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ORIGINAL PAPER

Degradation of phenanthrene by the endophytic fungus *Ceratobasidum stevensii* found in *Bischofia polycarpa*

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Abstract Some strains of white rot fungi, non-lignolytic fungi and litter-decomposing basidiomycetes have been recognized as PAH degraders. The purpose of our research was to enlarge the scope of PAH-degrading fungi and explore the huge endophytic microorganism resource for bioremediation of PAHs. In this study, phenanthrene was used as a model PAHs compound. Nine strains of endophytic fungi isolated from four kinds of plant from *Eupharbiaceae* were screened for degradation of phenanthrene. The endophytic fungus *Ceratobasidum stevensii* (strain B6) isolated from *Bischofia polycarpam* showed high degradation efficiency and was selected for further studies. Into the fungal culture, 100 mg l^{-1} phenanthrene was added, and after 10 days of incubation, about 89.51% of the phenanthrene was removed by strain B6. Extracellular ligninolytic enzyme activities of strain B6 were tested. The results showed that manganese peroxidase [MnP] was the predominant ligninolytic enzyme and that its production was greatly induced by the presence of phenanthrene. To confirm the involvement of MnP in phenanthrene degradation, promotion and inhibition studies on MnP in different concentration level of Mn^{2+} and NaN_3 were performed. Additionally, fungal mycelium-free and resuspended experiments were carried out. The results showed no apparent correlation between MnP activity and phenanthrene degradation. The mycelium and fresh medium were the crucial factors affecting the degradation of phenanthrene. To date, this is the first report on PAH degradation by *Ceratobasidum stevensii*. This study suggests that endophytic fungi might be a novel and important resource for microorganisms that have PAH-degrading capabilities.

Keywords Biodegradation · Polycyclic aromatic hydrocarbons · Manganese peroxidase · Ligninolytic enzyme · Mycelium

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one class of toxic environmental pollutants that have accumulated in the environmental due to a variety of anthropogenic activities (Juhasz and Naidu 2000). Most PAHs are toxic to living organisms, may reduce water activity and act as chaotropic stressors for microbial cells (Hallsworth and Nomura 1999; Hallsworth et al. 2003, 2007). Some of them and their metabolites are mutagenic and carcinogenic to

humans. Since the 1970s, there have been many studies on the biodegradation of various aromatic hydrocarbons, but their low water solubility and subsequent low degradation rates hamper the bioremediation of PAH-polluted environments (Cerniglia 1992; Han et al. 2004). Therefore, knowledge about microorganisms that have a high PAHs-degrading capability is essential for efficient remediation of PAHs contamination.

To date, most PAHs biodegradation studies have focused on degrading bacteria. However, research indicates that in soil habitats, filamentous fungi offer certain advantages over bacteria for bioremediation (Potin et al. 2004; Salvo et al. 2005). Fungi are seen as potential soil remediation organisms (Gramss et al. 1999), especially in arid and semi-arid environments (Kashangura et al. 2006). In the field of PAHs degradation by fungi, the majority of studies have focused on white rot fungi (Cerniglia 1992; Bezalel et al. 1996a; Magan and Mswaka 1998; Kim et al. 1998; Mori et al. 2003; Han et al. 2004), particularly *Phanerochaete chrysosporium* (Dhawale et al. 1992; Paszczynski and Crawford 1995; Zheng and Obbard 2002). These fungi produce ligninolytic enzymes, including lignin peroxidase [LiP] and manganese peroxidase [MnP], which are presumed to be involved in the PAH degradation process (Paszczynski and Crawford 1995). Other studies have focused on the ability of litter-decomposing fungi (Steffen et al. 2002, 2003, 2007) and non-lignolytic fungi (Cerniglia 1997), which could also metabolize a variety of PAHs to polar metabolites. Whereas, the rate of PAH degradation by these fungi is low, the full potential of biodegradation by filamentous fungi has not been fully investigated for bioremediation purpose. Table 1 lists the results concerning phenanthrene degradation by these fungi in liquid culture.

Endophytes include those fungi that can colonize internal plant tissues at some time in their life without causing apparent harm to their host (Petrini et al. 1991). The ecology of endophytic fungi has been studied intensively in terms of their interactions with plants, herbivores, and pathogens on living leaves. However, recent studies showed that endophytic fungi also play an important role in plant leaf litter decomposition, and some fungi even have marked ligninolytic activity and lignin degradation abilities (Koide et al. 2005; Jordaan et al. 2006). Based on these studies, we speculate that some endophytic fungi might also have the ability to degrade PAHs.

Up to now, some strains of white rot fungi, non-lignolytic fungi and litter decomposing basidiomycetes have been recognized as PAH degraders (Cerniglia 1992, 1997; Steffen et al. 2002). Nevertheless, the degradation rate of PAH by these fungi is low and is influenced significantly by a wide variety of abiotic and biotic factors (Cerniglia 1992; Field et al. 1993; Tekere et al. 2001). Different environments affect their capability to degrade PAHs, and even result in the death of the fungi (Steffen et al. 2007). However, it is worth noting that the specific symbiotic condition of endophytic fungi and its hosts offered fungi a stable position to accommodate the changing environment (Yuan et al. 2007). In addition, little has been known about the PAHs degradation by endophytic fungi. Study about the PAHs degradation by endophytic fungi and ligninolytic enzymes involved has not been reported. The main purpose of our study was to

Table 1 Phenanthrene degradation by reported fungus in liquid culture

Organism	Removal (%)	Transformation rate (mg day^{-1})	References
Coriolopsis gallica	42	0.42	Pickard et al. (1999)
Irpex lacteus	96	0.96	Cajthaml et al. (2002)
Phanerochaete chrysosporium	44.91	1.21	Zheng and Obbard (2002)
Phanerochaete chrysosporium	48	1.44	Kim et al. (1998)
Pleurotus ostreatus	94	2.14	Bezalel et al. (1996a)
Bjerkandera sp.	96	3.42	Terrazas et al. (2005)
Phomopsis sp. (B3)	52.54	5.25	Tian et al. (2007)
Phanerochaete chrysosporium	73.1	7.31	Dhawale et al. (1992)
Phlebia lindtneri	84	14.97	Mori et al. (2003)
Trametes versicolor	59.1	19.70	Han et al. (2004)

explore the huge endophytic microorganism resource for bioremediation of PAHs and develop a method for plant-microbial combined restoration of soil toxic environment.

Among PAHs, phenanthrene was selected because it is one of the most abundant PAHs in the environment, and it has been used as a model compound to study the biodegradation of PAHs (Bezalel et al. 1996a). In a former study, we screened endophytic fungus *Phomopsis* sp. strain B3 with phenanthrene degradation ability, and studied the enhancement of phenanthrene degradation when *Phomopsis* sp. strain B3 was co-cultured with the host plant in a symbiotic condition (Tian et al. 2007). However, the phenanthrene degradation efficiency by *Phomopsis* sp. strain B3 was relatively low. Previous research has already suggested that litter-decomposing fungi turned out to be moderate PAH degraders compared with wood rot fungi (Steffen et al. 2002). Some genera of this group are considered to be promising organisms for PAH bioremediation purposes (Steffen et al. 2007). In this experiment, nine fungal strains used were selected from 36 strains of endophytic fungi, which had been isolated from four kinds of plants of *Eupharbiaceae* (i.e. *Euphorbia pekinensis*, *Bischofia polycarpam*, *Euphorbia helioscopia* and *Sapium sebiferum*) (Shi et al. 2002). All of the nine fungal strains were shown, in our previous study, to have more efficient litter decomposition abilities (Shi et al. 2002). In this study, we screened one which demonstrated much higher degradation efficiency of phenanthrene from these strains. The ability of the screened fungus in degrading phenanthrene and the production of the ligninolytic enzymes were evaluated. Furthermore, the role of ligninolytic enzymes in phenanthrene degradation was discussed.

Materials and methods

Fungal strains inoculation and chemicals

Endophytic fungal strains (Table 3) were maintained on PDA (potato dextrose agar) slants. Petri dishes containing PDA were inoculated with a mycelium portion of each strain and incubated at 28°C for 8–10 days, after which the agar was cut and 1-cm^2 mycelial pieces were removed with a thin spatula and used as the inocula for the experiments of this study.

Phenanthrene, 2,2-ethylbenzothiazoline-6-sulfonic acid (ABTS) and veratryl alcohol were obtained from Sigma. Methanol was of HPLC-grade. All other reagents used in the present study were of analytical grade.

Screening of endophytic fungi for phenanthrene degradation

The experiment was conducted in 150-ml Erlenmeyer flasks containing 30 ml of medium made of (g l^{-1}): CMC-Na, 5; lignin, 1; sucrose, 2.5; 200 g potato extract; pH 5.5.

The required amount of phenanthrene was dissolved in methanol at 20 g l^{-1} and added to the flasks to give 100 mg l^{-1}. Once the solvent had evaporated, the crystalline phenanthrene was resuspended in 30 ml sterile culture medium, and the flasks were inoculated in the dark on a shaker (140 rpm) at 25°C. After 10 days of incubation, 200 µl samples were taken from each flask to measure the ligninolytic enzyme activities. The concentration of phenanthrene of each flask was then extracted and analyzed as described in "Extraction and analysis of phenanthrene" section. Control, without phenanthrene was processed in an identical manner. A culture autoclaved after inoculation with the fungus was used as an abiotic control.

Phenanthrene degradation in liquid cultures by strain B6

Inoculum preparation and culture conditions of strain B6 were identical to those described in "Fungal strains inoculation and chemicals" and "Screening of endophytic fungi for phenanthrene degradation" sections. After 0, 3, 6, 9, 12 days of incubation, the ligninolytic enzyme activities, the concentration of phenanthrene and fungal dry weight were monitored. In all experiments, cultures were grown in triplicate, and results were expressed in means and standard deviations.

Effect of Mn^{2+} and NaN_3 concentrations studies

Mn^{2+} was added separately as $MnSO_4 \cdot H_2O$ to obtain a serial concentration of Mn^{2+} in medium, i.e. 25, 50, 100, 250, 500 µM, while NaN_3 was added to obtain a serial concentration of 0.1, 1, 10, 100, 1000 µM. After 7 days of incubation as described in "Screening

of endophytic fungi for phenanthrene degradation" section, all cultures were harvested. Mycelial masses were used for dry weight determinations, and the extracellular fluid (0.20 ml) was taken for enzyme analysis. Phenanthrene was then extracted and analyzed as described in "Extraction and analysis of phenanthrene" section.

Fungal biomass estimation

Mycelium was filtered through pre-dried cellulose filters in a vacuum filtration apparatus. Filters were lyophilized and dry weight was measured. Then filtrate and lyophilized filters were recovered for phenanthrene extraction as described in "Extraction and analysis of phenanthrene" section.

Degradation of phenanthrene by fungi mycelium-free supernatant and resuspended biomass

The experiment was conducted in 150-ml Erlenmeyer flasks; 32 Erlenmeyer flasks (150 ml) containing 30 ml of sterile culture were inoculated with strain B6 and incubated in the dark on a shaker (140 rpm) at 25°C. After 5 days of incubation, the cultures were centrifuged at $5500\times g$ for 15 min to separate the medium from the biomass. The supernatant was then used as fungal extracellular fluid, with a certain MnP activity. Through the previous experiments, 32 samples of biomass and fungal extracellular fluid were obtained. Sixteen samples of biomass was resuspended with 25 ml of fresh medium, and the other 16 samples of biomass was resuspended with 16 samples of fungal extracellular fluid, respectively. The required amount of phenanthrene dissolved in methanol (based on the volume of 25 ml) was added to give a final result of 16 mg l^{-1}. The residual 16 samples of fungal extracellular fluid were also incubated with phenanthrene as previously mentioned. The three treatments, including (1) the fungi mycelium-free supernatant,(2) resuspended biomass with either fresh medium, (3) fungal extracellular fluid, were incubated on a shaker (120 rpm) at 25°C for 48 h. Sampling was undertaken four times at 0, 12, 24 and 48 h incubation. Three culture flasks of each treatment were taken at each sampling time to measure MnP activity and residual phenanthrene. The residual culture flasks of each treatment were incubated continuously for the next sampling. Four surplus culture flasks of each treatment acted as candidates to deal with unexpected accidents to this experiment.

Fungal strains incubation time

In order to obtain reasonable experimental results, the fungal strains incubation time for different purpose was various. For simplicity, Table 2 list the incubation time of all experiments in this study.

Extraction and analysis of phenanthrene

The whole replicate fungal culture was mixed with an equal volume of HPLC-grade methylene chloride in a 100-ml separatory funnel and vigorously extracted for 10 min. Here the whole fungal culture included both the filtrate and lyophilized mycelium, which had been utilized for dry-weight estimation. Extraction was repeated three times, and methylene chloride

Table 2 Incubation time of fungal strains

Experiment (corresponding section)	Incubation time (days)
Inocula preparation of all fungal strains (Fungal strains inoculation and chemicals)	8–10
Screening phenanthrene degradation fungi (Screening of endophytic fungi for phenanthrene degradation)	10
Phenanthrene degradation in liquid cultures by B6 (Phenanthrene degradation in liquid cultures by strain B6)	12
Effect of Mn^{2+} and NaN_3 concentrations studies (Effect of Mn^{2+} and NaN_3 concentrations studies)	7
Phenanthrene degradation by mycelium-free supernatant and resuspended biomass (Degradation of phenanthrene by fungi mycelium-free supernatant and resuspended biomass): biomass and extracellular fluid preparation	5
Phenanthrene degradation by mycelium-free supernatant and resuspended biomass (Degradation of phenanthrene by fungi mycelium-free supernatant and resuspended biomass): phenanthrene degradation of different treatments	2

phases were separated and combined. A tenth of a milliliter of extraction was evaporated in K–D concentrator using dry nitrogen gas. The residual was redissolved in methanol, and later passed through a 0.45-μm nylon filter prior to analysis for phenanthrene by HPLC.

All HPLC analyses were performed with an Agilent Eclips DC (4.6 × 250 mm) C_{18} reverse-phase column (Agilent Products). Separation was achieved by isocratic elution in methanol: water (90:10), with a flow rate of 0.8 ml min^{-1} and UV absorbance detector set at 252 nm. The detection limit achieved with this method was 0.01 mg l^{-1} for phenanthrene analysis.

Enzyme assays

Enzyme assays were conducted in 1-ml reaction mixtures at 30°C using the extracellular medium of fungal cultures as an enzyme source. Laccase activity was measured by monitoring the oxidation of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm. Specific activity was determined using $\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$ (Wolfenden and Wilson 1982). LiP activity was determined by the veratryl alcohol oxidation assay (Tien and Kirk 1983). MnP activity was assayed by the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate, pH 4.5, in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+}, form a complex with malonate, which absorbs at 270 nm ($\varepsilon = 11{,}590\ M^{-1}\ cm^{-1}$) (Wariishi et al. 1992).

Laccase, LiP and MnP Laccase activity were determined by the change of absorbance per minute on the condition of corresponding substrates preparation. In this study, lower than 0.002 of absorbance change within 1 min was considered as the consequence of instrument instability. Consequently, the detection limit of laccase activity, LiP activity and MnP activity achieved with our method was calculated as 1.17, 2.17 and 3.49 U l^{-1}, respectively.

Results

Screening of fungal isolates for phenanthrene degradation

Phenanthrene degradation capacity of the selected fungi and its ligninolytic enzymes were tested (Table 3). Only two strains, B3 and B6, showed phenanthrene degradation ability after 10 days of incubation—29.45% and 82.27% of the total phenanthrene initially present were degraded, respectively. However, ligninolytic enzymes secreted by strain B3 and B6 were completely different. Strain B3 produced laccase activity, while LiP and MnP activities were not detected under the given culture conditions. Interestingly, strain B6 showed LiP and MnP activities, laccase activity was not detected. Strain Y5 produced LiP and laccase but without phenanthrene degradation ability. A possible reason is that strain Y5 utilizes lignin or CMC-Na contained in the medium as its carbon source, and these enzymes are necessary for decomposing these compounds. The other six strains showed no ligninolytic enzymes activities.

Endophytic fungi B6 showed high degradation efficiency of phenanthrene following 10 days of growth with the degradation rate reaching 8.23 mg day^{-1}.

Table 3 Degradation of phenanthrene and associated enzyme activities of nine strain endophytic fungi

Host plant	Isolated tissue	Fungal strain	Phenanthrene removal (%)	LiP	MnP	Laccase
Euphorbia pekinensis	Leaf	*Alternaria* sp. (E1)	0	−	−	−
	Leaf	*Alternaria* sp. (E2)	0	−	−	−
	Stem's inner bark	*Fusarium* sp. (E4)	0	−	−	−
Bischofia polycarpam	Stem's inner bark	*Phomopsis* sp. (B3)	29.45 ± 2.52	−	−	+
	Stem's inner bark	*Ceratobasidum stevensii* (B6)	82.27 ± 0.01	+	+	−
Euphorbia helioscopia	Leaf	*Alternaria* sp. (H2)	0	−	−	−
	Leaf	*Alternaria* sp. (H3)	0	−	−	−
Sapium sebiferum	Stem's inner bark	*Chaetomium* sp. (Y5)	0	+	−	+
	Stem's inner bark	*Sclerotium* sp. (S2)	0	−	−	−

LiP, MnP and laccase activities are given as (+) above and (−) below the detection limits

Compared with the other screened phenanthrene that degraded fungi and some white rot fungi (Table 1), which had been used in the PAHs biodegradation, the degradation rate of phenanthrene by B6 was relatively high.

Phenanthrene degradation in liquid cultures by strain B6

Phenanthrene degradation by strain B6 was investigated in liquid cultures with 12 days of incubation. We conducted this kinetic experiment to determine the time course for phenanthrene degradation and the involvement of extracellular oxidative enzymes in phenanthrene degradation (Fig. 1).

Strain B6 showed a similar growth pattern whether in the absence or presence of phenanthrene, indicating that phenanthrene (100 mg l^{-1}) had no toxic effect. B6 exponential growth phase occurred within 3 days when the fungal biomass reached the highest level (3.8 g dry weight per 1,000 ml of culture). The depletion of phenanthrene was about 89.51% within 9 days of incubation, and further degradation did not occur after 9 days of incubation. The time course for extracellular ligninolytic enzyme in a liquid medium is also shown in Fig. 1. Compared with the cultures grown in absence of phenanthrene, LiP and MnP activity in cultures with phenanthrene were greatly induced. However, only negligible LiP activity was found in cultures of B6 (<20 U l^{-1}) whether in absence or in presence of phenanthrene. No laccase activity was detected in either treatment throughout the experiment.

Analysis of the correlation between phenanthrene degradation and MnP activity

MnP was the predominant ligninolytic enzyme of this fungus, and its activity was greatly induced when phenanthrene was present (Fig. 1). Some researchers have shown that MnP plays a crucial role in the degradation of PAHs (Bogan and Lamar 1996; Hofrichter et al. 1998; Clemente et al. 2001; Zheng and Obbard 2002; Steffen et al. 2003; Eibes et al. 2005). In order to confirm the role of MnP in the degradation of phenanthrene, promotion and inhibition of MnP activity were performed by using different concentration of Mn^{2+} (Fig. 2) and NaN_3 (Fig. 3).

Effect of Mn^{2+} concentrations ([Mn^{2+}]) on production of MnP and phenanthrene degradation

Production of MnP was significantly regulated by the concentration of Mn^{2+} in the culture medium—the

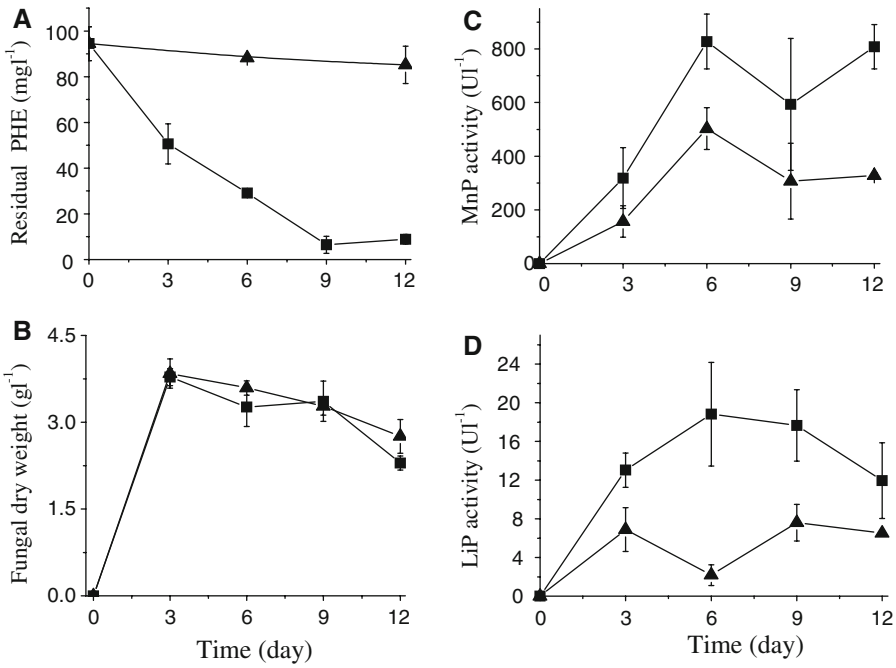

Fig. 1 Growth kinetics of the fungal strain B6 in liquid medium. Averages and standard errors of three replicates are shown. **a** Residual phenanthrene (*filled square*) and abiotic controls (*filled triangle*); **b** mycelium dry biomass in absence (*filled triangle*) or in presence (*filled square*) of phenanthrene; **c** MnP activity in absence (*filled triangle*) or in presence (*filled square*) of phenanthrene; **d** LiP activity in absence (*filled triangle*) or in presence (*filled square*) of phenanthrene

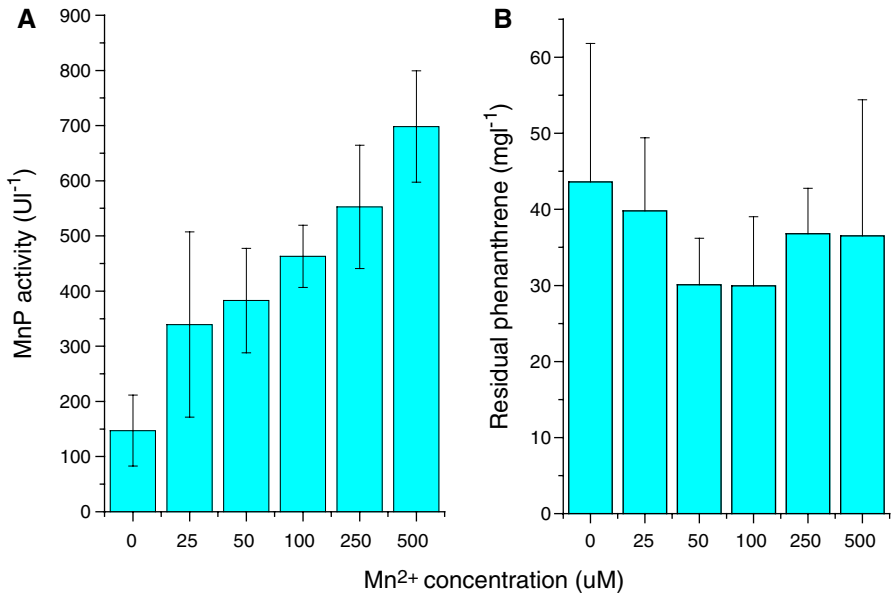

Fig. 2 Effect of [Mn^{2+}] on production of MnP (**a**) and phenanthrene degradation (**b**), supplied with 100 mg l^{-1} phenanthrene, inoculated with the strain B6, and incubated for 7 days. Values plotted are means of three replicates; *error bars* represent the standard deviation

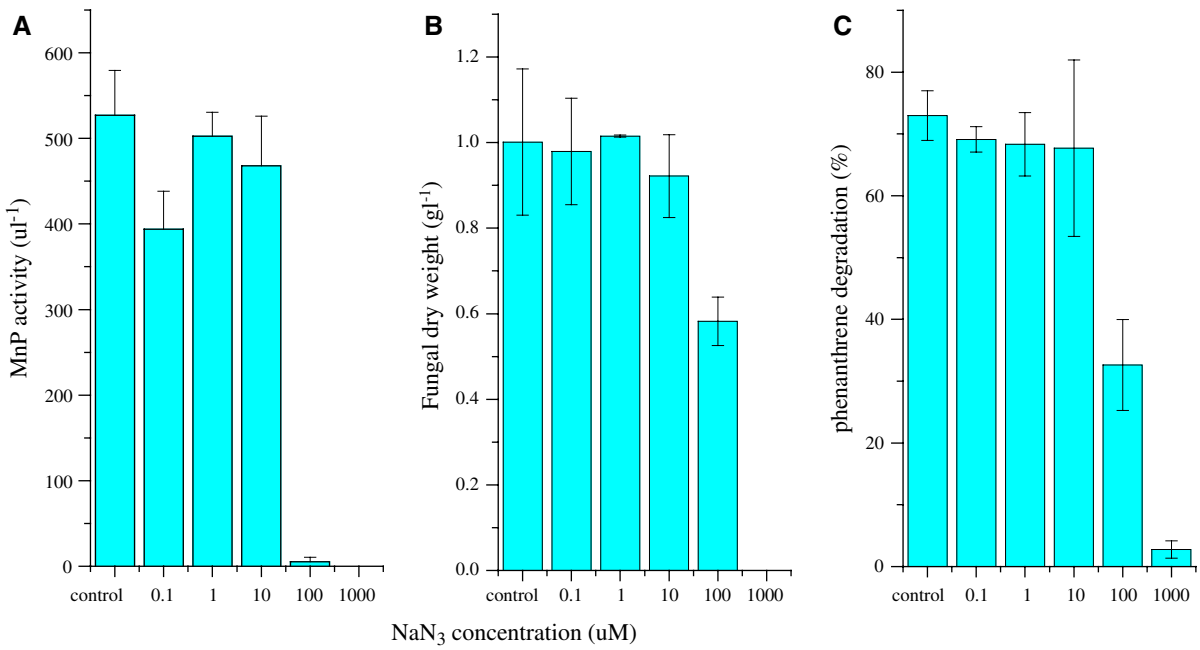

Fig. 3 Effect of sodium azide (NaN_3) concentration on MnP activity (**a**), biomass production (**b**) and phenanthrene degradation (**c**). Values plotted are means of three replicates; *error bars* represent the standard deviation

higher the concentration of Mn^{2+} in the test range (0–500 μM), the higher the maximum activity of MnP that appeared in culture solution (Fig. 2). The maximum activity of MnP occurred at a concentration of 500 μM Mn^{2+}, i.e. 689.36 U l^{-1}, compared to only 146.99 U l^{-1} in cultures without Mn^{2+} addition. This result was in agreement with previous studies on several white rot fungal species (Hamman et al. 1999; Zheng and Obbard 2002). Figure 2 also shows that the concentration of Mn^{2+} has no significant effect on the degradation of phenanthrene. In addition, the production of LiP by B6 was also detected to have

little activity (<16 U l^{-1}). Therefore, the influence by the different concentration of Mn^{2+} can be neglected (data not shown).

Effect of NaN_3 concentration on production of MnP, biomass and phenanthrene degradation

Sodium azide was used as an inhibitor of MnP. Figure 3 shows sodium azide at 100 μM, a concentration which inhibited about 99.01% MnP and induced 41.84% biomass production, decreasing about 55.31% of the phenanthrene degradation. This result suggests that MnP was not the first key enzyme in metabolizing PAH in this fungal strain.

Degradation of phenanthrene by fungi mycelium free and resuspended biomass

The present experiment was carried out to investigate the main factors effecting the phenanthrene degradation by strain B6. Figure 4a illustrates degradation of phenanthrene by fungi mycelium-free supernatant and resuspended biomass. The fungi mycelium-free supernatant, with an initial MnP activity of 395.08 U l^{-1}, did not result in the degradation of the phenanthrene. However, the separated biomass, resuspended in fresh medium, did remove phenanthrene efficiently. Approximately 74.87% of phenanthrene was degraded after 48 h of incubation. In addition, the separated biomass was resuspended with fungal extracellular fluid, which had an initial MnP activity of 395.08 U l^{-1} and degraded less phenanthrene than the resuspended in fresh medium. Moreover, the changes in MnP activity in all treatments were monitored (Fig. 4b). The change of MnP activity of mycelium-free supernatant treatment and resuspended treatment with fungal extracellular fluid showed a similar pattern: the MnP activity dropped rapidly within 12 h and then decreased relatively slowly. The treatment of separated biomass resuspended with fresh medium showed low levels of MnP activity (<20 U l^{-1}) within the first 12 h, but later increased rapidly and reached high level of 230.08 U l^{-1} after 48 h. Interestingly, the highest level of MnP production and phenanthrene degradation rate in the treatment of separated biomass with fresh medium occurred at the same time (Fig. 4a, b), which suggested that MnP was involved in the degradation of phenanthrene. It has been reported that MnP takes effect via the generation of Mn^{3+} ions from Mn^{2+}, which, when stabilized by chelators, act as low molecular weight redox mediators (Kishi et al. 1994;

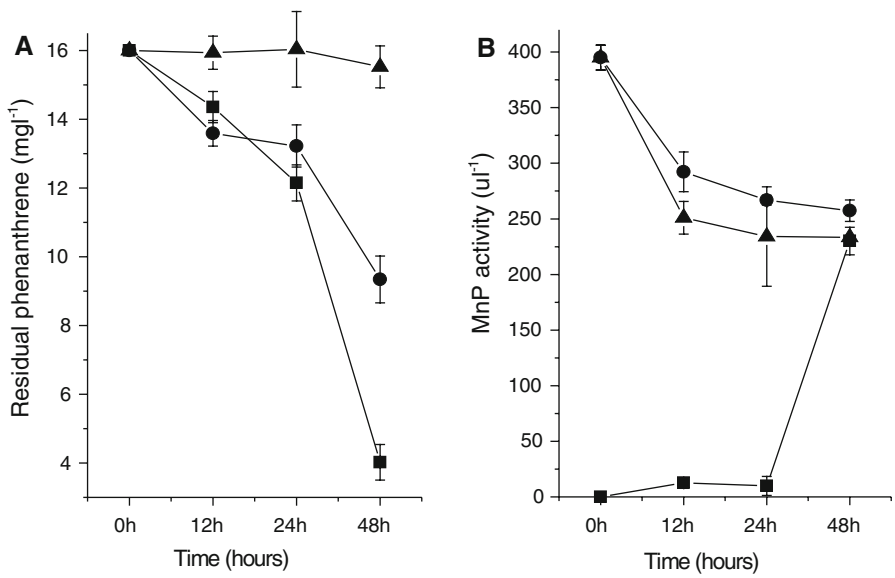

Fig. 4 Degradation of phenanthrene and the change of MnP activity. **a** Residual phenanthrene in fungi mycelium-free supernatant (*filled triangle*) and resuspended biomass with fresh medium (*filled square*) or with fungal extracellular fluid (*filled circle*); **b** change of MnP activity in fungi mycelium-free supernatant (*filled triangle*) and resuspended biomass with fresh medium (*filled square*) or with fungal extracellular fluid (*filled circle*); values plotted are means of three replicates; *error bars* represent the standard deviation

Zheng and Obbard 2002), and MnP attack PAHs via the formation of free radicals (Steffen et al. 2003).

Discussion

It is noteworthy that of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel and Daisy 2003). To exploit this huge resource, investigators have focused their attention on endophytes' potential for becoming bioactive metabolites with medical, agricultural and industrial potentials (Wang et al. 2006), as a food source, or for catalyzing ecological development (Kashangura et al. 2006). Recent studies show that endophytic fungi play an important role in the early stage of leaf litter decomposition—some even have marked ligninolytic activity and lignin degradation ability (Koide et al. 2005). Moreover, researchers had demonstrated that some litter-decomposing strains have been shown to oxidize PAHs (Steffen et al. 2003, 2007). In addition, recent reports show that natural phyllosphere bacteria are able to degrade monoaromatic hydrocarbons such as toluene, phenol, ethylbenzene, and xylene (Darlington et al. 2001; De Kempeneer et al. 2004; Sandhu et al. 2007). Some phyllosphere bacteria on plant leaves can even degrade phenanthrene (Waight et al. 2007). Based on these research results, we proposed that some endophytic fungi might also have the ability to degrade PAHs. Our present results confirm our hypothesis.

The degradation rate of phenanthrene by screened B6 was relatively high compared to the rate of other reported phenanthrene-degrading fungi. Furthermore, unlike that of some other fungi (Han et al. 2004; Terrazas et al. 2005), the degradation rate of phenanthrene by strain B6 was performed with the initial condition, without any optimization experiment on medium and culture conditions. This result indicates B6 might be a potential candidate for effective bioremediation of PAHs contamination.

Strain B6 showed good expelling and insecticide activity and was identified as *Ceratobasidum stevensii* in our previous study (Dai et al. 2006). To date, none of these genera have demonstrated the ability to degrade phenanthrene or other PAHs. Consequently, the opportunity to find new PAH degradation microorganisms among myriads of plants in different settings and ecosystems is promising.

MnP was the predominant ligninolytic enzyme of B6 and was greatly induced when phenanthrene was present. Some researchers have reported that MnP plays a crucial role in the degradation of PAH (Bogan and Lamar 1996; Hofrichter et al. 1998; Clemente et al. 2001; Zheng and Obbard 2002; Steffen et al. 2003; Eibes et al. 2005). Therefore, particular attention was paid to the role of MnP in this study in performing promotion and inhibition studies on MnP. It must be noted that the excretion of MnP by B6 was enhanced as $[Mn^{2+}]$ increased, while the degradation of phenanthrene was not notably effected by $[Mn^{2+}]$. With an addition of sodium azide at 100 μM, MnP activity was almost completely inhibited, while B6 still degraded phenanthrene at a relatively lower rate than the control. These findings agreed with previous studies that showed no distinct correlation between extracellular ligninolytic enzyme and PAH degradation (Bezalel et al. 1996b; Verdin et al. 2004), while Zheng and Obbard (2002), Steffen et al. (2003) reported high Mn^{2+} concentration beneficial for the degradation of PAH. These results indicated that MnP of these fungi, like laccase of *Fusarium solani* reported by Verdin et al. (2004), was not a first enzyme in metabolizing PAH.

This conclusion was supported by the fungi mycelium-free supernatant experiment (Fig. 4). Fungal biomass is a key factor for the degradation of phenanthrene, shown by the fact that the fungi mycelium-free supernatant did not result in the degradation of phenanthrene, despite the presence of MnP activity. Moreover, it must be noted that the treatment of separated biomass resuspended with fresh medium showed higher phenanthrene degradation potential than that resuspended with fungal extracellular fluid, although high activity levels of MnP existed in the fluid. High phenanthrene degradation rate in the treatment of separated biomass with fresh medium was observed after 24 h of the experiment (Fig. 4a), which coincided with the high activity levels of MnP production. On the basis of this result, we speculate that the key enzyme in metabolizing phenanthrene of these fungi is located in the fungal mycelium, and that the fungal growth in fresh medium facilitated the enzyme production. MnP was involved in the degradation of phenanthrene metabolites. Another possibility is that MnP oxidizes phenanthrene by using co-substrates like hydrogen peroxide, which is fungal mycelium-associated as reported by some researchers.

Although the phenomenon that PAHs are degraded only in the presence of fungal mycelium has been demonstrated by Zheng and Obbard (2002) using *Phanerochaete chrysosporium* and by Terrazas et al. (2005) using *Bjerkandera* sp., our work provide more information in the application of B6 and some other species for removal of PAH pollutants in the environment. Differences include an effort to obtain abundant fungal biomass and the usage of fresh medium rather than fungal extracellular fluid. Adding nutrition to the fungal extracellular fluid instead of fresh medium may be a candidate method.

From the results obtained, it can be confirmed that endophytic fungus B6 was very efficient in metabolizing phenanthrene. The potential for screening and optimizing more endophytic fungi for PAH degradation may be exploited in bioremediation applications. A preliminary experiment showed that no phenanthrene 9,10-dihydrodiol was found in the phenanthrene metabolites under non-ligninolytic conditions (data not shown), even though it is the important intermediate product as described by many researchers (Cerniglia 1992; Bezalel et al. 1996a; Cajthaml et al. 2002; Mori et al. 2003), indicating that B6 might utilize a different pathway to degrade phenanthrene. Further studies will be conducted to characterize phenanthrene metabolites to confirm the phenanthrene degradation pathway. In order to investigate endophytic fungi B6's potential use in bioremediation applications, the degradation ability of some other PAHs with more fused benzene rings like pyrene and benzo[a]pyrene, will be tested in both liquid culture and PAH-contaminated soils.

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