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Biodegradation of Polycyclic Aromatic Hydrocarbons by *Phanerochaete chrysosporium*

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The ability of the white rot fungus *Phanerochaete chrysosporium* to degrade polycyclic aromatic hydrocarbons (PAHs) that are present in anthracene oil (a distillation product obtained from coal tar) was demonstrated. Analysis by capillary gas chromatography and high-performance liquid chromatography showed that at least 22 PAHs, including all of the most abundant PAH components present in anthracene oil, underwent 70 to 100% disappearance during 27 days of incubation with nutrient nitrogen-limited cultures of this fungus. Because phenanthrene is the most abundant PAH present in anthracene oil, this PAH was selected for further study. In experiments in which [^{14}C]phenanthrene was incubated with cultures of *P. chrysosporium* containing anthracene oil for 27 days, it was shown that 7.7% of the recovered radiolabeled carbon originally present in [^{14}C]phenanthrene was metabolized to $^{14}CO_2$ and 25.2% was recovered from the aqueous fraction, while 56.1 and 11.0% were recovered from the methylene chloride and particulate fractions, respectively. High-performance liquid chromatography of the ^{14}C-labeled material present in the methylene chloride fraction revealed that most (91.9%) of this material was composed of polar metabolities of [^{14}C]phenanthrene. These results suggest that this microorganism may be useful for the decontamination of sites in the environment contaminated with PAHs.

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants. The contamination of air (10), soil (3), freshwater (surface water and groundwater) (1), and marine environments (1) by PAHs has been shown. Of concern to public health is the fact that many PAHs or their metabolites are mutagenic, carcinogenic, or both.

Apparently, the ability of microorganisms to degrade PAHs is a function of the number of aromatic rings in the compound. For example, microorganisms have been isolated that are able to use naphthalene, biphenyl, anthracene, and phenanthrene as their sole carbon source (9). However, there are no reports of microorganisms that are able to use PAHs containing four or more rings as their sole carbon source (9). This is not to say that PAHs containing more than four rings are not degraded, since there are numerous reports of microorganisms that are able to metabolize four- and five-ring compounds, provided that another carbon source is present (9).

Recent studies have shown that the white rot fungus *Phanerochaete chrysosporium* is able to degrade a wide variety of xenobiotic compounds, including PAHs, to carbon dioxide (2, 4–8, 11, 18). The biodegradation of these compounds has been shown to be dependent on the lignin-degrading system of this microorganism, and the initial oxidation of several PAHs has been reported to be catalyzed by ligninases isolated from *P. chrysosporium* (13, 14).

To date most biodegradation experiments (the studies of polychlorinated biphenyl degradation [11] and the degradation of phenolic wastes of paper pulping processes [16] are exceptions) with this microorganism have focused on its ability to degrade pure compounds. Unfortunately, chemical contamination by mixtures of chemicals is much more common than contamination by a single compound. Thus, in order for biodegradative processes to be of practical use, they must be able to degrade all or most of the individual components of these mixtures.

Anthracene oil is a complex mixture of PAHs that is obtained by the fractional distillation of coal tar (15). It is similar in composition to creosote and has been used in the environment as a wood preservative. In the present study it was demonstrated that *P. chrysosporium* is able to degrade PAHs present in anthracene oil.

MATERIALS AND METHODS

Fungi. *P. chrysosporium* BKM-F-1767 was obtained from the Forest Products Laboratory, U.S. Department of Agriculture, Madison, Wis. The fungus was maintained on malt agar slants at room temperature until use. Subcultures were routinely made every 30 to 60 days.

Chemicals. Phenanthrene, anthracene, naphthalene, acenaphthene, fluorene, 1-methylfluorene, fluoranthene, pyrene, and benzo[*a*]fluorene were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Standard Reference Material 1597 (SRM-1597), a partially characterized reference mixture of PAHs, was purchased from the National Bureau of Standards (Gaithersburg, Md.). Anthracene oil was a gift from Rutgerswerke Aktiengesellschaft (Castrop-Rauxel, Federal Republic of Germany). Partial characterization of this material by the gas chromatographic (GC) and mass spectroscopic (MS) techniques described below revealed that the major PAH components present in this sample were dibenzofuran (26 mg/g), fluorene (54 mg/g), dibenzothiophene (9 mg/g), phenanthrene (172 mg/g), anthracene (32 mg/g), carbazole (18 mg/g), 4*H*-cyclopenta[*def*]phenanthrene (15 mg/g), fluoranthene (74 mg/g), pyrene (37 mg/g), benz[*a*]anthracene (2 mg/g), and chrysene (1 mg/g). Trace amounts (less than 1 mg/g) of benzo[*a*]pyrene, benzo[*e*]pyrene, acenaphthylene, acephenanthrylene, 2-methylnaphthalene, 1-methylnaphthalene, naphthalene, biphenyl, cyclopenta[*cd*]pyrene, triphenylene, and perylene were also found. Other PAHs detected in this sample included methylbiphenyl, 9*H*-xanthene, methyldibenzofuran, 1-methylfluorene, 3-methylphenanthrene, 2-methylphenanthrene, 4- and/or 9-methylphenanthrene, phenylnaphthalene, benzo[*a*]fluorene, benzo[*b*]fluorene, and 1-methylpyrene. PAH concentrations were calculated on the basis of the chromatographic

peak area by using a standard curve for each PAH. It should be noted that concentrations were calculated only for those PAHs for which there was a corresponding compound of known concentration present in SRM-1597. [9-^{14}C]phenanthrene (10 mCi/mmol) was purchased from Pathfinder Laboratories, Inc. (St. Louis, Mo.).

Culture conditions. *P. chrysosporium* was incubated in the nutrient nitrogen-limited liquid culture medium (10 ml) described previously (8) at 39°C in 250-ml Wheaton bottles equipped with lids with a Teflon seal. This culture medium is a modification of that described by Fenn and Kirk (12). Cultures were established by inoculating the medium with spores as described previously (17). A total of 10 μl of anthracene oil (50 mg/ml) in acetone and 100 nCi (220,000 dpm) of [^{14}C]phenanthrene (in 9.2 μl of acetone) were added to cultures of *P. chrysosporium*. However, because this amount (final concentration, 50 mg/liter) of anthracene oil was lethal to *P. chrysosporium* when added on day 0, cultures were allowed to grow for 3 days before the addition of anthracene oil and [^{14}C]phenanthrene. This procedure circumvented the lethal effects of anthracene oil. During the first 3 days of incubation, cultures were allowed to grow in an atmosphere of air. After 3 days the cultures were flushed with oxygen and sealed for the remainder of the incubation period (a total of 30 days).

Mass balance analysis. Following incubation of [^{14}C]phenanthrene in nutrient nitrogen-limited cultures of *P. chrysosporium*, cultures were acidified with 2 drops of concentrated hydrochloric acid and homogenized in a Potter-Elvehjem tissue homogenizer equipped with a Teflon pestle (du Pont). The homogenized material was then extracted with three 10-ml portions of methylene chloride; these three portions were subsequently pooled. Following methylene chloride extraction, the mycelium was separated from the aqueous fraction by filtration through glass wool. Safety Solve (10 ml; Research Products International Corp., Mount Prospect, Ill.) was then added to 1-ml portions of the methylene chloride and aqueous fractions and to the entire mycelium fraction. Radioactivity was determined by liquid scintillation spectrometry with a liquid scintillation spectrometer (model LS-5801; Beckman Instruments, Inc., Fullerton, Calif.). The H-number technique was used to compensate for quenching.

The amount of $^{14}CO_2$ evolved from [^{14}C]phenanthrene in cultures of *P. chrysosporium* was determined in separate experiments by using culture conditions identical to those described above, except that culture bottles were equipped with gas-exchange manifolds and were flushed with oxygen at 3-day intervals during the $^{14}CO_2$ evolution assay. Details of this procedure have been described previously (8, 17).

HPLC. High-performance liquid chromatographic (HPLC) analysis of [^{14}C]phenanthrene, anthracene oil, and individual PAH standards was performed by using an HPLC system (System Gold Gradient; Beckman) equipped with a reversed-phase column (4.6 by 150 mm or 4.6 by 250 mm; VYDAC 201 TP5; The Sep/a/ra/tions Group, Hesperia, Calif.). The HPLC elution program consisted of isocratic elution for the first 5 min with a 35% mixture of acetonitrile in water. During the next 5 min the concentration of acetonitrile was increased linearly to 55% acetonitrile and was maintained at this concentration for 30 min. The flow rate was 2 ml/min. The retention times of PAH standards and of the components of anthracene oil were monitored at 254 nm. In a typical experiment, cultures of *P. chrysosporium* were extracted as described above. The methylene chloride extracts were evaporated under a gentle stream of argon and redissolved in 10 ml of acetonitrile. Twenty microliters of this material was then used for HPLC analysis. Percent disappearance was calculated by comparing peak areas by using a digital integrator (model 3390A Hewlett-Packard Co., Palo Alto, Calif.). Metabolite formation from [^{14}C]phenanthrene was monitored by liquid scintillation spectrometry of the HPLC fractions. In these experiments the methylene chloride fraction was evaporated and redissolved in 0.5 ml of acetonitrile. Twenty microliters of this material was then used for HPLC analysis. Fractions (1 ml) were collected in scintillation vials. Safety Solve (10 ml) was added to each fraction, and radioactivity was determined by liquid scintillation spectrometry.

GC-MS analyses were performed on the same samples that were used for HPLC, except that the samples were reduced in volume to ~50:1. To achieve this, 5 ml of the sample was evaporated under a gentle stream of argon to ~100 μl in a 5-ml vial (Reacti-vial; Pierce Chemical Co., Rockford, Ill.). The exact volume was then determined by using a 100-μl syringe (The Hamilton Co., Reno, Nev.). Controls were treated in the same manner. One-microliter portions were then used for GC-MS analysis. GC-MS analyses were performed by using a gas chromatograph (series 3400; Varian Instruments) equipped with an ion trap detector (MAT 7000; Finnigan). The ionization voltage was 70 eV. Separation of PAHs was achieved on a column (0.25 mm by 30 m; DB-5; J & W Scientific) by isothermal elution for 2 min at 100°C, followed by an increase of 4°C/min until a temperature of 270°C was achieved, and this was followed by isothermal elution at 270°C for 15 min. The temperature of the injection port was 300°C, and helium (1 ml/min) was the carrier gas. Because the mass spectra of several PAHs are virtually indistinguishable, the final structural assignments of several PAHs (3-methylphenanthrene and 2-methylphenanthrene; for example) were made on the basis of comparison of retention times of these compounds with those of compounds present in SRM-1597. Percent recovery was calculated for each PAH on the basis of peak area by using a standard curve generated for each PAH (except for two methylbiphenyl congeners) by using SRM-1597. Because the amounts of two methylbiphenyl congeners in anthracene oil exceeded the amounts of these compounds found in SRM-1597, the percent recovery of these compounds was calculated directly from the peak area by assuming that the GC response factor was linear.

RESULTS

The initial results obtained by HPLC indicated that only 2, 5, 2, 2, 26, and 20% of the fluorene, phenanthrene, anthracene, carbazole, fluoranthene, and pyrene initially present, respectively, remained in cultures of *P. chrysosporium* after 27 days of incubation (data not shown). Also, benzo [*a*]fluorene, 1-methylfluorene, and acenaphthalene were degraded to below the level of detectability. However, because some PAHs were inadequately separated by HPLC and because PAH identification was dependent solely on comigration with authentic standards, subsequent analyses by GC-MS were performed (Table 1).

The results obtained by GC-MS analyses showed that 22 PAH components present in anthracene oil underwent extensive disappearance during 27 days of incubation in nutrient nitrogen-limited cultures of *P. chrysosporium*. To control for nonbiological loss, control experiments were performed in which anthracene oil was added to cultures of *P. chrysosporium*, which were then immediately extracted

TABLE 1. Disappearance of PAH components of anthracene oil after incubation with nutrient nitrogen-limited liquid cultures of *P. chrysosporium*

Compound (mol wt)	GC retention time (min)	% Recovery from controls and 30-day-old cultures of *P. chrysosporium* in:			
		Control 1[a]	Control 2[b]	Expt 1[c]	Expt 2[c]
Dibenzofuran (168)	15.93	49 ± 23	46 ± 15	<1	<1
Fluorene (166)	17.87	75 ± 19	87 ± 12	<1	<1
Methylbiphenyl (168) (unidentified congener)	18.35	60 ± 18	65 ± 26	ND[d]	ND[d]
Methylbiphenyl (168) (unidentified congener)	18.55	43 ± 13	47 ± 26	ND	ND
9*H*-Xanthene (182)	18.88	80 ± 13	73 ± 20	ND	ND
Methyldibenzofuran (182) (unidentified congener)	19.25	88 ± 16	72 ± 16	2	ND
1-Methylfluorene (180)	21.05	97 ± 12	77 ± 15	ND	ND
Dibenzothiophene (184)	22.5	104 ± 9	91 ± 3	26	2
Phenanthrene (178)	23.43	100 ± 10	87 ± 12	4	4
Anthracene (178)	23.60	122 ± 14	103 ± 18	1	1
Carbazole (167)	24.70	111 ± 13	85 ± 13	2	2
3-Methylphenanthrene (192)	26.23	107 ± 11	93 ± 16	17	17
2-Methylphenanthrene (192)	26.37	110 ± 10	90 ± 10	15	13
4*H*-Cyclopenta[*def*]phenanthrene (190)	26.83	89 ± 8	73 ± 14	2	<1
4- and/or 9-Methylphenanthrene (192)	26.98	110 ± 12	96 ± 14	15	13
Fluoranthene (202)	30.52	108 ± 10	92 ± 14	21	28
Pyrene (202)	31.73	103 ± 7	87 ± 13	13	10
Phenylnaphthalene (204)	31.75	95 ± 13	72 ± 20	ND	ND
Benzo[*a*]fluorene (216)	33.82	96 ± 7	63 ± 11	4	7
Benzo[*b*]fluorene (216)	34.23	108 ± 12	58 ± 15	5	3
1-Methylpyrene (216)	35.05	104 ± 28	90 ± 20	ND	<1
Benz[*a*]anthracene (228)	39.03	120 ± 20	80 ± 10	14	10

[a] Anthracene oil (50 mg/liter) was added to 30-day-old nutrient nitrogen-limited cultures of *P. chrysosporium*. Cultures were then extracted and analyzed for PAHs by GC-MS.

[b] Anthracene oil (50 mg/liter) was added to the culture medium and incubated under the same conditions as those described for nutrient nitrogen-limited cultures of *P. chrysosporium* (see footnote *c*). Culture medium also contained $HgCl_2$ (70 mg per culture), to inhibit microbial growth. Culture medium was then extracted and analyzed for PAHs by GC-MS.

[c] Anthracene oil (50 mg/liter) was added to 3-day-old nutrient nitrogen-limited cultures of *P. chrysosporium*. Cultures were then extracted and analyzed for PAHs by GC-MS.

[d] ND, Not detected.

and analyzed for individual PAH components (control 1). In other control experiments, anthracene oil was incubated with sterile medium and $HgCl_2$ for 27 days prior to extraction and analysis (control 2). The results (Table 1) indicate that, in general, recoveries in most control experiments ranged between 70 and 100% for individual PAHs, whereas in experiments in which anthracene oil was incubated with *P. chrysosporium*, recoveries of individual PAHs ranged between 0 and 28%. In some cases substantial nonbiological disappearance of individual PAHs did occur. However, even in many of these cases the amount of biodegradation observed was substantially greater than the nonbiological disappearance observed in either control. For example, only 46 ± 15 and 49 ± 23% recovery was observed in the two controls for dibenzofuran disappearance, whereas in experiments in which anthracene oil was incubated with *P. chrysosporium*, less than 1% of the dibenzofuran originally present was found after 27 days of incubation. It should also be noted that because of very low recoveries in some control experiments it was not possible to assess by GC-MS the biodegradation of a number of PAH components present in anthracene oil. It was particularly difficult to assay those PAHs that are known to have relatively high vapor pressures (e.g., naphthalene, acenaphthene, and biphenyl) and many of those that were present in only trace amounts (e.g., perylene, triphenylene, chrysene, acenaphthylene, benzo[*a*]pyrene, and benzo[*e*]pyrene).

Figure 1 shows that [^{14}C]phenanthrene was mineralized by nutrient nitrogen-limited cultures of *P. chrysosporium*. Mineralization of [^{14}C]phenanthrene was greatest between days 3 and 21, after which the rate of mineralization declined such that after 30 days $^{14}CO_2$ evolution virtually ceased. Figure 1 also shows that like many organic compounds (5–8), mineralization of [^{14}C]phenanthrene depended on the availability of another carbon source to serve as a growth substrate since the addition of supplemental glucose after 30 days of incubation substantially increased the rate of [^{14}C]phenanthrene

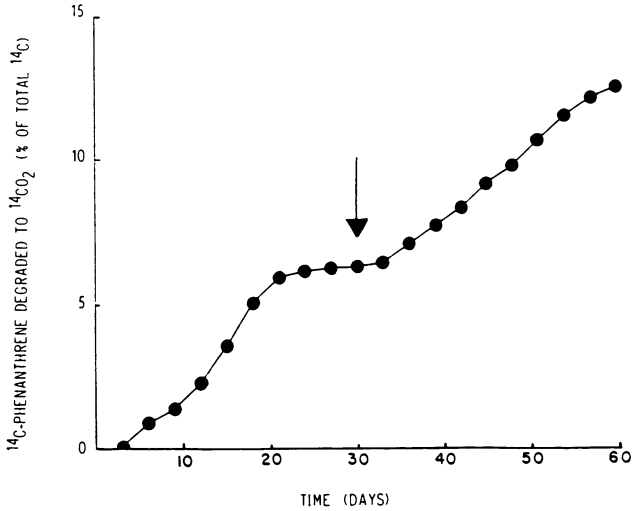

FIG. 1. Mineralization of [^{14}C]phenanthrene in nutrient nitrogen-limited cultures of *P. chrysosporium* containing anthracene oil (50 mg/liter). The arrow indicates the point at which supplemental glucose (56 mM) was added to each culture. Each point represents the mean of four determinations ± the standard deviation. The width of the error bars is less than the diameter of the circles.

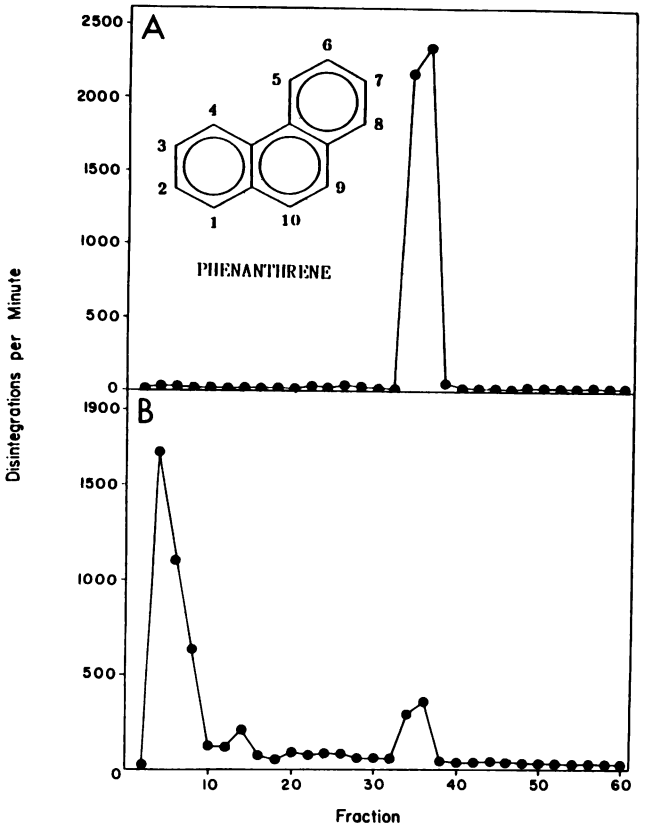

FIG. 2. HPLC elution profiles of [^{14}C]phenanthrene before incubation (A) and ^{14}C in a methylene chloride extract of a nutrient nitrogen-limited culture of *P. chrysosporium* that was incubated with [^{14}C]phenanthrene for 27 days in the presence of anthracene oil (50 mg/liter) (B).

mineralization. It should be noted that the ^{14}C label in [^{14}C]phenanthrene was located exclusively in the C-9 position (Fig. 2). Thus, it can only be stated with certainty that this carbon atom is mineralized. However, it should also be noted that mineralization of the C-9 carbon in [^{14}C]phenanthrene would require cleavage of the middle aromatic ring.

Mass balance experiments were performed after [^{14}C]phenanthrene was incubated for 27 days in a nutrient nitrogen-limited culture of *P. chrysosporium* containing 50 mg of anthracene oil per liter. The total radioactivity recovered was 81.9% of that which was initially present. Of this, 25.2% was water soluble, 56.1% was found in the methylene chloride fraction, 7.7% was evolved as $^{14}CO_2$, and 11.0% was present in the mycelium. In control experiments in which [^{14}C]phenanthrene was added to culture medium and extracted immediately, greater than 99% of the radioactivity was found in the methylene chloride fraction, and when the [^{14}C]phenanthrene was added to 30-day-old cultures, 97.1% of the radioactivity was found in the methylene chloride fraction, while 1.8% was found in the mycelium and 1.1% was found in the aqueous fraction.

When the methylene chloride fraction was analyzed by HPLC (Fig. 2), 91.9% of the radiolabeled material present in this fraction was found to be polar metabolities of [^{14}C]-phenanthrene, and the amount of residual [^{14}C]phenanthrene present (calculated based on radiochemical data) was found to be only 4.5% of that which was present initially and was in close agreement with that found in disappearance studies (i.e., 4 to 5% of phenanthrene remaining).

DISCUSSION

These results indicate that the wood-rotting fungus *P. chrysosporium* is able to extensively degrade many of the PAH components of anthracene oil. Degradation of the PAH components of anthracene oil was demonstrated by substrate disappearance, and corroborative data demonstrated that the radiolabeled carbon of [^{14}C]phenanthrene, which was the predominant component of this mixture, was oxidized to $^{14}CO_2$. Furthermore, mass balance analysis and HPLC experiments showed that [^{14}C]phenanthrene was converted to more polar and water-soluble metabolites.

That *P. chrysosporium* was able to degrade a wide variety of PAHs is consistent with previously published reports that have shown that, in addition to [^{14}C]phenanthrene, this fungus is able to degrade [^{14}C]2-methylnaphthalene, [^{14}C] biphenyl, and [^{14}C]benzo[*a*]pyrene to $^{14}CO_2$ (6, 8, 18). It is significant that, to date, no PAH has been reported to be completely resistant to degradation by *P. chrysosporium*.

It has been shown that the ability of *P. chrysosporium* to degrade a wide variety of structurally diverse organic compounds, including a number of PAHs, is dependent, at least in part, on the lignin-degrading system of this fungus that is expressed under nutrient-limiting conditions (2, 4–8, 11, 13, 14, 18). The lignin-degrading system of this fungus includes a family of lignin peroxidases, or ligninases as they are commonly known, which catalyze the initial oxidative depolymerization of lignin (19). Of particular interest are the findings of Haemmerli et al. (13), who have shown that a purified ligninase is also able to catalyze the initial oxidation of benzo[*a*]pyrene to the corresponding 1,6-, 3,6-, and 6,12-benzo[*a*]pyrene quinones. Similarly Hammel et al. (14) have shown that pyrene is oxidized to pyrene-1,6-dione and pyrene-1,8-dione by a pure ligninase. These results confirm a role for the lignin-degrading system in PAH biodegradation by *P. chrysosporium*. It has also been shown that the ability of ligninase H-8 to oxidize PAHs is dependent on the ionization potential of the PAH in question and that this ligninase isozyme has the ability to oxidize PAHs with an ionization potential of $\leq$7.56 eV (14). This is significant since horseradish peroxidase does not oxidize PAHs with ionization potentials greater than 7.35 eV and it has been suggested that the intermediate oxidation states of ligninase H-8 (i.e., compounds I and II) may be more electropositive (i.e., better oxidants) than corresponding intermediates in classical peroxidases (14). If true, this would mean that the ability of *P. chrysosporium* to degrade such a broad spectrum of environmentally persistent organic compounds may simply be a function of the ability of ligninase to catalyze the initial (and often most difficult) oxidation of these compounds.

Although Hammel et al. (14) have shown that ligninase H-8 does not have the ability to oxidize PAHs with ionization potentials greater than 7.56 eV, I have shown here that [^{14}C]phenanthrene, a compound with an ionization potential greater than 8.0 eV, is oxidized to $^{14}CO_2$ in nutrient nitrogen-limited cultures of *P. chrysosporium*. These results demonstrate that the fungus has the ability to oxidize PAHs with ionization potentials higher than 7.56 eV and suggest that fungal enzymes, possibly other ligninases, with oxidizing abilities even greater than those of ligninase H-8 may be present in this microorganism.

Because of its ability to degrade a wide variety of organopollutants, it has been proposed that *P. chrysosporium* may

be useful in some waste treatment systems. The development and study of systems designed to treat PAH-contaminated soil and water with this fungus are currently in progress.

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