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BIOREMEDIATION OF SOME POLYCYCLIC AROMATIC HYDROCARBONS (PAH) FROM SOIL USING *Sphingobium indicum, Sphingobium japonicum* AND *Stenotrophomonas maltophilia* BACTERIAL STRAINS UNDER AEROBIC CONDITIONS

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ABSTRACT

Bioremediation of some polycyclic aromatic hydrocarbons (PAHs) present in soil were studied using bacterial strain under aerobic conditions. PAHs used in this study were Anthracene (Anth), Benzo (a) anthracene (B(a)A), Benzo(a)pyrene (B(a)P). The degradation of selected PAHs was assessed by bioaugmentation process by using *Sphingobium indicum, Sphingobium japonicum* and *Stenotrophomonas maltophilia* as bacterial strain and biostimulation proceed by co-substrate. Effect of various parameters such as temperature, concentration and co substrate were studied. Maximum biodegradation was found at 30 ^{0}C for B(a)A, B(a)P and Anth. The percentage of biodegradation was maximum with bacterial strain and co-substrate. Percentage biodegradation was found to be increased with increase in concentration of PAHs.

Key Words : PAHs, *Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophillia,* Bioremediation

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are chemical compounds containing two or more fused benzene rings in linear, angular or cluster arrangement, containing only carbon and hydrogen. Polycyclic Aromatic carbons (PACs) include the unsubstituted hydrocarbons (PAHs) and substituted Polycyclic Aromatic Hydrocarbons (e.g. nitro-PAH, oxygenated PAH). They are usually generated under inefficient combustion conditions (e.g. in incomplete supply of oxygen).[1] The term polycyclic aromatic hydrocarbon (PAHs) refers to a ubiquitous group of several hundred chemically-related, environmentally persistent organic compounds of various structures and varied toxicity. Most of them are formed by a process of thermal decomposition (pyrolysis) and subsequent recombination (pyrosynthesis) of organic molecules. PAHs enter the environment through various routes and are usually found as a mixture containing two or more of these compounds. These compounds are widely distributed in the atmosphere and one of the first atmospheric pollutants to have been identified as suspected carcinogen. As molecular weight increases, the carcinogenicity of PAHs also increases and acute toxicity decreases. Benzo (a) Pyrene is notable for being the first chemical carcinogen to be discovered. PAHs are known for their carcinogenic and teratogenic properties.[2] Smaller vehicles (two three and four wheelers) which run mainly on different fuels have contributed a lot to PAHs in city atmosphere (air and soil).[3,4] Bioremediation is a promising option to completely remove PAHs from the environment or convert them to less harmful compounds. One of the main challenges in bioremediation of PAHs in a conventional roller bioreactor is the limitation on mass transfer due to the strong hydrophobicity and low water solubility of these compounds.[5]

There are different methods which can be applied for bioremediation of PAHs and every method has its own operation and favourable conditions.[6] Bioremediation is use of biological processes to

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degrade, break down, transform and/or it essentially remove contaminants or impairments of quality from soil and water.[7] Bioremediation is a natural process which relies on bacteria, fungi, and plants to alter contaminants as these organisms carry out their normal life functions. Metabolic processes of these organisms are capable of using chemical contaminants as an energy source, rendering the contaminants harmless or less toxic products in most cases.[8] It is one approach that has been used to remediate contaminated land and waters and promotes the natural attenuation of the contaminants using the *in situ* microbial community of the site.[9]

It refers to the use of microorganisms to degrade contaminants that pose environmental and especially human risks.[10] Due to its safety and convenience, it has become an accepted remedy for cleaning polluted soil and water. Bioremediation processes typically involve many different microbes acting in parallel or sequence to complete the degradation process. The ability of microbes to degrade a vast array of pollutants makes bioremediation a technology that can applied in different soil conditions.[11-14] The concentrations of PAHs in soil depend upon different environment condition.[15,16]

AIMS AND OBJECTIVES

The objective of this study was bioremediation of some polycyclic aromatic hydrocarbons (PAHs) in soil by using bacterial strain under aerobic conditions. PAHs used in this study was Anthracene (Anth), Benzo (a) anthracene (B(a)A), Benzo(a)pyrene (B(a)P). The degradation of selected PAHs was assessed by bioaugmentation process using *Sphingobium indicum*, *Sphingobium japonicum* and *Stenotrophomonas maltophilia* as bacterial strain and biostimulation proceed by co-substrate. Effect of various parameters such as temperature, concentration and co substrate were studied. Maximum biodegradation was achieved at 30 ^{0}C for B(a)A, B(a)P and Anth. The percentage of biodegradation was maximum with bacterial strain and co-substrate. Percentage biodegradation was found to be increased with increase in concentration of PAHs.

MATERIAL AND METHODS

Bacteria *Sphingobium indicum* MTCC 6364, *Sphingobium japonicum* MTCC 6362, *Stenotrophomonas maltophilia* MTCC 2446 were taken from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Soil

Soil collected from construction site of NIT, Jalandhar at the depth of 2743.2 mm. The texture analysis demonstrated that 66.8% sand, 32.8% silt, 0.4% clay, pH 6.3 (acidic soil), 46.45% porosity, 1.43 bulk density. Therefore, it was classified as sandy loam. It was dried and sieve to 2 mm. The soil was autoclaved at 121°C, 15 psi for 20 minutes to eliminate already present microorganisms and kept at 4°C before use.

PAHs contaminants

PAHs were purchased from Sigma Aldrich, Spain. PAH include Anthracene, Benzo(a)pyrene, Benzo(a)anthracene. Concentration of each PAH was maintained up to 50 ppm in their corresponding solvents (Cyclo hexane). All the three PAH mixed in equal ratio of 1:1:1. PAH stock solution was stored at 4^{o}C in the dark. They were used as contaminant during the experiment.

Co-substrate

Nutrients like carbon (C), nitrogen (N) and phosphorus (P), enhanced the microbial activities in composting process. The optimum ratio of C: N: P was 100:10:1.[14] Glucose, ammonia nitrate and potassium hydro phosphate were used as inorganic co substrate. These were added in the distilled water with constant stirring. Mixture was autoclaved for 20 minutes at 121°C, 15 psi. It enhanced the growth of microorganisms and prevent contamination.

Bacterial strain preparation

The strain was primary cultured in aerobic conditions in nutrient agar (Beef extract 1 g, yeast extract 2 g, peptone 5 g, agar 15 g, distilled water ,1 L) and Broth medium (beef extract 1 g, yeast extract 2 g, peptone 5 g, distilled water, 1 L) at pH 7 and

temperature 30°C. The *Sphingobium indicum, Sphingobium japonicum* strains were sub cultured after 15 days and *Stenotrophomonas maltophilia* was sub cultured after 30 days.

Experimental system

Concentration of each PAH's was made 50 ppm and PAH's solution were mixed in 1:1:1 for preparing the standard stock solution. This solution was sprayed over the surface of soil kept in petri dishes in laminar flow, where the concentration was 1g/kg (dry matter). PAH's contaminated soil was manually mixed with inorganic co substrate solution at a ratio of 1:0.25 (soil: co substrate) to maintained the moisture of the soil. Each inoculum (*Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophilia*) was poured separately in the petri plates. All the petri plates were kept in incubator for 7, 14 and 28 days for incubation purpose. The experimental program was as follows :

Treatment 1 : Soil samples at various temperature of 25°C, 30°C, 35°C, 40°C and total PAH (B(a)P, B(a)A, Anth) concentration of 100 µl containing co-substrate

Treatment 1.1: Contaminated soil, co-substrate, 60% water and *Sphingobium indicum*

Treatment 1.2: Contaminated soil, co-substrate, 60% water and *Sphingobium japonicum*

Treatment 1.3: Contaminated soil, co-substrate, 60% water and *Stenotrophomonas maltophilia*

Treatment 2 : Soil samples at temperature of 30°C and total PAH (B(a)P, B(a)A, Anth) concentration of 100 µl without co-substrate

Treatment 2.1: Contaminated soil, 60% water and *Sphingobium indicum*

Treatment 2.2: Contaminated soil, 60% water and *Sphingobium japonicum*

Treatment 2.3: Contaminated soil, 60% water and *Stenotrophomonas maltophilia*

Treatment 3 : Soil samples of total PAH (B(a) P, B(a)A, Anth) having various concentration of 100 µl, 200µl, 300µl, 400µl and at temperature of 30°C

Treatment 3.1: Contaminated soil, co-substrate, 60% water and *Sphingobium indicum*

Treatment 3.2: Contaminated soil, co-substrate, 60% water and *Sphingobium japonicum*

Treatment 3.3: Contaminated soil, co-substrate, 60% water and *Stenotrophomonas maltophilia*

Sampling

Biodegradation process was monitored during incubation period. The samples were collected in 1st day, 4th day, 7th day, 14th day and 28th day to measure the degradation rate of PAHs. All the contents were mixed manually for sampling before taking soil sample for further analysis. The petri plates were moistened with autoclaved distilled water and remixed to maintain the water content with in the optimum values.

PAHs analysis

To determine the PAHs concentration in these treatments, the 6-8 g of samples were extracted using a soxtlet extraction process for 24 hours using acetone /dichloromethane (1:1) as solvent. After extraction, the solvent was concentrated in rotaevaporator and remaining extract was dissolved in 1 ml of acetone/dichloromethane (1:1). The PAH's were identified and quantified by gas chromatograph. The 1µl of the extract solution were injected in a gas chromatograph (Nucon Gas Chromatograph) equipped with flame ionization detector (FID) and splitless injector. Initial temperature was maintained at 120ºC for 1 min and it was increased at the rate of 5ºC/min until 320ºC. The concentration of PAHs was determined after calibration method with standard PAHs sample of different concentration. Remaining PAHs percentages were calculated by dividing the PAHs residue concentration into the PAHs original concentration **(Table 1)**.

Table 1 : Physical and chemical properties of selected PAHs

S/N	PAHs	Melting-point (°C)	Vapour pressure (Pa at 25 °C)	n-Octanol : Water partition coefficient (log Kow)	Solubility in water at 25 °C d (mg/L)	Henry's law constant at 25 °C (Pa•m^3/mol)
1	Anth.	95	0.29	3.92	3.9	18.5
2	B(a)A	160.7	2.8×10^{-5}	5.91	0.0090	1.22
3	B(a)P	178.1	2.8×10^{-5}	6.35	0.00162	0.034

RESULTS AND DISCUSSION

Optimization of various reaction conditions took place for the percentage biodegradation of Anthracene, Benzo(a) anthracene, Benzo(a) pyrene by using three different bacterial strains (*Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophilia*).

Effect of temperature

Effect of temperature for the percentage biodegradation of Anthracene, Benzo(a)anthracene, Benzo(a)pyrene by using three different bacterial strains (*Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophilia*) have been shown in **Fig. 1(a)** to **Fig. 1(c)**, respectively. Laboratory microcosm experiment and parallel *ex situ* bioremediation studies were conducted to study the effect of temperature on *in situ* bioremediation systems. The optimum temperature for the maximum biodegradation of PAHs by *Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophillia* bacterial strain was found to be 30°C for seven days of incubation. The maximum degradation was as high as 76-78% at 30°C for B(a)A, B(a)P and Anth. This indicate that biodegradation of PAHs by bacterial strains is temperature dependent.The present studies show that optimizing temperature for *in situ* bioremediation technologies at sites can reduce time required for treatment of hazardous wastes; hence reduction in operational time and effort to ensure smooth running of the remediation process round the year (especially in cold regions and cold weather) can be realized.

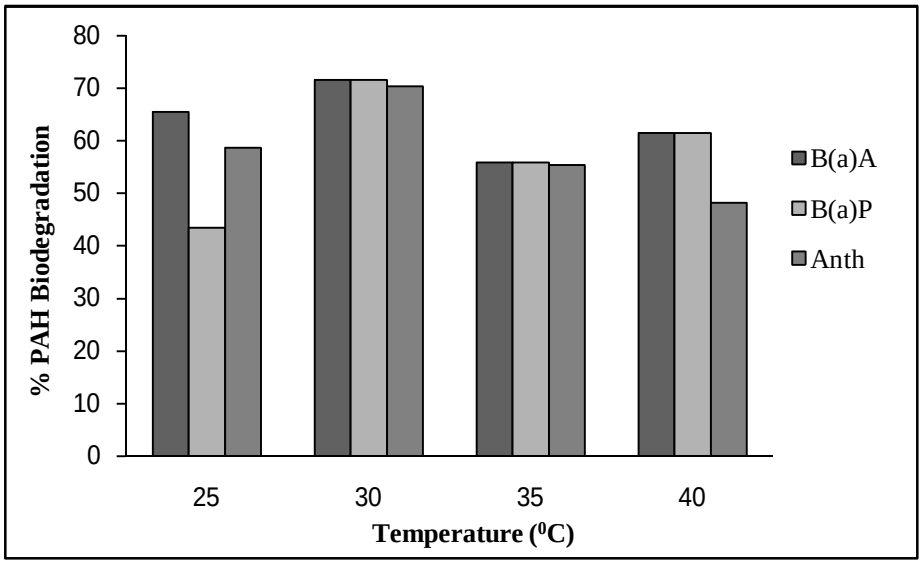

Fig. 1(a) : Percentage B(a)A, B(a)P, Anth biodegradation by *Sphingobium japonicum,* with temperature

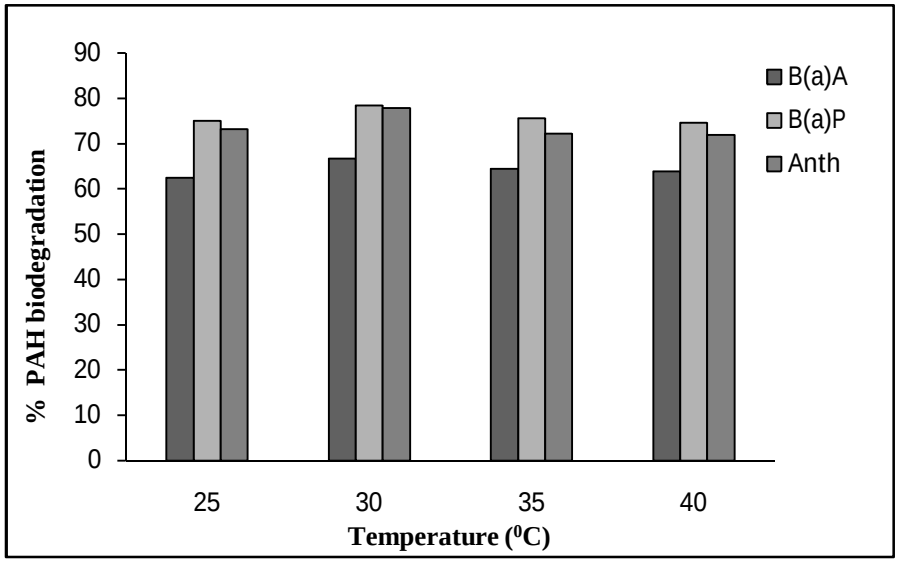

Fig. 1(b) : Percentage B(a)A, B(a)P, Anth biodegradation by *Sphingobium indicum*, with temperature

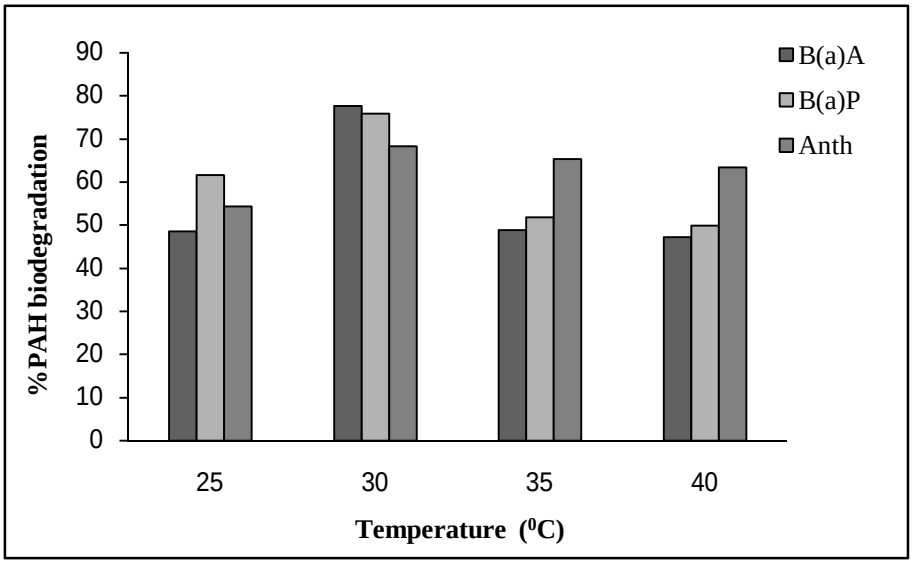

Fig. 1(c) : Percentage B(a)A, B(a)P, Anth biodegradation by *Stenotrophomonas maltophillia,* with temperature

Effect without co-substrate for the percentage biodegradation of Anthracene, Benzo(a)anthracene, Benzo(a)pyrene by using three different bacterial strains (*Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophilia*) have been shown in **Fig. 2(a)** to **Fig. 2(c)**, respectively. Percentage biodegradation of B(a)P, B(a)A, Anth by *Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophillia* with co-substrate was found to be better as compared to without co-substrate. Co-substrate may act as a catalyst for biodegradation of PAHs in the soil. Without substrate PAH degradation did not stimulate as high as with substrate. Substantial PAH degradation was only observed for Anth and B(a)P. This is the first study to our knowledge that revealed the importance of indigenous bacteria involved in determining degradation behaviour of three PAHs under different parameters.

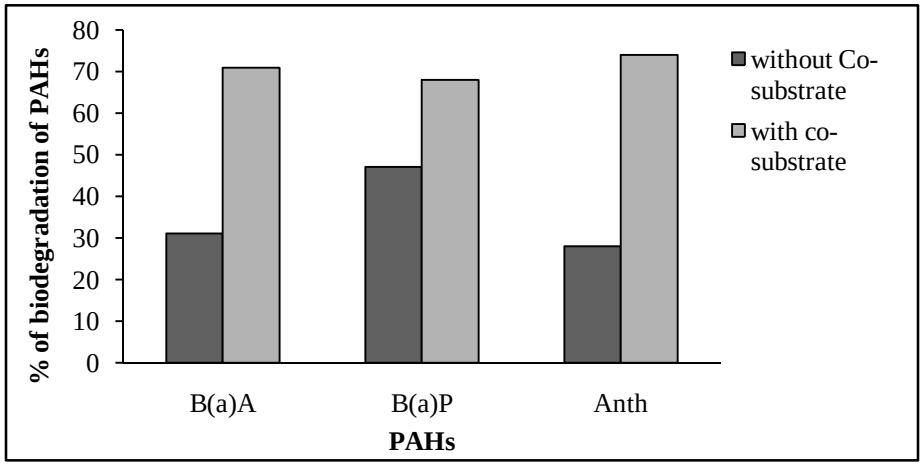

Fig. 2(a) : Percentage PAHs (B(a)A , B(a)P, Anth) biodegradation without co-substrate by *Sphingobium indicum*

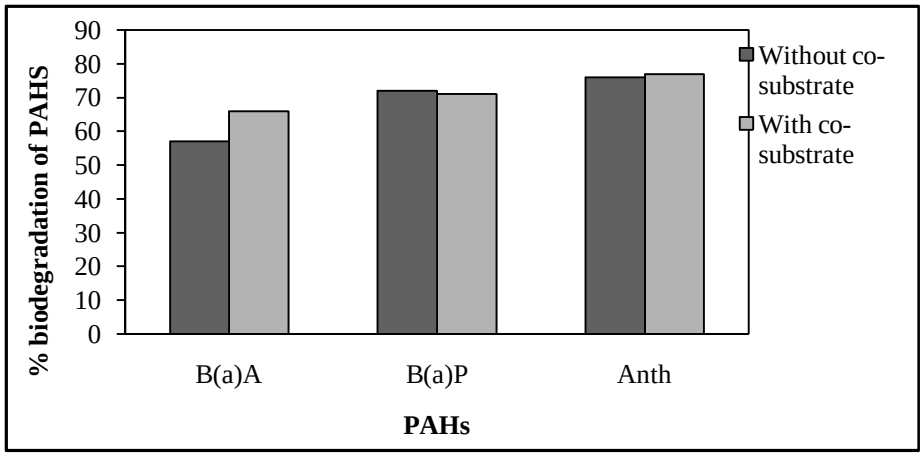

Fig. 2(b) : Percentage PAHs (B(a)A , B(a)P, Anth) biodegradation without co-substrate by *Sphingobium japonicum*

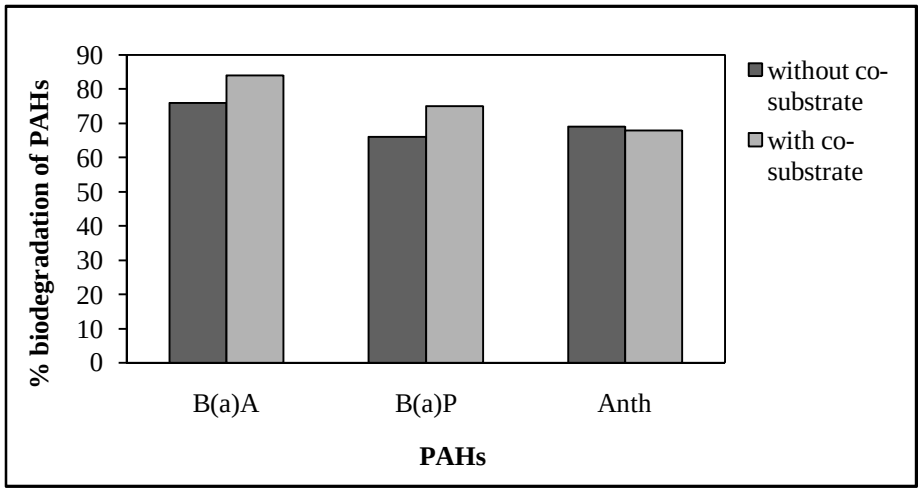

Fig. 2(c) : Percentage PAHs (B(a)A , B(a)P, Anth) biodegradation without co-substrate by *Stenoptrophomonas maltophillia*

Effect of PAHs concentration

Effect of PAHs concentration for the percentage biodegradation of Anthracene, Benzo(a) anthracene, Benzo(a)pyrene by using three different bacterial strains (*Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophilia*) have been shown in **Fig. 3(a)** to **Fig. 3(c**), respectively.

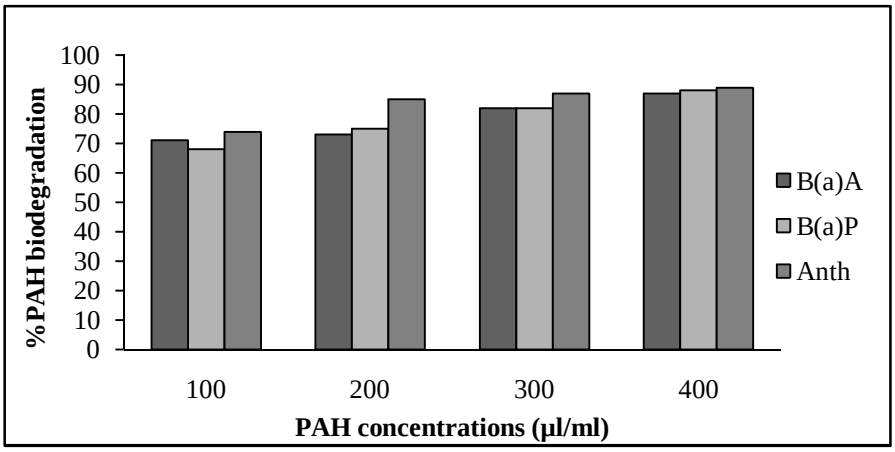

Fig. 3(a) : Percentage B(a)A, B(a)P, Anth biodegradation with concentration of PAH by *Sphingobium indicum*

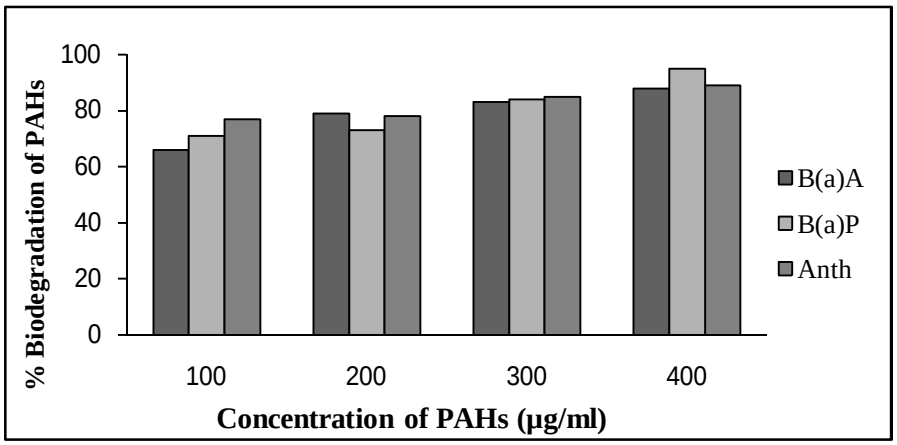

Fig. 3(b) : Percentage B(a)A, B(a)P, Anth biodegradation with concentration of PAH by *Sphingobium japonicum*

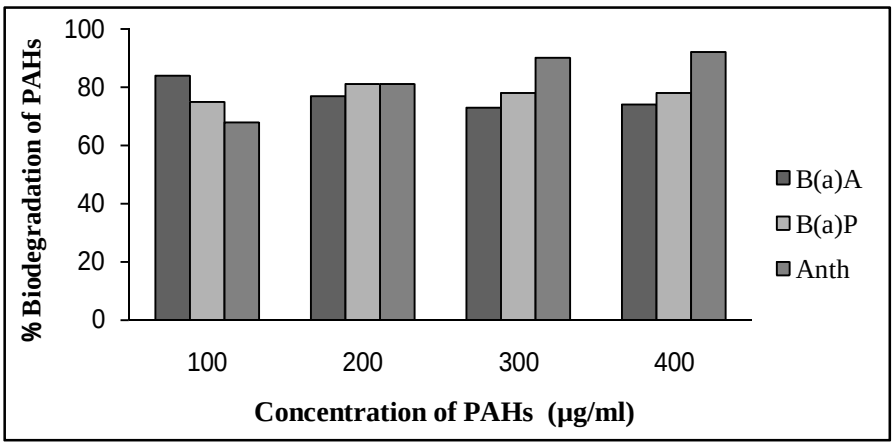

Fig. 3(c): Percentage B(a)A, B(a)P, Anth biodegradation with concentration of PAH by *Stenotrophomonas maltophillia*

When *Stenoptrophomonas maltophillia* was used than it was found that the percentage biodegradation of B(a)A, B(a)P, Anth was increases with increase even in the higher concentration of these PAHs. The maximum degradation was as high as 85-90% when the concentration of PAHs was 400 ug/ml. The reason may be *Stenotrophomonas maltophilia* is an aerobic, nonfermentative, Gram-negative bacterium. It is an uncommon bacterium and human infection is difficult to treat. Initially classified as *Pseudomonas maltophilia*, *S. maltophilia* was also grouped in the genus *Xanthomonas* before eventually becoming the type species of the genus *Stenotrophomonas* in 1993. *S. maltophilia* are slightly smaller (0.7–1.8 × 0.4–0.7 micrometers) than other members of the genus. They are motile due to polar flagella and grow well on MacConkey agar producing pigmented colonies

Effect of bacterial strains

Effect of bacterial strains for the percentage biodegradation of Anthracene, Benzo(a) anthracene, Benzo(a) pyrene by using three different bacterial strains (*Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophilia*) have been shown in **Fig. 4(a)** to **Fig. 4(d)**, respectively.

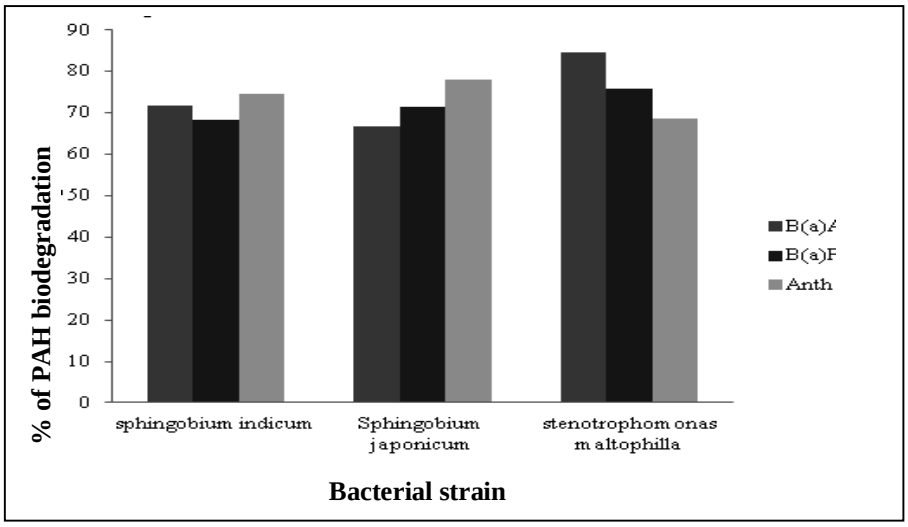

Fig. 4(a) : Optimization of bacterial strain for individual PAHs at 25^0C

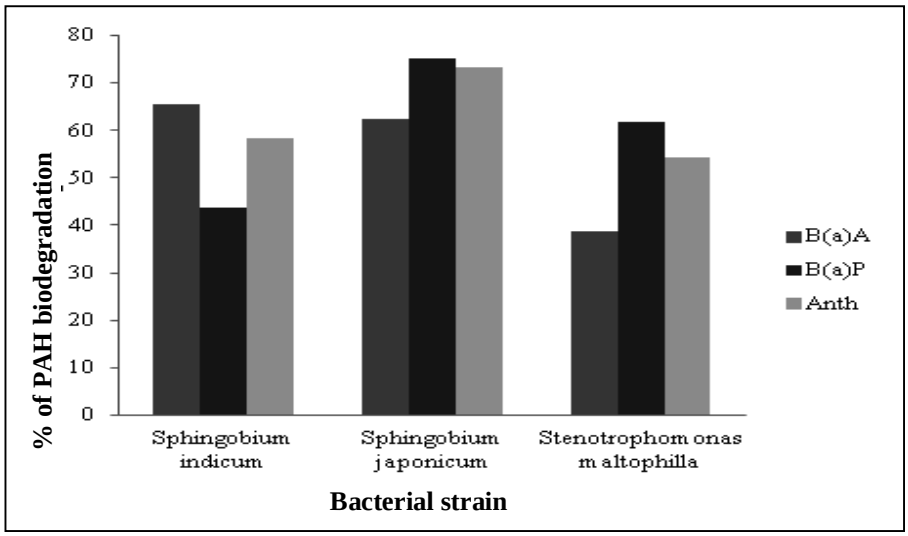

Fig. 4(b) : Optimization of bacterial strain for individual PAHs at 30^0C

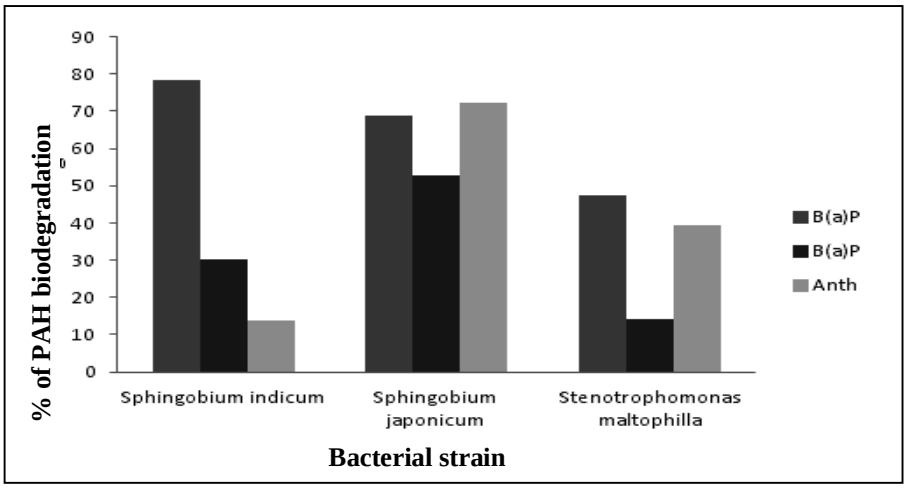

Fig. 4(c) : Optimization of bacterial strain for individual PAHs at 35^0C

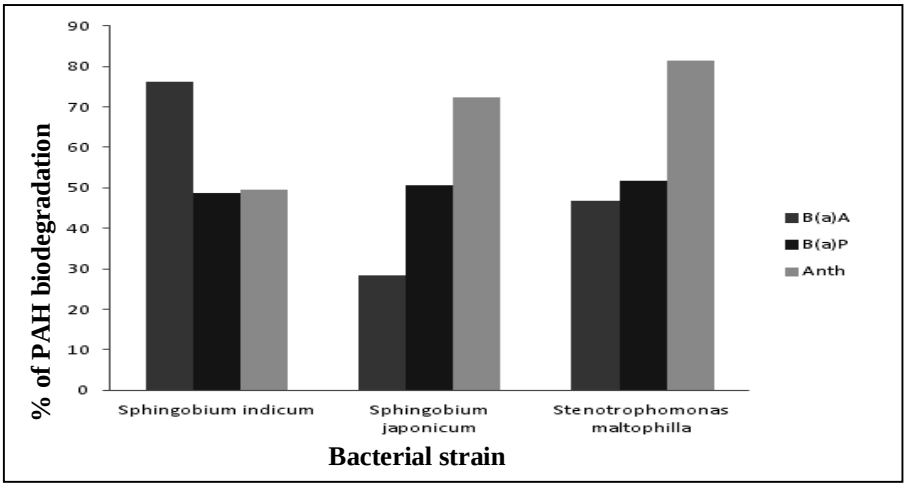

Fig. 4(d) : Optimization of bacterial strain for individual PAHs at 40^0C

The results indicated that at 25^0C, *Sphingobium indicum* showed best biodegradation for B(a)A and *Sphingobium japonicum* for B(a)P and Anth as compared to the other bacterial strains. This might be due to that its optimum growth occurred at this temperature. At 30^0C, *Sphingobium japonicum* show best biodegradation for Anth and *Stenotrophomonas maltophillia* for B(a)A and B(a)P as compared to the other bacterial strains. This was due to the fact that maximum growth of this bacteria occurred at this temperature and other bacteria was not grow properly at this temperature. At 35^0C, *Sphingobium indicum* show best biodegradation for B(a)A and *Stenotrophomonas maltophillia* for Anth and B(a)P as compared to the other bacterial strains due to the maximum growth of this bacteria at this temperature. At 40^0C, *Sphingobium indicum* show best biodegradation for B(a)P and *Sphingobium japonicum* show best biodegradation for Anth and B(a)A as compared to the other bacterial strains because this was the optimum temperature of this growth.

Proposed pathway for the degradation of PAHs by Mycobacterium

The proposed pathways for benzo(a)pyrene & Anthracene oxidation by some bacteria from the genus *Pseudomonas* are illustrated in **Fig. 5(a)** and **Fig . 5(b)**. A similar proposed pathway will be for B(a)A. Such pathways accelerate the evolution of modern catabolic pathways, providing new genetic material within the environment and resulting in an enhanced natural bioremediation potential.[17,18]

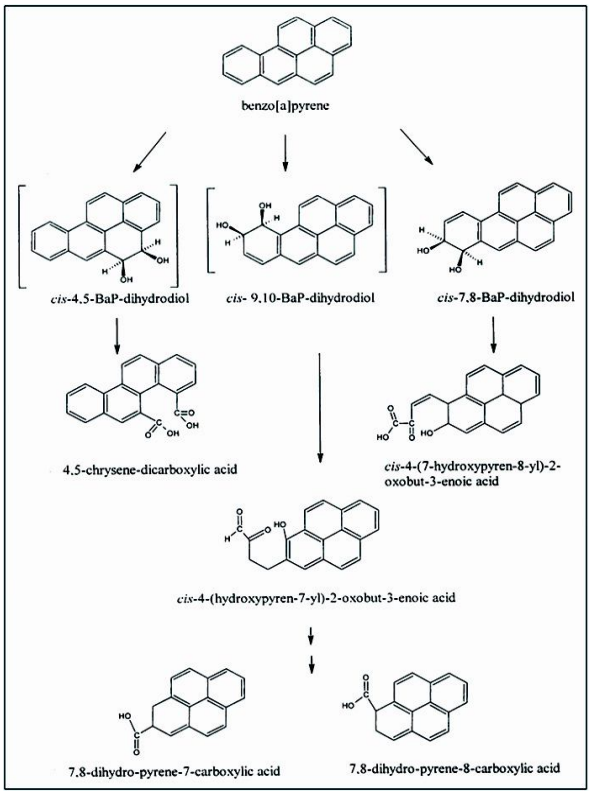

Fig. 5(a) : The proposed pathways for the metabolism of benzo[a]pyrene by *Mycobacterium*

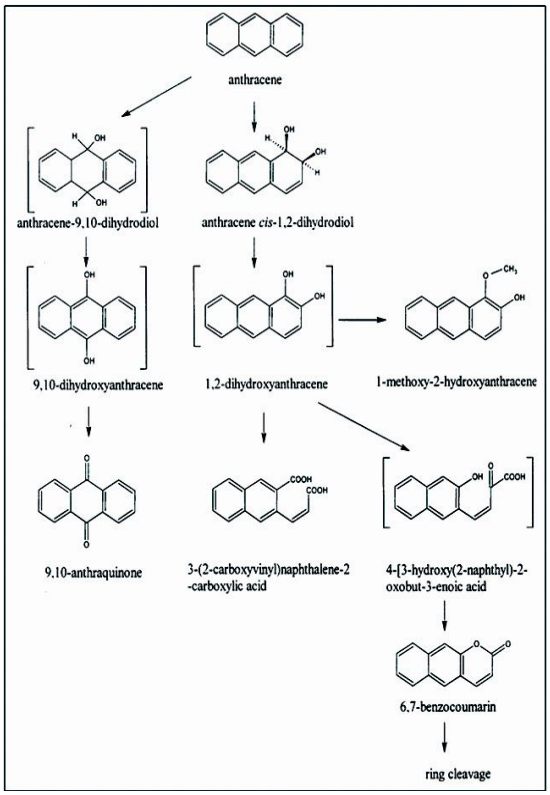

Fig. 5(b) : The proposed pathway for the degradation of anthracene by *Mycobacterium*

CONCLUSION

In this study optimization of various reaction condition for the percentage biodegradation of Anthracene, Benzo(a)anthracene, Benzo(a) pyrene by using three different bacterial strains (*Sphingobium indicum, Sphingobium japonicum, Stenotrophomonas maltophilia*) were carried out. In order to setup process conditions to reduce time required for treatment of hazardous wastes, hence reduce maintenance cost and smooth running of the remediation process round the year, especially in cold regions and cold weather. For this purpose a microcosm study and a real world-field investigation was designed to achieve the abovementioned objective. It was obvious from the results of the 30-day laboratory microcosm experiment that temperature, concentration and different bacteria is the important variables for determining the success of bioremediation of hazardous waste currently used at superfund sites. The results clearly indicated that efficiency of the bioremediation activity was enhanced with high temperature concentration and different bacteria.

1. The percentage of biodegradation of B(a)A was found to be increase with rise in temperature for *sphingobium indicum* but for B(a)P & Anth, the degradation rate was constant.
2. The percentage of biodegradation of B(a)A, B(a)P & Anth was showed an increase but after 30 ^{0}C, there is decrease in degradation rate and becomes constant at higher temperature for *Sphingobium japonicum, stenotrophomonas maltophilla.*
3. The percentage of biodegradation of PAHs is low without co-substrate as compare to with co-substrate for *sphingobium indicum.*
4. The percentage of biodegradation of PAHs is almost same in soil samples of without co-substrate and with co-substrate for *Sphingobium japonicum, Stenotropho - monas maltophilla.*
5. The percentage of biodegradation of PAHs was increased with increase of concentration for *sphingobium indicum, sphingobium japonicum, stenotr ophomonas maltophilla*

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