Marine Pollution Bulletin 58 (2009) 256–262

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Role of a moderately halophilic bacterial consortium in the biodegradation of polyaromatic hydrocarbons

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ARTICLE INFO

Keywords:
Biodegradation
Saline wastewater
Polycyclic aromatic hydrocarbons
Halophilic bacterial consortium

ABSTRACT

Polycyclic aromatic hydrocarbons are ubiquitous pollutants in the environment, and most high molecular weight PAHs cause mutagenic, teratogenic and potentially carcinogenic effects. While several strains have been identified that degrade PAHs, the present study is focused on the degradation of PAHs in a marine environment by a moderately halophilic bacterial consortium. The bacterial consortium was isolated from a mixture of marine water samples collected from seven different sites in Chennai, India. The low molecular weight (LMW) PAHs phenanthrene and fluorine, and the high molecular weight (HMW) PAHs pyrene and benzo(*e*)pyrene were selected for the degradation study. The consortium metabolized both LMW and HMW PAHs. The consortium was also able to degrade PAHs present in crude oil-contaminated saline wastewater. The bacterial consortium was able to degrade 80% of HMW PAHs and 100% of LMW PAHs in the saline wastewater. The strains present in the consortium were identified as *Ochrobactrum* sp., *Enterobacter cloacae* and *Stenotrophomonas maltophilia*. This study reveals that these bacteria have the potential to degrade different PAHs in saline wastewater.

1. Introduction

Biodegradation of polyaromatic hydrocarbons (PAHs) plays a vital role in cleaning the crude oil contaminated marine environment. PAHs have ubiquitous distribution and deleterious effects on human health. The hydrophobic nature of polyaromatic hydrocarbons makes their clean-up extremely difficult as they persist for a long period of time. In addition to increasing environmental persistence with increasing molecular size, evidence suggests that in some cases PAH genotoxicity also increases with size, up to at least four or five fused benzene rings (Cerniglia, 1992).

Low molecular weight (LMW) PAHs, such as anthracene, phenanthrene, and naphthalene, are easily biodegradable under laboratory conditions, since they are readily available in water for biodegradation, but high molecular weight (HMW) PAHs, such as benzo(*a*)pyrene and benzo(*a*)anthracene, show extensive resistance to degradation (Kanaly and Harayama, 2000). HMW polyaromatic hydrocarbons such as benzo(*a*)pyrene have been intensively studied for many years due to their potent genotoxic (Sutherland et al., 1995) and immunotoxic properties (Davila et al., 1995).

Several bacterial species were isolated from areas of different salt concentrations and their effects on biodegradation were analysed. The present article presents a detailed study on a moderately halophilic bacterial consortium grown on different PAHs at different concentrations. The ability of the consortium to treat PAH-contaminated oily saline wastewater was also studied.

2. Materials and methods

Chemicals: The PAHs were purchased from Sigma Aldrich and all other chemicals (analar grade) were purchased from Merck, India.

Samples: Water samples were collected from seven different sites (petroleum or coal contaminated) from the seaport of Chennai, India and stored at 10 °C for further use.

2.1. Composition of culture medium

The carbon-free mineral salts medium (MSM) contained NH_4Cl-2.5 g, KH_2PO_4-5.46 g, Na_2HPO_4-4.76 g, $MgSO_4$-0.20 g, NaCl-30.0 g, pH-7.4 ± 0.2, and distilled water-1 L. The final pH of the medium was adjusted to 7.4 with 0.1 N NaOH, and the medium was sterilized in an autoclave (121 °C for 15 min) prior to the addition of the PAH substrates. Stock solutions of each PAH (300 ppm) were prepared in ethyl acetate and stored.

2.2. PAHs

The PAHs were selected for the study based on their number of rings. The LMW PAHs phenanthrene and fluorene and the HMW PAHs pyrene and benzo(*e*)pyrene were used. LMW PAHs were used

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0025-326X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2008.09.017

at 5, 10, 20, 50, and 100 ppm concentrations, pyrene was used at 5, 10, 20, 50, and 100 ppm, and benzo(*e*)pyrene at 1, 2, and 5 ppm.

2.3. Enrichment of the bacterial consortium

PAH dissolved in ethyl acetate was added to a 250 mL conical flask and, after evaporation of the ethyl acetate, the mineral medium (100 mL) was added, with PAH as the sole carbon source. Five millilitres of 104–105 cfu/mL bacterial consortium was added. The conical flask was shaken at 150 rpm at 37 °C for 48 h. After growth, 5 mL of enriched culture was then transferred to fresh medium and incubated under the same conditions. The culture was passaged in the same PAH-containing medium to enrich the bacterial consortium.

2.4. Preliminary studies on PAH degradation by the bacterial consortium

PAH was added to the medium at different concentrations. The bacterial consortium isolated from the marine environment was grown and the plate count (cfu/mL) was checked daily. Cell morphology and the motility of cells in exponentially-growing liquid cultures were examined on freshly-prepared wet mounts by light microscopy. Plate counting (cfu/mL) was done on nutrient agar medium. The consortium was studied for its growth on LMW PAHs with phenanthrene/fluorene as the sole carbon source.

The spray-plate method, a qualitative assay, was used to check the degradation of PAH by the bacterial consortium. Clearing zones seen around the colonies indicated utilization of PAH sprayed on the medium (Kiyohara et al., 1982). This technique was also used to analyse pyrene utilization by the consortium.

For the degradation study, mineral medium containing PAH was inoculated with the bacterial consortium. Different conditions used for the degradation of PAH were (i) medium + PAH + bacterial consortium; (ii) medium + PAH and (iii) medium + bacterial consortium, with (ii) and (iii) serving as controls. The bacterial consortium was added to the medium at concentrations of 10^4–10^5 cfu/mL. The culture, in duplicate, was incubated at 37 °C with shaking at 150 rpm and extracted every 24 h for 5 days. Each culture was extracted twice with ethyl acetate (v/v) after acidification to pH 2.5 with 1N HCl. The extracts were filtered through anhydrous sodium sulphate and condensed to 1 mL with a rotavapour (Buchi, Germany) for further HPLC analysis.

2.5. HPLC analysis of PAH utilization

The condensed sample was filtered through a 0.2 mm syringe filter and analysed with high performance liquid chromatography (HPLC). HPLC analysis was performed on a KNAUER, (Germany) unit equipped with a PAH-specific column (Ultrasep ES, B590/02, 250 × 4 mm, Knauer, Germany) with a UV–vis detector connected to WINCHROME software to process the data. The mobile phase was acetonitrile. Standard solutions of different PAHs were used for reference. The samples were injected one by one and the utilization rate of PAH was calculated based on the peak area percent and retention time.

2.6. CO_2 evolution test

This study was conducted in mineral salts medium with both PAH and the bacterial consortium, while PAH + MSM and MSM + bacterial consortium served as controls. PAH (phenanthrene-3 mg/L) dissolved in ethyl acetate was added to sterile saline bottles (100 mL). After evaporation of the solvent, 25 mL of the mineral salts medium was added to the bottle. The bottles were sealed completely (airtight) with an aluminium stopper. The culture was kept shaking at 150 rpm at 37 °C. Samples were collected at 24 h intervals and analysed for CO_2 evolution in a gas chromatograph. Carbon dioxide content was measured with a thermal conductivity detector using a Porapak Q column (80/100 mesh, 2 m) with an external standard. The carrier gas was helium and the column temperature was 50 °C. The temperature of the injector and the detector was 100 °C. Samples (250 μL) of the headspace gas from the culture flasks were withdrawn with a gas-tight syringe and injected into the gas chromatograph for CO_2 determination. The samples in the saline bottles were extracted and analysed in the PAH-specific column by HPLC for degradation of PAH in the medium.

2.7. Metabolite formation-TLC and GC–MS

During the degradation study, the different kinds of metabolites formed were identified using thin layer chromatography. The condensed samples were loaded on TLC plates using a 10 μL capillary tube. The chromatograms were run in different solvents for migration of the PAH compounds. Solvent mixtures such as benzene/hexane (50:50), benzene/acetone (50:50) and benzene/acetone/acetic acid (80:10:10) were used to identify the metabolites. After removing the plates from the solvents, 2% giffs reagent was sprayed on the plate and observed under UV light at 265 nm.

A Hewlett–Packard 6890 gas chromatograph equipped with a 5973 mass spectrometer with a HP-5MS (30 m × 0.25 mm ID × 0.25 μm) fuse-silica capillary column was used for analysis. The column temperature program was set at 100 °C to hold for 1 min, 15 °C/min–160 °C and 5 °C/min–300 °C then held for 7 min. The GC injector was held isothermally at 280 °C for an undivided period of 3 min. Helium was used as the carrier gas, at a flow rate of 1 mL/min, controlled by an electronic pressure control. The GC/MS interface temperature was maintained at 280 °C. The MS was operated in electron impact (EI) ionization mode with an electron energy of 70 eV, and the scan to determine appropriate masses for selected ion monitoring ranged from 50 to 500 amu (atom to mass unit). Standards from Sigma Aldrich were used for the PAHs (phenanthrene/fluorene) and their metabolites. A GC–MS library search was used to confirm the metabolites without standards.

2.8. Estimation of PAHs in crude oil-contaminated saline wastewater

Crude oil-contaminated saline wastewater was collected from the Port of Chennai. 125 mL of 10^4–10^5 cfu/mL bacterial consortium was added to a 3 L reactor filled with 2.5 L of the saline crude oil wastewater as a seed. The inoculated saline crude oil–wastewater was aerated and mixed. Every 24 h, degradation of PAH in the saline crude oil wastewater was analysed by HPLC.

2.9. Scanning electron microscopy

The sample preparation for SEM was carried out according to the method of Prior and Perkins (1974). The isolates were grown on mineral salt medium (MSM) for 24 h. The cells were centrifuged at 8000× g for 10 min and the pellets were immediately resuspended in 2% glutaraldehyde with 0.05M phosphate buffer and 4% sucrose (pH 7.3). Cells were fixed overnight at 4 °C. The specimens were centrifuged at 8000 × g for 10 min, washed four times in distilled water, placed on aluminium foil disks, air dried, platinum coated and examined under SEM (JEOL JSM-6360).

2.10. Extraction and amplification of bacterial DNA

DNA from the bacterial cell was extracted using a Qiagen (QIAamp® DNA stool Mini kit Cat. No. 51504) DNA isolation kit.