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Isolation and characterization of polycyclic aromatic hydrocarbon degrading bacteria from contaminated soil sludge in Tamilnadu

(*Isolation of PAH degrading bacteria*)

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ABSTRACT

Eight bacteria capable of utilizing naphthalene, as their sole source of carbon and energy for growth were isolated from contaminated soil sludge in Chidambaram, Mayiladudurai,at Tamil nadu. By standard bacteriological methods, these bacteria were characterized taxonomically as belonging to the genus *Bacillus* and *Pseudomonas* .Four of the isolates (S5,S6,S2andS3), which showed the maximum growth during screening as demonstrated by an increase in their optical densities (OD610) and identified as *Pseudomonas* and *Bacillus* respectively, were also able to grow and degradation of naphthalene. There were visible changes in the colour of the growth medium of the isolates during their incubation, suggesting the production of different metabolites. There were also changes in their medium pH during growth. These studies demonstrate the possession by the bacterial species of novel degradative systems.

Keywords— *PAHs, persistence, biodegradation, Pseudomonas and Bacillus*

I. INTRODUCTION

PAHs are ubiquitous contaminants of aquatic and terrestrial ecosystems whose presence is attributable to a number of petrogenic and pyrogenic sources, which had increased since the end of the Second World War (Laflamme and Hite, 1978; NAS, 1983; Jonsen et al., 2005). Environments contaminated with PAHs are considered hazardous as studies using animals have shown the specific carcinogenic, mutagenic and teratogenic effects of some PAHs (Miller and Miller, 1974; Moore et al., 1989; Autrup, 1990). Their 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 (Jonsen et al., 2005). Even though higher molecular weight PAHs such as those containing four or more benzene rings are considered to be responsible for the majority of the potential hazards of these compounds to the environment and human health (EPA, 1984), lower molecular weight types such as naphthalene (the simplest containing two benzene rings), anthracene and phenanthrene (both of which contain three benzene rings) are known to have health effects that though are comparatively mild could be potentially hazardous (Klaasen, 2001). Furthermore, some like phenanthrene is considered as a model substrate in environmental PAHs degradation studies because its structure is found in the nucleus of carcinogenic PAHs such

as benzo[a]anthracene and 3-methylcholanthrene (Cerniglia and Yang, 1984). As a result of these hazardous effects of PAHs, there is much interest in their environmental effects. Although some physical processes msuch as volatilization, leaching, chemical and photo oxidation are often effective in reducing the environmental level of PAHs (Bossert and Bartha, 1984; Heitkamp et al., 1988), biodegradation using microorganisms is usually the preferred and major route of PAH removal from contaminated environments because of some inherent advantages such as its cost effectiveness and more complete cleanup (Pothuluri and Cerniglia, 1994). Moreover, the physical processes are often limited to aquatic environments only. The microorganisms should possess all the necessary enzymes needed to degrade PAHs. It is known that selection or adaptation of PAHdegrading microorganisms as with other chemicals occur as a result of their previous exposure to this substances in the environment (Lewis et al., 1984; Spain et al., 1980). However, these adaptations occur slowly, and usually depend on the recalcitrance or biodegradability of the particular substance involved (Spain et al., 1980). This is especially so considering that PAHs usually have low aqueous solubility and thus, are poorly available (low bioavailability) for microbial utilization. (Jonsen et al., 2005). A lot of isolated microorganisms have been successfully utilized in major hazardous waste clean-up processes, as for example, in industrial process streams and effluents (Levinson et al., 1994). Unfortunately, most of these studies were carried out in Western countries, and to a limited extent in South America and Asia (Kiyohara et al., 1982; Ghoshal et al., 1996; Prantera et al., 2002). In this work, we report the isolation and characterization of PAH (naphthalene)-degrading bacteria from soil sediments in Tamil nadu coastall environment, and their course of growth in naphthalene and other aromatic compounds..

II. Materials and Methods

Sample Collection

Sludge sample was collected with a sterile scoop from a layer 0 to 30 cm deep at contaminated soil sludge in Chidambaram, Mayiladudurai,at Tamil nadu. India. The sample was kept in a sterile container and stored in laboratory at 4°C (Refrigerator).

Bacterial Enrichment

Sludge sample of 10.0 g was put in to 250 ml Erlenmeyer flask having 150 ml of mineral salt broth (MSM) supplemented with 0.0250 gl-1naphthalene as the single source of carbon and energy. Mineral salt medium (MSM) used was composed of (NH4)2SO4 -1 gl-1, KH2PO4 -0.2 g l-1, K2HPO4 -1.6 gl-1, MgSO4.7H2O -0.2 gl-1, NaCl -0.1 g l-1, FeSO4 -0.1 g l-1 and CaCl2.2H2O -0.02 g l-1 (Sakata et al. 2004). Medium was prepared in deionized water and pH was maintained to 7-7.2 using 0.4M HCl or 0.4M NaOH. Medium was sterilized and kept at temperature31±2°C in an orbital shaker at 120 rpm for 7 days. Afterwards, 1.0 mL sample was taken from each culture and transferred into fresh enrichment medium, followed by incubation as described above for one week. The enrichment procedure was repeated for the third time, before their bacterial contents were isolated using a solid medium containing the enrichment medium and 15.0 g/L of pure agar. Inoculated plates were purified by repeatedly sub culturing. Pure cultures obtained by this procedure were stored in slants of enrichment medium with 15.0 g/L pure agar, and also in nutrient agar, and stored at 4 ^{o}C.

Screening of the isolates for the ability to use Naphthalene as sole source of carbon for growth

A loopful of each isolate was inoculated into large test tubes containing 25 mL of screening medium. The screening medium was the same as the enrichment medium, except that 15 mg of naphthalene dissolved in DMSO was added to each tube after autoclaving, as sole source of carbon. Thereafter, the test tubes were statistically incubated by keeping on the laboratory bench at room temperature (30 – 32 ^{o}C) for three days. The

ability of each isolate to utilize naphthalene was indicated by an increase in turbidity of the medium measured at 610 nm using a UV spectrophotometer.

Identification, characterization and standardization of isolates

The isolates was identified by colour, morphological, physiological, utilizhztion of carbon and biochemical test were tested in our laboratory as per Bergey's manual of systematic bacteriology. All isolated bacteria were scrutinized by Gram's staining reaction to differentiate between Gram positive and Gram negative bacteria. All the isolates were code-named and subsequently used for further studies. Before usage in subsequent works, cells were washed and standardized to the McFarland nephelometer standard of 0.5 (Baron and Finegold, 1990). In all cases, 1o/o v/v of standardized inoculum was used according to the volume of medium used. Isolates, which gave the highest OD readings, were identified to their species level.

Batch experiment of naphthalene degradation

For biodegradation studies, bacterial strain were pre inoculated into 100 ml of MSM containing 50 mg/l Naphthalene in Erlenmeyer flasks, and incubated for 24 h at room temperature, while shaking at 120 rpm in dark. From that medium the bacteria cell was harvested and diluted in sterile medium, Then, 3ml of, bacterial cell suspension of 0.06 optical densities (OD) at 610 nm were utilized as inoculums. All batch experiments conducted in 250 ml conical flasks containing 150 ml of (MSM) at pH-8-8.5 and added naphthalene as a substrate at concentration (100 mg 1^{-1}) individually as carbon source against respective un inoculated controls. The batch reactor were placed in a shaker (120rpm) at lab temperature of 31±1°C. The growth was examined over a period of 1 day by quantifying the OD values and biomass dry weight (DW). All the operations were done under sterilized conditions and testing were conducted in triplicate. The residual PAHs were determined by gas chromatography.

III. Results and Discussions

Screening and characterization of the isolates

Twenty-eight bacteria isolates were obtained from mixed soil sludge samples after screening,Eight microorganism which were found to degrade naphthalene in mineral salt broth medium (MSM) supplemented with 25 mg/L naphthalene, as the single source of carbon and energy. The pure cultures isolated were labelled sequentially as S1, S2, S3,S4,S5,S6,S7 and S8. The microbes were characterized based on Gram staining test and cell morphology. Among them two (S1,S2,S4andS6) were Gram negative and long, slender rods, others were Gram positive (S3,S5,S7 and S8). Each strain was further evaluated through batch experiments for s ability to degrade PAHs. From the "Fig .1" present the growth form of the isolates observed in the occurrence of PAHs. Out of eight, four organisms S2, S3, S5 and S6 exhibited growth by utilizing PAHs in the order of S6>S5>S3>S2. The isolates S2, S3, S5 and S6 were organisms were further isolated with high purity by repeated inoculation in PAH spiked media and plating them on petri plates. The morphological, physiological, and biochemical characteristics of these two microorganisms were tested as per the Bergey's Manual of Determinative Bacteriology (reference) and the results are summarized in Table 1 and Table 2.

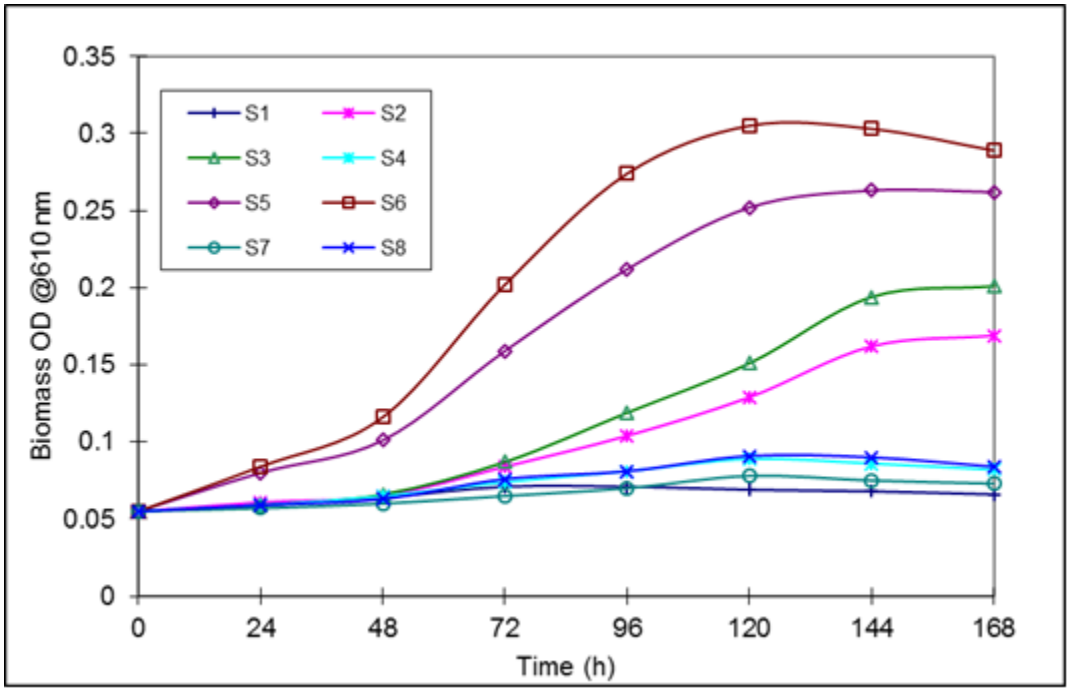

Fig. 1. Growth pattern of isolates in the presence of PAH mixtures

TABLE 1. Biochemical characteristics of the isolates

S. No	Biochemical characteristic	S2	S3	S5	S6
1	Catalase	+	+	+	+
2	Oxidase	+	+	+	+
3	Starchhydrolysis	+	+	+	+
4	Gelatinliquefaction	+	+	-	+
5	Citrate utilization	-	-	-	+
6	Denitrification	+	+	+	+
7	D-Glucose	+	+	+	+
8	Fructose	-	+	+	+
9	Gluconate	+	+	+	+
10	Glycerol	+	+	+	-
11	Tartrate	-	-	-	+
12	Malate	-	-	+	-
13	Mannitol	+	-	-	+
14	Pyruvate	-	-	+	+

TABLE 2. Morphological characteristics of the isolates

S.No.	Morphological characteristics	S2	S3	S5	S6
1	Typeofcolony	Small smooth convex andgrey	Large opaque, adherent irregular edges	Wrinkled	Smooth
2	Cell diameter	0.5-1μm	0.1-0.3 μm	0.7 – 0.8μm	Less than 1.0μm
3	Endospore	-	+	-	+
4	Pigmentation	-	-	Pale orange	-
5	Motility	+	+	+	+
6	Gram nature	Gram positive rods	Gram positive rods	Gram negative rods	Gram negative longrods

Morphologically the strain S2 and S3 were observed as rod-shaped, Gram- positive, alkaline tolarent bacterium. Optimal growth of the isolate occurred around 30°C, but growth was also observed at much normal pH(7.0-7.5). The results of bacteriological and biochemical characteristics of this organism suggested that the strain S2 and S3 belong to the genus BacillusMorphological examinations of S5 and S6 revealed that the isolate was aerobic rod-shaped, gram negative bacteria. Colonies were wrinkle and, smooth, Pale orange. The results of bacteriological and biochemical characteristics of this organism suggested that the strain S5 and S6 were belongs to the genus Pseudomonas and Bacillus.

Biodegradation of Polycyclic Aromatic Hydrocarbons

The naphthalene biodegradation efficiency of isolates was studied by the initial quantity of naphthalene spiked in the media. The efficiency of PAHs removal for naphthalene concentration by the isolates in batch studies as presented in "Fig. 2". It is observed that the isolates (S6,S5,S3 andS2) were degrades naphthalene about 95.1,42.67,26.45 and 18.45% at 100 mg l-1 initial concentration within 168h respectively. The increase in cell biomass against time for various initial naphthalene concentrations of isolates (S6,S5,S3 andS2) were presented in "Fig. 3".

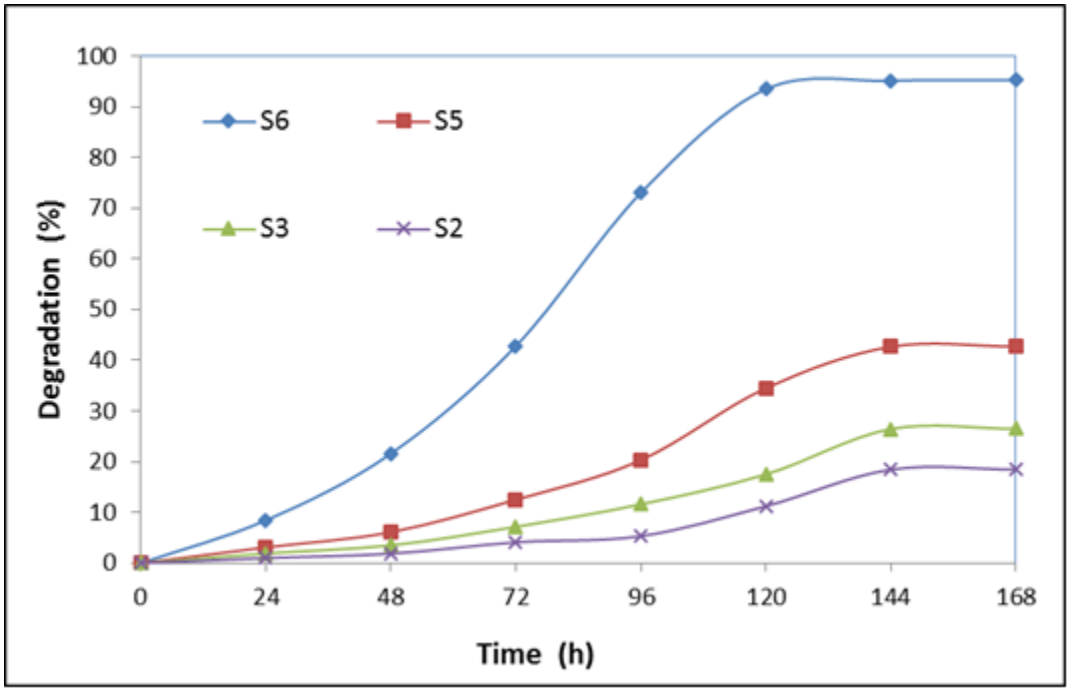

Fig. 2. Efficiency of naphthalene removal by isolates (S2,S3,S5 and S6)

PAHs are a complex class of organic compounds present in the environment. Bio degradation of PAHs by native microorganism is considered a safe and eco- friendly method to remove the contaminants. Soil contaminated with hydrocarbons are good sources for the isolation of PAHs degrading bacteria, (Jacques et al. 2009; Al-Thani et al. 2009) which can then be used for the removal of such compounds from the contaminated place. In this study, the bacterial strain was isolated from the marine sediment to utilize polycyclic hydrocarbons as single carbon energy source. Among the eight selected bacterial strains four (S2,S3,S5, and S6), showed high biomass growth in naphthalene as a sole Carbon energy. The selected four bacteria were recognized as; Bacillus and Pseudomonas. Previously different strains of Bacillus have been find out from PAHs contaminated soil (Das and Mukherjee, 2007; Jacques et al. 2009; Lin et al. 2010), which have the possible to biodegrade and utilize organic compounds. The present study the isolates(S6,S5,S3and S2) degrades naphthalene at 100 mgl^{-1} within 168 h were 95.1,42.67,26.45 and 18.45%.

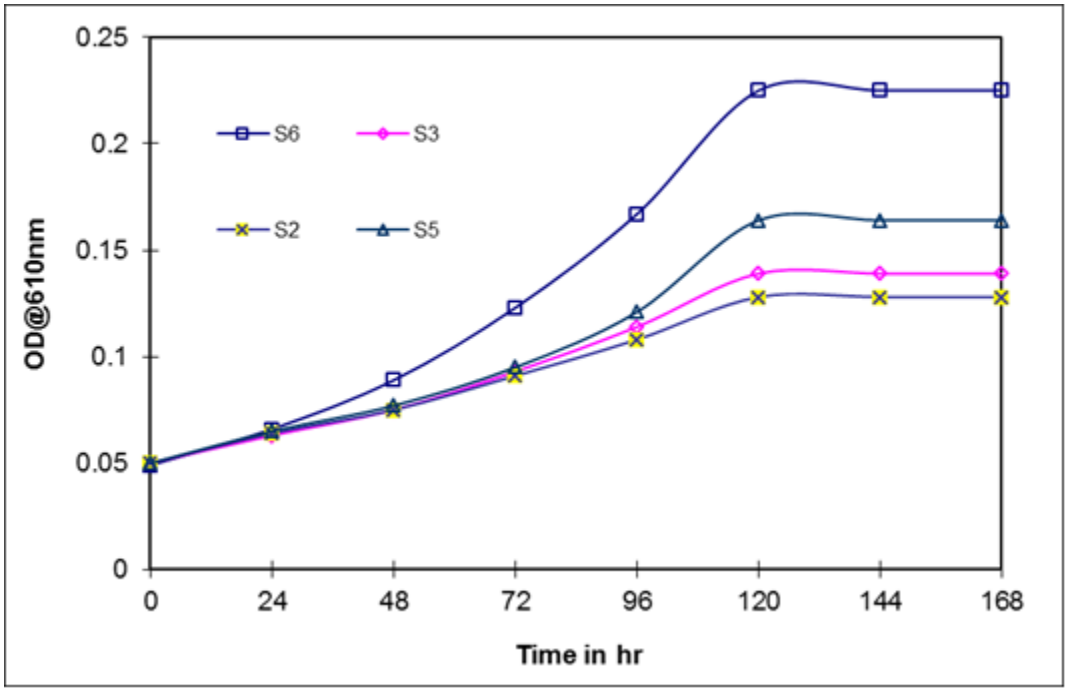

Fig. 3. Effect of naphthalene concentration(100mg l^{-1}) on growth of isolates (S2,S3,S5 and S6)

Conclusions

In the present study, we conclude the degradation efficiency of (PAH) by isolated aerobic bacteria(S2,S3,S5 and S6) was performed. The PAHs degradation tests were led in liquid medium, with the concentration of naphthalene (100 mg l^{-1}.The results exposed that the bacteria(S6) removed naphthalene completely 95.15 % at 168 h. Based on the results, the biodegradation potential of bacteria isolated from sludge should further be examined and optimized for bioremediation purpose. In future more studies on the interaction between different microorganisms, mixtures of PAHs, and effects of different environmental factors on biodegradation are essential

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