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DEGRADATION OF BENZO [A] PYRENE BY A NOVEL STRAIN *Bacillus subtilis* BMT4i (MTCC 9447)

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ABSTRACT

Benzo [a] Pyrene (BaP) is a highly recalcitrant, polycyclic aromatic hydrocarbon (PAH) with high genotoxicity and carcinogenicity. It is formed and released into the environment due to incomplete combustion of fossil fuel and various anthropogenic activities including cigarette smoke and automobile exhausts. The aim of present study is to isolate bacteria which can degrade BaP as a sole source of carbon and energy. We have isolated a novel strain BMT4i (MTCC 9447) of *Bacillus subtilis* from automobile contaminated soil using BaP (50 µg /ml) as the sole source of carbon and energy in basal salt mineral (BSM) medium. The growth kinetics of BMT4i was studied using CFU method which revealed that BMT4i is able to survive in BaP-BSM medium up to 40 days attaining its peak growth (10^{29} fold increase in cell number) on 7 days of incubation. The BaP degradation kinetics of BMT4i was studied using High Performance Liquid Chromatography (HPLC) analysis of BaP biodegradation products. BMT4i started degrading BaP after 24 hours and continued up to 28 days achieving maximum degradation of approximately 84.66 %. The above findings inferred that BMT4i is a very efficient degrader of BaP. To our best of knowledge, this is the first report showing utilization of BaP as a sole source of carbon and energy by bacteria. In addition, BMT4i can degrade a wide range of PAHs including naphthalene, anthracene, and dibenzothiophene therefore, it could serve as a better candidate for bioremediation of PAHs contaminated sites.

Key words: Benzo [a] Pyrene (BaP), polycyclic aromatic hydrocarbon (PAH), *Bacillus subtilis* BMT4i, colony forming units/ml (CFU/ml), high performance liquid chromatography (HPLC)

INTRODUCTION

High molecular weight (HMW) polycyclic aromatic hydrocarbons (PAHs) are considered environmentally significant because of their potential toxicity to the higher organisms and resistance to microbial attack (5). Usually, an increase in the number of fused rings increases the chemical stability and hydrophobicity of PAH molecules, making them more amenable to biodegradation. In mammals, PAHs are shown to possess high mutagenic, teratogenic or carcinogenic properties (9, 14, 24) therefore, many PAHs including phenanthrene, acenaphthene, acenaphthylene, fluoranthene, pyrene, benzo[a] pyrene (BaP) and benzo[a] anthracene are listed in the priority pollutant list of the United States Environmental Protection Agency (20, 26).

Benzo [a] Pyrene (BaP), a potent carcinogen is a

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pentacyclic high molecular weight PAH with high recalcitrant nature (4, 5, 24, 27). The natural sources of BaP are forest fires, volcanic eruptions, peat fires, burning of crude oil and shale oil, while anthropogenic sources include the incomplete combustion of fossil fuel, coke oven emissions, aluminum smelters, coal combustion and conversion industries, incinerators, vehicle exhausts, cigarette, cigar and marijuana smoke (2, 3). From contaminated soil and water, BaP enters into the food chain and metabolized to its ultimate genotoxic form which then interacts with nucleic acids and proteins forming highly reactive macromolecular adducts resulting in BaP induced toxicity, mutagenesis and carcinogenesis in mammals (8, 12, 23). In contrast to the mammalian system, some algal, fungal and bacterial species are shown to degrade BaP co-metabolically, alone or in a co-culture (16, 18, 22). However, bacteria capable of utilizing BaP as a sole source of carbon and energy have never been demonstrated.

In view of the above, the present study is focused on the isolation and characterization of bacterial species capable of degrading BaP as the sole source of carbon and energy.

MATERIALS AND METHODS

Chemicals and Reagents

The benzo (a) pyrene (BaP) (99.9%), benzo (a) pyrene cis- 7, 8 dihydrodiol, naphthalene, anthracene and dibenzothiophene (purity > 98%) were purchased from Supelco, USA. Tryptone, peptone, beef extracts, bacto-agar, yeast extracts and staining reagents were obtained from HiMedia Laboratory Pvt. Ltd. India. The general chemicals including constituents of basal salt mineral media (BSM) and solvents of analytical grade were purchased from Glaxo Pvt. Ltd. India and Merck Pvt. Ltd. India.

Preparation of Media

BSM (pH 7.0) was prepared by dissolving 0.38g KH_2PO_4, 0.6g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 1.0g NH_4Cl and 0.05g $FeCl_3$ in one liter double distilled water and autoclaved. Autoclaved nutrient broth contained 5g peptone, 5g NaCl, 3g beef extracts, 3g yeast extracts per liter double distilled water. All the solid media contained 1.5% agar along with BSM or nutrient broth. The stock solution (10 mg/ml) of BaP, benzo (a) pyrene cis- 7, 8 dihydrodiol, naphthalene, anthracene and dibenzothiophene were made in dimethylformamide and was sterilized by Millipore micro syringe filter assembly (0.45µm pore size).

Isolation of BaP degrading bacteria

The subsurface PAH-contaminated soil sample was collected from automobile workshop situated in Srinagar Garhwal, Uttarakhand, India.

The automobile contaminated soil was suspended in BSM (10% w/v) with vigorous stirring for 10 minutes and then allowed to settle at room temperature for one hour. The supernatant was serially diluted up to 10^{-9}. 100 µl inoculum of 10^{-7} diluted soil suspension was spread on BSM- agar plate supplemented with BaP (50 µg/ml) as sole source of carbon and energy and incubated for 1 week at 37^{o}C inside the clear plastic bags to conserve moisture. The bigger size bacterial colonies which appeared on BSM-BaP plate were aseptically removed and reselected on BSM-BaP plate to obtain their pure culture. Single colony of each isolate is then inoculated in 10 ml nutrient broth and grown for 24 h at 37 ^{0}C with constant shaking at 150 rpm (Remi, India, Model no. CIS-24). The cell suspensions were centrifuged at 8000 rpm for 10 minutes to obtain cell pellets of each soil isolate. The cell pellets were washed with BSM three times to remove the trace of nutrient broth and finally suspended in BSM. The optical densities of cell suspensions were adjusted to 1.0 approximately equal to the 10^8 cells per ml. The ability of each soil isolate to grow in the presence of BaP was checked by inoculating 1 ml of each soil isolates (approx. 10^8 cells/ml) into the 10 ml of BSM supplemented with BaP (50 µg/ml) as the sole source of carbon and energy. The cultures were incubated at 37^{0}C for 1 week with constant shaking at 150 rpm in dark to avoid photolysis of BaP. 100 µl each of grown culture were taken out and diluted serially upto 0- 10^{-20}. 100 µl of each serially diluted culture were spread over BSMG plate (BSM containing glucose 10gL^{-1}) and incubated

for 12-24 h at 37^0C. The colonies were counted and CFU/ml was determined for each culture. In addition, the cultures were virtually examined for the turbidity at 600 nm using UV-Vis Spectrophotometer (Systronics, India, model no. 119). The best BaP degrader (BMT4i) was selected on the basis of its highest growth in BaP-BSM as indicated by highest CFU/ml (Table 1) and turbidity at $A_{600.}$

Table 1. Characteristics and Viability of soil bacterial isolates in BaP-BSM after 7 days

Soil isolate	Morphology	CFU/ml
BMT128	Rod shaped, gram +	5×10^{16}
BMT637(R)	Fusiform rod shaped, gram +	4×10^{19}
BMT4i	Rod shaped, gram +	6×10^{35}
BMT5i	Rod shaped, gram +	3×10^{23}
BMT628	Fusiform rod shaped, gram +	2×10^{18}
DMT628	coccus, gram +	4×10^{13}
BMT137	Rod shaped, gram +	4×10^{16}
DMT137(R1)	Rod shaped, gram +	3×10^{20}
DMT137(R2)	Rod shaped, gram +	5×10^{20}
DMT128	coccus, gram +	6×10^{14}

Identification and characterization of the BaP degrading bacterium BMT4i

The BMT4i was maintained on BaP supplemented BSM agar plates. The identification and characterization of the BMT4i was done on the basis of the cell and colony morphology, growth characteristics, motility, various staining reactions and various biochemical tests as given by Bergey's Manual of Systematic Bacteriology (6) as mentioned in the results (Table 2). For molecular characterization, BMT4i genomic DNA was isolated and approximately 1.5 kb 16S rDNA gene fragment was amplified by PCR (Biometra, Germany, model Tpersonal) using the forward primers 5´-ACCACATGCAAGTGCAACG-3´ and reverse primer 5´-ACGGGCGGTGTGTAC-3´. The amplified product was purified by agarose gel electrophoresis and clone into the pGEM-T vector (Promega Scientific, Santa Barbara, California). Both the strands of cloned approximately 1.5 kb 16S rDNA gene fragment were sequenced using sequencing facility provided by Bangalore Genei, India. The approximately 1.5 kb region of the 16S rDNA gene was used for alignment and phylogenetic analysis. The sequences were compared to those in GenBank by using the Blast Alignment Tool and the software based on "Neighbor Joining Method" was used to construct phylogenetic tree. The aligned sequences were deposited to the GenBank, EMBL and DDBJ libraries. The culture of BMT4i was deposited in Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

Assessment of degradation ability of BMT4i

All the operations were carried out in dim yellow light in order to avoid photo degradation of BaP. All the experiments were set up in triplicates. A single colony of BMT4i maintained in BaP-BSM agar plate was inoculated in 10 ml nutrient broth and grown at 37^0C with constant stirring till the A_{600} reaches to 1.0 (approx. $1x10^8$ cells/ml). The cell culture was centrifuged at 8000 rpm for 10 minutes and washed three times with BSM to remove trace of nutrient broth. The BMT4i cell number was adjusted to 10^8 cells /ml in BSM. For the time course studies, 1 ml of BMT4i- BSM suspension was re-inoculated in various flasks (10^7 cells /ml) containing 10 ml BSM supplemented with 50 µg/ml of BaP as the sole source of carbon and energy and incubated at 37^oC in incubator shaker at 120 rpm for various time periods along with their respective controls devoid of BMT 4i. At various time points (0, 12 h, 1, 2, 3, 7, 14, 21, 28, 35, 42 days), first of all, the A_{600} was recorded and then CFU/ml of BMT4i was checked by removing 100 µl culture from all the experimental and control flask, diluting up to 10^{-40} and plating over BSM-glucose agar plate. After 24 h, the numbers of colonies were counted and the $\log_{10}$CFU/ml was calculated to obtain the growth curve. The respective cultures were then extracted twice with ethyl acetate (1:1 v/v), acidified to pH 4.0 with 0.1 N HCl and re-extracted twice with ethyl acetate

Table 2. Physical and biochemical characteristics of *Bacillus subtilis* BMT4i (MTCC 9447)

Test	Results
Morphological characterization	
shape and arrangement	short, chains
capsule	positive
gram staining	positive
spore staining	positive, round shaped, terminal
motility	motile
acid fast staining	non acid-fast
Culture characterization on agar plates	
colonies	white, abundant mucilage
temperature	optimum
growth	abundant
form	irregular
margins	serrate
elevation	flat
density	translucent
Growth on broth	
surface growth	pellicle
clouding	slight
sediment	flaky
Biochemical tests	
Oxidase	-
Catalase	+
Nitrate reduction	+
Litmus milk	-, alkaline
Urease	+
H_2S production	-
Methyl red	-
Vogues proskaeur	+
Citrate utilization	-
Indole production	-
Carbohydrate fermentation	
a. Lactose	-
b. Mannitol	+
c. Sucrose	+
d. Glucose	+
e. Maltose	+
Starch hydrolysis	+
Gelatin hydrolysis	+ (rapid)
Casein hydrolysis	+
Lipid hydrolysis	+

to enhance the recovery of acidic metabolites. The entire triplicate extracts of respective time period were separately dried in rotary evaporator (Perfit, Ambala, India, model no. 951) and finally suspended in 1.0 ml of methanol. The recovery of biodegraded products by ethyl acetate extraction was found to be more than 96 %. The extent of BaP biodegradation was checked by quantifying left over BaP in methanol suspension of biodegraded extracts by HPLC analysis (13, 21). The reverse phase HPLC was done on commercial basis from Herbal Research and Development Institute (HRDI), Selaqui, Dehradun, India (HPLC system: Waters, USA, model no. 600 E pump with Photo Diode

Array (PDA) detector). For that, 20 µl of the methanol dissolved extracts were injected into an HPLC system fitted with 5 µm particle diameter C_{18} column. The elution gradient was programmed as follows (shown as percent [by volume] of methanol in water, acidified with 0.76 ml of H_3PO_4 liter $^{-1}$): 50% for 2 min, linear gradient to 80% at 5% min^{-1}, holding at 80°C for 16 min. The flow rate was kept at 1ml min-^{1}. Peaks were measured at 254 nm. The percent degradation of BaP was assessed by subtracting left over BaP in experiments (with BMT4i) from the respective controls (without BMT4i; recovered products were considered as 100%) at various time intervals (0, 12 h, 1, 2, 3, 7, 14, 21, 28, 35, 42 days).

Viability of BMT4i in the presence of other PAH

To check the viability of BMT4i in the presence of other PAHs, the overnight grown nutrient broth culture of BMT4i was washed three times with BSM and spread over BSM plates containing 500 µg/ml each of naphthalene, anthracene and dibenzothiophene (DBT) as the sole sources of the carbon and energy. After 24-48 h of incubation at 37°C, the plates were observed for appearance of colonies. The CFU/ml was determined by inoculating thoroughly washed BMT4i culture (10^7cells/ml) in BSM broth containing naphthalene, anthracene and dibenzothiophene (DBT) (500 µg/ml) in separate flasks and CFU/ ml was calculated after 7 day of incubation at 37°C, 150 rpm.

RESULTS

Isolation and identification of a BaP degrading bacterium

The BaP degrading bacterial colonies were isolated from automobile contaminated soil by standard culture enrichment techniques with BaP as the sole source of carbon and energy. Among various BaP degrading bacterial isolates, the BMT4i was selected as best BaP degrader on the basis of its highest growth in BaP-BSM as indicated by highest CFU/ml (Table1) and turbidity at A_{600} after 7 days of growth. BMT4i was further characterized on the basis of cell and colony morphology, various staining and biochemical activities (Table 2) and 16S rDNA gene analysis. The cellular and colony morphology, various staining reactions and biochemical reactions identified BMT4i as a *Bacillus subtilis.* Two of its unique biochemical characteristics viz-abundant mucilage secretion and urease positive reaction indicated BMT4i as a novel strain of *Bacillus subtilis*. The sequence and comparative phylogenetic analysis of PCR amplified 16S rDNA gene fragment of BMT4i to those in GenBank confirmed BMT4i as the closest homologue and a novel strain of *Bacillus subtilis* (NCBI GenBank Accession No. DQ911348, MTCC 9447) (Figure 1).

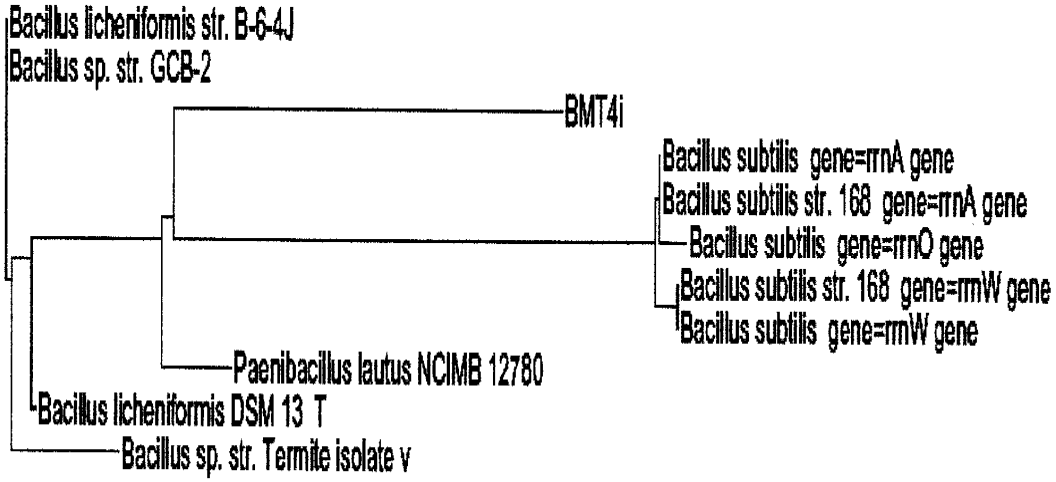

Figure 1. Phylogenetic analysis of *Bacillus subtilis* BMT4i based on 16S rDNA sequence analysis

Growth Kinetics of BMT4i in the presence of BaP

The growth kinetics experiment revealed that the BMT4i possess ability to utilize BaP for its biomass production. As shown in the plot of $\log_{10}$ of CFU/ml against incubation time, after 12 h of lag phase, $\log_{10}$ CFU/ml of BMT4i increases exponentially with increase in incubation duration up to 7^{th} day and there after it declines (Figure 2). At the beginning, 7.46 $\log_{10}$ CFU/ml (~ 2 x 10^7 CFU/ml) of BMT4i was added to the BaP-BSM medium, which almost remain constant (7.5 $\log_{10}$ CFU/ml) for 12 h, started increasing linearly after 24 h (8.4 $\log_{10}$ CFU/ml) attaining maximum 36.77 $\log_{10}$ CFU/ml (6.0 x 10^{36}) on 7^{th} day of incubation showing approx $4x10^{29}$-fold increase in the cell number. The CFU/ml declined slowly after 7^{th} day reaching to zero on 42 day. About $4x10^{29}$ fold increase in the CFU number is directly correlated with the ability of *Bacillus subtilis* BMT4i to utilize BaP as the sole source of carbon and energy which leads to high increase in cell number within just 7 days, which starts declining afterwards possibly due to the exhaustion of BaP.

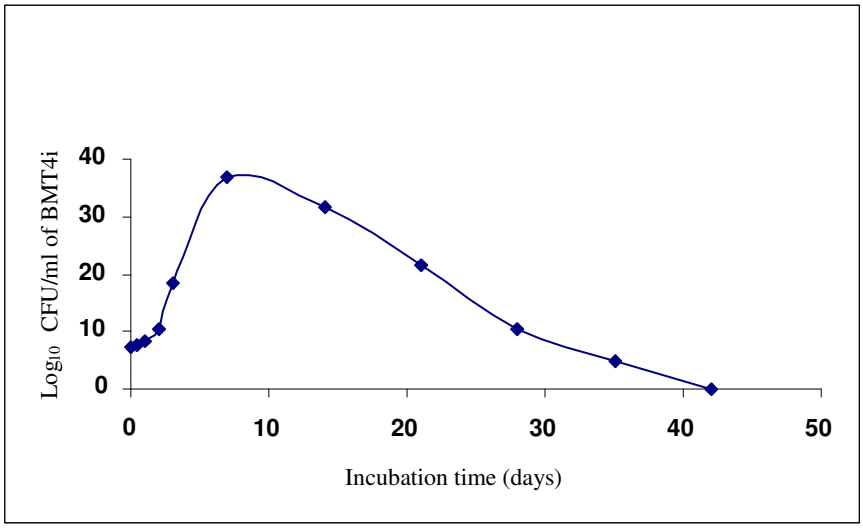

Figure 2. Counts (log $_{10}$ CFU/ml) of *Bacillus subtilis* BMT4i (MTCC 9447) in BSM with BaP against incubation time (days). Each point represents the average value obtained with triplicate flasks.

Assessment of BaP degradation

The HPLC profile for BaP and its metabolites after 7 days of growth of BMT4i in BaP medium was compared with that of negative control (without BMT4i) (Figure 3 A and B). The chromatogram showed six additional peaks (I, II, III, IV, V, VI) indicating different metabolites formed during the degradation of BaP. The analysis of metabolites is under study. The plot of percent BaP degradation against incubation time (Figure 4), demonstrated that *Bacillus subtilis* BMT4i possess ability to degrade BaP as shown by the linear increase in BaP degradation with time. BMT4i started degrading BaP after 24 h and continued up to day 28. On 12 h, no detectable amount of degradation was seen suggesting it as lag phase of degradation. On day 1, BMT4i mediated BaP degradation efficiency was about 5.18% that increased exponentially up to 7 day (about 48.34%), and there after with slow increase it became static on day 28 (84.66 %). Further incubation of 35 days and 42 days doesn't show any further increase in BaP degradation. Whereas, on day 2, 3, 14 and 21, BMT4i was able to degrade about 12.28%, 21.77%, 60.12% and 73.92% BaP respectively. Although, the number of viable cells ($\log_{10}$ CFU/ml) decreased considerably after 7 day of growth in BaP medium, the BMT4i continued to degrade BaP up to 28 days.

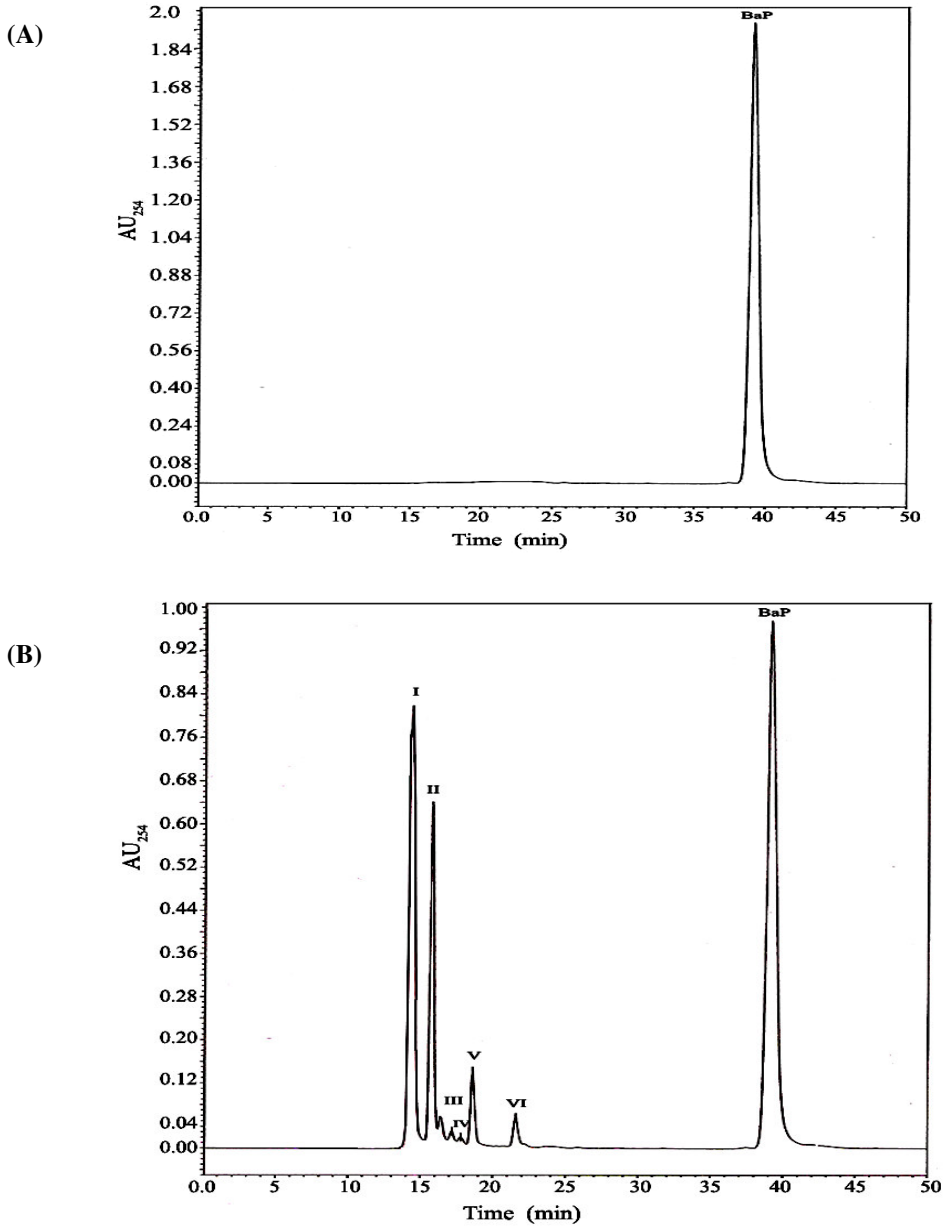

Figure 3. The HPLC profile for BaP and its metabolites after 7 days of incubation: (A) Without BMT4i (negative control); (B) with BMT4i in BSM- BaP medium

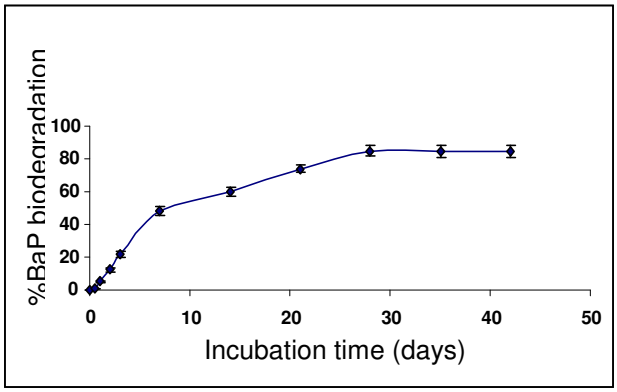

Figure 4. Percentage (%) degradation of BaP (50 µg/ml) by *Bacillus subtilis* BMT4i (MTCC 9447) with respect to time (days). Each point represents the average value obtained with triplicate flasks. The error bars represent the standard deviations of triplicate independent experiments.

Viability of BMT4i in the presence of other PAH

Bacillus subtilis BMT4i possess the ability to utilize naphthalene, anthracene and DBT as the sole source of carbon and energy as indicated by the appearance of numerous colonies after 48 h of growth over the respective PAH containing plates. This was further confirmed by CFU/ml after 7 days of growth in respective PAH containing broth. The CFU/ml was found to be about 10^{52} (naphthalene), 10^{49} (anthracene) and 10^{56} (DBT).

DISCUSSION

Present study is the first report demonstrating the utilization of BaP as a sole source of carbon and energy by bacteria. The novel strain *Bacillus subtilis* BMT4i (MTCC 9447) showed 10^{29} fold enhancement in biomass with in 7 days and about 84.66% of BaP degradation after 28 days of growth. The HPLC profile of 7 days incubation showed six additional peaks indicating intermediate metabolites. The identification of metabolites is under study. At present it is not clear whether the decrease in CFU/ml after 7 days is due to the accumulation of toxic metabolites or exhaustion of BaP as carbon and energy source. Our study is different from other studies in following three aspects. First, all the reported BaP degradation by bacteria have been shown to occur co-metabolically i.e., in the presence of additional carbon sources. Second, none of the bacterial species showed viability for such a long duration (40 days) in BaP-BSM medium. Third, this much of high BaP degradation efficiency has not been reported by earlier studies. Early observation of BaP biodegradation was made with mutant *Beijerinckia* sp. strain B8/36 and *Pseudomonas* sp. strain NCIB 9816 grown on succinate plus biphenyl and succinate plus salicylate (1, 7). *Mycobacterium vanbaalenii* PYR-1 using peptone, yeast extracts, and soluble starch as the additional carbon sources was able to degrade 24.7 % of BaP in pure culture where as in sediment water microcosm experiment it was able to mineralize 36 % of added BaP after 28 days (10, 11). In another study, *Mycobacterium* sp. Strain RJGII-135 showed 25 % co-metabolic mineralization of BaP in soil after 180 days (8). In a different study, *Mycobacterium* sp. strain RJGII-135 demonstrated about 40% degradation of BaP after 32 days (25). Co-metabolic degradation (41%) of BaP was also demonstrated by *Burkholderia cepacia* strains after 56 days (15). Some of the other studies involved use of bacterial consortium derived from soil which co-metabolically mineralizes BaP to greater than 95% in 150 days and 33-65% with in two weeks when provided with a complex hydrocarbon co substrate such as crude oil and diesel fuel separately in soil (17, 19). In a recent study, *Bacillus subtilis*-tgr3 isolated from PAH contaminated soil was able to transform 55% and 65% of BaP with in 48 h and 72 h at 30^{o}C when grown on minimal media supplemented with sodium citrate (13). However, during incubation, tgr-3 showed only 10^{2} fold enhancement in cell number after 48 hours after that it declined drastically becoming non-viable with in 96 hours.

Bacillus subtilis BMT4i also possess ability to degrade other PAHs such as naphthalene, anthracene and dibenzothiophene. Since *Bacillus subtilis* BMT4i has been isolated from automobile contaminated soil and is well adapted to PAHs including BaP contaminated environment, therefore it could serve as better model for bioremediation of PAH contaminated site and removal of PAHs including BaP from the environment.

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