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Structural Characterization of Lignin during *Pinus taeda* Wood Treatment with *Ceriporiopsis subvermispora*

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***Pinus taeda* wood chips were biotreated with *Ceriporiopsis subvermispora* under solid-state fermentation for periods varying from 15 to 90 days. Milled wood lignins extracted from sound and biotreated wood samples were characterized by wet-chemical and spectroscopic techniques. Treatment of the lignins by derivatization followed by reductive cleavage (DFRC) made it possible to detect DFRC monomers and dimers that are diagnostic of the occurrence of arylglycerol-β-*O*-aryl and β-β, β-5, β-1, and 4-*O*-5 units in the lignin structure. Quantification of these DFRC products indicated that β-*O*-aryl cleavage was a significant route for lignin biodegradation but that β-β, β-5, β-1, and 4-*O*-5 linkages were more resistant to the biological attack. The amount of aromatic hydroxyls did not increase with the split of β-*O*-4 linkages, suggesting that the β-*O*-4 cleavage products remain as quinone-type structures as detected by UV and visible spectroscopy. Nuclear magnetic resonance techniques also indicated the formation of new substructures containing nonoxygenated, saturated aliphatic carbons (CH_2 and CH_3) in the side chains of lignins extracted from biotreated wood samples.**

Lignin is a heterogeneous and highly cross-linked macromolecule that is particularly resistant to biological degradation (19). The bulk of lignin in wood consists of nonphenolic arylglycerol-β-*O*-aryl ether units. Other units, such as phenylcoumaran (β-5), resinol (β-β), and dibenzodioxocin (5-5/β-*O*-4,α-*O*-4) are also present in the lignin moiety (8, 30).

Although lignin is recalcitrant, some specialized fungi developed the ability to degrade this complex molecule. Most of these fungi belong to the basidiomycetes and are responsible for the white-rot decay process (19). White-rot fungi use an intricate oxidative complex to degrade lignin, which is based on oxidative enzymes and low-molecular-mass mediators (28). Several white-rot fungi produce a lignin peroxidase (LiP) that can initiate one-electron oxidation of nonphenolic arylglycerol-β-*O*-aryl ether units. This oxidation is followed by Cα-Cβ or β-*O*-aryl cleavage to give aromatic aldehydes or phenylglycerols, respectively, as primary products (19). On the other hand, some white-rot fungi degrade lignin efficiently without producing LiP (20). One of them, *Ceriporiopsis subvermispora*, has been extensively studied (15, 16, 17, 31, 32), owing to its suitability for biopulping (1, 27). Although the industrial applicability of this fungus has been recognized, its ligninolytic strategies are not completely understood. It has often been assumed that lignin degradation by *C. subvermispora* is dependent on manganese-dependent peroxidases and laccases (28). The occurrence of lignin degradation inside the wood matrix during the first stages of wood decay, when low cell wall permeability does not permit enzyme diffusion, has clearly indicated that some low-molecular-mass agents also act in the early stages of lignin biodegradation (2). Lipid peroxidation induced by manganese-dependent peroxidase has been proposed for lignin biodegradation by *C. subvermispora* (6, 17, 18, 32). Studies with lignin model compounds have shown that nonphenolic arylglycerol-β-*O*-aryl units are disrupted by means of one-electron oxidation mechanisms similar to the routes proposed for basidiomycetes that produce LiP (18, 32).

Under solid-state fermentation of wood, *C. subvermispora* extensively depolymerizes lignin. Lignin depolymerization has been demonstrated in studies based on the molecular mass distribution of milled wood lignins (MWLs) recovered from biotreated wood samples (15) and in studies using in situ derivatization followed by reductive cleavage (DFRC) of lignin in biotreated wood, which indicates lignin depolymerization as a consequence of aryl-ether degradation (16).

The present study provides additional data for evaluating lignin biodegradation by *C. subvermispora* during solid-state fermentation of *Pinus taeda* wood. The structural characteristics of MWLs extracted from sound (untreated) and biotreated wood samples were elucidated using wet-chemical and spectroscopic analyses.

MATERIALS AND METHODS

Fungus, inoculum preparation, and wood biodegradation. *C. subvermispora* (Pilat) Gilbertson et Ryvarden cultures were maintained on 2% (wt/vol) malt extract agar plates at 4°C. The wood chips (approximately 2.5 by 1.2 by 0.2 cm) used in this work came from a single log of a 28-year-old *P. taeda* tree.

Biodegradation experiments were carried out in 20-liter polypropylene bioreactors. Precolonized wood chips were used as inoculum seed to start the colonization of fresh wood chips. The inoculum seed was prepared by growing the fungus for 10 days in 2-liter Erlenmeyer flasks containing 200 ml of malt extract agar medium. After fungal growth on the medium surface (7 days), 50 g of sterilized wood chips was mixed with the cultures and incubated at 27°C for 30 days. Each 20-liter bioreactor was loaded with 2,000 g of freshly sterilized wood chips and 100 g of inoculum seed, shaken by hand, and stored at 27°C for 15, 30, 60, or 90 days (16). Humidified air, passing through a 0.2-μm-pore-size membrane, was provided to the bioreactors (23 liters h^{-1}) throughout biodegradation. Two cylindrical containers (9 cm in height and 5 cm in diameter) made of a 0.84-mm-pore-size steel screen were filled with 25 g of sterilized wood chips and inserted into each bioreactor just before inoculation. After the biotreatment, the bioreactors were opened, and the wood chips were washed with water to

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remove the superficial mycelium. The wood chips contained in the cylindrical containers were washed as well. These decayed wood chips were air dried, their moisture contents were determined, and the calculated initial and final dry weights were used to determine weight losses. The weight loss resulting from sterilization (1.0% ± 0.1%) was subtracted from the weight loss of each of the biodegraded samples. Component losses were calculated on the basis of the weight loss values and chemical analysis of the decayed wood chips recovered from the two cylindrical containers.

Chemical analysis of wood samples. The wood samples were milled in a knife mill to pass through a 0.75-mm-pore-size screen. Milled wood was treated with 95% ethanol for 6 h in a Soxhlet apparatus to remove extractives. Extracted wood samples were hydrolyzed with 72% (wt/wt) sulfuric acid at 30°C, as previously described by Ferraz et al. (10). The residual material was cooled and filtered through porous glass filter number 3 (Schott-Duran). Solids were dried to a constant weight at 105°C and determined to be Klason insoluble lignin. The soluble lignin concentration in the aqueous fraction was determined by measuring the absorbance at 205 nm (A_{205}) and using the value of 105 liters · g^{-1} · cm^{-1} as the absorptivity of soluble lignin. After acid hydrolysis, soluble sugars were quantified by high-pressure liquid chromatography with an HPX-87H column, heated at 45°C, and eluted with 5 mM sulfuric acid at 0.6 ml · min^{-1}. Total hemicellulose and glucan values were derived from a calculation considering the acid-released monomers glucose and xylose (10). Duplicate hydrolysis experiments were performed. The deviation between duplicates was lower than 3% of the average value.

Lignin isolation and characterization. MWL was extracted from ground extractive-free wood samples, according to the method of Lawther et al. (21). After drying over P_2O_5, 90 g of the extractive-free wood was moistened in toluene and ground in a rotary porcelain ball mill at room temperature for 130 h at 96 rpm with a porcelain ball/wood weight ratio of 20:1. The ball-milled samples were then extracted twice with dioxane-water solution (96%, vol/vol) in a shaker at 190 rpm for 24 h at 27°C in the dark. The extractions were performed by using 10 ml of solvent per g of ball-milled wood. The dioxane-water extracts were combined, and the solvents were removed at 35°C under reduced pressure. The remaining solids were dried under vacuum over P_2O_5. The yield of MWL recovered through this procedure was 4.4% ± 0.4% for the undecayed wood sample and 4.8 to 5.7% for the biotreated wood samples.

DFRC was performed as described by Lu and Ralph (25, 26). 4-Acetoxy-3-methoxycinnamyl acetates (DFRC monomers, both *cis* and *trans* isomers) were separated by gas chromatography-mass spectrometry (GC-MS) and identified based on their mass spectra, which were identical to those from authentic compounds, including coniferyl diacetate (*m/z* [relative intensity] = 264 [9], 222 [100], 179 [50], 163 [11], 151 [14], 131 [20], 119 [12], 91 [9]). The amounts of each monomer were determined by GC and flame ionization detection by using a response factor (1.40) derived from pure monomer standards, with 4,4′-ethylene bisphenol as the internal standard. DFRC products were also analyzed by GC and MS under conditions optimized to detect dimers. The GC-MS analysis was performed on a Finnigan MAT GCQ instrument equipped with a DB-5 column (30 m by 0.25 mm). The column temperature was maintained initially at 150°C for 1.0 min and then was heated up to 300°C at 10°C min^{-1}. This final temperature was maintained for 15 min. Helium at a linear velocity of 33 cm/s was used as a carrier gas. The injector, transfer line, and ion source temperatures were 280, 310, and 170°C, respectively. A sample volume of 1 μl was injected into the column by the splitless mode. The filament was turned on after 12 min, and the split valve was opened after 3 min. The structures proposed for the dimers detected were based on pattern recognition of MS spectral information (29) (see Fig. 3): for compound 1, *m/z* (relative intensity) 370 (9), 328 (34), 311 (1), 286 (100), and 255 (18); compound 2, *m/z* 400 (2), 358 (10), 298 (31), 209 (100), 167 (20), and 107 (90); compound 3, *m/z* 544 (2), 484 (29), 424 (10), 382 (33), 248 (52), 195 (50), and 137 (100); compound 4, *m/z* 530 (1), 488 (14), 446 (11), 386 (6), 326 (3), 189 (10), and 137 (100); and compound 5, *m/z* 484 (11), 382 (14), 322 (100), 289 (20), 207 (17), and 146 (12). The relative amount of individual dimers was estimated as the ratio between dimer and internal-standard (4,4′-ethylene bisphenol) peak heights divided by the mass (in milligrams) of Klason lignin in each sample.

UV and visible spectra were recorded in a Cintra 20-GBC spectrophotometer. The sample was dissolved in 96% dioxane-water solution, and the spectra between 205 and 600 nm were recorded. Fourier transform infrared (FTIR) spectra were obtained with a Nicolet 520 FTIR spectrophotometer and a KBr disk containing 0.5% of the sample. The elemental analysis and methoxyl contents were determined by following methods previously described (11).

^1H, ^{13}C, distortionless enhancement by polarization transfer with a θ of 135° (DEPT-135), and heteronuclear multiple quantum coherence (HMQC) spectra were obtained with a Bruker DRX-360 instrument. Approximately 80 mg of each acetylated sample was dissolved in 0.5 ml of acetone-d_6 (deuterium acetone), and the solvent peak was used as an internal reference (δ_H 2.04, δ_C 29.8). Acetylation of lignin samples was performed with acetic anhydride in pyridine as described by Lenz (22). DEPT subspectra were taken with θ equal to 135° and coupling constant $^1J_{^{13}C\text{-}^1H}$ equal to 150 Hz. HMQC experiments were performed by using the standard Bruker implementation.

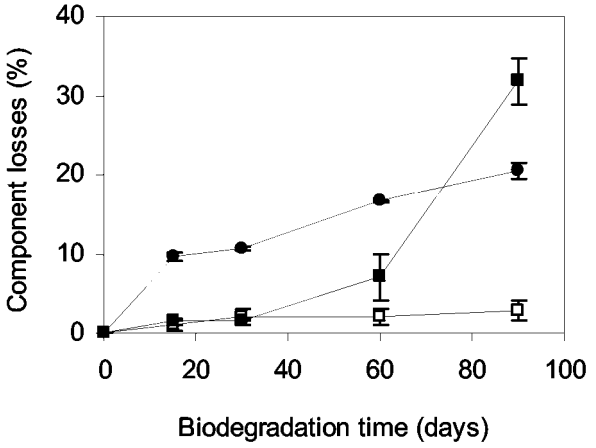

FIG. 1. Component losses of lignin (filled circles), hemicellulose (filled squares), and glucan (open squares) from *P. taeda* wood chips biotreated by solid-state fermentation with *C. subvermispora*.

RESULTS

Weight and component losses during the biotreatment of *P. taeda* with *C. subvermispora*. The weight losses of *P. taeda* biotreated with *C. subvermispora* were 2.3% ± 0.5%, 3.0% ± 0.4%, 9% ± 2%, and 13.8% ± 0.7% after 15, 30, 60, and 90 days, respectively. Lignin loss was high during these periods, reaching 9.6% ± 0.6% after 15 days and 22% ± 1% after 90 days of biodegradation. Glucan removal was lower than 2% during all biodegradation periods, while hemicellulose loss was high only after 60 days of biotreatment (Fig. 1). This pattern of wood decay, which is often described as selective lignin degradation, is consistent with previous studies reported for softwood biodegradation by *C. subvermispora* (1, 9, 27).

Characterization of lignin contained in biotreated wood samples. In previous work, DFRC was used to quantify *cis*- and *trans*-4-acetoxycinnamyl acetates (DFRC monomers) released from *P. taeda* samples biotreated with *C. subvermispora* (16). The use of in situ DFRC lignin analysis made it possible to demonstrate that aryl-ether linkages were cleaved during wood biodegradation, since the yield (based on the weight of Klason lignin) of such monomers decreased significantly with biodegradation time. In the present work, MWLs were extracted from biotreated samples for further characterization of the lignin degradation process. The extraction procedure was selected based on minimal damage to the lignin structure (21). The MWL yield resulting from this procedure was in the range of 4 to 5% (in grams per 100 g of Klason lignin). Despite the low yield, MWL proved to be representative of the overall lignin structure since the yields of DFRC monomers released from these lignins decreased with biodegradation time (Fig. 2), as previously observed by Guerra et al. (16) for the in situ DFRC technique. After detection of the primary monomers originating from DFRC degradation of MWL, the samples were rean-

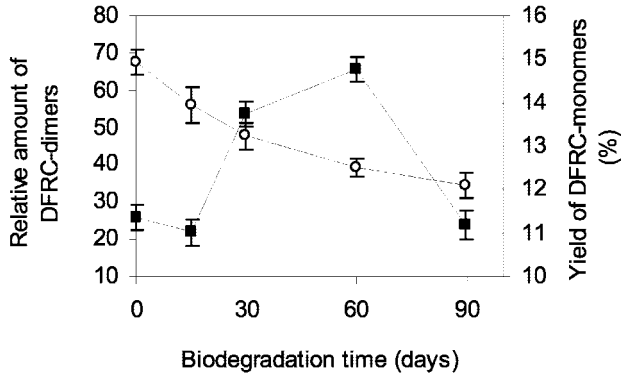

FIG. 2. Yields of DFRC monomers and dimers after DFRC degradation of MWLs extracted from sound and *C. subvermispora*-biotreated *P. taeda* wood chips. The yields of DFRC monomers (○) were calculated on the basis of Klason lignin content by use of response factors. The relative amount of DFRC dimers (■) was estimated as the ratio between dimer and internal-standard peaks divided by the Klason lignin content (in milligrams).

alyzed by GC and MS under conditions optimized to detect the main dimers connected by linkages other than β-*O*-aryl ether (see Materials and Methods). With this procedure, it was possible to quantify relative levels of the DFRC dimers (compounds 1 to 5) (Fig. 3) representing β-1, β-β, β-5, and 4-*O*-5 substructures in softwood lignins.

C. subvermispora degraded arylglycerol-β-*O*-aryl units (yield of DFRC monomers decreased), while the relative amount of dimers increased in 60 days of biotreatment (Fig. 2). These data suggest that carbon-carbon and 4-*O*-5 linkages were not cleaved during the first stages of the biological attack. However, the significant reduction in the amount of DRFC dimers after 90 days of biodegradation indicates that even C—C linkages are degraded after long biodegradation periods. This observation corroborates a previously published statement that β-5 models are degraded by *C. subvermispora* in submerged liquid cultures (5).

TABLE 1. Absorptivities in the UV and visible-light regions of MWLs extracted from sound and *C. subvermispora*-biotreated *P. taeda* wood chips

Biodegradation time (days)	Absorptivity (liter · g^{-1} · cm^{-1}) at the following wavelength (nm):			Absorptivity ratio at the following wavelengths (nm/nm):	
	280	310	420	280/310	280/420
0[a]	15.4	6.9	0.1	2.2	15.4
15	15.6	5.9	0.1	2.4	15.6
30	17.8	8.4	0.2	2.1	89
60	18.4	8.2	0.2	2.2	88
90	14.7	6.1	0.2	2.4	73

[a] Control.

The UV and visible spectral data on MWLs recovered from sound and biotreated wood samples are shown in Table 1. The ratios of absorptivity measured at 280 nm to absorptivity measured at 310 nm (a_{280}/a_{310}) were almost constant over 90 days of biodegradation, while the a_{280}/a_{420} ratios increased with biodegradation time. Constant values for a_{280}/a_{310} ratios indicate that α,β unsaturated and/or α-carbonyl groups did not change significantly during *P. taeda* biotreatment, while increases in a_{280}/a_{420} ratios suggest the formation of quinone-like substructures in biodegraded lignin (14).

The FTIR spectra of sound and biodegraded lignins were characteristic of a guaiacyl-type lignin (7). The absorbance ratios of bands at 1,730 and 1,660 cm^{-1} (assigned to aromatic-ring nonconjugated and conjugated carbonyl groups, respectively), with the reference band at 1,510 cm^{-1} (assigned to the aromatic ring), were almost unchanged over 90 days of bio-

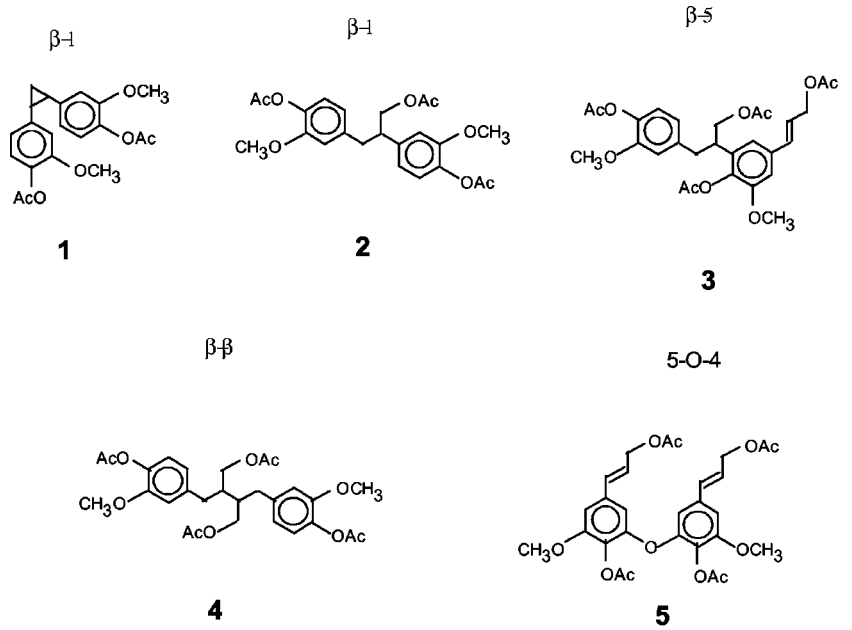

FIG. 3. Structures of dimers identified in the DFRC products of MWLs extracted from sound and *C. subvermispora*-biotreated *P. taeda* wood chips.

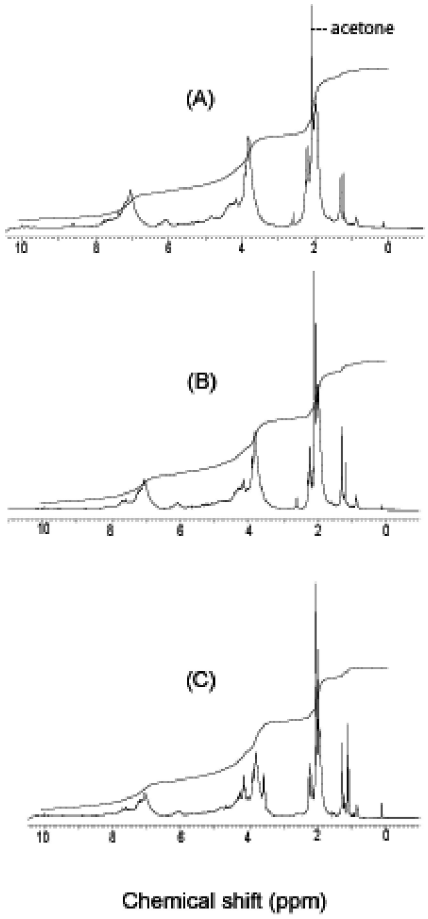

FIG. 4. ^{1}H-NMR spectra of MWLs extracted from sound and *C. subvermispora*-biotreated *P. taeda* wood chips. (A) MWL from sound wood; (B) MWL from 60-day biotreated sample, (C) MWL from 90-day biotreated sample.

degradation. The constancy of these ratios indicated that the biodegradation process was not followed by the formation of carboxyl or carbonyl groups in the side chains of the residual MWLs.

The ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra of acetate derivatives of MWLs were integrated in regions corresponding to different functional groups, as indicated in Fig. 4 and Table 2. Although some of these signals are not completely resolved, the integration of ^{1}H-NMR regions has long been applied to estimate the degree of aromatic-ring substitution and the contents of lignin-functional groups, such as aromatic and aliphatic hydroxyls, methoxyls, and β-*O*-4 structures (3, 12, 13, 22). Quantitative estimates were made by correlating the total integral values with the number of protons per C_9 unit in each MWL. The methoxyl contents as determined by wet-chemical analysis and ^{1}H-NMR were in good agreement, supporting the use of ^{1}H-NMR integrals for estimating the contents of functional groups in such lignins (Table 2). The amount of Hβ in β-*O*-4 units diminished with biodegradation, confirming the arylglycerol-β-*O*-4 aryl ether degradation detected by the DFRC method. Interestingly, the amount of aromatic hydroxyls did not increase with the split of β-*O*-4 linkages, suggesting that the β-*O*-4 cleavage products remain as quinone-type structures, as previously detected by UV and visible spectroscopy. MWLs from biodegraded wood samples also presented a reduced number of aromatic protons per C_9 unit, confirming that these lignins are more condensed than MWLs from sound wood. The contents of protons with chemical shifts lower than 1.5 ppm also increased with biodegradation time concomitantly with the reduction in the contents of aliphatic hydroxyl groups. Routine ^{13}C-NMR spectra of acetylated MWLs showed, in addition to the usual signals observed in the spectra of softwood lignins, a new signal at 14.7 ppm in lignins recovered from biotreated samples. This signal was assigned to a saturated carbon in the aliphatic side chain of the lignin moieties, in accordance with ^{1}H-NMR observations. Further structural information related to these substructures was provided by two-dimensional NMR. The most revealing two-dimensional technique was HMQC, which gives information about the correlation between ^{1}H and ^{13}C via $^1J_{C\text{-}H}$. The aliphatic region of the spectra of MWLs recovered from biodegraded wood samples differed from the spectra of MWLs from sound wood as illustrated in Fig. 5. The spectra of MWLs from biodegraded samples showed two signals (Fig. 5B, 1 and 2) that did not appear in the HMQC spectra of MWLs from sound wood (Fig. 5A). The chemical shifts of these signals are consistent with the presence of CH_3 (δ_C/δ_H, 14/1.2 ppm) and nonoxygenated CH_2 (δ_C/δ_H, 32/1.9 ppm) groups. The occurrence of these groups was also supported by DEPT-135 spectra of biodegraded lignins that presented positive and negative signals at 14 ppm (CH_3) and 32 ppm (CH_2), respectively; in DEPT-135 spectra, a negative signal indicates the presence of CH_2, while a positive signal derives from CH or CH_3 carbons.

DISCUSSION

The DFRC method specifically cleaves arylglycerol-β-*O*-aryl ether linkages in lignin (23–26) to yield 4-hydroxycinnamyl acetates (DFRC monomers) used to estimate the number of β-*O*-aryl ether linkages in lignin (16, 23–26). Peng et al. (29) also reported the feasibility of using DFRC dimers to identify β-1, β-β, β-5, and 4-*O*-5 substructures present in the lignin structure. In the present work, DFRC of MWLs extracted from *P. taeda* biotreated with *C. subvermispora* showed that the yield (based on the weight of Klason lignin) of DFRC monomers

TABLE 2. ^{1}H-NMR spectral data of MWLs extracted from sound and *C. subvermispora*-biotreated *P. taeda* wood chips

Chemical shift (ppm)	Functional group	No. of protons per C_9 unit of the MWLs at the following biodegradation time (days)[a]:				
		0[b]	15	30	60	90
6.2–7.9	Aromatic H	2.4	2.2	2.1	2.2	2.2
4.5–5.2	Hβ of β-*O*-4 aryl ether units	1.2	1.0	0.7	0.8	0.8
3.5–3.9	H in methoxyl groups[c]	2.7	2.5	2.7	2.5	2.5
2.2–2.5	Phenolic H	0.3	0.2	0.2	0.2	0.2
1.5–2.2	Aliphatic hydroxyl H	1.6	1.1	1.1	1.0	1.0
0.0–1.5	H in nonoxygenated saturated carbons	0.6	0.9	1.1	1.2	1.3

[a] Values were derived from spectral analysis of the acetylated lignins.
[b] Control.
[c] Methoxyl groups determined by wet-chemical analysis of these samples were in the range of 0.9 to 1.0 per C_9 unit, corresponding to 2.7 to 3.0 protons per C_9 unit.

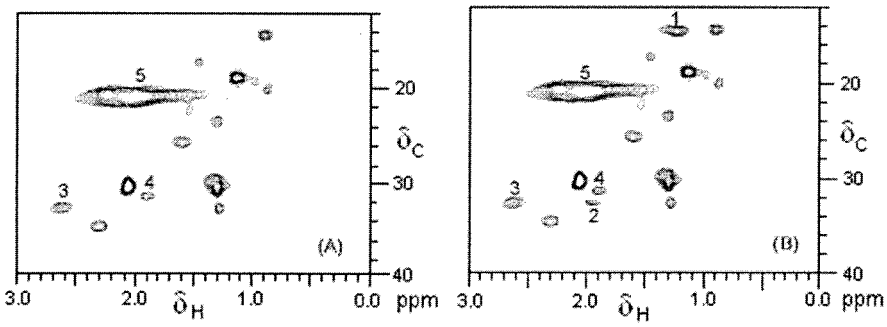

FIG. 5. Expanded aliphatic region of the HMQC spectra of MWLs extracted from sound and *C. subvermispora*-biotreated *P. taeda* wood chips. (A) MWL from sound wood; (B) MWL from 90-day biotreated sample. The spectra of all biodegraded samples were identical. Signal assignment: 1, CH_3; 2, nonoxygenated CH_2; 3, Cα-Hα in guaiacyl-$CH_2CH_2CH_2OH$; 4, Cβ-Hβ in guaiacyl-$CH_2CH_2CH_2OH$; 5, CH_3 in methoxyl. The origins of the other signals have not been elucidated.

decreased during biodegradation but that the yield of DFRC dimers increased in the first 60 days and decreased afterwards. These results indicate that β-1, β-5, β-β, and 4-*O*-5 substructures were more resistant to the biological attack and that arylglycerol-β-*O*-aryl units were degraded, resulting in a depolymerized residual lignin. A previous study with these MWLs also showed a similar trend, since the MWL average molecular mass decreased with biodegradation time (15).

Cleavage of β-*O*-aryl ether or even Cα-Cβ linkages might explain the yield decrease of DFRC monomers, since substructures like guaiacyl-4-*O*-CH_2CH_2OH, guaiacyl-CHO, and guaiacyl-COOH resulting from Cα-Cβ cleavage will not produce 4-acetoxy-3-methoxycinnamyl acetates (DFRC monomers) when subjected to DFRC. Actually, both oxidation reactions have been detected during biodegradation of lignin model compounds by *C. subvermispora* (32). The present study provided no evidence of accumulation of residual lignin substructures with α-carbonyls or benzaldehyde or benzoic acid end groups, the last two being diagnostic of Cα-Cβ cleavage. Data from UV and FTIR spectroscopy, ^{1}H-NMR and especially ^{13}C-NMR (absence of new signals from 168 to 194 ppm), and HMQC spectroscopy (absence of a cross-signal at δ_C/δ_H 82 to 5.6 ppm, which is characteristic of α-keto-β-aryl ether units) supported this observation. There are two possible explanations for the absence of these residual products. One is that these substructures may be intermediary products that do not persist in the cultures, being transformed beyond recognition or released as water-soluble fractions. The second possible explanation is that *C. subvermispora* did not induce Cα-Cβ cleavage under solid-state fermentation conditions.

The assessment of the structures of MWLs recovered from biotreated wood samples by the compilation of different characterization procedures also indicated the formation of new substructures containing nonoxygenated, saturated aliphatic carbons. The occurrence of such substructures can be assumed based on the following factors: (i) the increased contents of protons linked to saturated carbons observed by ^{1}H-NMR; (ii) the appearance of new saturated carbons as revealed by ^{13}C-NMR; (iii) the presence of signals 1 and 2 in the high-field region of the HMQC spectrum (Fig. 5); and (iv) the observation of positive and negative signals in the DEPT-135 spectrum corresponding to signals 1 and 2 in Fig. 5, respectively.

The possibility of assignment of these nonoxygenated, saturated aliphatic carbons to residual wood extractives or fungal metabolite contaminants in MWLs should not be ruled out. However, the formation of new substructures containing nonoxygenated β-CH_2 in biodegraded lignins was also observed in wood decayed by *Phanerochaete chysosporium* (4, 33) and by *Chrysonilia sitophila* (12). This substructure might be explained by a β-*O*-aryl ether cleavage or even by the reduction of minor amounts of cinnamyl alcohol end groups. However, the presence of CH_3 groups cannot be explained directly. This group suggests an ability of *C. subvermispora* to produce reductive reactions on the side chain of the lignin moiety or to incorporate new saturated chains in the lignin structure.

In conclusion, β-*O*-aryl ether cleavage was a significant route for lignin degradation during the solid-state fermentation of *P. taeda* by *C. subvermispora*, corroborating previous work by Srebotnik et al. (32), who identified diagnostic products of β-*O*-aryl ether cleavage during solid-state fermentation of a lignin model compound supported on cellulose sheets. On the other hand, the accumulation of new saturated aliphatic carbons instead of oxygenated by-products in the lignin remains to be explained for the lignin biodegradation resulting from the solid-state fermentation of wood chips by this fungus.

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REFERENCES

1. **Akhtar, M., R. A. Blanchette, G. Myers, and K. Kirk.** 1998. An overview of biomechanical pulping research, p. 309–383. *In* R. Young and M. Akhtar (ed.), Environmentally friendly technologies for the pulp and paper industry. Wiley, New York, N.Y.
2. **Blanchette, R. A., E. W. Krueger, J. E. Haight, M. Akhtar, and D. E. Akin.** 1997. Cell wall alterations in loblolly pine wood decayed by the white-rot fungus *Ceriporiopsis subvermispora*. J. Biotechnol. **53:**203–213.
3. **Chen, C. L., and D. Robert.** 1988. Characterization of lignin by ^{1}H-NMR and ^{13}C-NMR spectroscopy. Methods Enzymol. **161:**137–174.
4. **Chua, M. G., C. L. Chen, H. M. Chang, and T. K. Kirk.** 1982. ^{13}C-NMR spectroscopy study of spruce lignin degraded by *Phanerochaete chrysosporium*. Holzforschung **36:**165–172.
5. **Daine, S., M. Orlandi, G. Bestetti, C. Wiik, and G. Elegir.** 2002. Degradation of β-5 lignin model dimers by *Ceriporiopsis subvermispora*. Enzyme Microb. Technol. **30:**499–505.

6. **Enoki, M., T. Watanabe, S. Nakagame, K. Koller, K. Messner, Y. Honda, and M. Kuwahara.** 1999. Extracellular lipid peroxidation of selective white-rot fungus, *Ceriporiopsis subvermispora*. FEMS Microbiol. Lett. **180:**205–211.
7. **Faix, O.** 1991. Classification of lignins from different botanical origins by FTIR spectroscopy. Holzforschung **45:**21–27.
8. **Fengel, D., and G. Wegener.** 1989. Wood chemistry, ultrastructure and reactions. Walter de Gruyter, Berlin, Germany.
9. **Ferraz, A., J. Rodríguez, J. Freer, and J. Baeza.** 2001. Biodegradation of *Pinus radiata* softwood by white- and brown-rot fungi. World J. Microbiol. Biotechnol. **17:**31–34.
10. **Ferraz, A., J. Rodríguez, J. Freer, and J. Baeza.** 2000. Estimating chemical composition of biodegraded pine and eucalyptus by DRIFT spectroscopy and multivariate analysis. Bioresour. Technol. **74:**201–212.
11. **Ferraz, A., J. A. Souza, F. T. Silva, A. R. Gonçalves, R. E. Bruns, A. R. Cotrim, and R. Wilkins.** 1997. Controlled release of 2,4-D from granule matrix formulations based on six lignins. J. Agric. Food Chem. **45:**1001–1005.
12. **Ferraz, A., and N. Durán.** 1995. Lignin degradation during softwood decaying by the ascomycete *Chrysonilia sitophila*. Biodegradation **6:**265–274.
13. **Froass, P. M., A. J. Raugaskas, and J. Jiang.** 1996. Chemical structure of residual lignin from kraft pulp. J. Wood Chem. Technol. **16:**347–365.
14. **Goldshimd, O.** 1971. Ultraviolet spectra, p. 241–266. *In* K. V. Sarkanen and L. H. Ludwid (ed.), Lignin: occurrence, formation, structure and reaction. Wiley, New York, N.Y.
15. **Guerra, A., R. Mendonça, and A. Ferraz.** 2003. Molecular weight distribution of wood components extracted from *Pinus taeda* biotreated by *Ceriporiopsis subvermispora*. Enzyme Microb. Technol. **33:**12–18.
16. **Guerra, A., R. Mendonça, and A. Ferraz.** 2002. Characterization of the residual lignins in *Pinus taeda* biodegraded by *Ceriporiopsis subvermispora* by using in situ CuO oxidation and DFRC methods. Holzforschung **56:**157–160.
17. **Jensen, K. A., Jr., W. Bao, S. Kawai, E. Srebotnik, and K. E. Hammel.** 1996. Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis subvermispora* in the absence of lignin peroxidase. Appl. Environ. Microbiol. **62:**3679–3686.
18. **Kapich, A. N., K. A. Jensen, and K. E. Hammel.** 1999. Peroxyl radicals are potential agents of lignin biodegradation. FEBS Lett. **461:**115–119.
19. **Kirk, T. K., and D. Cullen.** 1998. Enzymology and molecular genetics of wood degradation by white-rot fungi, p. 273–308. *In* R. Young and M. Akhtar (ed.), Environmentally friendly technologies for the pulp and paper industry. Wiley, New York, N.Y.
20. **Kuhad, R. C., A. Singh, and K. L. Eriksson.** 1997. Microorganisms and enzymes involved in the degradation of plant fiber cell walls. Adv. Biochem. Eng. Biotechnol. **57:**45–126.
21. **Lawther, J., R. Sun, and W. Banks.** 1996. Extraction and comparative characterization of ball-milled lignin (LM), enzyme lignin (LE) and alkali lignin (LA) from wheat straw. Cellul. Chem. Technol. **30:**395–410.
22. **Lenz, B.** 1968. Application of nuclear magnetic resonance spectroscopy to characterization of lignin. TAPPI **51:**511–519.
23. **Lu, F., and J. Ralph.** 1998. The DFRC method for lignin analysis. 2. Monomers from isolated lignin. J. Agric. Food Chem. **46:**547–552.
24. **Lu, F., and J. Ralph.** 1998. The DFRC method for lignin analysis. 3. NMR studies. J. Agric. Food Chem. **18:**219–233.
25. **Lu, F., and J. Ralph.** 1997. Derivatization followed by reductive cleavage (DFRC method). A new method for lignin analysis: protocol for analysis of DFRC monomers. J. Agric. Food Chem. **45:**2590–2592.
26. **Lu, F., and J. Ralph.** 1997. The DFRC method for lignin analysis. Part 1. A new method for β-aryl ether cleavage: lignin model studies. J. Agric. Food Chem. **45:**4655–4660.
27. **Mendonça, R., A. Guerra, and A. Ferraz.** 2002. Delignification of *Pinus taeda* wood chips treated with *Ceriporiopsis subvermispora* for preparing high-yield kraft pulps. J. Chem. Technol. Biotechnol. **77:**411–418.
28. **Messner, K., K. Fackler, P. Lamaipis, W. Gindl, E. Srebotnik, and T. Watanabe.** 2003. Overview of white-rot research: where we are today. ACS Symp. Ser. **845:**73–96.
29. **Peng, J., F. Lu, and J. Ralph.** 1998. The DFRC method for lignin analysis. 4. Lignin dimers isolated from DFRC-degraded loblolly pine wood. J. Agric. Food Chem. **46:**553–560.
30. **Ralph, J., J. Marita, S. Ralph, R. Hatfield, F. Lu, R. Ede, J. Peng, S. Quideau, R. Helm, J. Grabber, H. Kim, G. Jimenez-Monteon, Y. Zhang, H. Jung, L. Landucci, J. Mackay, R. Sederoff, C. Chapple, and A. Boudet.** 2000. Solution-state NMR of lignins, p. 55–108. *In* D. Argyropoulos and T. Rials (ed.), Advances in lignocellulosic characterization. TAPPI Press, Atlanta, GA.
31. **Rüttimann-Johnson, C., L. Salas, R. Vicuña, and T. K. Kirk.** 1993. Extracellular enzyme production and synthetic lignin mineralization by *Ceriporiopsis subvermispora*. Appl. Environ. Microbiol. **59:**1792–1797.
32. **Srebotnik, E., K. A. Jensen, Jr., S. Kawai, and K. E. Hammel.** 1997. Evidence that *Ceriporiopsis subvermispora* degrades nonphenolic lignin structures by a one-electron-oxidation mechanism. Appl. Environ. Microbiol. **63:**4435–4440.
33. **Tai, D. S., M. Terazawa, C. L. Chen, and H. M. Chang.** 1990. Lignin biodegradation products from birch wood by *Phanerochaete chrysosporium*. Holzforschung **44:**185–190.