APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1996, p. 292–295 Vol. 62, No. 1
0099-2240/96/$04.00+0

Mineralization of Polycyclic Aromatic Hydrocarbons by the White Rot Fungus *Pleurotus ostreatus*

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Received 12 June 1995/Accepted 25 October 1995

The white rot fungus *Pleurotus ostreatus* was able to mineralize to $^{14}CO_2$ 7.0% of [^{14}C]catechol, 3.0% of [^{14}C]phenanthrene, 0.4% of [^{14}C]pyrene, and 0.19% of [^{14}C]benzo[*a*]pyrene by day 11 of incubation. It also mineralized [^{14}C]anthracene (0.6%) much more slowly (35 days) and [^{14}C]fluorene (0.19%) within 15 days. *P. ostreatus* did not mineralize fluoranthene. The activities of the enzymes considered to be part of the ligninolytic system, laccase and manganese-inhibited peroxidase, were observed during fungal growth in the presence of the various polycyclic aromatic hydrocarbons. Although activity of both enzymes was observed, no distinct correlation to polycyclic aromatic hydrocarbon degradation was found.

White rot fungi, including *Pleurotus ostreatus*, have the ability to efficiently degrade lignin, which is a naturally occurring aromatic polymer (9, 14–16). This capacity is assumed to result from the activities of lignin peroxidases, manganese peroxidases (MnPs), other oxidases, and laccases (14, 26). The polyaromatic structure of both lignin and polycyclic aromatic hydrocarbons (PAHs) prompted the suggestion that the same fungi might be able to degrade these ubiquitous pollutants (4). On the other hand, some nonligninolytic fungi, such as *Cunninghamella elegans*, also degrade PAHs (5).

Previous work with PAH degradation by white rot fungi found that these organisms were able to degrade PAHs and in some cases to mineralize them. Most of the work was done with *Phanerochaete chrysosporium*, and its ability to nonspecifically degrade a wide range of aromatic pollutants has recently been studied (1–3, 6, 8, 10–13, 19, 21–25, 27).

Under ligninolytic and nonligninolytic conditions, *P. chrysosporium* can metabolize a wide variety of PAHs, including the potent carcinogen benzo[*a*]pyrene (10, 23). However, *Trametes versicolor*, *Bjerkandera* sp., and *Pleurotus ostreatus* may be more promising than *P. chrysosporium* in their ability to mineralize PAHs to CO_2 (7, 22, 28). In addition, *Crinipellis stipitaria* has been reported for its ability to metabolize pyrene (17, 18). While screening for species able to degrade PAHs, Sack and Günther (22) showed that *P. ostreatus* is quite efficient in the degradation of phenanthrene and fluorene and is less efficient with fluoranthene. Pyrene was not degraded to any significant extent. Vyas et al. showed the ability of *P. ostreatus* to degrade anthracene (28). These studies indicate the potential of *P. ostreatus* to degrade PAHs.

P. ostreatus differs from *P. chrysosporium* in its lignin degradation mechanism in that it does not exhibit lignin peroxidase activity (14). Instead, its lignin degradation ability is assumed to be correlated with laccase activity (15, 16, 26, 29). For this reason, we were interested in investigating the ability of *P. ostreatus* to metabolize and mineralize PAHs. Laccase is a polyphenol oxidase, but it is also nonspecific as to its reducing substrate and the variety of substrates oxidized includes polyphenols, methoxy-substituted phenols, diamines, and a considerable range of other compounds. It also acts as a mediator, enabling the oxidation of nonphenolic lignin model compounds that are not laccase substrates on their own (26). We therefore hypothesized that this enzymatic activity might include oxidation of PAHs. In the present study, enzymatic activities were monitored during *P. ostreatus* growth in the presence of PAHs, and the fungus's ability to mineralize catechol and various PAHs was demonstrated.

Mineralization of PAHs. The potential of *P. ostreatus* to mineralize a wide range of ^{14}C-labeled compounds, including catechol and the PAHs [9,10-^{14}C]phenanthrene (19.3 mCi/mmol), [4,5,9,10-^{14}C]pyrene (59.5 mCi/mmol), [9,10-^{14}C]anthracene (58 mCi/mmol), [7,10-^{14}C]benzo[*a*]pyrene (21.7 mCi/mmol), [9-^{14}C]fluorene (14.2 mCi/mmol), and [3-^{14}C]fluoranthene (55.0 mCi/mmol), was investigated with two growth media, basidiomycetes rich medium (BRM) and basidiomycetes salt medium (BSM) (16). The most significant $^{14}CO_2$ production originated from the degradation of [^{14}C]catechol (7.4 mCi/mmol) in BRM, reaching 7.0% of the original added radioactivity within 11 days and reaching a plateau on the same day (Fig. 1). On BSM, a minimal medium on which only laccase activity was detected, catechol mineralization reached 3% within 8 days (Fig. 1 and Table 1). Catechol in BRM was mineralized, reaching 7% within 11 days, approximately 50% of the mineralization of [^{14}C]glucose in BSM on day 12 of incubation, the day at which it reached a plateau at 14.5%.

Formation of $^{14}CO_2$ by *P. ostreatus* during metabolism of the ^{14}C-labeled PAHs started on day 3 of incubation (Fig. 1) and reached different levels with different compounds (Table 1).

Phenanthrene degradation in BSM reached a plateau on day 11, when 3.0% of the [^{14}C]phenanthrene was mineralized to $^{14}CO_2$ (Fig. 1). On BRM, phenanthrene mineralization reached only 1.8% (Fig. 1 and Table 1). Pyrene was mineralized up to 0.4% of the original added radioactivity in BSM and reached a plateau on day 8 (Fig. 1). In BRM, the level of mineralization was lower and did not exceed 0.13% (Fig. 1 and Table 1).

Benzo[*a*]pyrene was mineralized to a lower level (0.19%) in BSM. Hardly any mineralization was observed in BRM (0.03%), but the degradation was still statistically significant (Table 1). Anthracene mineralization was not apparent up to 16 days of incubation but reached 0.6% on day 35. The level of fluorene mineralization was lower, and the rate was slower. Fluorene mineralization reached 0.19% in BSM and 0.15% in

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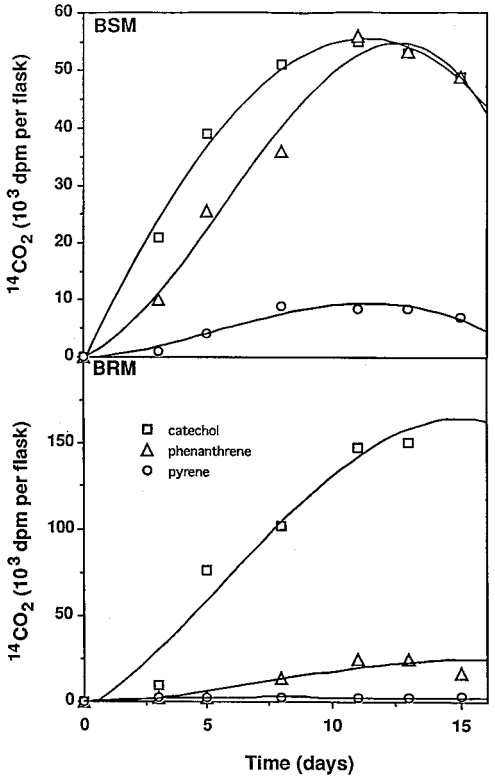

FIG. 1. Mineralization of catechol, phenanthrene, and pyrene by *P. ostreatus* in BSM and BRM.

BRM within 15 days. Fluoranthene, in contrast, was not mineralized by *P. ostreatus* to any significant degree.

Although the mineralization levels per gram (wet weight) of fungal mycelium were different, these differences were not consistent for any one type of medium (Table 1).

Other organisms that have been investigated for $^{14}CO_2$ formation have shown other mineralization values: *Chrysosporium lignorum*, *P. chrysosporium*, and *T. versicolor* did not mineralize benzo[*a*]pyrene without induction by a lignin component, and reached 2, 0.5, and 0.7% mineralization, respectively, between 40 and 100 days (20). *C. lignorum* and *P. chrysosporium* mineralized 1.3 and 4.5% of phenanthrene, respectively (21). Bumpus (2) showed 7.7% mineralization of phenanthrene after 21 days by *P. chrysosporium*. *P. chrysosporium* also mineralized 12% of anthracene on day 7 of incubation (12). Generally, *P. ostreatus* mineralized PAHs faster than other white rot fungi which were investigated. Lignin mineralization by *P. ostreatus* (16) started later than PAH mineralization (this work), starting on day 12 for lignin compared with day 3 for the PAHs, and lignin was still being mineralized even after 30 days. In contrast, PAH mineralization reached a plateau on day 8 or day 11, supporting the assumption that *P. ostreatus* uses different degradation systems for lignin and PAHs.

The mineralization levels were not associated with the different media (BRM or BSM). Preferences for one or the other varied. In BRM, for instance, the level of catechol mineralization was higher than that in BSM, but the level of phenanthrene mineralization was higher in BSM than in BRM (Fig. 1). Preliminary experiments have indicated that *P. ostreatus* metabolized the PAHs to organic solvent-extractable metabolites. Further research is in progress to identify these intermediates.

Extracellular ligninolytic enzymatic activity. Concomitant to the $^{14}CO_2$ measurements, the activities of the extracellular enzymes laccase (16), MnP, and manganese-inhibited peroxidase (MnIP) (27) in the BSM and BRM cultures were measured (Fig. 2 and 3). MnP activity was not detected under these experimental conditions. MnIP was not detected in BSM, because of the presence of Mn in the medium, but it was detected during incubation in the control cultures of *P. ostreatus* in BRM (Fig. 3). With catechol in BRM, the level of MnIP activity was high on day 5, decreased to almost no activity on days 8 and 11, and increased again to about a third of the 5th day's level of activity on days 13, 15, and 18. Phenanthrene-BRM cultures exhibited high levels of MnIP activity on days 3 and 5, lower levels of activity on day 8, and no activity later on. In pyrene-BRM cultures, a similar phenomenon was observed. Therefore, there appeared to be no correlation between MnIP activity and PAH-$^{14}CO_2$ evolution. MnIP activity decreased after a few days of incubation in growth media containing PAHs compared with MnIP activity in cultures growing under the same conditions without PAHs. PAHs did not reduce laccase activity.

Laccase activity reached levels of 46 U/ml in the catechol-BRM cultures, 57 U/ml in phenanthrene-BSM cultures, and 58 U/ml in pyrene-BSM cultures (Fig. 2 and Table 1), but the $^{14}CO_2$ levels were significantly different between these cultures. Catechol cultures, which had the lowest level of laccase activity, had the highest mineralization levels. Pyrene and phenanthrene cultures had the same laccase activity, but the

TABLE 1. Laccase and MnIP activities and specific mineralization produced by *P. ostreatus*

PAH	Total mineralization (% $^{14}CO_2$)[a]		Specific mineralization (% $^{14}CO_2$/g [wet wt])[b]		Activity of:		
					Laccase (U/ml)		MnIP (A_{610})
	BSM	BRM	BSM	BRM	BSM[c]	BRM[c]	BRM[c,d]
Control					56 (11)	33 (14)	1.66 (8)
Catechol	3.0	7.0	0.306	0.667	3 (13)	46 (8)	1.14 (5)
Phenanthrene	3.0	1.8	0.125	0.129	57 (13)	35 (3)	0.29 (3)
Pyrene	0.44	0.13	0.021	0.011	58 (8)	34 (8)	0.48 (3)
Fluorene	0.19	0.15	0.009	0.007	61 (5)	42 (3)	0.84 (13)
Fluoranthene					44 (14)	24 (14)	0
Benzo[*a*]pyrene	0.19	0.03	0.009	0.003	40 (13)	26 (8)	1.83 (8)

[a] Percentage of total radioactivity added.
[b] Percentage of $^{14}CO_2$ of original added radioactivity per gram (wet weight) of *P. ostreatus* mycelium.
[c] The numbers in parentheses represent the day of peak activity.
[d] MnIP was not detected in BSM cultures.

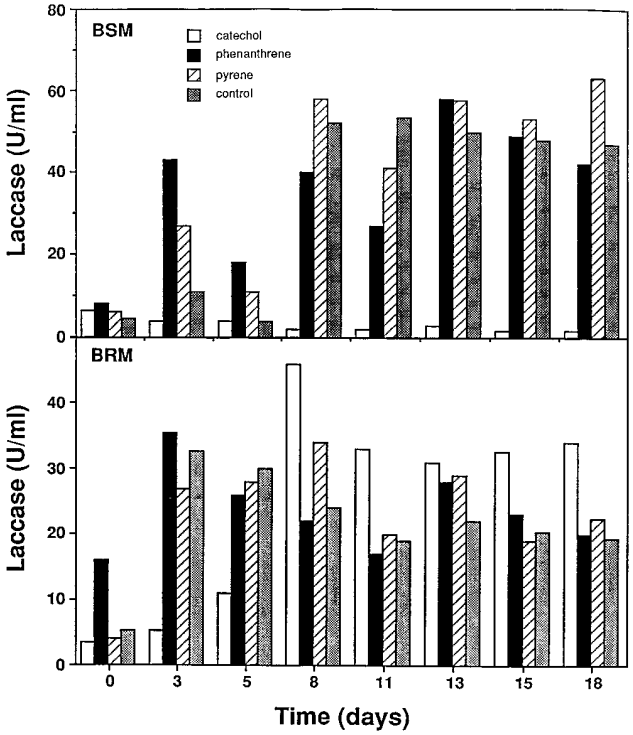

FIG. 2. Laccase activity in *P. ostreatus* in BSM and BRM with and without PAHs (catechol, phenanthrene, pyrene, and control).

level of mineralization of pyrene was much lower than that of phenanthrene.

The level of laccase activity was somewhat higher in BSM than in BRM in most treatments. However, in the catechol-amended BSM, the level of activity was much lower than that in BRM. Still, in most media, mineralization was efficient (Fig. 1 and 2).

In addition, enzymatic activities did not reach their peak at the same time for all cultures and did not reach their peak at the time that mineralization reached its maximum. Since laccase is found to be only a mediator in the oxidation of nonphenolic substrates (26), it did not come as a complete surprise that it may not have a direct role in oxidizing the PAHs in this system.

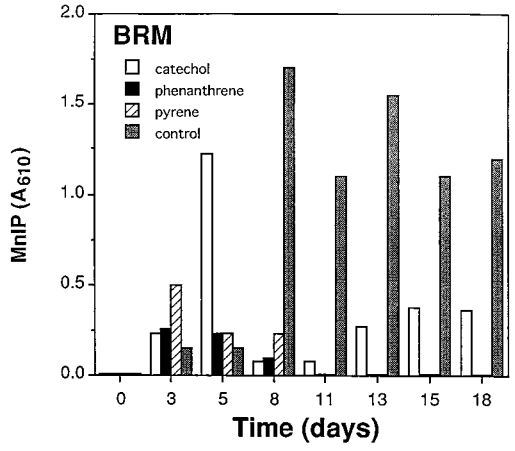

FIG. 3. MnIP activity of *P. ostreatus* in BRM with and without PAHs (catechol, phenanthrene, pyrene, and control).

The higher level of laccase activity measured simultaneously with increasing $^{14}CO_2$ levels may be related to a larger cell mass and physiological stages at which the fungus produces higher levels of laccase while at the same time being able to metabolize the PAHs through different mechanisms. The laccase may also be involved in a later degradation pathway of the PAH metabolites and not in the initial attack on the molecules.

MnIP also did not seem to be involved in PAH degradation. Its activity levels increased in the beginning of the incubation period but usually decreased around day 8. MnIP activity may be inhibited by one of the PAH metabolites, since activity decreased after only a few days of incubation in BRM cultures with PAH, whereas it did not change much in the control BRM cultures.

When catechol and phenanthrene were exposed to cell supernatant of *P. ostreatus*, no modification of the compounds occurred. This observation supports the hypothesis that the extracellular enzymes are not part of the initial attack on the molecules. On the other hand, anthracene was probably oxidized to anthraquinone by laccase or another extracellular, hydrogen peroxide-independent enzyme. Anthracene oxidation to anthraquinone by *P. chrysosporium* and *P. ostreatus* has been previously observed (12, 28). Since very little anthracene mineralization was observed in our study, the question of whether anthraquinone is part of the mineralization pathway or a dead-end product remains unanswered.

It is most probable that membrane-bound and intracellular enzymes are involved in the PAH degradation and mineralization processes. Ligninolytic enzymes, laccases, and peroxidases may be involved in PAH degradation after the initial attack by intracellular enzymes such as cytochrome P-450, since hydroxylated and epoxidated PAHs have structures similar to those of oxygenated lignins. Therefore, further investigations should include purification and characterization of metabolites from PAH degradation by *P. ostreatus* and identification and analysis of the enzymatic systems involved.

REFERENCES

1. **Barclay, C. D. D., G. F. Farquhar, and R. L. Legge.** 1995. Biodegradation and sorption of polyaromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **42:**958–963.
2. **Bumpus, J. A.** 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **55:**154–158.
3. **Bumpus, J. A., M. Tien, D. Wright, and S. D. Aust.** 1985. Oxidation of persistent environmental pollutants by a white-rot fungus. Science **228:**1434–1436.
4. **Cerniglia, C. E.** 1993. Biodegradation of polycyclic aromatic hydrocarbons, p. 227–245. *In* E. Rosenberg (ed.), Microorganisms to combat pollution. Kluwer Academic Publishers, Dordrecht, The Netherlands.
5. **Cerniglia, C. E., J. B. Sutherland, and S. A. Crow.** 1992. Fungal metabolism of aromatic hydrocarbons, p. 193–217. *In* G. Winkelmann (ed.), Microbial degradation of natural products. VCH Press, Weinheim, Germany.
6. **Dhawale, S. W., S. S. Dhawale, and D. Dean-Ross.** 1992. Degradation of phenanthrene by *Phanerochaete chrysosporium* occurs under ligninolytic as well as nonligninolytic conditions. Appl. Environ. Microbiol. **58:**3000–3006.
7. **Field, J. A., E. de Jong, G. F. Costa, and J. A. M. de Bont.** 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. **58:**2219–2226.
8. **Gold, M. H., and M. Alic.** 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57:**605–622.
9. **Hadar, Y., Z. Kerem, and B. Gorodecki.** 1993. Biodegradation of lignocellulosic agricultural wastes by *Pleurotus ostreatus*. J. Biotechnol. **30:**133–139.
10. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter.** 1986. Oxidation of benzo[a]pyrene by extracellular ligninase of *Phanerochaete chrysosporium*: veratryl alcohol and stability of ligninase. J. Biol. Chem. **261:**6900–6903.
11. **Hammel, K. E., W. Z. Gai, B. Green, and M. A. Moen.** 1992. Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**1832–1838.
12. **Hammel, K. E., B. Green, and W. Z. Gai.** 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA **88:**10605–10608.

13. **Hammel, K. E., B. Kalyanaraman, and T. K. Kirk.** 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]dioxins by *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**16948–16952.
14. **Hatakka, A.** 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13:**125–135.
15. **Higuchi, T.** 1990. Lignin biochemistry: biosynthesis and biodegradation. Wood Sci. Technol. **24:**23–63.
16. **Kerem, Z., D. Friesem, and Y. Hadar.** 1992. Lignocellulose degradation during solid-state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**1121–1127.
17. **Lambert, M., S. Kremer, O. Sterner, and H. Anke.** 1994. Metabolism of pyrene by the basidiomycete *Crinipellis stipitaria* and identification of pyrenequinones and their hydroxylated precursors in strain JK375. Appl. Environ. Microbiol. **60:**3597–3601.
18. **Lange, B., S. Kremer, O. Sterner, and H. Anke.** 1994. Pyrene metabolism in *Crinipellis stipitaria*: identification of *trans*-4,5-dihydro-4,5-dihydroxypyrene and 1-pyrenylsulfate in strain JK364. Appl. Environ. Microbiol. **60:**3602–3607.
19. **Moen, M. A., and K. E. Hammel.** 1994. Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol. **60:**1956–1961.
20. **Morgan, P., S. A. Lee, S. T. Lewis, A. N. Sheppard, and R. J. Watkinson.** 1993. Growth and biodegradation by white-rot fungi inoculated into soil. Soil Biol. Biochem. **25:**279–287.
21. **Morgan, P., S. T. Lewis, and R. J. Watkinson.** 1991. Comparison of abilities of white-rot fungi to mineralize selected xenobiotic compounds. Appl. Microbiol. Biotechnol. **34:**693–696.
22. **Sack, U., and T. Günther.** 1993. Metabolism of PAH by fungi and correlation with extracellular enzymatic activities. J. Basic Microbiol. **33:**269–277.
23. **Sanglard, D., M. S. A. Leisola, and A. Fiechter.** 1986. Role of extracellular ligninases in biodegradation of benzo[a]pyrene by *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **8:**209–212.
24. **Sutherland, J. B., A. L. Selby, J. P. Freeman, F. E. Evans, and C. E. Cerniglia.** 1991. Metabolism of phenanthrene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57:**3310–3316.
25. **Tatarko, M., and J. A. Bumpus.** 1993. Biodegradation of phenanthrene by *Phanerochaete chrysosporium*: on the role of lignin peroxidase. Lett. Appl. Microbiol. **17:**20–24.
26. **Thurston, C. F.** 1994. The structure and function of fungal laccases. Microbiology **140:**19–26.
27. **Tuor, U., H. Wariishi, H. E. Schoemaker, and M. H. Gold.** 1992. Oxidation of phenolic arylglycerol β-aryl ether lignin model compounds by manganese peroxidase from *Phanerochaete chrysosporium*: oxidative cleavage of an α-carbonyl model compound. Biochemistry **31:**4986–4995.
28. **Vyas, B. R. M., S. Bakowski, V. Sasek, and M. Matucha.** 1994. Degradation of anthracene by selected white rot fungi. FEMS Microbiol. Ecol. **14:**65–70.
29. **Youn, H. D., K. J. Kim, J. S. Maeng, Y. H. Han, I. B. Jeong, G. Jeong, S. O. Kang, and Y. C. Hah.** 1995. Single electron transfer by an extracellular laccase from the white-rot fungus *Pleurotus ostreatus*. Microbiology **141:**393–398.