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Anthracene degradation by novel strain of *Geobacillus stearothermophilus* "AAP7919"

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Abstract

Anthracene, a three fused benzene ring, polyaromatic hydrocarbons (PAH), released in the environment because of incomplete combustion of petroleum products, is potentially toxic to fishes, algae and environment. Due to its potential toxicity it is imperative to remove it from environment. The aim of present study is to isolate bacteria which can degrade anthracene as a sole source of carbon and energy. Furthermore, optimization of culture conditions with respect to temperature, pH, initial inoculum and agitations for the maximum anthracene degradation were determined. In present study we have isolated thirty four bacterial strains, with ability to degrade anthracene from oil contaminated soil. Among these bacterial isolate AAP7919 which was identified as *Geobacillus stearothermophilus* degraded maximum anthracene (64.09%) after 10 days of incubation. Maximum anthracene degradation by AAP7919 was observed at 50°C, pH 8.0, 5% initial inoculums size and 130 rpm agitation speed. The study leads to isolate a novel strain of *Geobacillus stearothermophilus* AAP7919 with anthracene degrading potential at higher temperature and which could be used for bioremediation of PAHs contaminated sites.

Keyword:- PAH, anthracene, *Geobacillus stearothermophilus* AAP7919, degradation, bioremediation.

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Introduction

PAHs are widespread pollutants and produced from anthropogenic process such as burning fossil fuels (Ni Chadhain et al., 2006). State and central pollution control boards identified PAHs as hazardous chemicals because of their toxic, carcinogenic and teratogenic effects on living organism (Ruma et al., 2007). Anthracene, a Polyaromatic hydrocarbon (PAH) could enter the environment because of incomplete combustion of petroleum products. Anthracene has proven toxic to endocrine, gastrointestinal, liver, skin and sense organs (Blumer, M., 1976, Kastner, M., 2000), thus, it is a potentially unsafe. The removal of this toxic chemical is a matter of great concern. Several physico-chemical methods could degrade and remove anthracene from environment. These methods need high temperature and pressure, and are expensive, labor-intensive with mounted risk of explosion and spread of pollution. Contrary to this, bioremediation is an economic, environment friendly and safe method for removal of these harmful chemicals. Earlier, strains *Pseudomonas fluorescens* showed to degrade anthracene with production of 2-hydroxy-3-naptholic acid as final product (Sanseverino *et .al*., 1993, Menn *et. al.,* 1993). Recent report showed plasmid associated anthracene degradation by *Pseudomonas sp* (Kumar *et. al.,* 2010). *Rhizobacteria* isolated from *Populus deltoides* also displayed anthracene degradation (Bisht *et. al.*, 2010). In such a bio diverse environment, there is further need to explore possibilities of newer,

more efficient and potential strains with degradative pathways leading from toxic substrates to eco friendly, safe and nontoxic products..

MATERIALS AND METHODS

Chemical and reagents:

Chemicals (> 98% purity) used in this study were procured from Himedia Laboratory Pvt. Ltd., India and Sigma Aldrich, USA. All chemicals were of analytical grades.

Preparation of media

Basal Salt Mineral (BSM) medium (pH 7.0) was prepared by dissolving 0.38 g KH_2PO_4,0.20 g $MgSO_4.7H_2O$, 1.0 g NH_4Cl, 0.05 g $FeCl_3$in one liter Milli-Q double distilled water and autoclaved. Solid media contained agar (1.8%) along with BSM. The stock solution (10 mg/ml) of anthracene was made in ethyl acetate.

Isolation of anthracene degrading bacteria

The subsurface (1-10 cm below the surface) oil contaminated soil samples were collected from different geographical locations in India. The oil contaminated soil was suspended (10%, w/v) in 100 ml of BSM supplemented with anthracene (500ppm) as the sole source of carbon and energy and incubated for 12 days at 37°C under constant stirring at 160 rpm in an incubator shaker. After incubation, 2 ml culture was withdrawn and re-inoculated in freshly prepared anthracene (500ppm)-BSM broth (100 ml) for 10 days under conditions as mentioned earlier. Afterwards, 1 ml of the culture was taken out and serially diluted in sterile BSM up to 10^{-7}. 100 µl of 10^{-7} dilution was spread on BSM agar plate supplemented with anthracene (500ppm) as the sole source of carbon and energy and incubated at 37°C for 3 days. The bacterial colonies which appeared in plates were aseptically removed and reselected on anthracene BSM plate to obtain their pure cultures. Single colony of each isolate was then inoculated in 10 ml nutrient broth and grown for 24 hr at 37°C with constant shaking. The degradation ability of each soil isolate was checked by inoculating 1.5% biomass of each soil isolate into the 30 ml of BSM broth supplemented with anthracene (50ppm) as the sole source of carbon. The cultures were incubated at 37°C for 10 days with constant shaking at 160 rpm. Simultaneously, control experiments were also setup, which lack soil isolates. After 10 days of incubation biomass from each flask was measured spectrophotometrically at 600 nm (y = 0.0808x-0.0021, R^2=0.9918). Seven bacterial isolates which showed highest biomass in anthracene-BSM medium were selected for further degradation study. The cultures of these isolates were extracted four times with equal volume of ethyl acetate for the recovery of metabolites (Manohar et al., 1999).The ethyl acetate extracts were vacuum dried on rotary evaporator and finally suspended in 1 ml of ethyl acetate. The ethyl acetate extracts of all the seven cultures were analyzed by UV-Visible spectrophotometer to check the extent of anthracene degradation. In brief, 20 µl of the ethyl acetate extracts were diluted in 3 ml of ethyl acetate and finally absorbance were measured at 254 nm and quantified by comparing with that of the standard curve of anthracene (y =0.041x-0.0473, R^2=0.9759).

The best anthracene degrader (soil isolate AAP7919) was selected on the basis of its highest growth in anthracene-BSM medium and by highest percent degradation.

Identification and characterization of the bacterial strain AAP7919

Identification of the strain AAP7919 was performed on the basis of the cell and colony morphology, growth characteristics, various staining reactions and different biochemical tests as given by Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986). For molecular characterization, the roughly 1.5 kb 16SrRNA gene fragment was PCR amplified using the forward(5'-AGAGTRTGATCMTYGCTWAC-3') and reverse primer (5'-CGYTAMCTTWTTACGRCT-3'). The presence of amplified product was checked on1% agarose gel and later purified by using DNA purification kit (Chromous Genomic DNA isolation teaching kit catalog number, RKT09). 1.5 kb 16S rDNA gene fragment was sequenced. The derived sequence was now compared with the sequence available in Gen Bank databases using the Basic Local Alignment Search Tool (BLAST). A phylogentic tree was constructed using sequences aligned with System Software aligner (Weighted Neighbor), and distance matrices formed using the Jukes-Cantor corrected distance model (Jukes et. al., 1969).

Characterization of anthracene degradation ability of AAP7919

All the experiments were set up in triplicates. 1.5% biomass of AAP7919 was inoculated in various flasks containing 30 ml BSM supplemented with anthracene (50ppm) as the sole source of carbon and incubated at 37°C in incubator shaker at 160 rpm for various time periods (0, 6, 12 hr, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days) along with their respective negative controls lacking the AAP7919 inoculum. At various time points (0, 6, 12 hr, 1, 2, 3, 4, 5, 6, 7, 8,9 and 10 days), first of all, the biomass was checked as mentioned earlier and then the degradation products were

extracted and subjected to quantification for assessment of anthracene degradation by spectrophotometer as mentioned earlier.

Culture conditions optimization for the anthracene degradation

To optimize the various parameters for maximum anthracene degradation AAP7919 was inoculated in various flasks, containing 30 ml BSM supplemented with anthracene (100 ppm) as a sole source of carbon and energy and kept at various temperature, pH, agitation and varied initial inoculums size for 10 days. The effects of temperature and pH on anthracene degradation were assessed by inoculating 1.5% biomass of AAP7919 at various temperatures ranging from 30°C to 70°C, and at various BSM pH values (2.0-11.0). To assess the effect of initial inoculums size on anthracene degradation, anthracene-BSM broth was inoculated with varied inoculum size (0.5 to 6.0%).

The effect of varied agitation on anthracene degradation was studied by inoculating AAP7919 in antracene-BSM medium at different agitating speed (100,110,120,130,140, and 160 rpm) at optimized temperature, pH and initial inoculum as obtained from previous experiment for 10 days.

Negative controls without AAP7919 were also set up for each experiment. After 10 days, percent anthracene degradation in the respective cultures flasks were quantified by UV-visible spectrophotometer as described earlier. The different parameters showing maximum antracene degradation were considered to be optimum parameters for antracene degradation by AAP7919.

Gas chromatography (GC) for the detection of anthracene degradation at optimum condition

Consumption of anthracene and corresponding metabolite formed was analyzed by gas chromatography (GC) with flame ionization detector. 30 ml of BSM anthracene (50 ppm) medium was inoculated with 5% biomass of AAP7919 and incubated at optimized value of culture condition for ten days. After incubation contents of the cultured flask were extracted thrice with ethyl acetate (1:1 v/v), acidified to pH 1.0 with 4.0 M H_2SO_4 and re-extracted thrice with ethyl acetate to enhance the recovery of acidic metabolites. The entire triplicate extracts of ethyl acetate and acidified ethyl acetate were pooled, dried in rotary evaporator and finally suspended in 1 ml of ethyl acetate. Extracted products were analyzed on DB-1 (30m x 0.25mm, 0.25µm film thickness) fused silica capillary column. Injector temperature at the time of sample injection was maintained at 275°C and flame ionization detector temperature was 300°C. Helium was used as the carrier gas, at a flow rate of 1 ml/min by using electronic pressure control. The temperature programming of GC was80°C (2 min isothermal), 80°C to 270°C (at the rate of 20°C/min) and 270°C (5 min isothermal).

RESULTS AND DISCUSSION

Isolation and identification of anthracene degrading bacteria

Thirty four bacterial colonies were isolated from oil contaminated soil by employing the standard culture enrichment techniques using anthracene as a sole carbon source. The thirty four bacterial colonies were checked for increase in biomass. Seven bacterial isolates which showed highest biomass in anthracene-BSM medium were selected for further degradation study. The spectrophometer analysis of the seven selected isolates showed that AAP7919 attain highest cell biomass and is the highest degrader of anthracene showing (64.09±0.0622%) degradation after 10 days of incubation (table 1), therefore, AAP7919 was selected as best anthracene degrader. The cell and colony morphology, various staining reaction sand biochemical activities revealed AAP7919 as Gram positive, motile and straight rod, spore forming and non acid fast. The isolate was able to ferment glucose, lactose, sucrose and was positive for starch hydrolysis, casein hydrolysis, gelatin hydrolysis while negative for urease activity, hydrogen sulphide production, indole test, methyl red test, Voges Proskauer test (table 2). After aligning and analyzing phylogenetic tree of 16S rDNA sequence with available databank was obtained using neighbors-joining method. It showed the position of strain "AAP7919"among the species of *Geobacillus* which fell into the phylogenetic group 5 of endospore forming bacteria. The distance matrix was compared with its closest neighbors. The phylogentic tree analysis predicted the closest bacterial organism as *Geobacillus stearothermophilus* with distance score 0.948 to strain AAP7919 (figure 1).

Table1: Percent degradation of anthracene by soil isolates in anthracene-BSM after 10 days

Soil isolate	% Degradation±SD
SPS7919	45.92±0.0776
SAS7919	33.95±0.0922
DAB7919	45.54±0.1653
DDD7919	58.94±0.0542
DAC7919	36.43±0.6510
AAP7919	64.09±0.0622
AAD7919	44.45±0.1949

Table 2: Identification of soil isolate AAP7919 based on morphological and biochemical

Characteristics	
Test	**Results**
Morphological characterization	
shape and arrangement	Straight rod
Gram staining	Positive
spore staining	Positive, round shaped, terminal
acid fast staining	non acid-fast
Biochemical tests	
Amylase production test	Positive
Degradation of pectin	Positive
Hydrolysis of gelatin	Positive
Casein hydrolysis	Positive
Urease test	Negative
Hydrogen sulfide production test	Negative
Carbohydrate catabolism	Negative (No colour change)
Carbohydrate fermentation	
i. Lactose	Positive
ii. Sucrose	Positive
iii. Glucose	Positive
IMVIC test	
i. Indole production	Negative
ii. Methyl red	Negative
iii. VogesProskauer test	Negative
iv. Citrate utilization test	Positive
v. Catalase test	Positive

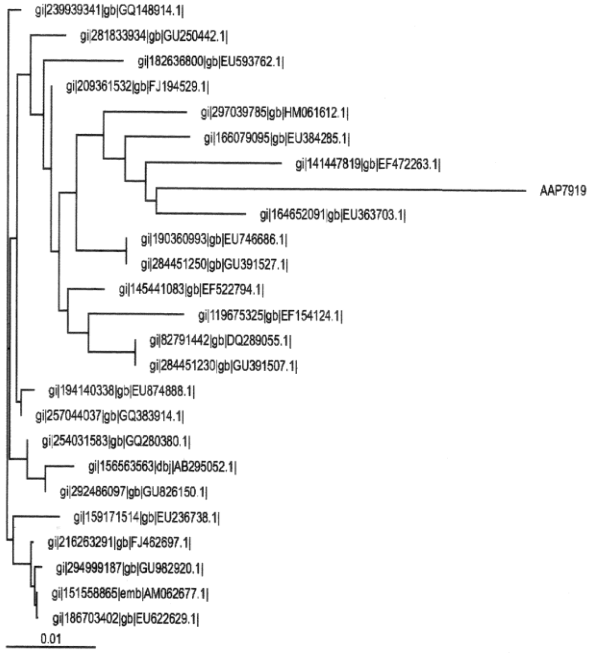

Figure1: Phylogenetic analysis of *Geobacillus stearothermophilus* AAP7919 based on 16S rDNA sequence analysis

The sequence of AAP7919 was deposited in NCBI Genbank USA, EMBL in Europe and the DNA Data Bank of Japan under the accession number GU459259.

Characterization of anthracene degradation ability of AAP7919

To study the degradation kinetics, AAP7919 was grown in anthracene-BSM broth for different time periods and the reaction products were extracted by ethyl acetate. The degradation of anthracene and the residual anthracene left in above purified extracts were evaluated by spectrophotometeric analysis and plotted against incubation times (figure 2). The plot evidently showed that the AAP7919 possesses the ability to degrade anthracene in time dependent manner. The degradation product of anthracene appeared first after the lag phase of 24 hr (8% degradation) and continued to increase up to the 7 days, leading to 64% degradation, afterwards, its production became stationary during further growth up to 10 days (64.5%). The biomass generation of AAP7919 was also studied in the above experiment which revealed an exponential increase in the growth of AAP7919 up to 9 days, attaining maximum biomass, thereafter, it became stationary

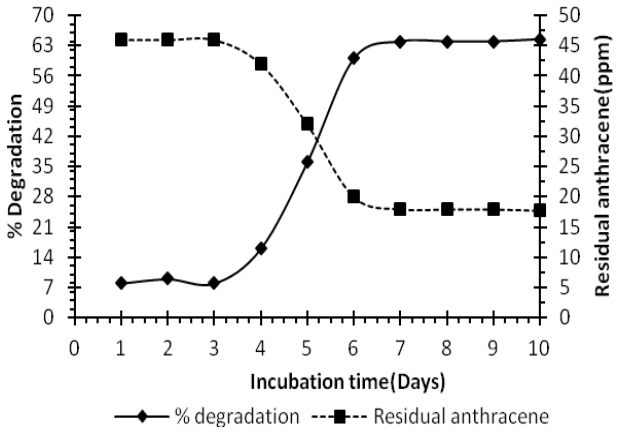

Figure 2: Degradation of anthracene with respect to time.

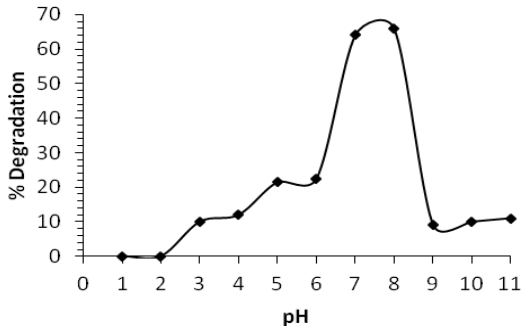

Figure 3: Anthracene degradation (%) at different pH.

Optimization of culture conditions of AAP7919

AAP7919 was subjected to optimization studies to evaluate optimal pH, temperature, inoculum size and agitation condition for maximum anthracene degradation. The optimum pH was found to be 8.0. At this pH maximum anthracene degradation was 65.96% (figure 3). AAP7919 mediated anthracene degradation activity was observed to be temperature dependent, with maximum anthracene degradation (68.32%) at 50°C, however, further increase in temperatures resulted in a gradual decline in anthracene degradation. Temperature optimization study revealed 50°C an optimum temperature which showed the thermohilic nature of the bacteria (figure 4). With increase in inoculum size (1% to 5%) of the AAP7919 the degradation increased significantly and reached maximum at 5% inoculum size (70.8% degradation). Further increase in the inoculum size resulted in decreased degradation (figure 5). The maximum degradation (72%) of anthracene was observed when the shake flasks were subjected to 130 rpm agitation speed and further increase in agitation decreased in the degradation (figure 6).

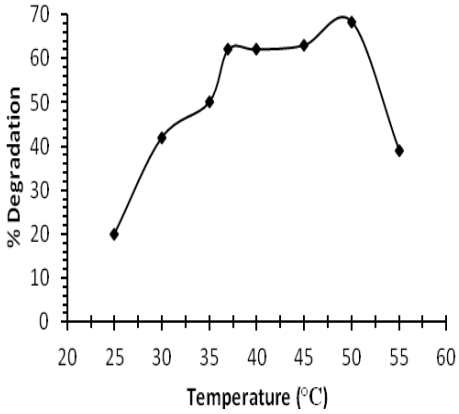

Figure 4: Anthracene degradation (%) at different Temperature.

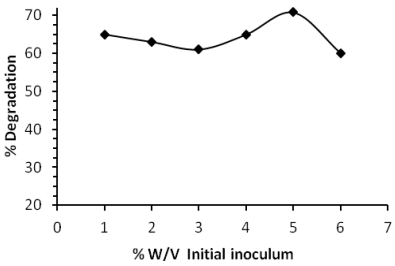

Figure 5: Anthracene degradation (%) at different initial inoculums size.

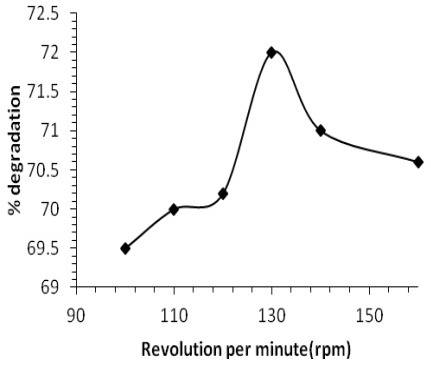

Figure 6: Anthracene degradation (%) at different agitation speed.

Gas chromatography (GC) analysis for anthracene degradation

Anthracene degradation was performed under optimized conditions and percent degradation was quantified by gas chromatography (GC). The GC profile of anthracene and its metabolite after 10 days growth of AAP7919 in anthracene-BSM was compared with that of negative control (without AAP7919) (figure 7 & 8). 76.15% anthracene degradation was reported under the optimized conditions of pH (8.0), temperature (50°C), inoculum size (5%w/v), and 130 rpm agitation speed.

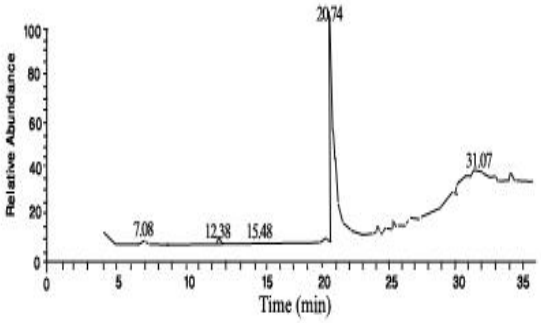

Figure7: GC profile for anthracene without *Geobacillus stearothermophilus* AAP7919 (control) after 10days.

Discussion:

AAP7919 strain was selected as best anthracene degrader showing 64.09% anthracene degradation. Current findings indicate that degradation of anthracene is time dependent and growth associated. There are several reports in the literature supporting these findings. The time profiles for anthracene degradation varied in different studies. In one study *Burkholderia* sp. showed complete degradation of added anthracene to autoclaved soil in 20 days (Somtrakoon et al., 2008).

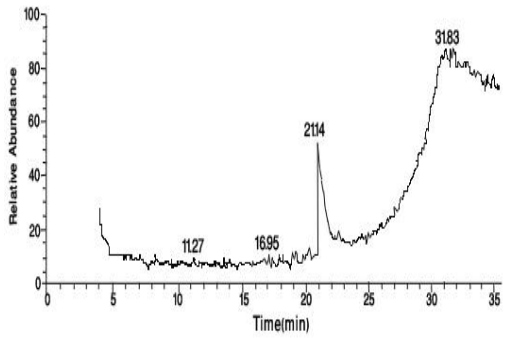

Figure 8: GC profile for anthracene and its metabolites after 10 days of incubation with *Geobacillus stearothermophilus*AAP7919 in BSM-anthracene medium

Our findings also corroborate with the finding of Matthew (2006), who isolated *Pseudomonas aeruginosa, Alcaligeneseutrophus, Bacillus subtilis* and *Micrococcus luteus* from crude oil polluted soils using 0.1% anthracene and calculated the residual oil concentration in the range of 22.2% to 91.7%. *Sphingomonas, Nocardia, Beijerinckia, Rhodococcus*and *Mycobacterium* also possess anthracene degradation potential (Dean-Ross et al., 2002; Moody et al., 2001). *Pseudomonas citronellolis* 222A degraded 72% of anthracene after 48 days (Eder et al., 2008). In the present study, optimization of pH, temperature, agitation and inoculum size led to increase in anthracene degrading ability of AAP7919, to the extent of almost 10% (64.09%to 74.18%). Kastner et al., (1998) documented the impact of pH, inoculum size, PAH content and PAH degrading bacteria on degradation of PAHs. Similar observations have been made by Jacobsen and Petersen, 1992 while working on growth and survival of *Pseudomonas* in soil amended with 2,4-dichlorophenoxyacetic acid. Ramadan et al., (1990) described inoculums size as a factor limiting success for biodegradation. Zaidi et al., (1988) also commented on chemical concentration and pH as factors limiting the success of inoculation to enhance biodegradation. Temperature also plays an important role in biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as affecting the physiology and diversity of the microbial flora. Based on extensive and intensive vast studies on biodegradation and biodiversity scientists have underlined the importance of search for thermophilic microorganisms that degrade environmental pollutants (Giedraityte et al., 2009). The high temperature tolerant and stable enzymatic

machinery of these organisms have attracted the attention of the scientific community to harness them as potential wealth for industrial applications (Pennisi, 1997, Landenstein et al., 1998). Besides, *Bacillus thermoleovorans* and, *B. stearothermophilus*, thermophilic *Geobacillus*strain also possess higher temperature optimuma (60ºC) for their enzymatic action (Zhang et al., 1998, Milo etal., 1999, Bubinas et al., 2007). During optimization of temperature conditions, AAP7919 showed highest anthracene degrading ability at 50ºC. This may be attributed to efficient thermozymes activity. Similar observations have been shown by Bubians et al. (2007) while they carried out studies on dioxygenase from thermophilic *Geobacillus* sp. The pH and temperature optima for enzyme activity were 8.0 and 60ºC. The values were close to that obtained in our studies on *Geobacillus stearothermophillus.* Similar kind of study performed by Lily et al. 2010 on degradation of Benzo [a] pyrene (BaP) by *Bacillus subtilis* BMT4i showed 30ºC temperature and pH 8.0 as a optimum value for degradation. The study led us to isolate and identify a thermo tolerant indigenous strain of *Geobacillus stearothermophilus* with potential to degrade anthracene. The novel strain could be a useful tool in bioremediation of polluted soils under adverse conditions.

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