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Biological Degradation of Tannins in Sericea Lespedeza (*Lespedeza cuneata*) by the White Rot Fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus* Analyzed by Solid-State ^{13}C Nuclear Magnetic Resonance Spectroscopy

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Leaves of sericea lespedeza exhibit a high proportion of condensed tannin, resulting in poor forage quality. The white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus* are known to preferentially degrade lignin in a variety of plants and were evaluated for their ability to degrade condensed tannin from sericea leaves with the aim of improving digestibility. Relative levels of condensed tannin, cutin, pectin, and cellulose were monitored as a function of fungal treatment by solid-state cross-polarization and magic angle spinning ^{13}C nuclear magnetic resonance spectroscopy. Total soluble phenolics, soluble tannins, and soluble and insoluble proanthocyanidin levels in fungus-treated and control samples were measured by established chemical techniques. Results indicate that both species of fungus preferentially degrade condensed tannin and that *C. subvermispora* is markedly superior to *C. stercoreus* in this capacity.

Tannins are a group of naturally occurring polyphenolic compounds which are common components of plants and comprise two major classes, i.e., condensed and hydrolyzable. Both classes of tannin inhibit microbial growth, but condensed tannins in particular limit the utilization of plants as feed for herbivores (8, 29). Condensed tannins present in high concentrations are antinutritive by binding to cell wall polymers, rendering the walls undegradable, as well as by binding digestive enzymes secreted by rumen microorganisms, rendering the enzymes inactive. Several previous studies have focused on the degradation of tannin-protein complexes by bacteria (7, 24, 26, 27). In these cases, the degradable tannins in question were hydrolyzable tannins. Previous studies (16, 17) have shown that no appreciable degradation of condensed tannins occurs in vitro by rumen microorganisms. Bacteria do not appear to be capable of appreciable degradation of condensed tannins but do have the capacity to develop tolerances to high concentrations.

Lignins, a group of polyphenols related to tannins, likewise are limited in their biodegradation by bacteria (10). In contrast to the bacteria, white rot fungi are well documented in their ability to degrade lignins and in fact are the only known microorganisms that are capable of mineralizing lignin in pure culture. The ability of white rot fungi to utilize lignin has been investigated for potential use in upgrading animal feeds (3, 12), pulp production (22), and environmental bioremediation (5). The breakdown of lignins by white rot fungi involves the enzymatic cleavage of ether bonds, the primary mode of linkage between monomeric units. The monomeric units of condensed tannins, composed largely of proanthocyanidins (Pas) and related compounds, are linked primarily by direct carbon-carbon bonds (13). It is therefore not obvious that the white rot fungi have the capacity for degrading condensed tannins. It has been shown, however, that the fungus *Sporotrichum pulverulentum* effectively degrades condensed tannins in oak (*Quercus incana*) leaves (20). This indicates that white rot fungi are capable of condensed tannin degradation, but the results also indicated a substantial removal of rumen-digestible carbohydrates, as the digestibility of the fermented oak leaves decreased relative to that of the unfermented oak leaves. *Ceriporiopsis subvermispora* and *Cyathus stercoreus* have both been shown to preferentially degrade the lignin component of plant cell walls and thereby improve the digestibility of the residual plant fiber (2). A preliminary study on leaves of sericea lespedeza pretreated with these fungi indicated that digestibility by rumen microbes was substantially improved (1a), but the possible correlation of this improvement with condensed tannins was not investigated.

Sericea lespedeza (*Lespedeza cuneata*), most varieties of which are high in condensed tannins, is a perennial legume grown in the southeastern United States both as an aid in soil conservation and as a forage crop and is valued for its productivity in acid soils and for its tolerance to drought (6). As is the case with most legumes, sericea lespedeza exhibits a high concentration of protein but is generally considered to be a poor forage crop because of the additional presence of large amounts of condensed tannins, which render the plant unpalatable to forage animals and the proteins undigestible. Leaves of sericea lespedeza were used as substrate for the fungi, since selective degradation of the tannin component from sericea lespedeza would increase its potential value as a forage crop.

In order to monitor the effects of fungal biodegradation, solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy was used as a probe. This technique is nondestructive of the material being analyzed; resolves many separate cell wall components including tannin, protein, carbohydrate, and cutin; and is capable of measuring relative changes in these components. In addition, nondegraded and degraded samples of sericea lespedeza were analyzed for total soluble phenolics, soluble tannins, soluble Pas, and total Pas (soluble and insol-

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uble) according to previously established isolation and chemical analytical methods (16).

MATERIALS AND METHODS

Plant substrate. A high-tannin cultivar of sericea lespedeza [*L. cuneata* (Dumont) G. Don, 'Interstate 76'] was collected as the second cutting from experimental fields at the Central Georgia Branch Station near Eatonton, Ga., in the vegetation stage. Leaves were manually separated from the stems and used as the sole substrate for the fungi.

Fungal treatment. The white rot fungi included two strains of *C. subvermispora*, CZ-3-8497 (CZ-3) and FP-90031-sp (FP) (courtesy of R. A. Blanchette, University of Minnesota), and *C. stercoreus* ATCC 36910 (courtesy of K. Karunanandaa, Pennsylvania State University). Fungi were maintained on malt extract agar or potato dextrose agar.

Twelve 500-ml flasks each with 25 g (wet weight) of sericea lespedeza leaves and 10 ml of distilled water were autoclaved (103.5×10^3 Pa, 121°C) for 25 min. After cooling, three flasks were each inoculated with each strain of *C. subvermispora* and *C. stercoreus*, and three flasks were left uninoculated to serve as controls. Inoculation was carried out by placing five 4-mm agar disks with fungal mycelia into the flasks. Flasks were swirled to distribute the disks in the flask, which were then incubated at 27°C for 3 weeks. After this period, the samples were feeze-dried and grounds to a 1-mm mesh size for solid-state NMR analysis.

Dry weight loss was determined from the weight difference between the freeze-dried "before treatment" sample and the freeze-dried "after treatment" samples. Results show that the *C. subvermispora* FP-treated sample experienced a 14.4% weight loss, *C. subvermispora* CZ-3 experienced a 21.6% weight loss, and *C. stercoreus* experienced a 20.7% weight loss relative to the control sample.

Tannin analysis. Total soluble phenolics and soluble tannins were determined colorimetrically as described in the work of Makkar et al. (18). Total soluble phenolics were quantified by Folin-Ciocalteu reagent, and tannins were quantified as the difference of phenolics before and after tannin removal from the extract with insoluble polyvinylpyrrolidone. Both soluble phenolics and tannins were measured as equivalent to tannic acid (Merck). Soluble Pas were measured by the butanol-HCl-iron reagent by the procedure of Porter et al. (27). The bound plus soluble Pas were measured according to the method described in the work of Makkar and Singh (19) except that iron was used in the butanol-HCl reagent as described by Porter et al. (27). Pas were expressed as leucocyanidin.

Protein analysis. Protein content was determined (4) by the method of combustion using a Leco FP-2000 protein-nitrogen analyzer.

Isolation of total acetone:H_2O-soluble component. Isolation of the total acetone:H_2O-soluble component of sericea lespedeza leaves was performed by refluxing 10 g of the leaves, ground to 1-mm mesh size, in 100 ml of 7:3 acetone:H_2O solution for 4 h. The resulting filtrate was evaporated to remove the acetone, and the resulting precipitate remaining in the aqueous phase was filtered and freeze-dried.

Isolation of condensed tannin. Isolation of condensed tannin was performed as reported previously (31). Leaves of sericea lespedeza were extracted with 7:3 acetone:H_2O solution. The filtrate was evaporated under vacuum at 30°C to remove the acetone, and the remaining aqueous residue was washed with petroleum ether to remove lipids and pigment. The resultant aqueous phase was freeze-dried, redissolved in 1:1 acetone:H_2O, and initially purified on a Sephadex G-50 column. The resultant collection of elutions was evaporated, freeze-dried, dissolved in 1:1 MeOH:H_2O, and further purified on a Sephadex LH-20 column.

NMR spectroscopy. ^{13}C cross-polarization and magic angle spinning (CPMAS) (11, 28) NMR spectra were obtained on a Bruker MSL 300 spectrometer utilizing a 7.05-T superconducting magnet and tuned to 75.47 MHz for ^{13}C observation. A 4-mm-diameter sample rotor, spun at 9 kHz, was used to obtain the spectra with a CPMAS probe. The spectral width in each spectrum was 30 kHz, with a 17-ms acquisition time. All experiments were performed with a 2-ms cross-polarization contact time, a recycle delay of 5 s, and a B_1 field of 55.5 kHz. Spectra were referenced to hexamethylbenzene and were processed without window functions. Correction factors for individual peaks in the ^{13}C CPMAS NMR spectra of treated and untreated samples were determined from variable contact time experiments. Observed intensities of peaks as a function of the contact time (t) were fitted (21) with equation 1:

$$I(t)/I^* = [T_{1\rho H}/T_{1\rho H} - T_{CH}]\,[\exp(-t/T_{1\rho H}) - \exp(-t/T_{CH})] \quad (1)$$

where $I(t)$ is the observed peak intensity at the cross-polarization time t, I^* is the theoretical maximum intensity, $T_{1\rho H}$ is the proton spin-locked longitudinal magnetization time constant, and T_{CH} is the cross-polarization time constant. The resultant fit produces characteristic values of $T_{1\rho H}$ and T_{CH} for individual peaks. The inverse of the right-hand side of equation 1 can then be used as the correction factor, K^{-1}, which can be applied to the observed intensities at a given cross-polarization time t to give I^*.

RESULTS AND DISCUSSION

The solid-state ^{13}C NMR spectrum of untreated sericea lespedeza is presented in Fig. 1, with peak assignments given in Table 1. Peak 1 is indicative of acetyl and ester moieties, which may be present in protein, phenolics, lipid (including cutin), hemicellulose, and pectin. Peaks 2, 3, and 4 are all indicative of aromatic ring structures present primarily in tannin and cutin. Peaks 5 and 7 to 12 are due primarily to the presence of cellulose, hemicellulose, and pectin carbohydrate components, with much weaker signals from other components also being present. Peak 13 is indicative of the methoxyl group present primarily in pectin. Peaks 6 and 14 are due to condensed tannin (see below). Peaks 15 to 17 are all indicative of —CH_2— and —CH_3 groups present in proteins and lipids.

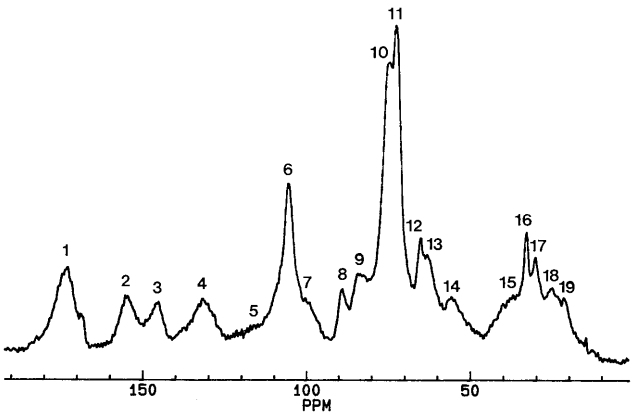

FIG. 1. CPMAS ^{13}C NMR spectrum of untreated sericea lespedeza.

Figure 2a shows the spectrum of the total acetone:H_2O-soluble phase of sericea lespedeza. Figure 2b is the spectrum of isolated condensed tannins, which are a component of the soluble phase. A previous report (23) on the solid-state NMR of tannic acid, which is an example of a hydrolyzable tannin, indicated that peaks are expected at δ 139.0 and δ 119.0 (C-4 and C-1, respectively, of the aromatic ring). These peaks are not observed either in the spectrum of the total acetone:H_2O-soluble phase or in the spectrum of condensed tannin, leading us to conclude that very little hydrolyzable tannin is present either in the sample of isolated condensed tannin or in the total acetone:H_2O-soluble phase. The absence of peak 14 at δ 55.0 in Fig. 2a suggests that monolignols are not present to any significant extent. The group of peaks from δ 45.0 to 0.0 further indicates a substantial amount of aliphatics, present primarily in cutin. Peak 16 at δ 33.3 in the spectrum of lespedeza is not present in the spectrum of the total acetone:H_2O-soluble phase and is therefore assigned in Table 1 as due primarily to the protein component, and peak 17 at δ 30.3 is assigned to cutin.

Prior to spectroscopic analysis of the *C. subvermispora*- and *C. stercoreus*-degraded samples, a determination of fungal biomass present in these samples was performed. To accomplish this, a ^{13}C NMR spectrum of isolated *C. subvermispora* biomass was obtained (Fig. 3). Comparison indicates that little residual fungal biomass is present in either *C. subvermispora* FP- or *C. stercoreus* ATCC 36910-treated sericea lespedeza samples (Fig. 4a and b, respectively), since the very sharp peaks at δ 185.0, δ 181.0, δ 176.0, δ 40.2, and δ 22.7 seen in Fig. 3 are not present to any substantial extent in the spectra of the fungus-treated samples.

$T_{1\rho H}$, T_{CH}, and the intensity correction factor K^{-1} for individual peaks were determined for the control sample of sericea lespedeza, with nearly identical results obtained for all of the fungus-treated samples. The fact that K^{-1} is unchanged after

TABLE 1. Chemical shift assignments for the CPMAS ^{13}C NMR spectrum of sericea lespedeza

Signal	δ (ppm)	Origin	Carbon atom	Reference(s)
1	173.8	Protein, hemicellulose, pectin, cutin	—C—C—O—	9, 14, 15
2	154.9	Tannin aromatic	UNK[a]	
3	145.4	Tannin aromatic	UNK	
4	131.4	Tannin aromatics	UNK	
		Cutin aromatics	UNK	9
5	115.0(sh)[b]	UNK	UNK	
6	105.3	Cellulose, hemicellulose, pectin	C-1	15, 30
		Tannin	UNK	
7	98.0(sh)	Tannin	UNK	
8	88.8	Cellulose (crystalline)	C-4	30
9	83.5	Cellulose (amorphous), hemicellulose, pectin	C-4	15, 30
10	74.5	Cellulose, hemicellulose, pectin	C-2, C-3, C-5	15, 30
11	72.4	Cellulose, hemicellulose, pectin	C-2, C-3, C-5	15, 30
12	64.8	Cellulose, hemicellulose, pectin	C-6	15, 30
13	62.4	Cellulose, hemicellulose, pectin	C-6	15, 30
14	55.5	Pectin	—OCH_3	15
15	35.0(sh)	Tannin	—CH_2—	
16	33.3	Protein, cutin	—CH_2—, —CH_3	9, 14
17	30.3	Protein, cutin	—CH_2—, —CH_3	9, 14
18	25.1	Protein, cutin	—CH_2—, —CH_3	9, 14
19	21.3	Protein, cutin	—CH_3	9, 14

[a] UNK, unknown.
[b] Sh, shoulder.

fungal treatment means that relative changes in various components can be monitored. Figure 4a shows the spectrum of *C. subvermispora* FP-treated sericea lespedeza overlaid with the untreated lespedeza. The intensity of the FP-treated sample spectrum is decreased until no component's intensity is higher than that of the untreated sample spectrum. The result indicates that the protein component, monitored by peak 16 at δ 33.3, is the least affected by fungal treatment. This is corroborated by the protein analysis, which indicates that protein is unaffected by fungal treatment and therefore that its weight percentage increases as a function of the fungal treatments. The tannin, cellulose, pectin, and cutin components, monitored at peaks 2, 8, 14, and 17, respectively, have all decreased relative to the protein. Table 2 shows the relative intensities of peaks indicative of protein, cutin, tannin, pectin, and cellulose as a function of fungal treatment. Each sample spectrum is normalized on the protein component, whose intensity as monitored by peak 16 at δ 33.3 is arbitrarily set to 6.0. The relative changes in the other components can be measured against this peak, so that the effects of the different fungal treatments can be compared. Results indicate that *C. subvermispora* CZ-3 degrades tannin most efficiently, removing 70%, while *C. subvermispora* FP or *C. stercoreus* ATCC 36910 removes 65 or 47%, respectively. Simultaneous degradation of cellulose, pectin, and cutin was also observed in each fungal treatment, though at smaller relative percentages compared with tannin.

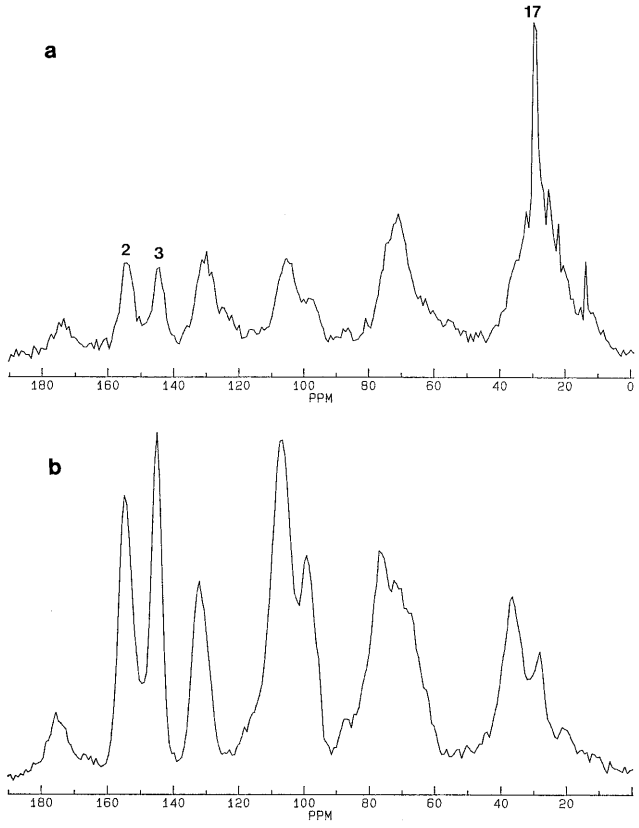

FIG. 2. CPMAS ^{13}C NMR spectra of acetone:H_2O-soluble materials from sericea lespedeza. (a) Total solubles; (b) condensed tannin.

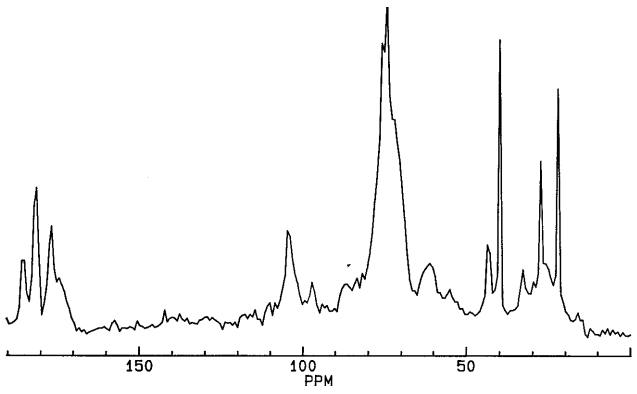

FIG. 3. CPMAS ^{13}C NMR spectrum of *C. subvermispora* FP biomass.

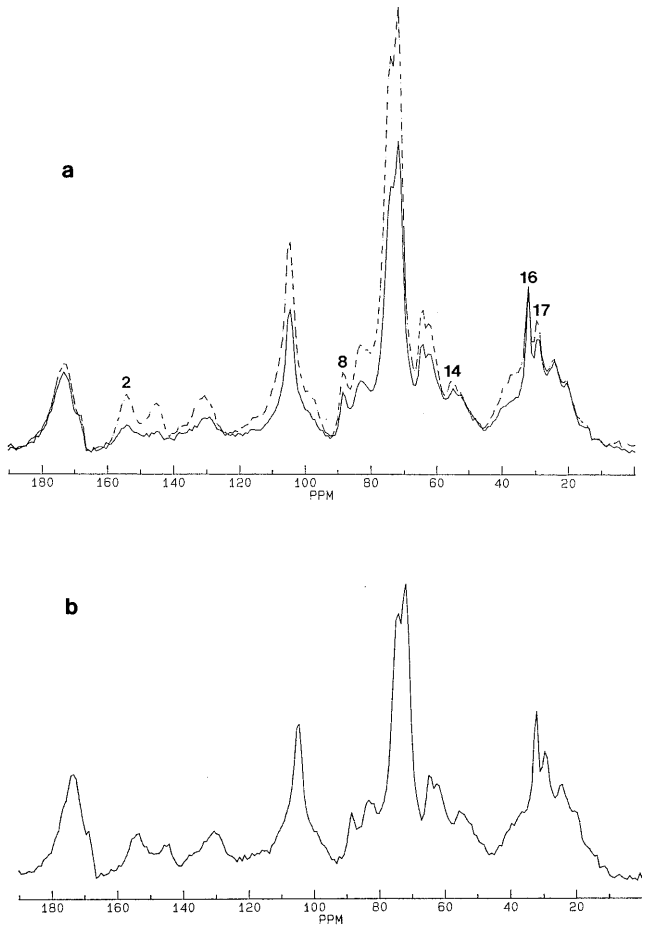

FIG. 4. CPMAS ^{13}C NMR spectra of fungus-treated sericea lespedeza. (a) *C. subvermispora* FP treatment (solid line) and control (dashed line); (b) *C. stercoreus* ATCC 36910 treatment.

Results from the analysis of total soluble phenolics, total soluble tannins, total soluble Pas, and total Pas (soluble and insoluble) for the control and the three fungus-treated samples indicated that each of the three white rot fungi removed these phenolic constituents (Table 3). *C. subvermispora* CZ-3 is the most effective of the three fungi studied at removing Pas. This strain removed 64% of bound and soluble Pas, while FP removed 63% and *C. stercoreus* removed 56%. These results together with the results obtained by NMR indicate that the two strains of *C. subvermispora* remove a higher amount of condensed tannin from the sericea substrate than does *C. stercoreus* in relation to other degraded cell wall components.

TABLE 2. ^{13}C CPMAS NMR spectral peak heights of resonances representative of chemical constituents of sericea lespedeza leaves treated with white rot fungi

Treatment	Peak ht (cm)				
	Peak 16, δ 33.3 (protein)	Peak 2, δ 154.9 (tannin)	Peak 8, δ 88.8 (cellulose)	Peak 14, δ 55.5 (pectin)	Peak 17, δ 30.3 (cutin)
Control	6.0	2.3 ± 0.3	3.0 ± 0.1	2.6 ± 0.1	4.8 ± 0.2
CZ-3	6.0	0.7 ± 0.1	1.7 ± 0.3	2.0 ± 0.1	4.2 ± 0.1
FP	6.0	0.8 ± 0.1	1.8 ± 0.3	2.1 ± 0.1	4.1 ± 0.1
C. stercoreus	6.0	1.2 ± 0.2	1.7 ± 0.3	2.2 ± 0.2	4.5 ± 0.1

TABLE 3. Total soluble phenolics, soluble tannins, and Pas in control and fungus-treated samples of sericea lespedeza as percentage of dry weight[a]

Treatment	% Dry matter			
	TP	T	Soluble Pas	Bound plus soluble Pas
Control	2.58 ± 0.14a	1.52 ± 0.11a	1.21 ± 0.12a	6.56 ± 0.15a
CZ-3	0.54 ± 0.04b	0.23 ± 0.03b	0.13 ± 0.02b	2.35 ± 0.11b
FP	0.62 ± 0.02b	0.34 ± 0.02b	0.20 ± 0.01b	2.44 ± 0.20b
C. stercoreus	0.57 ± 0.03b	0.27 ± 0.02b	0.25 ± 0.02b	2.88 ± 0.03c

[a] TP, total soluble phenolics; as tannic acid equivalent. T, soluble tannins; as tannic acid equivalent. Pas, as leucocyanidin equivalent. Values within columns with different letters differ at $P \leq 0.05$.

Previous work on sericea lespedeza leaves indicated that the white rot fungi *C. subvermispora* and *C. stercoreus* increased the low digestibility (about 20%) by nearly threefold (1). Results in the present study suggest that the fungi cause this increase in digestibility by chemically modifying the condensed tannins which are present, thereby improving the residue's biodegradability by rumen microorganisms. Previous reports on the degradation of lignin by white rot fungi indicated that specific fungus-substrate interactions occur, and this was found to be the case for the removal of condensed tannin from sericea as well. Both strains of *C. subvermispora* appear to exhibit a greater efficiency at removing condensed tannins than does *C. stercoreus*, which degrades a higher proportion of carbohydrates. Despite the fact that condensed tannins form direct C-C interunit linkages as opposed to the ether linkages exhibited by lignins, we have shown that the white rot fungi are capable of degrading both of these polyphenolic species, introducing the potential for further industrial applications.

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