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Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi

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Abstract

Selected strains of three species of white rot fungi, *Pleurotus ostreatus*, *Phanerochaete chrysosporium* and *Trametes versicolor*, were grown in sterilized soil from straw inocula. The respective colonization rates and mycelium density values decreased in the above mentioned order. Three- and four-ringed PAHs at 50 ppm inhibited growth of fungi in soil to some extent. The activities of fungal MnP and laccase (units per g dry weight of straw or soil), extracted with 50 mM succinate-lactate buffer (pH 4.5), were 5 to 20-fold higher in straw compared to soil. The enzyme activities per g dry soil in *P. ostreatus* and *T. versicolor* were similar, in contrast to *P. chrysosporium*, where they were extremely low. Compared to the aerated controls, *P. ostreatus* strains reduced the levels of anthracene, pyrene and phenanthrene by 81–87%, 84–93% and 41–64% within 2 months, respectively. During degradation of anthracene, all *P. ostreatus* strains accumulated anthraquinone. PAH removal rates in *P. chrysosporium* and *T. versicolor* soil cultures were much lower.

Abbreviations: DMAB – 3-dimethylaminobenzoic acid; IP – ionization potential; LiP – lignin peroxidase; MBTH – 3-methyl-2-benzothiazolinone hydrazone hydrochloride; MnP – manganese-dependent peroxidase; PAHs – polyaromatic hydrocarbons

Introduction

White rot fungi belong to the group of Basidiomycetes that colonize wood in nature and preferentially decompose lignin in lignocellulosic materials to cause white rotting of wood. Specialized for the decomposition of lignin, those fungi produce extracellular ligninolytic systems composed of peroxidases, H_2O_2-producing oxidases and laccase (Kirk & Farrell 1987). In addition to decomposition of lignin, the potential of white rot fungi for degradation of various organopollutants in both sterile and nonsterile soil has been well documented (e.g. Davis et al. 1993; Šašek et al. 1993). Therefore, they are considered promising for application to the clean-up of contaminated soils, but little is known about their ability to colonize various soils and their biochemical activities (Lamar et al. 1987; Boyle 1995).

Growth of *Phanerochaete chrysosporium* in sterile soil has been found to increase with both the soil nitrogen content up to 5% (W/W) and the soil water potential in the range of −1.5 to −0.03 MPa, as was demonstrated with various sandy and silty loam soils (Lamar et al. 1987). On the other hand, neither soil acidity nor the organic matter content of the soil played a significant role in the control of fungal growth in the soils tested. Other studies demonstrated that the addition of a carbohydrate carbon source, nitrogen source or a complex nutrient supplement such as straw, pine bark, alfalfa or bran to sterile or nonsterile

sandy loam soils increased the growth of *P. chrysosporium* and *Trametes versicolor* (Morgan et al. 1993; Boyle 1995). Direct objective measurement of fungal growth in soil is difficult since the hyphae stick to the solid substrate. Ergosterol content was found to be a suitable parameter describing fungal growth in soil as the compound is endogenous only to fungi and green algae (Newell et al. 1987; Davis & Lamar 1992).

White rot fungi have been demonstrated to attack a wide spectrum of polyaromatic hydrocarbons (PAHs) including carcinogenic benzo[*a*]pyrene (e.g. Bumpus 1989; George & Neufeld 1989, and the review papers Cerniglia 1992 and Paszczynski & Crawford 1995). Several enzymatic mechanisms are thought to be involved: (a) lignin peroxidase (LiP), and possibly also manganese-dependent peroxidase (MnP), directly catalyze one-electron oxidation of PAHs having the ionization potential (IP) values of $\leq$7.55 eV to produce PAH quinones (Hammel et al. 1986; Field et al. 1996) that can be further metabolized via ring-fission (Hammel et al. 1991); (b) laccase catalyzes one-electron oxidation of anthracene and benzo[*a*]pyrene (both having IP $\leq$7.45 eV), whose efficiency is enhanced in the presence of mediators such as 1-hydroxybenzotriazole or 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (Johannes et al. 1996; Collins et al. 1996); (c) some PAH compounds up to six rings were shown to be degradable via MnP-dependent lipid peroxidation reactions both *in vitro* and *in vivo* (Moen & Hammel 1994; Bogan & Lamar 1995); (d) intracellular cytochrome P450 monooxygenase activity followed by epoxide hydrolase-catalyzed hydration resulting in hydroxylation of 3-, 4-, and 5-ringed PAHs are believed to initially metabolize PAH molecules including phenanthrene having an IP of 8.03 eV (Sutherland et al. 1991; Bezalel et al. 1996a,b,c; Masaphy et al. 1996; Bezalel et al. 1997).

In spite of that the rates of PAH oxidation are rarely correlated with the above enzyme activities (Bogan et al. 1996a,b). In contrast to nonligninolytic fungi, white rot fungi are also able to mineralize PAHs, where the ligninolytic mechanism is supposed to be involved in later steps of metabolism leading to CO_2 evolution (Bezalel et al. 1996c).

In soil bioremediation, white rot fungi are normally applied in the form of mycelium grown on wood chips, sawdust, chopped straw or a similar biological material that is mixed with the contaminated soil (e.g. Loske et al. 1989; Šašek et al. 1993). Prerequisites for successful biodegradation of organopollutants in soil are: (i) survival of the introduced fungi in the soil, (ii) active fungal growth through the soil or massive introduction of a pre-grown fungus into the whole volume of contaminated soil, and (iii) production and persistence of fungal enzymes (extracellular and intracellular) attacking the pollutant molecule(s) during growth in the soil. Another important factor known to influence the biodegradability of PAH molecules is their sorption to soil particles that depends on the content of soil organic carbon and can negatively affect bioavailability of PAHs and consequently reduce the efficiency of degradation (Weissenfels et al. 1992; Shuttleworth & Cerniglia 1995). Compared to liquid-media cultures, the information on behaviour of white rot fungi in soil is rather scarce (e.g. Lamar et al. 1987; Morgan et al. 1993; Andersson & Henrysson 1996; in der Wiesche et al. 1996; Okeke el al. 1997) and the capability of enzyme production mostly unknown (Boyle 1995; Lang et al. 1997).

The purpose of our work was to study the capacity of selected white rot fungi to grow and produce extracellular ligninolytic enzymes in soil, compared to *P. chrysosporium*, and thus draw conclusions about their applicability to soil bioremediation. The individual enzyme productions on wheat straw and in soil were compared in order to document the effect of soil environment on the development of biochemical properties of the fungi. The ability of fungal soil cultures to remove PAHs from an artificially contaminated soil was evaluated in order to demonstrate their biodegradation efficiency.

Materials and methods

Microorganisms

Pleurotus ostreatus (Jacq.: Fr.) Kumm. strain F6 and related strains 14, 15 and 19 with varied levels of laccase and MnP, that were prepared by protoplasting from the wild strain, *Phanerochaete chrysosporium*, Burds. strain ATCC 34541, and *Trametes versicolor* (L.: Fr.) Pilát strain CCBAS 614 were maintained on malt extract (0.5%, W/V)/glucose (1%, W/V) medium (MEG) containing 2% (W/V) agar.

Cultivation and growth

Coarse milled wheat straw (ca. 30 g, particle size $<$1 mm, 77% moisture, W/W) was put into tube reactors (tube diam. 3.5 cm, length 24 cm) (Figure 1), sterilized

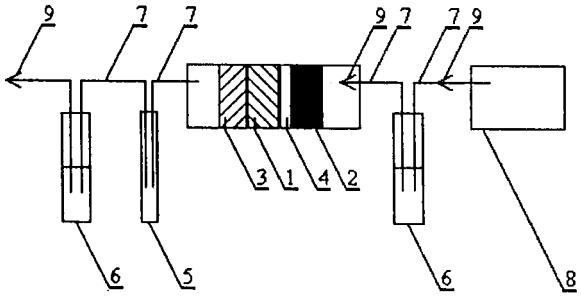

Figure 1. A scheme of tube reactor used for cultivation of white rot fungi in soil. 1 – fungus-inoculated chopped straw, 2 – sterile soil contaminated with PAHs, 3.– glass wool, 4 – nylon web separating the compartments, 5 – test tube to prevent return flow, 6 – washing bottle with distilled water, 7 – capillary tubing, 8 – pump, 9 – airflow.

in an autoclave three times (120 °C, 30 min) and inoculated with 2 agar discs (0.5-cm diam.) covered with 1 week-old mycelium of a MEG-grown tested fungus (Martens & Zadražil 1998). The fungus was growing through the straw for 1 week at 24 °C under aeration with sterile air (2–6 ml/min). Then an amount of soil (ca. 30 g dry weight), pre-sterilized three times (80 °C, 6 h) and moistened with sterile distilled water (soil Rossendorf – 25%, soil Braunschweig – 13%, W/W), was added to the colonized straw substrate in the tube reactor so that the fungus could colonize the soil. The compartments of straw and soil in the reactor were separated by a fine nylon web. The reactor was then connected to the aeration system again and the fungus was allowed to grow through the soil. The airflow was passing through a washing bottle filled with distilled water to maintain soil moisture during the experiment (soil moisture at the end of the experiment was 20–25%, W/W). Cultures for the estimation of ligninolytic enzymes in the straw and soil were harvested after 1, 2 and 3 weeks of growth, those for the measurement of depletion of PAHs after 8 weeks.

All cultures and controls in the study were run in triplicate including the biodegradation experiments.

Fungal growth was estimated by measuring the length (in cm) of straw and soil columns colonized in time and by the ergosterol method. Using the latter method, the amount of ergosterol in the soil was determined after extraction (with *n*-hexane/10% KOH in methanol (1:4) according to Davis & Lamar (1992)) of the soil from tube reactors and was different from those in which PAH biodegradation was measured. These authors critically evaluated quantitation of soil fungal biomass using fungal ergosterol and demonstrated that the ergosterol method could be used for routine analyses of fungal growth in soils, except for high-clay-content soils where ergosterol recovery by hexane/KOH-methanol extraction was incomplete.

Ergosterol was detected by HPLC under the following conditions: a WATREX 250 × 4 mm (Nucleosil 120-5 C18) column, temperature 50 °C, elution: acetonitrile/water gradient from 35 to 100% in 25 min, flow rate 1 ml/min, detection at 282 nm.

Soil

Soil Rossendorf (R). The chernozem soil was air dried and sieved to obtain four fractions of particles having size from 2 to 0.063 mm. The soil contained 11.7% (W/W) of total organic carbon and 0.45% of Kjehldal-determined nitrogen, the pH was 6.4. Before the experiments were started, the soil was reconstituted according to the percentages of the individual fractions, sterilized as mentioned above, and the moisture was adjusted using sterile distilled water.

Soil Braunschweig (B). The brown soil contained 0.8% of total organic carbon and 0.08% (W/W) of Kjehldal-determined nitrogen. The pH was 5.3.

Enzyme assays

An amount of ca. 8 g of lyophilized straw or soil from the tube reactor cultures, that were also used for quantification of growth by the ergosterol method, was extracted in a volume of 50 ml of 50 mM succinate/lactate buffer, pH 4.5 and desalted on a P10 (0.5 × 7 cm, Pharmacia, Sweden) Sephadex G25 column, using 1 ml of an enzyme sample and 3 ml of the above extraction buffer for elution. Laccase and manganese-dependent peroxidase (MnP) activities were determined with 3-dimethylaminobenzoic acid (DMAB)/3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) as the chromogen according to Vyas et al. (1994). MnP [Mn(II):hydrogen-peroxide oxidoreductase EC 1.11.1.13] activity was determined employing a reaction mixture containing in a final volume of 2 ml: 50 mM succinate/lactate buffer (pH 4.5), 50 μM MBTH (Fluka, Switzerland), 2.5 mM DMAB (Fluka, Switzerland), 100 μM $MnSO_4.4H_2O$, 50 μM H_2O_2, and the enzyme sample (50–500 μl). Reaction was initiated by adding H_2O_2. MnP activity was calculated from the increase of A_{590} after 1 min. Laccase (benzenediol:oxygen oxidoreductase EC 1.10.3.2.) activity was estimated using a similar mixture where Mn^{2+} + H_2O_2 were omitted.

Lignin peroxidase (LiP) (diarylpropane:oxygen, hydrogen-peroxide oxidoreductase EC 1.11.1.14)

activity was estimated with veratryl alcohol as the substrate according to Tien & Kirk (1988), but none was detected in any of the straw and soil cultures tested.

One unit of enzyme activity is defined as an activity producing 1 μmol of the product per min under the assay conditions.

Biodegradation of PAHs

Soil B was contaminated with a solution containing anthracene, phenanthrene and pyrene (33.37 mg each) in 5.5 ml of toluene, whose 250 μl aliquots were applied to 0.75 g of pasteurized dry soil. The solvent was then evaporated and the soil thoroughly mixed with a portion of 29.25 g of the same pasteurized dry soil. The final concentration of each PAH in the soil was 50 ppm. The soil moisture was adjusted as mentioned above and the amount of soil used as the filling of a tube reactor for the biodegradation experiment. Each tube reactor was thus contaminated separately to avoid inhomogeneities in PAH content in the individual reactors. The inoculation and growth were as described above. Two types of sterile controls were used: an uninoculated control that was aerated and kept at 24 °C as the inoculated cultures (aerated control) and an uninoculated control that was not aerated and was kept at 4 °C throughout the biodegradation experiment (cold room control). The latter control was used to follow the recovery of PAHs from soil matrix. The aerated control documented abiotic effects on PAHs taking place at the cultivation temperature in soil environment.

A two step extraction method was used. First, the soil samples (5 g) were extracted in a Soxhlet apparatus with hexane/acetone (3:1, V/V) mixture (25 ml) for 6 h as mentioned above, the extracts were vacuum evaporated at 40 °C and dissolved in 1 ml of methanol. They were filtered using cotton wool and analyzed by HPLC. Second, half of the hexane/acetone-extracted soil sample was refluxed for 4.5 h in a volume of 80 ml of 2 M KOH/methanol (1:4, V/V) mixture at a temperature slightly above the boiling point. The hydrolyzate was shaken 3 times with 10 ml of cyclohexanol, evaporated at 40 °C, dissolved in 1 ml of methanol and analyzed by HPLC. Extraction recoveries measured in nonaerated cold-room controls were the following: anthracene 69%, phenanthrene 75%, pyrene 86% of the original amounts added, reflecting sorption of aromatics to the soil (Means et al., 1980; Eschenbach et al., 1995) and glassware (Zachař et al., 1996; Beaudette et al., 1998).

PAH determination by HPLC

An apparatus consisting of a Perkin-Elmer 200 LC pump, Waters 990 UV photodiode array detector, and a 250 × 4 mm Merck LiChroCART^{R} column was used under isocratic conditions (methanol/water, 9:1, V/V) at a flow rate of 1 ml/min, pressure of 17.7 MPa and a column temperature of 40 °C.

Concentrations of the individual PAHs detected in the two extraction steps were combined and considered to represent the total amounts of the individual compounds present in the soil samples.

Chemicals

The standards of anthracene (98%; W/W), anthraquinone (97%), phenanthrene (98%), and pyrene (99%) were purchased from Aldrich (USA).

Results

Soil colonization by fungi

All three fungal species were capable of colonization of the two soils but the rate and intensity of colonization were different (Tables 1 & 2). The growth rates and mycelium densities in the soil B decreased in the order *P. ostreatus* > *P. chrysosporium* > *T. versicolor* (Table 1). The differences in growth between the fungi observed in a low-nutrient containing soil B were confirmed in the rich-nutrient containing soil R (Table 2) when growth was measured employing the ergosterol method modified for use in soils by including the ergosterol extraction under alkaline conditions (Davis & Lamar 1992).

Enzyme production in soil

As the ligninolytic enzyme system was shown to be involved in biodegradation of a broad range of PAHs via one-electron oxidation (review Cerniglia 1992), the production of individual enzyme activities secreted in soil was estimated and compared to those similarly extracted from the straw cultures, that demonstrated behaviour of the fungi growing on a natural lignocellulosic substrate (Table 3). The activities of individual enzymes in the straw (related to 1 g dry straw) were much higher than those in the soils (related to 1 g dry soil). Some enzymes were practically undetectable in the soils, even though produced in the straw at significant levels, e.g. MnP of *P. chrysosporium*

Table 1. Fungal growth at 24 °C under aeration in straw and in soil Braunschweig artificially contaminated with a mixture of anthracene, phenanthrene and pyrene.[1]

		Colonization of straw and soil in time (cm) Days						
		2	3	4	5	6	7	9
Phanerochaete	Straw			Weak growth				5.0
chrysosporium	B[2]	0	0.9	1.8	4.0	5.0	ND[4]	ND[4]
	B + A[3]	0	0.5	0.8	1.0	1.5	ND[4]	ND[4]
Trametes	Straw	0.7	ND[4]	ND[4]	1.8	ND[4]	3.1	5.0
versicolor	B[2]	0.4	0 5	0.8	Weak, irregular growth			
	B + A[3]			No growth into soil				
Pleurotus	Straw	0.8	ND[4]	ND[4]	1.8	ND[4]	3.1	5.0
ostreatus F6	B[2]	0.9	1.6	2.2	5.0	ND[4]	ND[4]	ND[4]
	B + A[3]	0.9	1.4	1.7	1.9	2.2	2.7	5.0

[1]Total length of straw- and soil columns to measure the rate of colonization was 5.0 cm.
[2](B), noncontaminated soil B; [3](B + A), soil B contaminated with anthracene, phenanthrene and pyrene (50 ppm each); [4]ND, not determined.

Table 2. Fungal growth under aeration at 24 °C in non-contaminated soil Rossendorf measured by the ergosterol method

Growth (μg ergosterol g^{-1} dry soil)[1,2]			
Days	7	14	21
Phanerochaete chrysosporium	0.66	2.21	6.20
Trametes versicolor	ND	0.61	2.22
Pleurotus ostreatus F6	2.6	13.67	10.96

[1]Each value represents a mean of three parallel samples that were combined after lyophilization prior to extraction; ND, not determined.
[2]The level of ergosterol present in the soil not inoculated with the fungi was undetectable by the method used.

and *T. versicolor* (Table 3). If the enzyme activities extracted from the soil R were related to the amount of ergosterol detected, that represented the fungal biomass produced in the soil, the maximal values of laccase and MnP found in *T. versicolor* were 0.33 and 0.12 μmol/min per μg ergosterol, respectively, whereas the respective enzyme amounts found in soil-growing cultures of *P. ostreatus* were only 0.02 and 0.06 μmol/min per μg ergosterol. The specific activity of MnP in *P. chrysosporium* growing in the soil R was even lower (0.04 μmol/min per μg ergosterol). These results demonstrate different productivity of the synthesis of ligninolytic enzymes with respect to growth yields of various white rot fungi growing in soil.

Degradation of PAHs in soil

Having documented the ability of the individual fungi to adapt to the conditions in soil, we also compared their biodegradation potential in the soil environment. Anthracene [3-ringed, IP = 7.43 eV (Pysh & Yang, 1963)], phenanthrene [3-ringed, IP = 8.03 eV (Pysh & Yang, 1963)], and pyrene [4-ringed, IP = 7.53 eV (Pysh & Yang, 1963)] were employed as model recalcitrant compounds in the biodegradation study. When the aerated controls were compared to the nonaerated, cold-room controls, it was found that the respective amounts of anthracene, pyrene and phenanthrene were reduced under the forced aeration of the tube reactors at 24 °C 1.7-fold, 1.7-fold and 8.5-fold, as related to the actual amounts measured in cold room controls (Tables 4 & 5). The airflow causing volatilization, important especially with phenanthrene, was directed from the soil compartment to the straw one, where the volatilized PAHs were at least partially adsorbed to the surface of straw. In addition to the volatilized portion of spiked PAHs, abiotic oxidation and irreversible sorption to the soil particles and/or glass surface of the reactors could be responsible for the PAH losses observed (Tables 4 & 5). Alkaline extraction of the soil matrix following the extraction with hexane/acetone provided only the respective additional recoveries of 3, 9 and 6% of the supplied concentrations of anthracene, pyrene and phenanthrene, which demonstrated that binding into the soil organic fraction was low (not shown).

Table 3. Ligninolytic enzyme activities of fungi growing in straw and soils under aeration at 24 °C

Enzyme	Laccase			MnP[1]		
	Units g^{-1} dry weight (straw or soil)					
Day	7	14	21	7	14	21
Phanerochaete chrysosporium						
Straw	0	0	0	0.43 ± 0.19	0.60 ± 0.40	0.40 ± 0.21
Soil R	0	0	0	0.03 ± 0.00	0	0
Straw	0	0	0	0	0.45 ± 0.11	0.34 ± 0.19
Soil B	0	0	0	0	0	0
Trametes versicolor						
Straw	1.22 ± 0.14	3.25 ± 1.85	4.14 ± 0.78	2.24 ± 1.14	3.20 ± 1.12	4.10 ± 0.10
Soil R	0.09 ± 0.02	0.20 ± 0.13	0.15 ± 0.10	0.04 ± 0.01	0.07 ± 0.05	0.18 ± 0.13
Straw	0.26 ± 0.03	0.20 ± 0.04	0.29 ± 0.12	3.32 ± 0.60	0	0
Soil B	0 02 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0	0
Pleurotus ostreatus F6						
Straw	0.40 ± 0.14	1.11 ± 0.20	0.52 ± 0.06	1.29 ± 0.68	1.06 ± 0.81	2.91 ± 1.18
Soil R	0.04 ± 0.03	0.25 ± 0.06	0.25 ± 0.08	0.10 ± 0.12	0.14 ± 0.09	0.65 ± 0.17
Straw	ND[2]	0.16 ± 0.03	0.24 ± 0.08	0.81 ± 0.09	2.27 ± 0.25	3.62 ± 0.07
Soil B	ND[2]	0	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.00

[1]MnP, manganese-dependent peroxidase.
[2]ND, not determined.

In the case of *P. ostreatus*, the wild strain F6 and three derived strains (14, 15 and 19) exhibiting different, stable levels of ligninolytic enzymes were employed, the rationale being to document whether such changes in the enzyme production pattern would result in modified rates of PAH biodegradation. The enzyme activities detected in the soil and related to those of the wild strain were as follows (not shown); strain 14: twofold to 1.5-fold reduction in laccase and MnP; strain 15: 1.5-fold reduction in MnP; strain 19: 1.5-fold reduction in laccase and twofold increase in MnP. Compared to the aerated controls, biodegradation by *P. ostreatus* strains reduced the concentrations of anthracene, pyrene and phenanthrene by 81–87%, 84–93% and 41–46%, respectively, whereas the removals by *P. chrysosporium* and *T. versicolor* were significantly lower (Tables 4 & 5). In case of the attack of anthracene molecule by *P. ostreatus*, PAH was transformed into anthraquinone, whose amount exceeded 5–7 times the concentration produced abiotically in the aerated control. No net production of anthraquinone in the soil cultures of *P. chrysosporium* and *T. versicolor* was detected (Table 4).

Discussion

In nature, white rot fungi are known to degrade predominantly wood (Tuor et al. 1995), however, much less is known about their behaviour in soil even though they are thought to be responsible for decomposition of lignin in forest soils (Killham 1994).

In our experiments, *P. ostreatus* was superior to other fungi in the ability to colonize the two presterilized soils (Tables 1 & 2). This superiority is in accordance with the observations of Martens & Zadražil (1998) who reported successful colonization of a low nitrogen-containing soil by 23 different strains of the genus *Pleurotus*, whereas other white rot and brown rot fungi were not able to colonize the soil amount throughout. Our results confirmed good growth of *P. chrysosporium* in nitrogen-rich soils also reported by Lamar et al. (1987). The poor ability of *T. versicolor* to colonize soil environment was emphasized in soils having low amounts of carbon and nitrogen (Table 1). Modest growth of *T. versicolor* in a sterilized unamended soil was reported by Boyle (1995), whereas Martens & Zadražil (1998) classified this fungus as unable to colonize nonsterile soil.

A mixture of anthracene, phenanthrene and pyrene (50 ppm each) was inhibitory to growth of the fungi in soil (Table 1). *P. ostreatus* was the most resistant to

Table 4. Residual concentrations of anthracene and formation of anthraquinone in soil Braunschweig after 8-week fungal degradation at 24 °C under aeration. Initial concentration of anthracene in the soil was 50 ppm

Fungus	Total PAHs (ppm)			
	Soil		Straw	
	ANT	ANQ	ANT	ANQ
Cold room control	34.4 ± 1.2	5.6 ± 0.4	ND	ND
Aerated control	20.1 ± 2.8	3.5 ± 0.6	1.7 ± 0.0	0.5 ± 0.4
P. chrysosporium	19.7 ± 3.1	2.3 ± 0.4	BDL[1]	0.2 ± 0.1
T. versicolor	19.7 ± 1.3	3.2 ± 0.4	BDL[1]	0.2 ± 0.1
P. ostreatus F6	2.6 ± 1.0	24.8 ± 7.4	BDL[1]	0.7 ± 0.1
P. ostreatus 14	3.7 ± 0.5	26.1 ± 0.7	BDL[1]	0.2 ± 0.1
P. ostreatus 15	3.5 ± 1.0	18.3 ± 0.9	BDL[1]	0.1 ± 0.0
P. ostreatus 19	3.8 ± 1.4	16.0 ± 6.1	BDL[1]	0.3 ± 0.0

ANT, anthracene; ANQ, anthraquinone.
[1]The value was below a detection limit of 0.0004 ppm.

Table 5. Residual concentrations of phenanthrene and pyrene in soil Braunschweig after 8-week fungal degradation at 24 °C under aeration. Initial concentrations of phenanthrene and pyrene in the soil were 50 ppm

Fungus	Total PAHs (ppm)			
	Soil		Straw	
	PHE	PYR	PHE	PYR
Cold room control	37.4 ± 2.2	43.1 ± 1.6	ND	ND
Aerated control	4.4 ± 1.4	25.0 ± 0.8	5.6 ± 0.4	2.6 ± 0.1
P. chrysosporium	3.4 ± 0.9	8.8 ± 4.8	0.3 ± 0.1	BDL[1]
T. versicolor	2.8 ± 0.9	23.5 ± 1.6	0.3 ± 0.1	0.3 ± 0.1
P. ostreatus F6	1.6 ± 0.0	1.7 ± 0.4	0.1 ± 0.0	0.1 ± 0.0
P. ostreatus 14	2.6 ± 0.1	4.0 ± 0.3	0.2 ± 0.1	BDL[1]
P. ostreatus 15	2.1 ± 0.1	1.9 ± 0.1	0.1 ± 0.0	BDL[1]
P. ostreatus 19	1.8 ± 0.1	2.5 ± 0.0	0.1 ± 0.0	BDL[1]

PHE, phenanthrene; PYR, pyrene.
[1]The value was below a detection limit of 0.0038 ppm.

the inhibition since the presence of PAHs negatively affected only the rate of soil colonization (Table 1) but not the final mycellum density (not shown). In *P. chrysosporium* cultures, both mycelium density (not shown) and growth rate were negatively affected. *T. versicolor* was not able to grow in the soil contaminated with PAHs. No inhibition with any fungus tested was observed when the soil was spiked with toluene free of the test PAHs.

Typical ligninolytic enzyme patterns of the white rot fungi in liquid cultures are the following (Hatakka 1994): *P. chrysosporium* – LiP, MnP, *P. ostreatus* – MnP, laccase, *T. versicolor* – LiP, MnP, laccase. Well growing in the soil, *P. ostreatus* produced both MnP and laccase at levels inferior to those on the straw (Table 3). *T. versicolor* that produced high levels of MnP and laccase on the straw synthesized the two enzymes also in soil, in spite of its limited growth in this environment (Tables 1 & 3). Production of the two enzymes by *T. versicolor* in both steamed and nonsteamed soil was also reported by Boyle (1995). No detection of LiP in the straw- or soil cultures of this fungus is in accordance with the observations of other authors but the reason for such behaviour remains unknown (Rogalski et al. 1991; Boyle 1995). Negligible production of ligninolytic enzymes by *P. chrysosporium* in soil is difficult to discuss but also the production in straw was rather low compared to the other fungi (Table 3).

P. ostreatus and *T. versicolor* have been shown to efficiently remove phenanthrene, anthracene and pyrene from liquid media up to a concentration of 100 ppm within several weeks (Field et al. 1992; Sack & Guenther 1993; Bezalel et al. 1996a,b; etc.). These fungi have been rarely tested in contaminated soil studies carried out to document that white rot fungi can also significantly reduce the amount of 3- and 4-ringed PAHs when present in soil (Baud-Grasset et al. 1993; Bogan et al. 1996a,b; Anderson & Henrysson 1996). The efficient removal of anthracene by *P. ostreatus* strains from soil observed in this work (Tables 4 & 5) confirmed the results of Andersson & Henrysson (1996) where *P. ostreatus* MUCL 29527 removed 99% of anthracene and 92% benz[*a*]anthracene within 65 days from a soil where PAHs were present at the respective concentrations of 100 and 20 ppm. The removal of anthracene was always accompanied by major accumulation of 9, 10-anthraquinone (Table 4; Andersson & Henrysson 1996), which is thought to be a dead-end metabolite in some fungi (Field et al. 1992; Bezalel et al. 1996a). Tenfold reduction in pyrene concentration (Table 5) documented the efficiency of *P. ostreatus* to remove this PAH molecule from soil and supported the data of in der Wiesche et al. (1996) on the ability of *Pleurotus* sp. Bhutan to mineralize pyrene in soil cultures. In contrast to the results of Andersson & Henrysson (1996) however, the removal rates of anthracene and pyrene in the *P. ostreatus* cultures measured under our conditions were much higher than those observed with *P. chrysosporium* and *T. versicolor* (Tables 4 & 5). Also, Bogan et al. (1996b) observed a significant removal of anthracene in soil cultures of *P. chrysosporium* accompanied by a massive accumulation of 9,10-anthraquinone. The reason for different behaviour of our soil cultures of *P. chrysosporium* and *T. versicolor* is unknown but it should be pointed out that, in our study, the biodegradation was performed by fungal exploratory mycelium whereas in most other studies, including the two above-mentioned ones, the solid lignocellulosic substrate-associated mycelium was directly mixed with contaminated soil. The difference between biodegradation capabilities of the exploratory and solid substrate-associated mycelium of the two latter fungi was also demonstrated by the finding that, in contrast to the situation in the spiked soil, the mycelia of the two fungi closely associated with the straw were able to remove efficiently anthracene sorbed to the straw surface (Table 4). A similar difference was also observed for the *T. versicolor* ability to remove pyrene (Table 5).

A significant removal of phenanthrene from the soil cultures was observed only with *P. ostreatus* strains but in all cases the results were obscured by massive volatilization of the compound caused by the aeration of reactors (Table 5). In partial contrast to the observed removal rates in the soil, all fungi tested efficiently removed the three PAH compounds when re-adsorbed to the straw in the reactor (Tables 4 & 5). It could point to either a different availability of PAH molecules for individual fungi when adsorbed to soil, or to some other limitation of the biodegradation process in soil resulting, for example, from a lack of nutrients and a different type of mycelium involved, etc.

MnP and laccase have been repeatedly implicated in biodegradation of PAHs including phenanthrene (Moen & Hammel 1994; Bogan et al. 1996a; Collins et al. 1996; Johannes et al. 1996). In the *P. ostreatus* cultures these enzymes were present at least during first 3 weeks of the experiment (Table 3) and thus their involvement in the removal of PAHs was possible, including the production of anthraquinone from anthracene (cf. Collins et al. 1996; Johannes et al. 1996). A similar correlation was also reported for the expression of MnP and the removal of fluorene and chrysene in soil cultures of *P. chrysosporium* (Bogan et al. 1996a). However, it should be kept in mind that another PAH biodegradation system probably operates in the organism simultaneously, i.e. intracellular cytochrome P-450 monooxygenase, that also may contribute to PAH degradation (Bezalel et al. 1996a,b), but the relative participation of the individual enzyme systems in PAH biodegradation is not known.

Use of various strains of *P. ostreatus* whose levels of MnP and laccase in soil differed maximally twofold (cf. Results) showed that such fluctuations in the enzyme activities did not affect the capacity of the fungus to degrade PAH molecules. It suggested that other enzyme(s) may play a major role in PAH biodegradation in white rot fungi or some other factor (e.g. bioavailability of PAH molecules, desorption from soil surface, H_2O_2 production, etc.) may represent the rate-limiting step in the biodegradation process. The efficient removal by all three fungi of PAHs resorbed on straw after volatilization during the experiments (Tables 4 & 5) indicated that various white rot fungi could be also employed in the form of straw-

based biofilters for capture and degradation of PAHs volatilized from biopiles used for soil remediation

Conclusions

The results show that *P. ostreatus* is a suitable candidate to apply to the clean-up of soils contaminated with recalcitrant pollutants because it is capable of robust growth and efficient extracellular enzyme production in soil even in the presence of relatively high concentrations of PAHs. Such a dense mycelial growth through contaminated soil and efficient enzyme expression are prerequisites for a good fungal capacity to remove the pollutant molecules from the whole bulk of soil. Rates of PAH degradation observed with *P. ostreatus* in artificially contaminated soil significantly exceeded those found with *P. chrysosporium* and *T. versicolor*, especially in the case of anthracene and pyrene.

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References

Andersson BE & Henrysson T (1996) Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. Appl. Microbiol. Biotechnol. 46: 647–652

Baud-Grasset F, Baud-Grasset S & Saffermann SI (1993) Evaluation of the bioremediation of a contaminated soil with phytotoxicity tests. Chemosphere 26: 1365–1374

Beaudette LA, Davies S, Fedorak PM, Ward OP & Pickard MA (1998) Comparison of gas chromatography and mineralization experiments for measuring loss of selected polychlorinated biphenyl congeners in cultures of white rot fungi. Appl. Environ. Microbiol. 64: 2020–2025

Bezalel L, Hadar Y, Fu PP, Freeman JP & Cerniglia CE (1996a) Initial oxidation products in the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 62: 2554–2559

Bezalel L, Hadar Y, Fu PP, Freeman JP & Cerniglia CE (1996b) Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 62: 2547–2553

Bezalel L, Hadar Y & Cerniglia CE (1996c) Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 62: 292–295

Bezalel L, Hadar Y & Cerniglia CE (1997) Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 63: 2495–2501

Bogan BW, Schoenike B, Lamar RT & Cullen D (1996a) Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 62: 2381–2386

Bogan BW, Schoenike B, Lamar RT & Cullen D (1996b) Expression of *lip* genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 62: 3697–3703

Bogan BW & Lamar RT (1995) One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 61: 2631–2635

Boyle CD (1995) Development of a practical method for inducing white rot fungi to grow into and degrade organopollutants in soil. Can. J. Microbiol. 41: 345–353

Bumpus JA (1989) Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysoporium*. Appl. Environ. Microbiol. 55: 154–158

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3: 351–368

Collins PJ, Kotterman MJJ, Field JA & Dobson ADW (1996) Oxidation of anthracene and benzo[*a*]pyrene by laccases from *Trametes versicolor*. Appl. Environ. Microbiol. 62: 4563–4567

Davis MW & Lamar RT (1992) Evaluation of methods to extract ergosterol for quantitation of soil fungal biomass. Soil Biol. Biochem. 24: 189–198

Davis MW, Glaser JA, Evans JW & Lamar RT (1993) Field evaluation of the lignin degrading fungus *Phanerochaete sordida* to treat creosote contaminated soil. Environ. Sci. Technol. 27: 2572–2576.

Eschenbach A, Kästner M, Wienberg R & Mahro B (1995) Microbial PAH degradation in soil material from a contaminated site – mass balance experiments with *Pleurotus ostreatus* and different ^{14}C-PAH, pp. 377–378. In: Van den Brink WJ, Bosman R & Arendt F (eds) Contaminated Soil '95. Kluwer Academic Publishers

Field JA, deJong E, Feijoo-Costa G & deBont JAM (1992) Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. 58: 2219–2226

Field JA, Vledder RH, van Zeist JG & Rulkens WH (1996) The tolerance of lignin peroxidase and manganese-dependent peroxidase to miscible solvents and the *in vitro* oxidation of anthracene in solvent:water mixtures. Enzyme Microb. Technol. 18: 300–308

George EJ & Neufeld RD (1989) Degradation of fluorene in soil by fungus *Phanerochaete chrysosporium*. Biotechnol. Bioeng. 33: 1306–1310

Hammel KE, Green B & Gai WZ (1991) Ring fission of anthracene by a eukaryote. Proc. Natl. Acad, Sci. USA 88: 10605–10608

Hammel KE, Kalyanaraman B & Kirk TK (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenzo[*p*]-dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. 261: 1648–1652

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. 13: 125–135

Johannes C, Majcherczyk A & Hüttermann A (1996) Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. Appl. Microbiol. Biotechnol. 46: 313–317

Killham K (1994) Soil Ecology, pp. 47–52. Cambridge University Press, UK

Kirk TK & Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. 41: 465–505

Lamar RT, Larsen MJ, Kirk TK & Glaser JA (1987) Growth of the white rot fungus *Phanerochaete chrysosporium* in soil, pp. 419–424. In: Land Disposal, Remedial Action, Incineration and Treatment of Hazardous Waste: Proceedings of the 13th Annual Research Symposium. U.S. Environmental Protection Agency

Lang E, Eller G & Zadražil F (1997) Lignocellulose decomposition and production of ligninolytic enzymes during interaction of white rot fungi with soil microorganisms. Microbial Ecology 34: 1–10

Loske D, Hüttermann A, Majcherczyk A, Zadražil F, Lorsen H & Waldinger P (1989) Use of white rot fungi for the clean-up of contaminated sites, pp. 311–322. In: Coughlan MP & Amaral Collaço MT (eds) Advances in Biological Treatment of Lignocellulosic Materials. Elsevier, London and New York

Martens R & Zadražil F (1998) Screening of white rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. Folia Microbiol. 43: 97–103

Masaphy S, Levanon D, Henis Y, Venkateswarlu K & Kelly SL (1996) Evidence for cytochrome P450 and P450-mediated benzo[*a*]pyrene hydroxylation in the white rot fungus *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. 135: 51–55

Means JG, Wood SG, Hassett JJ & Banwart WL (1980) Sorption of polynuclear aromatic hydrocarbons by sediments and soils. Environ. Sci. Technol. 14: 1524–1528

Moen MA & Hammel KE (1994) Lipid peroxidation by the manganese peroxidase of *P. chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol. 60: 1956–1961

Morgan P, Lee SA, Lewis ST, Sheppard AN & Watkinson RJ (1993) Growth and biodegradation by white rot fungi inoculated into soil. Soil Biol. Biochem. 25: 279–287

Newell SY, Miller JD & Fallon RD (1987) Ergosterol content of salt marsh fungi: effect of growth conditions and mycelial age. Mycologia 79: 688–695

Okeke BC, Paterson A, Smith JE & Watson-Craik IA (1997) Comparative biotransformation of pentachlorophenol in soils by solid substrate cultures of *Lentinula edodes*. Appl. Microbiol. Biotechnol. 48: 563–569

Paszczynski A & Crawford RL (1995) Potential for bioremediation of xenobiotic compounds by the white rot fungus *Phanerochaete chrysosporium*. Biotechnol. Progress 11: 368–379

Pysh ES & Yang NC (1963) Polarographic oxidation potentials of aromatic compounds. J. Am. Chem. Soc. 85: 2124–2130

Rogalski J, Lundell TK, Leonowicz A & Hatakka AI (1991) Influence of aromatic compounds on the production of ligninolytic enzymes by *Phlebia radiata*. Phytochemistry 30: 2869–2872

Sack U & Günther T (1993) Metabolism of PAH by fungi and correlation with extracellular enzymatic activities. J. Basic Microbiol. 33: 269–277

Shuttleworth KL & Cerniglia CE (1995) Environmental aspects of PAH biodegradation. Appl. Biochem. Biotechnol. 54: 291–302

Sutherland JB, Selby AL, Freeman JP, Evans FE & Cerniglia CE (1991) Metabolism of phenanthrene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 57: 3310–3316

Šašek V, Volfová O, Erbanová P, Vyas BRM & Matucha M (1993) Degradation of PCBs by white rot fungi, methylotrophic and hydrocarbon utilizing yeasts and bacteria. Biotechnol. Lett. 15: 521–526.

Tien M & Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. Methods in Enzymol. 161 238–249

Tuor U, Winterhalter K & Fiechter A (1995) Enzymes of white rot fungi involved in lignin degradation and ecological determinants for wood decay. Journal of Biotechnol. 41: 1–17

Vyas BRM, Bakowski S, Šašek V & Matucha M (1994) Degradation of anthracene by selected white rot fungi. FEMS Microbiol. Lett. 14: 65–70

Weissenfels WD, Klewer HI & Langhoff J (1992) Absorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. Appl. Microbiol. Biotechnol. 36: 689–696

in der Wiesche C, Martens R & Zadražil F (1996) Two-step degradation of pyrene by white-rot fungi and soil microorganisms. Appl. Microbiol. Biotechnol. 46: 653–659

Zachař P, Novotný Č, Vozňáková Z, Matucha M, Tesařová E, Sýkora D, Kubátová A, Popl M & Šašek V (1996) Physical factors negatively affecting evaluation of long-term biodegradation experiments of polychlorinated biphenyls. Chemosphere 33: 2411–2421.