

Oxidative Degradation of Benzo[a]pyrene by the Ligninolytic Fungi

Tony HADIBARATA^{1,2}

¹*Department of Applied Bioscience, Faculty of Agriculture, Ehime University,
3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan*

²*Laboratory of Pulp, Paper and Environment, Department of Forest Product Technology,
Mulawarman University, Kampus Gunung Kelua, Jl. Ki Hajar Dewantara No. 1,
Samarinda 75119, Indonesia*

(Received 9 January 2009; accepted 24 April 2009)

Abstract—A maximal degradation rate (76%) was obtained when *Polyporus* sp. S133 was cultured with 0.05% of tween 80, as compared to just 35% degradation in non-supplemented cultures. When fungus *Polyporus* sp. S133 was grown in presence of shitake's nutrient, the degradation rate increased up to 69% as compared to just 56 and 40% degradation in glucose and polypeptone supplemented cultures. One metabolite was identified as benzo[a]pyrene-6,12-quinone, using UV-VIS chromatography, gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC). Fungus *Polyporus* sp. S133 initiates benzo[a]pyrene degradation via deoxygenation at the C-6 and C12 ring positions to form quinone compound. For the first time, metabolism of benzo[a]pyrene in soil by *Polyporus* sp. S133 was investigated.

Keywords: benzo[a]pyrene, 5-rings PAH, ligninolytic fungus

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are highly toxic organic pollutants which are widely distributed in terrestrial and aquatic environments. They consist of benzene rings which have two or more fused aromatic rings in linear, angular, or clustered alignment and are mainly formed as products of incomplete combustion of fossil fuels. Until now, research on the microbial degradation of PAHs has shown that microorganisms such as bacteria, fungi and algae possesses catabolic abilities that may be utilized for the remediation of PAH-contaminated soil and water. Recently, bioremediation has been shown to be effective in remediating soils contaminated with low molecular weight PAHs (Mueller *et al.*, 1991; Kastner and Mahro, 1996). However, high molecular weight PAHs are generally recalcitrant to microbial attack (Park *et al.*, 1990; Cerniglia, 1992). Although the lack of microbial activity towards high molecular weight PAHs may be attributed to site specific environmental factors, such as bioavailability of the contaminant, nutrients, redox potential, etc., the limiting factor may be the scarcity of microorganisms capable degrading the more highly condensed compounds. For

bioremediation to be an effective tool for the clean up of PAH-contaminated soils, a greater understanding of the processes involved in limiting the degradation of high molecular weight PAHs, is required.

Although some low molecular weight PAHs such as the tricyclic anthracene, are not carcinogenic, their oxidation mechanisms are of considerable interest as the same arrangements of fused aromatic rings are found in the more complex carcinogenic PAHs such as benzo[a]pyrene and benzo[a]anthracene. The main methods utilized for eliminating BaP from the environment are: microbiological transformation and degradation, volatilization, photo-oxidation, chemical oxidation and bioaccumulation (Wilson and Jones, 1993). Conventional bioremediation methods are often ineffective or time consuming due to the inherent toxicity of the metabolites. For wastewater contaminated with phenolic compounds, the extent of degradation obviously increased after pretreatment with hydrogen peroxide which was then followed by biodegradation (Bowers *et al.*, 1989).

Many fungi have been shown to oxidize BaP by mechanisms similar to those observed in mammals. Fungal oxidation of BaP results in the formation of trans-dihydrodiols which supports the hypothesis that fungal BaP oxidation is mediated by cytochrome P-450 (Cerniglia and Gibson, 1980). Alternatively, the oxidation of BaP may be catalyzed by different enzymatic mechanisms involving extracellular peroxidases and laccase (Launen *et al.*, 1995). In the present study, we describe the results of an investigation on the degradation of BaP by *Polyporus* sp. S133, a fungus collected from petroleum-contaminated soil and capable of degrading polycyclic aromatic hydrocarbons (PAHs). The metabolic pathway for BaP was also examined.

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

Benzo[a]pyrene (BaP) 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 Merk (Darmstadt, Germany). 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).

Culture conditions and fungal inoculum

Polyporus sp. S133 was selected based on its ability to degrade PAHs in a

solid agar medium containing 20 ml of malt extract agar with the addition of BaP dissolved in dimethylformamide (DMF) and 300 mg l⁻¹ benomyl. It was then incubated at room temperature for two weeks and observed daily. A single colony of BaP-degrading fungus was transferred to a mineral salt broth medium containing BaP. The mineral salt broth (MSB) medium contained (in g/l distilled water, pH 5.6): glucose (10), KH₂PO₄ (2), MgSO₄·7H₂O (0.5), CaCl₂·2H₂O (0.1), ammonium tartrate (0.2) and trace elements (10 ml). The trace element solution comprised (mg l⁻¹): FeSO₄·7H₂O (12), MnSO₄·7H₂O (3), ZnSO₄·7H₂O (3), CoSO₄·7H₂O (1), (NH₄)₆Mo₇O₂₄·4H₂O (1) (Arora and Gill, 2001). 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 the mineral salt broth medium. Mycelia were then transferred to each vial containing fresh medium.

Soil treatment

Polyporus sp. S133, a fungus isolated from soil, was selected for its ability to degrade chrysene. Soil samples used were collected from an A (0–20 cm) horizon in the Matsuyama area and were air dried and sieved through 3 mm mesh. The soil was autoclaved at 120°C for 20 minutes to eliminate any microorganisms. To ensure the homogeneity of the treatments, soil samples were then sieved again through 3 mm mesh. The isolated fungus pregrown on wood meal was applied to the soil surface and then mixed thoroughly with a sterile spatula. The final concentration of BaP in treated soils was measured at 1 ppm plus a specified amount of the factor to be studied: effect of nutrients and some concentration of tween 80. After incubation for several days, soil was Soxhlet extracted for 16 hours. The extracts were purified by column chromatography and the rate of degradation was determined by GC-MS.

Analytical methods

After 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 bodies were extracted with ethyl acetate. Each extract was combined and purified by column chromatography using dichloromethane. The extracts were concentrated and analyzed by Gas chromatography-mass spectrometry (GC/MS, Shimadzu QP-5050). GC/MS was performed with a column 30 m in length and 0.25 mm in diameter, and a helium pressure of 100 kPa. The temperature was initially 80°C, held for 2 minutes, raised from 80°C to 200°C at 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 BaP between control and samples.

Detection of metabolites

The Mineral Salt Broth (MSB) medium was prepared as described above.

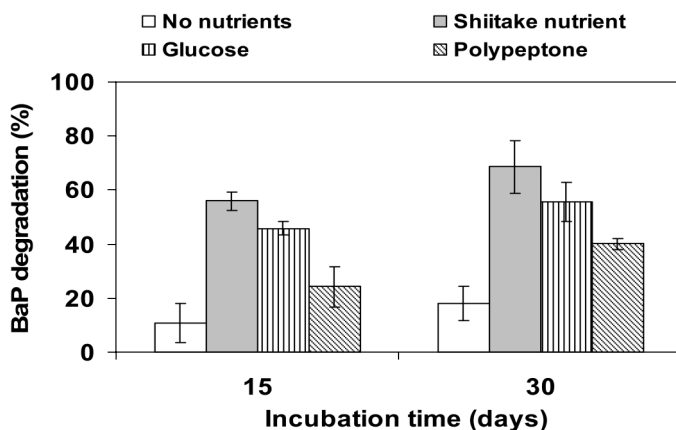


Fig. 1. Effect of nutrients on degradation of BaP in soil by *Polyporus* sp. S133 pre-grown in wood meal.

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. BaP dissolved in 100 μL of dimethylformamide (DMF) and 10 μL of tween 80 (1% solution) were added to the culture medium as described above. The incubation was conducted for 1–30 days at 25°C in the dark. UV-VIS absorption spectra were recorded on a UV-VIS Spectrophotometer (Shimadzu 1600). The metabolites were identified by comparing their retention times, mass spectra and absorption spectra with those of the corresponding authentic 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. The extracts from BaP-grown cultures were also analyzed by gas chromatography (GC) using a Shimadzu GC-17 equipped with a TC-1 capillary column (30 m \times 0.25 mm) ID 0.25 μm using a gradient of 60°C for 2 minutes, raised to 150°C at 15°C/min, then raised to 300°C at 25°C/min, and maintained at 300°C for 6 minutes. Injector and interface temperatures were 260°C. In order to confirm the metabolites of BaP 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. The mass spectra of individual total ion peaks were identified by comparison with the Wiley7 mass spectra database. Mass profiles were also compared with spectra of the authentic standards which were analyzed similarly. BaP degradation products in culture extracts were analyzed by TLC on silica gel 60 F₂₅₄ (20 \times 20 cm, thickness 0.25

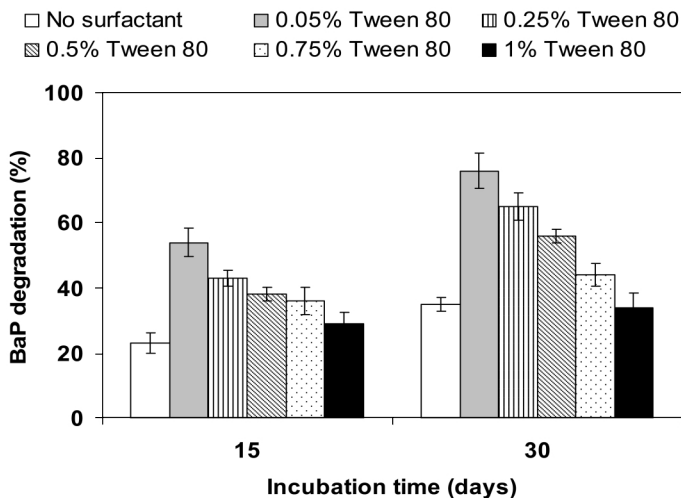


Fig. 2. Effect of surfactant concentration on degradation of BaP by *Polyporus* sp. S133 in a soil.

mm) using several solvent systems for short-term and long-term incubation. 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 in BaP degradation.

RESULTS AND DISCUSSION

Effects of nutrients and surfactants concentration on degradation of BaP in the soil.

Figure 1 shows the effect of different nutrients on the degradation of BaP in soil by *Polyporus* sp. S133 pre-grown in wood meal. The lowest degradation rate was observed with polyprptone (40%) after 30 days of incubation. Degradation of BaP with shitake's nutrients was best, at 69%.

Figure 2 shows the degradation of BaP in the absence or presence of the surfactant, tween 80 added at five different concentrations (0.05, 0.25, 0.50, 0.75, and 1%). The degradation of BaP in soil was affected by the presence of tween 80. *Polyporus* sp. S133 degraded 76, 65, 56, 44 and 34% of BaP at 0.05, 0.25, 0.50, 0.75, and 1% tween 80 in 30 days, respectively. Degradation was inhibited in the absence of tween 80 (35%).

This result is similar to that of Fava *et al.* (2004) that tween 80 is expected to increase the desorption rates of hydrophobic organic contaminants (HOC) from solid matrix of soil and to improve their pseudo-solubilization in the aqueous phase. The mass transfer rate of HOC to the aqueous phase is considered to be one of the key factors governing the biodegradation rate. Therefore, the use of tween 80 has been suggested to be a valuable approach to enhance microbial

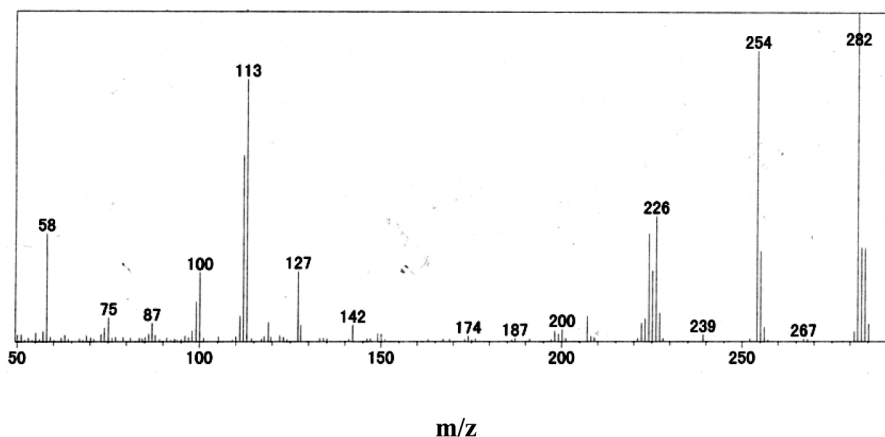


Fig. 3. Mass spectral profiles of Bap-6,12-quinone produced from BaP by *Polyporus* sp. S133.

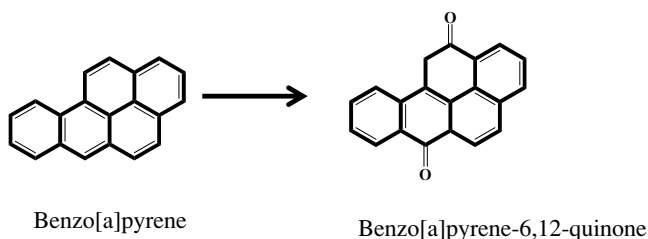


Fig. 4. Proposed pathway for the degradation of BaP by *Polyporus* sp. S133.

degradation of HOC (Bogan and Lamar, 1995). The present study was conducted to determine whether surfactants in water added to the soil surface would promote biodegradation of aromatic hydrocarbons present at a point beneath the soil surface. The means by which the surfactants enhance microbial destruction of the aromatic hydrocarbons was proven. Previous data suggest that surfactants at low concentrations promote biodegradation of aromatic hydrocarbons in soils of highly different organic matter content without altering the extent of desorption (Aronstein *et al.*, 1991). More recently, we found that surfactants at low concentrations substantially increased the biodegradation of aromatic hydrocarbons in inoculated samples of aquifer sand and soils. Emulsification is known to enhance hydrocarbon metabolism and surfactants may increase the enzymatic activity of microorganism or facilitate transport of the organic substrate into microbial cell. However, it is possible that the surfactants decrease the strength of binding of the organic molecules to the soil surface, making the compounds more readily available for microbial utilization (Berg *et al.*, 1990).

Identification of metabolites

One metabolite was detected during the degradation of BaP by *Polyporus* sp. S133. The TLC and UV-VIS spectrophotometer analyses were initially performed to indicate the presence of different intermediates in the degradation pathway by combining short- and long-term incubation extracts of the BaP-grown culture. The GC/MS and MS studies were carried out to conclusively prove the presence of these intermediates using short- and long-term incubation extracts separately. The identity of four of these metabolites was confirmed using authentic standards. One metabolite (I) having an R_f value of 0.5, gave an UV spectrum with λ_{\max} of 202, 252, 284, 296, 410 and 418 nm, similar to that of synthesized benzo[a]pirene-6,12-quinone. The spectrum of compound I (m/z 258, M^+) that had a retention time (t_R) 19.6 min is shown in Fig. 3. The GC retention time, MS properties of the M^+ at m/z 258, and the significant fragment ions at m/z 282, 267 and 230 ($M^+ - 28$), corresponding to the respective sequential losses of $-CO$, were identical to those of synthesized benzo[a]pirene-6,12-quinone.

The proposed degradation pathway of BaP by *Polyporus* sp. S133 is shown in Fig. 4. *Polyporus* sp. S133 grows in MSB medium with BaP as the source of carbon and energy. Analysis of the culture extracts showed that *Polyporus* sp. S133 did not accumulate large quantities of aromatic metabolites throughout the culture period. Low levels of metabolite production by some PAH-degrading fungi may be related to physical interaction between the cell and the hydrophobic substrates (Guerin and Boyd, 1992). Based on the identification of various metabolites produced during the initial ring oxidation and ring cleavage processes, the metabolism of BaP by *Polyporus* sp. S133 was explored.

These results showed that 6,12-oxidation and ring cleavage to give BaP-6,12-quinone is the major fate of BaP in ligninolytic *Polyporus* sp. S133. BaP metabolism in ligninolytic *Polyporus* sp. S133 differs from the pathway employed by most bacteria, which cleave this PAH between C3 and C4 (Gibson and Subramanian, 1984), and also differs from the process in nonligninolytic fungi and other eukaryotes, which are incapable of PAH ring fission (Gibson and Subramanian, 1984; Cerniglia and Yang, 1984). The identification of BaP-6,12-quinone in culture extracts indicates that *Polyporus* sp. S133 initiates its attack on BaP by dioxygenation at C6 and C12. Dehydrogenation of BaP-*cis*-6,12-dihydrodiol to the corresponding diol, followed by *ortho*-cleavage of the oxygenated ring, would yield the identified BaP-6,12-quinone.

REFERENCES

- Aronstein B. N., Y. M. Calvillo and M. Alexander (1991): Effect of surfactants at low concentration on the desorption and biodegradation of sorbed aromatic compounds in soil. *Environ. Sci. Technol.*, **25**, 1728–1731.
- Arora, D. S. and P. K. Gill (2001): Comparison of two assay procedures for lignin peroxidase. *Enzyme Microb. Technol.*, **28**, 602–605.
- Berg, G., A. G. Seech, H. Lee and J. Y. Trevor (1990): Identification and characterization of a soil bacterium with extracellular emulsifying activity. *J. Environ. Sci. Health A*, **25**, 753–764.
- Bogan, B. W. and R. T. Lamar (1995): One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.*

- 61**, 2631–2635.
- Bowers, A. R., P. Gaddipati, W. W. Eckenfelder and R. M. Monsen (1989): Treatment of toxic or refractory wastewater with hydrogen peroxide. *Water Sci. Technol.*, **21**, 477–486.
- Cerniglia, C. E. (1992): Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation*, **3**, 351–368.
- Cerniglia, C. E. and D. T. Gibson (1980): Fungal oxidation of benzo(a)pyrene and trans 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene: evidence for the formation of a benzo(a)pyrene 7,8-diol-9,10-epoxide. *J. Biol. Chem.*, **255**, 5159–5163.
- Cerniglia, C. E. and S. K. Yang (1984): Stereoselective metabolism of anthracene and phenanthrene by the fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol.*, **47**, 119–124.
- Fava, F., S. Berselli, P. Conte, A. Piccolo and L. Marchetti (2004): Effect of humic substances and soya lecithin on the aerobic bioremediation of a soil historically contaminated by polycyclic aromatic hydrocarbons (PAHs). *Biotechnol. Bioeng.*, **88**, 214–223.
- Gibson, D. T. and V. Subramanian (1984): Microbial degradation of aromatic hydrocarbons. p. 181–252. In *Microbial Degradation of Organic Compounds*, ed. by D. T. Gibson, Dekker, New York, U.S.A.
- Guerin, W. F. and S. A. Boyd (1992): Differential bioavailability of soil-sorbed naphthalene of two bacterial species. *Appl. Environ. Microbiol.*, **58**, 1142–1152.
- Kastner, M. and B. Mahro (1996): Microbial degradation of polycyclic aromatic hydrocarbons (PAHs) in soils affected by the organic matrix of compost. *Appl. Microbiol. Biotechnol.*, **44**, 668–675.
- Launen, L., L. Pinto, C. Wiebe, E. Kiehlmann and M. Moore (1995): The oxidation of pyrene and benzo[a]pyrene by nonbasidiomycete soil fungi. *Can. J. Microbiol.*, **41**, 477–488.
- Mueller, J. G., S. E. Lantz, B. O. Blattmann and P. J. Chapman (1991): Bench-scale evaluation of alternative biological treatment process for the remediation of pentachlorophenol and creosote contaminated materials: solid phase bioremediation. *Environ. Sci. Technol.*, **25**, 1045–1055.
- Park, K. S., R. C. Sims and R. Dupont (1990): Transformation of PAHs in soil systems. *J. Environ. Eng.*, **116**, 632–640.
- Wilson, S. C. and K. C. Jones (1993): Bioremediation of soils contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environ. Pollut.*, **88**, 229–249.