APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1980, p. 376-380
0099-2240/80/08-0376/05$02.00/0

Vol. 40, No. 2

Degradation of ^{14}C-Labeled Lignins and ^{14}C-Labeled Aromatic Acids by *Fusarium solani*

DALE M. NORRIS
Russell Laboratories, University of Wisconsin, Madison, Wisconsin 53706

Abilities of isolate AF-W1 of *Fusarium solani* to degrade the side chain and the ring structure of synthetic dehydrogenative polymerizates, aromatic acids, or lignin in sound wood were investigated under several conditions of growth substrate or basal medium and pH. Significant transformations of lignins occurred in 50 days in both unextracted and extracted sound wood substrates with 3% malt as the growth substrate and the pH buffered initially at 4.0 with 2,2-dimethylsuccinate. Degradation of lignin in such woods also occurred under unbuffered pH conditions when a basal medium of either 3% malt or powdered cellulose in deionized water was present. Decomposition of the lignin in these woods did not occur in cultures where D-glucose was present as a growth substrate. *F. solani* significantly transformed, as measured as evolved $^{14}CO_2$, both synthetic side chain (β, γ)-^{14}C- and *U*-ring-^{14}C-labeled lignins in 30 days under liquid culture conditions of only distilled deionized water and no pH adjustment. Degradation of dehydrogenative polymerizates by *F. solani* was reduced drastically when D_2 was the liquid medium. AF-W1 also cleaved the α-^{14}C from *p*-hydroxybenzoic acid and evolved $^{14}CO_2$ from the substrate, [3-^{14}C]cinnamic acid. Thus, the fungus cleaved side chain carbon from substrate that originally lacked hydroxyl substitution on the aromatic nucleus. Surprisingly, small amounts of ^{14}C cleaved from aromatic acids by *F. solani* were incorporated into cell mass. Initial buffering of the culture medium to pH 4.0 or 5.0 with 0.1 M 2,2-dimethylsuccinate significantly increased *F. solani* degradation of all lignins or aromatic acids. Results indicated that AF-W1 used lignin as a sole carbon source.

Efforts to elucidate the mechanisms by which microorganisms degrade lignin to simpler molecules have especially relied on experiments with white-rot fungi, e.g., *Phanerochaete chrysosporium* (5, 6). However, the metabolic pathways utilized by lignin-degrading microorganisms have not been defined (4, 11). Recent studies (11) with *Fusarium solani* M-13-1 indicated that it was the most vigorous microbial degrader of a synthetic lignin, i.e., a dehydrogenation polymer (DHP) of coniferyl alcohol, among organisms isolated from soils. Independent experiments with a different strain, AF-W1, of *F. solani* that is cultivated on wood by *Xyleborus* beetles (9) demonstrated the abilities of the fungus to degrade synthetic side chain (β, γ)-^{14}C- and *U*-ring-^{14}C-labeled DHPs (Haanstad et al., Arch. Microbiol., in press). Existing knowledge of the lignin-degrading capabilities of *F. solani* thus seemingly indicates that this non-white-rot fungus is a useful model to study some aspects of the mechanisms of microbial transformations of lignocelluloses.

This paper reports additional experimental findings on *F. solani* AF-W1 relative abilities to degrade the side chain and the ring structure of synthetic DHPs and aromatic acids or decompose lignin in sound wood under several conditions of growth substrate or basal medium and pH.

MATERIALS AND METHODS

Inocula. The *F. solani* inoculum was the AF-W1 isolate from our (i.e., Department of Entomology, University of Wisconsin, Madison) collection of microorganisms that are symbiotes of ambrosia beetles (e.g., Xyleborini). *Coriolus versicolor* and *Cryptoporus volvatus* were from the U.S. Forest Products Laboratory, Madison, Wis.

Standard culture. Our standard sound wood substrate, designated as no. 002, was composed of 93% *Ulmus* and *Acer*, 5% unidentified ring-porous wood, 1% *Betula*, and 1% *Populus*, and other softwood. It was commercially converted to chips (1 in by 2 to 3 in. [2.54 cm by 5.08 to 7.62 cm]) by Weyerhaeuser Corp. (Schofield, Wis.). The chips were dried at 38°C for 24 h and then were ground to a sawdust that passed through a 40-mesh screen. Sawdust was stored in sealed glass jars at −5°C until used. The benzene and ethanol extractables (Table 1) were removed from the sawdust before use in some identified experiments. Two grams of this lignocellulosic substrate were placed in each cotton-plugged 125-ml Erlenmeyer flask, and wood and flask were sterilized by autoclaving. The

appropriate liquid medium (i.e., growth substrate) was added aseptically to each flask.

Inoculum for a flask was obtained by dispersing aseptically a 6-mm plug of 2-week-old *F. solani*, *C. volvatus*, or *C. versicolor* grown on 2% malt agar into 2 ml of the chosen liquid medium, allowing the fungus to multiply in the 2 ml for 1 week, and then aseptically combining the resultant 2-ml inoculum with an additional 4 ml of the liquid medium on the wood substrate in the culture flask. Initial contents of a flask were acidified, as indicated in given experiments, with 1 N HCl and initially buffered with 0.01 M 2,2-dimethylsuccinate. Each flask was held at 28°C and 70% relative humidity in darkness. Each flask was aerated weekly with filter-sterilized air. Cultures were maintained for various periods as indicated in each experiment.

Liquid culture. The DHP substrates for these experiments were either synthetic *U*-ring-^{14}C- or side chain (β, γ)-^{14}C-labeled lignins obtained from T. K. Kirk, U.S. Forest Products Laboratory, Madison, Wis (4). Specific activities were 47,600 and 34,400 dpm/mg, respectively. Aromatic acid substrates were either *p*-[*carboxy*-^{14}C]hydroxybenzoic or [3-^{14}C]cinnamic acid. Specific activities were 8.88×10^8 and 6.96×10^8 dpm/mg, respectively, and the commercial source was Research Products International Corp., Elk Grove Village, Ill.

Inoculum was prepared as described above except that the inoculum plug was dispersed in 5 ml, instead of 2 ml, of the chosen liquid medium. The 5 ml of medium, inoculated or uninoculated, was incubated for 1 week at 28°C and 70% relative humidity in darkness. After 1 week, the radiolabeled substrate was added aseptically, in an additional 5 ml of the chosen liquid medium, to each sterilized, rubber-stoppered, 125-ml Erlenmeyer flask. There were three replicates of each treatment per experiment. The ^{14}C-labeled DHP was first suspended in a 10% dimethylformamide-in-water solution, and a 25-μl volume of this material, which yielded 47,600 dpm of *U*-ring-^{14}C- or 51,600 dpm of side chain (β, γ)-^{14}C-labeled lignin, was then dispersed into the 5 ml of liquid medium being added to each flask. The radiolabeled aromatic acid substrates were each diluted in small volumes of benzene, and 25 μl of the resultant solution was added to each flask by the method detailed for the ^{14}C-labeled DHPs. This constituted 3.0×10^6 dpm/flask for *p*-[*carboxy*-^{14}C]hydroxybenzoic acid or [3-^{14}C]cinnamic acid.

The contents of each rubber-stoppered flask were aerated for 15 min every 3 days with filter-sterilized air. The aeration system was a manifold type similar to that used by Kirk et al. (4). The system was composed of glass tubing, latex rubber tubing, appropriately located screw-type metal clamps, and a central empty glass flask that received the sterile air through an in-line packed cellulose-in-glass filter. The air flowed at the rate of 150 ml/min through a flask and via a syringe needle, into a scintillation vial that contained 10 ml of scintillation fluid composed of a 1:1 mixture of a toluene cocktail [4.0 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-(5-phenyloxazolyl)benzene per liter volume] and an anhydrous methanol-ethanolamine (4:1, vol/vol) solution. The trapped $^{14}CO_2$ per vial was counted in a 3380 Tri-Carb liquid scintillation spectrophotometer (Packard Instrument Co., Inc., Rockville, Md.). The latex tubing in the manifold was replaced after each experiment; all other components of the aeration system were cleaned of isotope, and all components were resterilized in the assembled-manifold state.

Liquid media and growth substrates. Sterilized liquid media included the following: deionized water; D_2, which was composed of D-glucose (2 g), $NaNO_3$ (20 mg), Wesson salts (2 g) (10), vitamin solution (50 ml) (10), and deionized water (150 ml); 3% malt extract; or the basal medium of Kirk et al. (5). Media were added aseptically in 6-ml volumes to standard cultures and in 10-ml volumes to liquid cultures. Finely powdered cellulose (i.e., 25 mg of Alphacel from ICN [Cleveland, Ohio] per flask) was added as a special growth substrate to indicated standard cultures. In given experiments, media were initially pH adjusted and buffered as detailed above.

Lignin determinations in standard cultures. Lignin per culture flask was quantitated both by the 72% H_2SO_4 hydrolysis method as detailed by Moore and Johnson (8) and the spectrophotometric technique of Johnson et al. (3). Control determinations for lignin were made on equally conditioned wood samples in each experiment, except that they were not exposed to the given fungus. Reported lignin degradation values thus represent the determined statistically significant differences between inoculated and uninoculated wood samples.

RESULTS

The AF-W1 isolate of *F. solani* significantly degraded lignin in both unextracted and extracted sound wood in 50 days under standard culture conditions (Table 1). This occurred un-

TABLE 1. *Degradation of lignin in sound wood by F. solani grown in standard cultures*

Wood treatment	Liquid medium	pH condition	Mean degradation[a] ± SD (%)
Extracted[b]	3% Malt	Buffered (pH 4.0)	6.87 ± 2.24[c]
Unextracted[b]	3% Malt	Buffered (pH 4.0)	5.04 ± 1.35[d]
Unextracted[b]	Alphacel + water	Unadjusted, unbuffered	3.98 ± 0.58[d]
Unextracted[b]	D_2	Buffered (pH 5.0)	1.47 ± 0.77[e]

[a] Mean degradation ± standard deviation (SD) was based on four replicates per treatment. Each experiment was run for 50 days.

[b] "Extracted" means that substances extractable with benzene/95% ethanol (2:1) followed by 95% ethanol have been removed from the wood.

[c] Significantly different from its uninoculated control, $P < 0.05$.

[d] Significantly different from its uninoculated control, $P < 0.01$.

[e] Not significantly different from its uninoculated control, $P < 0.05$.

der the combined conditions of added 3% malt growth substrate (i.e., liquid medium) and with the pH adjusted to, and buffered initially at, 4.0 with 0.01 M 2,2-dimethylsuccinate. The fungus also significantly degraded lignin in unextracted sound wood during the same period in standard culture under conditions of unadjusted and unbuffered pH when Alphacel in deionized water was used as the liquid medium. There was no significant ($P < 0.05$) lignin degradation in unextracted sound wood with an adjusted and buffered pH 5.0 and D_2 as the liquid medium (Table 1).

From a comparative study of lignin degradation from sound wood by *C. versicolor*, *F. solani*, and *C. volvatus* through 84 days under standard culture with 3% malt in deionized water as liquid medium and unadjusted and unbuffered pH, the results are presented in Fig. 1. In 84 days, the positive control white-rot fungus, *C. versicolor*, degraded 53.6% of the lignin. *F. solani* transformed 8.1%; and *C. volvatus*, an ectosymbiote of the bark beetle, *Dendroctonus psendotsugae* (2), degraded 6.2%.

F. solani, in the liquid culture with only distilled deionized water as the liquid medium and no pH adjustment or buffering, transformed 5.10 and 4.16% of synthetic side chain (β, γ)-^{14}C- and *U*-ring-^{14}C-labeled lignins in 30 days, respectively (Fig. 2). It degraded only 0.48 and 0.69%, respectively, in 30 days when D_2 was the liquid medium and no pH adjustment or buffering was used (Fig. 2).

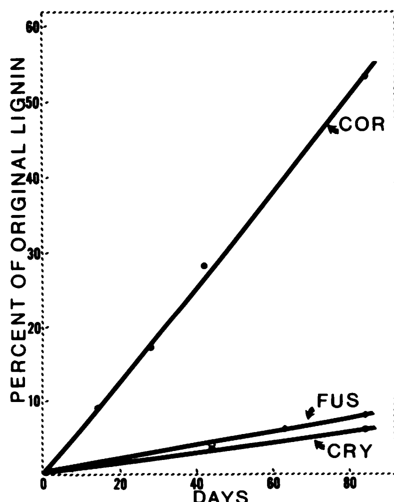

FIG. 1. *Comparative degradations of lignin from sound wood through 84 days by C. versicolor (COR), F. solani (FUS), and C. volvatus (CRY) under standard culture with 3% malt in deionized water as the liquid medium, and unadjusted and unbuffered pH.*

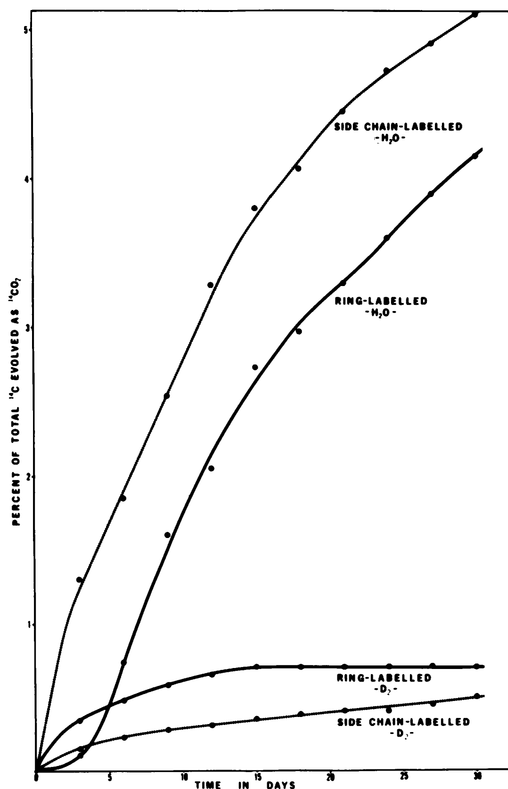

FIG. 2. *Percent of ^{14}C in side chain (β, γ)-^{14}C- and U-ring-^{14}C-labeled lignins evolved over time as $^{14}CO_2$ when exposed as substrate to F. solani in unbuffered water (H_2O) or D_2 liquid media.*

Isolate AF-W1 of *F. solani* growing on *p*-[carboxy-^{14}C]hydroxybenzoic acid in liquid culture, with distilled deionized water as the liquid medium and with no pH adjustment or buffering, evolved 67.5% of the ^{14}C as $^{14}CO_2$ in 20 days (Fig. 3). Under the same conditions, except that D_2 was the liquid medium, the fungus only evolved 53.0% of ^{14}C as $^{14}CO_2$. The relative amounts of the ^{14}C evolved as $^{14}CO_2$ by *F. solani* when grown on [3-^{14}C]cinnamic acid in liquid culture with water or D_2 as the liquid medium under several pH conditions are shown in Table 2. Percent of ^{14}C evolved as $^{14}CO_2$ with water as the liquid medium was 40.96 under conditions initially buffered at pH 7.3. This percent increased significantly to 48.65 under unbuffered conditions involving an initial pH 7.0. It increased markedly to 98.83% when the pH was initially adjusted to and buffered at 5.0, and then declined significantly in this experiment to 89.06% with an initially buffered pH 4.0. The unadjusted initial pH when D_2 was the liquid medium was 5.9. This situation yielded 46.08% of the ^{14}C evolved as $^{14}CO_2$ (Table 2). This value

was not significantly different from that observed with D_2 as the liquid medium and the initial pH buffered at 6.0. With D_2 as the liquid medium, the percent ^{14}C evolved as $^{14}CO_2$ increased significantly to 65.10 and 99.41% as the initial pH was buffered at 5.0 and 4.0, respectively.

DISCUSSION

The requirements for basal medium or growth substrate and pH in order for microbial degradation (biotransformation) of lignin to take place are extremely important considerations, and such conditions differ among microbial species (5, 11). Results presented in this paper further substantiate the abilities of isolate AF-W1 of *F. solani* to degrade significantly lignin and aromatic acids under several conditions of liquid medium and pH. Our overall results suggest that such parameters regarding microbial transformations of lignin surely deserve additional investigation. It seems clear now that the isolates, M-13-1 (11) and AF-W1, of *F. solani* can use DHPs as sole carbon sources. The only other possible carbon source in several of our tests was from the 6-mm plug of 2% malt agar that originally bore the inoculum. These culture conditions differ from the reported required carbon source situation with the white-rot fungus, *P. chrysosporium* (4).

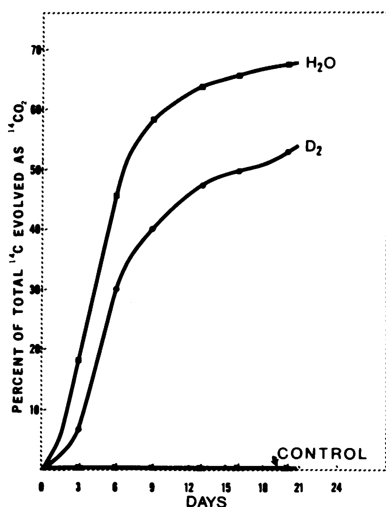

FIG. 3. *Percentage of ^{14}C in p-[carboxy-^{14}C]hydroxybenzoic acid evolved over time as $^{14}CO_2$ when exposed as substrate to F. solani in unbuffered water versus D_2 liquid medium.*

F. solani also can cleave both the ring structure and the side chain of substrate lignins. It accomplished significant degradations in cultures with the initial pH ranging from an unbuffered 7.0 to a buffered 4.0. However, studies of the effects of pH on *F. solani* degradation of lignin clearly indicated that decomposition was increased significantly by initially buffered pH's of 4.0 to 5.0. Thus, current knowledge on various fungal transformations of lignin provides strong evidence of the benefits of acidic (e.g., pH 4.0) media. Our findings on the usefulness of 0.01 M 2,2-dimethylsuccinate as a buffer in lignolytic systems confirm the previously published findings of Kirk et al. (7).

The roles of carbohydrate growth substances remain quite confused among studied microbial species. In our experiments, the liquid medium, 10 ml of D_2, which contains 2 g of D-glucose per 150 ml (i.e., 1.3%), markedly reduced the evolution of $^{14}CO_2$ from both *U*-ring-^{14}C- and side chain (β, γ)-^{14}C-labeled lignins exposed to *F. solani* under unbuffered conditions. It, however, only reduced *F. solani* evolution of $^{14}CO_2$ from *p*-[*carboxyl*-^{14}C]hydroxybenzoic acid by about 21% (Fig. 3). The quantity of glucose in this non-

TABLE 2. *Accumulative release of ^{14}C by F. solani as evolved $^{14}CO_2$ from the side chain of [3-^{14}C]cinnamic acid*

Liquid medium	pH	^{14}C release (% of dpm) at day:						
		3	6	9	12	15	18	21
Water	7.0[a]	42.60	47.14	47.99	48.23	48.41	48.54	48.65[b]
Water	7.3[c]	35.92	39.55	40.30	40.52	40.72	40.85	40.96[d]
D_2	5.9[a]	29.34	41.49	44.87	45.63	45.90	46.00	46.08[b,d]
D_2	6.0[c]	19.45	30.52	40.19	42.20	43.33	43.59	43.79[d]
Water	5.0[c]	39.09	79.68	91.42	97.13	98.15	98.65	98.83[e]
D_2	5.0[c]	5.68	44.25	55.48	62.04	64.76	64.84	65.10[f]
Water	4.0[c]	69.25	80.83	81.49	81.87	87.10	88.60	89.06[g]
D_2	4.0[c]	77.99	87.18	91.44	94.27	96.47	98.62	99.41[e]

[a] Unbuffered initial pH of the culture medium.
[b] Mean values not bearing the same superscript letter are significantly different ($P < 0.05$) based on the Duncan multiple-range statistical test.
[c] Initial pH of the culture medium buffered with 0.01 M 2,2-dimethylsuccinate.
[d-g] See footnote *b*.

pH-adjusted D_2 medium may have provided a level of readily available alternate substrate carbon that reduced the use of lignins or aromatic acids as carbon sources. The difference in apparent effects of D_2 on degradation of DHPs versus aromatic acid was striking. The high percentage of applied ^{14}C recovered from [3-^{14}C]-cinnamic acid as $^{14}CO_2$ when D_2 at initial pH 4.0 was the alternate carbon source (Table 2) implies that there is something unusual about *F. solani*'s metabolism. Essentially, no ^{14}C was incorporated into cell mass. Limited, but significant, degradation of lignin from sound wood did occur with *F. solani* in the presence of 25 mg of powdered cellulose (i.e., Alphacel) per 6 ml of liquid medium under unbuffered pH conditions. The 3% malt liquid medium, either with an initially buffered pH 4.0 or with an unbuffered and unadjusted pH, also was an alternate carbon source that allowed significant *F. solani* transformation of lignin from sound wood. It may be significant that 3% malt has a pH of approximately 4.0 to 4.2. The carbohydrates used as growth substrates in our reported experiments were chosen based on published positive findings of other investigators (4-7).

Our studies only involved air, not pure O_2, in aeration. The findings of other researchers with different microbes strongly indicate that aeration of *F. solani* cultures with O_2 could dramatically increase the rate and quantity of lignin degradation under most, if not all, otherwise suitable incubation conditions.

Our investigations of the mechanisms by which isolate AF-W1 of *F. solani* may degrade DHPs, aromatic acids, and lignin from sound wood are still rather preliminary, and the details will be presented in a subsequent paper. However, it does seem clear from the presented data (Fig. 2 and 3; Table 2) that different catabolic mechanisms are involved in the degradations of DHPs and aromatic acids. The observed products in our culture filtrates and substrate residues seem significantly supportive of the findings and suggestions of Ohta et al. (11). Our current knowledge confirms the ability of *F. solani* to cleave the α, β, or γ carbons of the side chain of the basic phenylpropanoid unit of lignin, or side chain remnants of aromatic acids. Our results also indicate that *F. solani* can cleave a benzene nucleus that initially lacks hydroxyl substitution. This apparently is different from the reported commonly required situation (1).

ACKNOWLEDGMENTS

The author acknowledges the generous contribution of the synthetic ^{14}C-labeled lignins (DHPs) by T. K. Kirk, U.S. Department of Agriculture Forest Products Laboratory, Madison, Wis. Research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison; and in part by the Wisconsin Department of Natural Resources; and research grant no. PFR 77-08279 from the National Science Foundation.

LITERATURE CITED

1. **Buswell, J. A., and K.-E. Eriksson.** 1979. Aromatic ring cleavage by the white-rot fungus *Sporotrichum pulverulentum*. FEBS Lett. **104**:258-260.
2. **Castello, J. D., C. G. Shaw, and M. M. Furniss.** 1976. Isolation of *Cryptoporus volvatus* and *Fomes pinicola* from *Dendroctonus pseudotsugae*. Phytopathology **66**: 1431-1434.
3. **Johnson, D. B., W. E. Moore, and L. C. Zank.** 1961. The spectrophotometric determination of lignin in small wood samples. Tappi **44**:793-798.
4. **Kirk, T. K., W. J. Connors, R. D. Bleam, W. F. Hackett, and J. G. Zeikus.** 1975. Preparation and microbial decomposition of synthetic [^{14}C] lignins. Proc. Natl. Acad. Sci. U.S.A. **72**:2515-2519.
5. **Kirk, T. K., W. J. Connors, and J. G. Zeikus.** 1976. Requirements for a growth substrate during lignin decomposition by two wood-rotting fungi. Appl. Environ. Microbiol. **32**:192-194.
6. **Kirk, T. K., W. J. Connors, and J. G. Zeikus.** 1977. Advances in understanding the microbiological degradation of lignin, p. 369-394. *In* F. A. Loewus and V. C. Runeckles (ed.), Recent advances in phytochemistry, vol. 11. Plenum Publishing Corp., New York.
7. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters of lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117**:277-285.
8. **Moore, W. E., and D. Johnson.** 1967. Procedure for the analysis of wood and wood products. U.S.D.A. Forest Service, Forest Products Laboratory, Madison, Wis.
9. **Norris, D. M.** 1979. The mutualistic fungi of Xyleborini beetles, p. 53-63. *In* L. Batra (ed.), Insect-fungus symbiosis. Allanheld and Osmun, Montclair, N.J.
10. **Norris, D. M., and J. K. Baker.** 1967. Symbiosis: effects of a mutualistic fungus upon the growth and reproduction of *Xyleborus ferrugineus*. Science **156**:1120-1122.
11. **Ohta, M., T. Higuchi, and S. Iwahara.** 1979. Microbial degradation of dehydrodiconiferyl alcohol, a lignin substructure model. Arch. Microbiol. **121**:23-28.