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Biodegradation of Benzo[a]pyrene by the mixed culture of *Bacillus cereus* and *Bacillus vireti* isolated from the petrochemical industry

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Abstract

Polycyclic aromatic hydrocarbons are a group of compounds that pose threat to humans and animal life. Methods to reduce the amount of PAHs in the environment are continuously being sought. The bacterial consortium capable of utilizing benzo(a)pyrene as the sole source of carbon and energy was isolated from petrochemical soil. The isolates were identified as *Bacillus cereus* and *Bacillus vireti* based on morphological characterization, and 16S rDNA gene sequence analysis. About 58.98 % of benzo(a)pyrene at concentration of 500 mg l^{-1} in mineral salts medium were removed by bacterial consortium. GC mass spectral analysis showed the presence of metabolite cis-4-(7-hydroxypyren-8-yl)-2-oxobut-3enoic acid. The results indicate that the bacterial consortium is a new bacterial resource for biodegrading benzo(a)pyrene and might be used for bioremediation of sites heavily contaminated by benzo[a]pyrene and its derivatives.

Key words

Benzo(a) pyrene, *Bacillus cereus*, *Bacillus vireti*, Biodegration, Mixed culture

Introduction

Polycyclic aromatic hydrocarbons are a class of toxic environmental pollutants consisting of two or more fused benzene rings. They contain two or more fused aromatic rings in linear, angular or cluster arrangements (Cerniglia, 1992). They are generated continuously by the incomplete combustion of organic matter, volcanic eruptions and forest fires. They are also distributed into the environment due to human activities such as cigarette smoking, automobile exhaust, and the processing, production and spillage of petroleum (Chauhan *et al*., 2008). PAHs are highly toxic, mutagenic and carcinogenic. They do not degrade easily under natural conditions. PAHs are hydrophobic compounds and their persistence in the environment is due to their low water solubility (Cerniglia, 1992). The solubility of PAH decreases and hydrophobicity increases with an increase in number of fused benzene rings. The volatility decreases with an increasing number of fused rings (Wilson and Jones, 1993). High molecular weight PAHs such as benzo[a]pyrene, benzo[a]fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene and indeno(1,2,3-cd)pyrene are potential carcinogens.

Benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) containing five fused benzene rings, is considered to be one of the 16 PAHs defined as priority pollutants by the US Environmental Protection Agency (EPA) due to its carcinogenicity, and acute toxicity (Boonchan *et al*., 2000). Benzo[a]pyrene may be removed from the environment through the biodegradative actions of bacteria and fungi. BaP has been detected in a variety of environmental samples (Juhasz and Naidu, 2000; Kanaly and Harayama, 2000). From

contaminated soil and water, BaP enters into the food chain and metabolized to its genotoxic form which then interacts with nucleic acid and proteins forming reactive macromolecular adducts resulting in BaP induced toxicity, mutagenesis and carcinogenesis in mammals. Studies have shown that there is a great diversity of microorganisms which are capable of degrading low molecular weight PAHs such as naphthalene, pyrene and phenanthrene. Relatively, few organisms have been observed to degrade high molecular weight PAHs. It has been reported that *Mycobacterium vanbaalenii* PYR-1 (Moody *et al*., 2004), *Bacillus subtilis* (Hunter *et al*., 2005) and *Sphingomonas yanoikuyae* JAR02 (Rentz *et al*., 2008) are involved in the degradation of BaP. There is limited information regarding the bacterial degradation of high molecular weight PAHs in both environmental samples and pure or mixed cultures (Kanaly and Harayama, 2000). It has been reported that benzo[a]pyrene biotransformation by bacteria occurs under cometabolic conditions (Juhasz and Naidu, 2000). BaP cannot be utilized both as a carbon and an energy source for single microorganisms (Cerniglia, 1992), it is necessary that a growth substrate be supplied to initiate growth of the organism and to induce the production of catabolic enzymes. Studies have shown that low molecular weight hydrocarbons are metabolized by pure strains and biodegradation of high molecular weight hydrocarbons requires the combined efforts of different populations (Kanaly *et al*., 2000). PAH compounds are transformed throughout a series of different metabolic reactions by microorganisms in the real environment. Therefore, the study of PAH metabolism by bacterial consortia is important. It will provide new insights for improving future studies on bioremediation of environmental pollutants. Aim of this study was isolation of the bacteria which could utilize the benzo(a)pyrene as its carbon source from petrochemical contaminated soil and identification of the degraded products of benzo(a)pyrene by the bacterial consortium carried out in gas chromatography - mass spectrometry.

Materials and Methods

Soil sampling : Soil samples were collected from soil contaminated with oil refinery wastes of petroleum industry (Chennai Petroleum Corporation Limited) situated in Chennai, Tamil Nadu, India. It was stored at -20°C.

Isolation and identification of benzo(a)pyrene degrading bacteria : The soil samples were collected from three sites in a petroleum industry situated in Chennai. 0.25 g of PAH contaminated soil was suspended, in 50 ml of mineral salt medium. Into the suspension 0.5 $\mu g ml^{-1}$ of benzo(a)pyrene were added as enrichment substrate and the suspension were incubated with shaking at 120 rpm at 30 °C in the dark for 14 days. After 14 days, 100 µl of inoculum was spread on MSM-agar plate supplemented with benzo[a]pyrene (0.5 mg ml^{-1}) as sole source of carbon and energy and incubated for 48 h at 37°C.The morphologically distinct bacterial colonies which appeared on MSM-agar plates were aseptically picked and streaked on minimal salt medium agar plates to obtain pure culture. All isolates were stored at -20°C as the liquid cultures containing 70% glycerol (v/v).

Total genomic DNA was extracted according to the method introduced by Kanaly and Harayama, (2000). The 16S rDNA gene fragment was amplified by PCR using the set of primers: 16S-8F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 16S-1495R (5'-C GGCTACCTTGTTACGA CTTC-3'). The PCR conditions (35 cycles of 3 min at 94 °C, 1 min at 94°C, 1 min 50°C, 2 min at 72°C and 2 min at 72°C) were performed in a thermal cycler (Mastercycler Gradient, Eppendorf, USA).The amplified products were eluted from the gel and sequenced using ABI 3130 XL (Gentic Analyser, Applied Biosystems ,USA). The 16S rDNA sequence of the isolates were compared using the BLAST program at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and the sequences analysis deposited at Genbank with the accession numbers HM135450 and HM135451.

Biodegradation of benzo(a)pyrene : The consortium was added into MSM-BaP (0.5mg ml^{-1}). Each culture flask containing 20 ml of medium plus bacterial inocula (8 x 10^{-7} CFU ml^{-1}) was placed on a rotary shaker (120 rpm) at 30 °C in the dark. MSM containing 0.5mg ml^{-1} BaP, but without the bacterial consortium, was used as a control. Samples were extracted from the experimental system at 0, 25 and 35 days.

Extraction and identification of benzo[a]pyrene metabolites: To obtain enough quantities of the possible metabolites for separation and identification with GCMS, the extraction of BaP was performed according to Li *et al*. (2008) and Kanaly and Harayama (2000). After desired period of incubation, samples from biodegradation experiments were used for the extraction of benzo[a]pyrene. The contents from each culture vials were centrifuged at 4000 rpm for 10 min and the particles were allowed to deposit for 5 min, and 20 ml of supernatant was transferred to separating funnels and extracted twice with an equal volume of dichloromethane. The organic extracts were pooled and dried over anhydrous Na_2SO_4. The extracts were condensed by evaporation of the dichloromethane under a stream of nitrogen, and remains were dissolved in 1ml hexane. Analyses of cultures by GC-mass spectroscopy (MS) were performed by using a Shimadzu GC 2010 instrument fitted with a fused silica capillary column (DB-5; 30m by 0.25mm; J & W Scientific). The temperature program was as follows: 50ºC for 2min, followed by an increase at a rate of 6ºC min^{-1} to 300ºC. The injection volume was 2 µl, and the carrier gas was helium (flow rate: 1.7ml min^{-1}). The mass selective detector was operated in the scan mode to obtain spectral data for compound identification (benzo(a)pyrene molecular ion at m/z 252).

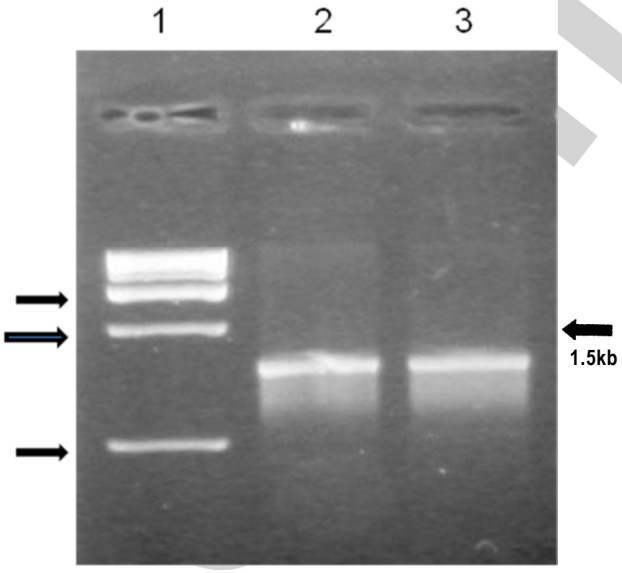
Results and Discussion

Isolation of benzo(a)pyrene degrading bacteria : The enriched soil samples obtained from the contaminated sites of petrochemical industry contained two morphologically distinct

colonies. The isolate 1 was Gram positive, facultative anaerobic and motile bacterium. It appeared as large rods and was creamy in color. Endo spores lied in central position. It showed negative results for oxidase and urease tests. However, the cells of isolate 2 were a Gram negative, facultative anaerobic, motile and round-ended rod. It appeared as circular, raised and dark cream color. Endo spores lie in central position.

Identification of bacterial strains: The bacterial strains capable of degrading benzo(a)pyrene were identified using 16S rDNA gene sequencing from which the nucleotide sequences of the strains were derived 16S rDNA gene fragments (1.5 kb) were amplified from the total DNA of the isolates (Fig.1). Blast results of the nucleotide sequence showed that isolate1 had 99% identity with *Bacillus cereus* and isolate 2 had 99% identity with *Bacillus vireti*. The nucleotide sequences of these two bacterial strains were submitted to the Gen Bank with the accession numbers HM135450 and HM135451.

Mineralization of benzo[a]pyrene and identification of its metabolites: By the end of the experimental process, 58.98% of BaP had been degraded by bacterial isolates. During the benzo[a]pyrene (0.5mg ml^{-1}) mineralization process, metabolites formed by 25 d and 35 d were analyzed using GC-mass spectral analysis (Table 1). The chromatogram of BaP showed a peak at retention time of 44.36 with m/z (mass/charge) ratio of 252 (Fig. 2a), which corresponds to the molecular weight of benzo[a]pyrene. In the chromatogram taken after 25th d, the original peak was not seen and new peak with retention time 22.9 was formed. Mass spectra showed mass ion at m/z value of 206 with characterized fragment ions as 191 and 57 (Fig.2b). After incubation for 35 d, one metabolite of benzo(a)pyrene (0.5mg ml^{-1}) was identified with mass ion at m/z of 314 by MS (Fig.2c). With the reference information of standard compounds and reported benzo[a]pyrene degradation pathway, this compound was proposed to be cis-4-(7-hydroxypyren-8-yl)-2-oxobut- 3enoic acid (Fig. 2c) and benzo[a]pyrene itself (Fig. 2a), respectively. The compounds corresponding to the other peaks were unidentified. Formation of *cis*-4-(7-hydroxypyren-8-yl)-2- oxobut-3enoic acid was consistent with meta fission of the hydroxylated BaP molecule (Schneider *et al*., 1996).



Lane 1: 1kb DNA Marker; Lane 2: The amplified product of isolate 1; Lane 3: The amplified product of isolate 2

Fig. 1: PCR amplification of genomic DNA of bacterial isolates by using 16S r DNA primers

Up to now, only a few strains capable of degrading benzo[a]pyrene have been reported, such as *Beijernickia* strain B836 (Gibson *et al*., 1975), *Mycobacterium vanbaalehii* PYR-1 (Moody *et al*., 2004), *Bacillus subtilis* (Hunter *et al*., 2005), *Sphingomonas paucimobilis* strain EPA 505 (Ye *et al*., 1996), *Sphingomonas yanoikuyae* JAR02 (Rentz *et al*., 2008), *Stenotrophomonas maltophilia* VUN 10,010 (Boonchan *et al*., 2002) and *Rhodanobacter* BPC1 (Kanaly *et al*., 2002). Early observation of benzo[a]pyrene degradation was made with mutant *Beijernickia* strain B8/36 and *Pseudomonas* sp. strain NCIB 9816 grown on succinate plus biphenyl and succinate plus salicylate (Barnsley, 1975). It had been observed that 25% of benzo[a]pyrene was mineralized by bacterial consortium (*Stenotrophomonas maltophilia* VUN 10,010 and *Penicillium janthinellum*) to CO_2 over 49 days, accompanied by transient accumulation and disappearance of intermediates as detected by high pressure liquid chromatography. Luo *et al*. (2009) reported that 44.07% of the 10 ppm benzo[a]pyrene was degraded after 14 d incubation by bacterial consortium which consists of *Ochrobactrum* sp., *Stenotrophomonas maltophilia* and *Pseudomonas fluorescens*. In previous studies, it had been reported that *Mycobacterium* strain RJGII-135, isolated from a coal gasification site, and was able to degrade [14C] benzo[a]pyrene, producing metabolites cis-4-(7-hydroxypyren-8-yl)-2-oxobut-3enoic acid, methylated 4,5-chrysene-dicarboxylic acid, cis-7,8-dihydrodiol-BaP and 7,8-dihydro-pyrene-8-carboxylic acid. In this study, bacterial consortium (*Bacillus cereus and Bacillus vireti*) isolated from petroleum contaminated soil was able to transform 58.98% of benzo[a]pyrene. The formations of cis-4-(7-hydroxypyren-8-yl)-2-oxobut-3enoic acid intermediates were found. This may be due to meta fission of the hydroxylated benzo[a]pyrene. The defined bacterial consortium provides a more versatile and effective way to clean up environmental pollution (Mrozik and Piotrowska-Seget, 2010). Microbial cooperation may promote broader and more efficient *in-situ* degradation of complex pollutant mixtures. Additional studies have to be made to reveal the optimum conditions (nutrients, temperature etc.) required to maximum benzo(a)pyrene degradation by bacterial consortium. With a better understanding of the degradation process of PAH by bacteria, strategies will be developed for the removal and containment of carcinogenic PAH from contaminated ecosystems and the reduction of health risks associated with exposure to PAH.

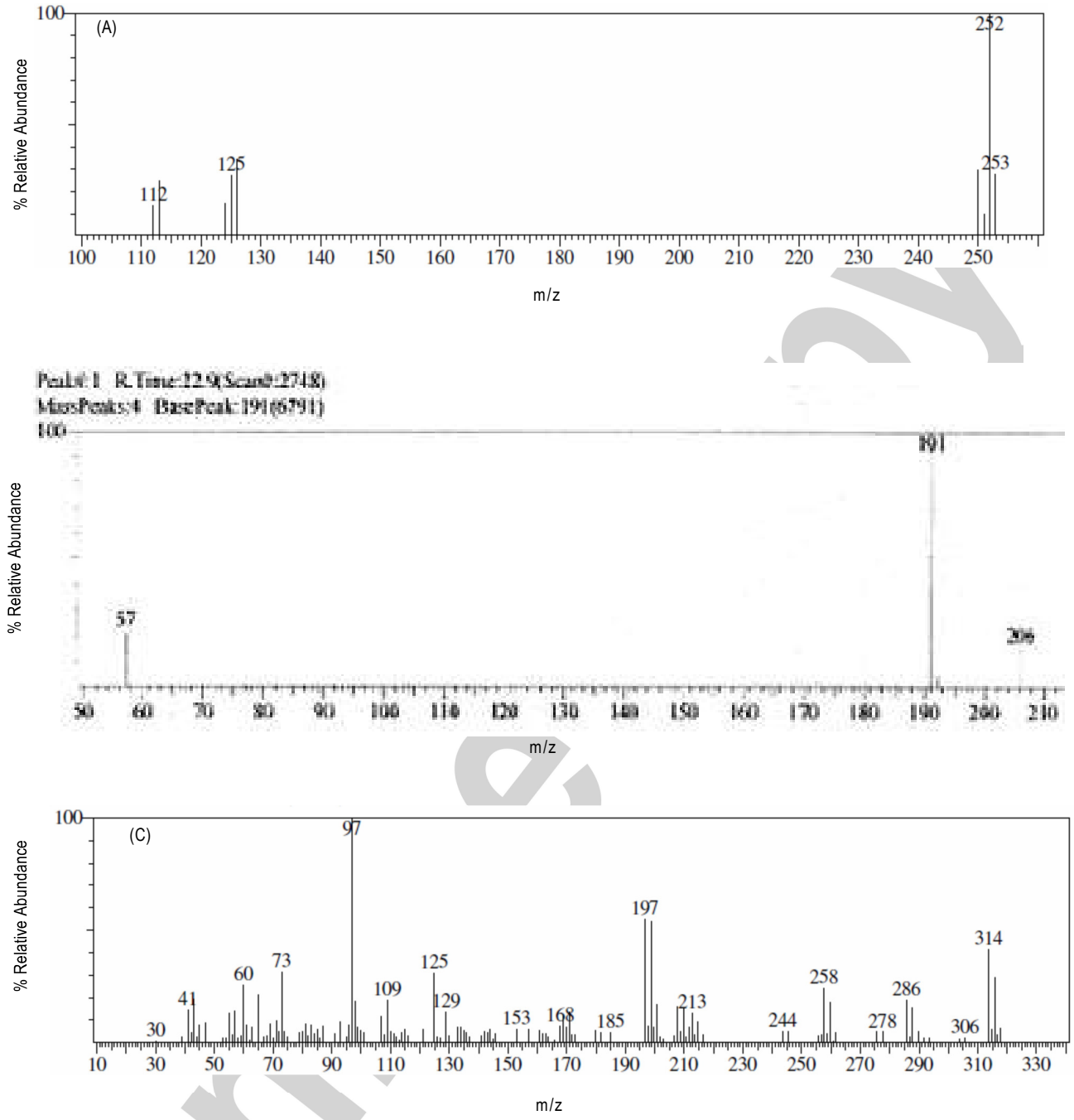


Fig. 2: Mass spectra of the metabolites of benzo[a]pyrene transformed by the bacterial consortium at (a) 0 days, (b) 25 days and (c) 35 days

Table- 1: GC mass spectral analysis of benzo[a]pyrene metabolites during its degradation by bacterial consortium

Metabolite	Molecular ion	Retention time(min)	m/z of fragments ions
cis-4-(7-hydroxypyren-8-yl)-2-oxobut-3enoic acid	314	31.8	306, 286, 278, 258, 244, 213

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