RESEARCH PAPER | Biochemistry | Volume : 4 | Issue : 5 | May 2014 | ISSN - 2249-555X

Isolation, Characterization and Anthracene Mineralization by Bacillus Cereus From Petroleum Oil Depot Soil

KEYWORDS: Anthraene, degradation, Bacillus cereus, mineralization pathway.

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ABSTRACT *Bacillus cereus strain, isolated from contaminated soil from IOCL depot Khapri, Nagpur, MS, India. Isolated species decomposes upto 1000 ppm of anthracene tried so far. Effects of environmental factors pH, temperature and substrate concentration over mineralization also studied. Temperature of 350C, substrate concentration 500 ppm & 7.5 pH are optimum environmental conditions studied so far in laboratory for mineralization. 2,3 dihydroxynapthalene, catechol, phthalate were reported as an intermediates, from HPLC analysis & possible mineralization pathway for anthracene through these intermediates were suggested.*

Introduction
Polycyclic aromatic hydrocarbons (PAHs) are global pollutants and pose a serious risk to the environment and human health because of their toxicological potency and resistance to degradation. PAHs pose a serious risk to the environment and human health. Microorganisms play an important role in the degradation of persistent organic pollutants in the environment. (Xue & Warshawsky, 2005 Kim et al., 2005). Anthracene mineralizations by species such as Pseudomonas, Sphingomonas, Nocardia, etc. are known (Evans et al 1965, Dean Ross et al 2001, Moody et al 2001). However, only few reports are available for anthracene degradation pathway by B. cereus . This study focus on anthracene degradation and catabolic pathway was proposed based on metabolites identified using HPLC.

Materials & methods
Chemicals
Bushnell Hass Agar and Bushnell Has broth (hi-media), Anthraccene (98% purity), 1-hydroxy-2-naphthoic acid, 2-hydroxy-1-naphthoic acid, 2 carboxycinnamic acid, phthalic acid, 2-formylbenzoic acid, and protocatechuic acid and other metabollies are of high purity were purchased. Chemicals employed in HPLC analysis were of HPLC grade.

Soil collection and enrichment.
Soil collected from the oil depot, bring to lab in preserved condition and subjected to enrichment, using anthracene as sole carbon and energy source. Minimal medium used for isolation is Bushnell Hass broth It is composed of (L-1): $MgSO_4$ 0.20 g, Ammonium Nitrate 1.00 g, Calcium chloride 0.02 g, $FeCl_3$ 0.05 g, Monopotassium Phosphate 1.00 g, K_2PO_4 Dipotassium Phosphate 1.00 g. The pH was adjusted to 7±0.2 after addition of trace element solution (was added at (L-1): CuSO4 0.4mg, KI 1.0 mg, $MnSO_4$.H20 4.0 mg, $ZnSO_4.7H_2O$ 4.0 mg. H_3BO_3 5.0 mg, H_2MoO4.2H2O 1.6 mg.) The pH was adjusted to 7±0.2. 1 g of soil sample was suspended in 100 ml of BH Broth containing 250 mg/L anthracene and incubated with shaking at 120 rpm at 28^0 C± 2^0 C on orbital Shaker (Remi). After 7 days, 5 ml aliquot from these flask were transferred to 100 ml of fresh BH Broth medium containing the same amount of these PAHs and incubated under the same condition as mentioned above.

Screening and isolation of anthracene degrading bacteria:-
Potential bacterial isolates able to degrade anthracene was screened using spray plate method. (Kiyohara et al 1982). Briefly 0.1 ml enrichment flask cultures after dilution spread on solid BH Agar plates and sprayed with anthracene (in 0.2% hexane) dissolved in it as sole carbon source. Plates incubated in dark at 30^0C. Anthracene degrading bacteria were visualized by a distinct sprayed-coated clear zone surrounding individual colonies. Representative colonies forming large clear zones were aseptically removed, purified.

Bacterial identification and phylogenetic analysis of 16S rDNA sequences
Morphological & phenotypic characters such as cell shape and colony morphology with biochemical characteristic was identified according to Bergy`s Manual of Determinative Bacteriology (Holt J. et al 1994). Genomic DNA extracted using Nucleo-Pore Genomic DNA Isolation kit (Genetix).16S rRNA gene was PCR amplified using following primers

16sF- 5'-AGAGTTTGATCCTGGCTCAG-3'
16sR- 5'ACGGCTACCTTGTTACACTT-3'.
Amplicon Concentration checked in a Nanodrop ND 2000 and purified using Nucleospin purification column (Macherey-Nagel). After sequencing of amplicon in ABI 3730xl cycle sequencer, forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The sequence analysis was carried out using BLAST program of NCBI to determine the closest available database sequences. Based on maximum identity score first few sequences were selected, aligned using multiple sequence alignment software and dendrogram was constructed (CLC genomics).

PAH degradation assays: The biodegradation in liquid culture was carried out in 250 ml capacity conical flasks. Stock solution of anthracene in acetone was added to have final concentration of 250 ppm. Flasks were kept for overnight to completely vent off acetone. 100 ml of autoclaved BH broth and 1ml aliquot of fresh Bacterial cultures used to inoculate the experimental flasks. For inoculation cells were collected by centrifugation (6000×g 10 min) washed twice with 0.5 M phosphate buffer (pH7) and re-suspended in the same to have OD of 1 at 600 nm. BH Broth with only anthracene used as control. Flasks incubated on rotary shaker (120 rpm, 28 ^{0}C).

Extraction of antharcene, metabolites & HPLC Analysis
Samples extracted twice for every 48 hours upto 8 days, with equal volume of diethyl ether after acidification to pH 2(4M H_2SO_4). The organic phase extractions combined, dried over with anhydrousNa_2SO_4, evaporated to dryness and dissolved in methanol. 10 µl of it was analyzed by HPLC. Degradation percentage was calculated from anthracene remain in the flask & its metabolites were identified by comparison of the retention time with those of authentic standards. HPLC analysis performed on Shimadzu SPD-20A prominence,

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equipped with uv-visible detector. For analytical purpose separation was achieved using a capillary column, Luna 5u C18 (2) 100A, with dimension of 150 mm length × 4.6 mm in diameter (Phenomenex), under binary mode. It Mobile phase consists methanol & water (70: 30) at a flow rate of 1.0 mL/ min. Standard anthracene solutions at different concentrations were used for reference. LC time solution software was used to collect and analyze data.

Result & discussion

Identification of the culture—

Isolate is a gram positive, motile, bacterium able to liquefy gelatin, positive catalase and MR-VP it fails to give positive reaction for citrate, indole production, D-gluose & D-mannose. Based on nucleotide homology, phylogenetic analysis, biochemical and morphological characteristics, the isolate identified as Bacillus cereus, (97% similarity) and referred by the same name in this study. E drposited the sequence in NCBI with accession No. KJ 622303. The phylogeny cluster of isolated B. cereus along with related species are depicted in fig 1. Dendrogram showing relationship with isolated B. cereus other closely related sequences collected from the Gene Bank.

Anthracene degradation

Bacillus cereus, could degrade anthracene to 60 % on eight day of incubation.7.2 % degradation was observed on second day, while 35.2 % and 53 % degradation for anthracene were noted on fourth and sixth day (Fig 2a- about biodegradation of anthacene by B. cereus at 250ppm).

The abiotic loss of napthalene and anthracene was only about 1% as evidenced from the control flask. Potential Bacillus species such as B.thermoleovorans, B. cereus 28BN, B. vireti etc are well known for PAHs degradation. (Anweiller et al 2000)

pH Variation- Highest degradation achieved with slight alkaline pH of 7.5 with degradation of 63% . 60% & 58.4% degradation obtained with pH 7 & pH 6.5 respectively.

(Fig. 2b about effects of pH on anthracene degradation by isolated B. cereus).

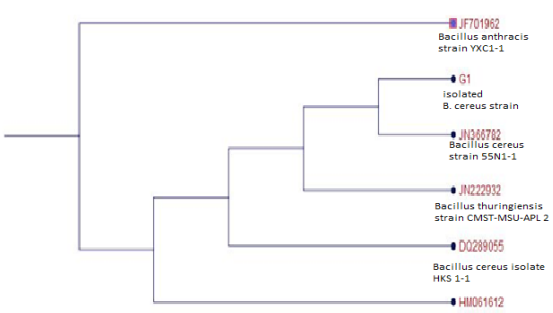


Fig 1. –Dendrogram showing relationship with isolated B. cereus other closely related sequences collected from the Gene Bank.

Substrate concentration- Degradation of 62.2 & 61% were observed with initial anthracene concentration of 500 and 750 ppm showed minute variation suggests it work with almost same efficiency in the given range of substrate. However at 1000 ppm degradation reduces to 57 %.

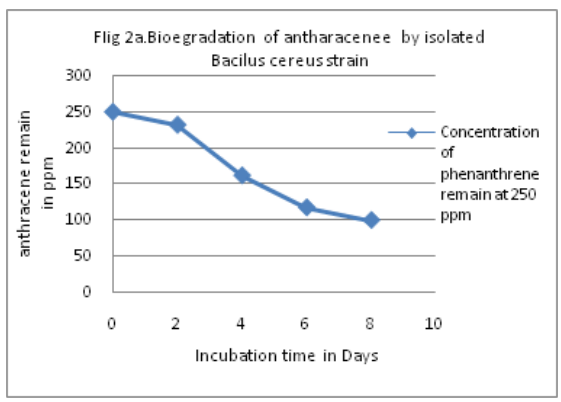
(Fig 2c about Effets of substrate concentrations on anthracene degradation by B. cereus).

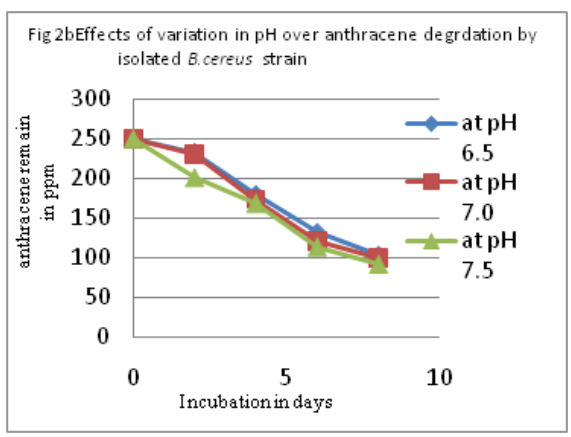
Temperature- Lowest degradation of 46.8 % reported at 25^{0}C. Highest degradation at 35^{0}C observed with 64% degradation. 59.6% degradation obtained at 30^{0}C (Fig2d about Effets of temperature on anthracene degradation by B. cereus).

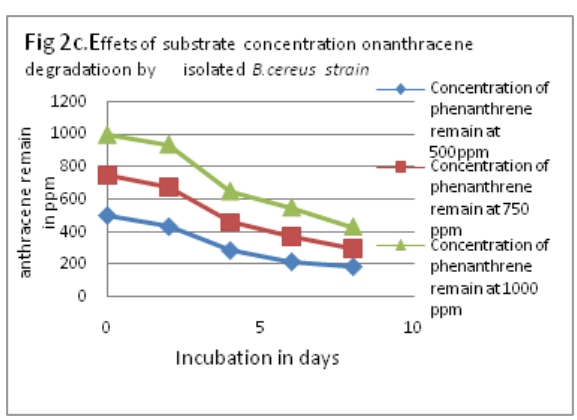
The thermophilic aerobic bacterium Bacillus thermoleovorans Hamburg 2 grows at 60°C on naphthalene as the sole source of carbon and energy (Annweiler et al 2000).

Identification of metabolic intermediates & pathway –

Elution profile for the B. cereus for metabolites revealed many peaks with anthracene as sole carbon and energy source. Anthracene eluted with Retention Time 11.999 minutes. We found a metabolite that elute from the column with RT 4.371 min and identified as 2,3-Dihydroxynaphthalene from comparison of HPLC chromatogram with same pure chemical compound. We unable to detect 1,2-dihydroxynaphthalene, but found 3 hydroxy2-napthoic acid, a peak with RT 3.021min. Pthalate and catechol with RT 9.864 &12.952min respectively are the others detected metabolites.







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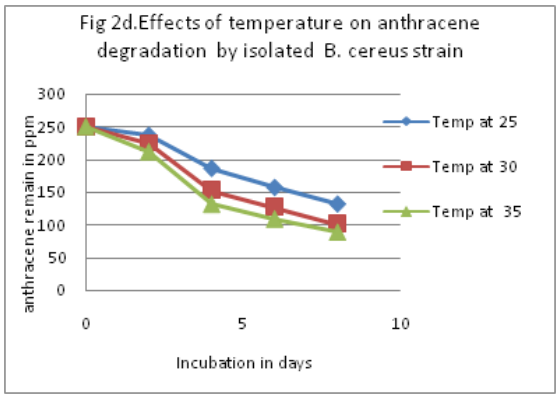

Anthracene mineralization ell known and reported in many gram negative and positive bacteria. (Mrozik et al 2003). First step in degradation of anthracene is Hydroxylation at 1, 2 positions to 1, 2-dihydroxyanthracene through cis-1,2-dihydroxy- 1,2-dihydroanthracene diol (Evans et el 1965). In the next step these bacteria oxidize 1,2-dihydroxyanthracene to the ring fission product cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-enoic acid with subsequent conversion to 2-hydroxynaphthoic acid, which is metabolized to salicylate and catechol through 2,3-dihydroxynaphthalene. Catechol is degraded to simple aliphatic compounds by a similar pathway to catechol conversion in the naphthalene degrading pathway. (Cerniglia 1984, Evans et al 1965). Antharcene mineralization by B. cereus may take this pathway for mineralizationof anthraene converting it to 2, 3 dihydroxynapthalene, another detected metabolite.

Identification of catechol as intermediated indicates 2, 3dihydroxy napthalene catabolism in the bacterium may occur through catechol and further by its conversion to cis-cis muconate due to reported activity of catechol1,2 but not 2, 3 dioxygenase in the extract (data not shown). Ortho cleavage of catechol is not new, but routine mechanism employed in hydrocarbon degradation reported in Bacillus cereus CP001746 (Bajkic et al 2011). Detection of pthalate in extract also suggests possibility that 1, 2 dihydroxyanthracene may converted to phthalate that further metabolized by phthalate degradation pathway (Ahmed et al 2012). Possibility may also exist that isolated B. cereus able to utilize both pathway for anthracne degradation and or have some common metabolites linking these two pathaway, which we unable to detect.

Fig 3.about Proposed pathway for anthracene mineralization by isolated B. cereus strain.

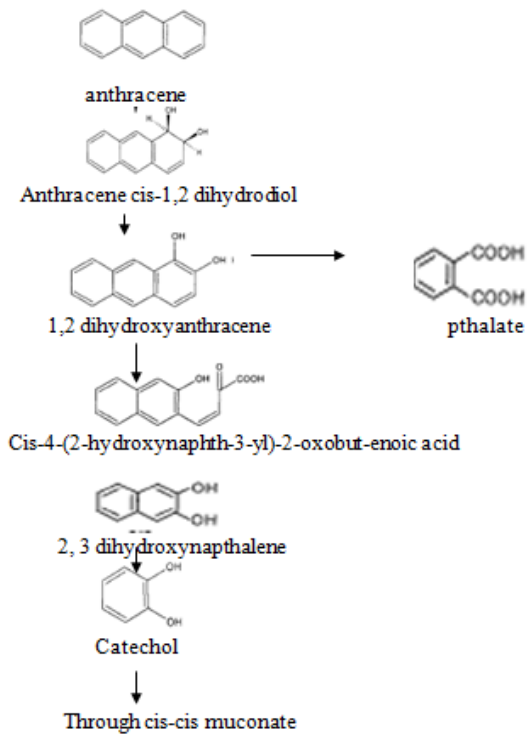

Fig.3. Proposed pathway for anthracene mineralization by isolated B. cereus.

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