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Biodegradation of Polycyclic Aromatic Hydrocarbons Using Sponge *Biemna Fortis* Associated Bacteria

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Abstract: *In the present investigation, the degradation potential of sponge Biemna fortis associated bacteria sponge was carried out on 5 polycyclic aromatic hydrocarbons (PAH's). The five PAH's used were naphthalene, phananthrene, fluoranthene, pyrene and anthracene. The degradation studies were conducted in minimal broth media with concentration of PAH of 20mg/l. After 4 days of incubation at 28 ± 2°C, solvent extraction of the aqueous broth media was carried. TLC analysis of the broth extracts revealed that 4 bacteria were able to degrade 3 PAHs. The morphological and biochemical identification of these 4 bacteria revealed that they belong to Paenibacillus spp., Corynebacteium spp., Kocuria spp., Micrococcus spp.,*

Keywords: *Polycyclic aromatic hydrocarbons, Biemna fortis, fluoranthene, pyrene and phenanthrene.*

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are the recalcitrant pollutants that have accumulated in the environment due to anthropogenic as well natural activities where the major contribution is due to humans. In nature, PAHs arises due to the incomplete combustion of organic matter, volcanic eruptions and forest fires. Whereas PAHs released due to anthropogenic activities are as result of processing, production, transportation and spillage of petroleum, refuse burning, gasification and plastic waste incineration, cigarette smoking, automobile exhaust [1, 2].

Due to their hydrophobic nature and low water solubility they persist as in the environment for a long time [3]. Low-molecular-weight (LMW) PAHs composed of two or three fused benzene rings are readily degraded by bacteria, high-molecular-weight (HMW) PAHs consisting of four rings or more are recalcitrant to biodegradation and persist in the environment [4, 3]. Many of these compounds have been found to have toxic, mutagenic, neurotoxic and carcinogenic properties [2]. Due to their serious effects on flora and fauna, the US Environmental Protection Agency (US EPA) has listed 16 PAHs as priority pollutants for remediation [5].

Since 1970s, bioremediation has proven as attractive and cost effective tool for the restoration of PAHs contaminated sites [6]. Microorganisms have been found to degrade PAHs either via metabolism or cometabolism. Many biodegradation studies have been carried using pure as well as mixed culture of microorganisms in laboratory experiments [7]. But it is still difficult to study the biodegradation of PAHs in natural environment due to environmental factors like temperature, pH, oxygen concentration, salinity, light intensity, sediment type, the presence of co-substrates, and season. Moreover the biodegradation of PAHs also depends on the concentration and bioavailability of PAH to the microorganisms [1].

In recent years the ocean has been considered as a rich source of compounds possessing novel structures and biological activities [8]. Sponge harbors a rich diversity of microorganisms in their tissues. These organisms include a diverse range of green algae, heterotrophic bacteria, cyanobacteria, archaea, cryptophytes, red algae, dinoflagellates and diatoms [9]. Many sponges contain chemical compounds with bioactive properties, several of which are promising as pharmaceutical leads, and sponge-associated microorganisms are of interest due to their potential role in producing some of these compounds [10]. Most of marine sponges harbor dense and diverse microbial communities of bioactivity importance. Four Gram positive bacterial cultures (HA-21, HA-68, HAMS- 105 and HA-

MS-119) were isolated from the sponge *Amphimedon ochracea*, collected from the Red Sea coast of Egypt [11].

The selection of appropriate bacterial species is critical in the successful application of biodegradation techniques. The present study is an attempt to isolate and identify new indigenous bacterial species from the sponge *Biemna fortis* and to assess their capability to degrade high concentrations of polycyclic aromatic hydrocarbons (PAHs).

2. Materials and Methods

2.1. Collection of Sponge

The sponge *Biemna fortis* was collected from the Mhapan region, Maharashtra, India. The sponge was stored at -20^{0}C and was used for further analysis.

2.2. Isolation of Sponge Associated Bacteria

Approximately 1 gram of sponge tissue was excised out and was washed with sterile sea water aseptically (Fig.1). The sponge tissue was homogenized with sterile sea water and up to 10^{-3} dilutions were made using sterile sea water. 0.1ml of dilutions from 10^{-1} to 10^{-3} was spread plated on Zobell marine agar (ZMA) and Nutrient agar (NA) media plates. The plates were incubated at 28 ± 2°C for 24-48 hours.

2.3. Purification and Storage

The isolates were sub-cultured on the ZMA media to get pure colonies (Fig. 2). The plates were sealed and stored at 4^{0}C till further use.

Fig1. *Sponge Biemna fortis used for isolation of sponge associated bacteria.*

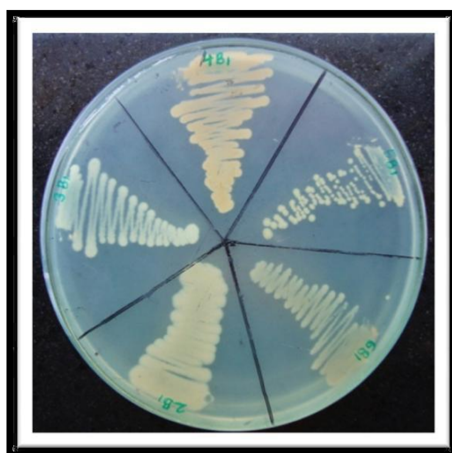

Fig2. *Purified cultures of isolates*

2.4. Biodegradation Studies

A loop full of pure isolates was added to 10ml of Zobell Marine broth (ZMB) and was allowed to grow for 48 hours incubated at 28 ± 2 °C in a shaker incubator. After 48 hours of incubation, the bacterial cell mass was separated from broth using centrifugation. The broth was discarded and the cell pellet was washed twice using Phosphate buffered saline (PBS) to remove the media components.

The pelleted cells were transferred to 250ml of PBS (50mM, pH of 7.2) and PAH were added to it with a concentration of 20mg/l. Each of the PAH was subjected separately to all the bacterial isolates. The flasks were incubated for 4 days in shaker incubator at 120rpm at 28 ± 2°C (Fig.3). Meanwhile, blank (sterile PBS) and control (PBS + PAH) were also maintained with the same conditions. After the period of incubation the PBS containing the culture was centrifuged to separate out the supernatant and the cell pellet mass.

2.5. Solvent Extraction

The supernatant was transferred to a separating funnel for solvent extraction to which distilled chloroform was added, 30% of the total volume of broth. More solvent was also added to get better separation. The mixture was shaken vigorously in a separating funnel and was allowed to stand undisturbed till two distinct layers were formed, that is the upper aqueous layer and bottom organic layer. Since the polycyclic aromatic hydrocarbons used are soluble in chloroform they readily dissolved in it and get separated out from the aqueous layer and move into organic (chloroform) layer. The chloroform layer formed below the aqueous layer was separated out distinctively. This step was repeated twice to remove any solutes if present. The chloroform layer was then washed with distilled water twice to remove any salt impurities (Fig. 4).

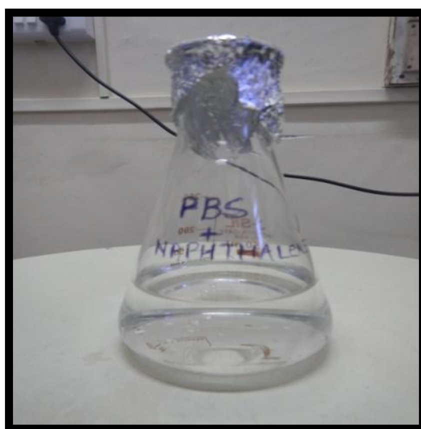

Fig3. *PBS containing PAH*

Fig4. *Chloroform extraction of broth.*

2.6. Concentration of Chloroform Extract

The concentration of the chloroform layer was carried out in a rocket evaporator which uses the principle of vacuum to evaporate a large volume of solvent leaving behind the dry component extracts (Fig. 5). To these dry extracts, a minimal volume of distilled chloroform was added and the solvent was then transferred into dry glass vial and subjected to rota-evaporator which also uses the principle of vacuum to evaporate the solvent from the vial (Fig. 6). The vials containing the extracts were then stored at 40C till further use.

Fig5. *Rocket evaporator used for concentration of high volume of solvent*

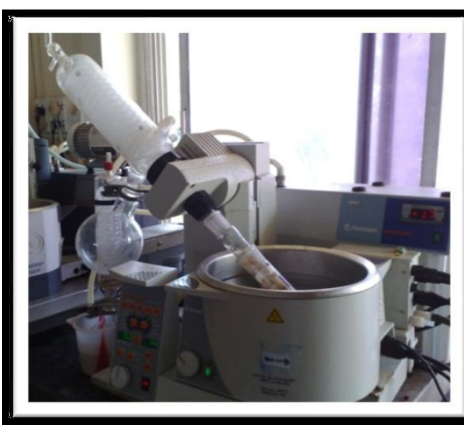

Fig6. *Rotor evaporator used for concentration of solvent*

2.7. Thin Layer Chromatography (TLC) Analysis

The dried extracts were dissolved in minimal volume of chloroform. The extracts were spotted on the TLC plates along with blank (sterile PBS), control (PBS + PAH) and standard (PAH). Petroleum ether was used as a mobile phase to run the plate. The plates were dried and then observed under UV light to visualize the presence of PAH. The plate was sprayed with methanolic sulfuric acid; air dried and then kept in hot air oven for visualization of spots. The bacterial cultures found to degrade any of the PAH were identified using morphological and biochemical tests according to the Bergey's Manual of Determinative Bacteriology.

3. Results and Discussion

Table1. *Morphological and biochemical properties of PAH degrading bacteria*

	Colony morphology			
Properties	*Paenibacillus macquariensis*	*Kocuria varians*	*Micrococcus luteus*	*Corynebactreium xerosis*
Gram Reaction	+	+	+	+
Cellular Morphology	Rods	Cocci	Cocci	Rods
Spore Test	+	-	-	-
Pigmentation	-	Yellow	Yellow	-
Biochemical Test				
Catalase	+	+	+	+
Oxidase	+	-	-	+
Methyl Red	+	-	-	-
Voges-Proskauer	-	-	-	-
Starch Hydrolysis	+	-	-	-
6.5% NaCl Growth	+	-	-	-
Acid Production from				
Glucose	+	+	-	-
Mannitol	-	-	-	-
Arabinose	-	-	-	-

(-) Negative Result *(+) Positive Result*

The morphological characteristics and biochemical analysis of these 4 bacteria revealed that they belong to *Paenibacillus macquariensis, Kocuria varians, Micrococcus luteus, and Corynebactreium xerosis* (Table 1).

The bacterial cultures isolated from *Biemna fortis* were found to biodegrade PAHs: particularly anthracene, fluoranthene, naphthalene, phenanthrene and pyrene. 4 bacterial isolates were found to be utilizing of naphthalene, fluoranthene and pyrene and as sole carbon and energy source for growth.

According to TLC analysis, it was observed that naphthalene was degraded by all 4 bacterial cultures from 20mg/l to non-detectable levels in 4 days. The bacterial cultures which degraded naphthalene were *Paenibacillus macquariensis, Kocuria varians, Micrococcus luteus, Corynebactreium xerosis* (Fig.7). This may be due to the fact that naphthalene is the simplest PAH having a fused pair benzene rings with highest water solubility of 31mg l^{-1} at 25°C that will result in more availability of the substrate to the bacteria.

In contrast, anthracene and phenanthrene showed no scene biodegradation in the minimal media by any of the Biemna fortis associated bacteria after subjecting 4 days of incubation time. The TLC analysis of culture extracts containing anthracene and phenanthrene from the bacterial culture showed prominent spots corresponding to the standard anthracene and phenanthrene spot and having the same Rf value respectively (Fig. 11 & Fig. 12).This may be due to the fact that anthrance and phenanthrene have less solubility in medium i.e. anthracene and phenanthrene have water solubility of 0.444mg/l at 250C and 1.6mg/l at 250C. This makes the PAH not available for degradation, since the bacteria are well know to degrade those compounds which are soluble in water [12]. But it is not the case in all because the PAH also depends on other factors like presence of co-substrates and bio-availability. However solubility itself does not play a prominent role in all the cases, unavailability of co-substrates or low concentration of inorganic nutrients may adversely affect the uptake of PAH [13].

Fluoranthene & pyrene were degraded by the Paenibacillus macquariensis from approximately 20mg/l to non-detectable levels in 4 days (Fig. 8 & Fig. 9)

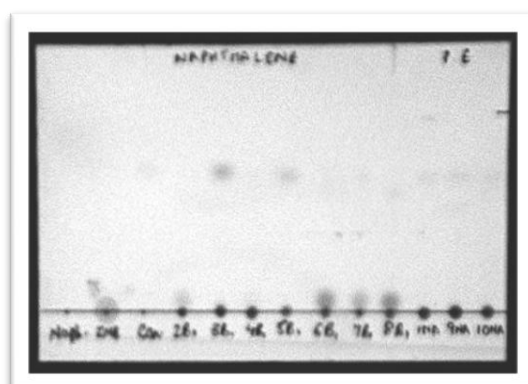

Fig7. *TLC analysis of naphthalene*

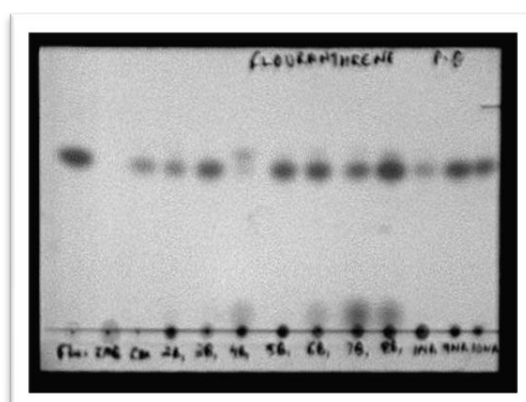

Fig8. *TLC analysis of fluoranthene*

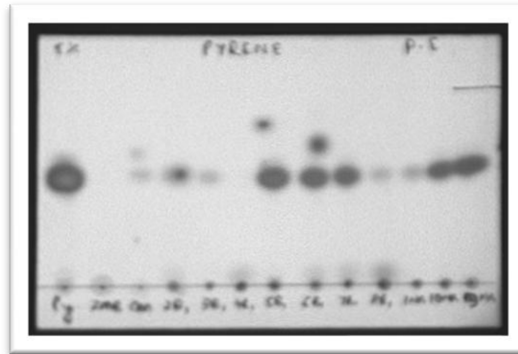

Fig9. *TLC analysis of pyrene*

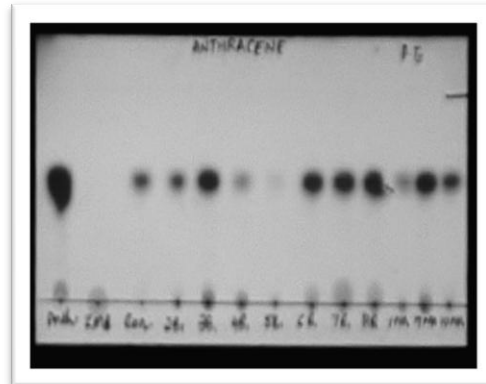

Fig11. *TLC analysis of phenanthrene*

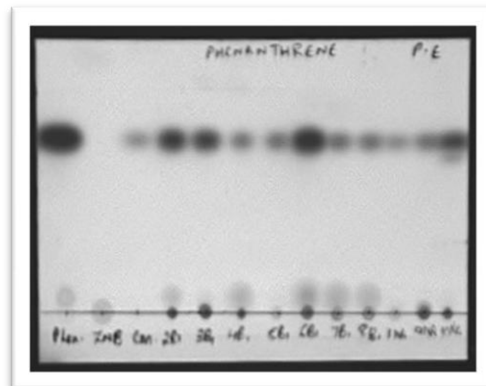

Fig12. *TLC analysis of anthracene*

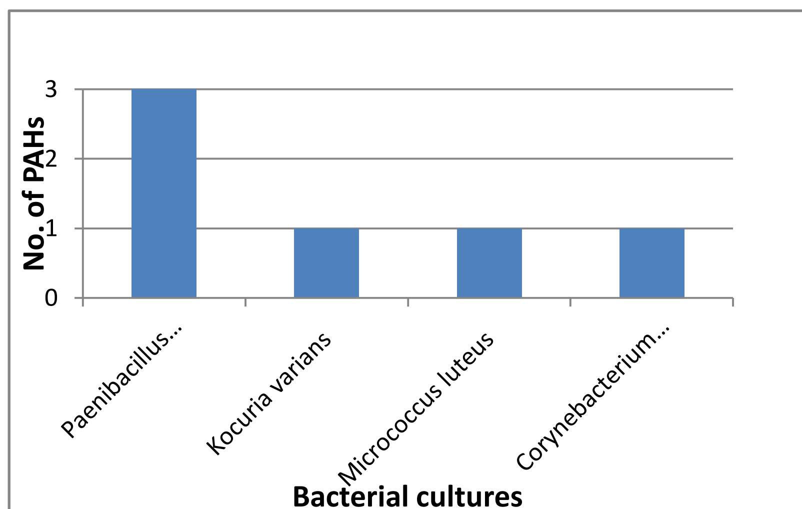

Fig13. *Depicts the number of PAHs degraded by the bacteria*

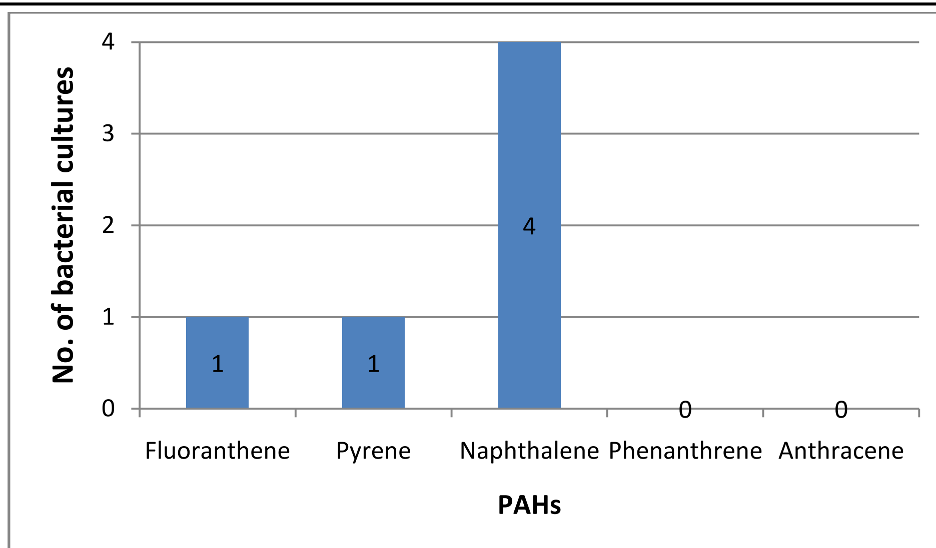

Fig14. *Depicts the number of bacterial cultures degrading the PAH.*

4. Conclusion

Bacteria associated with the sponge are complex and diverse. They live in a highly competitive environment in which access to space and nutrients are limited. Hence we exploited sponge *Biemna fortis* associated bacteria to utilize the PAH as the sole carbon source for their growth.

Considering the capability to metabolize 3 PAHs i.e., naphthalene, flouranthene and pyrene, *Paenibacillus macquariensis* is a potent degrader which was also capable of utilizing flouranthene and pyrene with more complex structure and lesser water solubility of 265µg/l at 25^0C and 0.135mg/l at 25^0C respectively. Hence *Paenibacillus macquariensis* can therefore be employed in metabolizing other recalcitrant PAHs having more complex structure and lesser solubility. With these promising results *Paenibacillus macquariensis* can itself play an effective role in the contaminated sites in cleaning up of more number of PAHs at a time.

These novel associations within the sponge may contribute to their ability to specialize in such oil-rich and potentially toxic environments. Whether PAH-degrading bacteria may act as detoxifiers or even contribute to the nutrition of the sponge via degradation of aromatic hydrocarbons remains unclear. Further studies to determine the function of these bacteria and determine the nature of their associations with the sponge, as well as to examine other invertebrates for associations with PAH-degrading bacteria in oil-seep or contaminated environments should yield interesting results.

In conclusion, our findings revealed associations of a complex bacterial community in sponge Biemna fortis capable of degrading PAHs. While our study suggests that these communities may contribute to the success of this sponge living in such unique ecosystems.

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