# 2D-NMR DEMONSTRATION OF LIGNIN REMOVAL FROM WOOD AND NON-WOOD PLANT FEEDSTOCKS BY FUNGAL VERSATILE PEROXIDASE

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## ABSTRACT

The ability of versatile peroxidase to modify/degrade lignin (in conjunction with veratryl alcohol as a mediator) in both wood (*Eucalyptus globulus*) and non-wood (*Pennisetum purpureum*) lignocellulosic feedstocks was demonstrated using the *Pleurotus ostreatus* enzyme heterologously expressed in *Escherichia coli* and "in vitro" folded. Two-dimensional nuclear magnetic resonance (NMR) of the treated materials, swollen in dimethylsulfoxide-$d_6$, revealed a reduction in the number of lignin side-chain linkages after both treatments. However, a significant removal of lignin by the enzymatic treatment was only observed in the case of eucalypt wood, as shown by the decrease of lignin aromatic signals referred to carbohydrate anomeric signals.

## I. INTRODUCTION

Lignin removal is a key step for recycling the carbon fixed by land photosynthesis and a limiting issue for the use of lignocellulosic biomass by the pulp and paper industry and in the sustainable production of fuels and other chemicals. Only a group of basidiomycetes, the so-called white-rot fungi, are efficient lignin degraders in nature [1]. According to the genomic data, ligninolytic peroxidases - the so-called lignin peroxidase (LiP, EC1.11.1.14), manganese peroxidase (MnP, EC1.11.1.13) and versatile peroxidase (VP, EC 1.11.1.16) - are exclusive of lignin-degrading white-rot basidiomycetes and play a central role in lignin degradation [2]. The presence of LiP has been associated with the ability to degrade lignin in white-rot fungi and *in vitro* depolymerization of lignin by LiP was demonstrated [3]. On the other hand, a recent study show the presence in the model agaric *Pleurotus ostreatus* of a peroxidase repertoire in which VPs play the role that LiPs do in white-rot polypores, being able to depolymerize lignin in the presence of VA [4].

In the present study, eucalypt (*Eucalyptus globulus*) and Elephant grass (*Pennisetum purpureum*), two fast growing plant species, were treated with VP from *P. ostreatus* in the presence of VA to investigate its ability to modify natural lignin in lignocellulosic feedstocks, as it has been previously demonstrated for the laccase-mediator system [5,6]. The eventual modification of the cell-wall polymers during the enzyme treatment of the whole plant material was analyzed by hetereonuclear single quantum correlation (HSQC) solution NMR of gels prepared by lignocelluloses swelling in dimethylsulfoxide-$d_6$ [7,8].

## II. EXPERIMENTAL

*Recombinant VP*

VP (isoenzyme VP1) from the basidiomycete *P. ostreatus* was produced heterologously in *Escherichia coli*. The mature protein-coding sequence of gene model 137757 from the PC9 monokaryon genome, after manual curation, was synthesized by ATG:biosynthetics (Merzhausen, Germany) after verifying that all the codons had been previously used for expressing other genes in the same *E. coli* strain (and substituting them when required). Mature protein-codifying sequence was cloned in the expression vector pET23a (+) (Novagen). The resulting plasmid pET23a-137757, was directly used for expression. The peroxidase was produced in *E. coli* BL21(DE3)pLysS. Cells were grown for 3 h in Terrific Broth, induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and grown further for 4 h. The apoenzyme accumulated in inclusion bodies, as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was solubilized using 8 M urea. Subsequent *in vitro* folding was performed using 0.16 M urea, 5 mM $Ca^{2+}$, 20 µM hemin, 0.5 mM oxidized glutathione, 0.1 mM dithiothreitol, and 0.1 mg/ml protein, at pH 9.5. Active enzyme was purified by Resource-Q chromatography using a 0-300 mM NaCl gradient (2 ml·min$^{-1}$, 20 min) in 10 mM sodium tartrate, pH 5.5.

*Wood and grass treatments with VP*

Milled Elephant grass and eucalypt wood samples (100 mg) were treated with 5 mg of VP from *P. ostreatus* in 10 mM Na acetate (pH 4.5) containing 10 mM VA and 60 mM $H_2O_2$ at 25 ºC for 24 h. Treatments without

enzyme were also performed. For NMR analysis, the whole samples recovered after washing (70-85 mg) were swollen in dimethylsulfoxide-$d_6$ and HSQC spectra were acquired at the gel state [7,8]. A Bruker Biospin (Billerica, MA) AVANCE 500 MHz spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe with inverse geometry (proton coils closet to the sample) was used. The $^{13}C$-$^{1}H$ correlation experiment was an adiabatic HSQC experiment (Bruker standar pulse sequence ´hsqcetgpsisp.2´; phase-sensitive gradient-edited-2D HSQC using adiabatic pulses for inversion and refocusin). Spectra were acquired from 10 to 0 ppm in F2 ($^{1}H$) with 1000 data points for an acquisition time (AQ) of 100 ms, an interscan delay (D1) of 500 ms, 200 to 0 ppm in F1 ($^{13}C$) with 400 increments (F1 acquisition time 8 ms) of 40 scans. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in 1H and a squared cosine bell in $^{13}C$. Prior to Fourier transformation, the data matrices were zero filled up to 1,024 points in the $^{13}C$ dimension. The central solvent peak was used as an internal reference ($\delta_C/\delta_H$ 39.5/2.49). The aromatic $^{13}C$-$^{1}H$ correlation signals of the different lignin units were used for estimation of its composition in guaicyl (G) and syringyl (S) lignin units. The *p*-coumaric and ferulic acid contents and the percentages of main lignin substructures (including β-O-4' and β-β' side-chain linkages) were referred to total lignin. Signal assignement was based on previous studies on *E. globulus* and *P. purpureum* lignins [9,10].

## III. RESULTS AND DISCUSSION

The detailed assignments of aliphatic-oxygenated (top) and aromatic-unsaturated (bottom) signals in the control and VP-VA treated eucalypt wood and Elephant grass samples are shown in the spectra expansions included in **Fig. 1**. The main lignin and cinnamic structures identified are shown in **Fig. 2**.

The aliphatic oxygenated region of the spectrum of control (**Fig. 1a** and **c**) showed signals of both lignin and carbohydrates, the latter mainly corresponding to xylan units (X). In addition to methoxyl signals, signals of lignin side-chains were observed, the latter corresponding to $C_\alpha$–$H_\alpha$ correlations ($A_\alpha$) in β-O-4´ alkyl–aryl ether substructures, and $C_\beta$–$H_\beta$ correlations in β-O-4 alkyl–aryl ether substructures including a second S-unit ($A_\beta$(S)). The main signals in the aromatic-unsaturated region of the HSQC spectrum of control eucalypt wood (**Fig. 1a**) corresponded to the lignin benzene rings, including the G and S whereas in the case of the Elephant grass **(Fig. 1c)** *p*-coumaric acid signals are also present together with lignin signals.

The oxidation of VA, used as mediator, results in the production of veratraldehyde which remains attached to the sample, despite the exhaustive washing, overlapping some signals. This can be due to the coupling of VA radical on the lignin units.

*Enzymatic modification of eucalypt lignin as shown by 2D NMR*

As shown by the NMR spectra of the whole treated sample at the gel state, a single step VP-VA treatment (without a subsequent alkaline extraction) led to a significant reduction in the molar frequency of both β-β' ($B_\alpha$ signal) and β-O-4' ($A_\beta$ signal) inter-unit linkages (per 100 lignin units) in the treated sample (40% and 1%, respectively, in **Fig. 1b**) with respect to the control (44% and 4%, respectively, in **Fig. 1a**). Moreover, a reduction of the lignin content was produced, as shown by the lignin-to-carbohydrate molar ratios (L/CH) estimated from the intensity of the lignin aromatic signals referred to the carbohydrate anomeric signals ($X_1$ and $X'_1$), passing from a L/CH ratio of 2.3 in the control to a L/CH ratio of 1.6 in the treated sample (**Fig 1**). Finally, a slightly preferential removal of G units was observed, resulting in higher S/G molar ratio in the treated sample (1.5) compared with the control (1.4), in agreement with related studies [5,6].

*Enzymatic modification of Elephant grass lignin as shown by 2D NMR*

The single step VP-VA treatment (without subsequent alkaline extraction) resulted in a reduction (>10%) of β-O-4' linkages per 100 lignin units in the treated sample (**Fig. 1d**) with respect to the control (**Fig. 1c**) (β-β linkages were not detected in the Elephant grass samples). However, in contrast with that observed for eucalypt wood, no significant reduction of lignin content was produced, as shown by the unchanged lignin-to-carbohydrate molar ratios (1.0 in both samples), and the *p*-coumaric acid (PCA signals) signals remained basically unchanged.

## IV. CONCLUSIONS

In the present study, the ability of a high-redox ligninolytic peroxidase from *P. ostreatus* (VP) to remove lignin from two lignocellulosic samples is demonstrated by NMR analysis, in agreement with its central role in lignin degradation in Agaricales and with our previous results in which VP was able to degrade both a non phenolic lignin-model dimer and synthetic polymeric lignin (DHP). The results obtained showed that VP was more effective against eucalypt wood lignin than against Elephant grass lignin, leading to both a significant reduction in the frequency of main inter-unit linkages and a moderate reduction of the lignin content, while the carbohydrate signals remain basically unchanged.

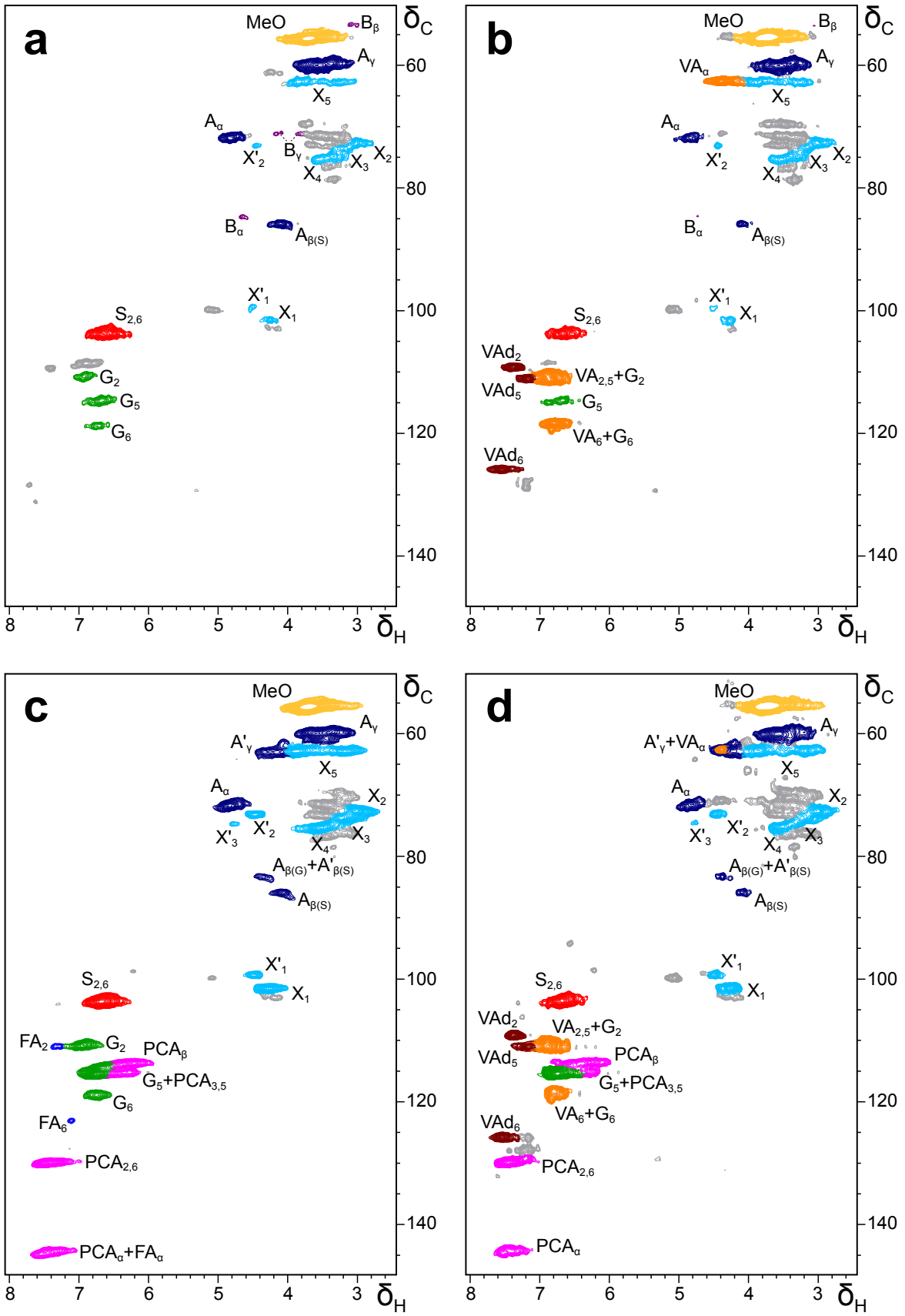


**Figure 1.** Aromatic region of the HSQC spectra of eucalypt wood (top) and Elephant grass (bottom) treated with *P. ostreatus* VP and VA (as mediator). **A** and **C** controls without enzyme; **B** and **D** treatments with enzyme. See **Fig. 2** for identification of the lignin, cinnamic acid and mediator-derived structures assigned (MeO correspond to methoxyl groups). X and X' signals correspond to normal and acetylated carbohydrates, respectively.

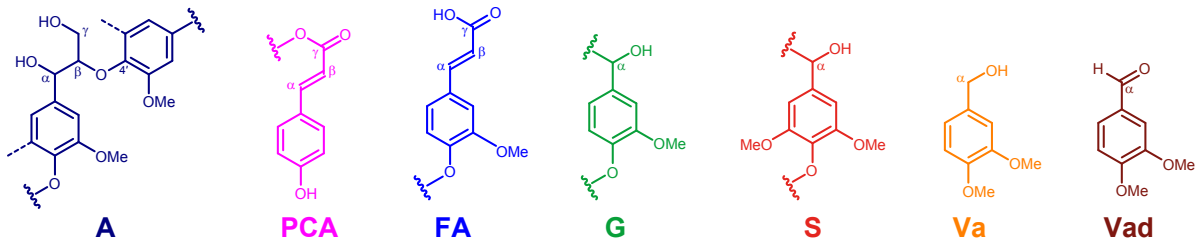


**Fig. 2.** Main lignin, cinnamic acid and mediator-derived structures identified in the Elephant grass and eucalypt samples analyzed by HSQC NMR (Fig. 1): **A**, β-O-4´ lignin structure (aliphatic region of the spectra); **PCA**, p-coumaric acid; **FA**, ferulic acid; **G**, guaiacyl units; **S**, syringyl units; **Va**, veratryl alcohol (mediator); and **Vad**, veratraldehyde (Va oxidation product).

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