Biodegradation of Polycyclic Aromatic Hydrocarbons by Bacteria

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) are the pollutants having ubiquitous distribution in the surroundings derived from both anthropogenic and natural resources. PAHs biodegradation has been receiving constant scientific consideration. Highly potential, PAHs degrading bacteria were isolated and idendified from crude oil contaminated sludge enriched with naphthalene. Among the isolates *Bacillus foraminis* is an aerobic, Gram positive, alkaliphilic. The present study was report for bacterial isolates *Bacillus foraminis* utilizing the PAHs (naphthalene, and anthracene,) as sole carbon and energy source. The PAHs degradation study were conducted in liquid medium, with the different substrate concentration of naphthalene (100 ,200 and 300mg l^{-1}) and anthracene (50,100 and 150 mg l^{-1}) .The results revealed that the bacteria degraded naphthalene completely 96.66±0.018% at120 h, and anthracene 99.07 ± 0.449 % at144 h for the substrate concentration (100 and 50 mg L^{-1}) . From the outcomes, the biodegradation potential of *Bacillus foraminis* isolated from the polluted sludge should be examined and Various factors optimized for bioremediation purpose.

Keywords: *Bacillus, Biodegradation, PAH, Naphthalene, Anthracene,*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are the most prevalent and persistent pollutants in the environment. PAHs occur as hybrids encompassing various structural and chemical components, They are composed of two or more fused aromatics rings.. They occur as common constituents of petroleum , coal, tar and naturally formed by incomplete combustion of fossil fuels, waste incineration, accidental spilling of crude oils, coal gasification, organic oil seepage and natural biogeochemical process (Bamforth and Singleton, 2005). The movement of PAHs in the environment is a great ecological alarm because of their Physical and Chemical properties. Due to their lipophilic nature they stick at in the surroundings as recalcitrant for very long periods of time and are photocyto toxic to both floras and faunas and are capable to enter the plants and marine organism that persons will finally consume. PAHs compounds have been found to be noxious, mutagenic and cancer-causing in nature (Netto et al. 2000, Villemur et al. 2000). Due to their harmful effects activities they are classified as priority environmental pollutants by United states Environmental Protection Agency (USEPA) and European community (Bayoume, 2009). Though, the major process for effective deduction and exclusion of PAHs from the surroundings is the bioconversion and biodegradation (Dagher et al. 1997; Shyong et al. 2003). Biodecotamination is the general term used for biological breakdown of their hydrocarbon rings and complete biodegradation leads to mineralization. Bioremidiation metod have been comprehensively used in several features of the decontamination of the polluted aera. Using of Plants, fungi and microorganisms were very effective method in the deduction of toxic organic xenobiotics from the surroundings. Bacteriological activity is the greatest substantial cause of PAH elimination from the surroundings, and the facility of microorganism to detoxification of PAHs were reported and well documented.

Microbial action has been believed the best efficient, economical and ecologically safe elimination of PAHs in contaminated soil, sediment and water (Carniglia.1993, Juhaz et al. 2000, Arulazhagan et al. 2010). During the past few eras diversity of microorganisms capable to removal of PAHs particularly low molecular weight compound such as naphthalene, phenantherene and anthracene have been discovered (Juhaz et al. 2000, Doane et al. 2002). The degrading strains that have been characterized so far in the literature are taxonomically diverse and mainly belong to the genera Pseudomonas, alcligenes, sphingomonous, bacillus and mycobacterium etc. (Juhaz et al. 2000;Jonsen et al. 2005; Yu et al. 2005,velayutham et al 2011). The biodegradation of (PAHs) was testified in the alkaline-saline soil of Lake Texcoco in Mexico (Betancur et al., 2006), and a soot-contaminated alkaline soil in Italy (Moretto et al. 2005). Furthermore, several researcher reported the isolated alkaliphiles bacteria , that are capable to growth alkanes (Yumoto et al. 2002), phenol (Kanekar et al. 1999), pyrene and other PAHs (Gerbeth et al. 2004,Habe et al. 2004), benzene and its by-products (Li et al. 2006, Fahy et al. 2006).The main objective of this experiment is to isolate and identify PAHs reducing microorganisms from the sludge of the oil drills. Bacterial cultures were isolated and subjected to removal efficiency the PAHs in a batch process. From this the efficient PAHs degrading microorganisms were identified by amplification and sequencing of 16S rRNA genes. Moreover, the obtained results could be very useful for the upcoming design of an efficient aerobic treatment process for treating PAHs containing wastewater.

2. Materials and Methods

2.1. Sample Collection

Sludge sample was collected with a sterile scoop from a layer 0 to 30 cm deep at an oil exploration drill site in Mayiladuthurai, Tamilnadu, India. The sample was kept in a sterile container and stored in laboratory at 4°C (Refrigerator).

2.2. 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^{-1}naphthalene 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 7-7.5 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.

2.3. Isolation of naphthalene degrading bacteria

One ml of sample from enrichment medium was consecutively diluted up to 10^{-5} dilution and every dilution was plated onto minimal salt agar (15g l^{-1}) agar with minimal salt broth composition, (pH -7-7.5) by spread plate method. 0.0250 g l^{-1}naphthalene was speckled over the agar plates seeded as energy source. Plates were incubated and colonies were purified by reiterated streaking on the same medium with sprinkled naphthalene and stored at 4° C.

2.4. Screening of bacteria on the basis of naphthalene utilizing ability

A loop full of each bacteria was immunized into flask containing 150 ml sterile (MSM) supplemented with 0.050 g l-1 naphthalene as energy source. Flasks were kept at temperature 31±2°C for 7 days. The ability of every bacteria to consume naphthalene was related to increase in growth of the medium was measured at 600 nm using UV Spectrophotometer. Isolates that exposed no significant growth in medium were discouraged for further experiment.

2.5. Identifications of bacteria

The bacteria 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. The organism further identification was done using 16srRNA gene sequencing and compared with known gene bank database and confirmed from Aristogene Bioscience Pvt Ltd. Bangalore, India.

2.6. Analysis of PAH in Mineral Salt Broth by Gas Chromatography

A known volume of the suspension was acidified and centrifuged at 20,000 rpm for 20 min and filtered through a 130 Whatman filter paper. The filtrate was extracted with dichloromethane (Rodrigo et al. 2007). Extracted material was quantified in a gas chromatograph (Chemito GC model 7610) equipped with 5% phenyl polysiloxase-packed capillary column (BP-5) (30 m×0.25 mm×0.25 mm) used in FID mode. The injector and detector temp 280 °C and 290 °C and temperature program was 100 °C, ramp to 390 °C maintaining isotherm for 1 minute. A 0.2 µml aliquot was injected at split rate of 1:50. The degradation quantity of naphthalene and anthracene was determined relative to PAH concentration in flask without inoculums (control).

2.7. Batch experiment of PAHs 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 600 nm were utilized as inoculums. All batch experiments conducted in 250 ml conical flasks containing 150 ml of (MSM) at pH-7-7.5 and added naphthalene as a substrate at three different concentration (100 mg l-1,200 mg 1-1,300 mg l^{-1}) and anthracene (50 mg l^{-1},100 mg l^{-1} 150 mg l^{-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.

3. Results and Discussions

Bioremediations of area polluted with crude oil and other derivatives of PAHs is viable due to their diversity of degrading microbes present in these area. PAHs removal bacteria are commonly spread in contaminated soil and water. The adaptation or decontamination of PAHs by microorganisms depends on the benzene ring of compounds and environmental conditions (Atlas 1989).

3.1. Isolation and screening of PAHs degrading bacterial culture

Four microorganism were isolated from mixed sludge samples inoculated 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 A1, A2, A3 and A4. The microbes were characterized based on Gram staining test and cell morphology. Among them two (A1andA3) were Gram negative and long, slender rods, others were Gram positive (A2 and A4). Each strain was further evaluated through batch experiments for its ability to degrade PAHs. From the figure (1) present the growth form of the isolates observed in the occurrence of PAHs.The four organisms A1, A2, A3 and A4 exhibited growth by utilizing

PAHs in the order of A2>A1>A4>A3. The isolate A2 organism were further isolated with high purity by repeated inoculation in PAH spiked media and plating them on petri plates. The morphological, biochemical characteristics and physiological, of the microorganism were tested as per the Bergey's Manual of Determinative Bacteriology (reference) and the tested results are shwon in Table 1 and Table 2. Bacterial isolate were analysed by the 16srRNA gene sequencing and homology searches receded isolate A2 belongs to the genus *Bacillus* sps, and the closest species are bacterial strains namely *foraminis*, *boranophillis* and *chandigharensis* (accession **No. T_DQ013307**), was presented in Figure 2. In this content, the *bacillus* species were the predominant microorganisms in highly polluted soil samples.

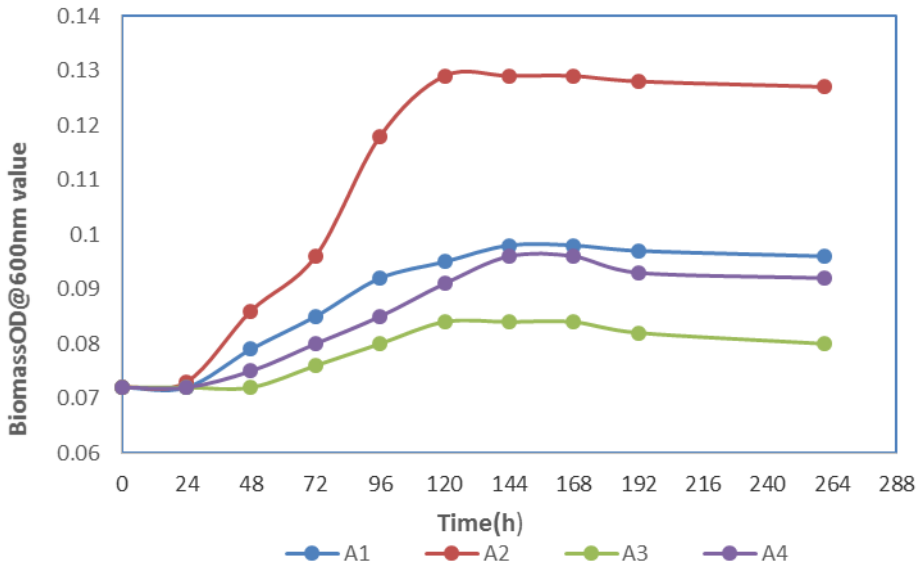

Figure 1 Growth pattern of isolates in the presence of PAH mixtures

Table 1 Biochemical characteristics of the isolates

Sl. No.	Biochemical characteristics	A2
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

Sl. No.	Morphological Characteristics	A2
1	Type of colony	Small smooth convex and grey
2	Cell diameter	0.5-1μm
3	Endospore	-
4	Pigmentation	-
5	Motility	+
6	Gram nature	Gram positive

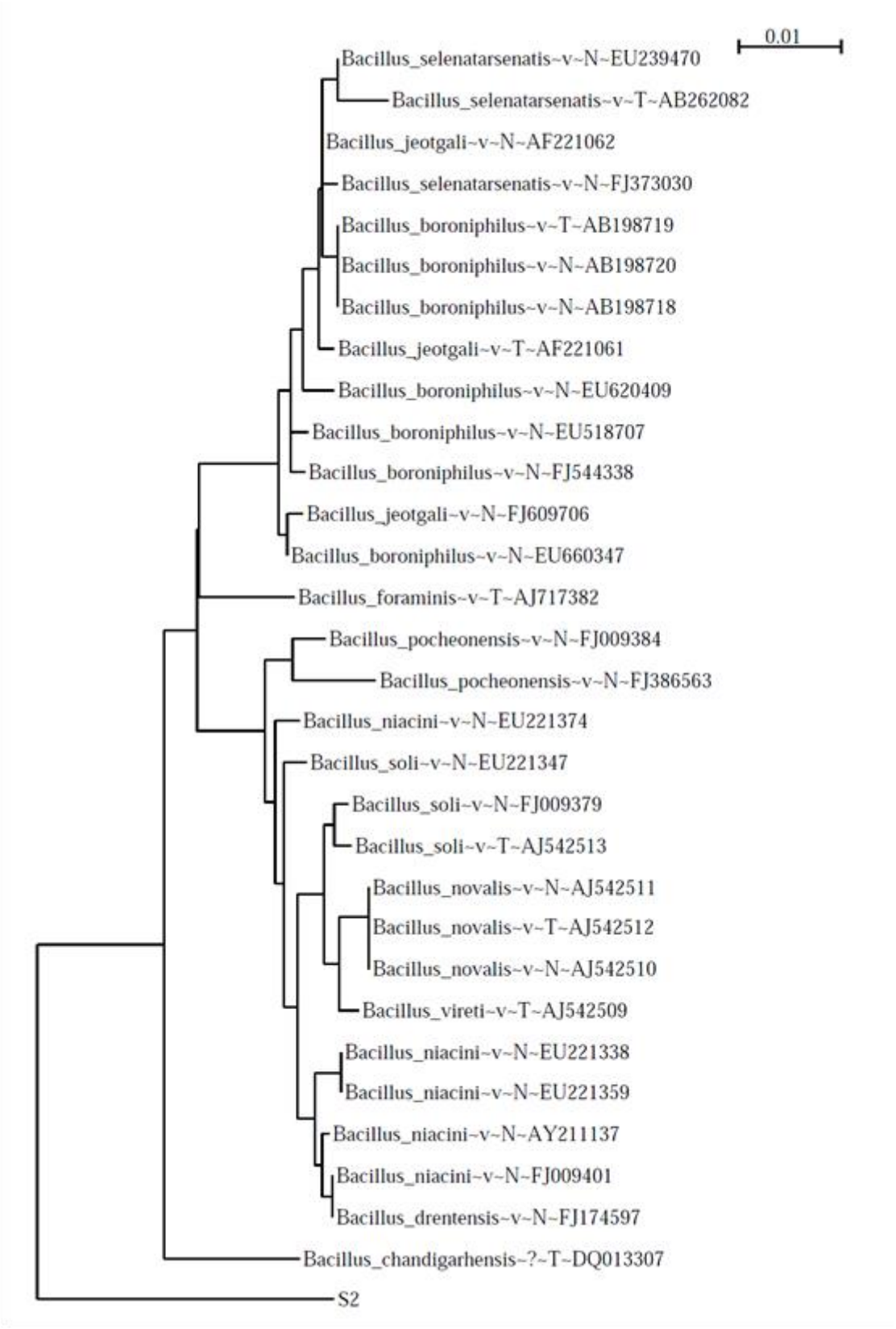

Figure 2. Phylogenetic tree for taxonomic location of strain A2

3.2. Biodegradation of Polycyclic Aromatic Hydrocarbons

3.2.1 .Effect of initial naphthalene concentration(quantity)

The naphthalene biodegradation efficiency of *Bacillus foraminis* was studied by varying the initial quantity of naphthalene spiked in the media. The efficiency of PAHs removal for varing naphthalene concentration by *Bacillus foraminis* in batch studies as presented in Figure 3. It is observed that the *Bacillus foraminis* degrades napthalene about 96.66 ± 0.018 % at 100 mg l^{-1} initial concentration within 120 h respectively. The increase in cell biomass against time for various initial naphthalene concentrations of *Bacillus foraminis* was presented in Figure 4. Increase in quantity of naphthalene proportionally extended the lag phase. As the naphthalene concentration increases (200 and 300 mg l^{-1}), the efficiency of degradation decreases 48.1% and 30.12%.

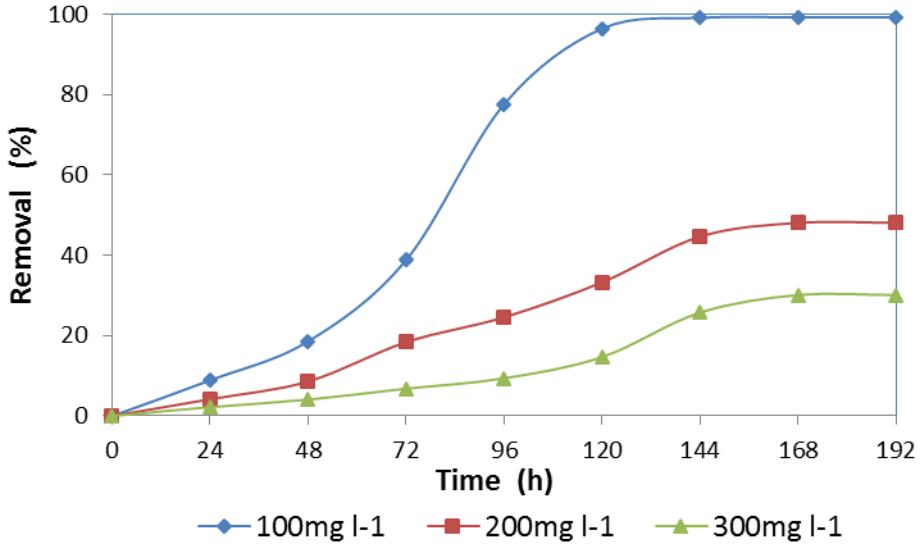

Figure 3 Efficiency of naphthalene removal by *Bacillus foraminis*

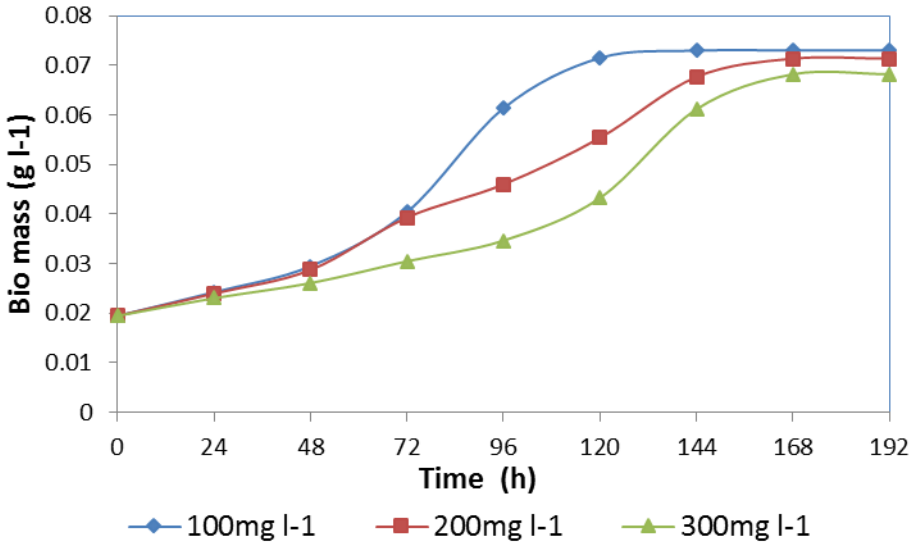

Figure 4 Effect of naphthalene concentration on growth of *Bacillus foraminis*

3.2.2 .Effect of initial concentration of anthracene

Removal of anthracene with respect to time in batches spiked with various concentration of anthracene varying from 50 to 150 mg l^{-1} as shown in Figure.5. For given initial concentration of 50, 100 and 150 mg l^{-1} removal efficiencies corresponding to 99.07 ± 0.449 % of the anthracene at concentration 50 mg l^{-1} in 144 h, 76.65±0.12 and 29.58±0.15 % of the anthracene at concentration 100 and 150 mg l^{-1}

respectively was observed in 168 h. The growth pattern of the isolate during the degradation process in various initial concentration of anthracene was shown in Figure 6. As observed in the case of anthracene, lag phase increased with higher concentration of anthracene. Maximum growth attained by the microorganisms also decreased with the increase in anthracene concentration. The average removal rates of anthracene were 0.298 ±0.009 mg hr^{-1} at 50 mg l^{-1} and 0.25±0.012 mg hr^{-1} at 100 mg l^{-1}.

Time-course studies generally have shown that compound with low molecular weights tend to degrade at faster rates than those with high molecular weights. The biodegradability depends on the complication of carbon arrangements and physico-chemical properties of PAHs.

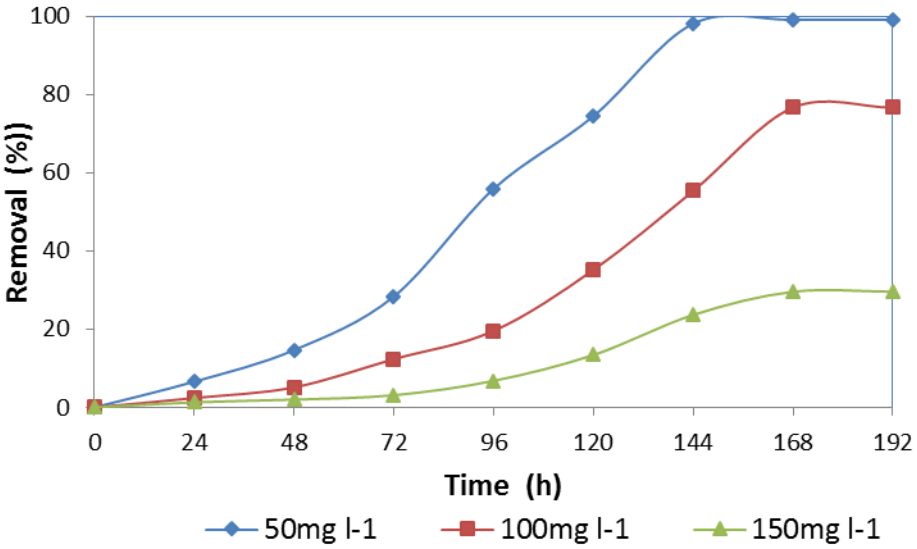

Figure 5 Efficiency of anthracene removal by *Bacillus foraminis*

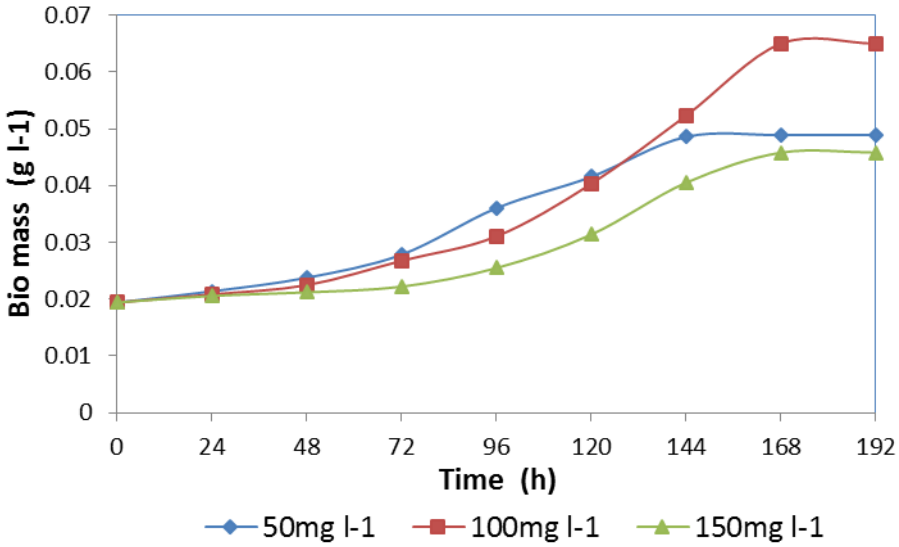

Figure 6 Growth of *Bacillus foraminis* in various anthracene concentrations

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 crude oil contaminated soil to utilize polycyclic hydrocarbons as single carbon energy source. Among the four selected bacterial strains

(A1, A2, A3 &A4), A2 showed high biomass growth in naphthalene as a sole Carbon energy. The selected A2 bacteria were recognized as; *Bacillus foraminis* .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 strain *Bacillus foraminis* increasing the biomass in different quantity of naphthalene within 140 h and about 99.15±0.125 % of naphthalene degraded at the concentration of100 mg l^{-1}.

In biodegradation studies of anthracene (50 mg l-1) by *Bacillus foraminis* 99.07 ± 0.449 % reduction in anthracene concentration was observed at pH 7.5 after six days. These results are reliable with other reports on the efficiency of PAHs biodegradation by *Bacillus* . For example, Lin et al.,(2010) showed optimal naphthalene degradation at pH 7 by *Bacillus fusiformis* isolated from wastewater sludge of an oil refinery. The isolation of two bacterial strains identified as *Bacillus sp*. having the ability to degrade PAH individually as well as in consortium and optimally at 37ºC at pH 7 reported (Shuyu et al., 2007). A novel strain of *Bacillus subtilis* was isolated from a contaminated soil at an automobile workshop. Which can be biodegraded some PAHs including benzo (a) pyrene, anthracene, naphthalene and dibenzothiophene as single carbon energy source (Lily et al., 2009). The significant reduction in total petroleum hydrocarbon (TPH) quantity in the soil after treatment was reported by a consortium of *Bacillus subtilis* and two strains of *Pseudomonas aeruginosa* (Das and Mukherjee, 2007). The present study bacteria *Bacillus foraminis* was able to degrade 99.25±0.04% of naphthalene at a concentration of 100mg l^{-1} with in 168h and anthracene 99.3±0.048ata concentration of 50 mg l^{-1} in the range of pH 7.0 to 7.5. Bacteria of the genera *Sphingomonas, Burkholderia, Pseudomonas, BacillusAcinetobacter, Rhodococcusand Mycobacterium* are well known PAH degraders. They successfully mineralized both low molecular weight PAHs (LMW-PAHs) and high molecular weight PAHs (HMW-PAHs) which has been reported by several workers (Catterall et al. 1971; Cerniglia. 1984; Heitkamp et al. 1987; Mueller et al. 1990; Kastneretal. 1994; Mueller et al. 1997; Bosch et al. 2000; Jonsenet al. 2002; Jonsen et al. 2005; Yu et al. 2005). Although several PAH-degrading pure cultures bacteria can eagerly consume PAHs as a carbon energy (Rodrigues et al. 2005; Kim et al. 2007). In all the cases, the ability of the microorganisms to solely utilize the PAH substrates as sources of both carbon and energy were emphasized. The present study has demonstrated that the bacteria *Bacillus foraminis* effectively mineralized both naphthalene and anthracene and also carry out rapid removal of naphthalene anthracene in the liquid medium. It therefore provides concrete evidence of the usefulness of the bacteria *Bacillus foraminis* for bioremediation of PAHs contaminated environment.

5. Conclusions

In the present study, we conclude the degradation efficiency of two (PAHs) by aerobic bacteria *Bacillus foraminis* was performed. The PAHs degradation tests were led in liquid medium, with the concentration of naphthalene (100 mg l^{-1}) and, anthracene (50mg l^{-1}) .The results exposed that the bacteria removed naphthalene completely 96.66±0.018% at120 h, and anthracene 99.07 ± 0.449 % at144 h . The other PAHs results shows, the degradation efficiency deceases with increase in the number of benzene rings. 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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