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Biodegradation of chrysene, an aromatic hydrocarbon by *Polyporus* sp. S133 in liquid medium

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ABSTRACT

Polyporus sp. S133, a fungus collected from contaminated-soil was used to degrade chrysene, a polycyclic aromatic hydrocarbon (PAH) in a mineral salt broth (MSB) liquid culture. Maximal degradation rate of chrysene (65%) was obtained when *Polyporus* sp. S133 was incubated in the cultures supplemented with polypeptone (10%) for 30 days under agitation of 120 rpm, as compared to just 24% degradation rate in non-agitated culture. Furthermore, the degradation of chrysene was affected by the addition of carbon and nitrogen sources as well as kind of surfactants. The degradation rate was increased with increase in added amount of carbon and nitrogen sources, respectively. The degradation rate in agitated cultures was enhanced about 2 times higher than that in non-agitated cultures. The degradation mechanism of chrysene by *Polyporus* sp. S133 was determined through identification of several metabolites; chrysenequinone, 1-hydroxy-2-naphthoic acid, phthalic acid, salicylic acid, protocatechuic acid, gentisic acid, and catechol. Several enzymes (manganese peroxidase, lignin peroxidase, laccase, 1,2-dioxygenase and 2,3-dioxygenase) produced by *Polyporus* sp. S133 were detected during the incubation. The highest enzyme activity was shown by 1,2-dioxygenase (237.5 U l^{-1}) after 20 days of incubation.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants commonly found in soil, surface waters, sediments and their fates in nature are of great environmental concern due to their hazardous or potential toxicity, mutagenicity, and carcinogenicity [1]. Human may be exposed to these compounds from a wide variety of sources, such as through occupation, natural environment, diets, and so on [2,3]. PAHs may undergo photolysis, volatilization, adsorption, bioaccumulation, and chemical oxidation; biodegradation is the main process affecting PAH persistence in nature [4]. Indeed, biodegradation has begun to gain wider acceptance to be an economic and efficient alternative method to other degradation processes such as chemical or physical ones for purification of environment contaminated with PAHs [5]. Recently, some white rot fungi have the ability to degrade a wide variety of PAHs such as *Chrysosporium P.*, *Bjerkandera adusta*, *Irpex lacteus*, and *Lentinus tigrinus* [6–8].

Chrysene, a symmetrical PAH consisting of four condensed benzene rings, is produced by incomplete combustion of organic materials such as fossil fuels, other industrial processes, and natural occurrence such as forest fire. It is toxic, mutagenic and carcinogenic to humans [9]. Microbial degradation is believed to be one of the major ways to clean up chrysene-contaminated environments. Microbial communities could have considerable potential to remedy oil-contaminated sediment and remove chrysene from aqueous solution [10,11]. High-molecular-weight PAHs such as chrysene and benzo[*a*]pyrene are hard to be biodegraded whereas lower-molecular-weight PAHs such as phenanthrene and naphthalene are efficiently degraded [12–14]. The efficiency in which PAH is biodegraded in sediment differs from that in liquid medium. Some reports showed that the biodegradation was reduced by sorption to sediments as highly lipophilic PAHs tended to sorb tightly limiting their availability to microorganisms [15,16]. In addition, fungi also play an important role in the degradation of many chemicals, including aromatic hydrocarbons. Fungal oxidation of aromatic hydrocarbons results in the production of metabolites with higher aqueous solubility and less biological reactivity than the parent compound. However, several metabolites generally still toxic as same as the parent compound. White rot fungi possess a number of advantages not associated with other bioremediation systems. The key components of their degrading system are extracellular so the fungi can degrade compounds that are not easily taken up by the cell such as lignin and many hazardous environmental pollutants [17,18]. A diverse group of ligninolytic and non-ligninolytic fungi have the ability to oxidize PAHs. Of these, a number of isolates have been shown to transform chrysene to polar metabolites after

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grow on an alternative carbon source; however only a few isolate can mineralize chrysene [19]. The bioavailability of chrysene in liquid medium may be increased by the application of surfactants. Surfactants increase the bioavailability of organic contaminants by solubilisation of the molecule into hydrophobic core of micelles in solution. This has been observed for surfactants in excess of their critical micelle concentration [20].

In order to eliminate chrysene, degraders should be able to get enough biomass and ideally mineralize and grow on chrysene as carbon and energy source [21]. The present study aims to investigate the method of using fungus isolated from oil-contaminated soil to degrade chrysene in liquid medium. The production of enzyme which plays an important role and metabolites produced during the degradation process were also investigated to evaluate the availability of fungus for bioremediation in soil.

2. Materials and methods

2.1. Microorganism

Polyporus sp. S133 isolated from a petroleum contaminated-soil in Matsuyama city, Ehime, Japan was used for experimentation. The strain was maintained on malt extract agar (2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) polypeptone, and 1.5% (w/v) agar) in a plastic Petri disk at 4 °C prior to use.

2.2. Chemicals

Chrysene used in this study was purchased from Alfa Aesar (Lancaster, UK). 1-Hydroxy-2-naphthoic acid, gentisic acid, protocatechuic acid and catechol were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Malt extract and polypeptone were purchased from Difco (Detroit, USA). Thin layer chromatography (TLC) aluminium sheets (Silica gel 60 F_{254}, 20 cm × 20 cm) were obtained from Merk (Darmstadt, Germany). Salicylic acid, phthalic acid, the silica gel used for column chromatography (wakogel S-1), and all other chemicals were purchased from Wako Pure Chemical Industry Co. Ltd. (Osaka, Japan) at the highest purity available. Chrysene-quinone was prepared from chrysene by dichromate oxidation [22]. Chrysene was dissolved in hot glacial acetic acid (approximately 90 °C) that was saturated in $Na_2Cr_2O_7$. The solution was tightly capped, stirred at 110 °C for 23 h and allow to cool. After ethyl acetate and H_2O was added then the mixture was shaken. The ethyl acetate fraction was collected, and the H_2O fraction was extracted five times more with ethyl acetate. The concentrated, crude chrysene-quinone, obtained in about 60% yield, was purified by column chromatography.

2.3. Culture conditions and fungal inoculums

Polyporus sp. S133 was selected based on its ability to degrade chrysene in a solid medium containing 20 ml of malt extract with the addition of chrysene dissolved in dimethylformamyde (DMF) and 300 mg l^{-1} chloramphenicol or benomill followed by incubation at room temperature for two weeks and observed daily. A single colony of chrysene-degrading fungus was transferred to mineral salt broth medium containing chrysene. The fungus used in the present research was capable of utilizing chrysene as determined using mineral salt broth (MSB) medium containing (in g/l distilled water): glucose (10), KH_2PO_4 (2), $MgSO_4{\cdot}7H_2O$ (0.5), $CaCl_2{\cdot}2H_2O$ (0.1), ammonium tartrate (0.2), and trace elements (10 ml) [23]. The pH of the medium was adjusted to 5.7. The fungal inoculum was prepared by growing each fungus on malt extract agar plates at 25 °C for 7 days. The inoculum was added to a flask containing mineral salt broth medium. Flasks were shaken at 120 rpm for 3 days at 25 °C, and filtered through filter paper under sterile conditions. Mycelia were then added to each vial.

2.4. Experimental design

Experiments were performed in 100-ml Erlenmeyer flasks containing 20 ml of liquid medium plus 1 mM chrysene dissolved in DMF to 1 ml. In addition, as the strains have different growth rates, the period of incubation was varied from 5 to 7 days in order to obtain a similar radial growth and to minimize variation in the starting inoculums. Mycelia plugs of a selected fungus were cut from the outer edge of an actively growing culture on an inoculums plate. Three 5-mm disks obtained by punching out with a cork-borer from the outer edge of an actively growing culture of a particular fungus were inoculated into a flask containing 20 ml of liquid medium supplemented with 1 mM of substrates. The flasks were incubated at 25 °C. Growth and substrate consumption were determined at 7-day intervals. One set of inoculated flasks was incubated stationary. The effect of different carbon source concentrations on the degradation of chrysene was studied using glucose in the concentration range 2–10%. The effect of varying the nitrogen source concentration on the degradation of chrysene was studied by replacing ammonium tartrate with polypeptone in the concentration range 2–10%. The effect of varying the surfactant on chrysene's degradation was studied using tween 80 and tween 20. Agitation at 120 rpm was conducted to enhance the degradation of chrysene in the liquid medium. All media were sterilized by autoclaving at 121 °C for 20 min. Control experiments were performed by incubating chrysene in autoclaved cultures (121 °C for 20 min) and by incubating MSB medium with chrysene without an inoculum. All assays were conducted in triplicate. Before the incubation, a flask of each treatment was selected for immediate extraction. All remaining flasks were incubated for 15 and 30 days. The culture broth was blended with ethyl acetate to extract the aromatic hydrocarbon and metabolites from the mycelia.

2.5. Analytical methods

After the incubation, the culture broth was blended with ethyl acetate and acidified with 1N HCl. The filtrate (liquid medium) and residue (fungal body) were separated by filtration, and the liquid medium and fungal body were extracted with ethyl acetate, respectively. Each extract was combined and purified by silica gel column chromatography using dichloromethane (150 ml). With this method, all substrates initially present in the liquid medium were recovered. The extracts were concentrated and analyzed by gas chromatography–mass spectrometry (GC–MS Shimadzu QP-5050). The amount of substrate was determined using 4-chlorobiphenyl as an internal standard. GC–MS was performed with the following conditions: column TC-1; 30 m in length and 0.25 mm in diameter, helium pressure 100 kPa. The temperature program was started at 80 °C, held for 2 min, raised from 80 to 200 °C at 20 °C/min, then to 260 °C at 7.5 °C/min, then held for 4 min. The flow rate was 1.5 ml/min, interface temperature was 260 °C, and injection volume was 1 μl. The degree of degradation was determined by comparing the amount of chrysene remaining between the control and samples.

2.6. Enzyme assays

The extracellular enzyme production was investigated in mineral salt broth medium. After homogenization at 10,000 rpm, the enzymatic activities in the crude supernatant were determined using an UV–vis spectrophotometer. All activities were expressed in U, defined as the amount of enzyme required to oxidize 1 μmol

of substrate in 1 min. Manganese peroxidase activity was assayed using 50 mM malonate buffer and dimethoxyphenol in 20 mM $MnSO_4$ [24]. One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of dimethoxyphenol per minute and activities were expressed in U l^{-1}. Laccase activity was assayed using syringaldazine in 100 mM sodium acetate buffer [25]. The enzymatic reaction was carried out at room temperature and one unit of activity was defined as the amount of enzyme oxidizing 1 μmol of substrate in 1 min. Lignin peroxidase activity was determined using veratryl alcohol as a substrate [26]. One unit (U) was defined as the amount of enzyme that oxidized 1 μmol of veratryl alcohol per minute and the activity was reported as U l^{-1}. 1,2-Dioxygenase and 2,3-dioxygenase were measured by a modified previous method [27]. 1,2-Dioxygenase and 2,3-dioxygenase activities were assayed using catechol as a substrate. One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute and the activity was expressed in U l^{-1}.

2.7. Detection of metabolites

The MSB medium was prepared as described above. After inoculation of the medium with *Polyporus* sp. S133, the culture was pre-incubated by standing for 7 days at 25 °C in the dark. Chrysene dissolved in 100 μl of DMF and 10 μl of tween 80 (1% solution) was added to each culture medium as described above. The incubation was conducted for 7-30 days at 25 °C in the dark. Thin layer chromatography (TLC) to determine the metabolites of chrysene degradation was performed on a silica gel 60 F_{254} (20 cm × 20 cm, thickness 0.25 mm). Authentic chrysene, 1-hydroxy-2-naphthoic acid, salicylic acid, phthalic acid, protocatechuic acid, catechol, and gentisic asid were used as standards. The extracts were purified using silica gel column chromatography by successive elution with several solvent combinations. The metabolites were tentatively identified by comparing R_f values and UV properties (i.e. quenching under short UV_{254nm} or blue–green fluorescence under long UV_{365nm}) of the samples to those of authentic compounds. As six authentic compounds, viz. 1-hydroxy-2-naphthoic acid, salicylic acid, phthalic acid, protocatechuic acid, catechol, and gentisic asid, could not be detected directly by GC–MS, an analytical derivatization procedure was used to detect these compounds with GC–MS; these compounds were subjected to trimethylsilylation (TMS). Similarly, extract from chrysene-grown cultures were also derivatized and subjected to test for the presence of these six compounds. After the vacuum drying of each eluate from chrysene-grown cultures (100 μl) in a vial, *N*,*O*-bis-trimethylsilyl acetamide (40 μl), pyridine (40 μl), and trimethylchlorosilane (20 μl) were added. Trimethylsilylation of the eluate was conducted for 10 min at 80 °C without contact with moisture. The trimethylsilyl derivatives of the extract were analyzed by gas chromatography (GC) using a Shimadzu GC-17 equipped with a TC-1 capillary column (30 m × 0.25 mm) i.d. 0.25 μm using a gradient of 60 °C for 2 min, raised to 150 °C at 15 °C/min, then raised to 300 °C at 25 °C/min, and maintained at 300 °C for 6 min. Injector and interface temperatures were 260 °C. In order to confirm the metabolites of chrysene degradation and to determine the degradation pathway, a gas chromatograph-mass spectrophotometer, Shimadzu QP5050, was used in this experiment. The conditions for GC–MS consisted of the use of a detector at 1.3 eV, scan intervals of 1 s, and a mass range of 50–500.

3. Results and discussion

3.1. Isolation and identification of fungi

A total of 10 samples of soil were inoculated on a malt extract agar medium containing chrysene. Among the 10 samples isolated, 20 fungi were selected for further screening by the method described above. Six fungi that grew well on the agar medium were selected among the 20. Other isolates were discarded based on their comparatively poor growth on agar medium containing chrysene. One fungus, named S133, was found to be most capable of degrading chrysene. S133 has a cap with an indented cell similar to a honeycomb white in color. Its stem is short, unequal, flared upward, and bent with the cap. Its spore is white, grows in groups of several specimens or in tuft of two or three individuals sometime with the caps welded together, and has clamp connection between hyphae [28,29]. Based on these macroscopic morphological characteristics, S133 was identified as *Polyporus* sp. S133.

3.2. Investigation of degradation of chrysene by selected fungi

The rate of degradation was above 20% for 30 days of incubation with *Polyporus* sp. S133. *Polyporus* sp. S133 degraded 24% of chrysene at 1 mM in 30 days. It was observed that by 30 days in the stationary cultures, *Polyporus* sp. S133 formed filamentous mats at the surface of the growth medium, while in the set incubated with agitation, uniform pellets were formed. From Fig. 1, it could be seen that 65% degradation was achieved in 120 rpm agitated cultures in 30 days, as compared to 24% degradation in stationary cultures. The increased efficiency of degradation could be due to the physiological state of the fungus as pellets and increased mass transfer between the cells and the medium. Biodegradation by white rot fungi has been attributed to the extracellular activity of oxidative enzymes such as laccase. Laccase production was greatest in agitated cultures and hence maximum degradation was achieved in agitated cultures. The oxygen concentration is directly dependent on the air flow rate. Stirring increases the contact between the reagents (substrate, oxygen, and biomass), thus enhancing mass transfer and, as a consequence, the biodegradation rate [30]. In stationary culture, the formation of a mat at the surface restricts the transfer of oxygen to the cells beneath the surface and in the medium resulting in oxygen limitation, which inhibits the oxidative enzymes and prevents degradation.

Fig. 1 also shows the effect of surfactants on the degradation of chrysene. The highest degradation rate was obtained with tween 80 (49%) after 30 days of incubation. When the present cultures were supplemented with tween 80, degradation rates were about 2-fold higher than those obtained in control cultures (without tween 80). Furthermore, enzyme activity levels were maintained throughout the culture, which might be correlated with a greater stability of the isoenzyme produced. One of the benefits of using surfactants like tween 80 is a better dissolution of the very hydrophobic substrates [31]. On the other hand, tween 80 promotes both the uptake

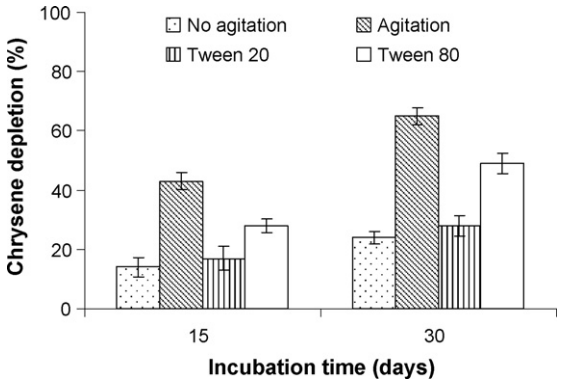

Fig. 1. Effect of agitation and kind of surfactants on degradation of chrysene by *Polyporus* sp. S133.

and release of compounds from the cells through modifications of plasma membrane permeability [32]. Moreover, tween 80 increases the solubility of petroleum components such as chrysene, or lowers the interfacial tension to enhance the mobility of petroleum. Typical desirable properties include solubility enhancement, surface tension reduction, critical micelle concentrations, wet-ability, and foaming capacity [33].

3.3. Investigation of effects of carbon and nitrogen sources on degradation of chrysene

Fig. 2 shows the effect of different concentrations of carbon and nitrogen sources. *Polyporus* sp. S133 degraded 35, 42, 47, 54 and 56% of chrysene at 2, 4, 6, 8 and 10% glucose in 30 days, respectively. Of all the concentrations of carbon sources tested, the lowest rate of degradation of chrysene was observed at 2% glucose (35%). At 8% glucose, the rate of degradation in 30 days was only 54%. Among all the concentrations tested, 10% glucose was the best with 56% degradation. Furthermore, *Polyporus* sp. S133 degraded 44, 46, 50, 64, and 65% of chrysene at 2, 4, 6, 8, and 10% polypeptone in 30 days, respectively. The highest rate of degradation was 65% when *Polyporus* sp. S133 was incubated in the culture with 10% polypeptone. Polypeptone as a nitrogen source, when added to the MSB medium, remarkably increased the rate of degradation of chrysene. The chrysene concentration reached a low level within 30 days. It has been reported that production of laccase by *Pleurotus ostreotus* in the medium supplemented with glucose and polypeptone was high [32]. This could be attributed to the fact that the degradation of chrysene was mainly due to the extracellular enzyme activity. Also glucose as the carbon source and peptone as the nitrogen source have been reported to give high laccase activity [34,35]. Thus with an increase in carbon and nitrogen concentrations, the levels of laccase activity increased. Laccase production increased during cultivation in media with all the investigated carbon and nitrogen sources. Hence, 10% glucose and polypeptone were selected as the best concentrations of carbon and nitrogen sources for degradation of chrysene. No degradation was observed in the control flasks without inoculum.

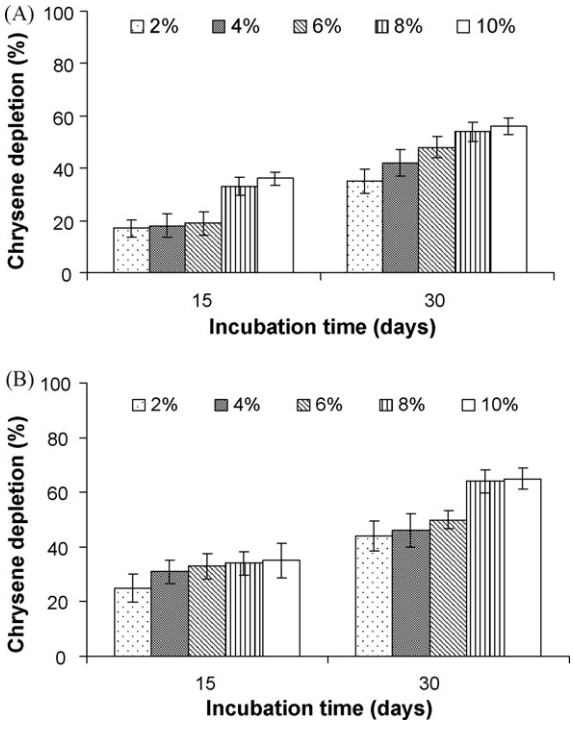

Fig. 2. Effect of nutrients on degradation of chrysene by *Polyporus* sp. S133. (A) Glucose, (B) polypeptone.

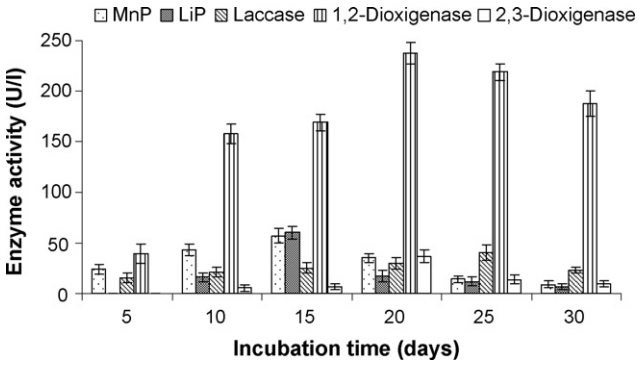

Fig. 3. Change in enzyme activity during incubation of *Polyporus* sp. S133.

3.4. Investigation of enzyme activity of selected fungi in the liquid medium

Several enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP), laccase, 1,2-dioxygenase and 2,3-dioxygenase were detected in the culture produced by *Polyporus* sp. S133. The levels of MnP and LiP activity were highest after 15 days of cultivation (57.1 and 60.3 U l^{-1}) while 1,2- and 2,3-dioxygenase showed the highest level after 20 days (237.5 and 36.8 U l^{-1}) (Fig. 3). *Polyporus* sp. S133 showed the greatest laccase production after 25 days (40.4 U l^{-1}). Those ligninolytic and dioxygenase enzymes play an important role in the oxidization of various environmental pollutants such as chlorophenol, aromatic dyes, and polycyclic aromatic hydrocarbons including chrysene [36]. LiP is able to oxidize various aromatic compounds, while MnP oxidizes almost exclusively Mn(II) to Mn(III), which then degrades phenolic compounds [36]. Laccase is a copper-containing oxidase that reduces molecular oxygen to water and oxidizes phenolic compounds. In most species, peroxidase and laccases are expressed as several isoenzymes. Both types of ligninolytic enzymes are glycosylated, which may increase their stability [37]. Pinyakong et al. showed that dioxygenase is the mechanism to degrade polycyclic aromatic hydrocarbons by bacteria [38]. Dioxygenase initially attacked the aromatic compound at both the 1,2-position and the 2,3-position.

3.5. Identification of metabolites

Seven metabolites were detected during the degradation of chrysene by *Polyporus* sp. S133 (Fig. 4) (Table 1). The TLC and UV–vis spectrophotometer analysis were initially performed to indicate the presence of different intermediates in degradative pathway by combining short- and long-time extracts of the chrysene-grown culture. The GC/MS and MS studies were taken carried out to conclusively prove the presence of these intermediates using short- and long-time extract separately. The identity of six of these metabolites was confirmed using authentic standards. The other compound was chrysenequinone based on the possible initial oxidation reaction with chrysene. By comparing the Total Ion Chromatography (TIC) profiles of III, IV, V, VI, and mass spectra of II and VII with standards, the identity of these compounds could be confirmed. *Polyporus* sp. S133, grown in MSB with chrysene for 7 days, was able to degrade chrysene to chrysenequinone. An analysis of the ethyl acetate-extractable metabolites was conducted using GC/MS, under conditions described in the materials and methods. Compound I (m/z 258, M^+) was possibly chrysenequinone as

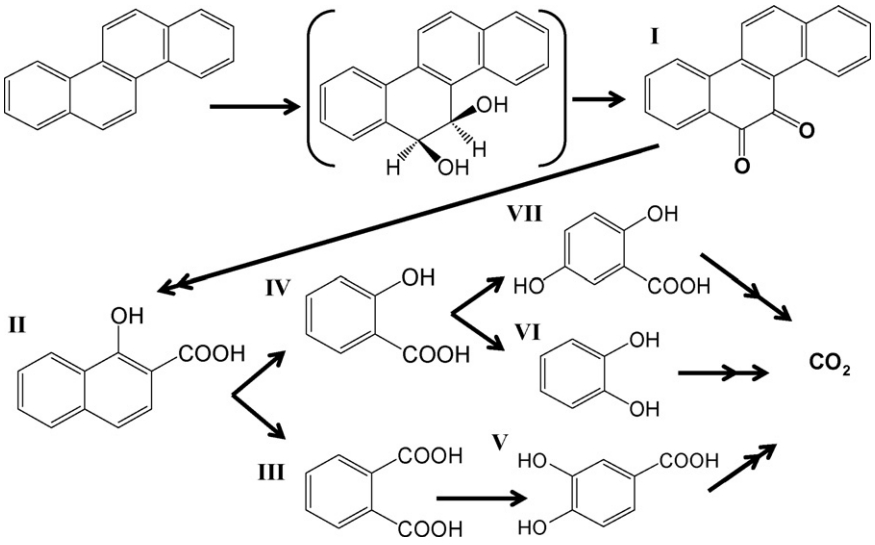

Fig. 4. A proposed pathway for the degradation of chrysene by *Polyporus* sp. S133.

reflected by a major peak at 3.1 min. The GC retention time, MS properties of the M^+ at *m*/*z* 258, and fragment ions at *m*/*z* 230 (M^+-28), corresponding to the respective sequential losses of –CO, were identical to those of synthesized chrysenequinone. *Polyporus* sp. S133, grown in MSB for 15 days with chrysene, was also able to degrade chrysene to compound II. An analysis of compound II isolated from the ethyl acetate-extractable metabolites was measured by mass spectrum. The mass spectrum of compound II shows a molecular ion (M^+) at *m*/*z* 188 and fragment ion at *m*/*z* 170 (M^+-18), were identical to those of authentic 1-hydroxy-2-naphthoic acid.

Polyporus sp. S133 degraded chrysene via 1-hydroxy-2-naphthoic acid to phthalic acid and salicylic acid in similar incubation time. When the short-time extract was subjected to GC/MS analysis, only chrysenequnone could be identified whose retention time and mass-fragmentation pattern matched with the synthesized compound. However, when a long-time extract was analyzed, many peaks from the short-time extract disappeared and new peaks appeared, the retention time and fragmentation pattern of compounds III and IV matched with authentic phthalic acid and salicylic acid, respectively. No chrysene peak was apparent, probably because of the total degradation of chrysene. Phthalic acid and salicylic acid in the extracts were identified using the method described in the experimental section. In the TIC profile, the retention time of these compounds coincided with those of the authentic compounds. The Mass Spectrum (MS) of the TMS ether of these compounds also coincided with those of the authentic TMS ethers of the compounds. Two peaks designated III and IV were obtained from compound II. Compound III was possibly phthalic acid as reflected by a major peak at 8.9 min and its mass spectrum. MS analysis of the phthalic acid produced from 1-hydro-2-naphthoic acid gave an apparent molecular ion [M^+] at *m*/*z* 310 for TMS-derivatives and apparent losses of [M^+-15] at *m*/*z* 295 corresponding to the respective sequential losses of methyl ($-CH_3$), as well as the expected fragment ions at *m*/*z* 147 and 73. Based on the data above, the compound should be phthalic acid. The pattern of the changes of the peak areas of phthalic acid and compound IV during degradation were similar, suggesting that a different metabolic pathway and enzymatic system were utilized for their formation. Compound IV was attributed to be salicylic acid given its major peak at 8.7 min and mass ion spectrum. MS of the salicylic acid produced from 1-hydroxy-2-naphthoic acid gave an apparent loss of [M^+-15] for TMS-derivatives at *m*/*z* 295 corresponding to the respective sequential losses of methyl ($-CH_3$), as well as the expected fragment ions at *m*/*z* 73. Based on the data above, the compound should be salicylic acid.

In order to determine the further metabolites, *Polyporus* sp. S133 was grown in phthalic acid and then the extract were subjected to GC/MS. In phthalic acid grown culture, we found one fraction (compound V), which had a GC retention time of 11.3 min. GC/MS revealed this metabolite to have a molecular ion [M^+] at *m*/*z* 370 and fragment ion [M^+-15] at 355, representing a probable loss of CH_3. The mass spectral fragmentation pattern suggested that this metabolite was protocatechuic acid. This indicates that 1-hydroxy-2-naphthoic acid was converted into protocatechuic acid via phthalic acid.

Table 1
Mass spectra analysis of the principal metabolites detected during the degradation of chrysene by *Polyporus* sp. S133.

Metabolites	*m*/*z* of fragment ions (% relative abundance)	Possible structures
I	258 (12, M^+), 230 (100), 231(21), 202 (20), 200 (18), 201 (13), 228 (5)	Chrysenequinone (confirmed with a synthesized compound)
II	188 (40, M^+), 170 (100), 114 (71), 115 (23), 77 (19)	1-Hydroxy-2-naphthoic acid (confirmed with a standard)
III	310 (3, M^+), 147 (100), 73 (79), 148 (57), 140 (34), 59 (31), 149 (29), 295 (22), 75 (22), 76 (21)	Phthalic acid-TMS derivatives (confirmed with a standard)
IV	267 (92, M^+-15), 73 (100), 268 (37), 147 (22), 74 (21), 75 (18), 149 (18), 135 (17), 91 (15), 209 (14)	Salicylic acid-TMS derivatives (confirmed with a standard)
V	370 (72, M^+), 73 (100), 193 (98), 355 (44), 311 (35), 194 (28), 371 (26), 74 (17), 165 (16), 223 (15)	Protocatechuic acid- TMS derivatives (confirmed with a standard)
VI	254 (5, M^+), 73 (100), 151 (11), 74 (10), 135 (10), 75 (7)	Catechol-TMS derivatives (confirmed with a standard)
VII	154 (100, M^+), 136 (87), 108 (15), 137 (14)	Gentisic acid (confirmed with a standard)

On the other hand, *Polyporus* sp. S133 degraded salicylic acid to catechol and gentisic acid in similar incubation time. An analysis of the ethyl acetate-extractable metabolites was conducted using GC/MS, under normal conditions. By the GC analysis of metabolites after silylation, we detected one TMS-derivative peak. The mass spectrum of the major compound VI having a retention time of 7.1 min exhibited a molecular ion [M^+] at m/z 254 and fragment ion at [M^+-15] at 239 representing a probable loss of CH_3. This metabolite was identified as catechol based on a comparison with the authentic compound. Compound VII (m/z, 154) was possibly gentisic acid as reflected by mass spectrum. Mass spectrum properties of the M^+ at m/z 154, and fragment ions at m/z 136 [M^+-18], were identical to those of authentic gentisic acid.

Many PAHs contain a "bay region" and a K-region". The bay- and K-regions, which can be formed metabolically, are highly reactive both chemically and biologically. As chrysene contains bay- and K-regions, it has been used as a model substrate for studies on the metabolism of bay-region- and K-region-containing carcinogenic PAHs such as benzo(a)pyrene and benzo(a)anthracene [39,40].

Based on the identification of various metabolites produced during the initial ring oxidation and ring cleavage processes, the metabolism of chrysene by *Polyporus* sp. S133, a fungus screened from nature, was successfully explored. The pathways for chrysene's degradation were proposed based on the identification of various metabolites. It is possible that a fungal culture could utilize the dioxygenase system to transform chrysene to *cis*-chrysene or *trans*-chrysene dihydrodiol, and further to dihydroxy chrysene, respectively. However, only chrysenequinone was detected in the present study, suggesting that the fungus utilized the dioxygenase system to transform chrysene. Chrysenequinone was further degraded to 1-hydroxy-2-naphthoic acid. The chemical oxidation of this quinine is a facile reaction, and chrysenequinone is formed as a side product when chrysene is chemically oxidized to produce 1-hydroxy-2-naphthoic acid. GC analysis showed that the overall pattern of chrysenequinone oxidation in vivo resembled the pattern found for chrysene and the major product formed from chrysenequinone was 1-hydroxy-2-naphthoic acid. Our results show that ligninolytic *Polyporus* sp. S133, does not accumulate dihydrodiol. The explanation for this result cannot simply be that the dihydrodiol was both formed and rapidly degraded in our experiments, because we did not find this compound in the medium for several days incubation. These results indicate that *cis*-chrysene or *trans*-chrysene dihydrodiol is not a major intermediate in 1-hydroxy-2-naphthoic acid production from chrysene in ligninolytic culture. Two different classes of enzymes are presumably involved in the degradation of 1-hydroxy-2-naphthoic acid. *Polyporus* sp. S133 can degrade chrysenequinone through a highly complex initial metabolic pathway but this pathway converged into 1-hydroxy-2-naphthoic acid. This reaction is presumably catalyzed by salicylate hydroxylase or equivalent enzymes [37,41]. 1-Hydroxy-2-naphthoic acid can be further degraded via two routes. In one route, it undergoes ring cleavage to form phthalic acid and protocatechuic acid, which is finally cleaved to from pyruvic acid and ultimately enter the tricarboxylic acid cycle. In the other route, 1-hydroxy-2-naphthoic acid undergoes oxidative decarboxylation to form 1,2-dihydroxynaphthalene, which is then subject to *meta*-cleavage to form saliclylic acid. Salicylic acid can also be further degraded via the formation of either catechol or gentisic acid. Both catechol and gentisic acid undergo ring fusion to form tricarboxylic acid-cycle intermediates [40,42]. Some degradation products of PAHs are toxic (e.g., carcinogenic) and incomplete degradation of chrysene may still have threat to ecosystem or even worse in case degradation products would be more toxic than chrysene. The role of manganese peroxidase, lignin peroxidase, and laccase in chrysene's degradation is still unclear.

4. Conclusions

The results shows that chrysene, a PAH was degraded by *Polyporus* sp. S133 in a MSB liquid culture. The maximum degradation rate (65%) was obtained when *Polyporus* sp. S133 was incubated in the agitated cultures supplemented with polypeptone (10%) for 30 days under 120 rpm. Furthermore, the degradation rate was affected by carbon and nitrogen sources as well as surfactants. The degradation mechanism for chrysene by *Polyporus* sp. S133 was determined through identification of several metabolites, chrysenequinone, 1-hydroxy-2-naphthoic acid, phthalic acid, salicylic acid, protocatechuic acid, gentisic acid, and catechol. Several enzymes such as manganese peroxidase, lignin peroxidase, laccase, 1,2-dioxygenase and 2,3-dioxygenase, produced by *Polyporus* sp. S133 were detected during the incubation. The highest level of enzyme activity was shown by 1,2-dioxygenase (237.5 U l^{-1}) after 20 days incubation. The capability of *Polyporus* sp. S133 to degrade chrysene can be used for bioremediation of PAHs-contaminated environments. Further study on potential biodegradability of *Polyporus* sp. S133 to various PAH compounds is needed.

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References

[1] L.H. Keith, W.A. Telliard, Priority pollutants I—a perspective view, Environ. Sci. Technol. 13 (1979) 416–423.
[2] D.J.L. Prak, P.H. Pritchard, Solubilization of polycyclic aromatic hydrocarbon mixtures in micellar nonionic surfactant solutions, Water Res. 36 (2002) 3463–3472.
[3] Y. Liu, L. Zhu, X. Shen, Polycyclic aromatic hydrocarbons (PAHs) in indoor and outdoor air of Hangzhou, China, Environ. Sci. Technol. 35 (2001) 840–844.
[4] C.E. Cerniglia, Biodegradation of polycyclic aromatic hydrocarbons, Curr. Opin. Biotechnol. 4 (1993) 331–338.
[5] O. Potin, E. Veignie, C. Rafin, Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from aged PAH contaminated soil, FEMS Microbiol. Ecol. 51 (2004) 71–78.
[6] J.A. Field, E. DeJong, G.F. Costa, J.A.M. DeBont, Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi, Appl. Environ. Microbiol. 58 (1992) 2219–2226.
[7] T. Eggen, A. Majcherzykb, Removal of polycyclic aromatic hydrocarbon (PAH) in contaminated soil by white rot fungi *Pleurotus ostreotus*, Int. Biodeter. Biodegr. 41 (1998) 111–117.
[8] L. Valentin, T.A. Lu-Chau, C. Lopez, G. Feijoo, M.T. Moreira, J.M. Lema, Biodegradation of dibenzothiophene, fluranthene, pyrene, and chrysene in a soil slurry reactor by the white-rot fungus *Bjerkandera* sp. BOS55, Process Biochem. 42 (2007) 641–648.
[9] R.G. Harvey, Polycyclic Aromatic Hydrocarbons: Chemistry & Carcinogenicity, Cambridge University Press, Cambridge, 1991.
[10] M.A. Ramsay, R.P.J. Swannell, W.A. Shipton, N.C. Duke, R.T. Hill, Effect of bioremediation community in oiled mangrove sediments, Mar. Pollut. Bull. 20 (2000) 413–419.
[11] N.F.Y. Tam, C.L. Guo, W.Y. Yau, Y.S. Wong, Preliminary study on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong, Mar. Pollut. Bull. 45 (2002) 316–324.
[12] L.C. Massie, A.P. Ward, J.M. Davies, The effect of oil exploration and production in the northern North Sea: part 2—microbial biodegradation of hydrocarbons in water and sediments, Mar. Environ. Res. 15 (1985) 235–262.
[13] M.A. Heitkamp, C.E. Cerniglia, Effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in fresh water and estuarine ecosystem, Environ. Toxicol. Chem. 6 (1987) 535–546.
[14] M. Yamada, H. Takada, K. Toyoda, A. Yoshida, A. Shibata, H. Nomura, M. Wada, M. Nishimura, K. Okamoto, K. Ohwada, Study on the fate of petroleum-derived polycyclic aromatic hydrocarbons (PAHs) and the effect of chemical dispersant using an enclosed ecosystem, mesocosm, Mar. Pollut. Bull. 47 (2003) 105–113.
[15] W.F. Guerin, S.A. Boyd, Differential bioavailability of soil-sorbed naphthalene of two bacterial species, Appl. Environ. Microbiol. 58 (1992) 1142–1152.

[16] Y. Laor, P.F. Strom, W.J. Farmer, Bioavailability of phenanthrene sorbed to mineral-associated humic acid, Water Res. 7 (1999) 1719–1729.
[17] R. Martens, F. Zadrazil, Screening of white rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil, Folia Microbiol. 43 (1998) 97–103.
[18] D.P. Barr, S.D. Aust, Mechanisms white rot fungi use to degrade pollutants, Environ. Sci. Technol. 28 (1994) 78–87.
[19] C.E. Cerniglia, Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation, J. Ind. Microbiol. Biotechnol. 19 (1997) 324–333.
[20] S.C. Wilson, K.C. Jones, Bioremediation of soils contaminated with polynuclear aromatic hydrocarbons (PAHs): a review, Environ. Pollut. 88 (1993) 229–249.
[21] M. Alexander, Biodegradation and Bioremediation, Academic Press Inc., San Diego, 1999.
[22] A.I. Vogel, Vogel's Textbook of Practical Organic Chemistry, fifth ed., John Wiley & Sons, Inc., New York, 1989.
[23] D.S. Arora, P.K. Gill, Comparison of two assay procedures for lignin peroxidase, Enzyme Microb. Technol. 28 (2001) 602–605.
[24] H. Wariishi, K. Valli, M.H. Gold, Manganese (II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*-kinetic mechanism and role of chelators, J. Biol. Chem. 267 (1992) 23688–23695.
[25] M. Tien, T.K. Kirk, Lignin-degrading enzyme from *Phanaerochaete crysosporium*. Purification, characterization and catalytic properties of an unique H_2O_2-requiring oxygenase, Proc. Natl. Acad. Sci. 81 (1984) 2280–2284.
[26] M. Kuwabara, J.K. Glen, M.A. Morgan, M.H. Gold, Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanaerochete chrysosporium*, FEBS Lett. 169 (1984) 247–250.
[27] T. Nakazawa, A. Nakazawa, Pyrocatechase (Pseudomonas), in: H. Tabor, W. Tabor (Eds.), Methods in Enzymology, vol. 17a, Academic Press, New York, 1970, pp. 518–522.
[28] T. Laessoe, G. Lincoff, E.H. Edwards, N. Fletcher, Mushrooms, Dorling Kindersley Publishers Ltd., London, 2002.
[29] G. Pace, Mushrooms of the World: With 20 Photographs and 634 Full Color Illustrations of Species and Varieties, Firefly Books Ltd., New York, 1998.
[30] E. Collina, G. Bestetti, P. Di Gennaro, A. Franzetti, F. Gugliersi, M. Lasagni, D. Pitea, Naphthalene biodegradation kinetics in an aerobic slurry-phase bioreactor, Environ. Int. 31 (2005) 167–171.
[31] A. Kapich, M. Hofrichter, T. Vares, A. Hatakka, Coupling of manganese peroxidase-mediated lipid peroxidation with destruction of non-phenolic lignin model compounds and ^{14}C-labelled lignins, Biochem. Biophys. Res. Commun. 259 (1999) 212–219.
[32] M. Asther, G. Corrieu, R. Drapron, E. Odier, Effect of Tween 80 and oleic acid on ligninase production by *Phanaerochaete chrysosporium* INA-12, Enzyme Microbiol. Technol. 9 (1987) 245–249.
[33] D.M. Falatko, Effect of biologically reduced surfactants on the mobility and biodegradation of petroleum hydrocarbons, MS thesis, Virginia Polytechnic Institute and State University, Blackburg, 1991.
[34] M. Stajic, L. Persky, D. Friesem, Y. Hadar, S.P. Wasser, E. Nevo, J. Vukojevic, Effect of different carbon and nitrogen sources on lacacase and peroxidases production by selected Pleurotus species, Enzyme Microbiol. Technol. 38 (2006) 65–73.
[35] J.E.E. Kaal, A.J. Field, W.T. Joyce, Increasing ligninolytic enzyme activities in several white-rot Basidiomycetes by nitrogen-sufficient media, Bioresour. Technol. 53 (1995) 133–139.
[36] T. Mester, M. Tien, Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants, Int. Biodeter. Biodegr. 46 (2000) 51–59.
[37] G. Nie, N.S. Reading, S.D. Aust, Relative stability of recombinant versus native peroxidase from *Phanerochaete chrysosporium*, Arch. Biochem. Biophys. 365 (1999) 328–334.
[38] O. Pinyakong, H. Habe, N. Supaka, P. Pinpanichkarn, K. Juntongjin, T. Yoshida, K. Furihata, H. Nojiri, H. Yamane, T. Omori, Identification of novel metabolites in the degradation of phenanthrene by *Sphingomonas sp.* Strain P2, FEMS Microbiol. Lett. 191 (2000) 115–121.
[39] J.K. Fawell, S. Hunt, The polycylic aromatic hydrocarbons, in: J.K. Fawell, S. Hunt (Eds.), Environmental Toxicology: Organic Pollutants, Ellis Horwood, West Sussex, 1988, pp. 241–269.
[40] D.T. Gibson, V. Subramanian, Microbial degradation of aromatic hydrocarbons, in: D.T. Gibson (Ed.), Microbial Degradation of Organic Compounds, Dekker, New York, 1984, pp. 181–252.
[41] N.V. Balashova, A. Stolz, H.J. Knackmuss, I.A. Kosheleva, A.V. Naumov, A.M. Boronin, Purification and characterization of a salicylate hydroxylase involved in 1-hydroxy-2-naphthoic acid hydroxylation from the naphthalene and phenanthrene-degrading bacterial strain *Pseudomonas putida* BS202-P1, Biodegradation 12 (2001) 179–188.
[42] J.E. Houghton, M.S. Shanley, Catabolic potential of Pseudomonas: a regulatory perspective, in: G.R. Caudhry (Ed.), Biological Degradation and Bioremediation of Toxic Chemicals, Chapman & Hall, London, 1994, pp. 11–32.