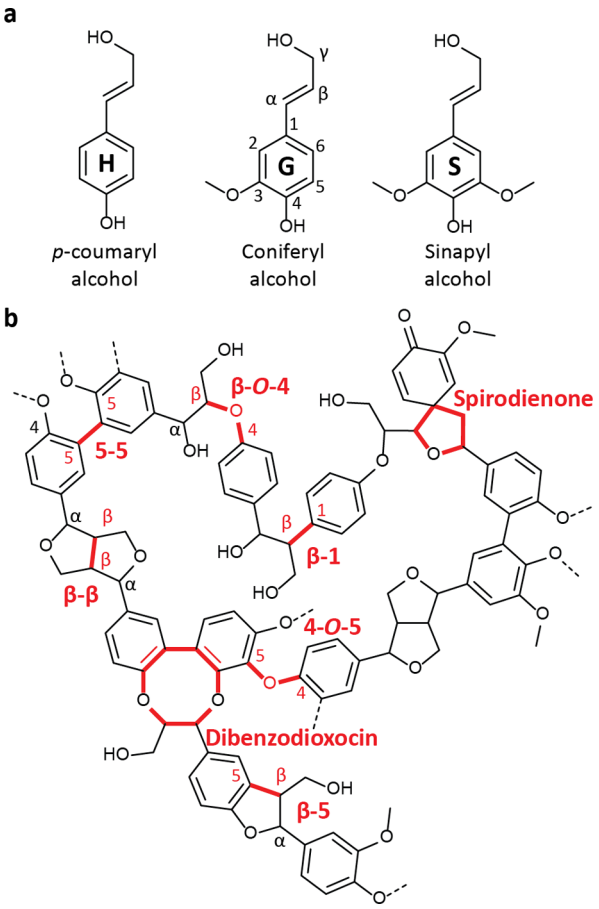

Figure 1. (a) Three constitutive monomers of lignin: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol; and (b) structure illustrating the principal bonds in lignin, a three-dimensional highly cross-linked biopolymer.

are a few studies in the literature describing enzymes preferentially degrading some types of bonds rather than others in lignin small model.[14−17]

While peroxidases can work alone in lignin oxidation, LA tends to polymerize lignin when by itself, especially in the case of plant laccases. In the presence of small aromatic molecules defined as "mediators", this phenomenon reverses, which improves lignin degradation.[18] A wide range of mediators, natural or synthetic, have been identified.[19] It was also reported that laccases from different microorganisms interact differently with mediators.[20] However, the mechanism remains identical for all strains, with LA using molecular oxygen to oxidize the mediator, which then acts as a chemical oxidant for lignin (Figure 2). It was hypothesized that mediators can diffuse into the intricate lignin structure, thanks to their small size. The presence of mediators also allows LA to oxidize the type of bonds that would otherwise remain untouched due to the size of laccase and selectivity.[18] The addition of mediators should further improve lignin depolymerization by cleaving some new bonds of the heterogeneous structure.

In this study, we select three of the major ligninolytic enzymes, two peroxidases and LA, as well as a white rot fungi external enzyme cocktail from *Grammothele fuligo*,[21] to compare the differences in lignin depolymerization. A set of three industrial lignins, Kraft, alkali, and organosolv, are used to represent the different structures and bonds in lignin. Finally, six mediators, including two new, are investigated for laccase mediated systems.

In this study, we aim at improving our understanding of the mechanism underlying the enzymatic degradation of lignin by monitoring changes in its structure by 2D $^{1}H-^{13}C$ quantitative heteronuclear multiple-quantum correlation (Q-HMQC) NMR analysis. Three new and three well reported mediators will be investigated to study the different interactions and mechanism involved in laccase mediated systems. Furthermore, the impact of lignin structure and composition on its enzymatic depolymerization will be determined to optimize a biocatalytic process for the production of small phenolic molecules from lignins. To the best of our knowledge, this is the first report comparing the effect of different types of mediators for lignin enzymatic degradation using different types of lignin and analyzing the depolymerized structures using Q-HMQC NMR to understand the oxidation reaction on the different bonds of lignin and the degradation mechanism.

EXPERIMENTAL SECTION

Materials. Enzymes: Lignin peroxidase (LiP) from *Phanerochaete chrysosporium* (EC: 1.11.14), manganese peroxidase (MnP) from *Nematoloma frowardii* (EC: 1.11.1.13), and laccase (LA) from *Cerrena unicolor* (EC: 1.10.3.2) were purchased from Jena Bioscience (Jena, Germany). Laccase from *Trametes versicolor* was purchased from Sigma-Aldrich (Sydney, NSW, Australia).

Chemicals and solvents: Chemicals were purchased from Sigma-Aldrich (Sydney, NSW, Australia) (hydrogen peroxide, manganese sulfate, citric acid monohydrate, sodium phosphate dibasic dehydrate, succinic acid, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate), violuric acid monohydrate, 1-hydroxybenzotriazole, KF, triton X, bovine serum albumine, potassium fluoride, 2,6-dimethoxyphenol (DMP), lithium bromide, sodium hydroxide, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP)), from Acros Organics (Geel, Belgium) (3-hydroxyanthranilic acid), and from TCI (Tokyo, Japan) (aluminum chloride, DMP, *tert*-butyl chloride, 2,4,6-tri-*tert*-butylphenol) and used as received. Reagent grade solvents (propanol, dimethylformamide, acetonitrile, hexane, chloroform) were purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia).

Fungus strain: *Grammothele fuligo* strain was obtained from UMR BBF (Biodiversité et Biotechnologie Fongiques, Marseille, France). The cultures were grown at 30 ± 1 °C during 7 days on potato dextrose agar (PDA) medium.

Lignins: Organosolv lignin from corn cob from *Stigmata maydis* was purchased from Chemical Point (Munich, Germany) and was washed with methanol before use to remove low molecular weight fraction. This precaution aimed at reducing dispersity of the lignin sample so that degradation detection by GPC analysis is facilitated: the pre-emptive removal of small molecular weight fraction allows the

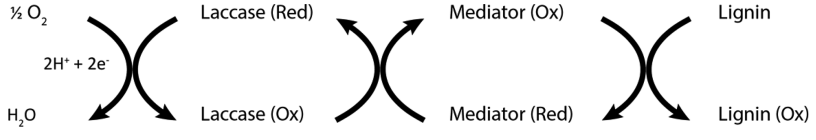

Figure 2. Catalytic cycle of laccase and mechanism of laccase mediated biodegradation of lignin.

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future detection of small oligomers to be identified as an indicator of bond cleavage and degradation of the studied sample. Lignin was set in methanol at 1 g/100 mL overnight under vigorous stirring. The resulting insoluble part was filtered and dried to yield about 20% high molecular weight lignin. Gel permeation chromatography analysis indicated M_n = 6000 and $Đ$ = 7.3. Alkali lignin was purchased from Sigma-Aldrich (Sydney, NSW, Australia) and was washed by the same process to remove low molecular weight. GPC analysis indicated M_n = 5600 and $Đ$ = 7.2. Kraft lignin was isolated through the process described later from black liquor provided by the Visy paper mill from Tumut, NSW, Australia, which mainly uses *Pinus radiata* (softwood). After isolation, the lignin is washed with methanol to yield some Kraft lignin with M_n = 6000 and $Đ$ = 7.2. ^{31}P NMR analysis has been performed on the lignins to determine the aliphatic hydroxyl, phenolic, and carboxylic acid content. Results can be found in Figure S1 and Table S1.

Methods. Buffers: Each enzyme was associated with its best buffer solvent to improve activity as follows: LiP in 300 mM citric acid 400 mM sodium phosphate dibasic adjusted to pH = 4.5, MnP in 500 mM succinic acid adjusted to pH = 4.5, and LA in 300 mM citric acid 400 mM sodium phosphate dibasic adjusted to pH = 5. Experiments involving several enzymes used the latter buffer.

Isolation of lignin from black liquor: Kraft lignin was isolated from black liquor according to the following procedure. Water was added to dilute the black liquor to 12 w/w% solid content to reduce viscosity. Under stirring, hydrochloric acid was added to acidify the solution to pH around 1. The resulting solid was filtered out, washed/rinsed with deionized water, and then dried overnight at 40 °C under vacuum. The dried lignin was dissolved in 3 w/w% sodium hydroxide solution to a total concentration of 12 w/w% lignin. The solution was heated at 100 °C under reflux for an hour to further break down, dissolve, and remove remaining hemicellulose from the lignin sample. After being cooled, the solution was once again acidified, filtered, rinsed with water, and dried overnight to isolate lignin. Elemental analysis of carbon, hydrogen, nitrogen, and sulfur gave the following result (average over three measurements): carbon 65.30%, hydrogen 6.07%, nitrogen <0.3% (detection limit), sulfur 2.57%.

Isolation of fungal external secretome: *Grammothele fuligo* was chosen as it exhibited activity of LiP, MnP, and LA. Production of extracellular enzyme cocktail from *Grammothele fuligo* was carried out using a solid-state fermentation. Experiments were performed in 500 mL Erlenmeyer flasks containing 10 g of ground *Miscanthus* straw. Flasks were autoclaved, impregnated to 80% moisture content with deionized water, and inoculated with five agar plugs (diameter, 0.7 cm). The Erlenmeyer flasks were incubated at 30 ± 1 °C without shaking. The samples extractions were performed after 7 days of incubation by adding 100 mL of deionized water per flask. Flasks were incubated on a rotary shaker (200 rpm) for 1 h. The mixture was then centrifuged at 5000 rpm for 15 min at 4 °C to isolate the secretome and stored at −20 °C.

Peroxidases assays: Enzyme assays were performed by following the procedure of Zhou et al.[22] Peroxidase (LiP and MnP) activities were measured regarding the oxidation of DMP. One unit (1 U) of these enzymes is the amount of enzyme that will oxidize one micromole (1 μmol) of DMP per minute.

To a UV cuvette were added 1 mL of buffer, 524 μL of enzyme solution, 256 μL of 17.2 mM KF solution, and 200 μL of 18 mM DMP solution, and the reaction was started by addition of 20 μL of 0.1 M H_2O_2 solution. Absorbance at λ = 468 nm was then followed for 5 min, while the temperature was maintained at 30 °C. Oxidized DMP has an extinction coefficient of ε = 49 600 M^{-1} cm^{-1}.[23] Blank experiment with no enzyme was also used as reference. For MnP, 40 μL of the buffer solution was replaced with 40 μL of 5 mM manganese sulfate solution. The KF solution inhibited the activity of laccase; it is mostly useful when dosing the peroxidase activity in *Grammothele* enzyme cocktail.

Laccase assays: LA activity were measured regarding the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). One unit (1 U) of laccase is the amount of enzyme that will oxidize one micromole (1 μmol) of ABTS per minute.

To a UV cuvette were added 1.5 mL of buffer and 1.2 mL of enzyme solution, and the reaction was started by addition of 300 μL of 5 mM ABTS solution. Absorbance at λ = 405 nm was then followed for 5 min, while the temperature was maintained at 30 °C. Oxidized ABTS has an extinction coefficient of ε = 36 800 M^{-1} cm^{-1}.[24] Blank experiment with no enzyme was also used as reference.

Lignin degradation: Degradation experiments of lignin by peroxidases (LiP, MnP, and *Grammothele* cocktail) were performed as follows. 50 mg of the selected lignin was added to a test tube, along with 0.5 mL of isopropanol, 100 μL of 5 mM $MnSO_4$ solution in the case of MnP, the enzyme solution, and was topped up to 4.95 mL with buffer. Degradation was started by addition of 50 μL of 0.1 M hydrogen peroxide. Reaction mixture was stirred gently and maintained at 30 °C. All experiments were at least performed in duplicate.

Degradation experiments of lignin by LA were performed as follows. 50 mg of selected lignin was added to a test tube along with 0.5 mL of isopropanol. Additives were added in different amounts. For ABTS, violuric acid (VA), 1-hydroxybenzotriazole (1-HBT), and 3-hydroxyanthranilic acid (HAA), the ratio mediator to lignin was chosen according to research from Bohlin et al. on degradation of *β-O*-4 model bond by LA and a mediator. A ratio of lignin:mediator = 1:1 where lignin was regarded to the molecular weight of 334.37.[25] The optimum amount of mediator was also confirmed by changing the ratio of LA/mediator to higher and lower values and by measuring lignin degradation over 24 h (Supporting Information Figure 5). For both TBDMP and its tri-*tert*-butyl equivalent, TTBP (2,4,6-tri-*tert*-butylphenol), ratios were chosen similarly as if lignin was PPO and with a ratio of 1:1 regarding the repeating unit.[26]

Degradation experiments involving LiP were performed with a total of 0.06 U, MnP with a total of 0.02 U, *Grammothele* cocktail exhibited an activity of 0.01 U of laccase, 0.25 U MnP, and 0.03 U of LiP, and laccase experiment with a total of 0.3 U. Yields ranged between 95% and 99%.

The synthesis of *tert*-butyl-dimethyl-phenol (TBDMP) is described in the Supporting Information.

Characterization. The molecular weights of lignin samples were analyzed at 40 °C by gel permeation chromatography (GPC) performed on a Tosoh EcosHLC-8320 equipped with double detectors (RI, UV 280 nm) using Tosoh alpha 4000 and 2000 columns. DMF/LiBr 0.1 M was used as a mobile phase (flow rate 1 mL/min). Polystyrene standards were used for the calibration.

UV measurements for enzyme assays were interchangeably performed on a Thermo Scientific MultiskanFC microplate photometer or an Agilent Cary 60 UV−vis cell reader spectrophotometer.

2D HMQC NMR experiments were performed on a Bruker Avance 500 MHz NMR spectrometer equipped with a CryoProbe Prodigy 5 mm ^{1}H/2H−^{13}C/^{15}N probe. NMR experiments were performed with the sample held at 25 ± 0.1 °C. Chemical shifts for all experiments are referenced using the Unified Scale relative to 0.3% tetramethylsilane in deuterochloroform.[27,28] Multiplicity edited HSQC experiments used the standard hsqcedetgpsisp2.2 Bruker pulse program. There were 512 experiments with 64 scans collected over 6010 Hz in the ^{1}H dimension and 20 139 Hz in the ^{13}C dimension with a 1.5 s relaxation delay. The data were processed in phase-sensitive (echo-antiecho) mode to 2048 × 2048 data points using a π/2-shifted sine-squared window function in both dimensions.

^{31}P NMR experiments were performed on a Bruker Advance III 400 (9.4 T magnet) with a 5 mm broadband probe with *z*-gradient and BACS 60 tube autosampler. TMDP was used as derivatizating agent for phosphylation of hydroxyl groups, following procedures reported in the literature.[29] Experiments were performed with 180 scans and a 25 s relaxation delay.

RESULTS AND DISCUSSION

Degradation by Ligninolytic Enzymes. LiP, MnP, LA, and an extracellular enzyme cocktail from *Grammothele fuligo* were investigated for the degradation of three different sources of lignin: organosolv, alkali, and Kraft lignin. As shown in

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Figure 3, lignins' molecular weight tended to decrease rapidly in the first minutes of the reaction, followed by a slow repolymerization over time, reaching even higher molecular weight than the original sample for reaction time over 48 h. This recondensation happening for longer reaction times was expected as ligninolytics enzymes can both depolymerize and polymerize lignin.[30] It is also believed that the oxidation of some lignin bonds leads to a homolytic cleavage, as observed on numerous model bond degradation studies.[31−33] After some time, the resulting free radicals can react together or form a more stable bond.

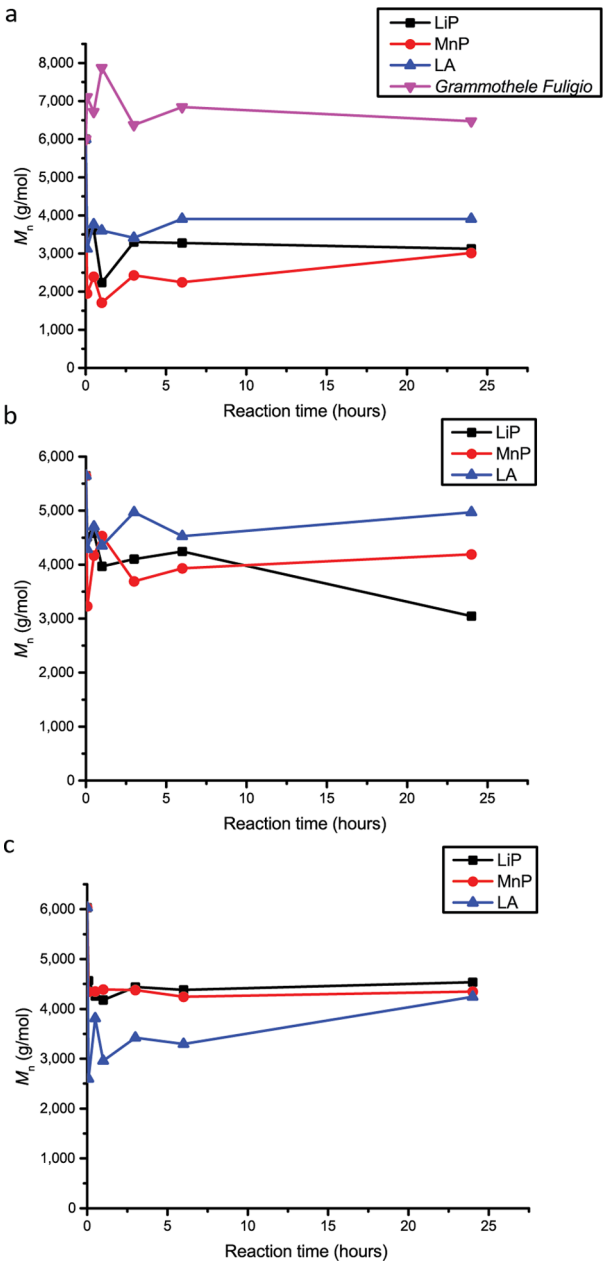

Figure 3. Evolution of average molecular weight by number versus reaction time, measured by GPC. Three sources of technical lignin are compared: (a) organosolv, (b) alkali, and (c) Kraft, degraded by LiP, MnP, LA, and *Grammothele fuligo*.

LiP is reported in the literature as the enzyme with the highest redox potential (1.2 V versus standard hydrogen electrode[1]) followed by MnP (0.8 V) and LA (0.5−0.8 V versus SHE). Therefore, oxidation and degradation of lignin should be more easily achieved by LiP, then MnP, and finally LA. However, lignin depolymerization with MnP reveals the best overall bonds' cleavage ability, followed by LiP and LA. The extracellular enzyme cocktail extracted from *Grammothele fuligo* exhibits activity of both peroxidases and LA. However, when exposed to the enzyme cocktail, organosolv lignin showed a rapid polymerization (Figure 3a).

These results highlight the complexity of the lignin enzyme degradation mechanism by enzyme cocktails. The combination of multiple enzymes could not improve the depolymerization efficiency. The three different lignins reacted differently to enzymatic degradation. Overall, organosolv was much easier to degrade than the Kraft or Alkali lignin; the organosolv lignin was thus chosen for the subsequent studies. Literature recently highlighted how different processes such as Kraft and alkali pulping can change the amount of *β-O*-4 bonds in treated lignins.[34] 2D HMQC analysis of the organosolv, alkali, and Kraft lignin used for our depolymerization indicated a *β-O*-4 content of 62%, 67%, and 37%, respectively. Although this *β*-ether bond is considered to be the easiest lignin bond cleavable through oxidation,[4,35] the *β-O*-4 richest lignin, alkali lignin, is actually the least prone to degradation. Other factors must influence the enzymatic depolymerization. Besides the structure difference, Kraft lignin also possesses a non-negligible amount of sulfur (2.5 w/w%, measured by elemental analysis), about 30% of which is in the sulfate form and the remaining as organically bound sulfur.[36] The higher degradation recalcitrance of Kraft lignin as compared to organosolv might be partially due to enzyme inhibition by these species.

Mediators for Enhanced Enzymatic Activity. The use of additives to enhance LA lignin-degradation properties has been vastly reported.[18,37−39] It was hypothesized that these agents facilitate the degradation mechanism by improving diffusion into the lignin network and by widening the range of lignin bonds that can be cleaved. Bourbonnais and Paice highlighted the indirect mechanism of degradation showing the importance of small intermediate molecules, called mediators.[40] Molecular oxygen is used by LA in the catalytic cycle to oxidize the mediator, which then acts as chemical oxidant for lignin. This mechanism eliminates the steric limitation of enzymes that could otherwise highly hinder oxidation of bulky molecules. The mediated process also allows laccases to overcome their phenolic-substrate restriction, expanding the range of lignin units that can be oxidized. Typical laccase mediators are small phenolic compounds such as vanillin, syringaldehyde, veratryl alcohol, but also synthetic mediators such as 1-hydroxybenzotriazole (1-HBT), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), or violuric acid (VA).[41] The choice of mediator can influence the oxidative potential of laccase and also induce stereopreference in the substrate.[42] 3-Hydroxyanthranilique acid (HAA) was investigated by our group for the degradation of lignin-like polymer (unpublished results). In nature, this mediator can favor lignin degradation by laccase and hinder the repolymerization process.[43] 2,4,6-Tri-*tert*-butylphenol (TTBP) and 4-*tert*-butyl-2,6-dimethylphenol (TBDMP) are involved in the redistribution mechanism to cleave the 4-*O*-5 bonds in lignin.[26] The initial step is an oxidation and the creation of a phenoxy radical, cleaving the bond by replacing the aromatic at the C4 position. If laccase can oxidize TBDMP and activate it as a mediator, a strong decrease in 4-*O*-5 bonds and concomitant drop in M_n would be

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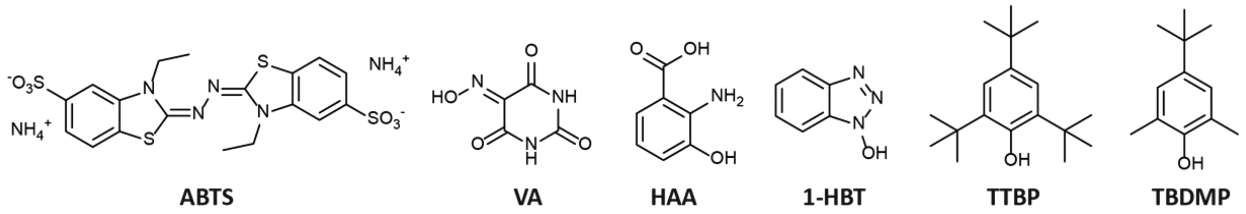

Figure 4. Different agents used to improve laccase degradation of lignin.

expected. Those six compounds (see Figure 4) were used as a mediator for screening reaction and to determine which could best improve lignin depolymerization (Figure 5).

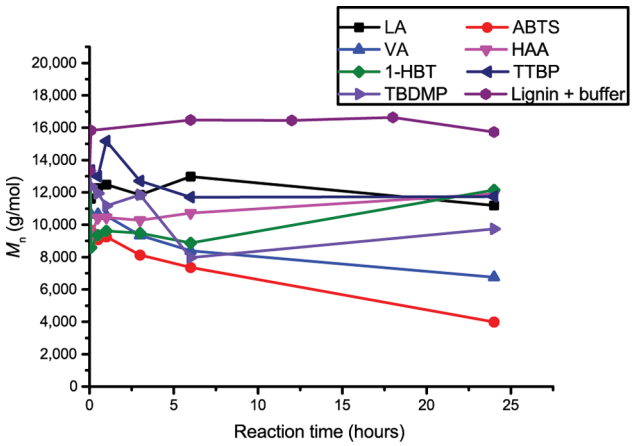

Figure 5. Lignin degradation by LA and different mediators. Evolution of average molecular weight by number versus reaction time, measured by GPC. Molecular weights are overestimated due to experimental constraint.

Organosolv lignin has a high amount of *β-O*-4 linkages and proved to be a better substrate for oxidative degradation (previous section). Thus, organosolv lignin was selected for the following study. Table 1 summarizes the changes in molecular weight of lignin with the different mediators. Molecular weights were overestimated as compared to the first section due to a cut off of the low molecular weight fraction as the residual mediator area was removed from the molecular weight analysis using GPC (Figure S2).

Table 1. Summary of Lignin Degradation Experiment by LA Enzyme with the Different Mediators

entry	enzyme	mediator	M_n at max degradation (g/mol)	time at max degradation	$Đ$
0	untreated original lignin		14000[a]		3.8
1	none	none	15700[a]	24 h	9.3
2	LA	none	11200[a]	24 h	3.2
3	LA	ABTS	4000[a]	24 h	1.5
4	LA	VA	6800[a]	24 h	3.9
5	LA	HAA	9400[a]	5 min	4.5
6	LA	1-HBT	8600[a]	5 min	4.2
7	LA	TTBP	11700[a]	6 h	3.6
8	LA	TBDMP	8000[a]	6 h	3.5
9	none	ABTS	15500[a]	24 h	9.9
10	none	VA	14600[a]	24 h	7.3

[a]Overestimation due to analysis constraints; see Figure S2.

The six mediators were compared altogether and versus LA alone. Molecular weight was clearly reduced with VA and ABTS (Figure 5 and Table 1, entries 3 and 4). In comparison, other mediators had less effects on lignin molecular weight, but still provided a better degradation than LA alone. VA and ABTS presented a slow degradation process as compared to the four other mediators, with maximum depolymerization reached at 24 h. Some repolymerization of the lignin fragments was also observed. Higher reaction time led to molecular weight higher than that of the untreated original lignin by recondensation reaction. Full information is shown in Figure S3. TBDMP, a new synthetic agent for laccase mediated systems, was more effective to depolymerize lignin than was 1-HBT, a well-reported mediator (Table 1, entries 6 and 8). HAA had an effect similar to that of 1-HBT, with a rapid depolymerization within the first 5 min, followed by recondensation. As for TTBP, no significant improvement of depolymerization was observed. TTBP and TBDMP required a longer reaction time than HAA and 1-HBT, suggesting a different pathway or slower mechanism. TTBP has however a much weaker impact on lignin degradation; this is likely due to the additional steric hindrance around the phenol group caused by the two *tert*-butyl side groups. After this screening, ABTS and VA showed the best improvement of lignin degradation by LA. The biodegradation products of these two systems require further study to elucidate the degradation mechanism.

Analysis of Degradation Products. From the previous screening reactions, we were able to identify VA and ABTS as the two best candidates for a more thorough investigation. GPC analysis data through lignin depolymerization using both mediators with LA are shown in Figure 6.

The clear shift of the lignin peak to right indicates a change toward lower molecular weight. At this point, as the commercial production of LA from *Cerrena unicolor* had been discontinued, LA from *Trametes versicolor* was used instead. Although both LA have similar redox potentials (Table 3), they present a similar trend but slight differences in lignin degradation. Overall depolymerization had similar efficiency, with lignin M_n decreased by 49% (7200 g/mol) and 73% (3800 g/mol) from the original value (14 000 g/mol) for ABTS and VA, respectively, using LA from *C. unicolor*. 2D Q-HMQC NMR analysis was conducted to investigate the structural changes in lignin during the LA action. Four regions in 2D proton-carbon NMR allow insight on the lignin structure: (a) the aliphatic oxygenated side chain region (δ_C/δ_H 50−90/2.5−5.8), (b) the aromatic/unsaturated region (δ_C/δ_H 90−150/5−8.5), (c) the aliphatic side chain region (δ_C/δ_H 5−38/0.5−2.8), and (d) the aldehyde region (δ_C/δ_H 170−210/9−10), although only the first two are of interest for this study.[39] Correlations regions were compared to previous literature and peaks were assigned accordingly.[34,44−48] Q-HMQC NMR allows quantification of carbon bonded protons

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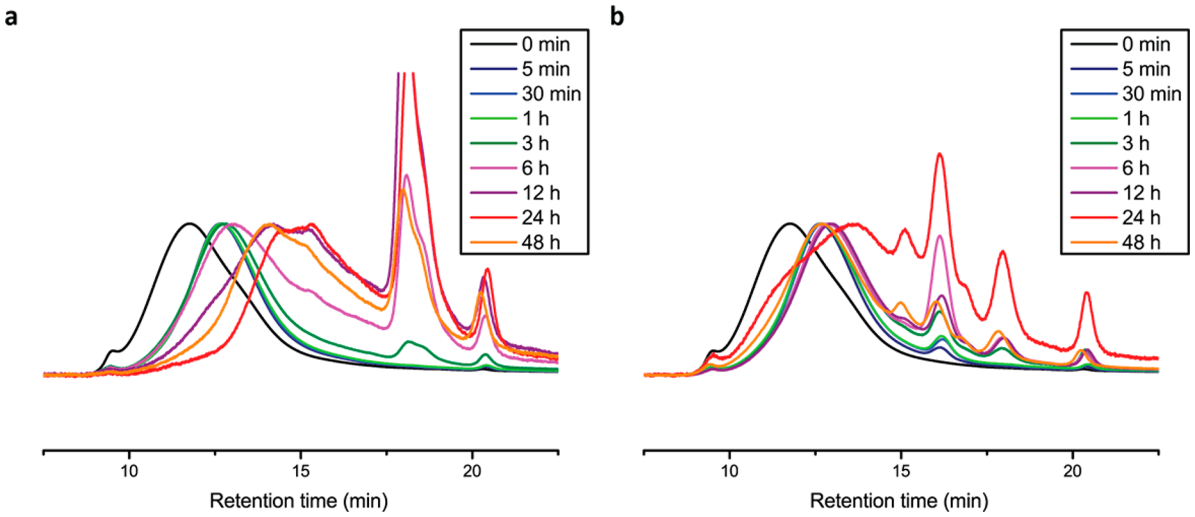

Figure 6. GPC analysis of organosolv lignin degraded by Laccase from *Trametes versicolor* and (a) ABTS and (b) violuric acid, at different reaction times. The clear shift to the right indicates a decrease in lignin molecular weight. The peak at 16.5 min retention time corresponds to ABTS, while that at 17.5 min is violuric acid. The peak at 20.5 min retention time represents the solvent. Other peaks appearing in high retention time area may be lignin oligomers or side products from reaction of the mediator with enzyme.

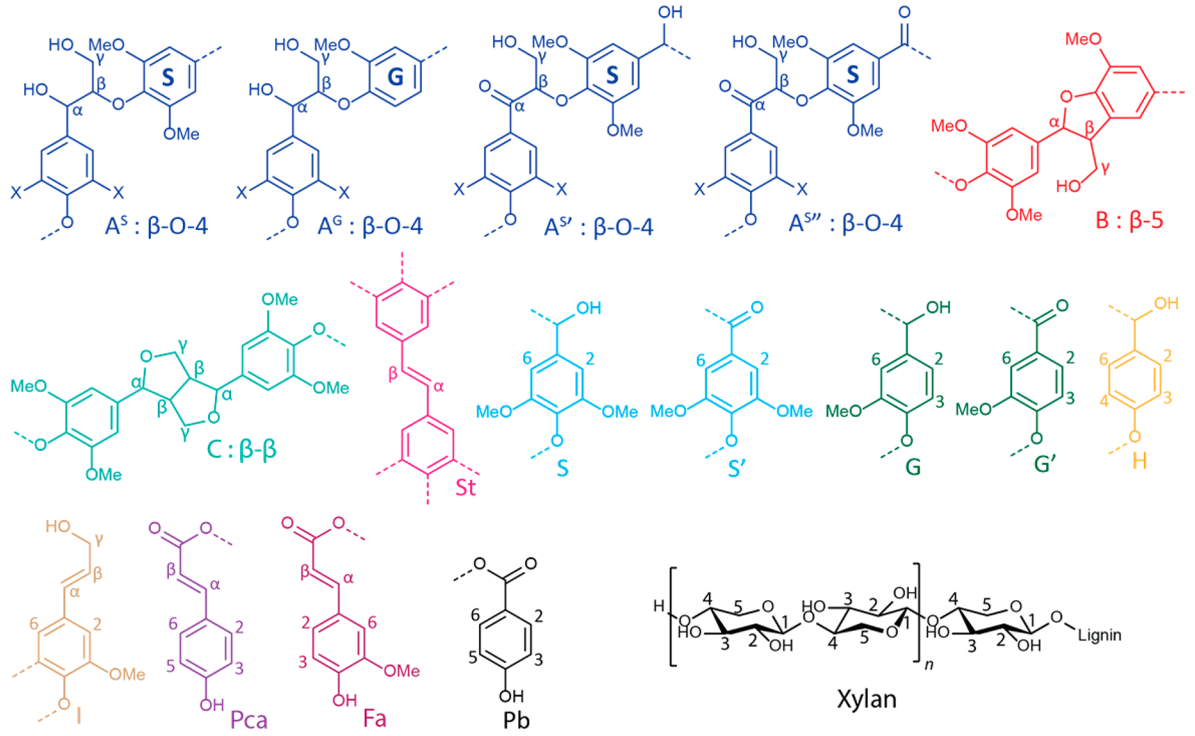

Figure 7. Summary of the different lignin linkages and other structures identified by $^{13}C/^{1}H$ correlation 2D NMR. X = H/OCH_3.

by integration of the C−H correlation patterns. Examination of the aromatic/unsaturated region allows distribution of aromatic units inside lignin between the three possible forms: syringyl (S), guaiacyl (G), or *p*-hydroxyphenyl (H) units. These units differ in structure by the presence of two (S), one (G), or the absence (H) of methoxy group on the aromatic ring (Figure 7). The ratio between these units can vary from one lignin to another and can allow the determination of its origin (hardwood, softwood, plant).

In the aliphatic oxygenated side chain region, correlation patterns allow quantification of several bonds between the aromatic units in lignin, including the *β-O*-4, the *β*-5, and the *β*−*β* bond (Figure 7). Furthermore, in the case of *β-O*-4 bonds, the type of unit at the position C4 can also be differentiated between S (A^S) or G (A^G) units. Finally, oxidation of those bonds can be monitored in both aliphatic oxygenated ($A^{S'}$, $A^{S''}$) and aromatic regions (G′, S′).

The initial organosolv lignin structure was characterized (Figure 8a and d). The analysis of the aliphatic oxygenated side chain region indicates the presence of *β-O*-4 and a few *β*-5 bonds; however, no *β*−*β* bonds could be detected. Integration and percentage of the bonds forming lignin (that can be detected with HMQC NMR) are summarized in Table 2. Peaks in the aromatic region show evidence of the presence of

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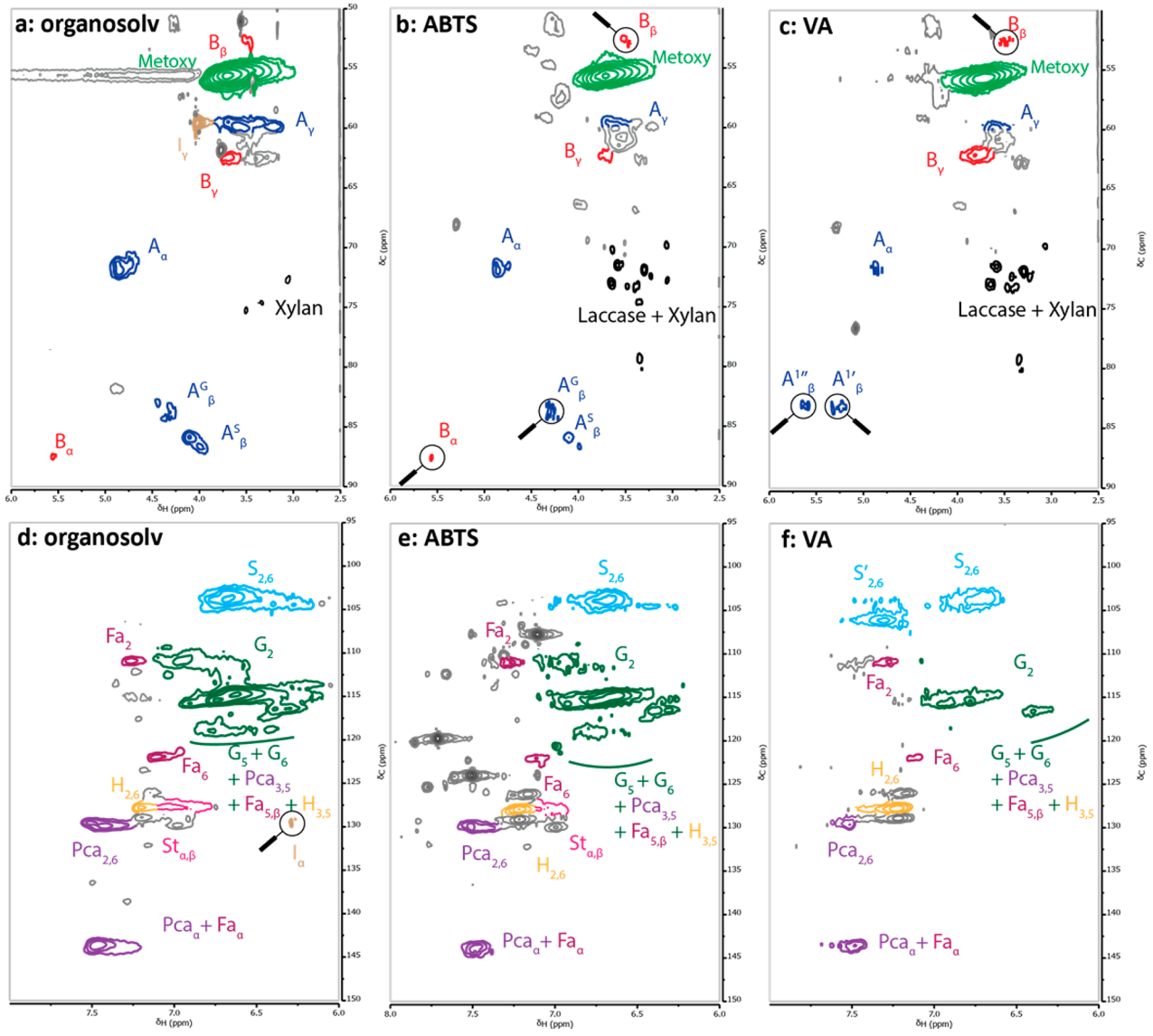

Figure 8. $^{13}C/^{1}H$ correlation HMQC 2D NMR analysis. (a–c) Aliphatic oxygenated side chain region of original lignin, ABTS-LA degraded lignin, and VA-laccase degraded lignin, respectively. (d–f) Aromatic hydrogen region of the same samples, respectively.

Table 2. Characterization of Lignin Sample Structures from GPC and 2D NMR Integration

	original lignin	treated LA + ABTS	treated LA + VA
M_n (g/mol)	14000	7200	3800
S/G/H units ratio	44%/53%/3%	45%/44%/11%	58%/26%/16%
oxidized S unit/S unit[a]	n.d.[b]	9%	47%
oxidized G unit/G unit[a]	n.d.	n.d.	n.d.
β-5[a]	8%	10%	12%
β–β[a]	n.d.	n.d.	n.d.
non ox. β-O-4[a]	62%	59%	50%
β-O-4 (A^G)	22%	21%	20%
β-O-4 (A^S)	34%	33%	22%
β-O-4 ox. ($A^{S'}$)[a]	n.d.	n.d.	14%
β-O-4 ox. ($A^{S''}$)[a]	n.d.	n.d.	15%
St[a]	13%	13%	n.d.
DC	0.00	0.10	0.53

[a]Per aromatic circle. [b]n.d., not detected.

S, G, and H units, with a ratio of 44/53/3. The origin (grass lignin) of the organosolv lignin was confirmed by the HMQC analysis. Syringyl units (S, δ_C/δ_H 102–108/6.1–6.8) can be found in both hardwood and herb lignin. The presence of *p*-hydroxyphenyl units (H, δ_C/δ_H 128/7.2, non-negligible amount in herb lignin) and the absence of *p*-hydroxy benzoate group (Pb, δ_C/δ_H 132/7.5–7.9, typical of hardwood) also confirm that observation.[38] The presence of some protein residue was found from plant cell wall typical of many grasses. Kim et al. reported that two proteins (phenylalanine and tyrosine) have a distinct correlation pattern in the same area as *p*-hydroxyphenyl units, therefore causing its slight overestimation.[49] Organosolv process involving mild acid ethanol/water treatment was reported to create β-O-4 ethoxy groups in the Cα position (instead of hydroxyl), which could hinder these bond oxidations; however, no corresponding peak could be observed in this case (δ_C/δ_H 81/4.55 and 64/3.3), suggesting an even milder treatment was used for this lignin.[43] Furthermore, no polysaccharide peaks could be observed in the carbohydrate region, nor the usual ether bond between C_α in a

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β-*O*-4 and a C6 of polysaccharide (δ_C/δ_H 82/4.6), indicating the absence of cellulose from the lignin sample.

Next, depolymerized lignin products using both mediators ABTS and VA with LA were characterized by 2D HMQC NMR (Figure 8b,e and c,f). After the enzymatic treatment of lignin samples, some clear changes were observed in their structure. These changes appear to be mediator-dependent, suggesting a more complex mechanism from the mediator than a simple intermediate. A common behavior observed was a general decrease in the relative amount of G units in both lignins. This decrease suggests that some new bonding was created, such as condensation reaction in position 5 of the aromatic ring, for example. Such reactions would cause a shift in the resonance frequency of protons in positions 2 and 6. Previous literature proved that 4-*O*-5 condensation reaction can induce a shift of G_6 protons (C6 protons of G structure) into the $S_{2,6}$ (C2 and C6 protons of S structure) correlation area (Figure 8), which would explain a slight increase in the calculated relative amount of S units.[50−52] An increase in H units' amount could be a result of superimposition with signal from phenylalanine protein, which is present in LA from T. *versicolor*.

Furthermore, calculation for a condensation degree was formulated by modifying the Capanema equation on ^{13}C quantitative NMR spectroscopy.[53] The degree of condensation (DC) refers to the average number of aromatic protons per aromatic ring being substituted by condensation reactions. It can be calculated from integrating the correlation region δ_C/δ_H 100−125/6−7.5. The contribution of the S, G, and H units in the region of interest is 2 protons for syringyl units, 3 for guaiacyl units, and 2 for H units.

$$\mathrm{DC} = \frac{\mathrm{Ar{-}H_{theory}} - \mathrm{Ar{-}H_{measured}}}{\mathrm{Ar{-}H_{theory}}} \quad (1)$$

$$\mathrm{Ar{-}H_{theory}} = 2 \times \mathrm{S} + 3 \times \mathrm{G} + 2 \times \mathrm{H} \quad (2)$$

The condensation degree of the original and degraded lignins can be found in Table 2 with detailed calculations in the Supporting Information. As expected, a clear increase in condensation degree follows the drop in relative amount of G units, thus corroborating the expected recondensation phenomenon by action of ligninolytics enzymes, also observed in the first section of this study. Initially, organosolv lignin samples contained about 62% of β-*O*-4 bonds, 8% of β-5 bonds, and an amount of β−β bonds below detection levels (Table 2); this is typical of organosolv herb lignin.[32] 2D HMQC analysis can differentiate if a β-*O*-4 bond involves an S (syringyl) or G (guaiacyl) unit in the C_4 position (Figure 7). When a β-*O*-4 bond involving a syringyl unit at the C_4 position (A^S) gets oxidized (C_α ketone) during depolymerization, the β proton shifts to lower fields, thus allowing quantification of oxidized β-*O*-4. In the case of ABTS mediated degradation, no oxidized β-*O*-4 ($A^{S\prime}$ nor $A^{S\prime\prime}$) bonds were observed in $^1H-^{13}C$ NMR, while action of the mediator VA oxidized many β-*O*-4 bonds with a high amount of $A^{S\prime}$ and $A^{S\prime\prime}$, 14% and 15%, respectively (Table 2). The concomitant decrease of the overall S units involved in nonoxidized β-*O*-4 bonds (A^S and A^G units) and the increase of oxidized S units (δ_C/δ_H 104−110/7.1−7.5) confirm the ability of VA to efficiently mediate lignin oxidation. In both cases, no oxidized G units were observed, suggesting that β-*O*-4 involving S units are easier to oxidize/cleave than that involving G. This corroborates literature on β-*O*-4 model bonds' degradation by LA and mediator.[5,16,25,39] An overview of S/G type simple phenolic compounds and mediators redox potential is shown in Table 3.

Table 3. Redox Potential of Mediators and Different S/G Model Molecules[a]

ox	red	E^0 (V)	S/G type molecule	ref
O_2	H_2O	1.2−0.8		
$ABTS^{+\bullet}$	$ABTS^{+2}$	1.09		37
VA	VA_{Ox}	0.91		37
ferulic $acid_{Ox}$	ferulic $acid_{Red}$	0.79[b]	G1	56
conyferyl $aldehyde_{Ox}$	conyferyl $aldehyde_{Red}$	0.79[b]	G2	56
LA $T.V._{Ox}$	LA $T.V._{Red}$	0.78		57
LA $C.U._{Ox}$	LA $C.U._{Red}$	0.75		58
$guaiacol_{Ox}$	$guaiacol_{Red}$	0.71	G3	59
ABTS	$ABTS^{+\bullet}$	0.68		37
synapic $acid_{Ox}$	synapic $acid_{Red}$	0.68[b]	S1	56
synapyl $aldehyde_{Ox}$	synapyl $aldehyde_{Red}$	0.65[b]	S2	56
$syringol_{Ox}$	$syringol_{Red}$	0.56	S3	59

[a]Potentials are reported versus standard hydrogen electrode. [b]Anodic potential. S/Gx are aromatic with the same structure with one (G) or two (S) methoxy groups.

As a general trend, two simple phenols with similar structure differing only by the substitution of one (G) or two (S) methoxy groups on the aromatic ring have different redox potentials, with the disubstituted (S) phenol having the lowest. This seems to indicate that G units are more stable toward oxidation than S units. With a lower redox potential by 0.2 V as compared to VA, ABTS is less efficient in oxidizing S type units. VA could oxidize S units easier, thus depolymerizing lignin further. However, it is known that ABTS can undergo a second oxidation (from $ABTS^{+\bullet}$ to $ABTS^{+2}$) at a higher redox potential of 1.09 V. It was suggested that due to the Nernst law, some $ABTS^{+2}$ and oxidized VA should be present in the medium as LA from *Trametes versicolor* and have a strong redox potential around 0.8 V.[54] Some VA and, to a less extent, $ABTS^+$ might have been oxidized by LA. Both mediators will then oxidize lignin proportionally to their concentration. Through this mechanism, laccase could perform the thermodynamically unfavored oxidation of mediator, which in turn led to lignin bonds' oxidation and cleavage. S units being seemingly easier to oxidize, lignin with a high amount of syringyl units should therefore be favored as a feedstock for enzymatic degradation of lignin and products recovery. Although β-*O*-4 bonds have proved to be targeted by the LA mediated systems, it is not a sufficient condition for high performance lignin depolymerization.

In addition, not only β-*O*-4 bonds but also stilbene bonds (St, see Figure 7) also seem to be oxidized during the depolymerization. Stilbene bonds are believed to be linkages resulting from reaction of β-1 or β-5 bonds during the pulping process, and are present in the original lignin. With LA−VA treatment, stilbene bonds disappeared from NMR, suggesting an oxidation reaction of the alkene bond or a condensation reaction. Enzymatic oxidation of resveratrol, a lignin model stilbene compound, was previously reported. Crestini et al. observed that oxidation of stilbene on model bond led to ketone with up to 29% yield by the action of ABTS mediated laccase; a higher yield can be expected from VA.[55]

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In addition, infrared spectroscopy analysis was performed to confirm the overall oxidation of the structure (Figure 9).

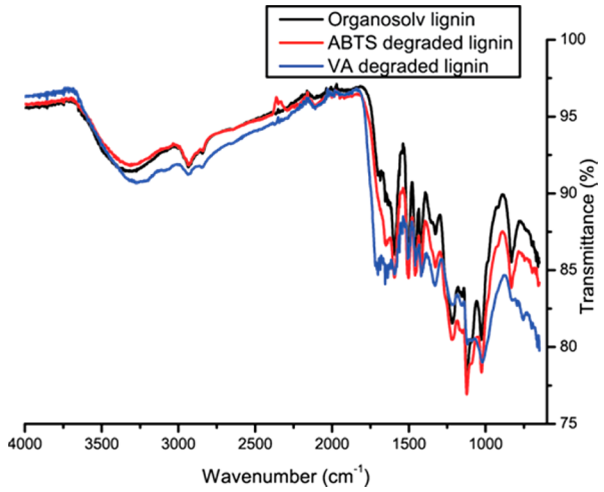

Figure 9. Infrared spectroscopy of organosolv lignin (black) and the degradation products of the laccase ABTS mediated (red) and violuric acid mediated (blue) biodegradation of lignin.

Increase of absorption in the region $\nu \approx 1600-1800$ cm^{-1}, corresponding to the C=O stretch vibration, indicates an overall increase in ketones and carboxylic acids in lignin after enzymatic treatment.

CONCLUSION

While both LA and peroxidases could degrade lignin individually, the enzyme cocktail from *Grammothele fuligo*, which contained both enzymes, induced straight repolymerization of lignin, highlighting the complexity of enzymes' synergistic interactions that still require a better understanding. Of the three lignins, organosolv is the most prone to biodegradation even though it presented an amount of *β-O*-4 bonds similar to that of alkali lignin. The amount of syringyl (S) units in lignin was identified as one of the main factors affecting the efficiency of enzymatic depolymerization, thus explaining the weak results with the Kraft and alkali lignins.

TBDMP, a novel mediator for LA mediated systems, has an efficiency in lignin degradation similar to that of ABTS, the most used LA mediator. ABTS and VA are the best agents for lignin depolymerization in this study, with up to a 73% decrease in molecular weight as compared to original lignin. 2D HMQC NMR analysis highlighted that the mediator that depolymerized lignin the most also induced more oxidation. Lignin S units were clearly targeted by the enzymatic oxidation, as well as the *β-O*-4 bonds involving S units in the C_4 position. Therefore, when considering chemical production from lignin biodegradation, a lignin feedstock with both high amounts of S units and *β-O*-4 bonds should be considered; hardwood and herb lignin are working best, while softwood lignin would be the most recalcitrant. A strong correlation was established between the redox potential of lignin model molecules and the lignin oxidation by LA mediated systems.

Highly purified and concentrated enzymes are costly, and single enzymatic systems have limited efficiency in degrading lignin. Enzymatic cocktail from white fungi secretome is a cheaper alternative for biorefineries implementation, but complex interactions between the different agents highly hinder the depolymerization process. LA mediated systems present another approach for lignin valorization in biorefineries. VA and TBDMP are good candidates as mediator for depolymerization. The future of lignin valorization by enzymatic degradation might lie in a combination of mediators instead of a combination of enzymes, the mechanisms of which are still too poorly understood.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b01426.

Description of GPC analysis of organosolv lignin degraded by LA mediated systems, different lignin linkages, structures identified by $^{13}C/^{1}H$ correlation 2D NMR, $^{13}C/^{1}H$ correlation HMQC 2D NMR analysis, calculation of S/G/H ratio and degree of condensation, and analysis of three lignin structures (PDF)

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Notes

The authors declare no competing financial interest.

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