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RESEARCH ARTICLE

Novel Polyaromatic Hydrocarbon (PAH) degraders from oil contaminated soil samples

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Abstract

Background: Polycyclic aromatic hydrocarbons (PAHs) are generally a hazardous class of organic compounds. They may be produced as a result of combustion or pyrolysis of fossil fuels or other organic matter. Microbial degradation is majorly responsible for ecological recovery of PAHs from sites contaminated with it. The present study was conducted to isolate microbial strains capable of degrading naphthalene with the aid of biosurfactant production. **Methods:** Oil contaminated soil samples collected from Oil and Natural Gas Corporation (ONGC), Karaikal, Tamil Nadu, India were enriched, serially diluted and spread plated in Bushnell Haas (BH) medium with naphthalene as a carbon source. The isolates grown from the enriched samples were further screened for naphthalene degraders and for biosurfactant production by appropriate protocols.
Results: A total of 13 naphthalene degrading strains extended among 9 genera namely *Morganella*, *Actionomyces*, *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, *Corynebacterium*, *Actinomadura*, *Serratia* and *Staphylococcus* were isolated from the samples. This is the first report which describes new strains capable of degrading naphthalene. These include species of *Actinomadura*, *Morganella*, *Stenotrophomonas* and *Serratia*. *Morganella morganii* subspecies *morganii* was found to be the best PAH degrader. Strains from the genera *Serratia* and *Stenotrophomonas* were identified as the best biosurfactant producers.
Conclusion: The isolates obtained were screened for their ability to utilize naphthalene as a sole source of carbon and energy with the aid of biosurfactant production. This confirms high degradative ability and ubiquity associated with these isolates as it concerns biodegradation of both soil and water environments polluted with petroleum and its many products. Further studies in this direction can reveal interesting options in bioremediation of PAH.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are unique class of organic compounds. These compounds are generated inadvertently and continuously in the environment by incomplete combustion of organic matter; for instance in forest fires, home heating, traffic and waste incineration. As hydrocarbons, these compounds are composed of carbon and hydrogen; the carbon atoms being arranged in a series of adjoining six-member benzene rings. Thus, all PAHs have in common a singular feature that is based on two or more fused benzene rings. This biochemical persistence in the environment arises from dense clouds of π-electrons on both sides of the ring structures, making them resistant to nucleophilic attack. Besides this, they possess physical properties, such as low aqueous solubility

and high solid water distribution ratios, which stand against their ready microbial utilization and promote their accumulation in the solid phases of the terrestrial environment (Johnsen et al., 2005). These PAHs which are major fractions of petroleum mixtures have high toxic, mutagenic or carcinogenic effects to humans and animals. As a result, the large areas of oil-contaminated soil pose threats to the ecosystem and humans (Mulligan et al., 1984).

There are physical, chemical and biological methods for cleaning up the PAHs-contaminated soils. However, biological methods which involve microbial transformation and degradation are considered an effective and environmentally benign clean-up technology as it involves the partial or complete bioconversion of these pollutants to microbial biomass, carbon dioxide and water (Head et al., 1999; Xia et al., 2005). Even though biological degradation is widely accepted, the degrading capacities of microorganisms are dependent upon many factors which include concentration, bioavailability, toxicity, mobility, activated enzymes and access to other nutrients (Kumara et al., 2006). Biodegradation of a given hydrocarbon depends on its dispersion state and is generally maximized when the water-insoluble substrate is solubilized or emulsified. Many microorganisms which utilize these hydrocarbons as sole-carbon source often produce surface-active compounds. These surface-active molecules contain hydrophilic and hydrophobic components that enable such molecules to concentrate at interfaces and to reduce the surface tensions of aqueous media. These surface-active agents that are produced by certain microorganisms are called as biosurfactant (Koch et al., 1991). Biosurfactants have several advantages over the chemical surfactants. These include lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH, and salinity; in addition to its ability to be synthesized from renewable feed stocks (Desai et al., 1997). The main objective of the present study was to isolate naphthalene degrading bacteria from oil contaminated soil and further screen them for biosurfactant production.

MATERIALS AND METHODS

Collection of Soil Samples

Oil contaminated soil samples were collected from Oil and Natural Gas Corporation (ONGC), Karaikal, Tamil Nadu, India. The samples were collected in sealed airtight plastic bags and were brought to the laboratory and immediately processed.

Enrichment of naphthalene degraders from the soil samples

Enrichment of oil contaminated soil samples was done in Bushnell Haas (BH) medium described by (Bushnell et al., 1941; Nnamchi et al., 2006). The composition of BH medium in g/L is: $MgSO_4$ - 0.2, $CaCl_2$ - 0.02, KH_2PO_4 - 1.0, $(NH_4)_2HPO_4$ - 1.0, KNO_3 - 1.0, $FeCl_3$ - 0.05, pH - 7.2 ± 0.2. The sole carbon source, naphthalene was added at a concentration of 2 % (w/v) to the BH medium after sterilization (Deziel et al., 1996). 1.0 g of each soil sample was inoculated into medium and incubated at room temperature for 7 days at 120 rpm in an orbital shaker.

Isolation of naphthalene degraders from the enriched soil samples

The enriched samples were serially diluted and spread plated on BH agar medium plates with 2% hexane solution (v/v) of naphthalene. The plates were incubated at 25°C for 3 days. Different types of colonies with halo zone formation indicative of naphthalene degradation in the agar plates after incubation were identified and designated as PAH 1, PAH 2, PAH 3, etc. The colonies were pure cultured subsequently onto fresh BH medium agar plates and stored in nutrient agar slants at 4°C for further studies.

Screening test for naphthalene degraders

A loopful of each PAH isolate was inoculated in BH broth medium with 2% naphthalene. The culture tubes were incubated at room temperature (25°C – 30°C) for three days. The ability of each isolate to utilize naphthalene was indicated by an increase in turbidity of the medium measured at A_{600} nm using Double Beam UV-VIS Spectrophotometer SL 164 (Shimadzu Corporation, Kyoto, Japan) (Nnamchi et al., 2006).

Identification of naphthalene degraders

Naphthalene degrading isolates were identified to their generic level by morphological studies like microscopic examination, Gram staining, motility and biochemical tests (Murray 2003).

Screening for Biosurfactant production

The PAH isolates were screened for biosurfactant production by four methods namely, semi-quantitative agar plate method, drop collapse test, determination of emulsification activity i.e. *EI-24* index and haemolysis of erythrocytes.

Semi-quantitative agar plate method

This is one of the best methods to detect microorganisms producing biosurfactants. Biological anionic tensides (biosurfactants) form an insoluble ion pair with the cationic tensides ($CaCl_2$) indicated by formation of pink zone around the well by the basic dye (fuchsin) present in the medium. Minimal Broth Davis (MBD) medium described by Siegmund et al was used for screening biosurfactant producers (Siegmund et al., 1991). The composition of the MBD medium in g/L is: $C_6H_{12}O_6$ – 1.0; K_2HPO_4 – 7.0; KH_2PO_4 – 2.0; $NaH_2C_6H_5O_7$ – 0.5; $MgSO_4$ – 0.1; $(NH_4)_2SO_4$ – 1.0; Agar – 20; $CaCl_2$ – 0.2; fuchsin – 0.005, pH 7.0 ± 0.2. A volume of 10 µL culture broth was added to the wells cut in the medium by a cork borer. The plates were incubated at 37°C for 2 – 3 days.

Drop collapse test

The drop collapse technique depends on the principle that a drop of a liquid containing a biosurfactant will collapse and spread completely over the surface of oil (Youssef et al., 2004). Many microorganisms produce extracellular or membrane-associated surface active compounds that play essential roles in the survival of the producing microorganisms either through facilitating nutrient transport, providing microbe-host interaction or playing role as biocides (Tugrul et al., 2005). The drop collapse test was done for both culture broth and culture supernatant to check for intracellular and extracellular biosurfactant production respectively. Collapse of the drop for culture broth indicates intracellular (cell-adhered) biosurfactant production whereas collapse of the drop for culture supernatant indicates extracellular production of biosurfactant. A drop of coconut oil was placed on a glass slide followed by a drop of the culture broth and supernatant to check for intracellular and extracellular biosurfactant production respectively.

Assessment of emulsification activity by Emulsification index (EI) i.e. EI-24 index

The emulsification activity i.e. *EI-24* index of the isolates was estimated to check the stability of biosurfactant produced. As in case of drop collapse test, the *EI-24* index was estimated for both culture broth and culture supernatant respectively. A 0.5 mL of the culture broth / culture supernatant and 0.5 mL of kerosene was added to 4.0 mL of distilled water. The tube was vortexed for 10 s and held stationary for 1 min. The emulsion stability was determined after 24 h and *EI-24* index was calculated as per the formula given below (Tuleva et al., 2002; Płaza et al., 2006).

$$EI\text{-}24(\%) = \frac{\text{emulsion height (cm)}}{\text{Total liquid height (cm)}} \times 100$$

Haemolysis of erythrocytes

Since it has been observed that biosurfactant haemolyse erythrocytes, this method was used for screening biosurfactant producing microorganisms (Carrillo et al., 1996). The isolates were streaked on blood agar plates containing 5% (v/v) blood and incubated at room temperature (RT) for 24 - 48 h. Haemolytic activity was detected by the presence of a definite clear zone around a colony indicative of biosurfactant production.

RESULTS AND DISCUSSION

Isolation, Identification and Screening for potential naphthalene degraders

This study describes the isolation and characterization of naphthalene degrading microorganisms obtained from oil-contaminated soil samples. Thirteen isolates spanning among 9 genera which include an *Actinomadura*, *Actinomyces*, *Bacillus*, *Corynebacterium*, *Morganella*, *Pseudomonas*, *Staphylococcus*, *Serratia* and *Stenotrophomonas* (Table 1) were isolated capable of utilizing naphthalene as a carbon source based on formation of halo zone around the colony. Twelve naphthalene degraders were found to be of bacterial origin and one was identified as *Actinomadura pellettieri*, an actinomycete. On screening for best among the isolated naphthalene degraders, *Morganella morganii* subspecies *morganii* showed highest optical density value at A_{600} nm followed by *Serratia marcescens* and *Corynebacterium* species (Fig. 1) (Nnamchi et al., 2006; Pizzul et al., 2007).

Screening for biosurfactant production

The naphthalene degraders were screened for biosurfactant production using four methods.

Semi-quantitative agar plate method

Chemical anionic tensides in aqueous solutions can be determined by formation of an insoluble ion pair with various cationic substances, for example organic metal salts, soluble amines, basic dyes and cationic tensides. Biosurfactant production was confirmed by the presence of a pink zone around the well. Of the 13 isolates, *Actinomyces naselundii*, *Serratia marcescens*, *Serratia odorifera* (Biogroup I) and *Stenotrophomonas maltophilia* were found to be excellent biosurfactant producers followed by *Bacillus sphaericus* and *Morganella morganii* subspecies *morganii* and *Staphylococcus* species (Table 2).

Drop collapse test

The drop collapse method is a sensitive and easy method to test for biosurfactant production. In this study, the drop collapse technique was applied as a qualitative method to detect biosurfactant production. *Actinomyces naselundii* was observed as extracellular biosurfactant producer whereas *Morganella morganii* subspecies *sibonii* and *Serratia marcescens* were found to be producers of intracellular biosurfactant producers. It was also noted that *Serratia odorifera* (Biogroup I) and *Stenotrophomonas maltophilia* produced both extracellular and intracellular (cell-adhered) biosurfactant as indicated by positive results for both culture broth and culture supernatant (Table 2).

Determination of emulsification activity

The emulsification activity (*EI-24* index) of the 13 isolates is as shown in Fig. 2 and Fig. 3. Many isolates showed an *EI-24*index of more than 50%. Out of the 13 isolates *Morganella morganii* subspecies *morganii* and *Stenotrophomonas maltophilia* showed the highest *EI-24*index of 53.57% in cell broth. *Serratia* species showed the highest *EI-24* index of 57.14% followed by *Actinomadura pelletieri*, *Actinomyces naselundii* and Staphylococcus species of 55.17% in cell supernatant. An emulsion to be stable if, the *EI-24* index is 50% or better (Jain et al., 1991). Hence the biosurfactants produced in thus study have an *EI-24* index more than 50% and therefore can be said to be stable (Viramontes-Ramos et al., 2010).

Haemolysis of erythrocytes

Haemolysis has been used as a criterion for the isolation of surfactant-producing organisms. Carrillo et al found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen for biosurfactant activity (Carrillo et al., 1996). Haemolytic activity was observed in strains of *Morganella morganii* subspecies *sibonii*, *Serratia marcescens* and *Serratia odorifera* (Biogroup I) (Table 2).

Table 1: List of isolates identified capable of utilizing naphthalene as a carbon source

Isolate Number	Isolates identified
PAH 1	*Actinomadura pelletieri*
PAH 2	*Actinomyces naselundii*
PAH 3	*Bacillus sphaericus*
PAH 4	*Corynebacterium* species
PAH 5	*Morganella morganii* subspecies *morganii*
PAH 6	*Morganella morganii* subspecies *sibonii*
PAH 7	*Pseudomonas* species
PAH 8	*Serratia marcescens*
PAH 9	*Serratia odorifera* (Biogroup I)
PAH 10	*Serratia* species
PAH 11	*Staphylococcus aureus*
PAH 12	*Staphylococcus* species
PAH 13	*Stenotrophomonas maltophilia*

Table 2: Comparative analysis of screening methods for biosurfactant production

Isolate Number	Semi-quantitative Agar Plate Method	Drop collapse test		Hemolysis of erythrocytes
		CB	CS	
PAH 1	–	–	–	–
PAH 2	+++	–	+	–
PAH 3	++	–	–	–
PAH 4	–	–	–	–
PAH 5	++	–	–	–
PAH 6	–	+	–	+
PAH 7	–	–	–	–
PAH 8	+++	+	–	+
PAH 9	+++	+	+	+
PAH 10	–	–	–	–
PAH 11	–	–	–	–
PAH 12	+	–	–	–
PAH 13	+++	+	+	–

Legend: +++: Excellent biosurfactant producer, ++: Good biosurfactant producer, +: Biosurfactant producer, –: Negative result, CB: Cell broth

Fig. 1 – Screening for naphthalene degraders

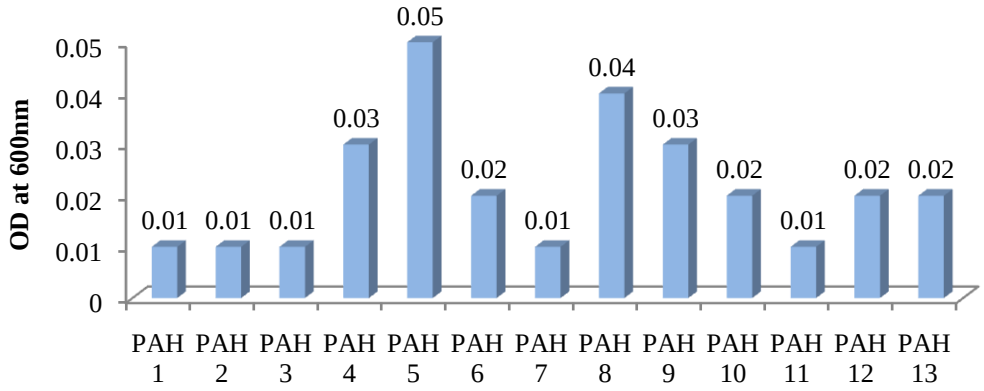


Fig. 2: *EI-24* index for culture broth

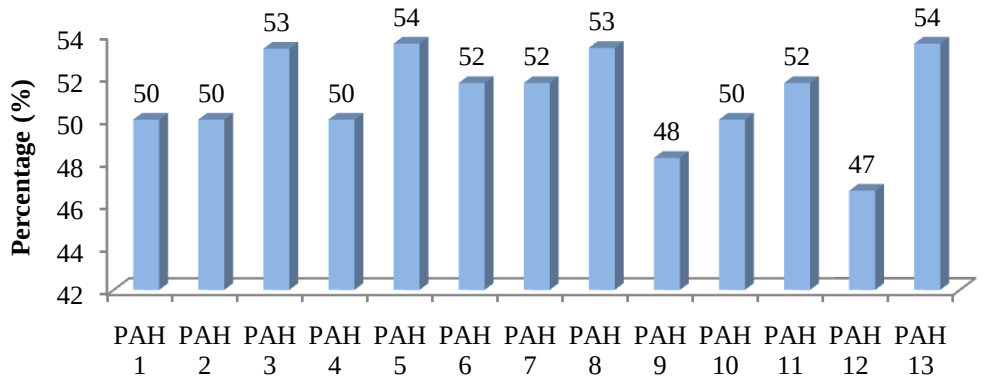
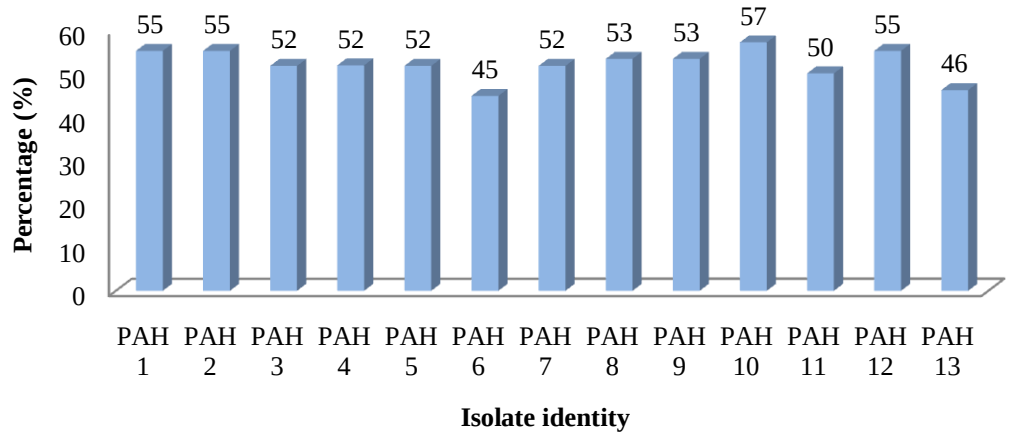


Fig. 3: *EI-24* index for culture supernatant



CONCLUSION

Polycyclic Aromatic Hydrocarbons (PAHs) are unique contaminants in the environment because they are generated continuously by the inadvertently incomplete combustion of organic matter. In spite of the presence of microorganisms in the soil, the capacity to degrade these compounds is limited and slowly transformed due to low aqueous solubility and hence low bioavailability, physical constraints such as temperature, availability of oxygen salinity, pH, type of ecosystem and nutritional factors (Atlas 1981; Bisht et al., 2010). A successful bioremediation strategy would require an in-depth understanding of the factors that influence the biodegradation process and the ecology of pollutant-degrading bacteria. The present work was undertaken to study the biodegradation ability of PAH compound viz., naphthalene by bacteria isolated from the oil contaminated soil samples. Thirteen naphthalene degraders were isolated and identified based on formation of halo zone around the colony. One isolate (PAH 8) was identified as *Actinomadura pellettieri*, an actinomycete. This is the first report which describes a newly isolated strain of *Actinomadura pellettieri* capable of degrading naphthalene by utilizing it as a carbon source. Only *Nocardia* species and other genera of actinomycetes have been reported as PAH degraders. Five other new strains previously not reported as being capable of degrading naphthalene were isolated and identified as *Morganella morganii* subspecies *sibonii*, *Stenotrophomonas maltophilia*, *Morganella morganii* subspecies *morganii*, *Actinomyces naselundii*, and *Serratia odorifera*. These novel strains form a basis for study of PAH degradation. The isolates obtained were screened for their ability to utilize naphthalene as a sole source of carbon and energy. This confirms high degradative ability and ubiquity associated with these isolates as it concerns biodegradation of both soil and water environments polluted with petroleum and its many products. All the isolates exhibited high degradative capacity. In addition, microbial populations that are found in higher concentrations of hydrocarbon contaminated areas bring about biosurfactant production. In this study *Serratia marcescens*, *Serratia odorifera* (Biogroup I) and *Stenotrophomonas maltophilia* exhibited good amount of biosurfactant production. This is novel finding in this study with these isolates also capable of degrading PAH. The strains identified in this study as potential PAH degraders represent a valuable source of new compounds with surface-active properties, and potential application for bioremediation. A further study on the physiology and genetics of these strains will benefit the industries on a large scale with their huge applications and can reveal interesting options in bioremediation of PAH.

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