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Enhanced Chrysene Biodegradation in Presence of a Synthetic Surfactant

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Abstract—To degrade chrysene, a polycyclic aromatic hydrocarbon (PAH) found in crude oil contaminated soil by bioremediation the *Polyporus* sp. S133, a fungus collected from soil was used. Maximal degradation (86%) was obtained when *Polyporus* sp. S133 was incubated in 0.5% tween 80 for 30 days, as compared to 30% degradation rate without tween 80. Chrysene was indeed degraded by *Polyporus* sp. S133 through several intermediates, chrysenequinone and 1-hydroxy-2-naphthoic acid were detected among the reaction products.

Keywords: chrysene, biodegradation, metabolites, *Polyporus* sp. S133

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds in the environment and they are known or suspected to be genotoxic or carcinogenic (White, 1986). The study of their fate in nature is therefore a matter of great environmental concern. Persistence of PAHs in the environment is linked to their hydrophobic character and low water solubility. Furthermore, they often get adsorbed to soil or sediments. As a consequence, they are poorly available for degrading microorganisms. One possible way of enhancing their bioavailability for their easy biodegradation is the addition of biological or chemical surface active agents (surfactants). There amphipathic molecule are known to be effective for solubilization of hydrophobic components by reducing surface and interfacial tension and by increasing desorption. However, the very low solubility of more complex PAH, in fact, strongly reduces their bioavailability and makes microbial growth and biodegradation difficult. The degradation is also limited by substrate bioavailability in laboratory conditions, where the PAH are supplied in solid form (Boldrin *et al*., 1993).

Chrysene is a symmetrical polycylic aromatic hydrocarbon consisting of four condensed benzene rings. Chrysene is a by-product formed from the

incomplete combustion or pyrolysis of coal and crude oil. At high temperatures the reaction produces fragmented molecules and radicals that together form the basis of PAH's. In crude petroleum refining, chrysene is formed from the catalytic cracking in the high boiling fraction. The distillation or pyrolysis of many fats and oils can also produce chrysene in very small quantities. The following exposure limits have been set for all polycyclic aromatic hydrocarbons including chrysene (Harvey, 1991).

Microbial degradation is believed to be one of the major processes to clean up PAH-contaminated environments. Microbial communities could have a considerable potential to remedy the oil-contaminated sediments and remove PAHs from aqueous solution (Ramsay *et al*., 2000; Tam *et al*., 2002). The efficiency of PAH biodegradation in sediment was different from that in liquid medium. Some reports showed that PAH biodegradation was reduced by sorption to sediments as highly lipophilic PAH tended to sorb tightly on sediments and limited its availability to microorganism (Guerin and Boyd, 1992; Laor *et al*., 1999). Fungi 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 generally less biological reactivity than the parent compounds.

The purpose of this study is to investigate the effect of nonionic surfactant in chrysene degradation by the white rot fungus *Polyporus* sp. S133. The metabolic products in the degradation of chrysene were also investigated.

MATERIALS AND METHODS

Fungus

The fungus *Polyporus* sp. S133 was isolated from a petroleum contaminated soil. Isolation and culture media are described elsewhere (Hadibarata *et al*., 2008).

Chemicals

Chrysene used in this study was purchased from Alfa Aesar (Lancaster, UK). The nonionic chemical surfactant tween 80 [poly(oxyethylene)$_{20}$ sorbitan monooleate]. 1-Hydroxy-2-naphthoic acid was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Malt extract and polypeptone were purchased from Difco. Thin layer chromatography (TLC) aluminium sheets (Silica gel 60 F_{254}, 20 × 20 cm) were obtained from Merck (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) at the highest available purity. Chrysene-quinone was prepared from chrysene by dichromate oxidation (Vogel, 1989). 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 allowed to cool. After cooling ethyl acetate and water was added and the mixture was shaken. The ethyl acetate

fraction was collected, and the water fraction was extracted five times more with ethyl acetate. The concentrated, crude chrysenequinone (about 60% yield), was purified by column chromatography.

Chrysene degradation in soil

The air dried soil was passed through a 3-mm sieve prior to use. Biodegradation assay were carried out for 15 and 30 days in plastic boxes containing 200 g of sieved soil. These boxes were inoculated to give an initial concentration of 15% white rot fungus pre-grown on wood meal for each inoculation treatment. Three replicate were run for each control. All soil samples were moistened to approx. 60% of their water holding capacity. After inoculation, the boxes were closed with sterile cotton plugs and incubated in the dark at room temperature.

Extraction of chrysene

Optimum extraction conditions were determined after preliminary assays. The following conditions were selected. After 15 and 30 days incubation, soil were oven-dried (30°C, overnight) before extraction. Samples (30 g) were extracted in a Soxhlet apparatus for 16 h with dichloromethane. With this method, 96% of chrysene initially present in soils was 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. GCMS was used with the following conditions: column 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 minutes, raised from 80°C to 200°C at 20°C/min, then to 260°C at 7.5°C/min, then held for 4 minutes. The flow rate 1.5 ml/min, interface temperature 260°C, and injection volume 1 μl. Degree of degradation was determined by comparison of the remaining chrysene between control and samples.

Detection of metabolites

The soil was prepared as described above. After inoculation of the soil with *Polyporus* sp. S133, the soil was pre-incubated upon standing for 4 days at 25°C in the dark. Chrysene dissolved in 100 μL of dimethylformamide (DMF) and 10 μL of tween 80 (1% solution) were added to each culture medium as described above. The incubation was conducted from 7–20 days at 25°C in the dark. The extracts were purified using silica gel column chromatography by successive elution with several solvent combinations. After the vacuum drying of each eluate (100 μL) in a vial, N, O-bis-trimethylsilyl acetamide (40 μL), pyridine (40 μL), and trimethylchlorosilane (20 μL) were added, in succession. Trimethylsilylation of the eluate was conducted for 10 minutes at 80°C without contact with moisture. The trimethylsilyl (TMS) derivatives of the extract were analyzed by gas chromatography (GC) using 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

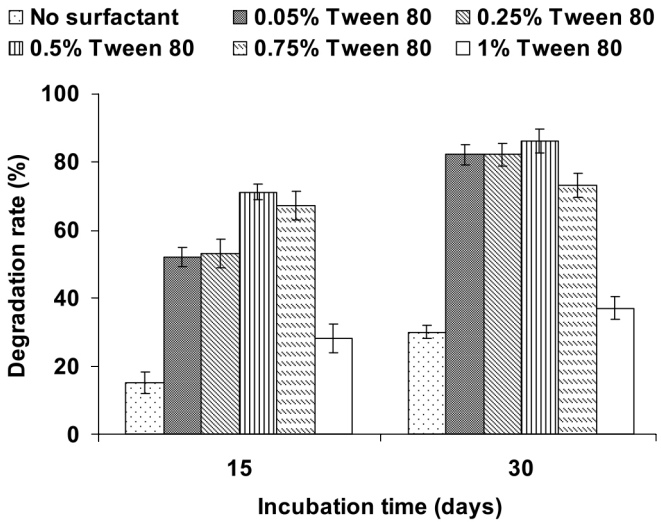

Fig. 1. Effect of surfactant concentration on degradation of chrysene by *Polyporus* sp. S133 in a soil.

300°C for 6 minutes. Both injector and interface temperature was 260°C. In order to confirm the intermediates/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 analysis consisted of a detector at 1.3 eV, scan intervals of 1 s, and mass range of 50–500.

RESULTS AND DISCUSSION

Effect of tween 80 on degradation rate

The impact of addition of tween 80 was assessed during the *Polyporus* sp. S133 growth on chrysene. Figure 1 shows the degradation of chrysene in the absence and presence of surfactants added at five different concentrations (0.05, 0.25, 0.50, 0.75, and 1%) of tween 80. The degradation of chrysene in soil was affected by the presence of tween 80. *Polyporus* sp. S133 degraded 82, 82, 86, 73 and 37% of chrysene 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 (30%).

This result shows that biodegradation activity of chrysene-degrading fungi is influenced by the bioavailability of chrysene in aqueous phase. By increasing the solubility of solid chrysene, tween 80 facilitates the transport of this organic substrate to microbial cells and enhance the metabolism of this hydrocarbon. This positive effect on biodegradation rate is not a general response to the addition of surfactant since some surfactants may also inhibit biodegradation (Laha and Luthy, 1992; Thiem, 1994; Stelmack *et al*., 1999). This result is also similar to

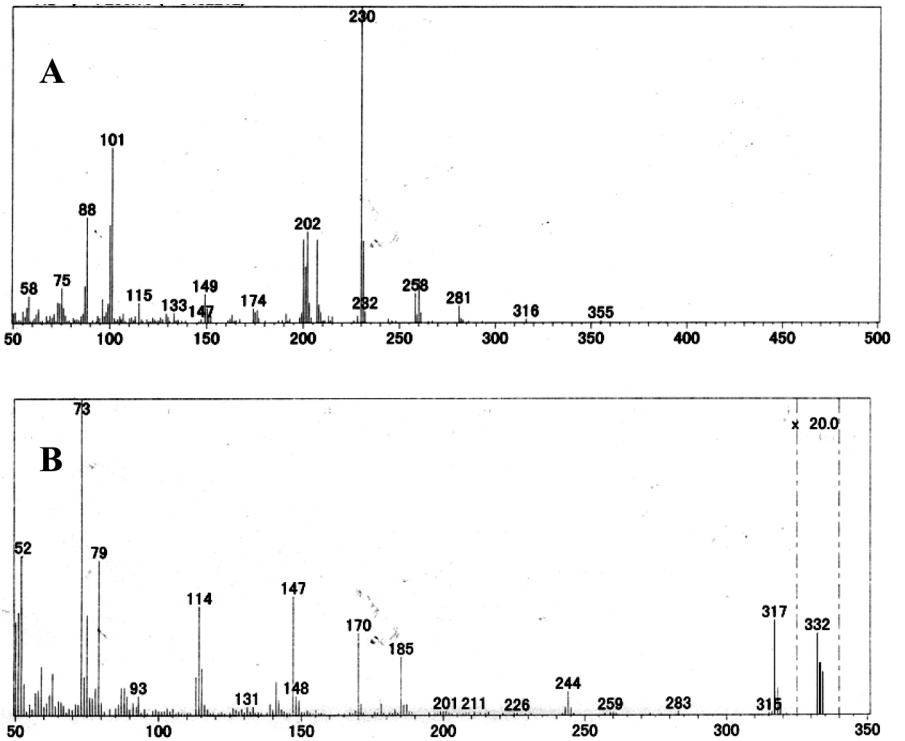

Fig. 2. Mass spectral profiles of chrysene metabolites: chrysenequinone (A) and 1-hydroxy-2-naphthoic acid-TMS derivative (B).

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 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 degradation of HOC (Bogan and Lamar, 1995). The present study was conducted to determine whether a surfactant 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 surfactant enhances microbial destruction of the aromatic hydrocarbons was proven. Previous data suggest that surfactants at low concentration 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 note that surfactants at low concentration substantially increased the biodegradation of aromatic hydrocarbons in inoculated samples of aquifer sand and soils (Aronstein and Alexander, 1992). Emulsification is known to enhance hydrocarbon metabolism and surfactants may increase the enzymatic

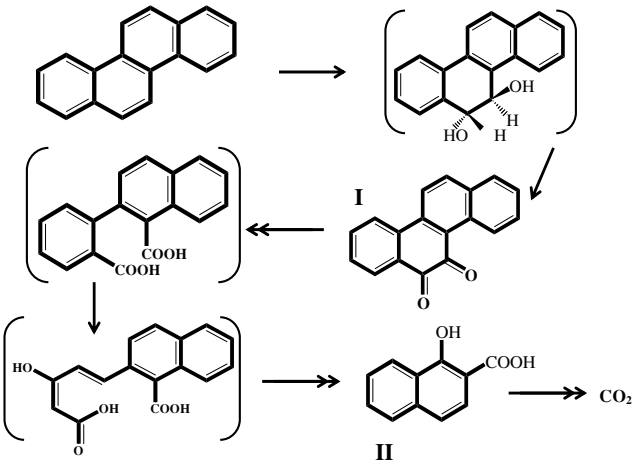

Fig. 3. A proposed pathway for the degradation of chrysene by *Polyporus* sp. S133. Compounds in brackets were not identified in our culture extracts.

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). It seem that the potential use of surfactants for improved hydrocarbon pollutants elimination depends mainly on physiological capabilities of fungi communities, the chemical structure of the hydrocarbon and on environmental factors such as soil characteristics.

Identification of metabolites

Two metabolites were detected during the degradation of chrysene by *Polyporus* sp. S133. The identity of two metabolites was confirmed by their corresponding authentic standards and finally synthesized compound. Based on the possible oxygenase initial reaction of chrysene, the synthesized compound was confirmed as chrysenequinone as follows. By comparing the GC/DI elution profile of I and II with standard, the identity of these compounds could be confirmed (Fig. 2). The possible structure of the compound I (*m*/*z*, 258) was chrysenequinone as reflected by its major peak at 16.9 minutes, mass ion spectrum and the presence of dihydroxy chrysene, a dehydrogenation product from dihydrodiol. The GC retention time, MS properties of the M^+ at *m*/*z* 258, fragment ions at *m*/*z* 230 [M^+-28], corresponding to the respective sequential losses of -CO, were identical to chrysenequinone. *Polyporus* sp. S133, grown in MSB with chrysene was able to mineralize chrysene to compound II. An analysis of the ethyl acetate-extractable metabolites was conducted using GC-MS, under normal conditions. A major peak at 9.6 minutes, which represented all the

metabolites, was identified as 1-hydroxy-2-naphthoic acid. The GC retention time, MS properties of M^+ at *m/z* 188, fragment ions at *m/z* 170 [M^+-18] were identical to that of authentic 1-hydroxy-2-naphthoic acid.

Identification of these compounds in the extracts using the method described in the experimental section was conducted. In the Total Ion Chromatography (TIC) profile, the retention time of these compounds coincided with that of these authentic compounds. The Mass Spectrum (MS) of the trimethylsilyl (TMS) ether of these compounds also coincided with that of the authentic TMS ether of the compound. 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 is also used as a model substrate for studies on metabolism of bay-region- and K-region-containing carcinogenic PAHs such as benzo(a)pyrene and benzo (a)anthracene (Gibson and Subramanian, 1984; Fawell and Hunt, 1988).

Based on the identification of various metabolites produced during initial ring oxidation and ring cleavage processes, the metabolism of chrysene by the *Polyporus* sp. S133, a fungus screened from nature was successfully explored (Fig. 3). The chrysene degradation pathways were proposed based on the identification of various metabolites. It is possible that a fungal culture could utilize 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 dioxygenase system to transform chrysene. Chrysenequinone was further degraded to 1-hydroxy-2-naphthoic acid. Two different classes of enzymes are presumably involved in the degradation of 1-hydroxy-2-naphthoic acid. *Polyporus* sp. S133 can degrade chrysenequinone through 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 (Pinyakong *et al*., 2000; Balashova *et al*., 2001).

Polyporus sp. S133, that can transform a high-molecular-weight chrysene, belongs to versatile PAH-degrading white rot fungi. It can be used for degradation of PAHs in contaminated environment, where the initial reactions of PAH oxidation often limit the rate of bioremediation. The fungus may also be useful for chemical production by bioconversion, as it performed a selective oxidation of only one aromatic ring. In addition, the fungus is good for investigation of PAH mixture bioconversion and high-molecular-weight PAHs degradation.

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