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Preferential Degradation of Phenolic Lignin Units by Two White Rot Fungi

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The differential biodegradation of phenolic and nonphenolic (C-4-etherified) lignin units in wheat straw treated with the white rot fungi *Pleurotus eryngii* and *Phanerochaete chrysosporium* was investigated under solid-state fermentation conditions. Two analytical techniques applied to permethylated straw were used for this purpose, i.e., alkaline CuO degradation and analytical pyrolysis (both followed by gas chromatography-mass spectrometry for product identification). Despite differences in the enzymatic machinery produced, both ligninolytic fungi caused a significant decrease in the relative amount of phenolic lignin units during the degradation process. Nevertheless, no differences in the biodegradation rates of phenolic and etherified cinnamic acids were observed. Changes in lignin composition and cinnamic acid content were also analyzed in the phenolic and nonphenolic lignin moieties. The results obtained are discussed in the context of the enzymatic mechanisms of lignin biodegradation.

Lignin polymerization is initiated by plant peroxidases transforming *p*-hydroxycinnamyl alcohols into the corresponding phenoxy radicals. These are involved in many subsequent coupling reactions, yielding a high proportion of C-4-etherified lignin units. However, a certain amount of phenolic units exist in lignin, especially in the family Gramineae (18). This characteristic, together with cinnamic bridges (16), is responsible for the solubility of grass lignins in sodium hydroxide (18). Moreover, it should affect degradability by the enzymes produced by white rot fungi. Among ligninolytic enzymes produced by white rot fungi (12, 23, 25), only lignin peroxidase can directly oxidize nonphenolic lignin models, whereas Mn-dependent peroxidase and laccase are supposed to degrade mainly phenolic units. Some white rot fungi from the genus *Pleurotus* are being investigated with reference to their ability to remove grass lignin with only a minor attack on cellulose (13, 20). This preferential removal of lignin is of great interest for the development of environmentally friendly biotechnological processes to be applied in feed production and paper manufacture.

Several methods for estimating the phenolic content in lignin have been described (2, 15); most of them have been applied to isolated lignin preparations. However, lignin extraction presents several methodological constraints, and it is especially problematic when biodegraded lignins are being analyzed. The present study includes the optimization of two analytical methods, including permethylation followed by (i) alkaline CuO oxidation and (ii) analytical pyrolysis (Py), for the estimation of the phenolic content of lignin in whole-straw samples. CuO depolymerization of grass lignins is better than the classical nitrobenzene oxidation, since it permits the separate estimation of cinnamic acids, which are very abundant in herbaceous plants (9). Cupric oxide oxidation breaks mainly ether bonds, which constitute the most frequent intermonomer linkage in lignin, but C-C linked moieties exist in every lignin type (10). When the strength of chemical agents is increased to analyze this condensed lignin, the risk of artifacts increases. However, a generalized depolymerization of lignin with minimal generation of artifacts can be attained by analytical Py (3). In this study, the alteration of lignin during wheat straw treatment with the white rot fungi *Pleurotus eryngii* and *Phanerochaete chrysosporium* is analyzed by both techniques.

MATERIALS AND METHODS

Straw degradation by fungi. Solid-state fermentation (SSF) of wheat straw samples (5 to 20 mm long) was performed in a rotary fermentor, containing six horizontal bottles with a capacity of 1 liter (27, 28). Each bottle contained 25 g of straw and 75 ml of inoculum solution and was aerated with 166 ml of wet air per ml. Two different SSF experiments were carried out with the ligninolytic fungi *Pleurotus eryngii* IJFM A-169 (=

TABLE 1. Modification of straw composition by fungi

Parameter	Value for:		
	Control straw	*Pleurotus eryngii*[a]	*Phanerochaete chrysosporium*[b]
Straw fractions (g/100 g of straw)			
Extractives	4.3	6.6	11.1
Water soluble	5.4	9.1	15.4
Lignin	15.9	10.2	14.4
Xylan	17.4	12.2	14.3
Arabinan	3.1	2.4	2.4
Cellulose	40.7	42.8	24.6
Fungal degradation (% of initial matter)			
Wt loss	0	18	39
Lignin loss	0	47	45
Xylan loss	0	43	50
Cellulose loss	0	14	63

[a] 80-day incubation.
[b] 30-day incubation.

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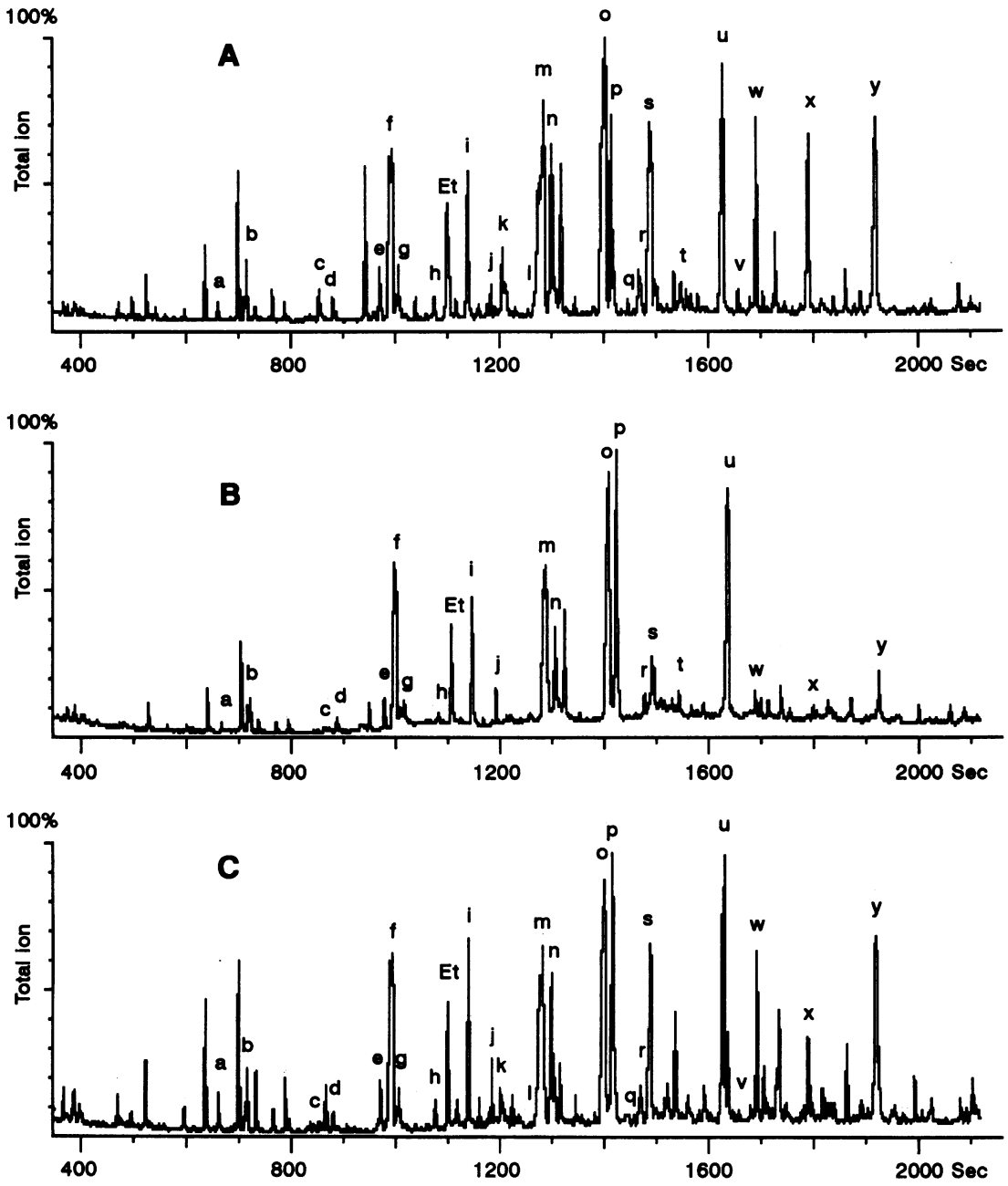

FIG. 1. Gas chromatograms of the products obtained after methylation and CuO depolymerization of wheat straw from the untreated control (A) and degraded by *Pleurotus eryngii* (B) and *Phanerochaete chrysosporium* (C). Peaks: a, 4-MeO-acetophenone; b, 4-TMSO-benzaldehyde; c, 3,4-diMeO-benzaldehyde; d, 4-TMSO-acetophenone; e, 4-MeO-benzoic TMS ester; f, 3-MeO, 4-TMSO-benzaldehyde; g, 3,4-diMeO-acetophenone; h, 3,4,5-triMeO-benzaldehyde; Et, 4-TMSO-ethylvanillin (internal standard); i, 3-MeO, 4-TMSO-acetophenone; j, 4-TMSO-benzoic TMS ester; k, 3,4,5-triMeO-acetophenone; l and s, *cis*- and *trans*-4-MeO-cinnamic TMS ester; m, 3,5-diMeO, 4-TMSO-benzaldehyde; n, 3,4-diMeO-benzoic TMS ester; o, 3,5-diMeO, 4-TMSO-acetophenone; p, 3-MeO, 4-TMSO-benzoic TMS ester; q and w, *cis*- and *trans*-4-TMSO-cinnamic TMS ester; r, 3,4,5-triMeO-benzoic TMS ester; t and x, *cis*- and *trans*-3,4-diMeO-cinnamic TMS ester; u, 3,5-diMeO, 4-TMSO-benzoic TMS ester; v and y, *cis*- and *trans*-3-MeO,4-TMSO-cinnamic TMS ester. Abbreviations: MeO, methoxy; TMS, trimethylsilyl; TMSO, trimethylsilyloxy.

ATCC 90787, CBS 613.91) and *Phanerochaete chrysosporium* ATCC 24725. For inoculum preparation, stationary cultures in 500 ml-flasks containing 50 ml of medium as described elsewhere (6), were used. After development of a fungal mat (20 days for *Pleurotus eryngii* and 7 days for *Phanerochaete chrysosporium*), the mycelia were recovered, homogenized, and incubated at 170 rpm for 2 to 3 days in 500-ml flasks containing 100 ml of the same medium. The pellets from each flask were washed, suspended in 75 ml of water, and used to inoculate the straw in one bottle. Samples were taken after 7, 20, 30, and 80 days of SSF. Each SSF experiment included a flask with uninoculated straw, which was separately analyzed as a control.

General analyses. The straw samples treated with and

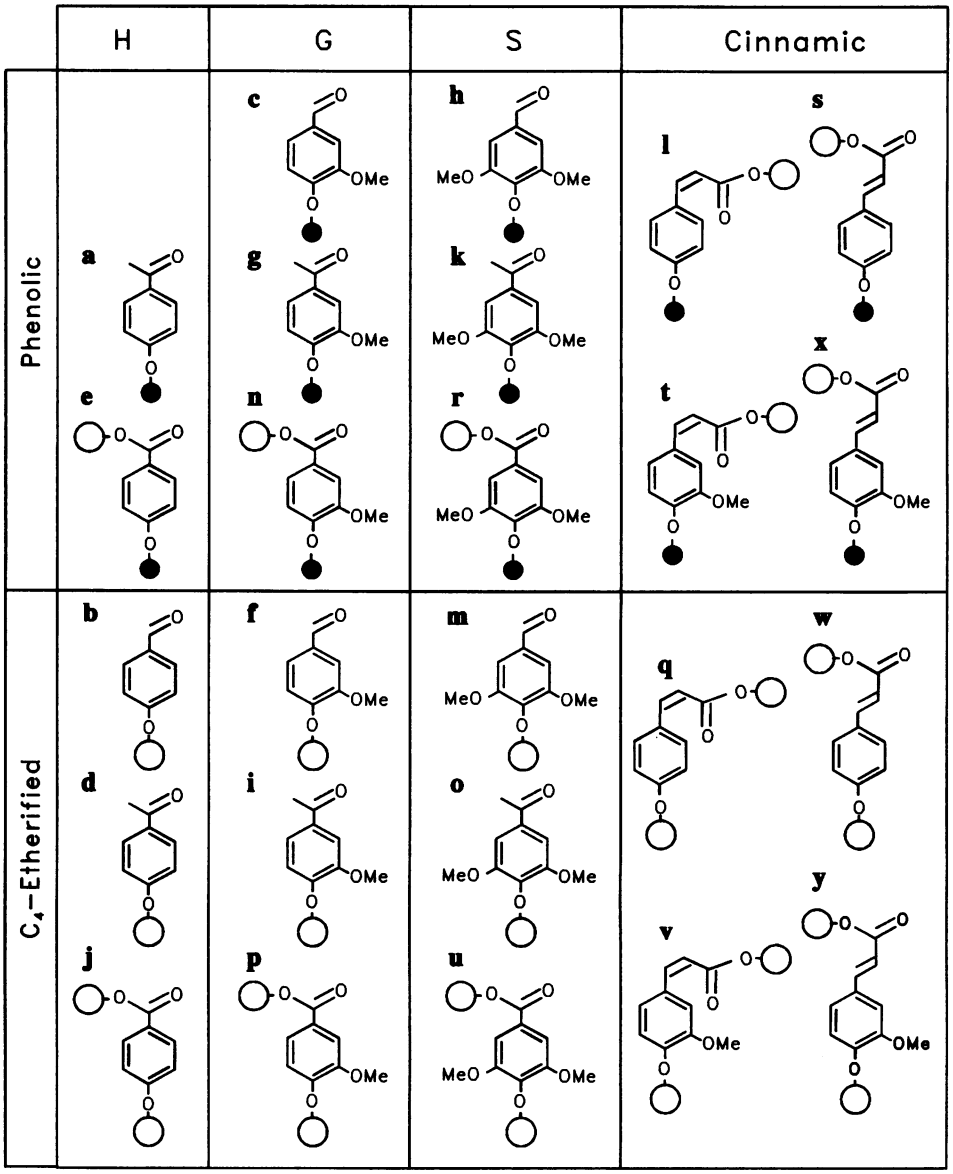

FIG. 2. Different aromatic compounds from phenolic and nonphenolic H, G, and S lignin units and cinnamic acids, recovered after CuO depolymerization of methylated wheat straw (letters are defined in the legend to Fig. 1). Solid circles, methyl; open circles, trimethylsilyl.

without fungi were freeze-dried, weighed, and milled (size, <0.2 mm). General analyses and phenolic content estimations were carried out in triplicate, and 95% confidence limits were calculated. Extractives, hot-water-soluble material, Klason lignin contents, and the polysaccharide composition (from monosaccharides released by acid hydrolysis) were determined by standard Tappi methods T 211, T 264, T 207, T 236, T 222, and T 249 (26).

Methylation of degraded samples. Methylation was carried out by a modification of previously described procedures (4, 19). Samples of milled straw were extracted with light petroleum ether for 18 h in a Soxhlet to remove lipid materials. Dried samples (200 mg) were shaken for 7 days at room temperature in 20 ml of dioxane, and then 5 ml of 0.25 M ethereal diazomethane and 10 ml of methanol were added to the straw suspension, which was kept on a magnetic stirrer for 5 days. Methylation was repeated every day by addition of fresh diazomethane (2 ml per sample). Finally, the methylated straw was washed with ether-methanol (1:1), centrifuged, and dried under N_2.

Alkaline CuO oxidation. Samples of 100 mg of straw were degraded at 170°C for 3 h under N_2 in Teflon bombs with 2 g of CuO, 200 mg of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 14 ml of 2 M NaOH (9). The degradation products were recovered with ether, suspended in 100 μl of pyridine (containing 33 μg of ethylvanillin as the internal standard), and derivatized with bis(trimethylsilyl)trifluoroacetamide. They were analyzed by gas chromatography-mass spectrometry (GC-MS) with an SP-2100 column (30 m by 0.25 mm) and a temperature program from 100 to 270°C at 4°C/min. The lignin degradation products were identified from the mass spectra obtained with an ion trap detector under electron impact at 70 eV from 40 to 350 *m/z* at 1 scan per s. The molar abundances of lignin degradation

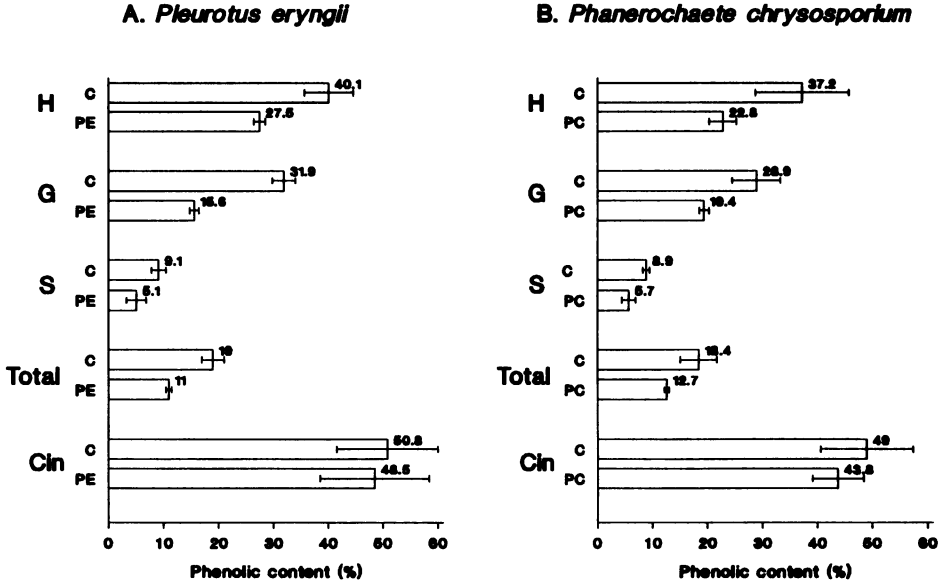

FIG. 3. Decrease of phenolic content in lignin (H, G, S, and total units) and cinnamic acids (Cin) after treatment with *Pleurotus eryngii* (PE) and *Phanerochaete chrysosporium* (PC), compared with controls (C), as estimated by CuO depolymerization of methylated wheat straw (mean values and 95% confidence limits).

products were calculated from peak areas, using response factors from standard compounds.

Py-GC-MS. Straw samples (ca. 1 mg) were pyrolyzed in a quartz holder at 600°C for 5 s, using a Pt coil pyrolyzer coupled to a gas chromatograph interfaced to an ion trap detector. The gas chromatograph was fitted with an SPB-5 column programmed from 50 to 260°C at 5°C/min, holding the initial temperature for 10 min. Mass spectra were obtained under the conditions described above. Lignin Py products were identified on the basis of standards, the National Bureau of Standards library of spectra, and retention times and spectra reported after Py-GC-MS of lignocellulosic materials (24). The molar abundances of Py products were calculated from the relative areas of the peaks.

RESULTS

The ligninolytic fungi studied were chosen as representative of two different patterns of wheat straw degradation. The kinetics of SSF transformation of straw constituents by these white rot fungi have already been reported (13, 27, 28), and samples from different incubation times were subjected to fractionation and lignin analysis. The samples showing around 50% lignin removal were considered the most suitable to illustrate the alteration of lignin by the two fungi, and the corresponding results are presented in the tables and figures. Differences in straw degradation patterns are shown by the results of general analyses presented in Table 1. *Phanerochaete chrysosporium*, which has been largely characterized from the enzymatic and molecular points of view, caused considerable loss of weight and initiated a simultaneous attack on lignin and polysaccharides. However, *Pleurotus eryngii* caused a preferential degradation of lignin and xylan and attacked cellulose only slightly.

The two degradation methods used for analyzing lignin break down intermonomer linkages and produce partial degradation of lignin side chains, but they keep intact the substituents of the aromatic rings, enabling the identification of the products originated from *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units. Gas chromatograms of the products obtained after methylation and alkaline CuO depolymerization of the straw samples treated with both fungi and the control straw are presented in Fig. 1. The H-, G-, and S-type aromatic aldehydes, ketones, and acids are characteristic products of CuO depolymerization of lignin. The three compounds obtained from each phenylpropanoid unit represent different degrees of oxidative alteration of the lignin side chains after the cleavage of intermonomer ether linkages. In addition, a substantial amount of cinnamic acids, which could be linked to lignin, carbohydrate, or lipid polymers, was obtained after CuO depolymerization of straw. As a consequence of straw methylation, the above-described lignin degradation compounds were obtained in two different forms, as shown in Fig. 2. Those with a methoxy group at C-4 (compounds a, c, e, g, h, k, n, and r) come from phenolic units, whereas those with a trimethylsilyloxy group at C-4 (compounds b, d, f, i, j, m, o, p, and u) corresponded to the C-4-etherified units. The same can be applied to the cinnamic acids, which can be phenolic (giving rise to compounds l, s, t, and x) or etherified at C-4 (giving rise to compounds q, v, w, and y). Although their *trans* isomers were the most abundant

TABLE 2. Lignin composition and cinnamic acid content in the straw treated with fungi and the control straw[a]

Treatment	Composition (mol %) of:			
	Total lignin		Phenolic moiety	
	H/G/S	Cinnamate/ (H + G + S)	H/G/S	Cinnamate/ (H + G + S)
None (control)	7:39:55	0.4	12:61:27	0.7
Pleurotus eryngii	6:39:55	0.2	14:56:30	1.3
Phanerochaete chrysosporium	7:43:50	0.3	13:65:23	1.1

[a] Molar percentages estimated by CuO depolymerization.

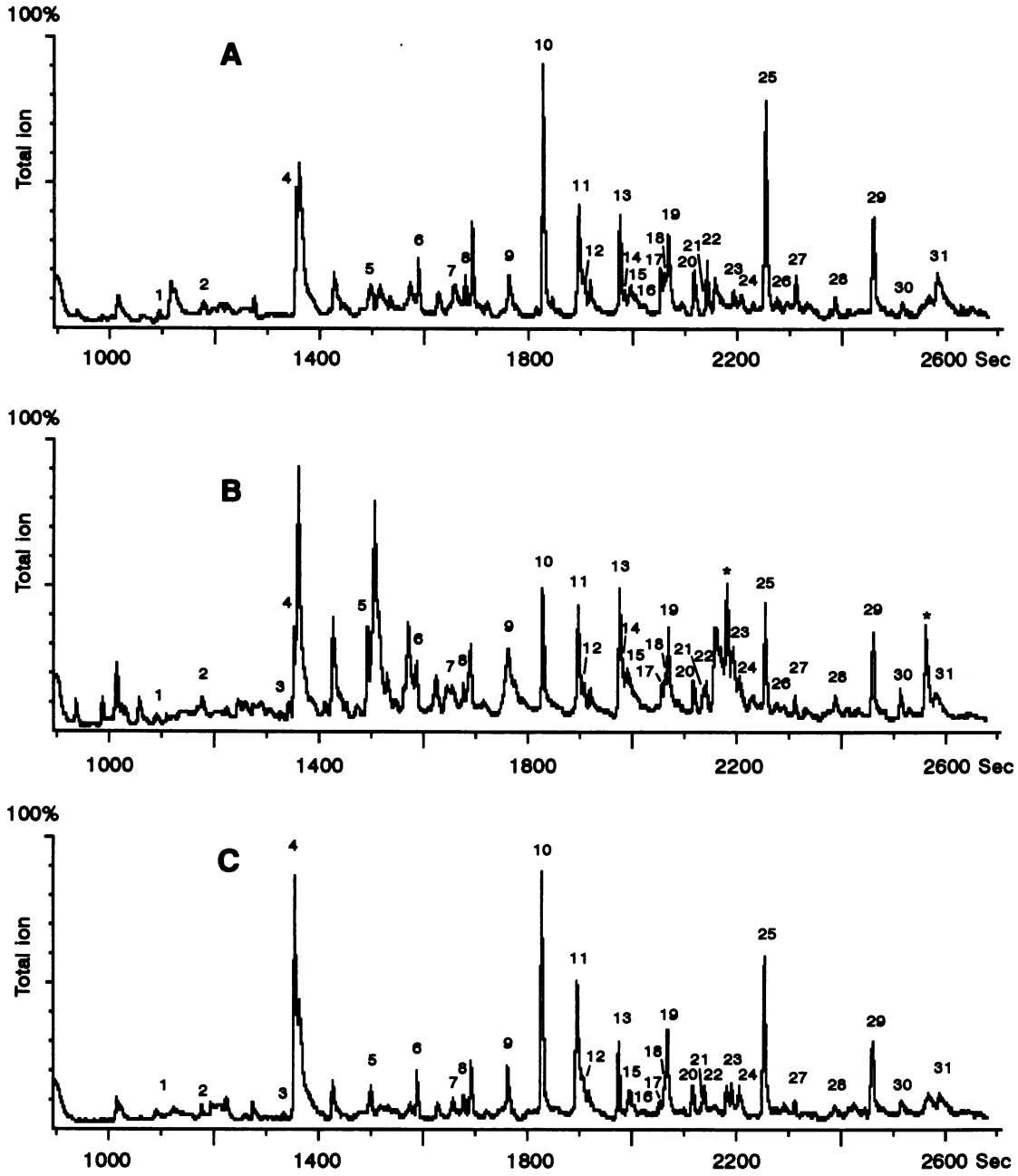

FIG. 4. Gas chromatograms of the products obtained after methylation and Py-GC-MS of the wheat straw from the untreated control (A) and degraded by *Pleurotus eryngii* (B) and *Phanerochaete chrysosporium* (C). Peak identification: 1, phenol; 2, 4-MeO-toluene; 3, 4-methylphenol; 4, guaiacol; 5, 4-MeO-vinylbenzene; 6, 4-methylguaiacol; 7, 4-vinylphenol; 8, 3,4-diMeO-toluene; 9, 4-ethylguaiacol; 10, 4-vinylguaiacol; 11, syringol; 12, eugenol; 13, 1,2-diMeO-4-vinylbenzene; 14, 1,2-diMeO-4-(2-propenyl)benzene; 15, vanillin; 16 and 19, *cis*- and *trans*-isoeugenol; 17 and 22, *cis*- and *trans*-1,2-diMeO-4-(1-propenyl)benzene; 18, 4-methylsyringol; 20, veratraldehyde; 21, acetovanillone; 23, 4-ethylsyringol; 24, guaiacylacetone; 25, 4-vinylsyringol; 26, 4-(2-ketopropyl)-1,2-diMeO-benzene; 27, 4-allylsyringol; 28 and 29, *cis*- and *trans*-4-(1-propenyl)syringol; 30, acetosyringone; 31, 1,2,3-tri-MeO-5-vinylbenzene.

(peaks s, x, w, and y), the *cis* isomers, in both phenolic and etherified forms, were also detected as small peaks (peaks l, t, q, and v) (Fig. 1). From peak areas in gas chromatograms of the products released by CuO depolymerization (Fig. 1), the molar percentages were estimated. Figure 3 shows the average phenolic content in H, G, S, and total lignin units and cinnamic acids in straw treated with both fungi, compared with the controls. Moreover, the composition (H:G:S ratio) of phenolic and total lignin and the amounts of phenolic and total cinnamic acids were estimated. The values found in the straw treated with *Pleurotus eryngii* and *Phanerochaete chrysosporium*, compared with the control straw, are presented in Table 2.

Gas chromatograms after methylation and Py-GC-MS of the samples treated with both fungi and the control straw are presented in Fig. 4. A series of pyrolysis products from total phenylpropanoid compounds (i.e., lignin and cinnamic acids),

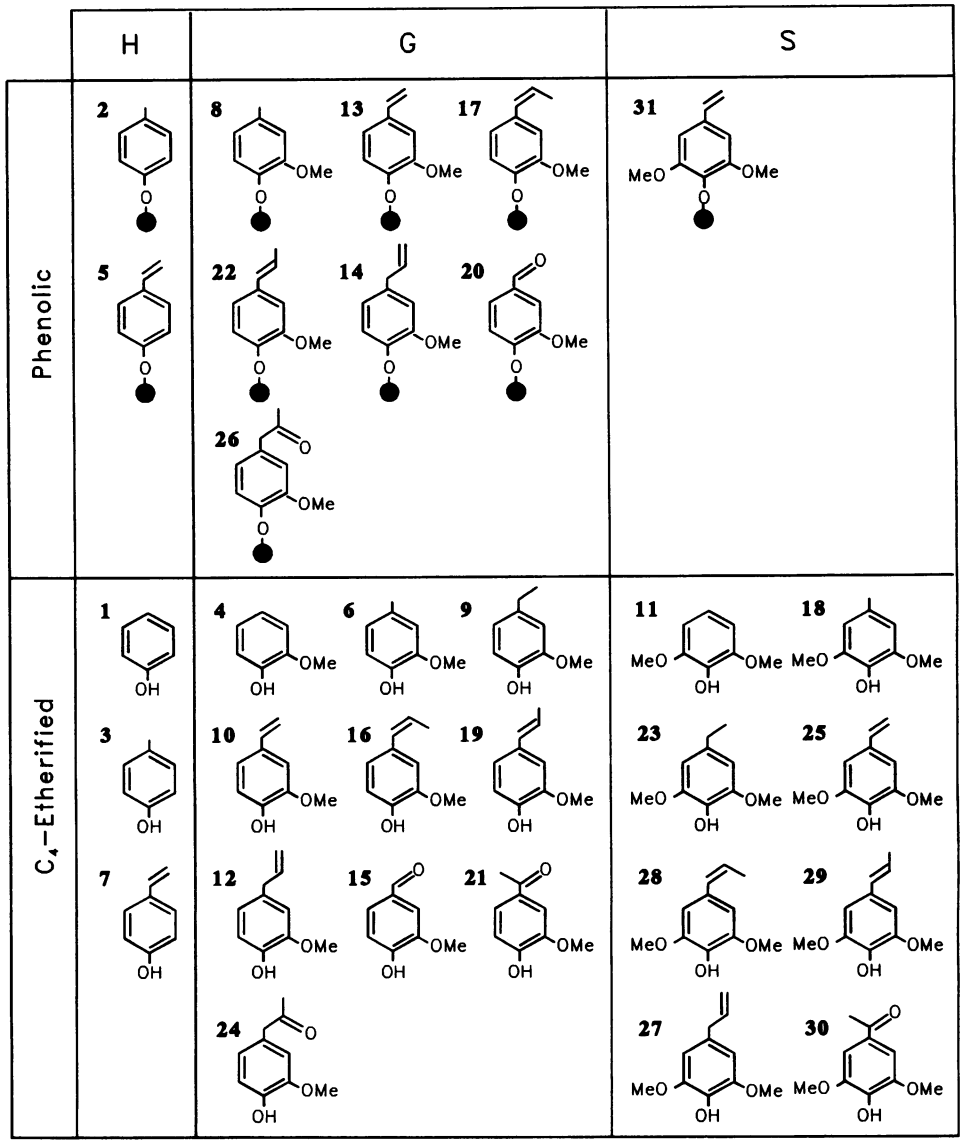

FIG. 5. Different aromatic compounds from phenolic and nonphenolic H-, G-, and S-type lignin units and cinnamic acids, recovered after Py-GC-MS of methylated wheat straw (numbers are defined in the legend to Fig. 4). Solid circles, methyl.

including phenol, guaiacol, and syringol and their *para* methyl, ethyl, vinyl, and propenyl derivatives, were identified. Some aromatic aldehydes (peaks 15 and 20) and ketones (peaks 21, 24, and 26) have also been reported as lignin Py products. In addition, the two peaks indicated with asterisks in Fig. 4 correspond to the methyl esters of vanillic and syringic acids. As with CuO depolymerization, the methylation of straw gave rise to two types of Py products, shown in Fig. 5, which were derived from phenolic and nonphenolic phenylpropanoid compounds. In the Py-GC-MS analysis, no additional derivatization was used, so the products derived from phenolic units (compounds 2, 5, 8, 13, 14, 17, 20, 22, 26, and 31) had a methoxy group at C-4 while those that originated from etherified units (compounds 1, 3, 4, 6, 7, 9 to 12, 15, 16, 18, 19, 21, 23 to 25, and 27 to 30) had a hydroxyl group at the same position. Despite the diversity of the above Py products, their origin can be straightforwardly deduced as derived from H-, G-, and S-type phenylpropanoid compounds. Nevertheless, separate analysis of lignin and cinnamic acids is not possible by this technique, since they give rise to the same Py fragments. This was confirmed by Py of ferulic acid and G-type dehydrogenation polymer, both yielding vinylguaiacol (compound 10) as the main Py product. However, when the acids are esterified they are quite stable under Py conditions, as verified with vanillic and ferulic methyl esters. According to the above considerations, the phenolic contents of total phenylpropanoid compounds (lignin plus cinnamic acids) were also calculated from the CuO depolymerization results and compared with those obtained by Py-GC-MS (Table 3) in order to contrast the two degradation techniques.

DISCUSSION

Although most of the actual knowledge of the enzymatic degradation of lignin comes from the use of simple model compounds, useful information can be obtained from the

TABLE 3. Comparison of CuO depolymerization and Py-GC-MS for estimation of phenolic phenylpropanoid compounds in the straw treated with fungi and control

Treatment	Composition (mol %) obtained by:							
	CuO oxidation				Py-GC-MS			
	H	G	S	Total	H	G	S	Total
None (control)	55	31	9	26	55	46	6	30
Pleurotus eryngii	40	16	6	13	33	38	6	25
Phanerochaete chrysosporium	49	21	6	20	45	18	6	16

analysis of molecular alterations of the lignin polymer during fungal attack. This approach is limited by the availability of suitable analytical methods, but it will permit validation of the degradation mechanisms postulated from results obtained with model compounds. In this way, the results from CuO oxidation and Py-GC-MS provide valuable information on the changes in the composition of phenolic and nonphenolic lignin moieties during wheat straw biodegradation.

The phenolic content of wheat straw lignin was estimated by CuO depolymerization as 19% of total units, a value similar to that reported by Lapierre et al. (17) using thioacidolysis. This result suggested that a substantial portion of the lignin polymer could be directly oxidized by ligninolytic enzymes acting on phenolic compounds. The phenolic percentages varied for the different lignin units (39% of H, 30% of G, and 9% of S), in accordance with the distribution of methoxy groups. On the other hand, the amount of C-4-free cinnamic acids was around 50%, with the major percentage as phenolic *p*-coumaric acid. Py-GC-MS gave a larger phenolic content for the total phenylpropanoid compounds (28% of the total). Nevertheless, when these values were compared with the CuO percentages for total phenylpropanoid compounds, the differences were slight. Thus, it becomes apparent that the higher phenolic content attained by Py-GC-MS can be attributed to the joint estimation of Py products from lignin and cinnamic acids.

During the whole SSF period with both ligninolytic fungi, a progressive decrease of the phenolic percentages at the different lignin units was found by CuO depolymerization, but no significant changes in phenolic cinnamic acids were found. Moreover, decreases in the phenolic content of H-, G-, and S-type phenylpropanoid compounds after fungal attack were also observed by Py-GC-MS. The ligninolytic enzymes lignin peroxidase and Mn-dependent peroxidase, but not laccase, are produced by *Phanerochaete chrysosporium* (12, 23, 25). Lignin peroxidase has not been reported in *Pleurotus* species, but these fungi produce the last two enzymes (7, 20, 21). In addition, both fungi secretes two different H_2O_2-producing oxidases (5, 6, 11). In spite of these differences, the results obtained show a preferential degradation of the lignin phenolic moiety by both fungi. The decreases in the amounts of phenolic lignin units could be due to the action of laccase or Mn-dependent peroxidase, both of which can easily oxidize these phenolic structures. Extensive depolymerization of the dehydrogenation polymer of *p*-hydroxycinnamyl alcohols by purified Mn-dependent peroxidase has been demonstrated (29), but the oxidation of phenolic substrates can be considered the most important role of Mn-dependent peroxidase. The same can be said for laccase, although this enzyme was shown to oxidize certain nonphenolic lignin models in the presence of 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (1, 22). In the case of lignin peroxidase, the phenolic substrates are not good reducing agents of compound II, which is transformed into compound III by H_2O_2, causing lignin peroxidase inactivation (8). However, in the presence of veratryl alcohol, which closes the catalytic cycle, lignin peroxidase could oxidize phenolic in competition with nonphenolic substrates. Since veratryl alcohol is synthesized by *Phanerochaete chrysosporium* as a secondary metabolite (25), oxidation of phenolic units in lignin could also be caused by lignin peroxidase.

Additional evidence for the preferential degradation of the phenolic lignin moiety by fungal enzymes has been recently provided by Kurek and Monties (14). These authors described milled-wood lignin oxidation by lignin peroxidase from *Phanerochaete chrysosporium*, in the presence of veratryl alcohol, as a two-stage process. During the first phase, enzyme oxidation of phenolic lignin units was predominant and no veratryl alcohol oxidation was detected. After this period, veratraldehyde formation was observed, although at rates attenuated by competitive inhibition by milled-wood lignin. Our results, obtained during fungal attack of complex lignocellulosic material, support the results described above obtined with polymeric lignin (14) and those obtained with simple compounds (8). The evidence provided by these studies indicates that the preferential degradation of phenolic substructures constitutes an initial phase of natural degradation of lignin by white rot fungi.

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