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Phenolic Compound Utilization by the Soft Rot Fungus *Lecythophora hoffmannii*

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Nine phenolic compounds were metabolized by the soft rot fungus *Lecythophora hoffmannii* via protocatechuic acid and subsequently cleaved by protocatechuate 3,4-dioxygenase as determined by oxygen uptake, substrate depletion, and ring cleavage analysis. Catechol was metabolized by catechol 1,2-dioxygenase. Fungal utilization of these aromatic compounds may be important in the metabolism of wood decay products.

The soft rot fungi are a large group of soil-inhabiting ascomycetes and fungi imperfecti that cause a characteristic decay of the surface layers of timber, occurring even when the wood has been treated with preservatives (9). Soft rot fungal species of the genera *Chaetomium*, *Humicola*, *Petriellidium*, and *Phialophora* are known to metabolize low-molecular-weight aromatic compounds (1, 3, 8) which are the degradation products of lignin (6, 18, 21, 33). Well over 20 single-ring phenolic compounds are metabolized by one or more soft rot fungi. Among these are caffeic acid (5), catechol and *p*-coumaric acid (5, 27), ferulic acid (5, 19, 22, 27), *p*-hydroxybenzoic acid (5, 19, 22, 27, 29), protocatechuic acid (5, 22, 27), vanillin (19, 22, 27), and others (13, 23, 24). *Lecythophora hoffmannii* (van Beyma) Gams & McGinnis (11) [= *Phialophora hoffmannii* (van Beyma) Schol-Schwarz] is one of the most important fungi causing soft rot in preservative-treated wood (10, 12, 20, 28). Although the process of hyphal invasion of wood and soft rot cavity formation from release of cellulolytic and ligninolytic enzymes by this fungus has been well studied (14–17), the metabolism of lignin and related aromatic compounds was not reported.

The objective of this investigation was to evaluate the utilization of low-molecular-weight phenolic compounds, similar to those found in decaying wood, by the soft rot fungus *Lecythophora hoffmannii*. Utilization of phenolic substrates was determined by measuring the specific activity of dioxygenase enzymes through oxygen uptake analysis of cell-free homogenates derived from *L. hoffmannii* cells incubated with the substrates. Ring cleavage was also assayed by the Rothera reaction.

L. hoffmannii (van Beyma) Gams & McGinnis (11) was obtained from the American Type Culture Collection (ATCC 34158) and maintained on slants of potato dextrose agar. The liquid culture medium (pH 5.5) included NH_4NO_3 (2.0 g), KH_2PO_4 (2.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), and Bacto-yeast extract (0.5 g; Difco) per liter of distilled water and was supplemented with 0.01% of either a phenolic compound or succinate. Fungal inoculum for shake cultures was prepared by cutting sections from the edges of hyphae that were growing on plates of potato dextrose agar and incubating these hyphae for 30 to 36 h in a liquid medium supplemented with succinate. A 1-ml portion of this liquid medium was used to inoculate the experimental flasks, which were incubated at 27 ± 2°C and 70% relative humidity with shaking on a Gyrotory shaker at 150 rpm. Fungal dry weight was determined gravimetrically after filtering a 10-ml sample of medium through a polycarbonate membrane (0.2 μm pore size). Soluble protein content was determined by the method of Lowry et al. (25).

Phenolic substrates from culture filtrates were analyzed at 270 nm by high-pressure liquid chromatography (HPLC) (Varian model 5020) with a C_{18} reverse-phase Varian Micro-Pak MCH-10 column (30 cm by 4 mm inner diameter) at a flow rate of 1.5 ml min^{-1} at ambient temperature. The mobile phase was a 12.5 mM KH_2PO_4 buffer adjusted with perchloric acid to pH 3.0 (870 ml) with acetonitrile added to make 1 liter. Cell-free homogenates were prepared from collected mycelia on polycarbonate membranes (0.6 μm pore size) which were ground with a mortar and pestle in 2.0 ml of 50 mM KH_2PO_4 buffer (pH 7.0) for 3 min (2, 23). This homogenate, along with a rinse in 2 to 3 ml of buffer of the mortar, was centrifuged at 1,150 × *g* for 15 min, and the supernatant was used for oxygen uptake analysis. Oxygen uptake analysis was performed with a Yellow Springs model 53 oxygen monitor, and reaction mixtures contained 2.8 ml of 50 mM KH_2PO_4 buffer (pH 7.0) and 0.2 ml of crude cell homogenate at 30°C (32). Samples were equilibrated for 5 min, the oxygen probe was inserted into the chamber, and oxygen uptake was monitored for 10 min. Then, 20 μl of either a catechol, gallic acid, gentisic acid, or protocatechuic acid solution was added to the chamber (final concentration, 70 μM), and oxygen uptake was monitored for an additional 10 min to determine oxygen uptake due to a dioxygenase. Independent measurements were performed on duplicate samples. Oxygen concentration was determined by using 5.84 μl ml^{-1} (7.53 ppm) as the solubility of oxygen at 30°C (34). For stoichiometric analysis, the amount of protocatechuic acid metabolized was also determined. A total of 1.5 ml of 5% HCl was added to the reaction chamber, either immediately after the addition of protocatechuic acid or 10 min later. Extraction of protocatechuic acid was performed three times with ethyl ether (2 ml each) subsequently back-washed to neutrality and evaporated, and the residue was dissolved in ethanol and analyzed by HPLC. Ring cleavage was determined by the colorimetric test of Ottow and Zolg, which yields a deep purple color by the Rothera reaction as a positive test for the presence of β-ketoadipate (30).

Protocatechuic acid supported the growth of *L. hoffmannii* as measured by an increase in fungal dry weight of 50, 110, and 210 mg l^{-1} of medium and a soluble protein increase of 0.58, 0.66, and 3.33 mg l^{-1} in medium supplemented with

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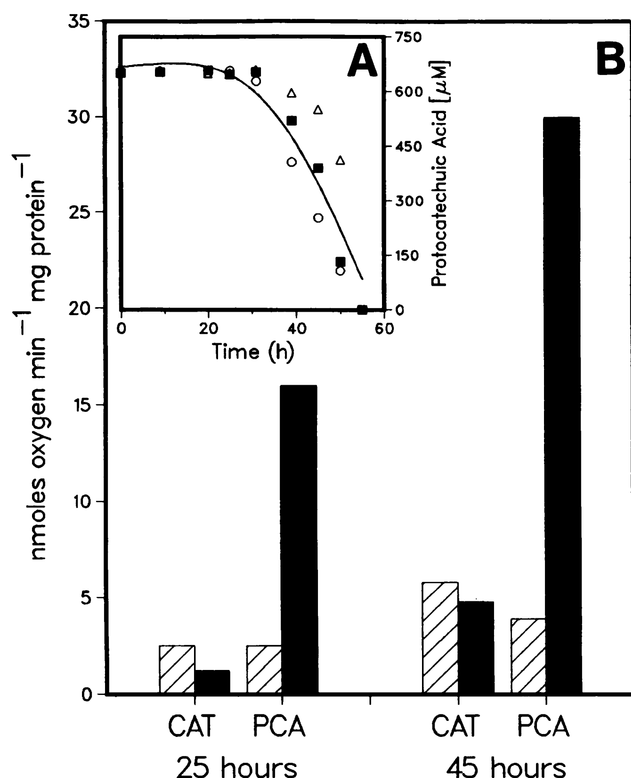

FIG. 1. (A) Depletion of protocatechuic acid by *L. hoffmannii* in three separate determinations, with the curve determined by the root-mean-square minimization procedure (computational). (B) Oxygen uptake by cell-free homogenates derived from *L. hoffmannii* cells initially cultured with protocatechuic acid. Cells were collected at 25 and 45 h, and measurements were made on control homogenates (hatched bars) and on homogenates after addition of catechol (CAT) or protocatechuic acid (PCA) (solid bars).

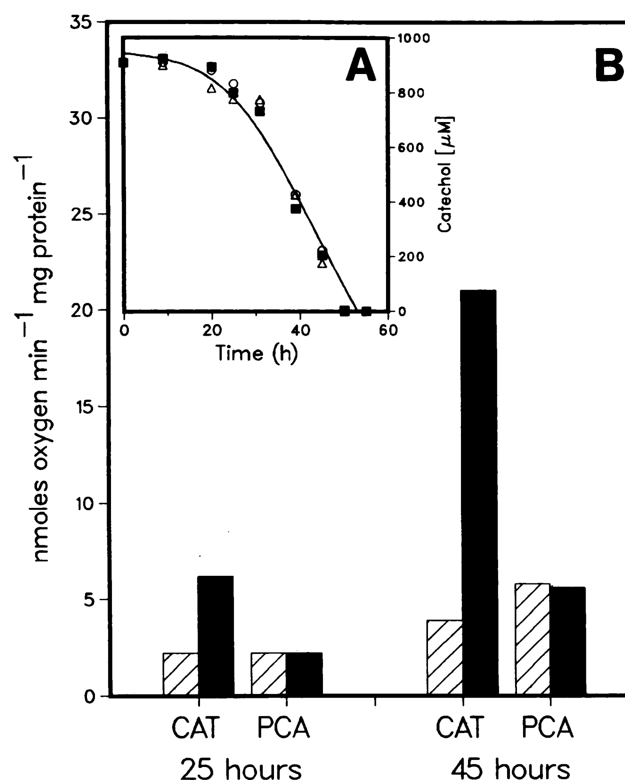

FIG. 2. (A) Depletion of catechol by *L. hoffmannii* in three separate determinations, with the curve determined by the root-mean-square minimization procedure (computational). (B) Oxygen uptake by cell-free homogenates derived from *L. hoffmannii* cells initially cultured with catechol. Cells were collected at 25 and 45 h, and measurements were made on control homogenates (hatched bars) and on homogenates after addition of catechol (CAT) or protocatechuic acid (PCA) (solid bars).

0%, 0.01% (650 μM), and 0.05% (3250 μM) protocatechuic acid, respectively. The concentration of substrate decreased 510 μM (79%) and 1,160 μM (36%) in medium supplemented with 0.01 and 0.05% protocatechuic acid, respectively. A concomitant increase in oxygen uptake was observed with the decrease in medium substrate concentration for cell-free homogenates derived from cells initially cultured with protocatechuic acid (Fig. 1). Oxygen uptake upon addition of 70 μM protocatechuic acid was increased by 13.5 and 26.1 nmol of O_2 min^{-1} mg of protein^{-1} for homogenates derived from cells incubated for 25 and 45 h, respectively. Oxygen uptake activity was destroyed by boiling the homogenate. Cells grown with protocatechuic acid for 45 h produced a deep purple color by the Rothera reaction, indicating that protocatechuic acid is metabolized by *ortho* cleavage through protocatechuate 3,4-dioxygenase. Similarly, a concomitant increase in oxygen uptake was observed with the decrease in medium substrate concentration for cell-free homogenates derived from cells initially cultured with catechol (Fig. 2). Oxygen uptake upon addition of catechol was increased by 4 and 17 nmol of O_2 min^{-1} mg of protein^{-1} for homogenates derived from cells incubated for 25 and 45 h, respectively. Catechol is metabolized by catechol 1,2-dioxygenase, since a positive Rothera reaction was observed from cells grown with catechol for 45 h. Addition of the enzyme inhibitor KCN (0.5 mM) or NaN_3 (0.5 mM) decreased the oxygen uptake ability of cell-free homogenates derived from cells initially cultured with protocatechuic acid for 36 h. A 75% decrease in oxygen uptake, i.e., by 15 nmol of O_2 min^{-1} mg of protein^{-1}, was observed after the addition of either inhibitor in the presence of 70 μM protocatechuic acid. When succinate and acetate were added to 70 μM each, i.e., at concentrations comparable to that produced by the metabolism of 70 μM protocatechuic acid by the β-ketoadipate pathway, a decrease in oxygen uptake of 35 and 55% was observed for the two inhibitors, respectively.

Since protocatechuic acid is metabolized by fungi via the β-ketoadipate pathway, in which the compound is converted enzymatically to succinate and acetyl coenzyme A (acetyl-CoA) (4, 7, 31), the only oxygen-requiring enzyme in this pathway to succinate and acetyl-CoA is protocatechuate 3,4-dioxygenase. Therefore, the difference in the oxygen uptake values determined upon addition of protocatechuic acid and similar values determined upon addition of succinate and acetate will be the result of oxygen uptake due to the dioxygenase. Upon addition of 70 μM protocatechuic acid to cell-free homogenates derived from cells initially cultured with protocatechuic acid, oxygen uptake values were 4.2 and 4.6 nmol of O_2 min^{-1} for samples A and B, respectively, whereas oxygen uptake values upon addition of 70 μM succinate plus 70 μM acetate were 1.4 nmol of O_2 min^{-1} for both samples. Oxygen uptake due to protocatechuate 3,4-dioxygenase is therefore 2.8 and 3.2 nmol of O_2 min^{-1} for samples A and B, respectively. Substrate deple-

TABLE 1. Oxygen uptake upon addition of catechol and protocatechiuc acid by cell-free homogenates of *L. hoffmannii* cultured with various aromatic compounds

Growth substrate[a]	Mean increase in O_2 uptake[b] (nmol/min per mg of protein) ± SEM	
	Catechol	Protocatechuic acid
Caffeic acid	5.2 ± 1.8	**17** ± 1.0
Catechol	**19** ± 1.0	3.5 ± 0
p-Coumaric acid	4.2 ± 0.8	**29** ± 1.0
Ferulic acid	6.0 ± 0.8	**18** ± 1.5
m-Hydroxybenzoic acid	3.8 ± 0.6	**19** ± 0.5
p-Hydroxybenzaldehyde	6.7 ± 1.1	**49** ± 2.0
p-Hydroxybenzoic acid	3.6 ± 0.6	**35** ± 0
Protocatechuic acid	0.9 ± 0.9	**22** ± 1.0
Vanillic acid	7.8 ± 0.4	**42** ± 1.5
Vanillin	2.2 ± 0.8	**41** ± 1.0
Vanillyl alcohol	7.0 ± 1.2	**55** ± 3.0
Succinic acid (control)	0.6 ± 0.6	1.8 ± 0.6

[a] All substrates added at 0.01%, and cells were harvested between 30 and 34 h.

[b] Increase in oxygen uptake with addition of the indicated dioxygenase substrate at 70 μM. Numbers in boldface type indicate the preferred metabolic intermediate.

tion for samples A and B was 3.1 and 3.5 nmol of protocatechuic acid metabolized min^{-1}, respectively. Therefore, the ratio of oxygen uptake to substrate depletion was 0.90:1 for sample A and 0.91:1 for sample B. This stoichiometry is in close agreement with the 1:1 theoretical ratio expected for this metabolism, since dioxygenases incorporate both atoms of diatomic oxygen into the aromatic substrate (7).

The major pathways for metabolism of phenolic compounds by *L. hoffmannii* were determined by assessing oxygen uptake of cell-free homogenates prepared from cells initially cultured with the various aromatic compounds. The fungus depleted all 11 aromatic compounds from the culture medium within 90 h. Cells cultured with succinate served as a control, because this substrate does not appear to induce aromatic dioxygenases. The oxygen uptake values for succinate control cell-free homogenates upon the addition of protocatechuic acid and catechol were low (1.8 and 0.6 nmol of O_2 min^{-1} mg of $protein^{-1}$), suggesting that dioxygenases were not being induced or activated to any significant extent (Table 1). In addition, cells grown with succinate for 45 h produced a negative Rothera reaction. Oxygen uptake was stimulated upon the addition of 70 μM protocatechuic acid to cell-free homogenates derived from the cells initially cultured with the following nine compounds: caffeic acid, *p*-coumaric acid, ferulic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, vanillic acid, vanillin, and vanillyl alcohol (Table 1). When either 70 μM catechol, 70 μM gallic acid, or 70 μM gentisic acid was added to the homogenates, oxygen uptake was stimulated to only 16, 13, and 13% of the levels attained by protocatechuic acid addition, respectively. These low levels of oxygen uptake stimulation upon addition of catechol, gallic acid, or gentisic acid may be due to low levels of other dioxygenases being induced or to constitutive levels of other dioxygenases (26). The nine phenolic compounds listed above are therefore metabolized to protocatechuic acid and enzymatically cleaved with protocatechuate 3,4-dioxygenase by *L. hoffmannii.* Metabolism by this enzyme is also supported by the production of a deep purple color by the Rothera reaction from cells grown with each of the nine substrates for 45 h, except cells grown with caffeic acid, in which the mycelia produced a black pigment that obscured the color reaction.

L. hoffmannii, one of the important soft rot fungi (10, 12, 20, 28), possesses the enzymatic activity for metabolizing phenolic compounds via protocatechuic acid, particularly *p*-hydroxybenzoic acid, vanillic acid, and vanillin, which are putative metabolites of lignin degradation (6, 18). This ability may help it utilize part of the amorphous granular material, probably containing lignin breakdown products, that is produced in soft rot activities (17). The oxygen uptake method used in this study for assaying homogenates of fungi cultured with phenolic substrates is rapid and provides results that putatively identify the major metabolic pathway used by the particular organism for the substrate.

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