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Enhanced Biodegradation of Phenanthrene in Oil Tar-Contaminated Soils Supplemented with *Phanerochaete chrysosporium*

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In recent years, the white rot fungus *Phanerochaete chrysosporium* has shown promise as an organism suitable for the breakdown of a broad spectrum of environmental pollutants, including polynuclear aromatic hydrocarbons (PAHs). The focus of this study was to determine whether *P. chrysosporium* could effectively operate in an actual field sample of oil tar-contaminated soil. The soil was loaded with [^{14}C]phenanthrene to serve as a model compound representative of the PAHs. Soil with the native flora present under static, aerobic conditions with buffering (pH 5.0 to 5.5) displayed full mineralization on the order of 20% in 21 days. The addition of *P. chrysosporium* was synergistic, with full mineralization on the order of 38% in 21 days. In addition to full mineralization, there was an increase in the proportion of radiolabelled polar extractives, both soluble and bound, in the presence of *P. chrysosporium*. From this study, it is apparent that the native soil microflora can be prompted into full mineralization of PAHs in some contaminated soils and that this mineralization can be enhanced when supplemented with the white rot fungus *P. chrysosporium*. With further refinement, this system may prove an effective bioremediation technology for soils contaminated with PAHs.

Improper disposal methods and inadequate control of toxic materials have led to widespread contamination of soils, groundwater, and surface water bodies. Achieving permanent cleanup of these sites is problematic in that some remediation technologies are not always viewed positively by the public or may not be amenable to particular sites. Many methods of treating recalcitrant wastes, including various types of physical, chemical, and biological processes, have been developed. Current strategies for disposing of heavily contaminated soils include excavation followed by incineration and/or secure landfilling. Land farming has also been used for less contaminated soils (2). Bioremediation often appears the most acceptable remediation technology, although it is still viewed largely as a black box (1). Some of the most difficult bioremediation problems involve the decontamination of soils contaminated with oil and coal tar residues. These problems arise because of the recalcitrant nature of polynuclear aromatic hydrocarbons (PAHs) and the difficulties in achieving effective contact with these substances when they are associated with the soil.

In situ bioremediation of soils involves employment of naturally occurring microorganisms. Enhancement may be achieved through a variety of approaches, including nutrient supplementation, control of moisture or pH, or addition of other microorganisms (16). During the past several years, one organism which has been examined for its ability to degrade recalcitrant pollutants is the white rot fungus *Phanerochaete chrysosporium* (4, 7). Although the natural substrate degraded by this organism is lignin, the enzyme complement secreted by *P. chrysosporium* can degrade a variety of recalcitrant pollutants, including PAHs (3, 8). It has also been shown that *P. chrysosporium* can degrade the more recalcitrant PAHs containing four or more rings (10, 11). It appears that this degradation is due to the structural similarities of these pollutants to portions of the lignin substructure and the low-level specificity of the complement of ligninases produced by *P. chrysosporium*.

In moving towards the practical application of this organism, we have examined its use as a supplement for enhanced in situ biodegradation of PAHs in oil tar-contaminated soil obtained from a former oil gasification plant. Phenanthrene was one of the principal PAHs in the soil and was therefore used as a tracer to monitor mineralization (i.e., complete degradation to CO_2 and water) and metabolism under various conditions.

MATERIALS AND METHODS

Soil. The contaminated soil used in this study was from a former oil gasification site and was contaminated with tarlike oil. The soil was classified as a sandy-clayey silt by particle size distribution tests (20% sand, 20% clay, and 60% silt). The primary soil contaminants and their average concentrations were as follows (in micrograms per gram): phenanthrene, 34; anthracene, 10; benzo[*a*]pyrene, 10; total PAHs, 335; and oil and grease, 1,540. Studies were conducted with both native soil (natural flora present) and sterile soil. For the sterile soil, a bulk sample (100 g) was heat sterilized in an autoclave at 121°C and 15 lb/in^2 for 30 min. This soil sample was distributed aseptically into 20-ml vials for use in various experiments.

Organism and culture conditions. *P. chrysosporium* INA-12 was obtained from the Collection Nationale de Culture de Microorganisms, Institute Pasteur, Paris, France. Cultures were maintained on potato dextrose agar slants (Difco Laboratories) at 4°C and subcultured every 3 months. The inoculum was prepared by growing subcultures at 37°C for 5 days in 1-liter Roux bottles containing 100 ml of potato dextrose agar. On the 5th day, 69 ml of sterile medium was added to the bottle with gentle agitation to suspend the spores. A 2.0-ml volume of this suspension was used to

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inoculate each flask, which normally resulted in a final concentration of 2×10^8 spores per ml after inoculation. A modified version of the high-nitrogen–glycerol-based medium described by Buswell et al. (6), which contained oleic acid at a final concentration of 0.045% (vol/vol), 0.047% (vol/vol) Tween 80, and 0.42 mM veratryl alcohol, was used. All cultures were grown under stationary conditions in 500-ml Erlenmeyer flasks containing 50 ml of medium in an incubator at 37°C. The flasks were aseptically flushed with 100% oxygen for 1.5 min at 10 liters/min on the day of inoculation and every 3 days thereafter. Flasks were sealed with rubber stoppers which had been wrapped in aluminum foil to minimize adsorption of radiolabelled compounds.

Toxicity studies. Toxicity studies with various soil loadings were conducted in a manner analogous to the growth studies, with and without pH adjustment. Soil was added on day 3 of growth and harvested on day 7. Reported fresh weights are for filterable solids (biomass plus enmeshed soil) resulting from filtration through 202-μm-pore-size Nitex monofilament cloth (B. & S. H. Thompson, Scarborough, Ontario, Canada). Dry weights represent samples weighed after drying at 105°C until a constant weight was achieved. Accurate measurements of dry weight were confounded because of the presence of soil enmeshed in the biomass. Dry weight determinations were corrected by using the ash content. Biomass samples were ashed at 1,000°C for 12 h. On the basis of experiment, the total organic content of the soil was found to be negligible. Corrected values were obtained with the formula

$$M_{B_D} = M_{T_D}\left(\frac{\%A_S - \%A_T}{\%A_S - \%A_B}\right)$$

where M_{B_D} is the dry weight of biomass, M_{T_D} is the dry weight of biomass plus enmeshed soil, $\%A_T$ is the percent ash content of biomass plus soil, $\%A_S$ is the percent ash content of soil (~95% as determined by experiment), and $\%A_B$ is the percent ash content of biomass (~5% as determined by experiment).

Biodegradation studies. Radiolabelled phenanthrene ([9-^{14}C]phenanthrene; specific activity, 418 MBq/mmol) was purchased from Amersham Canada Ltd., Oakville, Ontario, Canada. A stock solution of [^{14}C]phenanthrene was prepared in dimethyl sulfoxide and used to preload the soil samples. Aliquots (30 μl) containing 50 to 54 kBq were added dropwise to each soil sample in a 20-ml vial. The samples were then homogenized with a glass rod, and the soil samples were sealed for 24 h prior to being added to the culture flasks on day 3. This method is not unlike that used by Herbes and Schwall (12) to load petroleum-contaminated sediments with ^{14}C-labeled compounds. Residual holdup in the vials was less than 1% of the amount initially added.

The $^{14}CO_2$ liberated in the flasks was trapped by the method of Haider and Martin (9). Sterile 20-ml vials containing 5 ml of 0.5 M NaOH were positioned in the bottoms of the 500-ml Erlenmeyer flasks to collect the liberated $^{14}CO_2$. The vials were changed every 3 days prior to being flushed with O_2, and the pH was adjusted to approximately 5.0. Aliquots (0.2 ml) of the NaOH were taken and added to 0.5 ml of H_2O and 4.5 ml of Universol ES liquid scintillation cocktail (ICN Biomedicals Inc., Irvine, Calif.), and then the radioactivity was determined with a Wallac 1410 liquid scintillation counter (Wallac Oy, Turku, Finland).

A general outline of the culture-harvesting method for experiments in which soil was added is given in Fig. 1. The culture was decanted carefully and filtered through Whatman no. 1 filter paper. Because the biomass floated on the surface of the medium the soil was easily retained in the bottom of the flask. The filtered biomass was lifted from the filter paper, and each sample was transferred to a separate 20-ml vial. The filter paper and biomass were extracted with 15 and 10 ml, respectively, of 50:50 (vol/vol) methanol-methylene chloride (MeOH-$MeCl_2$) for 24 h with periodic shaking. Aliquots (0.5 ml) of the extract were added to 4.5 ml of scintillation cocktail, and radioactivity was counted. Control studies with biomass gave >90% recovery of the adsorbed activity. The filtrate was used to wash the soil particles from the flask into a centrifuge tube. The soil and medium were centrifuged in a clinical centrifuge at $1,000 \times g$ for 10 min. The medium was decanted off, a 0.5-ml aliquot was added to 4.5 ml of scintillation cocktail, and radioactivity was counted. The soil pellet was divided into two samples, which were transferred to separate 20-ml vials and extracted with 10 ml of 50:50 (vol/vol) MeOH-$MeCl_2$ for 24 h with periodic shaking, and radioactivity was counted as

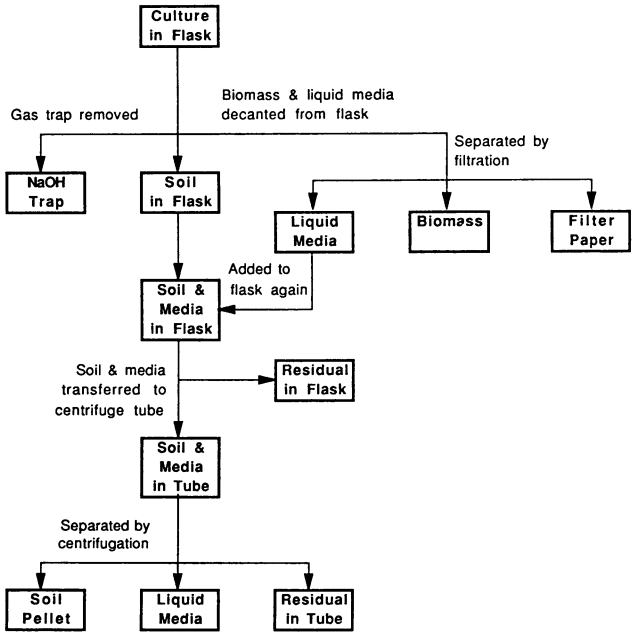

FIG. 1. Flow diagram of culture-harvesting method.

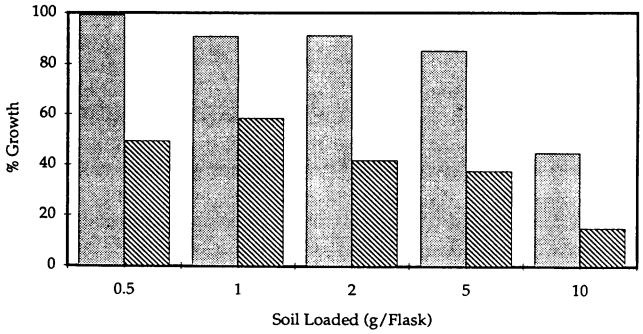

FIG. 2. Percent growth (based on dry weight) relative to control growth without soil, as a function of soil loading. The average yield (dry weight) for a soil loading of 0 g/50 ml of medium was 0.2 g/50 ml. Shaded bars, pH adjusted; hatched bars, pH not adjusted.

TABLE 1. Effect of acid addition on medium pH for various soil loadings

Time	Acid addition	Medium pH with soil loading (g of soil/50 ml of medium) of:				
		0.5	1	2	5	10
Start	No	5.08	5.13	5.23	5.32	5.42
Day 3		5.38	5.45	5.61	5.85	6.10
Start	Yes	4.96 (0.1)[a]	5.02 (0.2)	4.98 (0.4)	4.95 (1.0)	4.98 (2.0)
Day 3		5.07	5.18	5.22	5.26	5.38

[a] Values in parentheses designate the volume (in milliliters) of 1.0 M HCl added.

described above. The culture flasks were washed with 25 ml of a 5% Conrad 70 (Canlab, Mississauga, Ontario, Canada) solution, a 0.5-ml aliquot was added to 4.5 ml of scintillation cocktail, and radioactivity was counted. Residual activity in the centrifuge tube was recovered with two 10-ml washes with 50:50 (vol/vol) MeOH-$MeCl_2$; these were combined, and the radioactivity in a 0.5-ml aliquot was counted as described above. Counts for the filter paper extract, soil extract, and centrifuge tube wash were summed and are referred to as the radioactivity in the soil. This procedure for control studies yielded 86 to 99% recovery of [^{14}C]phenanthrene for both sterile and native soils.

Adsorption and desorption studies. For adsorption studies, the medium was loaded with 50 to 54 kBq of [^{14}C]phenanthrene per 50 ml and then the appropriate weight of soil was added. Samples of medium (0.5 ml) were taken periodically for counting. For desorption studies, preloaded soil was added to the medium and sampled as described above. For both adsorption and desorption studies, the flasks were briefly agitated after each sample was taken.

Phenanthrene biotransformation. Samples (5.0 ml) of medium (polar) were partitioned against 5.0 ml of hexane (nonpolar), and radioactivity in 0.5-ml aliquots of each phase was counted. For the MeOH-$MeCl_2$ samples, 0.5 ml of the MeOH-$MeCl_2$ extract was added to 4.5 ml of H_2O (polar) and partitioned against 5.0 ml of hexane (nonpolar), with radioactivity counted as described above. The pH of the medium was approximately 5.0.

The data in most cases are the means of duplicate samples.

RESULTS AND DISCUSSION

Establishment of experimental conditions for bioremediation studies. Before the biodegradative capacity of *P. chrysosporium* in soil could be evaluated, a number of practical problems had to be solved. Preliminary experiments were run to provide some guidelines for appropriate conditions under which bioremediation experiments with contaminated soil could be conducted. The primary purpose of these experiments was to provide a starting point for evaluating the suitability of *P. chrysosporium* as a supplement to enhance bioremediation in soils contaminated with PAHs. The toxicity of the soil to *P. chrysosporium* was tested in a series of experiments (Fig. 2). Toxicity effects were seen at soil loadings of 0.5 g/50 ml of medium in which biomass production was reduced approximately 50% without pH adjustment. Growth progressively decreased at soil loadings greater than 1 g/50 ml.

The soil was found to have considerable natural buffering capacity in the range of pH 7.0, probably because of the carbonate content. This is outside the range for both optimal growth and induction of the ligninolytic system of *P. chrysosporium* (5). The concentration of 2,2-dimethylsuccinic acid in the growth medium was insufficient to buffer to the pH of 5.0 with soil loadings greater than 0.5 g/50 ml of medium. Experiments were conducted to control pH in this range by the periodic addition of HCl at 3-day intervals. The resulting effects on pH of this addition are given in Table 1. This technique was effective in maintaining the pH in a range of 5.0 to 5.3 for up to 5 g of soil per 50 ml of medium. The effect of pH regulation minimized the toxicity effects reflected by biomass production (Fig. 2). With pH regulation, reductions in biomass production were relatively small at soil loadings up to 5 g/50 ml. As a consequence, bioremediation studies were conducted at soil loadings no greater than 5 g/50 ml of medium.

Experiments were conducted to establish the conditions for preloading the soil with [^{14}C]phenanthrene. Isotherms for adsorption of [^{14}C]phenanthrene from medium onto soil are

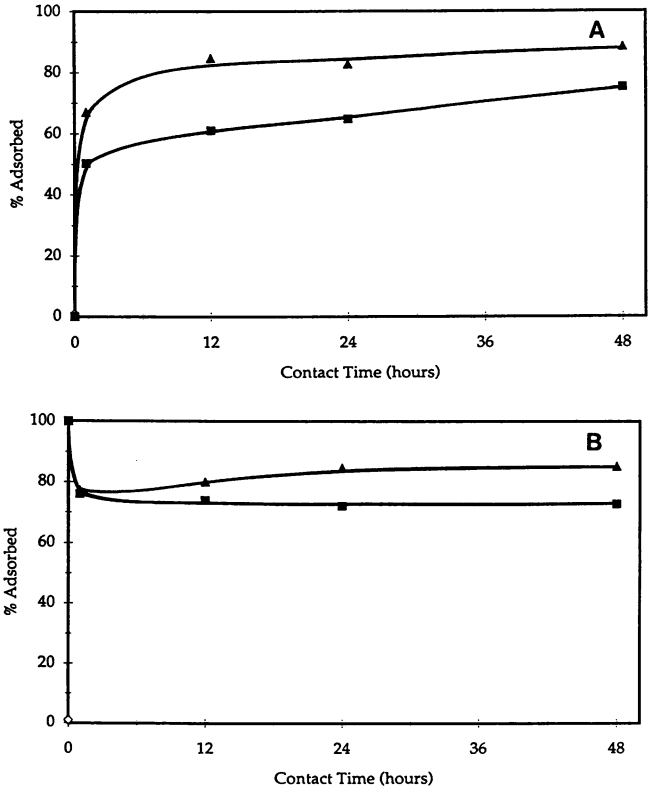

FIG. 3. (A) Isotherms for adsorption of [^{14}C]phenanthrene from medium onto soil. ■ and ▲, 2 and 5 g of soil, respectively, per 50 ml of medium. (B) Isotherms for desorption of [^{14}C]phenanthrene from preloaded soil. ■ and ▲, 2 and 5 g of soil, respectively per 50 ml of medium.

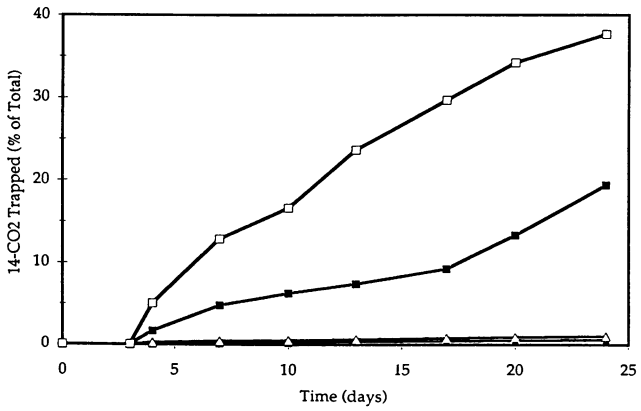

FIG. 4. Cumulative $^{14}CO_2$ trapped, expressed as a percentage of the total activity recovered from soil cultures loaded with [^{14}C]phenanthrene. Results are shown for sterile soil (2 g/50 ml of medium) alone (▲) and with *P. chrysosporium* (△) and for native soil (2 g/50 ml of medium) alone (■) and with *P. chrysosporium* (□).

reported in Fig. 3A. The curves closely resemble the Langmuir isotherm form that represents monolayer adsorption phenomena. Desorption studies were conducted with soil preloaded with [^{14}C]phenanthrene, and the results are reported in Fig. 3B. The bulk of the desorption occurred during the first hour, with a steady-state level of approximately 80% adsorption being maintained after this point. Similar levels of desorption were observed for sterile soil samples (data not shown).

Biodegradation studies. On the basis of the experiments described above, a series of soil degradation experiments was run. Soil loadings of 2 and 5 g/50 ml of medium were used. The cumulative $^{14}CO_2$ trapped as a percentage of the total recovered for the biodegradation experiments is shown in Fig. 4. It is apparent that considerable full mineralization of the phenanthrene is possible in native soil alone and in native soil supplemented with *P. chrysosporium*. The final distribution of radioactivity 21 days after the addition of the soil is given in Table 2. For soil alone, at a loading of 2 g/50 ml of medium, full mineralization was 20% of the total activity originally added. When soil was supplemented with *P. chrysosporium*, full mineralization increased to approximately 38% of the total activity originally added. This result is to be compared with that for pure cultures of *P. chrysosporium*, in which full mineralization was only 1.7%. The rate of mineralization was still constant in native-soil cultures with and without *P. chrysosporium* present at day 21, and mineralization would be expected to continue thereafter.

In cultures supplemented with *P. chrysosporium*, enhanced mineralization occurs. Considering the extents of mineralization for soil alone and for *P. chrysosporium* independently, it is apparent that the effect is not additive but synergistic. Results in Table 2 indicate that the activity associated with the soil decreases for both native soil and sterile soil in the presence of *P. chrysosporium*. In sterile soil alone, 62% of the activity, which decreased to 9% when *P. chrysosporium* was added, was found to be associated with the soil. Because this decrease could be due partly to soil enmeshed in the biomass, the total bound activity (activity associated with the soil, biomass, and residual holdup on the flask) was considered. Bound activity was also observed to decrease for both native soil and sterile soil when *P. chrysosporium* was added. For example, for the sterile soil alone, 67% bound activity and 30% medium activity shifted to 42% bound activity and 44% medium activity when *P. chrysosporium* was added. *P. chrysosporium* appears to mediate a redistribution of activity from that associated with the soil and/or biomass to that solubilized and/or mineralized.

This redistribution may be explained in part if *P. chrysosporium* was responsible for partially metabolizing the [^{14}C]phenanthrene. There are numerous reports on the ability of *P. chrysosporium* to degrade or modify PAHs, and they are supported by the full mineralization observed for the pure culture in this study. For example, Bumpus (3) showed that phenanthrene could be fully mineralized as well as converted to more-polar and water-soluble metabolites in cultures containing anthracene oil. To determine whether the phenanthrene was being chemically transformed, an experiment was conducted to characterize the nature of the activity associated with the medium, soil, and biomass on the basis of polarity (Table 3). It is apparent that after 21 days in the presence of viable soil floras, there is an increase in activity associated with all polar fractions for medium, soil, and biomass. For example, there is an increase from 2.3 to 27.5% in the total polar activity recovered. In the presence of *P. chrysosporium*, this is further increased to 46.6%. For sterile soil, there was no change in the activity associated with the polar fraction after 21 days (data not shown).

In conclusion, supplementation of native soil with *P. chrysosporium* coupled with regulation of some of the environmental factors (e.g., nitrogen, carbon source, and pH, etc.) allows improved bioremediation of soils contaminated with PAHs. In this study, the addition of *P. chrysosporium* was found to be synergistic. The basis of this synergism is related to *P. chrysosporium*'s ability to partially metabolize the phenanthrene to more-polar compounds. The consequence(s) of this metabolism may be that the polar products are more readily metabolized by the native soil flora and/or

TABLE 2. Final distribution of radioactivity 21 days after the addition of soil[a]

Treatment	% of total activity[b]					
	$^{14}CO_2$	Medium	Soil	Biomass	Bound[c]	Recovered[d]
Medium + *P. chrysosporium*	1.7	73.0	0.0	21.6	24.4	99.1
Sterile soil	0.6	29.6	61.6	0.0	66.5	96.6
Sterile soil + *P. chrysosporium*	1.0	43.7	8.7	31.5	41.8	86.5
Soil	19.5	24.3	24.1	29.9	55.3	99.1
Soil + *P. chrysosporium*	37.7	21.9	15.5	19.8	36.4	96.0

[a] 2 g of soil per 50 ml of medium.
[b] The percentage of total activity is based on activity recovered from appropriate controls for soil samples at day 3, when soil was added.
[c] Bound activity includes activity associated with the soil, biomass, and residual holdup in the flask.
[d] Recovered activity includes residual holdup in the flask.

TABLE 3. Distribution of radioactivity in polar and nonpolar fractions 21 days after the addition of soil[a]

Treatment (day)	% of total radioactivity[b]							
	Medium		Soil		Biomass		Total recovered[c]	
	NP	P	NP	P	NP	P	NP	P
Medium	95.3	4.7	0.0	0.0	0.0	0.0	95.3	4.7
Soil (0)	94.9	5.1	98.6	1.4	0.0	0.0	97.7	2.3
Soil (21)	8.5	91.5	86.6	13.4	88.2	11.8	72.5	27.5
Soil + *P. chrysosporium* (21)	5.7	94.3	79.3	20.7	76.9	23.1	53.4	46.6

[a] 5 g of soil per 50 ml of medium.
[b] NP, nonpolar; P, polar.
[c] Includes medium, soil, and biomass.

that the change in polar character results in a redistribution to the aqueous phase, making the polar products more accessible for degradation. Lamar et al. (13, 14) have found that *P. chrysosporium* can be applied under field conditions for mineralizing and metabolizing pentachlorophenol. Ryan and Bumpus (15) demonstrated the biodegradation of 2,4,5-trichlorophenoxyacetic acid in soil supplemented with *P. chrysosporium* and ground corncobs. Considerable work in assessing the feasibility of applying *P. chrysosporium* under field conditions remains, but its practical application looks promising.

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