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Bioremediation of PAH by *Streptomyces sp*

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Abstract An actinobacterium designated as PAH-13 was isolated from bitumen (heavy crude oil) soil collected from Mathura refinery, India. The isolate was screened for its ability to degrade 3–4 ring PAH compounds (anthracene, fluorene, phenanthrene and pyrene) in minimal medium supplemented with 0.1% yeast extract as a co-substrate. The isolate was found to degrade four PAHs between 28%–92% within 15 days at 100 ppm level. In addition, the strain showed good extracellular production of biosurfactant measured as emulsification index (55%) in the presence of glucose as co-metabolic source. The isolate PAH-13 was identified as *Streptomyces rochei* by phylogenetic analysis of 16S rDNA sequence. The above findings indicated the potential of *S.rochei* for bioremediation of PAHs. This is first report confirming the involvement of *S.rochei* in PAHs degradation.

Keywords Biodegradation · *Streptomyces* sp · PAHs · HPLC · Bitumen

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants found in various ecosystems. They are considered as priority pollutants due to their potential toxicity, mutagenicity and carcinogenicity. PAHs occur as natural constituents and combustion products of crude and refined fossil fuels. In recent decades, the major sources of PAHs pollution are industrial production, vehicular discharge, gasification and plastic waste incineration. Due to their hydrophobic nature, most PAHs bind to particulate in soil, rendering them less available for biological degradation (Cerniglia 1992; Mueller et al. 1996). Microbial metabolism plays an important role in bioremediation of contaminated sites.

Various microbial groups participate in degradation of PAHs in soil but actinomycetes are good candidates for soil bioremediation because they utilize a wide range of carbon sources including aromatic lignin molecules. Actinomycetes possess peculiar characteristics i.e. resistance to drought, survival and growth in alkaline pH and production of extracellular enzymes which make them suitable candidates for bioremediation (Goodfellow and Williams 1983). In the present study an actinobacterium strain was isolated from bitumen sample collected from Mathura refinery of India and evaluated for degradation of PAHs.

Materials and Methods

Bitumen samples were collected from Mathura refinery, India. Four PAHs (anthracene, fluorene, phenanthrene and pyrene) were purchased from Merck India Ltd. Different culture media i.e. Bushnell and Haas (B&H) and Glucose-yeast-malt extract were prepared using standard protocols (Bushnell and Haas 1941).

Isolation of actinobacterium was done by enrichment technique. Bitumen unit soil (10 g) was spiked with a mixture of PAHs (anthracene, fluorene, phenanthrene and pyrene 100 μg/g each) in Bushnell and Haas medium and incubated for 15 days at 30 ± 1°C. Subsequently 1 ml of sample was withdrawn, serially diluted and plated on Bushnell and Haas medium plates spiked with PAH

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mixture containing 100 ppm each of anthracene, fluorene, phenanthrene and pyrene. Typical powdery colonies were picked up and purified on glucose-yeast-malt extract medium. The purified isolate was characterized for different phenotypic traits following standard procedures described in Bergey's manual of Systematic Bacteriology (Kreig and Holt 1984).

DNA isolation of culture was done by growing culture overnight in glucose-yeast extract medium at 30 ± 1°C using the standard protocol of Charles and Nester (1993) with slight modification. PCR amplification of 16S rRNA region was carried out using primers (16S F 5′AGAGT TTGATCCTGGCTCAG3′, 16S R5′ ACGGCTACC TTG TTACGACTT-3′). Phylogenetic analysis of partial 16S rRNA gene sequence of PAH-13 was done by homology search with the standard 16S rDNA sequences available in Genbank using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov). The nucleotide sequences with maximum homology were aligned with the partial 16S rDNA sequence of the isolate PAH-13 using ClustalW multiple sequence alignment program (version 1.83).

Emulsification activity, cell surface hydrophobicity and growth dependent changes were estimated in the culture grown with individual and mixture of PAHs. The emulsification index (EI 24) of isolate with individual PAHs and glucose was determined by method of Cooper and Goldenberg (1987). Cell affinity towards different PAH compounds were measured by the method of Rosenberg et al. (1980). Biosurfactant production was measured by method of Mukherjee et al. (2009). The nature of biosurfactant was analysed by method of Sawhney and Singh (2000).

For evaluation of degradation potential of isolate PAH-13, the microbe was grown under controlled conditions in B&H medium containing 0.1% yeast extract as N-source fortified with 100 ppm each (anthracene, phenanthrene, fluorene and pyrene) of PAH compound under submerged conditions. The medium was inoculated with 10% v/v of spore suspension of strain PAH-13 containing 10^7 spores/mL. The flasks were incubated at 30 ± 1°C on rotary shaker at 150 rpm. Samples were withdrawn at 3, 5, 7, 15 day, extracted with ethyl acetate and analysed for residual PAHs by HPLC. Growth in terms of total protein was also estimated by the method of Lowry et al. (1951).

HPLC analysis was done using Hewlett Packard HPLC instrument (series 1100) equipped with degasser, quaternary pump, photo diode-array detector connected with rheodyne injection system (20 µL loop) and a computer (model Vectra). The stationary phase consisted of Lichrospher on C-18 packed stainless steel column (250 mm × 4 mm i.d). Chromatogram was recorded in a Windows' NT based HP chemstation programme. Acetonitrile: water in the ratio of 60:40 at 1 mL/min flow rate was used as mobile phase. HPLC analysis was performed at wavelength of 246 and 208 nm, which was detected for absorption maxima of compounds using photodiode array (PDA) with isoplot option. Each run was repeated thrice and the detector response was measured in terms of peak areas.

All the estimations were performed in triplicate and mean was calculated as per standard procedures. Correlation analyses were undertaken using Microsoft Excel package. Error values at 5% level are depicted in the graphs as bars.

Results and Discussion

The PAH degrading soil actinobacterium was isolated using enrichment culture technique, from bitumen unit soil of Mathura refinery. Bitumen (Heavy crude) is extremely viscous black sticky bottom feature of crude oil, containing high concentrations of highly condensed polycyclic aromatic hydrocarbons. It contains high concentration of asphaltenes resins, nitrogen and sulphur containing heteroaromatics and several metals (Leon and Kumar 2005). The characteristics of bitumen soil are given in Table 1. The actinobacterium PAH-13 was identified as *Streptomyces sp* on the basis of various physiological and biochemical tests as described in Bergey's Manual of Determinative Bacteriology. The isolate was found to be gram positive rods. The culture was catalase positive, exhibited the ability to reduce nitrate to nitrite and exhibited the ability to degrade esculine, casein, gelatin, starch and L-tyrosine. Phylogenetic analysis of 16S rDNA showed greatest homology (99%) with the members of *Streptomyces rochei.* The sequence has been submitted to NCBI with accession number GQ904711.

The culture was evaluated for emulsification index at 24, 48 and 72 h with individual PAHs as well as PAH mixture. Emulsification index of individual PAH as well as PAH mixture ranged from 45 to 52% (Fig. 1). The PAH mixture was also evaluated after addition of glucose (0.1%) and emulsification index increased to 55% at 72 h. The amount of biosurfactant produced was 1.8 g/L and found to be carbohydrate in nature. Affinity studies indicated maximum affinity of bacterial cells towards fluorene (3.3%)

Table 1 Characteristics of bitumen soil

S·No	Characteristic (unit)	Values
1	pH	7.1
2	EC (mS/cm)	0.50
3	Organic C (%)	0.54
4	N (Kg/ha)	37.63
5	P (Kg/ha)	241.92
6	K (Kg/ha)	478.91

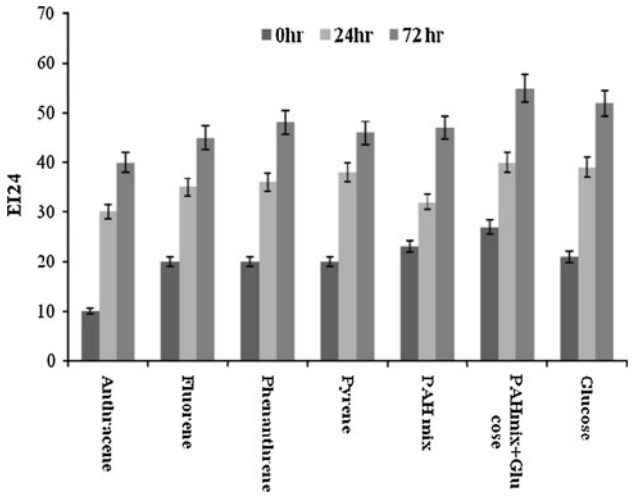

Fig. 1 Emulsification index of *Streptomyces sp.* for different PAH compounds

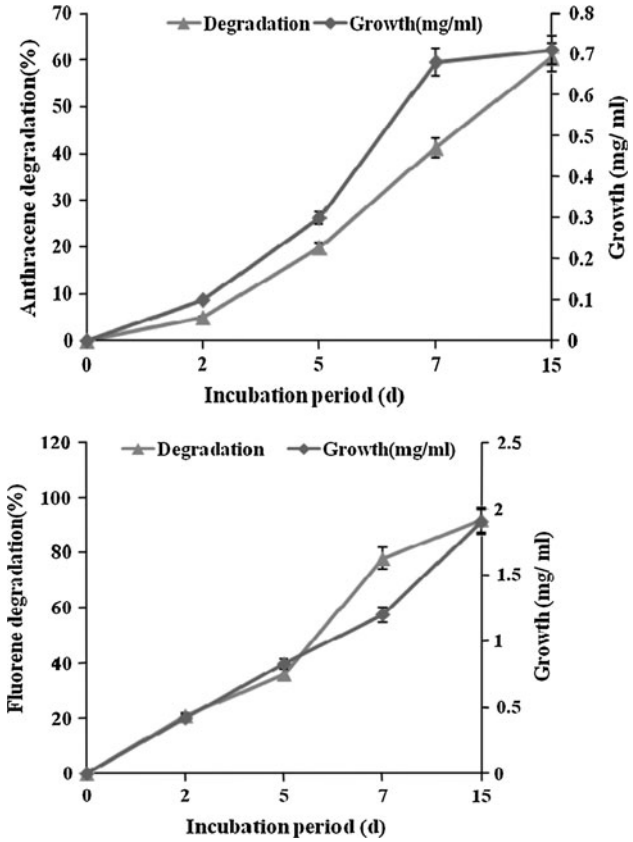

Fig. 2 Growth versus degradation of anthracene and fluorene by *Streptomyces sp* in submerged medium containing PAH as sole carbon source

followed by phenanthrene, pyrene and anthracene (2.9%, 1.2% and 0.6%, respectively).

The PAH degradation potential of *S. rochei* was estimated by HPLC analyses of the samples at 3, 5, 7, 15 day. The four PAHs i.e. fluorene, phenanthrene, anthracene and

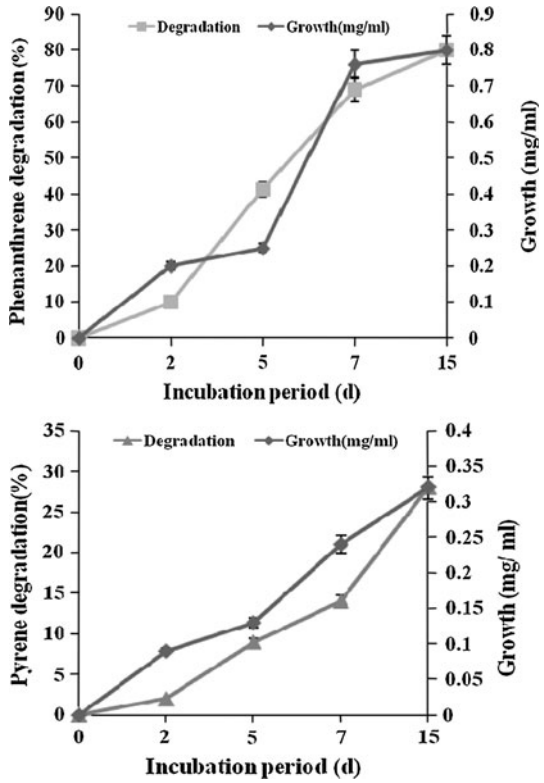

Fig. 3 Growth versus degradation of phenanthrene and pyrene by *Streptomyces sp* in submerged medium containing PAH as sole carbon source

pyrene were well separated at retention time 7.18, 8.08, 8.91 and 13.06 min, respectively. At 100 ppm level, degradation of all the four PAHs was highest on 15 day. The degradation of different PAHs ranged from 28–92%. Within 15 days fluorene, phenanthrene, anthracene and pyrene were degraded to 92, 80, 60.2 and 28%, respectively (Fig. 2). The degradation of 3-ring PAHs was found to be more than that of 4-ring PAH i.e. pyrene (Fig. 3). Maximum degradation (92%) was observed with fluorene where two metabolites, cis muconic acid (RT = 1.265 min) and catechol (RT = 2.239 min) were also detected during HPLC analysis by comparison with authentic standards. Growth was significantly correlated with degradation (anthracene; r = 0.970, fluorene; r = 0.971, phenanthrene; r = 0.864, pyrene; r = 0.957). Generally, bacteria and non lignolytic fungi metabolize PAHs via initial PAH ring oxidation involving dioxygenases to form cis-dihydrodiols, which are transformed to catechol compounds by dehydrogenases. Several other mono and dioxygenases also oxidised substituted catechol and non-catechol compounds. Finally, all ortho- or meta-cleaved metabolites are further oxidized to simpler compounds (Haritash and Kaushik 2009). This actinobacterium also produces bio-surfactant, leading to the formation of true micelles, and enhancing the bioavailability of PAH for

degradation. Further, the presence of yeast extract as a co-substrate in minimal medium significantly stimulated the degradation of 3–4 rings PAH.

Our study shows that *Streptomyces rochei* is a good PAH degrader and the bioavailability of PAHs may be attributed to its biosurfactant production and emulsification ability. To the best of our knowledge, this is first report on degradation of PAH by *Streptomyces rochei.* This strain could be used for bioremediation of PAHs contaminated soils.

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