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Characterization of Anthracene Degrading bacteria from Drug Industry Effluent Polluted Soil

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ABSTRACT

Anthracene, a tricyclic aromatic hydrocarbon is used as an indicator compound to detect PAHs contamination in environment. Its removal from polluted sites is important as it causes health associated problems and being carcinogenic in nature. PAH degrading microorganism are present in various industry effluent contaminated sites. In the present study, thirty six anthracene degrading bacteria were isolated from drug industry effluent polluted soil using Bushnell Haas medium supplemented with anthracene as sole carbon and energy source. The anthracene degradation ability of these isolates were confirmed by 2, 6-dichlorophenol indophenol (2, 6-DCPIP) qualitative assay. Three bacterial isolates having highest anthracene degradation potential were selected and identified as Aeromonas hydrophila, Bacillus polymyxa and Streptococcus mutans on the basis of morphological and biochemical characteristics. The growth and pH change in the medium were assessed periodically at 1mg/ml concentration of anthracene for individual isolates as well as with consortium. The pH was decreased in all flasks; however highest decrease was observed in the medium inoculated with bacterial consortium. Similarly 94% degradation of anthracene was observed with consortium whereas B. polymyxa, A.hydrophila, and S. mutans degraded 80%, 52% and 44% of anthracene respectively. The study indicated consortium mediated biodegradation of anthracene is more effective in bioremediation of anthracene.

Keywords: Anthracene, Bioremediation, 2, 6-DCPIP assay, Percent Degradation, Consortia.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of recalcitrant compounds consisting of more than one benzene rings and composed solely of carbon and hydrogen atoms [17]. PAHs are used as chemical intermediate for dyes diluents, wood preservatives and pesticides. They are originated from natural (biogenic and geochemical) and anthropogenic activities and are highly carcinogenic, genotoxic and cytotoxic [2]. PAHs are ubiquitous in the environment and released as a result of incomplete combustion of fossil fuels and therefore present in a variety of products such as tar, coal, soot, petroleum and tobacco smoke (International Agency for Research on Cancer (IARC), [10]. PAH and their derivatives have been recognized as the major carcinogenic agent and causes human lung cancer, anaemia, asthma, splenomegaly, bladder cancer and Brest cancer [3, 21].

Anthracene is one of the important priority PAH pollutant among 16 PAHs listed by US-EPA. It consists of three fused benzene rings and it is toxic soil contaminant [11]. It is included in substances of Very High Concern list (SVHC) by European Chemicals Agency (ECHA) [6]. It is used in wood preservatives, insecticides and coating materials. Humans are mainly exposed through tobacco smoke and ingestion of food contaminated with combustion products. It also exhibits toxicity to fish, algae and shows bioaccumulation in the food chain [25]. It is used as a model PAH to determine the factors that affect the bioavailability, biodegradation potential and the rate of microbial

degradation of the PAHs in the environment [4, 13]. Physical, chemical and biological methods are used to remove the PAHs from polluted sites.

Microbial degradation plays major role in the removal of PAHs from contaminated sites. The ability to degrade PAHs is shown by various groups of organisms including bacteria, fungi and algae [28]. The bacteria can be isolated from soil polluted by effluent of paper, pesticides, petrochemical, polymer, rubber and printing, shoe, drug, industry, as effluent containing carbon source plays significant role in bioagumentation [8, 22]. These microorganisms also have role in biodegradation of lignin, resin, organophosphrous pesticides, phenols, azo dyes, and phorate [12]. The biodegradation rate and fate varies in aerobic and anaerobic condition. In aerobic biodegradation the genera like Pseudomonas, *Bacillus, Alkaligens, Acenetobacter, Streptococcus, Aeromonas, Bacillus* and *Mycobacterium* might have potential role in biodegradation of anthracene. These contaminated sites may act as source for isolation of potentional anthracene degraders.

The drug industry effluent may contain microorganism which might have high potentional to degrade anthracene effectively in individually or in consortium, hence these isolates will be promising for biodegradation of anthracene or some other complex structure PAHs. With the aim of remediation of anthracene polluted sites by potent isolates, the present study has planned to isolate and characterize anthracene degrading bacterial isolates from drug industry effluent polluted soil and to characterize anthracene degradation ability individually as well as in consortium.

MATERIALS AND METHODS

Chemicals and media

Anthracene was obtained from Sigma Aldrich, Bangalore. 2, 6-dichlorophenol indophenols, Bushnell Haas Broth (BHB) medium and other media ingredients were procured from Hi-Media, Mumbai. All chemicals used under study were of analytical grade.

Isolation of anthracene degrading bacteria

Soil samples were collected from drug industry effluent polluted site, Nanded, (M.S.) India. Soil samples were collected in sterile polythene bag, air dried in the dark and stored at 4^{o}C until use. Soil (1gm) was inoculated in BHB amended with 1 mg/ml anthracene as sole source of carbon and incubated at 32^{o}C under shaking conditions (120 rpm). After 8 days of incubation growth was observed in the form of turbidity and 5 % enriched broth was used as inoculums for re-enrichment in BHB supplemented with 1 mg/ml of anthracene repeated transfer was carried out in fresh BHB amended with increased anthracene concentration till a stable and consistent growth of anthracene degrading bacteria were obtained. The enriched broth was serially diluted upto 10^{-7} and 0.1 ml each dilution was spread on BH agar containing anthracene (1 mg/ml) and incubated at 32℃ for 48 hrs. The anthracene was dissolved in dichloromethane and allowed to evaporate before inoculation. Different colonies were selected on the basis of morphological characteristics.

2, 6-DCPIP (2, 6-Dicholorophenol indophenol) assay

The assay mixture containing 2250 µl of Fe-free W medium [14], 150 µl of $FeCL_3.6H_2O$ solution (150 µg/ml) and 150 µl of 2, 6-DCPIP solution (50 µg/ml) was mixed with 240 µl of bacterial cell suspension (O.D. 1.0 at 600 nm) and 25 µl of Anthracene (1 mg/ml in dichloromethane) the reaction mixture was incubated at 32℃ under shaking conditions (120 rpm) for 3 days. Anthracene degradation ability of the isolates was indicated by change in the colour of the medium from blue to colourless [16].

Identification of the anthracene degrading bacterial isolates

The identification of bacterial isolates with anthracene degradation ability was performed on the basis of microscopic observation and biochemical characterisation as per the standard criteria given in *Bergey's Manual of Systematic Bacteriology* (9th Edition). The tests used for identification were colony morphology, ability to hydrolyse starch, casein and gelatine, urease and catalase production, oxidase test, utilization of sucrose, mannitol, dextrose, lactose and fructose as carbon source, indole production and citrate utilization tests.

Anthracene degradation studies

Anthracene degradation studies were performed in flasks containing 50 ml BHB medium supplemented with 1 mg/ml anthracene. The individual isolates were grown in 5 ml of LB broth at 32^{o}C at 120 rpm. The culture broth was centrifuged at 5000 rpm for 5 min. The cell pellets were washed with sterile saline and resuspended in phosphate buffer to attain O.D. of 0.1 at 600 nm. 1 ml of each cell suspension was inoculated into 50 ml BHB. The cultures were incubated at 32^{o}C on orbital shaker at 120 rpm for 12 days.

The samples were removed periodically to observe the growth of isolates in terms of O.D at 600 nm and change in

pH of medium and residual anthracene. The 10 ml of ethyl acetate was added to 50 ml of BHB medium in capped test tubes and shaken vigorously for several times. The 5 ml of organic phase was transferred to quartz cuvetts. The λmax of anthracene was determined at 254 nm against ethyl acetate as blank. The % degradation of anthracene was determined by using following formula [18, 19].

$$\% \, degradation = \frac{A - B}{A} \times 100$$

A- Initial weight of anthracene.
B- Final weight of anthracene.

RESULTS

Isolation of anthracene degrading bacteria

The growth of anthracene degrading microorganism were observed in incubated BHB medium after 8 days of incubation of soil sample and the BH agar plate showed 36 well isolated, morphologically distinct colonies after spreading incubated browth at 32^{o}C.

2, 6-DCPIP assay

The 36 anthracene degrading bacterial isolates showed decolourization of 2, 6-DCPIP dye in varying time of incubation where as 6 bacterial isolates have to fast decolourization of DCPIP dye. However among the DCPIP decolourizing bacterial isolates, GS4, GD6 and GD7 had maximum rate of dye decolourization within 48 hrs of incubation (Fig1).

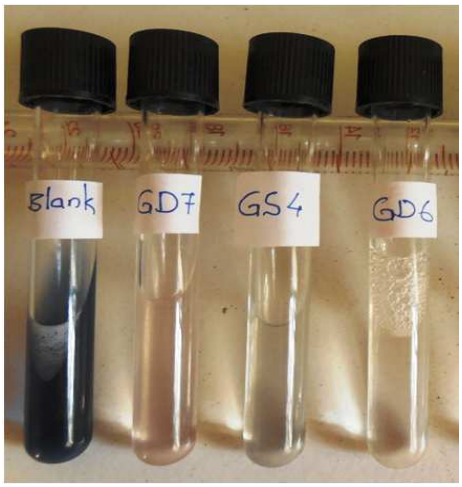

Fig.1. DCPIP assay of selected isolates showing dye discoloration within 48 hrs.

Identification of anthracene degrading bacterium

The anthracene degrading bacterial isolates GS4, GD6 and GD7 having maximum decolourization rate have identified as *Aeromonas hydrophila, Bacillus polymyxa* and *Streptococcus mutans* respectively using identification criteria given in *Bergey's Mannual of Systematic Bacteriology* (9th edition) i.e. catalase, oxidase, hydrolyzing starch, gelatine and casein, nitrate reduction, fermentative utilization of maltose, lactose, ribose, fructose, dextrose, mannitol and arabinose and IMViC tests (Table.1).

Table: 1. Biochemical characteristics of bacterial isolates.

Sr. No.	Isolates	GS4	GD6	GD7
1	Nitrate reduction tests	+	+	−
2	Catalase tests	+	+	−
3	Oxidase tests	+	−	+
4	Hydrolysis of			
	Starch	+	+	−
	Casein	−	+	+
	Gelatine	−	+	−
5	Fermentation of			
	Sucrose	+	+	+
	Dextrose	+	+	+
	Mannitol	+	+	+
	Fructose	+	+	+
6	Indole tests	−	−	+
7	Methyl red tests	+	−	+
8	VP tests	−	+	+
9	Citrate utilization	−	−	−
	Tentative identification	*Aeromonas hydrophila*	*Bacillus polymyxa*	*Streptococcus mutans*

Anthracene degradation studies

The anthracene degradation by *Aeromonas hydrophila***,** *Bacillus polymyxa* and *Streptococcus mutans* were studied with reference to biomass and change in pH of degradation medium. The biomass of all bacterial isolates used for degradation studies increases from 1st day to 12 th day of incubation whereas change in pH trend was also observe from 1st day to 12 th day of incubation. The slight decrease in pH was observed (Fig.2, Fig.3 and Fig.4).

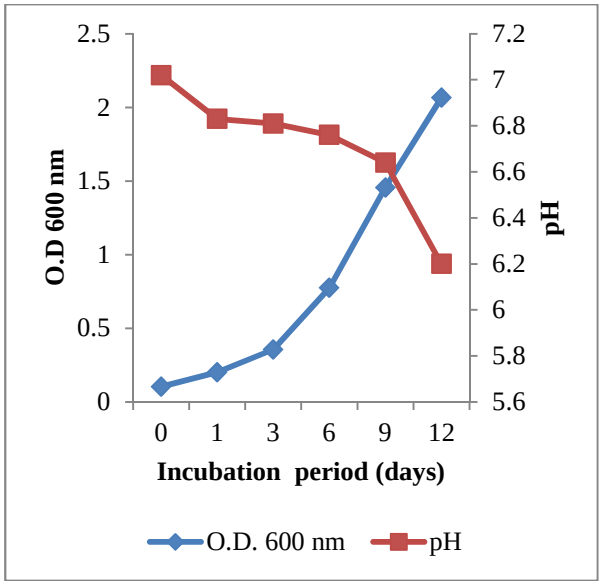

Fig.2. Changes in biomass and initial pH of medium during anthracene degradation by *Aeromonas hydrophila.*

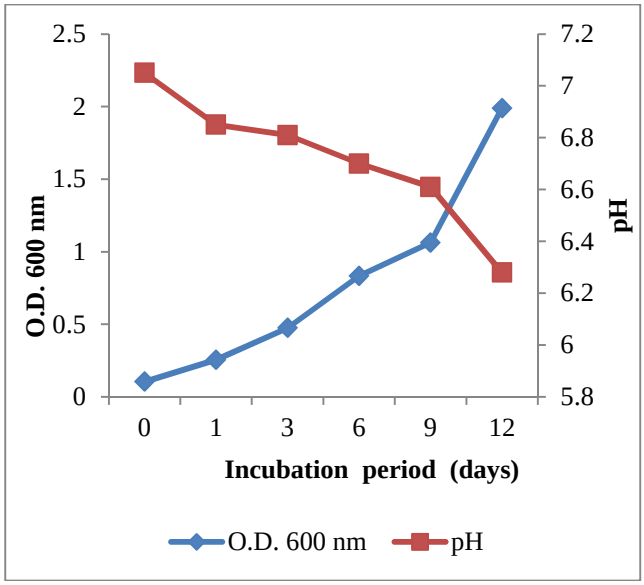

Fig.3. Changes in biomass and initial pH of medium during anthracene degradation by *Bacillus polymyxa.*

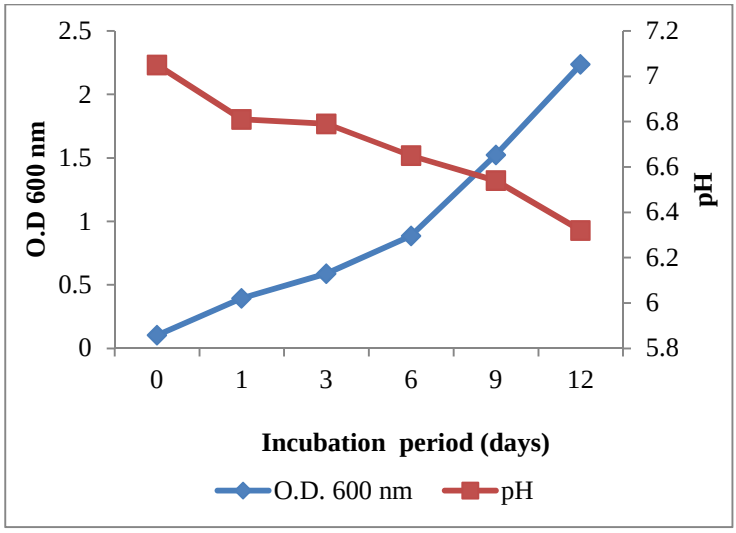

Fig.4. Changes in biomass and initial pH of medium during anthracene degradation by *Streptococcus mutans*

Percentage (%) degradation of anthracene

The residual anthracene after degradation studies were used for determination of % degradation of anthracene. The consortia of three bacteria showed 94% degradation of anthracene. Whereas 80%, 52% and 44% anthracene degradation was observed with *Bacillus polymyxa*, *Aeromonas hydrophila* and *Streptococcus mutans* respectively (Fig.5).

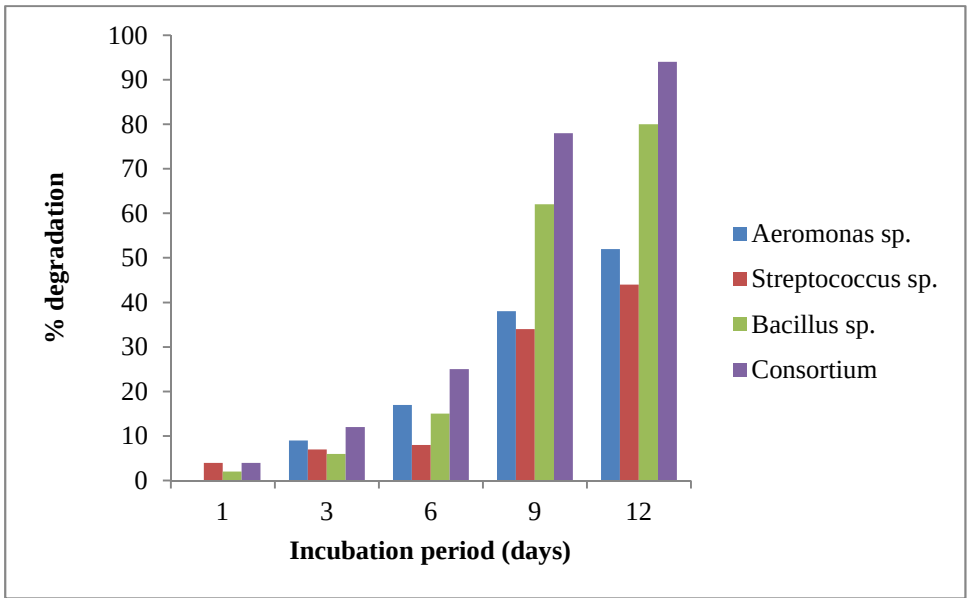

Fig.5. Percentage (%) degradation of Anthracene (1 mg/ml) *Aeromonas hydrophila, B. polymyxa, S. mutans* and Consortium with respect to time (Days). Each point represents the average value obtained with triplicate obtained with triplicate flasks.

DISCUSSION

Soil contaminated with PAHs poses serious environmental concerns because of their toxic nature and harmful effects. Anthracene is known to be a model substrate for environmental studies due to their wide distribution, toxicity to biological functions and their presence as a structural part of carcinogenic PAHs, benzopyrene, benzoanthracene and 3 –methyl cholanthrene. However their low solubility in aqueous medium and organic solvents and bioavailability is a major challenge in anthracene degrading studies.

In the present study, three anthracene degrading bacterial isolates, *Aeromonas hydrophila*, *Bacillus polymyxa* and *Streptococcus mutans* were selected from 36 anthracene degrading bacteria as potential anthracene degraders on the basis of their highest growth on anthracene supplemented media and 2, 6-DCPIP assay. Growth on anthracene containing media indicated the ability of selected isolates to utilize anthracene as sole source of carbon and energy.

Different groups of bacterial species including *Mycobacterium* sp., *Sphingobium* sp., *Nocardia* sp., *Rhodococcus* sp., *Micrococcus* sp., *Acinetobacter* sp., *Alcaligens* sp. and *Pseudomonas* sp. [5, 24, 26, 27] have shown to utilize anthracene for their growth. There is no report of degradation of anthracene by *S. mutans* and *Aeromonas* sp. Continuous increase in O.D. at 600 nm on anthracene supplemented media at concentration 1000 mg/lit confirmed that these isolates have ability to utilize anthracene.

The 2, 6-DCPIP assay is considered suitable for hydrocarbon degradation studies as it can sensitively detect the primary oxidation NADH to $NAD^{+,}$ which is related to hydrocarbon degradation by bacteria. In the present study the selected isolates showed reduction of DCPIP dye within 48 hrs of incubation. Similarly Koma et al., [15] isolated long chain hydrocarbon degrading isolates *Acinetobacter* sp ODDK71, *Rhodococcus* sp NDKK48 and *Gordonia* sp. NDKY76A during bioremediation studies. This assay has also been applied for selecting hydrocarbon degrading autochthonous microorganism in contaminated soils. In similar study Bidoia et al. [1] reported that *B. subtilis* can completely reduce DCPIP at 138 hrs, 125 hrs, 75 hrs and 87 hrs for synthetic, semi-synthetic, mineral and used oil respectively.

The anthracene in ethyl acetate shows typical λ_{max} of 254 nm [23] using UV spectrophotometer. Hence it was used to determine the anthracene concentration during anthracene degradation studies. In biodegradation studies of anthracene by *B. polymyxa*, 80% reduction in anthracene concentration was observed at 12^{th} day of incubation. It was higher than the *B. cereus* KWS2 it reduces only 45% of anthracene at same condition after 7 days of incubation [20]. The three isolates, *Aeromonas hydrophila*, *Bacillus polymyxa* and *Streptococcus mutans* showed 94% anthracene degradation when used as consortium. This amount was higher than those obtained during individual studies with these isolates. Several studies have showed that biodegradation caused by mixed culture is more effective than those when pure cultures are used. This may be due to broader enzymatic capability and counter action of toxic intermediates by co-metabolic processes [7, 9].

CONCLUSION

The present study indicated importance of *Aeromonas* sp, *Bacillus polymyxa* and *Streptococcus mutans* in anthracene degradation studies. The more effective degradation was obtained by consortium of these isolates. However, detail studies regarding various factors affecting on anthracene degradation are required before its in situ release for PAH degradation application.

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