

Identification of Phenanthrene Metabolites Produced by *Polyporus* sp. S133

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Abstract—*Polyporus* sp. S133, a fungus collected from contaminated-soil, was used to degrade phenanthrene, a polycyclic aromatic hydrocarbon (PAH), in soil. A maximal degradation rate (89%) was obtained when *Polyporus* sp. S133 was cultured with shitake's nutrient addition for 30 days, as compared to just 44% degradation in non-supplemented cultures. When the fungus *Polyporus* sp. S133 was grown in presence of phenanthrene, two metabolites, 9,10-phenanthrenequinone and 2,2'-diphenic acid were identified using UV-VIS chromatography, gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC). Fungus *Polyporus* sp. S133 initiates phenanthrene degradation via dioxygenation at the C-9 and C10 ring positions to form 9,10-phenanthrenequinone and the *ortho*-cleavage of the 9,10-diol leads to the formation of 2,2'-diphenic acid. Metabolism of phenanthrene in soil by *Polyporus* sp. S133 was investigated for the first time, in this study.

Keywords: phenanthrene, Polycyclic Aromatic Hydrocarbon (PAH), white rot fungus

INTRODUCTION

Many polycyclic aromatic hydrocarbons (PAHs) are considered to be environmental pollutants that can have a damaging effect on the flora and fauna of affected habitats (Sudip *et al.*, 2002). Phenanthrene is a tricyclic compound found in PAH-contaminated environments (Wilson and Jones, 1993). Bioremediation is one of the primary ways for eliminating PAHs from contaminated sites. However, biodegradation of PAHs is restricted by their inadequate bioavailability (Thomas *et al.*, 1986).

During the past decade, a variety of microorganisms have been recognized as capable of catabolizing phenanthrene as a source carbon and energy (Churchill *et al.*, 1999; Bogan *et al.*, 2003). Especially, various white rot fungi such as *Pleurotus* sp. and *Phanerochaete* sp. seem to be involved in the degradation of PAHs containing two or more rings in soil or aquatic environment. During the

past decade, variety of microorganisms capable of catabolizing phenanthrene as a source of carbon and energy has been recognized (Balashova *et al.*, 1999; Churchill *et al.*, 1999). However, degradation pathways for phenanthrene's catabolism by *Polyporus* species are not well known. Under mesophilic conditions, white rot fungi species metabolize phenanthrene at different sites of the molecule, presumably via ligninolytic enzymes and dioxygenase on the aromatic nucleus. The initial attack in the K-region, presumably by a ligninolytic enzyme, leads to the formation of 9,10-phenanthrene quinone (Hammel *et al.*, 1992).

Due to its extremely low water solubility and adsorption on soil particles, bioavailability of phenanthrene is low and hence, biodegradation is slow (Barr and Aust, 1994). Biodegradation is one of the primary means of eliminating phenanthrene from contaminated sites. However, the biodegradation of phenanthrene is restricted by its limited bioavailability (Thomas *et al.*, 1986). The bioavailability of phenanthrene in liquid medium may be increased by the application of surfactants. Surfactants increase the bioavailability of organic contaminants through solubilization into the hydrophobic core of micelles in solution. This has been observed for surfactants in excess of their critical micelle concentration (Wilson and Jones, 1993).

However, further degradation products of 9,10-phenanthrene quinone have not been elucidated by using white rot fungi. The objective of the present study is to investigate the capability of fungi screened from nature to degrade phenanthrene in soil. The metabolites during the degradation process were also investigated to evaluate the availability of fungi for bioremediation in soil.

MATERIALS AND METHODS

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.

Chemicals

Phenanthrene was purchased 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 F₂₅₄, 20 × 20 cm) were obtained from Merck (Darmstadt, Germany). Silica gel used for column chromatography (wakogel S-1), organic solvents and all other chemicals were purchased from Wako Pure Chemical Industry Co. Ltd. (Osaka, Japan). 9,10-Phenanthrenequinone was prepared from phenanthrene by chromate oxidation (Vogel, 1989). Phenanthrene was dissolved in sulfuric acid that was saturated in Na₂Cr₂O₇. The solution was heated to 90–95°C in a water bath and purified phenanthrene and potassium dichromate were added. The solution was then heated in a boiling water bath for further 30 minutes. 9,10-Phenanthrenequinone

was filtered with suction and washed with water until it is free from chromium salt. The concentrated, crude 9,10-phenanthrene quinone, obtained in about 60% yield, was purified by column chromatography.

Culture conditions and fungal inoculum

Cultures of *Polyporus* sp. S133, were grown in 100-ml Erlenmeyer flasks containing 20 ml of mineral salt broth (MSB) medium containing (in g/l distilled water, pH 5.6): glucose (10), KH_2PO_4 (2), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1), ammonium tartrate (0.2), and trace elements (10 ml). The trace element solution comprised (mg l^{-1}): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (12), $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ (3), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (3), $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (1), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (1) (Arora and Gill, 2001). Phenanthrene-containing medium was prepared by adding an aliquot of dimethylformamide-dissolved phenanthrene to sterile flask, and medium and cell were added after the solvent was evaporated. The culture was incubated on a rotary shaker at 25°C. The culture were kept shaking for seven days.

For the degradation studies, soil samples used were collected from an A (0–20 cm) Horizon from Matsuyama and were air dried and sieved through 3 mm mesh. The soil was autoclaved at 120°C for 20 minutes to eliminate microorganisms. To ensure the homogeneity of the treatments, soil samples were then sieved again through 3 mm mesh. The isolated fungus pre-grown on wood meal was applied to the soil surface and then mixed thoroughly with a sterile spatula. The final concentration of phenanthrene in treated soils was measured at 1 ppm plus a specified amount of the factor to be studied: effect of addition of carbon and nitrogen sources. After incubation for several days, soil was extracted using soxhlet for 16 hours. The extracts were purified by column chromatography and the rate of degradation was determined by GC-MS.

Preparation and identification of metabolites

After 7 days of growth, the cells were removed by centrifugation and the supernatants were extracted with three volumes of ethyl acetate. The aqueous fractions after extraction were acidified with concentrated hydrochloric acid to pH 2 and extracted again with three volumes of ethyl acetate. The organic extracts were dried over anhydrous sodium sulfate and the solvent was removed in vacuum at room temperature. Phenanthrene and their degradation products were separated by column chromatography using S-1 silica gel. 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). GC-MS quantification was performed using a column 30 m in length and 0.25 mm in diameter, and with a helium pressure of 100 kPa. The temperature was initially 80°C, held for 2 minutes, raised from 80°C to 200°C at a rate of 20°C/min, then to 260°C at 7.5°C/min, and held for 4 minutes. The flow rate was 1.5 ml/min, interface temperature was 260°C, and injection volume was 1 μl . Degree of degradation was determined by comparison of the remaining phenanthrene between control and samples.

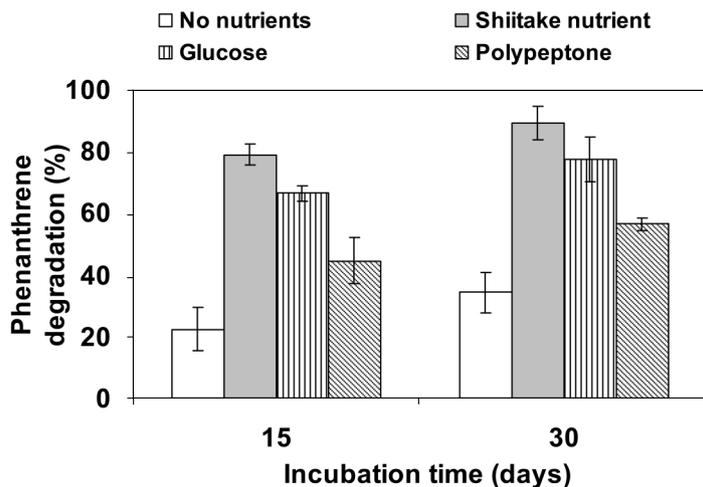


Fig. 1. Effect of nutrients on degradation of phenanthrene in soil by *Polyporus* sp. S133 pre-grown in wood meal.

Authentic phenanthrene and 2,2'-diphenic acid 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 a short wavelength (UV_{254nm}) or blue-green fluorescence under a long wavelength (UV_{365nm})) of the samples to those of authentic compounds. As authentic compounds, viz. 2,2'-diphenic acid, could not be detected directly by GC/MS, an analytical derivatization procedure was used to detect these compounds in GC/MS; these compounds were subjected to trimethylsilylation (TMS). Similarly, extracts from phenanthrene-grown cultures were also derivatized and subjected to tests for the presence of these three compounds.

The mass spectra of individual total ion peaks were identified by comparison with the Wiley mass spectra database. Mass profiles were also compared with spectra of the authentic standards which were analyzed in a similar way. Phenanthrene degradation products in culture extracts were analyzed by TLC on silica gel 60 F₂₅₄ (20 × 20 cm, thickness 0.25 mm) using hexane : chloroform (20:10 v/v) as the solvent system. The locations of the compounds on the TLC plates were detected by the use of UV light. The R_f values of the detected spots were compared with those of authentic compounds known or suspected to be metabolites of phenanthrene degradation.

RESULTS AND DISCUSSION

Degradation of phenanthrene by selected fungi

Figure 1 shows the effect of different nutrients on the degradation of

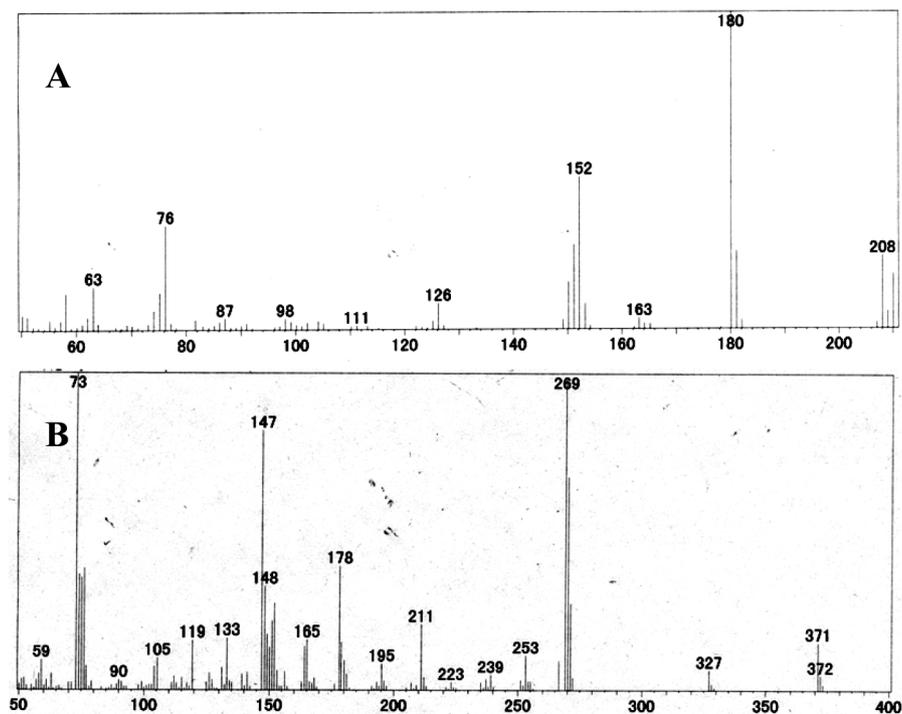


Fig. 2. Mass spectral profiles of phenanthrene metabolites: 9,10-phenanthrenequinone (A) and 2,2'-diphenic acid-TMS derivative (B).

phenanthrene in soil by *Polyporus* sp. S133 pre-grown in wood meal. The lowest degradation rate was observed with polyprptone (56%) after 30 days of incubation. Degradation of phenanthrene with a shitake's nutrient was the best, at 89%. It has been reported that production of laccase by *Pleurotus ostreotus* in the medium supplemented with nutrients was high (Asther, 1987). This could be attributed to the fact that the degradation of phenanthrene was mainly due to the extra cellular enzyme activity. Shitake's nutrient, glucose as the carbon source and peptone as the nitrogen source have also been reported to give high laccase activity (Kaal *et al.*, 1995; Stajic *et al.*, 2006). 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. No degradation was observed in the control flasks without inoculum.

Identification of metabolites

TLC which was obtained using neutral ethyl acetate extractable metabolites of phenanthrene produced by the white rot fungus *Polyporus* sp. S133 showed the

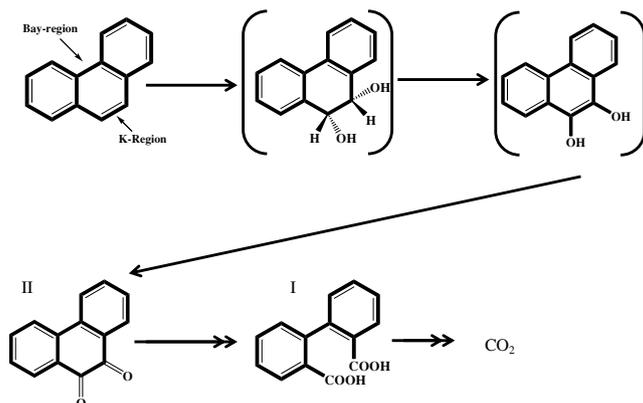


Fig. 3. A proposed pathway for the degradation of phenanthrene by *Polyporus* sp. S133. Compounds within brackets were not identified in our culture extracts.

presence of several metabolites. One metabolite (I) having an R_f value of 0.44, gave an UV spectrum with λ_{\max} of 282, 346, and 416 nm, similar to that of synthesized 9,10-phenanthrenequinone. The spectrum of compound I (m/z 208, M^+) that had a retention time (t_R) 13.4 min is shown in Fig. 2A. The GC retention time, MS properties of the M^+ at m/z 208, and the significant fragment ions at m/z 152 and 180 (M^+-28), corresponding to the respective sequential loss of $-CO$, were identical to those of synthesized 9,10-phenanthrenequinone. Another metabolite (II) with an R_f value of 0.38, gave an UV spectrum with λ_{\max} of 225 and 285 nm, similar to that of the authentic 2,2'-diphenic acid standard. The GC/MS analysis of compound II (Fig. 2B) with a GC retention time of 14.7 min indicated a di-TMS compound. MS analysis of the 2,2'-diphenic acid produced from phenanthrene gave an apparent molecular ion at m/z values of 371 (M^+-15), sequential losses of methyl ($-CH_3$), 269 (M^+-117), sequential loss of $-COOSi(CH_3)_3$, as well as the expected fragment ions at 147, 117 and 73 [$(CH_3)_3Si$].

The white rot fungus *Polyporus* sp. S133 grows in soil with phenanthrene as one of the sources for carbon and energy. Analysis of the extracts showed that fungus *Polyporus* sp. S133 grown on PAH did not accumulate large quantities of aromatic intermediates throughout the culture period. Low levels of metabolites production by some PAH-degrading microorganisms may be related to a physical interaction between the cells and the hydrophobic substrates (Guerin and Jones, 1988).

Based on the identification of various metabolites produced during initial ring oxidation and ring cleavage process, growth substrate range experiments, the metabolism of phenanthrene by *Polyporus* sp. S133 was explored. The degradation pathway of phenanthrene by *Polyporus* sp. S133 is shown in Fig. 3. Identification of 9,10-phenanthrenequinone and 2,2'-diphenic acid, in culture extracts indicate

that *Polyporus* sp. S133 initiates its attack on phenanthrene either by dioxygenation at C9 and C10 to give *cis*-9,10-dihydrodiol. Dehydrogenation of phenanthrene-*cis*-9,10-dihydrodiol to the corresponding diol, followed by *ortho*-cleavage of the oxygenated ring, would yield the identified 2,2'-diphenic acid via 9,10-phenanthrenequinone.

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