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Catabolic Fate of *Streptomyces viridosporus* T7A-Produced, Acid-Precipitable Polymeric Lignin upon Incubation with Ligninolytic *Streptomyces* Species and *Phanerochaete chrysosporium*†

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Degradation of ground and hot-water-extracted corn stover (*Zea mays*) lignocellulose by *Streptomyces viridosporus* T7A generates a water-soluble lignin degradation intermediate termed acid-precipitable polymeric lignin (APPL). The further catabolism of T7A-APPL by *S. viridosporus* T7A, *S. badius* 252, and *S. setonii* 75Vi2 was followed for 3 weeks in aerated shake flask cultures at 37°C in a yeast extract-glucose medium containing 0.05% (wt/vol) T7A-APPL. APPL catabolism by *Phanerochaete chrysosporium* was followed in stationary cultures in a low-nitrogen medium containing 1% (wt/vol) glucose and 0.05% (wt/vol) T7A-APPL. Metabolism of the APPL was followed by turbidometric assay (600 nm) and by direct measurement of APPL recoverable from the medium. Accumulation and disappearance of soluble low-molecular-weight products of APPL catabolism were followed by gas-liquid chromatography and by high-pressure liquid chromatography, utilizing a diode array detector. Identified and quantified compounds present in culture media included *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, protocatechuic acid, vanillic acid, and vanillin. The further catabolism of these APPL-derived aromatic compounds varied with the culture examined, and only *S. setonii* and *P. chrysosporium* completely degraded all of them. Some new intermediates of APPL metabolism also appeared in culture media, but the patterns were culture specific. Additional evidence from high-pressure liquid chromatography analyses indicated that one strain, *S. badius*, converted a water-soluble fraction evident by high-pressure liquid chromatography (7 to 10 min retention time range) into new products appearing at shorter retention times. Mineralization of a [^{14}C-lignin]APPL was also followed. The percent ^{14}C recovered as $^{14}CO_2$, ^{14}C-APPL, ^{14}C-labeled water-soluble products, and cell mass-associated radioactivity, were determined for each microorganism after 1 and 3 weeks of incubation in bubbler tube cultures at 37°C. *P. chrysosporium* evolved the most $^{14}CO_2$ (10%), and *S. viridosporus* gave the greatest decrease in recoverable ^{14}C-APPL (23%). The results show that *S. badius* was not able to significantly degrade the APPL, while the other microorganisms demonstrated various APPL-degrading abilities. The significance of these findings relative to the fate of APPLs in nature was discussed.

The degradation of lignocellulose by *Streptomyces viridosporus* results in the production of a modified lignin polymer, which is a water-soluble lignin degradation intermediate that precipitates from aqueous solution upon acidification. This modified lignin polymer, which has been named acid-precipitable polymeric lignin (APPL), is the initial intermediate of lignin degradation by *S. viridosporus* (6). Chemically, APPLs are lignin fragments which have an increased number of free phenolic hydroxyl, α-carbonyl, and carboxylic acid groups as compared with native lignin (6). The appearance of APPL as a water-soluble metabolite during lignocellulose degradation by *S. viridosporus* correlates closely with lignin loss from the insoluble substrate (1). The accumulation of APPL in the aqueous phase of culture media suggested that APPLs could be a terminal end product of lignin degradation by *Streptomyces* spp. If so, ligninolytic *Streptomyces* spp. might be solubilizing lignin to access plant polysaccharides which they readily degrade with extracellular cellulases and xylanases (5).

It was the objective of this investigation to determine whether three lignin-degrading *Streptomyces* spp. (*S. badius* 252, *S. setonii* 75Vi2, and *S. viridosporus* T7A) and the white-rot fungus *Phanerochaete chrysosporium* could degrade an APPL produced previously during the degradation of corn stover lignocellulose by *S. viridosporus*. Catabolism of soluble APPL by each organism was followed by turbidometric and gravimetric assays for a 3-week incubation period under cultural conditions optimal for lignin degradation by each strain. Appearance or disappearance, or both, of low-molecular-weight intermediates of APPL metabolism were followed by high-pressure liquid chromatography (HPLC), utilizing a diode array detector, and by gas-liquid chromatography (GLC). The ability of each microorganism to degrade a [^{14}C-lignin]APPL, prepared from ^{14}C-lignin-labeled corn lignocellulose by bioconversion with *S. viridosporus*, was also determined over a 3-week incubation period. The results demonstrate that this modified lignin polymer was slowly degraded by each of the microorganisms except *S. badius* and that there were culture-specific patterns of metabolism that clearly differentiated the organisms from one another.

MATERIALS AND METHODS

Organisms. Flasks were inoculated with spores from 2- to 12-week-old stock slants of cultures grown on yeast extract-malt extract agar (18). *Streptomyces* sp. strains used were *S. badius* 252 (ATCC 39117) (14), *S. setonii* 75Vi2 (ATCC 39116) (16) and *S. viridosporus* T7A (ATCC 39115) (19). *P. chrysosporium* ME446 was obtained from R. L.

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Crawford (University of Minnesota) and was maintained on malt extract agar (Difco Laboratories, Detroit, Mich.).

Production of T7A-APPL. Corn stover (*Zea mays*) was dried, ground in a Wiley mill to pass a 20-mesh screen, and hot water extracted. Five-gram portions of air-dried extracted corn lignocellulose were sterilized by autoclaving in 1-liter reagent bottles and then inoculated with 50 ml of active *S. viridosporus* cells which had been grown aerobically for 48 h at 37°C in a 0.6% (wt/vol) mineral salts-yeast extract medium (1). Cultures were incubated at 37°C for 6 weeks as aerobic solid-state fermentations (6). The APPL produced by *S. viridosporus* T7A (T7A-APPL) was then harvested by extraction of the partially decomposed lignocellulose in each bottle with 100 ml of 0.1 N NaOH. The residual insoluble lignocellulose residue was removed by filtration (Whatman no. 54 filter), and the filtrate was then acidified to pH 1 to 2 with concentrated H_2SO_4. The T7A-APPL which precipitated was collected by centrifugation at 16,000 × *g* for 30 min, air dried, and ground to a fine powder with a mortar and pestle. A standard stock solution of T7A-APPL was prepared by adding 10 g of APPL to 800 ml of 1 N NaOH and stirring until the APPL completely dissolved. The pH was adjusted to 9.5 to 10.0 with concentrated HCl, and the volume was brought to 1 liter. This solution was stored at 4°C until needed.

Catabolism of T7A-APPL. Spores of each *Streptomyces* sp. were inoculated into a liquid APPL medium containing 0.05% (wt/vol) T7A-APPL, 0.1% (wt/vol) yeast extract (Difco Laboratories), 0.1% (wt/vol) glucose, and mineral salts solution (5.3 g of Na_2HPO_4, 1.98 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.05 g of $CaCl_2 \cdot 2H_2O$, plus trace elements [18] per liter of deionized H_2O; pH 7.1 to 7.2). Inoculated flasks were incubated with shaking at 100 rpm and 37°C, as were sterile uninoculated controls. For *P. chrysosporium* the defined basal medium described by Kirk et al. (11) was used with 0.05% (wt/vol) T7A-APPL and 1.0% (wt/vol) glucose. Incubation was by stationary cultures, as recommended by Kirk et al. (11), at 37°C, pH 5.5. Inoculated control cultures for HPLC analysis were incubated as above for each microorganism, using the same medium minus the T7A-APPL.

Utilization of the T7A-APPL by each culture in 1-liter volumes of the APPL medium was followed over time by monitoring changes in turbidity (600 nm) of acidified 2-ml samples (1). In addition, changes in recoverale APPL were determined weekly for 3 weeks, using 1-liter cultures. In this procedure flasks were harvested, and cells were removed by filtration onto preweighed filter paper disks (Whatman no. 54). The dry weight of cell mass was determined by washing, drying, and reweighing the disks. The filtrate was acidified with 1 to 3 ml of concentrated H_2SO_4, and the precipitate (APPL) was collected by centrifugation at 16,000 × *g* for 30 min in preweighed centrifuge bottles. After air drying at 50°C overnight the amount of recoverable APPL was determined by reweighing the bottles. The Kjeldahl nitrogen content of each of the APPLs was determined to estimate their crude protein content (12). Therefore, correction for protein contamination of recovered APPL could be performed.

After removal of the APPLs, the acidified supernatants were extracted twice with ether and once with ethyl acetate (16). The solvent-extractable fraction of each filtrate was examined for low-molecular-weight aromatic compounds by GLC of their trimethylsilyl derivatives (2, 17) and by HPLC (16).

Chromatographic analyses. For GLC, trimethysilyl derivatives of solvent-extractable samples, 2 to 3 mg each, were prepared with 100 μl of *p*-dioxane containing internal standard (0.1 mg of 3,4-dimethylbenzaldehyde), 10 μl of pyridine, and 50 μl of *N,O*-bis(trimethylsilyl)-acetamide. Each sample was held at 35°C for 2 h prior to injection. GLC was performed on a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Bellevue, Wash.) with a flame ionization detector and RSL-150 capillary column (30 m by 0.25 mm) (Alltech Associates, Inc., Deerfield, Ill.). Column conditions were as follows. The oven temperature was 120°C for 2 min followed by a 20°C/min gradient to 260°C, holding there for 3 min. The injector temperature was 240°C and the detector temperature was 280°C.

HPLC was performed on a Hewlett-Packard 1090A high-pressure liquid chromatograph, using an HP-1040A diode array detector. During each run, chromatograms for 258, 280, and 310 nm were recorded. UV absorbance spectrum (250 to 350 nm) of each peak was recorded at its front, apex, and back side. A 100-mm Hewlett-Packard microbore reverse-phase column (2.1-mm inside diameter) of Hypersil ODS with 5-μm particle diameter was used with a 40°C column temperature, 5-μl sampling loop, and 0.4-ml/min flow rate. A gradient was used for solvent delivery, using pH 3.2 water (H_2SO_4) and 10% acetonitrile initially for 2 min, followed by the acetonitrile concentration increasing to 50% over the next 8 min, increasing to 100% over the following 4 min, and held at 100% acetonitrile for the final 2 min, with a total run taking 16 min.

Standard compounds used for identification and quantification by GLC and HPLC were *p*-coumaric acid (4-hydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid), and vanillin (3-methoxy-4-hydroxybenzaldehyde) (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The specific retention times and absorption maxima for these standards when separated by HPLC, and their relative retention times by GLC, are given in Table 1.

[^{14}C-lignin]APPL degradation. L-[U-^{14}C]phenylalanine was treated with L-phenylalanine ammonia-lyase as described by Pometto and Crawford (15). The procedure produced a mixture containing *trans*-[U-^{14}C]cinnamic acid (60%) and L-[U-^{14}C]phenylalanine (40%). A 10-μCi portion of this mixture was fed to a 2-month-old corn plant by phloem injection with a syringe. The plant was then kept in a growth chamber for 1 week to allow growth and incorporation of the ^{14}C-labeled lignin precursors. Then the plant

TABLE 1. Relative retention time and absorption maximum of each standard single-ring aromatic compound as determined by GLC and HPLC

Compound	HPLC		GLC (relative retention time [min])[a]
	Retention time (min)	Absorption maximum (nm)	
p-Coumaric acid	4.75	308	1.54
Ferulic acid	5.48	320	1.86
p-Hydroxybenzoate	2.09	254	1.23
p-Hydroxybenzaldehyde	2.83	286	0.89
Protocatechuic acid	1.51	260	1.43
Vanillic acid	2.66	260	1.37
Vanillin	3.94	278	1.10

[a] Calculation of the relative retention time was based on an internal standard of 3,4-dimethoxybenzaldehyde which had an actual retention time of 6.04 min.

was harvested, and a ^{14}C-lignin-labeled lignocellulose was prepared from its air-dried tissues as previously described (3, 4, 9). Medium containing this extracted ^{14}C-lignin-labeled corn lignocellulose was then sterilized, inoculated with a concentrated cell suspension of *S.viridosporus*, and incubated for 4 weeks at 37°C, using the solid-state fermentation system, as described above. The culture was then harvested, and the residue containing partially degraded ^{14}C-labeled lignocellulose, biomass, and ^{14}C-APPL was extracted with hot water and then filtered (6). The [^{14}C-lignin]APPL, which was extracted into the aqueous phase, was collected by acid precipitation from the filtrate, centrifugation, and air drying as described above. The specific activity (934.6 dpm/mg) was determined by combustion of milligram-size samples to $^{14}CO_2$ at 750°C in a tube furnace. $^{14}CO_2$ was quantified by liquid scintillation counting on a Beckman LS-7000 (Beckman Instruments Inc., San Jose, Calif.) after trapping in a scintillation cocktail (3). A stock solution of [^{14}C-lignin]APPL containing 100 mg of ^{14}C-APPL in 10 ml of 0.1 N NaOH was prepared.

An APPL medium was prepared for growth of the *Streptomyces* spp. as described above, containing 0.05% (wt/vol) [^{14}C-lignin]APPL, 0.1% (wt/vol) yeast extract, and 0.1% (wt/vol) glucose in the mineral salts solution. [^{14}C-lignin]APPL was added volumetrically to the medium from the stock solution prepared previously. The medium for *P. chrysosporium* contained 0.05% (wt/vol) [^{14}C-lignin]APPL plus 1.0% (wt/vol) glucose in a low-nitrogen medium at pH 4.5 as recommended by Kirk et al. (11). Five replicates of each culture were inoculated into 10 ml of appropriate medium and incubated aerobically at 37°C as bubbler tube cultures (7, 14). Five uninoculated controls for each of the two media were also incubated. After 1 and 3 weeks of incubation with continuous aeration, the percentage of the initial ^{14}C-APPL that had been evolved as $^{14}CO_2$ was determined (14). The cell mass produced by each culture was collected by filtration after 1 and 3 weeks of incubation. The filtrates were acidified to precipitate the remaining [^{14}C-lignin]APPL, which was recovered by centrifugation. The percent recovery of ^{14}C in the cell mass and APPL fractions was determined by combustion. The percent recovery of ^{14}C in the supernatant was determined in a water-tolerant liquid scintillation cocktail (1-ml sample per 6 ml of Aquasol-2; New England Nuclear, Boston, Mass.).

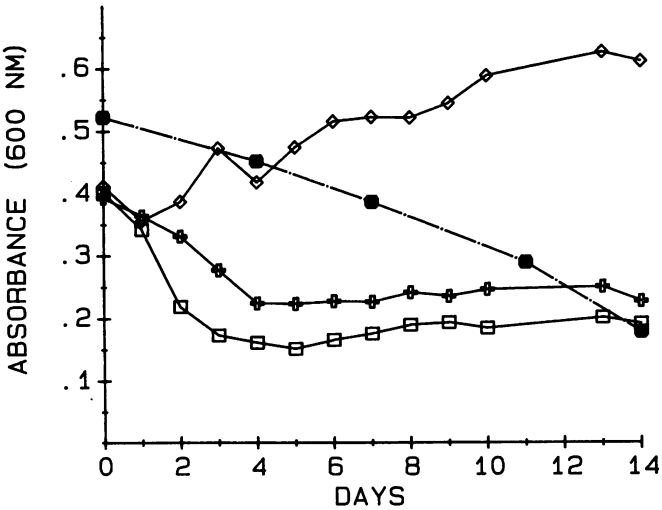


FIG. 1. APPL loss over a 2-week incubation as measured by changes in A_{600} of acidified 2-ml samples for cultures incubated at 37°C in APPL medium. Symbols: *S. badius* (◆); *S. setonii* (✤); *S. viridosporus* (□); *P. chrysosporium* (■).

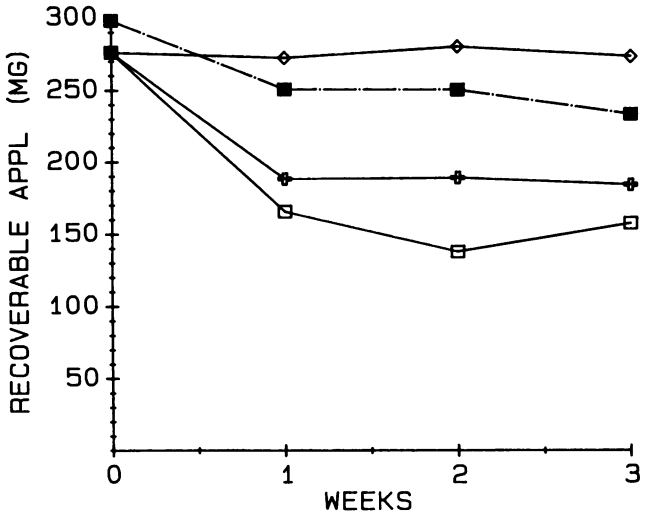


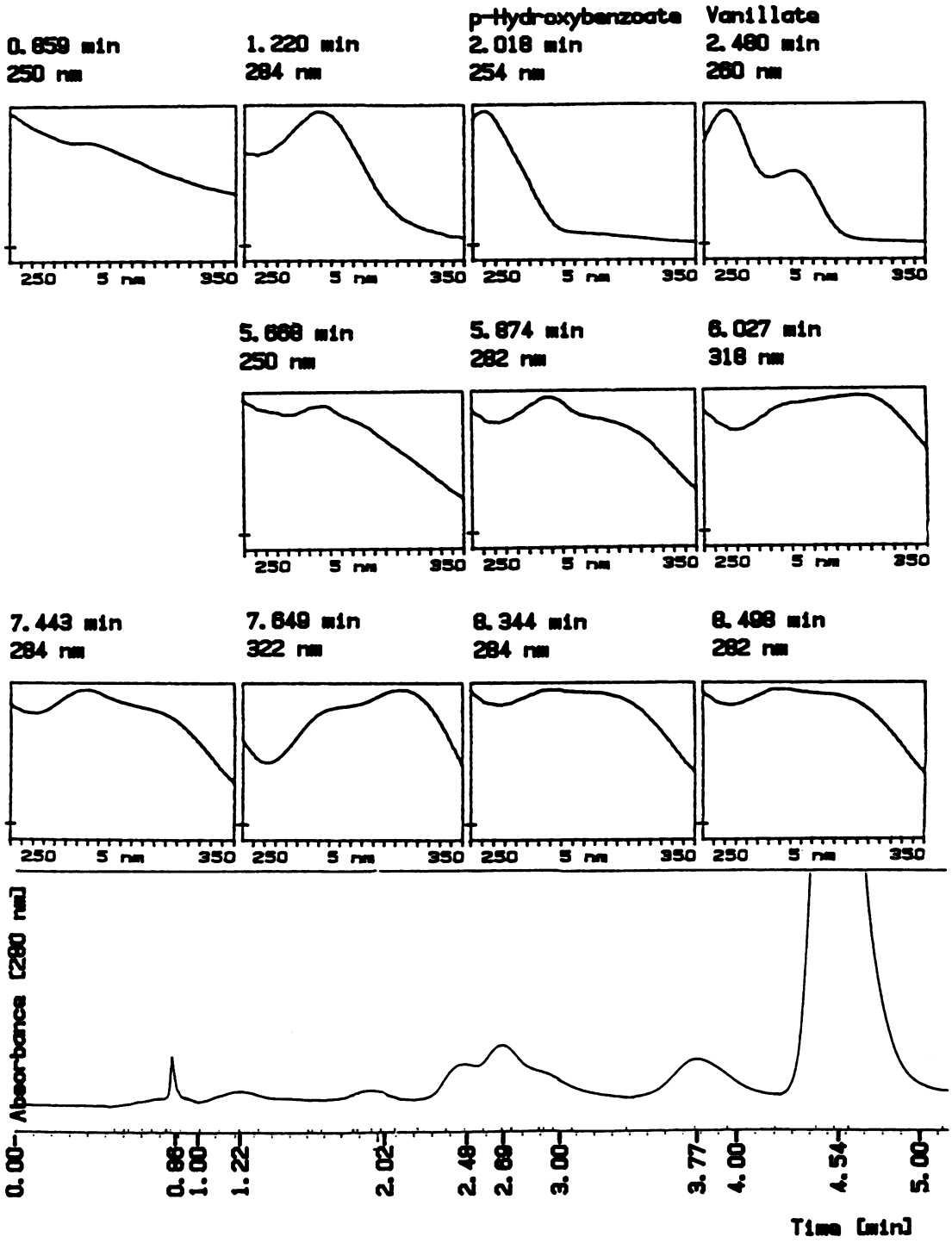
FIG. 2. Changes in APPL recoverable from the APPL media over a 3-week incubation. Results are for 1 liter of APPL medium with an initial content of 500 mg of APPL. The percent protein was subtracted from each value. Symbols: *S. badius* (◆); *S. setonii* (✤); *S. viridosporus* (□); *P. chrysosporium* (■).

RESULTS

Turbidometric assay of APPL degradation. Over a 14-day incubation each of the microorganisms except *S. badius* removed APPL from the growth medium as measured by a decrease in absorbance of acidified medium samples at 600 nm (Fig. 1). *S. setonii* and *S. viridosporus* removed APPL rapidly during the first 4 to 5 days, but there was little change thereafter. In contrast, *S. badius* showed an increase in acid-precipitable material over time. This was due to an increase in extracellular protein (see later discussion) which coprecipitated with the APPL. In stationary culture *P. chrysosporium* catalyzed the slow continual removal of precipitable material. Turbidity of samples from the uninoculated controls also declined from 0.409 to 0.318 for the *Streptomyces* sp. medium (control-S) and from 0.561 to 0.436 for the *Phanerochaete* sp. medium (control-PC), probably as a result of partial depolymerization and autooxidation of the APPLs during the long period of aerobic incubation at 37°C. These results indicate that APPLs were subject to a low level of nonbiological degradation under aerobic conditions.

Cell mass production and APPL loss during growth on APPL. Cell mass accumulation for *S. badius*, *S. setonii*, and *S. viridosporus* in the shake flask cultures entered a stationary growth phase after 1 to 2 days and remained there for the rest of the 3-week incubation. In standing culture *P. chrysosporium* reached stationary growth phase at day 9. However, most of the apparent cell mass increase after day 4 or 5 was probably due to the accumulation of polysaccharide slime, not cell mass (T. K. Kirk, personal communication).

The weight of recoverable APPL decreased over the 3-week period for each of the microorganisms except *S.*



badius, which showed no change (Fig. 2). Control-S and control-PC showed relatively little decrease (<5%) in recoverable APPL during the 3-week incubation period. These relatively unchanged gravimetric values demonstrate that the control APPL losses indicated by the turbidometric assay were actually rather minor. With *S. badius* the amount of crude protein associated with the precipitated APPL was 26 to 27%, while for the other *Streptomyces* spp. and control-S the values ranged between 19 and 23%. For *P. chrysosporium* and control-PC crude protein content was 11 to 12%.

Changes in low-molecular-weight aromatic compounds present in the APPL medium. APPLs produce a range of low-molecular-weight, water-soluble aromatic components upon autoclaving. Figure 3 shows an HPLC chromatogram for control-S supernatant and the UV absorption spectrum

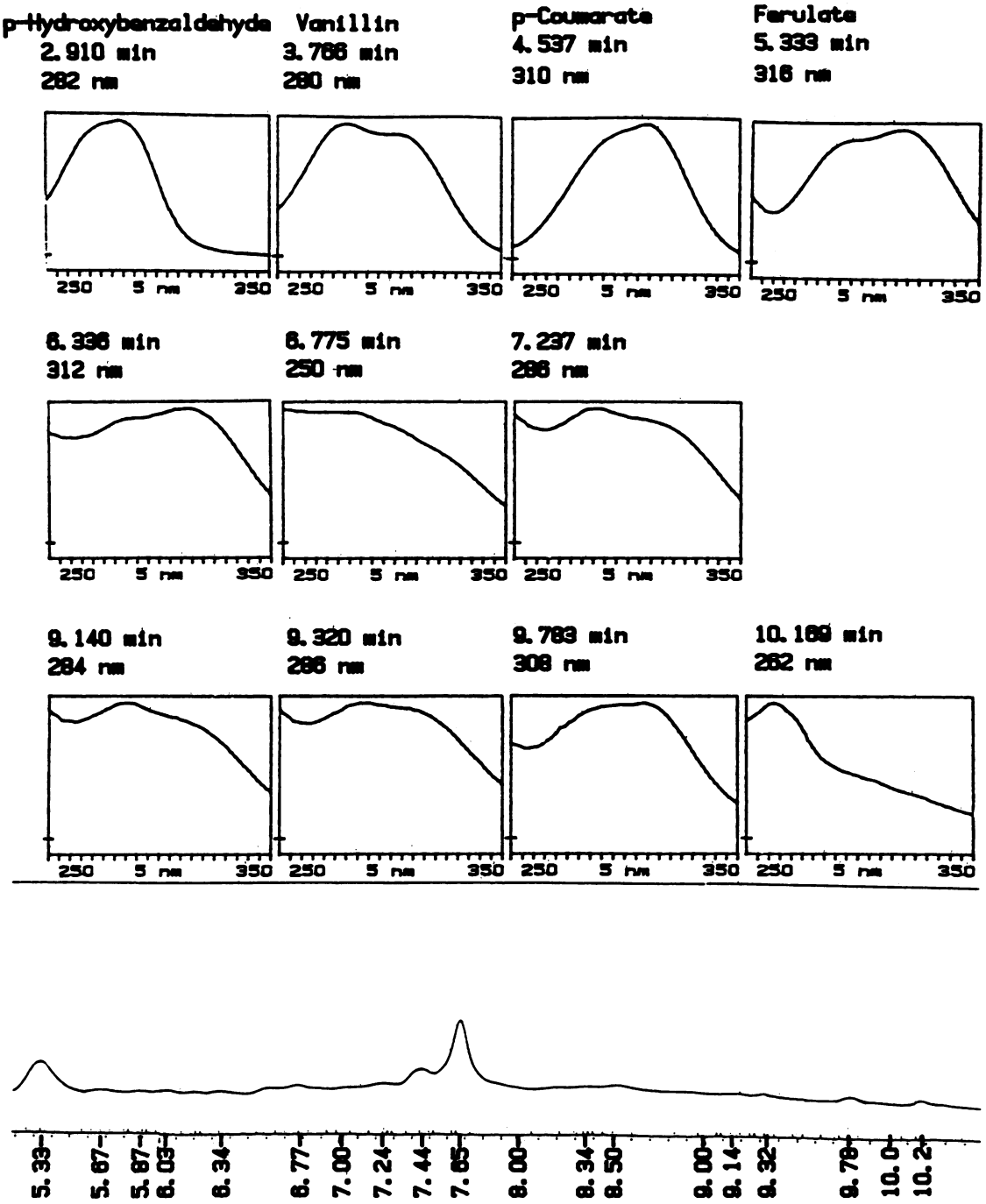


FIG. 3. HPLC at 280 nm and 0.2 absorbance unit with retention time and UV absorption spectrum with λ_{max} for each peak present from the solvent extract of acidified uninoculated control-S APPL medium supernatant after 1 week of shaken incubation at 37°C.

for each of the significant peaks. Several of these peaks were identified and quantified by comparisons to authentic standard compounds chromatographed by both HPLC and GLC (Table 2). Each of the microorganisms caused changes in the amounts and kinds of low-molecular-weight aromatic products detected by HPLC in culture supernatants as compared with the appropriate control (Fig. 4 and 5). *S. setonii* almost completely removed all of the initial compounds in the control HPLC chromatogram (Fig. 3). Those specifically identified were substituted single-ring aromatic acids and aldehydes that *S. setonii* is known to catabolize (Table 2) (17, 20). *S. badius*, on the other hand, catabolized none of these identifiable single-ring aromatics (Table 2), but it did cause a decrease in the concentration of other products present in the control-S supernatant, which were present in HPLC chromatograms as compounds with retention times ranging between 7 and 10 min (Fig. 4). Most of the peaks in the 7- to 10-min range have absorption maxima of 312 to 314

TABLE 2. Identification and quantification by GLC of low-molecular-weight aromatic compounds solvent extracted from acidified culture broths after 3 weeks of incubation of the ligninolytic microorganisms in the APPL medium as compared with an uninoculated control[a]

Compound	mg/500 mg of initial APPL					
	S. badius	*S. setonii*	*S. viridosporus*	Control-S[b]	*P. chrysosporium*	Control-PC[c]
p-Coumaric acid	3.97[d]	0.06	2.72[d]	3.92[d]	0.59[d]	2.86[d]
Ferulic acid	ND[e]	0.13	0.19[d]	0.20[d]	0.19	0.62[d]
p-Hydroxybenzoate	0.52[d]	0.01	0.12	0.26[d]	0.13[d]	0.15[d]
p-Hydroxybenzaldehyde	0.11	0.02	0.08	0.86[d]	ND	0.69[d]
Vanillic acid	1.29[d]	0.03	1.08[d]	0.66[d]	0.11	0.33[d]
Vanillin	ND	0.06	ND	0.32[d]	0.03	0.24

[a] Values were determined from an internal standard of 3,4-dimethoxybenzaldehyde of known concentration (10 mg/ml) and a standard curve for each compound.
[b] Sterile uninoculated control for *Streptomyces* sp. APPL medium, pH 7.1.
[c] Sterile uninoculated control for *Phanerochaete* sp. APPL medium, pH 5.5.
[d] Compounds also confirmed by HPLC.
[e] ND, Not detected.

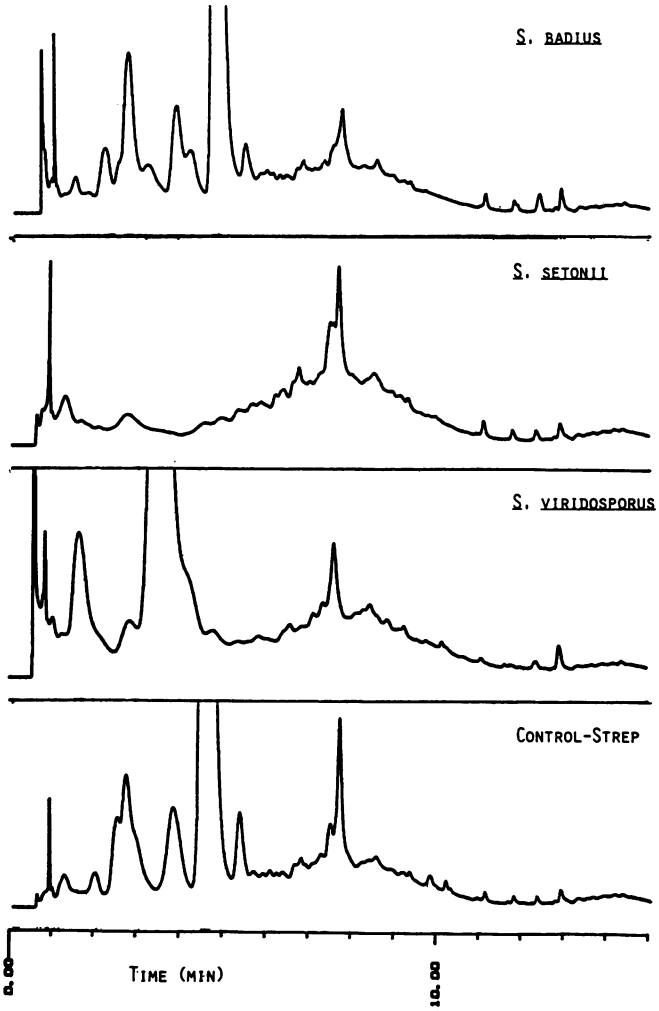


FIG. 4. HPLC at 280 nm and 0.2 absorbance unit for solvent extracts of acidified culture supernatants from each of the lignin-degrading *Streptomyces* spp. and from an acidified uninoculated control-S supernatant harvested after 3 weeks as a shake flask culture at 37°C in APPL medium.

nm (Fig. 3), an absorption often associated with aldehyde or carbonyl groups or both. The appearance of four new products was observed during APPL degradation by *S. badius* (Fig. 6). The 1.51-min peak was identified as protocatechuic acid (0.03 mg). *S. viridosporus* also altered the pattern of peaks observed with retention times of under 5 min as compared with the control-S (Fig. 4). Vanillin, which was present in control supernatants, was converted to vanillic acid by *S. viridosporus* (Table 2). This streptomycete is known to produce an aromatic aldehyde oxidase that readily oxidizes vanillin (8). *S. viridosporus* has also been shown to catabolize *p*-hydroxybenzaldehyde via *p*-hydroxybenzoic acid (8). The oxidation of *p*-hydroxybenzaldehyde present in control-S supernatants to the corresponding acid by *S. viridosporus* was also observed here (Table 2). The production of four new unidentified products during APPL metabolism was observed for *S. viridosporus* (Fig. 6). As shown in the HPLC chromatogram

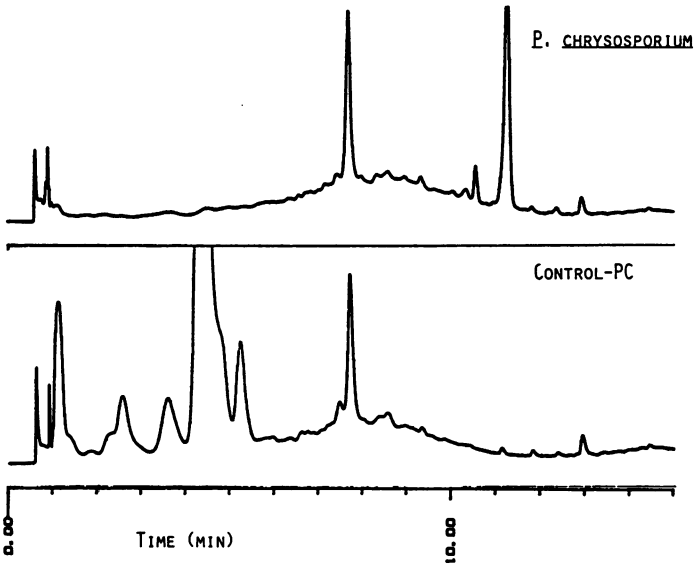


FIG. 5. HPLC at 280 nm and 0.2 absorbance unit for solvent extracts of an acidified culture supernatant from *P. chrysosporium* culture and for an acidified uninoculated control-PC supernatant harvested after 3 weeks as a stationary culture at 37°C in APPL medium.

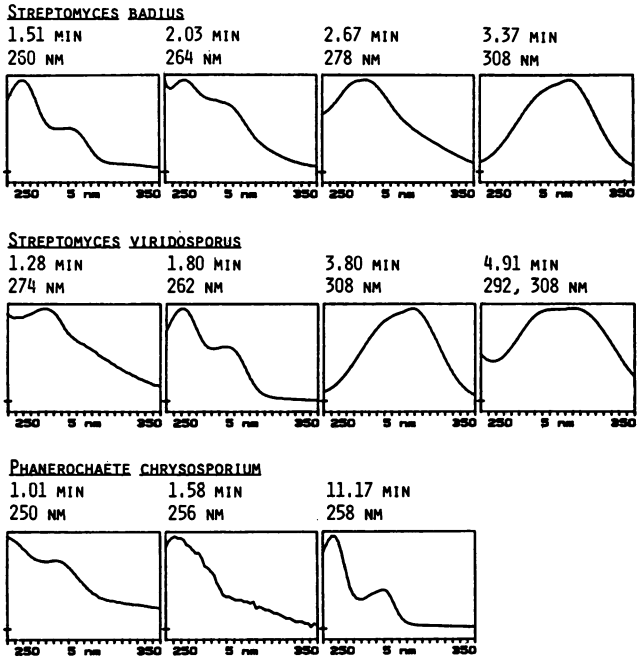


FIG. 6. HPLC data showing the retention times and UV absorption spectra with λ_{max} for new solvent-extractable low-molecular-weight aromatic compounds detected during incubation of the lignin-degrading microorganisms in APPL medium.

in Fig. 5, *P. chrysosporium* in stationary culture removed virtually all of the compounds initially present in the control-PC supernatant. Every one of the identified compounds was dramatically reduced as the result of fungal growth (Table 2). *P. chrysosporium* has been shown to degrade each of the single-ring aromatic compounds listed in Table 2, plus many other lignin substructure model compounds (21). The production of three new unidentified products was observed during APPL degradation by *P. chrysosporium*. The major product formed had a retention time of 11.17 min (Fig. 6). In each of the cases where new product peaks were observed by HPLC, new peaks were also detected by GLC. None of these chromatographic peaks were detected in any culture filtrate when each culture was incubated in the same medium minus the APPL.

Degradation of [^{14}C-lignin]APPL. Recoveries of each ^{14}C-APPL fraction resulting from the catabolism of [^{14}C-lignin]APPL by the four microorganisms were determined after 1 and 3 weeks of incubation (Tables 3 and 4). After 1 week of incubation each of the *Streptomyces* spp., except *S. viridosporus*, had converted a greater percentage of the ^{14}C-APPL to $^{14}CO_2$ than was observed for the uninoculated control-S (Table 3). *P. chrysosporium* mineralized the most ^{14}C-APPL to $^{14}CO_2$ in 3 weeks (10.2%) followed by *S. setonii* (6.4%) (Table 4). However, for *S. setonii* most (70%) of the $^{14}CO_2$ evolution occurred during week 1 of incubation (Table 3). *S. viridosporus* mineralization of ^{14}C-APPL was more rapid during weeks 2 and 3 than during week 1 of incubation (Tables 3 and 4). *S. badius* showed only a slight increase in $^{14}CO_2$ recovered over that observed in the uninoculated control-S (Table 4).

TABLE 3. Catabolism of ^{14}C-[lignin]-APPL by the three lignin-degrading *Streptomyces* spp. after 7 days of incubation in bubbler tubes at 37°C[a]

Culture	% Recovered			
	$^{14}CO_2$	^{14}C-water soluble	^{14}C-APPL	^{14}C-cell mass
S. badius	1.8	47.8	44.7	5.7
S. setonii	4.5	46.6	33.8	14.8
S. viridosporus	0.9	56.1	21.2	21.8
Control-S	1.1	54.5	44.3	

[a] Values are averages of five replicates. Cultures were grown in an APPL medium containing 0.05% (wt/vol) [^{14}C-lignin]APPL (4,680 dpm/5 mg), 0.1% (wt/vol) yeast extract, and 0.1% (wt/vol) glucose in mineral salts solution.

TABLE 4. Catabolism of [^{14}C-lignin]APPL by the three lignin-degrading *Streptomyces* spp. and *P. chrysosporium* after 3 weeks of incubation in bubbler tubes at 37°C[a]

Culture	% Recovered			
	$^{14}CO_2$	^{14}C-water soluble	^{14}C-APPL	^{14}C-cell mass
S. badius[b]	3.2	42.6	50.4	3.8
S. setonii[b]	6.4	39.3	40.5	13.7
S. viridosporus[b]	4.0	43.0	35.1	17.9
Control-S[b]	2.2	45.9	51.8	
P. chrysosporium[c]	10.2	31.2	48.2	10.5
Control-PC[c]	0.4	45.2	54.5	

[a] Values are averages of five replicates.
[b] Cultures were grown in an APPL medium containing 0.05% (wt/vol) [^{14}C-lignin]APPL (4,680 dpm/5 mg), 0.1% (wt/vol) yeast extract, and 0.1% (wt/vol) glucose in mineral salts solution. Uninoculated controls were also incubated for the 3-week period.
[c] The low-nitrogen *P. chrysosporium* medium of Kirk et al. (11) was used, with 0.05% [^{14}C-lignin]APPL (4,680 dpm/5 mg) supplied as the source of lignin. Uninoculated controls were also incubated for the 3-week period.

After 3 weeks of incubation, decreases in the amount (percent) of ^{14}C water-soluble radioactivity in culture supernatants as compared with uninoculated controls were greatest for *P. chrysosporium* (14.0% removed) and *S. setonii* (6.6% removed) (Table 4). *S. badius* and *S. viridosporus*, in contrast, showed only slight decreases in water-soluble ^{14}C after 3 weeks, and with *S. viridosporus* an increase was actually observed at 1 week.

The greatest decrease in the percentage of recoverable ^{14}C-APPL, as compared with the appropriate uninoculated control, was for *S. viridosporus* after both 1 and 3 weeks of incubation (23.1 and 16.7% lower, respectively) (Tables 3 and 4). *S. setonii* was second with ^{14}C-APPL recoveries after 1 and 3 weeks of 10.5 and 11.3%, respectively. *P. chrysosporium* showed only a 6.3% lower value than its control after 3 weeks of incubation (Table 4), and *S. badius* showed no significant change as compared with its controls after 1 or 3 weeks (Tables 3 and 4).

The APPL-derived ^{14}C which was associated with the cell mass fraction of cultures exhibited microorganism-specific patterns. *S. viridosporus* cultures had the highest percentage of ^{14}C associated with cell mass after 1 and 3 weeks of incubation (21.8 and 17.9% of the initial ^{14}C-APPL, respectively; Tables 3 and 4). These values correspond to APPL which was either biosynthetically incorporated into cell mass or became adsorbed to cell mass. *S. setonii* showed only a slight change between the first and third weeks, with values averaging 14.8 and 13.7%, respectively. With *P. chrysosporium*, cell mass-associated radioactivity was 10.5% of the initial ^{14}C-APPL after 3 weeks of incubation, approximately equivalent to the 10.2% recovered as $^{14}CO_2$. *S. badius* gave the smallest ^{14}C-cell mass-associated recovery after both 1 and 3 weeks of incubation (5.7 and 3.8%, respectively).

DISCUSSION

Each of the microorganisms, with the possible exception of *S. badius*, was able to slowly metabolize the APPL or APPL-derived aromatic compounds or both. Growth of *S. badius* resulted in no decrease in the amount of APPL recoverable from culture media as compared with controls (Fig. 2), and this streptomycete generated only a minor amount of $^{14}CO_2$ (3.2%) from the [^{14}C-lignin]APPL in 3 weeks as compared with the control (2.2%) (Table 4). However, there was a definite change in the pattern of low-molecular-weight products present in supernatants from *S. badius* cultures as compared with uninoculated controls. During incubation, a quantitatively significant product appeared (with a retention time of 2.67 min in HPLC chromatograms), and there was a simultaneous reduction of some of the other low-molecular-weight products present in control chromatograms in the 7- to 10-min retention time range as observed by HPLC (Fig. 4). This reduction was confirmed by GLC (Table 2). This indicates that *S. badius* has the ability to modify certain as yet unidentified aromatic compounds derived from the APPL.

S. setonii, *S. viridosporus*, and *P. chrysosporium* each had the ability to degrade at least a portion of the APPL as demonstrated by their abilities to remove APPL from culture media and by their abilities to mineralize a significant amount of the [^{14}C-lignin]APPL. *S. setonii* and *P. chrysosporium* efficiently degraded low-molecular-weight products present in the culture media as confirmed by both the HPLC data and reductions in the percentage of ^{14}C-labeled water-soluble components recovered after growth of these organisms on APPL. The rate of [^{14}C-lignin]APPL mineralization by *S. setonii* was greatest during week 1 of growth (4.5% as $^{14}CO_2$), with a more limited rate in the last 2 weeks (6.4% final recovery). Over the 3-week incubation period *S. setonii* did not produce any new products that could be detected by HPLC or GLC. Readily degradable single-ring aromatics had been decomposed by the end of 1 week, and no new products accumulated, an indication that any low-molecular-weight APPL degradation intermediates produced by *S. setonii* were almost immediately catabolized after release. Alternatively, the culture may not appreciably attack the APPL polymer, though it will rapidly degrade many low-molecular-weight APPL-derived aromatic compounds. HPLC and GLC showed that *P. chrysosporium* also rapidly removed single-ring aromatic compounds from culture media as it decomposed APPL. In addition, the much more significant mineralization of the [^{14}C-lignin]APPL by this fungus (10.2% as $^{14}CO_2$ in 3 weeks) as compared with the *Streptomyces* spp. (3.2 to 6.4% mineralization in 3 weeks) indicates that *P. chrysosporium* also has a greater ability to attack the APPL polymer. However, the conditions of bubbler tube cultures may not have been optimal for lignin degradation by *P. chrysosporium* and probably underestimate the organism's ability to mineralize APPL lignin. We did not detect veratryl alcohol, which is known to be produced as a secondary metabolite accumulating during lignin degradation by *P. chrysosporium* (9, 10, 13), in any fungal supernatants by either HPLC or GLC. However, all of the data clearly show that the culture was ligninolytic when growing on APPL. The high concentration of [^{14}C-lignin]APPL associated with *S. viridosporus* cell mass (17.9%; Table 4) indicates that this actinomycete had a high affinity for its APPL and adsorbed it strongly. In comparison, the other cultures had between 3.8 and 13.7% of the initial radioactivity associated with their cell mass after 3 weeks of incubation (Table 4). The strong adsorption of APPL by *S. viridosporus* indicates that its ligninolytic enzyme system could be cell surface associated. As compared with the other microorganisms, *S. viridosporus* was intermediate in its ability to mineralize the [^{14}C-lignin]APPL (Tables 3 and 4), and it had less ability to degrade the low-molecular-weight aromatic compounds initially present in the APPL medium (Fig. 4). The culture's main activity was the oxidation of APPL-derived aromatic aldehydes to their corresponding carboxylic acids, an activity also observed with *S. badius*.

The data indicate that these ligninolytic *Streptomyces* spp. have only a limited ability to further metabolize APPL once it is released as a lignin degradation intermediate. This is the case even for *S. viridosporus*, the organism which originally generated the APPL used in this study. A limited ability to further metabolize APPLs indicates also that these ligninolytic *Streptomyces* spp. may be releasing lignin in the form of APPL as a mechanism to gain access to plant polysaccharides which they degrade with active cellulases and xylanases (5).

APPLs, particularly [^{14}C-lignin]APPL, may be useful as a substrate in studies to determine whether specific microorganisms have the ability to attack polymeric, water-soluble lignin degradation intermediates. Since APPLs become available to other soil microorganisms upon their release from lignocellulose by *Streptomyces* spp., this is an important question relating to the microbial ecology of lignin biodegradation. However, before using other *Streptomyces* spp.-produced APPL-like polymers in such studies, one should do the chemical characterizations needed to confirm their lignin origin (6) and to determine if they have already been modified to a point where they are not sufficiently lignin-like anymore (1).

The HPLC with diode array detector analysis procedure is a valuable tool for the study of lignin biodegradation. When standards are available for confirmation of compound identities, the combination of retention time and UV absorption spectrum for each peak permits rapid identification of products. Also, peak purity can be evaluated by comparing UV spectra obtained from the front, apex, and back side of each chromatographic peak. This is very important for accurate quantification of compounds. In the present research, spectral analysis of each of the peaks shown in Fig. 3, 4, and 5 confirmed very clean separation of the peaks. The detection of new products can be determined by appearance of new chromatographic peaks and UV absorption spectra (Fig. 6). In addition, the spectra of peaks in the chromatogram shown in Fig. 3 indicate that many of the unidentified peaks probably have similar structures. Possibly the spectra of peaks around 7-min retention time (Fig. 3) were those of aromatic dimers which *S. setonii* cannot utilize (Fig. 4) but which were catabolized by *P. chrysosporium* (Fig. 5). The aromatic structures of the lignin molecule lend themselves to this type of analysis, and we expect that the HPLC with diode array analysis technique will play an increasing role in future studies of lignin biodegradation.

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