



Structural Modifications Induced During Biodegradation of Wheat Lignin by *Lentinula edodes*

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Abstract—The structural modifications occurring during wheat straw lignin biodegradation were evaluated by the concerted use of ³¹P-, ¹H- and 2D homo- and heteronuclear NMR spectroscopies. Straw lignin was found to be oxidatively degraded via stereoselective side-chain oxidation as evidenced by a lower *erythro/threo* ratio. Significantly lower amounts of phenolic hydroxy and methoxy groups in the decayed lignin may be indicative that its structure after the fungal treatment contained a lower amount of aromatic units. In addition an increase in carboxylic acids content, that cannot be explained only on the basis of side-chain oxidation reactions, was also apparent. This evidence, coupled with pertinent data collected during this effort, suggests the occurrence of aromatic ring cleavage reactions. In fact the oxidative degradation of lignin model compounds by fungi has been reported to occur via the 3-oxoadipate pathway which is known to cause aromatic ring cleavage with the formation of aliphatic chains. The presence of aliphatic moieties in the decayed lignin was confirmed by detailed ¹H- and 2D NMR spectroscopic analyses. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Introduction

Lignin is a three-dimensional phenylpropanoid polymer mainly linked by arylglycerol ether bonds between monomeric phenolic units most of which are not readily hydrolyzable. In view of increasing interest in the development of alternative pulping and bleaching processes, the use of biotreatments has been considered aimed at reducing chemical demand and pollution load, and enhancing papermaking characteristics.¹ At the same time, an increasing interest in utilizing renewable resources for pulp making is apparent and the possibility of using non-woody plants or cereal residues such as wheat straw is considered as an attractive alternative to wood.² White-rot basidiomycetes are the most efficient lignin degrading microorganisms. The structural modifications induced by fungal treatments have been

extensively studied both on model compounds and directly on lignin by using mainly traditional oxidative degradation methods and other spectroscopic techniques.³ From these studies a general degradation mechanism involving mainly the oxidative cleavage of the lignin side-chain and its aromatic rings has emerged.^{4,5}

Among white-rot fungi, *Lentinula edodes* has been reported to produce two classes of ligninolytic enzymes: laccase and manganese peroxidase.^{6–9} It has been shown that *L. edodes* extracellular enzymes are able to perform the degradation of lignin models by both depolymerization through oxidative cleavage of the lignin side-chain and cleavage of the aromatic moieties.^{10,11}

In this study our attention was focused on the process of wheat straw biodegradation by *L. edodes*. In order to evaluate the chemical modifications induced on the lignin by the fungal treatment the detailed characterization of the lignin structure before and after solid-state fermentation was performed using advanced NMR

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techniques. There are many examples where magnetic resonance techniques when applied to lignin have proved to be excellent analytical tools for the structural elucidation of these complex biopolymers. Accordingly the work of our laboratory has been focused on the development of ^{31}P based novel magnetic resonance methods aimed to expand the frontiers of application of NMR to lignin analysis.^{12–16} These NMR techniques are capable of detecting and quantitatively determining all functional groups in lignin possessing reactive hydroxyl groups, i.e. aliphatic OH, the various forms of phenolic OH, and carboxylic acids. Using this methodology combined with 2D homo- and heteronuclear correlated NMR experiments, an extensive study of the overall structure of wheat straw lignin before and after fungal decay was made possible.^{12,17}

Results and Discussion

Both milled wheat straw (ML) and biotreated milled wheat straw (ML-*Le*) lignins were submitted to phosphorylation with 2-chloro-1,3,2-dioxaphospholane **1**, or 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane **2** and analyzed by quantitative ^{31}P NMR in the presence of a known amount of bisphenol A or cyclohexanol as internal standards, respectively (Scheme 1).^{12–16}

The obtained spectra showed well resolved signals for the different hydroxyl groups present within the two lignin preparations. In particular ^{31}P NMR spectra of samples phosphitylated with dioxaphospholane **1** show a good resolution of the different aliphatic OH groups, (Figure 1A) while ^{31}P NMR spectra of samples phosphitylated with dioxaphospholane **2** show a good resolution of the different phenolic OH groups (Figure 1B).

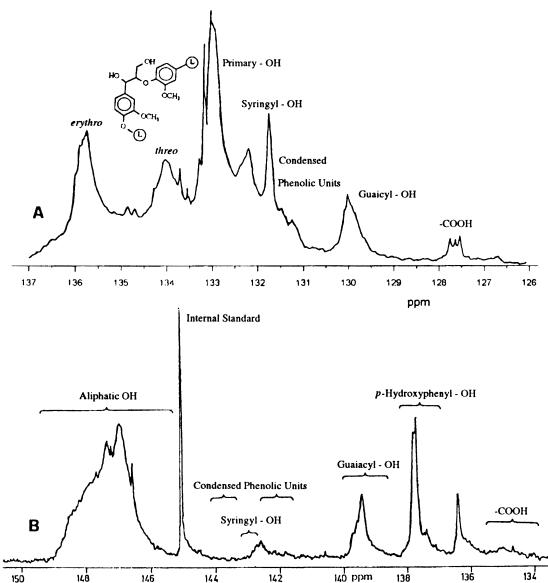
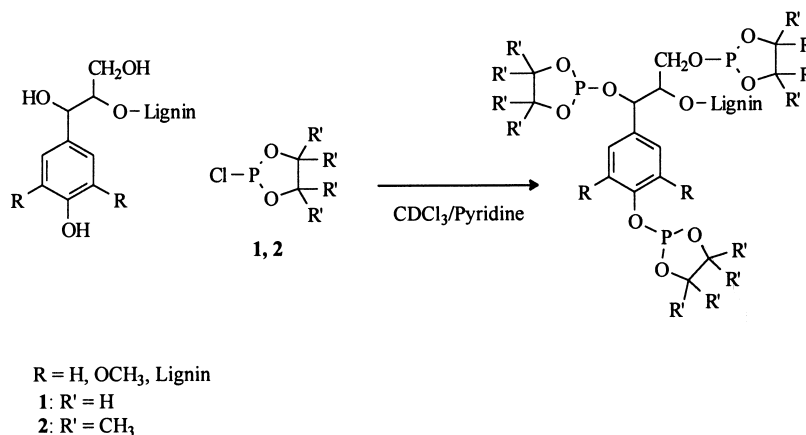


Figure 1. Quantitative ^{31}P NMR spectra of lignin samples derivatized with (A) 2-chloro-1,3,2-dioxaphospholane **1** and with (B) 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane **2**. For signal assignment see Refs 13–16.

Table 1 displays the quantitative data on the distribution of the various OH groups for all the experiments carried out during this effort. The spectra of samples derivatized with dioxaphospholane **2** showed a reduction of the overall aliphatic OH groups from 3.49 to 3.40 mmol/g (Figure 2, 145.5–149.5 ppm). The distribution and quantitative evaluation of phenolic OH and carboxylic acids were obtained by ^{31}P -NMR for the samples derivatized with dioxaphospholane **2** (Figure 2). The biotreatment caused a reduction of the overall phenolic content, that is, from 1.53 to 1.32 mmol/g



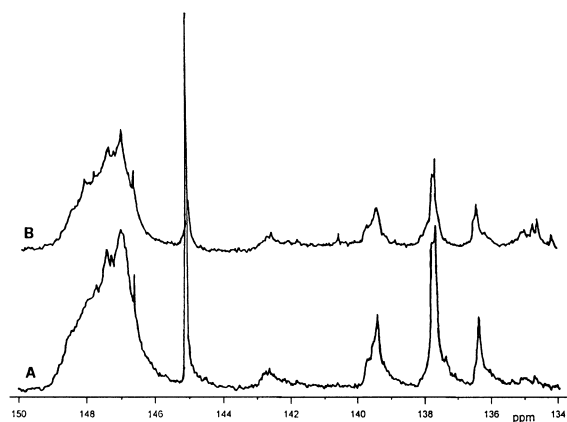
Scheme 1. Phosphitylation of lignin hydroxy moieties with 2-chloro-1,3,2-dioxaphospholane **1** or 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane **2**. For signal assignment see references 13, 14 and 16.

Table 1. Phenolic, aliphatic, and carboxylic hydroxyl groups present on the examined lignins determined by quantitative ^{31}P NMR spectroscopy

Functional group (mmol/g)	Milled straw lignin (ML) ^a	Biotreated milled straw lignin (ML-Le) ^b
Aliphatic OH ^c	3.49	3.40
Phenolic OH ^c	1.53	1.32
H ^{c,d}	0.68	0.55
G ^{c,e}	0.51	0.43
S ^{c,f}	0.16	0.14
Condensed phenolic OH ^c	0.18	0.20
COOH ^c	0.12	0.26
E/T ^{g,h}	1.82	1.64

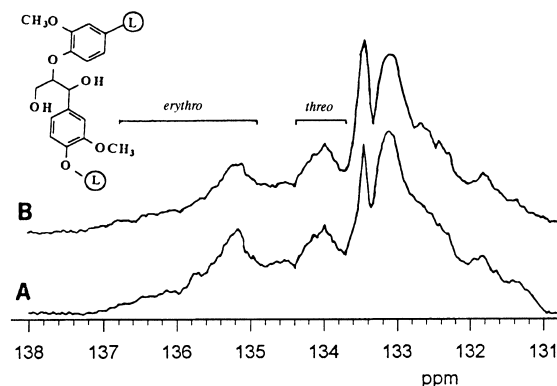
^aML, Milled wheat straw lignin.^bML-Le, Milled straw lignin from wheat straw biotreated with the white-rot fungus *L. edodes*.^cPhosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane **2**.^dH, *p*-Hydroxyphenyl phenolic OH.^eG, Guaiacyl phenolic OH.^fS, Syringyl phenolic OH.^gE/T, *erythro*/*threo* ratio.^hPhosphitylated with 2-chloro-1,3,2-dioxaphospholane **1**.

(136.6–143.5 ppm). Among the different phenolic groups the *p*-hydroxyphenyl moieties (H) decreased by 0.13 mmol/g (136.6–138.2 ppm). However, it should be noted that these units are mainly ester linked to the lignin backbone, and this decrease could be simply due to the hydrolysis of ester bonds.¹² Similarly, the frequency of guaiacyl (G) phenolic units also decreased after the biotreatment, while the syringyl (S) and condensed phenolic structures are evidently more recalcitrant to biological degradation since their frequency remained

**Figure 2.** Quantitative ^{31}P NMR spectra of lignin samples before (A), and after (B) fungal decay, derivatized with dioxaphospholane **2**. For signal assignment see refs 12 and 15.

almost unchanged after the biotreatment (Table 1). The aliphatic OH/aromatic OH ratio was found decreased after fungal decay (Table 1), suggesting a preferential degradation of aromatic units with respect to side chains. The reduction of phenolic groups could be attributed either to aromatic ring cleavage or to extensive condensation reactions. The latter, however, are not evident in our analytical data unless fully etherified condensed structures are produced. Similarly, the biotreatment also caused a decrease in both oxygen and methoxy contents. The obtained reduction in methoxy content, coupled with the observed decrease in phenolic unit content may be indicative that the structure of lignin after fungal treatment contained a lower amount of aromatic units. The reduction of aliphatic OH groups indicates the parallel occurrence of side-chain oxidation reactions. The carboxylic acids showed a clear increase, (0.14 mmol/g) that can not be explained only with the occurrence of side chain oxidation reactions. In fact the carboxylic acid increase was found higher than the aliphatic hydroxyl decrease, confirming the hypothesis of the occurrence of both side-chain oxidation and aromatic ring cleavage reactions. The occurrence of aromatic ring cleavage during lignin decay by white-rot fungi had been previously reported on the basis of IR and NMR data.³ Aromatic ring cleavage reactions were detected upon incubation of a dimeric lignin model compound in the presence of extracellular enzymes produced by *L. edodes*.¹¹ The occurrence of such reactions on the lignin biopolymer was evidenced by the recovery of a product of aromatic ring cleavage upon incubation of a lignocellulose complex with *L. edodes* extracellular enzymes.¹¹

From the spectra of samples derivatized with dioxaphospholane **1** it was possible to quantify the relative amounts of *erythro* and *threo* secondary OH groups (Figure 3). The data showed a decrease of the *erythro*/*threo* ratio

**Figure 3.** Quantitative ^{31}P NMR spectra of lignin samples before (A), and after (B) fungal decay, derivatized with dioxaphospholane **1**. For signal assignment see ref. 13.

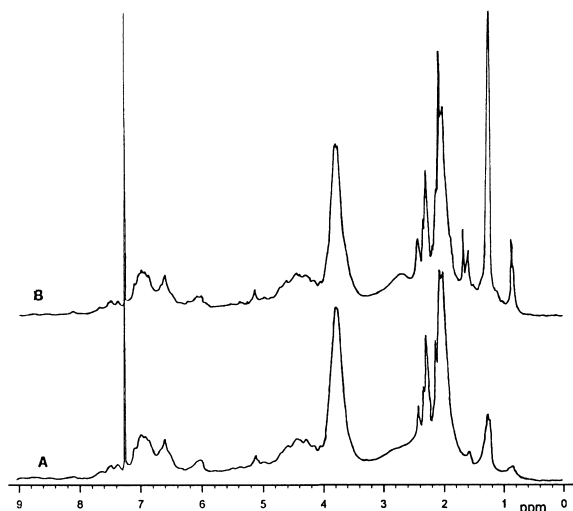


Figure 4. ^1H NMR Spectrum of acetylated wheat straw lignin before (A), and after (B) fungal decay. For signal assignment see Refs 19 and 20.

(Table 1). This possibly implies that the enzymatic oxidation of lignin side-chain is stereoselective and facilitates the degradation of the *erythro* arylglycerol- β -aryl ethers. The stereoselective degradation of the *erythro* forms of β -O-4 arylethers has been previously reported to occur during kraft pulping processes.¹⁸

As opposed to what has been reported before about heavily decayed lignins,³ the residual wheat straw lignin after the fungal treatment showed lower oxygen and methoxyl contents and a higher hydrogen content. These data seem to suggest an initial overall reduction of the residual lignin after treatment with *L. edodes*.

In order to further investigate this aspect of wheat straw lignin biodegradation we obtained the ^1H NMR spectrum of the acetylated lignin, before and after fungal treatment (Figure 4). From the comparison of the spectra it is evident a decrease of the signals due to aromatic groups as compared to aliphatic acetyl moieties. Careful integration for quantitative aim was not possible due to overlapping of the signals which did not allow an accurate

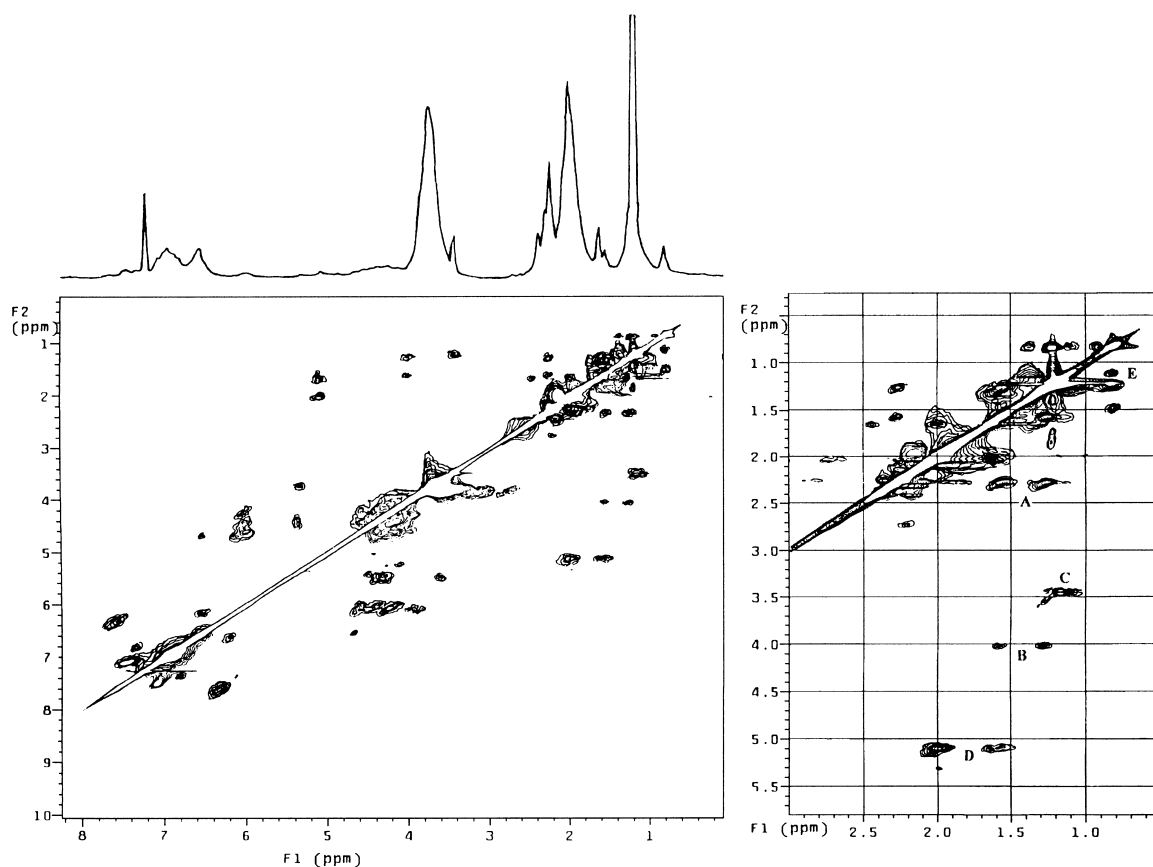


Figure 5. (A) Phase sensitive 2D HOHAHA spectrum of acetylated wheat straw lignin after fungal decay, acquired with 80 ms spin-lock. (B) Phase sensitive 2D HOHAHA spectrum of acetylated wheat straw lignin after fungal decay: enlarged aliphatic section.

measure of the ratios. The most interesting piece of information arising from these spectra was the presence of two groups of signals centered at 0.8–0.9 and 1.1–1.6 ppm, respectively, in the biotreated lignin. In the untreated lignin these signals were present only as traces.^{19,20} These signals are due to aliphatic protons. The occurrence of such signals in lignin ¹H NMR spectra had been attributed to the presence of fatty acids as lignin contaminants.^{19,20} The samples of wheat straw that we used had been previously submitted to exhaustive extraction with acetone prior to isolation, thus precluding the presence of such contaminants. Moreover such signals were present in appreciable amounts only in the biotreated sample. In addition, the occurrence of aliphatic chains has been reported,²¹ in lignin biotreated with *Chrysomya sitophila* with no explanation as to their occurrence. The oxidative degradation of lignin models by fungi has been reported to take place via the 3-oxoadipate pathway which leads to aromatic ring cleavage with concomitant formation of aliphatic chains bearing carboxylic acids. Furthermore the growth of white-rot fungi is associated with the production of hydroxyl radicals, hydrogen peroxide and superoxide anion radicals.²² It is also known that the presence of superoxide anion radical can induce aromatic ring cleavage.²³

With the aim to characterize in more detail the structure of wheat lignin and to identify the nature of the observed signals by establishing the complete spin systems responsible for those signals, a 2D homocorrelated NMR experiment (HOHAHA), providing enhanced spin lock lengths, enough to detect the full spin system, was performed on samples of acetylated lignins. Figure 5 shows the HOHAHA spectrum of ML-*Le*. The spectrum presents the correlation peaks previously reported for the main lignin interunit linkages, and is analogous to that of non-treated wheat straw lignin.¹² Nevertheless there is a significant difference: the aliphatic region shows several strong correlations. In particular, five different spin systems, A–E containing aliphatic signals were identified and tentatively assigned (Figure 5B, Table 2). The HOHAHA experiments showed that the signals centered at 1.1–1.6 and 0.8–0.9 ppm are part of a more complex spin system. The correlations A–D are

Table 2. Correlation peaks derived from the phase sensitive 2D HOHAHA spectrum of acetylated wheat straw lignin after fungal decay

Cross peak	δ (ppm)
A	1.25–1.56–2.30–4.10
B	1.27–1.57–2.26
C	1.17–3.45–3.50
D	1.60–2.02–2.21–5.10
E	0.85–1.22–1.31

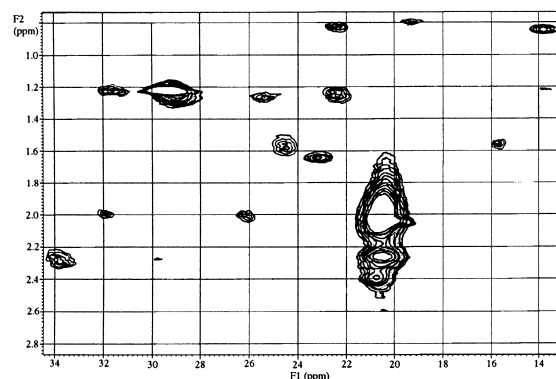


Figure 6. 2D HMQC NMR spectrum of acetylated milled straw lignin after fungal decay: enlarged aliphatic section.

due to aliphatic chains ether or ester bonded. Their presence could be tentatively explained considering possible radical reactions occurring on the muconate moieties initially generated by the 3-oxoadipate pathway. In fact such double bonds conjugated to carboxylic or carboxylate groups could easily undergo radical addition reactions²⁴ and/or oxidative decarboxylation to yield the aliphatic chains ether or ester linked to lignin residues, responsible for the observed spin systems. The persistent presence of aliphatic methyl groups in such correlations and the occurrence of correlation E, due to pure aliphatic chains, could be attributed to possible reductive stages occurring during the degradation of the lignin backbone. The aliphatic nature of such peaks was further confirmed by a 2D heteronuclear NMR experiment: HMQC. The peaks centered at 0.8–0.9 ppm and 1.1–1.6 ppm show ¹³C chemical shifts consistent with the proposed assignment (Figure 6).

Conclusions

On the basis of these data it is possible to speculate that, at least during the early stages of wheat lignin biodegradation, the lignin backbone might undergo an oxidation leading to aromatic ring cleavage followed by the loss of low molecular weight oxidized fragments such as formic or oxalic acids. This hypothesis is further substantiated by the recovery of formate and oxalate esters in lignin obtained from bagasse degraded by *L. edodes*.¹⁰

The white-rot basidiomycete *L. edodes* is an effective degradation agent of wheat straw lignin. The biodegradation was also characterized by some stereoselective side-chain oxidation processes. In addition to the presence of new aliphatic acids and ethers the residual lignin polymer contained a higher carboxylic acid content,

and lower phenolic OH, aliphatic OH and methoxy contents.

Experimental

Quantitative ^{31}P NMR spectra were obtained on a Varian XL-300 spectrometer by using methods identical to those described by Argyropoulos.^{12,15} The chemical shifts were referenced to phosphoric acid. The ^{31}P NMR data reported in this effort are averages of three phosphorylation experiments followed by quantitative ^{31}P NMR acquisitions. The maximum standard deviation of the reported data was 2.10^{-2} mmol/g, while the maximum standard error was 1.10^{-2} mmol/g. ^1H - and 2D NMR spectra were acquired at 25°C on a Varian Unity 500 NMR spectrometer using a 5 mm inverse detection probe (DHP). The chemical shifts were referenced to tetramethylsilane (TMS). Elemental and methoxy groups analyses were carried out by Schwarzkopf Microanalytical, Woodside, N.Y. UV spectra were recorded on a Perkin–Elmer Lambda 2 spectrophotometer.

Biodegradation of wheat straw. Wheat straw was submitted to fungal colonization by solid-state fermentation at Tuscia University. *Lentinula edodes*, strain SC 495, was kindly provided by Prof. T. H. Quimio (Los Banos, Philippines). Inoculum was produced by growth at 30°C in shaken 250 mL flasks in a liquid medium described previously.^{2,25} A liquid salt medium was added to wheat straw (cut into 5 cm pieces), so as to obtain a final moisture content of 75%. After steam-sterilization of 1 h, the substrate was inoculated aseptically (5% w/w) and incubated at 30°C for 7 days in a rotary bioreactor at the mixing rate of one revolution per day.²⁴ The carbon dioxide concentration was kept constant at 5.0%. After biotreatment the straw was dried in a vacuum oven at 40°C . During the fungal treatment about 15% of the initial straw weight was lost. The lignin content, determined by the Van Soest method,²⁶ showed a reduction from 8.17 to 7.12 g for 100 g of dry weight. As an index of the content of mycelium, the quantification of glucosamine from the hydrolysis of fungal chitin was performed and was found to be 1.8 mg/g.

Preparation of milled lignins. Milled lignins were prepared from ultra ground, extractives free powder according to Bjorkman's procedure with some modification. Extractive free powders, prepared by milling the straw samples at 40 mesh followed by exhaustive extraction with acetone:water (9:1, v/v), were ultra ground for 3 weeks in a rotary ball mill. The ML fractions were then extracted with dioxane:water (96:4, v/v). The residues were concentrated under reduced

pressure and freeze dried. Purification ensued by dissolving the lignins in 90% acetic acid. The solution were then added dropwise to stirred water. The precipitated lignins were centrifuged and freeze dried. They were then dissolved again in a mixture of 1,2-dichloroethane: ethanol (2:1, v/v) and precipitated in diethyl ether. The resulting products were about 82% lignins as evidenced from UV lignin content measurements.

Elemental and methoxy groups analyses. ML (Milled wheat straw Lignin) (%): C (54.58), H (5.56), O (31.02), OCH_3 (14.45). ML-*Le* (Milled Lignin from wheat straw biotreated with *L. edodes*) (%): C (60.41), H (6.48), O (29.43), OCH_3 (13.00).

Lignin acetylation. Acetylation was carried out with pyridine:acetic anhydride (1:1) at 25°C for 48 h.¹²

Quantitative ^{31}P NMR. Derivatization of the lignin samples with 2-chloro-1,3,2-dioxaphospholane (I)^{13,16} and 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (II)^{12,14,15} were performed as previously described. Samples of lignin, (30 mg) accurately weighed, were dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1 v/v ratio (0.5 mL). The phospholane (100 μL) was then added, followed by the internal standard and the relaxation reagent solution (100 μL each).

^1H - and 2D homonuclear NMR spectroscopy (HO-HAHA).^{12,17} Such NMR spectra were recorded using lignin (30 mg) dissolved in CDCl_3 (0.6 mL). HOHAHA spectra were acquired over a 9.5 ppm window in both F_2 and F_1 , with MLEV-17 spin lock length 80 ms. $2\text{K} \times 256$ Increments were acquired. After F_1 zero-filling, Fourier transformation and squared cosine-bell apodization, the transformed data matrix was $1024 (F_2) \times 256 (F_1)$ real points.

2D Heteronuclear NMR spectroscopy (HMQC). HMQC spectra were acquired over a 9.5 ppm window in F_2 (^1H) and 140 ppm in F_1 (^{13}C) with GARP-1 decoupling. BIRD presaturation of ^1H - ^{13}C magnetization and a pre-HMQC delay of 400 ms were used. $2\text{K} \times 256$ increments were acquired with simultaneous data acquisition. After F_1 zero filling, Fourier transformation and squared cosine bell apodization, the transformed data matrix was $1024 (F_2) \times 512 (F_1)$ real points.^{17,27,28}

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