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ORIGINAL PAPER

Enhancement of bioconversion of high-molecular mass polycyclic aromatic hydrocarbons in contaminated non-sterile soil by litter-decomposing fungi

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Abstract With the focus on alternative microbes for soil-bioremediation, 18 species of litter-decomposing basidiomycetous fungi were screened for their ability to grow on different lignocellulosic substrates including straw, flax and pine bark as well as to produce ligninolytic enzymes, namely laccase and manganese peroxidase. Following characteristics have been chosen as criteria for the strain selection: (i) the ability to grow at least on one of the mentioned materials, (ii) production of either of the ligninolytic enzymes and (iii) the ability to invade non-sterile soil. As the result, eight species were selected for a bioremediation experiment with an artificially contaminated soil (total polycyclic aromatic hydrocarbon (PAH) concentration 250 mg/kg soil). Up to 70%, 86% and 84% of benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene, respectively, were removed in presence of fungi while the indigenous microorganisms converted merely up to 29%, 26% and 43% of these compounds in 30 days. Low molecular-mass PAHs studied were easily degraded by soil microbes and only anthracene degradation was enhanced by the fungi as well. The agaric basidiomycetes *Stropharia rugosoannulata* and *Stropharia coronilla* were the most efficient PAH degraders among the litter-decomposing species used.

Keywords *Agrocybe* · Biodegradation · Litter-decomposing fungi · Polycyclic aromatic hydrocarbons · Soil bioremediation · *Stropharia*

Abbreviations

BaP	Benzo(a)pyrene
LiP	Lignin peroxidase
LPO	Lipid peroxidation
LDF	Litter-decomposing fungi
MnP	Manganese peroxidase
PAHs	Polycyclic aromatic hydrocarbons

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Introduction

The contamination of soils with various organic xenobiotics and organopollutants is of general

and public interest, especially for authorities who will have to decide whether an area is habitable or not. With new laws and stricter regulations more and more sites will have to be cleaned either with harsh methods like incinerating the soil or by the ''softer'' way of bioaugmentation or natural attenuation. A typical group of soil contaminants still frequently found in threatening concentrations are polycyclic aromatic hydrocarbons (PAHs), which are present especially at filling stations and other areas where fossil fuels have been used. The most harmful among the PAHs are those with more than four rings; they are often mutagenic and carcinogenic which emphasizes the importance of their removal from the environment (Cerniglia 1993).

Biodegradation of PAHs has been studied for more than 20 years, and already in the 1980s a basidiomycete, the white-rot fungus *Phanerochaete chrysosporium*, was shown to be capable of degrading PAHs (Bumpus et al. 1985; Bumpus 1989). Since then, several promising white-rot fungi—all of them belonging to the wood-decaying fungi—have been used for PAH degradation studies in liquid culture or using contaminated soils, among others *Pleurotus* spp. (Eggen and Majcherczyk 1998; Martens et al. 1999; Baldrian et al. 2000), *Irpex lacteus* (Bhatt et al. 2002; Cajthaml et al. 2002), and *Trametes* spp. (Novotny et al. 1999). The degradation potential of white-rot fungi has been attributed mainly to their extracellular ligninolytic enzymes: lignin peroxidase (LiP) (Hammel et al. 1986), manganese peroxidase (MnP) (Sack et al. 1997) and laccase (Collins et al. 1996). High levels of these enzymes have already been used as one selection criterion within several fungal screenings (Field et al. 1993; Gramss et al. 1999a).

There is, however, also a clear disadvantage of these fungi. Their preference to colonize compact wood (logs, trunks, etc) limits their capability to grow in a completely different environment such as soil (Sasek 2003). Litter-decomposing fungi (LDF) are another eco-physiological group of basidiomycetes, which can cause the so-called white-rot of humus (Hintikka 1970). Some genera of this group are promising organisms for soil bioremediation purposes for the following reasons: (i) They produce powerful ligninolytic enzymes similar to those produced by white-rot fungi (Steffen et al. 2002b) and degrade the recalcitrant biopolymer lignin (Steffen et al. 2000), which has been proposed to be a prerequisite for the unspecific biodegradation of organopollutants by basidiomycetous fungi (Bogan and Lamar 1996; Sasek 2003); (ii) They have been shown to degrade different PAHs in liquid culture (Wunch et al. 1997; Steffen et al. 2002a) and even to mineralize them to a certain extent (Wunch et al. 1999; Steffen et al. 2003). MnP, which is produced by many LDF, is involved in the oxidation of PAHs, which has already been demonstrated for selected litter-decomposers (Steffen et al. 2002a; Steffen et al. 2003); (iii) Since the soil-litter layers in forests and grasslands are the natural habitats of LDF, it is to expect that they actively grow in and into soil and survive there for a prolonged time. They can compete with other soil microorganisms but also co-exist with bacteria and molds, which can additionally stimulate PAH degradation as shown for *Mycobacterium* sp. (Schneider et al. 1996) and *Cladosporium sphaerospermum* (Potin et al. 2004). On the other hand, white-rot fungi such as *Pleurotus* and *Trametes* spp. have been reported to reduce the number of bacteria during growth in soil, a fact that could negatively influence the conversion of low-molecular mass PAHs (Gramss et al. 1999b; Andersson et al. 2003; Baldrian 2004). The survival of fungi supplemented to soil depends on the inoculation procedure, which requires additional carbon sources preferably lignocellulosic materials (Morgan et al. 1993).

In our present approach, we screened a number of LDF for their ability to produce MnP and laccase in liquid culture, to grow in different lignocellulosic substrates suitable for inoculation and to invade non-sterile soil. The most promising species were tested to degrade five PAHs (anthracene, pyrene, benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene) in an artificially contaminated sandy soil, which is frequently used as filling material in industrial areas.

Materials and methods

Chemicals

Cyclohexane, dichloromethane and acetonitrile were purchased form Merck KGaA (Darmstadt, Germany) and were of HPLC grade. A PAH mixture (Macherey-Nagel) according to EPA (U.S. Environmental Protection Agency) standards was used as a reference for HPLC analysis. All other chemicals were obtained from Merck or Sigma Aldrich (Steinheim, Germany) and were of the highest chemical purity available.

Fungi

Basidiomycetous wood- and litter-decomposing fungi used in this study (Table 1) are deposited in the culture collection of the Department of Applied Chemistry and Microbiology, University of Helsinki (designation K), in the culture collection of the International Graduate School of Zittau, Germany (designations MW and TM or a plain number), and in the German Collection of Microorganisms and Cell Cultures Braunschweig, Germany (designation DSM). All strains were maintained on 2% (w/v) malt extract agar (MEA) in culture slants at 4°C.

Culture conditions

Liquid cultures

For the detection of ligninolytic enzyme activities, a semi-synthetic medium was used containing 5.0 g glucose, 2.0 g KH_2PO_4, 0.5 g $MgSO_4 \times 7H_2O$, 0.1 g $CaCl_2$, 0.5 g ammonium tartrate, 1.8 g sodium succinate, 0.2 g yeast extract, and 0.04 g $MnCl_2 \times 4H_2O$ in 1 l of distilled water. The pH was adjusted to 5.0 prior to autoclaving. Four agar-plugs (10 mm diameter) covered with pre-grown mycelium from 20 days old MEA cultures

Table 1 Litter-decomposing fungi screened for enzymatic activities in liquid cultures and growth on lignocellulosic materials compared with two wood-rotting *Pleurotus ostreatus* strains

Fungus	Strain	Enzyme activity		Growth on lignocellulose		
		Laccase>50 U/l	MnP >100 U/l	Straw	Flax	Bark
Agrocybe dura	MW 71–2	+	–	+	+	++
Agrocybe praecox	TM 70.84	+	+	++	+++	+++
Clitocybe gibba	K 32	–	–	–	––	––
Clitocybe odora	K 168	–	–	––	+	–
Clitocybe odora	TM 3	–	–	––	+	––
Collybia dryophila	K 209	–	+	––	n.d.	++
Collybia peronata	K 220	–	–	–––	–––	–
Hypholoma capnoides	K 87	+	–	+	+	+++
Kuehneromyces mutabilis	K 22	–	+	+++	+++	+++
Lepista nebularis	K 103	–	–	–	–	––
Lepista nuda	TM 14	–	–	+	–	–––
Marasmius alliaceus	TM SW1	–	–	+	++	+
Marasmius scorodonius	TM SW2	–	–	–	+++	+++
Mycena epipterygia	K 72	–	–	––	++	++
Pleurotus ostreatus	DSM 11191	–	–	+++	+++	+++
Pleurotus ostreatus	K Ax3	+	–	+++	+++	+++
Stropharia aeruginosa	K 208	–	+	––	+	++
Stropharia aeruginosa	K 218	–	+	––	+	+
Stropharia coronilla	TM 47-1	+	+	––	+++	+++
Stropharia cubensis	TM SW3	+	+	+++	++	+
Stropharia hornemanii	K 122	+	+	n.d.	++	+
Stropharia hornemanii	K 138	+	+	––	–	+
Stropharia hornemanii	K 205	+	+	–––	++	+
Stropharia rugosoannulata B2	11372	+	+	+++	+++	+++
Stropharia rugosoannulata G	11373	+	+	+++	+++	+++

The scale is between no growth (–––) and excellent growth (+++) with (+) representing 50% coverage of the material. Enzyme activities are given as (+) over and (-) under the indicated limits

were used as the inoculum. Fungal cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of the medium. They were kept as surface cultures at 25°C in the dark for 6 weeks. Aliquots of 1 ml were taken once a week for enzyme activity measurement.

Fungal mycelium for the inoculation of solid materials (lignocelluloses, soil) was pre-cultured in 250-ml Erlenmeyer flasks, containing 75 ml of a 2% malt-extract medium, for 2 weeks at 25°C in the dark.

Solid state cultivation

This culture method was used to select suitable growth substrates and to test the ability of fungi to grow into non-sterile soil. Three different lignocellulosic waste materials from agriculture or forestry were used. Air dried chopped wheat straw or flax (both from Hildesheim, Germany; <1 cm) were swelled in distilled water (ratio straw or flax:water; 1:3, w/w). Chopped pine bark (Kitee, Finland; <2 cm), a waste product from the debarking of timber, was saturated with water and allowed to drain off. Thirty grams of the wetted material was added to 250-ml Erlenmeyer flask and autoclaved for 20 min at 121°C. Fungal inoculation material was obtained from liquid cultures (malt extract) after filtration and washed with sterile water to remove residual malt extract. The mycelium was divided into two equal portions (but not mixed) and used to inoculate solid substrates. During 30 days of incubation at 25°C in the dark, fungal growth was followed visually with respect to mycelial density as well as hyphae distribution on and in the solid substrate.

Soil experiments

Those fungal strains, which colonized at least one of the lignocellulosic substrates thoroughly, and had activities of MnP (>100 U/l) or laccase (>50 U/l) at least in one measurement, were used in a second experiment to evaluate their capability to grow into natural non-sterile soil. For this, the best growth substrate for each selected fungus was used as described above but in 150 ml glass bottles (diameter 5 cm). Bottles were filled with 6–7 g of the lignocellulosic substrate (filling level ~2.5 cm), inoculated and later covered with an equally thick layer of sandy soil.

The soil selected for the experiment had been used as a land filling material (Helsinki, Finland) and it was excavated from a depth of 20 cm. The humidity of this soil was 4.8%, the maximum water holding capacity (WHC_{max}) 24%, and the pH 5.1. The soil was sieved (<2 mm) and stored prior to the experiment at 4°C. A soil column of 2.5 cm (5 cm diameter) contained 40 g of soil with 50% of the WHC_{max}. The cultivation lasted 30 days and proceeded at 25°C in the dark; fungal growth was visually estimated and monitored during the whole time. Results are summarized in Table 2 distinguishing between no, poor, moderate, good, very good and excellent growth.

An identical experiment was performed with artificially contaminated soil. A mixture of five PAHs, comprising of anthracene, pyrene, benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene, was dissolved in acetone (115 ml) and added to the soil (1,600 g total weight). The soil was mixed thoroughly and excess acetone was allowed to evaporate. The WHC_{50} was adjusted according to non-contaminated water evaporation controls. The final concentration of each PAH in the soil was 50 mg/kg (total PAH contamination 250 mg/kg). Non-inoculated samples contained either non-sterile or autoclaved soil as well as autoclaved lignocellulosic material. They served as controls to measure the PAH biodegradation by indigenous soil microbes as well as to estimate their possible autooxidation and adsorption to soil particles. The bottles were incubated at 25°C in the dark. During this time, the bottles were constantly weighed in order to monitor the water content, which was readjusted with sterile water if necessary. After 69 days of incubation, 10 g of the soil of each bottle were separately extracted with an Accelerated Solvent Extractor ASE 200 (Dionex, USA). The extraction was performed using cyclohexane at 100°C with a pressure of 10.3 MPa (1500 psi).

Enzymatic activities

Manganese peroxidase (MnP) and laccase activities were measured with a Shimadzu UV-1700

Table 2 Litter-decomposing fungi screened for the growth into non-sterile soilTheir growth was compared with two wood-rotting Pleurotus ostreatus strains. The scale ranges from no growth (−−−) to poor (−−), moderate (−), good (+), very good (++) and excellent (+++) growth with (+) meaning a 50% coverage of the lignocellulosic material. Visual evaluation was done after 30 days (compare also Fig. 1)

Fungus	Strain	Growth substrate	Growth into soil
Agrocybe dura	MW 71–2	Flax	+++
Agrocybe praecox	TM 70.84	Flax	+++
Hypholoma capnoides	K 87	Bark	+
Kuehneromyces mutabilis	K 22	Bark	++
Lepista nuda	TM 14	Flax	−−
Marasmius alliaceus	TM SW1	Flax	−−−
Marasmius scorodonius	TM SW2	Flax	−−−
Mycena epipterygia	K 72	Flax	−−
Pleurotus ostreatus	DSM 11191	Straw	+++
Pleurotus ostreatus	K Ax3	Straw	+++
Stropharia aeruginosa	K 218	Flax	−−−
Stropharia coronilla	TM 47–1	Bark	+++
Stropharia cubensis	TM SW3	Straw	−−
Stropharia hornemanii	K 122	Bark	+++
Stropharia rugosoannulata B2	11372	Bark	+++
Stropharia rugosoannulata G	11373	Bark	+++

Pharma Spec photometer (Kyoto, Japan). MnP activity was determined at 270 nm by following the formation of Mn^{3+}-malonate complexes (Wariishi et al. 1992). Laccase activity was measured by the oxidation of ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulphonate)) at 420 nm (Eggert et al. 1996). All activities were expressed in units (U) defined as 1 μmol of substrate oxidized per minute at 25°C.

Physical and chemical analysis

The extract obtained after accelerated solvent extraction (ASE) was concentrated under a nitrogen atmosphere with a rotavapor (Turbo Vap, Zymark, USA) and transferred into a cyclohexane containing silica gel cartridge (Alltech, Germany). The immobilized PAHs were eluted with duplicate addition of a cycloheaxane/dichloromethane mixture (50/50, v/v). Afterwards, a second concentration step using the evaporator was performed but the PAHs were eluted with acetonitrile. The samples were analyzed by HPLC using a model W 600S (Waters, USA) chromatograph equipped with a 3.0 × 250 mm Ultrasep-ES-PAH column (Sepserv, Germany) at a flow rate of 0.9 ml/min with a linear gradient of acetonitrile in water (50%–100%; vol/vol) for 20 min followed by an isocratic run (100%) for 15 (total of 35 min). PAH detection occurred both with a photo-diode array detector (Waters 996) and a scanning fluorescence detector (Waters 474).

Results

The first screening of 25 basidiomycetous fungal strains belonging to 19 different species was performed to find an optimal lignocellulosic growth substrate as well as to select organisms with sufficient levels of ligninolytic enzymes (Table 1). Among these 25 strains, 21 belong to the group of LDF, two species—namely *Hypholoma capnoides* and *Kuehneromyces mutabilis*—colonize wood that has tight contact to soil (roots, stumps) and two strains represent a typical wood-decayer (*Pleurotus ostreatus* used as positive control for comparison). Only 15 strains including the control strains were able to produce sufficient activities of either laccase (>50 U/l) or MnP (>100 U/l; Table 1). *Stropharia coronilla* produced the highest level of laccase (660 U/l; reached on day 42 d; data not shown) and all other strains at least 5 U/l. MnP was secreted by the majority of strains at least at low levels around 10 U/l with *K. mutabilis* producing the highest activity of 327 U/l (on day 42; data not shown). Only *Lepista* spp. did not produce any MnP activity and both *Clitocybe odora* strains only traces (< 5 U/l). Surprisingly,

the control strains of *P. ostreatus* showed only moderate enzymatic activities (< 100 U/l).

Since the growth into soil requires a well-established fungal mycelium on an utilizable lignocellulosic substrate, straw, flax and bark were tested with all strains. Only three species, namely *K. mutabilis*, *P. ostreatus* and *Stropharia rugosoannulata*, were able to grow rapidly and thoroughly on all substrates (Table 1). Five other species grew at least on one of the substrates sufficiently and three species showed no or poor growth on the substrates (Table 1).

On the basis of the production of ligninolytic enzymes—either more than 50 U/l laccase or 100 U/l MnP—as well as on a good growth on at least one of the three lignocellulosic substrates, 16 strains were selected and tested in a further screening to evaluate their growth behavior in non-sterile, uncontaminated soil. In this test, also two strains of *Marasmius* and one *Mycena* strain were included because they grew well on flax and bark, although their enzyme secretion was poor. For each strain its optimal growth substrate was used (Table 2). Eight of the chosen strains showed a good active growth into the non-sterile soil and two further ones grew moderately. Only the *Marasmius* strains, *Mycena epipterygia* as well as *Stropharia aeruginosa* were incapable of or poorly invading the soil (Table 2). There was no significant difference in fungal growth between flasks of the same strain, which were differently inoculated (on the top or beneath the soil; Fig. 1A and C). The inability to colonize the non-sterile soil became apparent by the formation of mycelial barriers at the soil contact zone (Fig. 1B) or by the standstill of growth. Those strains showing good growth in the soil colonized it completely within 15 days. After 30 days, the mycelial growth slowed down, which can be attributed to hyphae autolysis and the exhaustion of nutrients.

Eleven strains belonging to nine fungal species including both *P. ostreatus* control strains were chosen for the PAH degradation experiment. Each strain was pre-grown on its preferred substrate and added to flasks with an artificially contaminated soil supplemented with five different PAHs (total amount 250 mg/kg). In contrast to the growth in natural soil, fine hyphae were less developed and thicker rhizomorphs were formed by most of the strains. The colonization of the contaminated soil took generally more time compared to the clean soil. There were also differences in the growth of the particular fungal species ranging from 20% (*Stropharia cubensis*) to total colonization by *Pleurotus* spp., *S. coronilla* and *S. rugosoannulata* (Table 2).

After the treatment of the soil samples (10 g from each flask) with an ASE apparatus, the extracts obtained were analyzed for the PAH concentration. After 69 days, the degradation of low-molecular mass PAHs by the autochthonous microflora (bacteria, yeasts and molds) amounted to 60% and 90% for anthracene and pyrene, respectively (data not shown). Pyrene extraction from sterile controls yielded only 80% of the initial concentration, which pointed to its partial

Fig. 1 Active growth of litter-decomposing fungi into soil when inoculated from top (**A**: *Stropharia hornemannii*) or from the bottom (**C**: *S. rugosoannulata* G). Formation of a protective barrier by *Marasmius scorodonius* against the soil layer with no growth into the soil (**B**)

volatilization during the extraction. Recovery of other PAHs was higher than 96%. These findings demonstrate the good bioavailability and degradability of freshly added small PAH molecules. In the case of anthracene, degradation was considerably enhanced in the presence of fungi, ranging between 68% for *H. capnoides* and 96% for *P. ostreatus* DSM 11191 (the autochthonous microflora converted 60%; Fig. 2). True litter-decomposers, especially *Agrocybe* spp. and *Stropharia* spp., were equally successful in removing anthracene (about 90%). None of the fungal strains tested significantly stimulated the degradation of pyrene (data not shown) due to its efficient degradation by the indigenous microorganisms (90%).

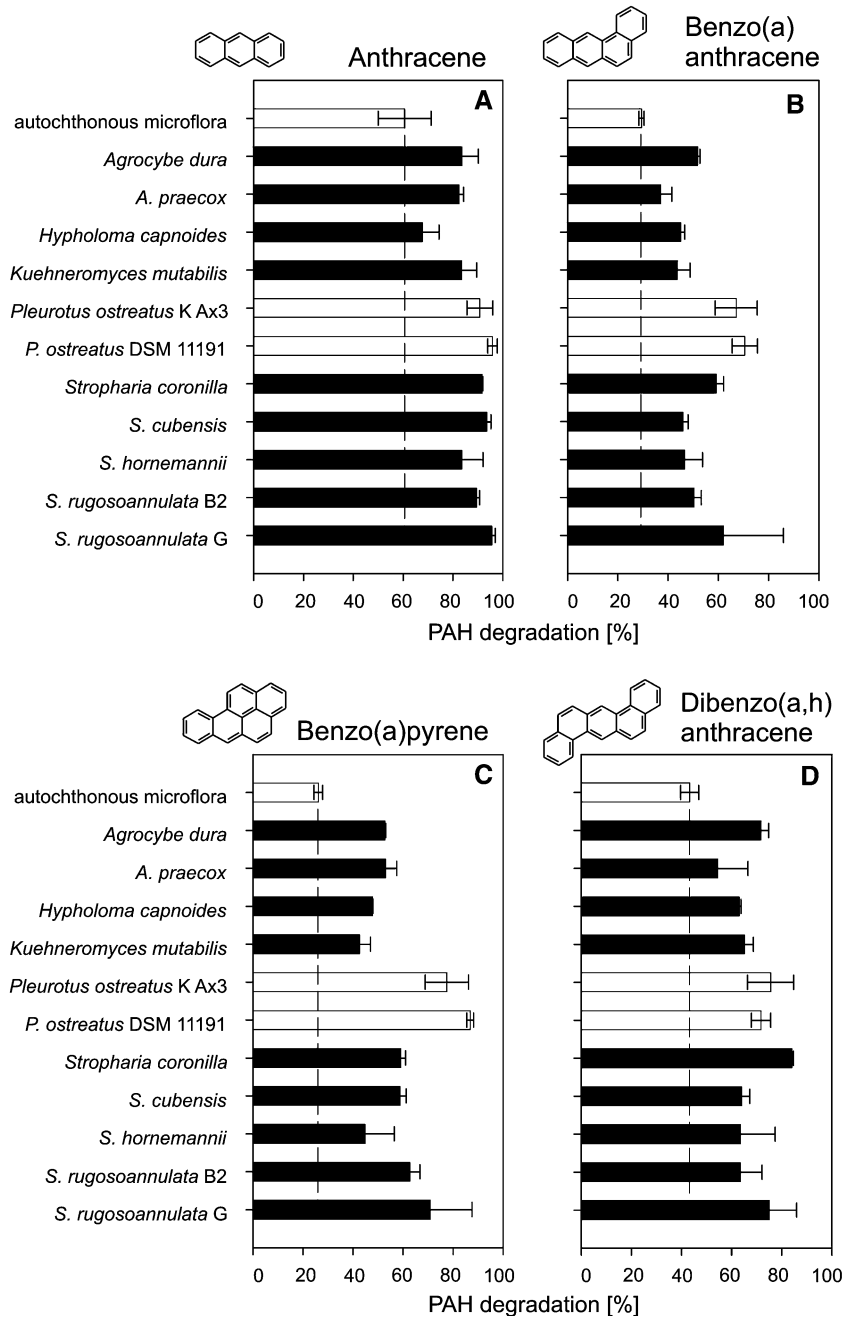

Fig. 2 Degradation of anthracene, benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene in non-sterile soil by the autochthonous microflora, litter-decomposing fungi and, as a comparison, by one white-rot fungus species (two *Pleurotus ostreatus* strains) after 69 days. (Means of three replicates with standard deviations)

The stimulation of PAH conversion by fungal inoculation was particularly pronounced in the case of high-molecular mass PAHs benzo(a)anthracene, benzo(a)pyrene (BaP) and dibenzo(a,h)anthracene; the indigenous microbes removed only 29%, 26% and 43% of them, respectively (Fig. 2). Both *P. ostreatus* strains showed strong degradative activities and achieved the highest extent PAH degradation (e.g., 70%–86% for the strain P1; Fig. 2). Interestingly, the litter-decomposing fungus *S. coronilla* removed also up to 84% of dibenzo(a,h)anthracene. The most promising litter decomposer, however, was *S. rugosoannulata* G that converted all high-molecular mass PAHs to at least 60% (Fig. 2).

Discussion

The present results demonstrate that both, selected wood- and litter-decomposing basidomycetes, have a well-founded potential to be used in soil bioremediation. The degradation of PAH in soil by them was improved by selecting those species, which are able to produce appreciable amounts of ligninolytic enzymes and in particular, to colonize non-sterile soil. A further positive effect was most probably achieved by supplying the preferred growth substrate of each fungus, i.e. providing an easy assimilable lignocellulosic carbon source along with sufficient water and shelter.

Some LDF enhanced the degradation of high-molecular mass PAHs in the artificially contaminated soil noticeably compared to the indigenous microflora. The general potential of LDF to degrade PAHs was already reported by Gramss et al. (1999b) but using other and less hazardous compounds. Apparently, soil microbes (primarily bacteria) attack only low-molecular mass PAHs such as anthracene and pyrene, whereas the substantial degradation of benzo(a)anthracene, benzo(a)pyrene and dibenzo(a,h)anthracene required the presence of ligninolytic fungi. The explanation may be that small PAHs serve as sole carbon source for certain bacteria and are intracellularly metabolized, whereas the larger PAHs enter the cell to a minor degree and cannot be utilized as growth substrate. This phenomenon is analogous for the inability of bacteria to degrade high molecular weight lignin and lignin model compounds (Hatakka 2001). Eggen and Majcherczyk (1998) clearly demonstrated an eight-fold increase of BaP degradation by *P. ostreatus* in creosote-contaminated soil compared to uninoculated samples. The assumption is further supported by the fact that the bioavailability of small PAHs (naphthalene, phenanthrene) is higher due to their better solubility in water.

Only few bacterial genera have been found to use four-ring PAHs as a sole carbon source and five-ring PAHs are only partially degraded in the presence of another primary carbon source (cometabolism) (Juhasz and Naidu 2000; Kästner 2000). Mostly soil bacteria and molds merely modify the molecules in a way that enables the subsequent binding to the humic matrix (Sack and Fritsche 1997). On the other hand, there are indications that white-rot fungi are capable of mineralizing high-molecular mass PAHs substantially (Sack and Fritsche 1997; Steffen et al. 2003). Simultaneously, they stimulate the humification of organopollutants and can even mineralize the already humified material afterwards (Tuomela et al. 1999).

The clean up of non-sterile soil, however, may enable consortia of fungi and bacteria to develop where fungi can attack especially the high-molecular mass PAHs that will be further degraded by bacteria (Boonchan et al. 2000; Sasek 2003). Such a joined conversion and detoxification of BaP has been demonstrated in contaminated soil with a *Bjerkandera* sp. (Kotterman et al. 1998) implementing a possible succession of microbes—first fungi, then bacteria—in the degradation process.

The key role of ligninolytic enzymes in the degradation of PAHs has been demonstrated in several studies (Hammel et al. 1986; Sack et al. 1997; Steffen et al. 2003; Rodriguez et al. 2004). Our results strengthen the assumption that MnP and laccase are involved in the breakdown of PAHs by LDF. The tested fungi converted the PAHs efficiently and all eight species studied produced both enzymes. Low activities of MnP in *P. ostreatus* cultures might be due to the fact that they produce versatile peroxidases with lower affinity towards manganese (Martinez 2002) or that the used medium was not optimal. This medium contained a sufficient amount of nitrogen,

which is preferred by most LDF but might negatively influenced both *Pleurotus* strains. However, the actual levels of ligninolytic enzymes do not always correlate with PAH degradation (Schützendübel et al. 1999). Thus, they surely function together with intracellular oxidoreductases (e.g., cytochrome P450 enzymes) at least in some species (Bezalel et al. 1996; Masaphy et al. 1996). Last but not least, also the contribution of lipid peroxidation (LPO) or other redox mediator systems cannot be ruled out. LPO can be initiated by MnP (Bogan and Lamar 1996) and has been also observed for MnPs of LDF (Kapich et al. 2005).

Since a successful colonization of soil requires active growth and formation of large mycelia, carbon must be available in a suitable form. For ligninolytic fungi, organic materials containing cellulose and hemicelluloses are the natural choice (Hatakka 2001). However, fungi cannot grow in all lignocellulosic substrates, as shown in this work. With an optimal growth substrate, soil invasion and consequently pollutant degradation can be enhanced. Most wood-decayers (white-rot fungi) utilize straw or wood shavings as preferred substrates (Morgan et al. 1993). We could show that straw was only useful for a few LDF species like *S. rugosoannulata* or *S. cubensis* but most of the LDF tested showed a better growth on less lignified materials such as flax or pine bark. Bark has a lower density than straw or flax, is often part of natural soil litter and due to the presence of cutin and suberin, it is degraded more slowly than wood or other lignocellulosic materials. For a biotechnological application, bark is feasible, as it is inexpensive and available in large quantities at least in countries producing pulp or timber. Last but not least, bark does not attract molds and other undesired microbes as much as straw does. It hence reduces the risk of microbial contamination when it is used as a substrate for the fungal inoculum.

The production of ligninolytic enzymes enabled the tested fungi to colonize their substrate faster and to invade the non-sterile soil rapidly. The selection of fungi according to their ability to produce MnP or laccase is therefore an important screening tool to find applicable organisms and to improve fungal bioremediation techniques. The use of pre-grown lignocelluloses allows the fungus to get a good start position to penetrate afterwards deeply into the soil. In this context, bark or mulch serve not only as a growth substrate, but store also water and protect the mycelium. This will enhance the survival of a fungus in soil where it encounters other microorganisms or toxic compounds (Canet et al. 2001).

When comparing white-rot fungi and LDF, it seems that the latter are more efficient in colonizing soil, but less efficient in converting PAHs. However, differences in the degradative potential are marginal (Gramss et al. 1999b), which was confirmed for *S. rugosoannulata* in this work. In the end, the active growth into the soil is decisive. Morphological differences as observed in our work may not be decisive and might be a defensive measure against a toxic environment. Grimm et al. (2005) already stated that a correlation of morphology and productivity is not possible. Thus, LDF can be considered as a good alternative for soil bioremediation. Nonetheless, the efficiency of LDF varies among the different species, and the degradative potential of LDF may be even considerably higher since only a limited number of species has been tested so far. We assume that more efficient LDF species can be found, which will be even more suitable for bioremediation applications. An adaptation of certain species to special soil types is also to expect, as they dwell various habitats. Different strategies to compete with other soil microorganisms in soil may be also important (e.g. antibiosis). Moreover, certain LDF could be the organisms of choice for bioaugmentation purposes since they naturally colonize soil environments.

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References

Andersson BE, Lundstedt S, Tornberg K, Schnurer Y, Oberg LG, Mattiasson B (2003) Incomplete degradation of polycyclic aromatic hydrocarbons in soil inoculated with wood-rotting fungi and their effect on the indigenous soil bacteria. Environ Toxicol Chem 22:1238–1243

Baldrian P (2004) Increase of laccase activity during interspecific interactions of white-rot fungi. FEMS Microbiol Ecol 50:245–253

Baldrian P, in der Wiesche C, Gabriel J, Nerud F, Zadrazil F (2000) Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. Appl Environ Microbiol 66:2471–2478

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

Bhatt M, Cajthaml T, Sasek V (2002) Mycoremediation of PAH-contaminated soil. Folia Microbiol 47:255–258

Bogan BW, Lamar RT (1996) Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl Environ Microbiol 62:1597–1603

Boonchan S, Britz ML, Stanley GA (2000) Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl Environ Microbiol 66:1007–1019

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

Bumpus JA, Tien M, Wright D, Aust SD (1985) Oxidation of persistent environmental pollutants by a white-rot fungus. Science 228:1434–1436

Cajthaml T, Moder M, Kacer P, Sasek V, Popp P (2002) Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. J Chromatogr 974:213–222

Canet R, Birnstingl JG, Malcolm DG, Lopez-Real JM, Beck AJ (2001) Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by native microflora and combinations of white-rot fungi in a coal-tar contaminated soil. Biores Technol 76:113–117

Cerniglia CE (1993) Biodegradation of polycyclic aromatic hydrocarbons. Curr Opin Biotechnol 4:331–338

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

Eggen T, Majcherczyk A (1998) Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white-rot fungus *Pleurotus ostreatus*. Int Biodet Biodegr 41:111–117

Eggert C, Temp U, Eriksson KEL (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl Environ Microbiol 62:1151–1158

Field JA, de Jong E, Feijoo-Costa G, de Bont JAM (1993) Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol 11:44–49

Gramss G, Kirsche B, Voigt K-D, Günther T, Fritsche W (1999a) Conversion rates of five polycyclic aromatic hydrocarbons in liquid cultures of fifty-eight fungi and the concomitant production of oxidative enzymes. Mycol Res 103:1009–1018

Gramss G, Voigt KD, Kirsche B (1999b) Degradation of polycyclic aromatic hydrocarbons with three to seven aromatic rings by higher fungi in sterile and unsterile soils. Biodegradation 10:51–62

Grimm LH, Kelly S, Krull R, Hempel DC (2005) Morphology and productivity of filamentous fungi. Appl Microbiol Biotechnol 69:375–384

Hammel KE, Kalyanaraman B, Kirk TK (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium* ligninase. J Biol Chem 261:16948–16952

Hatakka A (2001) Biodegradation of lignin. In: Hofrichter M, Steinbüchel A (eds) Lignin, humic substances and coal. Wiley-VCH, Weinheim, Germany, pp 129–180

Hintikka V (1970) Studies on white-rot humus formed by higher fungi in forest soils. Communicationes Instituti Forestalis Fenniae 69:2

Juhasz AL, Naidu R (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. Int Biodet Biodegr 45:57–88

Kapich AN, Steffen KT, Hofrichter M, Hatakka A (2005) Involvement of lipid peroxidation in the degradation of a non-phenolic lignin model compound by manganese peroxidase of the litter-decomposing fungus *Stropharia coronilla*. Biochem Biophys Res Comm 330:371–377

Kotterman MJ, Vis EH, Field JA (1998) Successive mineralization and detoxification of benzo[a]pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. Appl Environ Microbiol 64:2853–2858

Kästner M (2000) Degradation of aromatic and polyaromatic compounds. In: Rehm H-J, Reed G (eds) Biotechnology. Wiley-VCH, Weinheim, Germany, pp 211–239

Martens R, Wolter M, Bahadir M, Zadrazil F (1999) Mineralization of ^{14}C-labelled highly-condensed polycyclic aromatic hydrocarbons in soils by *Pleurotus* sp. Florida. Soil Biol Biochem 31:1893–1899

Martinez AT (2002) Molecular biology and structure-function of lignin- degrading heme peroxidases. Enzyme Microb Technol 30:425–444

Masaphy S, Levanon D, Henis Y, Venkateswarlu K, Kelly SL (1996) Evidence for cytochrome P-450 and P-450-mediated benzo(a)pyrene hydroxylation in the white-rot fungus *Phanerochaete chrysosporium*. FEMS Microbiol Lett 135:51–55

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

Novotny C, Erbanová P, Sasek V, Kubátová A, Cajthaml T, Lang E, Krahl J, Zadrazil F (1999) Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. Biodegradation 10:159–168

Potin O, Veignie E, Rafin C (2004) Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. FEMS Microbiol Ecol 51:71–78

Rodriguez E, Nuero O, Guillen F, Martinez AT, Martinez MJ (2004) Degradation of phenolic and non-phenolic aromatic pollutants by four *Pleurotus* species: the role of laccase and versatile peroxidase. Soil Biol Biochem 36:909–916

Sack U, Fritsche W (1997) Enhancement of pyrene mineralization in soil by wood-decaying fungi. FEMS Microbiol Ecol 22:77–83

Sack U, Hofrichter M, Fritsche W (1997) Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii*. FEMS Microbiol Lett 152:227–234

Sasek V (2003) Why mycoremediations have not come into practice. In: Sasek V, Glaser A, Baveye P (eds) The ultilization of bioremediation to reduce soil contamination: problems and solutions. Kluiwer Academic Publishers, Netherlands, pp 247–266

Schneider J, Grosser R, Jayasimhulu K, Xue W, Warshawsky D (1996) Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl Environ Microbiol 62:13–19

Schützendübel A, Majcherczyk A, Johannes C, Hüttermann A (1999) Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. Internat Biodet Biodegr 43:93–100

Steffen KT, Hatakka A, Hofrichter M (2002a) Removal and mineralization of polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. Appl Microbiol Biotechnol 60:212–217

Steffen KT, Hatakka A, Hofrichter M (2003) Degradation of benzo[a]pyrene by the litter-decomposing basidiomycete *Stropharia coronilla*: role of manganese peroxidase. Appl Environ Microbiol 69:3957–3964

Steffen KT, Hofrichter M, Hatakka A (2000) Mineralisation of ^{14}C-labelled synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidiomycetous fungi. Appl Microbiol Biotechnol 54:819–825

Steffen KT, Hofrichter M, Hatakka A (2002b) Purification and characterization of manganese peroxidases from the litter-decomposing basidiomycetes *Agrocybe praecox* and *Stropharia coronilla*. Enzyme Microb Technol 30:550–555

Tuomela M, Lyytikäinen M, Oivanen P, Hatakka A (1999) Mineralization and conversion of pentachlorophenol (PCP) in soil inoculated with the white-rot fungus *Trametes versicolor*. Soil Biol Biochem 31:65–74

Wariishi H, Valli K, Gold MH (1992) Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. J Biol Chem 267:23688–23695

Wunch KG, Alworth WL, Bennett JW (1999) Mineralization of benzo[a]pyrene by *Marasmiellus troyanus*, a mushroom isolated from a toxic waste site. Microbiol Res 154:75–79

Wunch KG, Feibelman T, Bennett JW (1997) Screening for fungi capable of removing benzo(a)pyrene in culture. Appl Microbiol Biotechnol 47:620–624