Journal of Scientific & Industrial Research
Vol. 67, July 2008, pp. 538-542

Biodegradation of polycyclic aromatic hydrocarbons by white rot fungi *Phanerochaete chrysosporium* in sterile and unsterile soil

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Received 23 July 2007; revised 28 April 2008; accepted 07 May 2007

Ligninolytic white rot fungus, *Phanerochaete chrysosporium,* isolated from soil sample of petroleum refinery, was used for degradation of five polycyclic aromatic hydrocarbons (PAHs: acenaphthene, anthracene, phenanthrene, fluoranthene and pyrene), simultaneously and individually in sterile and unsterile soil. For maximum biodegradation, after 42 days of incubation, optimum conditions were pH 7.0, 30°C and 5µg/g PAHs concentration. In sterile soil, degradation in 42 days of incubation of five PAHs was as follows: phenanthrene, 98.96; anthracene, 92.6; pyrene, 92.2; acenaphthene, 83.8; and fluoranthene, 79.8%. In unsterile soil, PAHs degradation was low (38.94-62.89%).

Keywords: Bioremediation, *Phanerochaete chrysosporium*, Polycyclic aromatic hydrocarbons (PAHs), Sterilized soil, Unsterilized soil

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed[1,2], owing to both natural and anthropogenic sources[3,4]. PAHs are highly recalcitrant molecules and can persist in environment due to their hydrophobicity and low water solubility[5]. US Environmental Protection Agency (USEPA) has classified PAHs among priority pollutants[6]. Bioremediation of hydrocarbon-contaminated soils has been established as an efficient, economic, versatile, and environmentally sound treatment[7]. Principal processes for biodegradation of PAHs from environment, however, are thought to be microbial transformation and degradation[3,5]. Degradation of PAHs by local microorganisms at polluted sites is a bioremediation strategy[8]. In PAHs contaminated soil, microorganisms capable of utilizing and degrading hydrocarbons could be employed for PAHs elimination[9]. Microorganisms of different origin can be used; nevertheless particular attention is devoted to indigenous ones improving their *in situ* degradation capability through optimization of temperature, pH, water-content and oxygen concentration[10]. Effect of introduced species on degradation of petroleum hydrocarbon contaminated soil[11] has been discovered. White-rot fungi (*Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor*) produce extra-cellular enzymes, including lignin peroxidase (LiP) and manganese peroxidase (MnP), which are presumed to be involved in PAH degradation process[12].

This study investigates biodegradation potential of PAHs from sterilized and unsterilized soil spiked simultaneously and individually with five PAHs using adapted white rot fungal strain *P. chrysosporium* and indigenous microflora.

Materials and Methods

Chemicals

All chemicals, except PAHs, were purchased from Merck (Darmstadt, Germany) in highest purity grade available. Hexane, acetone and acetonitrile were AR grade and redistilled in glass apparatus. Phenanthrene, acenaphthene, anthracene, fluoranthene and pyrene (all with 99.0% analytical standards) were purchased from Sigma Aldrich Chemicals (Germany).

Collection of Samples

Soil samples were collected from PAHs noncontaminated soil of district Hisar, Haryana. Soil was shade dried, powdered and sieved (2 mm) to remove plants and debris. Noncontaminated soil was sterilized in autoclave at 15 psi and 121°C for 20 min and artificially spiked with five PAHs (5µg/g). For isolation

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of fungus, soil samples were collected from a petroleum refinery.

Culture Medium

A basal salt media (BSM) containing [$(NH_4)_2SO_4$, 2.4; K_2HPO_4, 1.55; $NaH_2PO_4.H_2O$, 0.85; NaCl, 0.5; and $MgSO_4.7H_2O$, 0.26 g/l] + [1 ml of trace element solution containing: nitrilotriacetic acid, 15.0; $CaCl_2.2H_2O$, 15.0; $MnCl_2.2H_2O$, 6.0; $FeSO_4.7H_2O$, 1.0; $CO(NO_3)_2.6H_2O$, 1.0; $ZnSO_4$, 1.0; $CuSO_4$, 0.1; H_3BO_3, 0.1; Na_2MoO_4, 0.1; and $Al(SO_4)_2.H_2O$, 0.1 mg/l] + mixture of five PAHs (0.01%) as carbon source, was used for enrichment. Medium pH was adjusted to 5.0 with 0.1N HCl. Rose Bengal Agar (RBA) Medium [dextrose, 10; peptone, 5; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1.0; Rose Bengal, 0.033; and streptomycin, 0.033 g/l; and agar, 2% (pH 5.0)] was used for fungus growing on plates.

Isolation of Fungi from Soil Sample

Soil (10 g) sample from petroleum refinery at Panipat, Haryana, was shaken overnight in deionized water (100 ml) at 28°C and 120 rpm and then supernatant (5 ml) was added to BSM (45 ml). Enrichment was made by serially sub-culturing in the same medium using 10% of inoculum from previous culture. Medium was enriched with single PAH phenanthrene for three months and next three months in 0.01% mixture of five PAHs. PAHs enriched culture was diluted 10-fold and 0.1 ml culture were spread on RBA plates supplemented with streptomycin. After 5 days of incubation at 28°C, fungal colonies were selected and replated on the same medium until pure colonies of fungal species were obtained and identified as white rot fungus *P. chrysosporium* from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Optimization Study

Parameters [(pH (5-9), temperature etc.)] were optimized for 14 days. Effects of varying PAHs concentration (5-20µg/g) were used for biodegradation at optimized pH and temperature.

Biodegradation Study

Experiment was performed in 9 ml culture media having 1.0 g soil using 30 ml vials. PAHs mixture (5µg/g) was inoculated with *P. chrysosporium* culture (1 ml) already optimized with PAH. Control was prepared by autoclaving 1.0 g soil in 30 ml vials at 15 psi and 121°C for 20 min. Degradation rate of *P. chrysosporium* in sterile (control) and unsterile soil spiked containing mixture of five PAHs was studied at 30°C and 7.0 pH for 42 days at 120 rpm. Biodegradation rate of individual PAHs was also studied and compared with biodegradation rate of PAHs mixture. All these experiments were performed in triplicate under darkness. Vial was withdrawn at a regular interval of 7 days for measuring residual PAHs concentration. Biodegradation efficiency was calculated as

$$\text{Biodegradation efficiency (\%)} = \frac{(C_0 - C_e)}{C_0} \text{ X } 100$$

where, C_0, initial concentration of PAHs (µg/g); Ce, equilibrium concentration of PAHs (µg/g)

Analytical Methodology

Residual PAHs were extracted on ultrasonicator (Transsonic) and on rotary evaporator (Variac) twice with n-hexane, centrifuged for 10 min at 12000g and filtrated with syringe filters (0.45µm). Cleanup procedure was carried out using chromatographic column. Extracts were analysed by high performance liquid chromatography (HPLC) (Water 600) equipped with UV detector. Analytical column (250 mm long, 4.6 m diam) was packed with totally porous spherical C-18 material (packed size, 5µm). Acetonitrile-water mixture (75: 25) was used as mobile phase at a flow rate of 1.0 ml min^{-1}. Sample (20 µl) was injected into column through sample loop. UV- detector was set at 254 nm for compound detection. Stock solutions of PAHs were prepared by dissolving PAHs analytical standard (200-1000 ppm) in acetonitrile. Working standard (10 ppm) PAHs mixture was prepared by suitable dilution of stock solution with acetonitrile. Calibration graph at several dilution of standard mixture of individual compounds of PAHs were used for determining retention time and studying linearity of detector (Fig. 1). Concentration of PAHs was calculated by comparing peak areas of sample chromatogram with that of peak area of standard chromatogram[2].

$$\text{Concentration of PAHs in sample (µg/ml)} = \frac{\text{Peak area of chromatogram of sample}}{\text{Peak area of chromatogram of standard PAHs compound}} \times \text{Conc. of standard PAHs compound}$$

Results and Discussion

Environmental Factors Affecting Biodegradation of PAHs

After a series of mixture of five PAHs, degradation tests at 14 days incubation were conducted at different pH (5-9), temperature (20-40°C) and PAHs (5-20 µg/g).

PAH degradation (Fig. 2A) with *P. chrysosporium* increased as pH increases (5-7) and after that degradation decreased as pH increases (7-9). Kastner *et al*[13] observed that *Sphingomonas paucimobilis* was more sensitive to pH of growth media, with degradation of PAHs phenanthrene and anthracene. Phenanthrene removal was only 40% at pH 5.5 after 16 days, whereas at circum-neutral pH values, phenanthrene removal was >80%. Biodegradation of PAHs can occur over a wide temperature range; however, most studies tend to focus on mesophilic temperature rather than efficiency of transformations at very low or high temperature. Optimal temperature for degradation of PAHs was observed at 30°C (Fig. 2B) whereas, biodegradation efficiency decreased as temperature increased or decreased. Solubility of PAHs increased with an increase in temperature[14], which increased bioavailability of PAH molecules. In addition, oxygen solubility decreased with increasing temperature, which will reduce metabolic activity of aerobic microorganism. Higher the concentration of PAHs, slower the degradation rate; perhaps a reflection of increased levels of PAHs concentration or other metabolite toxicity (Fig. 2C). Microbial community growth is affected with increase in toxicity[15].

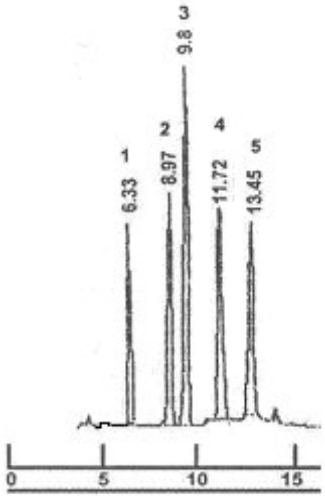

Fig. 1 — Typical chromatogram of standard PAHs mixture (1-acenaphthene, 2-anthracene, 3-phenanthrene, 4-fluoranthene, and 5-pyrene)

Biodegradation of Sterile and Unsterile Soils Simultaneously Spiked with Five PAHs

Experiment was designed for biodegradation of PAHs in sterile and unsterile soils with and without addition of inoculant *P. chrysosporium.* In each case, initial concentration of five PAHs added to the soil was 5µg/g. Operating conditions for experiment designs were set at 30°C and pH 7.0 in 42 days incubation period. Degradation of five PAHs (Fig. 3) from sterilized soil was only 6.79-8.59% and native soil having indigenous microorganisms without inoculant was 9.14-14.91%. Maximum degradation of PAHs was 90.61-100% in sterilized soil and 48.87-70.08% in unsterlized native soil with inoculant of fungal strain. Degradation of PAHs in sterilized soil and unsterilized native soil without inoculant has been observed very much less as compared to with PAHs adapted fungal inoculant. This strongly supports

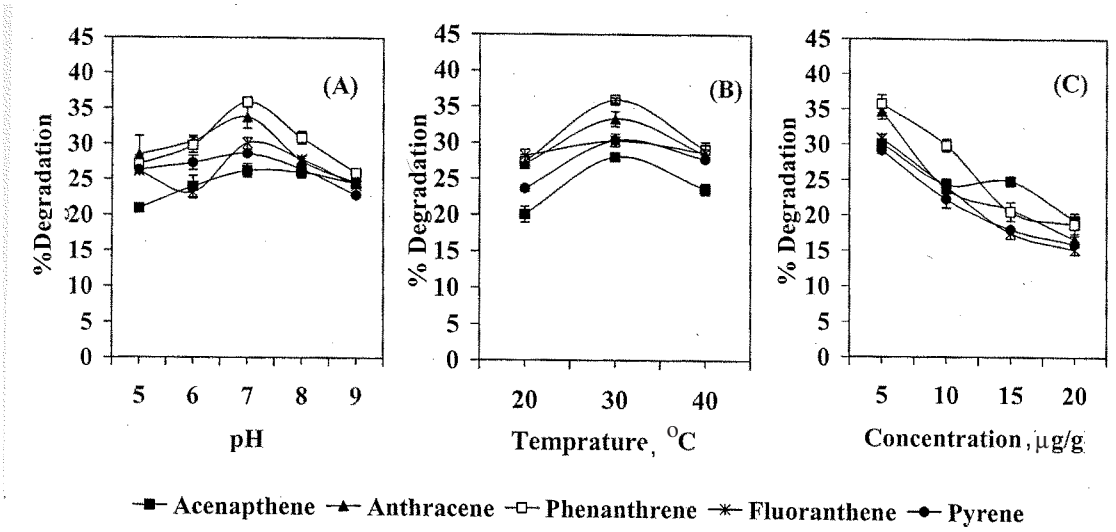

Fig. 2 — Environmental factors affecting biodegradation of PAHs: A) effect of pH; B) temperature (°C); and C) PAHs concentrations (µg/g)

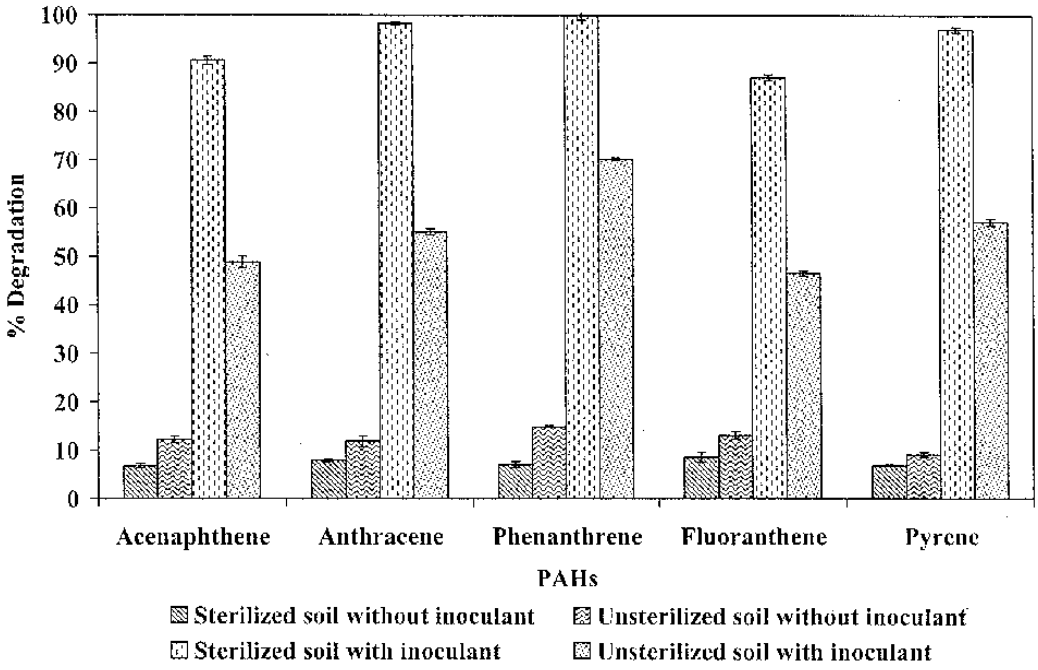

Fig. 3 — Biodegradation of PAHs in sterile soil and unsterile soil spiked with simultaneously 5 PAHs with and without inoculant of fungal strain *P. chrysosporium* in 42 days incubation time

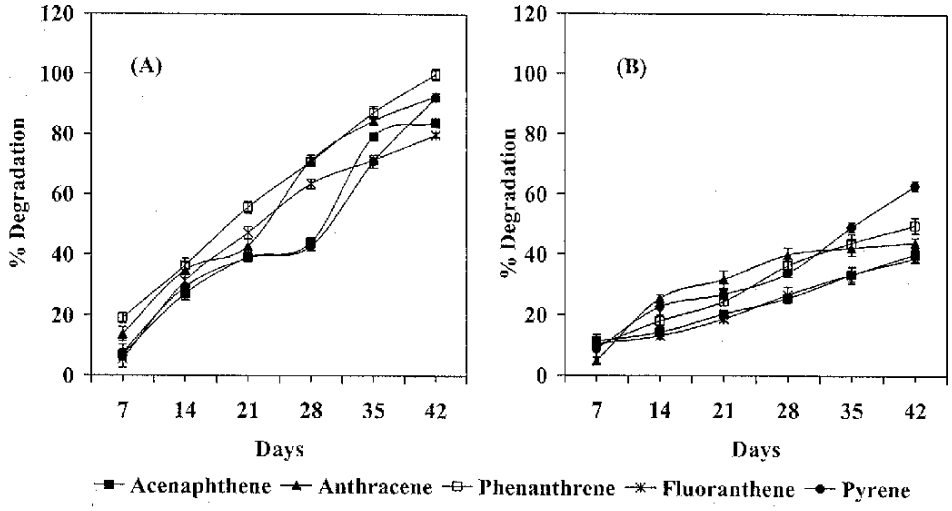

Fig. 4 — Biodegradation of PAHs in 42 days incubation time with inoculant fungal strain *P. chrysosporium*: A) sterile soil spiked with individually 5 PAHs; (B) unsterile soil

that PAH degradation in soil is the result of microbial action, which is enhanced by treating soil with PAH-adapted microbes. Unlike unsterilized soils, newly introduced fungal strain may be in competition with indigenous soil microbes in natural soil samples and consequently may impact on degrading potentials[16].

Biodegradation of Sterile and Unsterile Soil Individually Spiked with Five PAHs

In biodegradation study, sterilized and unsterilized soils artificially spiked with individually PAH (5µg/g) namely acenaphthene (3-ring), anthracene (3-ring), phenanthrene (3-ring), fluoranthene (4-ring) and pyrene (4-ring) was used for biodegradation with PAHs adapted isolated fungal strain *P. chrysosporium* in 42 days incubation period at optimum conditions (Fig. 4). Degradation rate of anthracene, pyrene, acenaphthene, and fluoranthene was 92.6%, 92.2%, 83.8%, and 79.8% respectively, whereas phenanthrene was completely degraded in sterilized soil at optimum condition (Fig. 4A). In unsterilized soil, biodegradation of five PAHs observed as (Fig. 4B): fluoranthene (38.94%) < acenaphthene (40.25%) < anthracene (44.02%) < pyrene (49.79%) and < phenanthrene (62.89%). Biodegradation rate was

found maximum in sterilized soil than unsterilized soil. Degradation of pyrene, acenaphthene, anthracene and fluoranthene was less than phenanthrene in sterilized and unsterilized soil because in adaptation experiment, individual microbial culture was enriched with phenanthrene for three months and then with a combination of five PAHs for another three months. Similarly, degradation of phenanthrene was faster than any other PAHs because consortium enriched in phenanthrene for 3 years and in combination of other PAHs for 1 year[17]. Many introduced microorganism, including fungi and bacteria have shown to possess degradative enzyme for the oxidation of PAHs[18]. However, conditions for degradation may be vastly different in sterilized and nonsterilized soil. In sterilized soil, introduced microorganism will act as the dominant species. When new microorganism is applied into unsterilized soil, then there will be competition between introduced and indigenous microorganism[19].

Conclusions

PAHs adapted fungal strain *P. chrysosporium*, isolated from the soil of petroleum refinery, have ability to degrade phenanthrene, anthracene, acenaphthene, fluoranthene and pyrene in sterilized as well as unsterilized soil in optimum conditions. Degradation rate was found high when all five PAHs were present simultaneously rather than individually.

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