Environment and Ecology 37 (1B) : 398—409, January—March 2019
Website: environmentandecology.com ISSN 0970-0420

Isolation and Biochemical Characterization of Bacteria from Petroleum Hydrocarbon Contaminated Soils from Maharashtra, India

Vaidehi B. Khobragade, Satish D. Kulkarni

Received 1 October 2018; Accepted 3 November 2018; Published on 24 November 2018

Abstract For standardization of the bioremediation process, information regarding the indigenous microbial population from petroleum hydrocarbon contaminated soil is significant. The diversity of indigenous bacteria able to utilize PAHs, from several sites of Maharashtra, was explored by the use of typical selective enrichment (MSM supplemented with PAHs) technique and their identification using morphological and biochemical characterization was carried out. Twelve soil samples were collected from petroleum hydrocarbon contaminated sites with their heterotrophic bacterial count in the range of 3.2×10^5 (SI) to 2.4×10^7 (SH) indicated the ability of the microorganisms to adapt and grow in contaminated soils. After enrichment, 21 bacteria were isolated from the culture broths, of which, 19 isolates were rods, whereas, only 2 isolates were cocci: Fifteen isolates were Gram + ve; whereas, only 6 were Gram –ve in nature. The phenotypic characterization facilitate identification of the isolates upto generic level representing 13 genera viz., *Bacillus. Arthrobacter, Serratia, Microbacterium Staphylococcus. Georgenia, Exiguobacterium, Ochrobactrum, Kocuria, Brachybacterium, Cellulomonas, Pseudomonas* and *Oerskovia.* The screening of these bacteria in liquid and solid MSM media supplemented with PAHs namely anthracene, phenanthrene and pyrene showed varying PAHs utilizing capacities. Out of the 21 isolates, six isolates showed excellent growth, which indicated their potential to degrade and utilize PAHs; and thus may aid in bioremediation of petroleum hydrocarbon contaminated sites.

Keywords Polycyclic aromatic hydrocarbons, Indigenous bacteria, Biochemical characterization, Biodegradation.

Introduction

The unprecedented population increase and industrial development during the 20th century not only increased conventional solid and liquid waste pollutants to critical levels, but also produced a range of previously unknown pollution for which the society was unprepared (Ahamed et al. 2010). Petroleum hydrocarbons, although are not xenobiotics, by their large scale use and accidental release, have occasionally inflicted serious environmental damage (Atlas and Bartha 1998). Economic growth in developing countries have many effects, such as increasing oil exploitation as one energy resource and also increasing

Vaidehi B. Khobragade*, Satish D. Kulkarni
Department of Environmental Science, New Arts, Commerce and Science College, Ahmednagar 414002, Maharashtra, India
e-mail: vaidehi.khobragade@ gmail.com
*Corresponding author

growth of other industries, such as paper, pesticide and petrochemical industries, which produce large amount of Polycyclic Aromatic Hydrocarbons (PAHs) (Abd-Elsalam et al. 2009).

Petroleum is a complex mixture of many thousands of compounds mainly consisting of carbon and hydrogen. These can be divided into 4 major groups: Alkanes, aromatics, resins and asphaltenes. In general, an alkane fraction is the most biodegradable, whereas the polar fraction (i.e. resins and asphaltenes) is resistant to biological degradation. The aromatic compounds, especially the PAHs, are of intermediate biodegradability, but these are of most concern owing to their toxicity and tendency to bioaccumulate (Wrenn and Venson 1996). PAHs are major fractions of petroleum mixtures with highly toxic, mutagenic or carcinogenic effects to human and animal (Doddamani and Ninnekar 2000).

PAHs are pollutants produced via natural and anthropogenic sources, generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities (Gemma et al. 2006). They are a large group of organic compounds with 2 or more fused aromatic rings in linear, angular or cluster arrangements. They have a relatively low solubility in water, but are highly lipophilic (Hafez et al. 2008, Johnsen et al. 2005) and have the potential to biomagnify through the food chain (Kanaly and Harayama 2000). PAHs are considered as priority pollutants by the United States Environmental Protection Agency (USEPA) because of their toxicity; in fact, some PAHs are human carcinogens (Cerniglia 1993).

Once PAHs enter the environment, they are subjected to 5 different processes: Volatilization, leaching, degradation, bioaccumulation and sequestration. Because of structural complexity and stability (Harvey 1991), they are not easily removed by the process like volatilization, photo–oxidation or chemical reaction. On the other hand, the use of conventional remediation approaches, such as dredging and incineration, can be costly and may cause further damage to the environment by dispersing PAHs and making them more bioavailable (Kanaly and Harayama 2000, Kastner et al. 1994, Meyer et al. 1999. Mueller et al. 1994).

Despite some physical processes such as volatilization, leaching, chemical and photo-oxidation are often effective in reducing the environmental level of PAHs (Heitkamp et al. 1988), biodegradation using microorganisms is usually the preferred and major route of PAH removal from contaminated environments because of its cost effectiveness and complete cleanup (Pothuluri and Cerniglia 1994). Interest in the microbial biodegradation of pollutants has increased in recent years. Bioremediation and biotransformation aroused to eliminate a wide range of pollutants and wastes from the environment (Watanbe 2001). By the consent of nature, these are microorganisms, ubiquitously distributed in soil and aquatic environment, which have hydrocarbon degrading capabilities and considered to be the major agents of remediation of contamination sites (Leahy and Colwell 1990, Boonchan et al. 2000, Widada et al. 2002, Zhong et al. 2007, Lin and Cai 2008). Contamination of hydrocarbons, either terrestrial or aquatic, truly acts as selection pressure for these indigenous microorganisms (Dagley 1987, Lawrence and Lynda 1999).

There have been several successful investigations on indigenous microorganisms with degrading capability in sediments contaminated with pure, low molecular weight PAHs. These PAHs are transformed into other aromatic substrates and the results suggest that biodegradation plays an important role in degradation of mixtures in the environment (Grifoll et al. 1995 and Ramirez et al. 1996). Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon and energy for metabolic activities and these microorganisms are omnipresent and widely distributed in nature. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants (Adeline et al. 2009). Previous studies have resulted in the successful isolation of PAH-degrading bacteria from contaminated environmental samples (Bastiaens et al. 2000, Boldrin et al. 1993, Dagher et al. 1996, Uyttebroek et al. 2007), including aquatic sediments (Churchill et al. 1999, Daane et al. 2001, Geiselbrecht et al. 1998, Geiselbrecht et al. 1996, Hedlund et al. 1999, Hedlund and Staley 2006). For effective bioremediation, information about the indigenous microbial community of the contaminated site is essential (Vidali 2001).

In this view, the present study was focused to explore and isolate indigenous bacterial population from petroleum hydrocarbon sites from Maharashtra, India; to identify them on the basis of their morphological and biochemical characterization and to test their ability to utilize PAHs as their sole carbon and energy source.

Materials and Methods

Soil microbial analyses

Indigenous microorganisms were extracted from the contaminated soil sample by mixing 1 g of soil with 10 ml of sterile saline solution in 50 ml Erlenmeyer flask for 2 h on a shaker at 120 rpm as per Kastner et al.(1998). The soil particles were allowed to sediment for 30 minutes, the supernatant was diluted 10^{-1} to 10^{-5} dilutions and the dilutions were plated on sterile nutrient agar plates. The inoculated plates were incubated at 37^0C for 24 h. The colony forming units per g (cfu/g) of soil sample were determined using serial dilution agar plate method (Dubey and Maheshwari 2012).

Enrichment of PAH degrading soil bacteria

The cultivable bacterial strains from soil samples contaminated with PAHs were isolated by enrichment culture technique. Mineral salts medium (MSM) was used for the enrichment of the PAHs degrading bacterial strains from the soil samples. Five gram of each soil sample was inoculated in 100 ml of MSM in separate 250 ml Erlenmeyer flask. The MSM had the following composition: K_2HPO_4 (1.8 g/l ; NH_4Cl (4g/l); $MgSO_4$. $7H_2O$) (0.2 g/l); NaCl (0.1 g/L); Na_2SO_4. $7H_2O$ (0.01 g/L) ; Agar for MSM plates (20 g/L). The medium without hydrocarbons was sterilized by autoclaving at 121^0C for 15 minutes. The MSM was supplemented with 0.01% of anthracene, phenanthrene or pyrene to serve as the sole source of carbon and energy as per Grifoll et al. (1992). The hydrocarbons were dissolved in acetone and added to sterilized flasks forming a thin film of either of the PAHs on the flask bottom. After evaporation of acetone,100 ml of MSM was added. The supplemented MSM was inoculated with 5 g of soil sample collected from petroleum hydrocarbon contaminated sites. The flasks were incubated aerobically at 37^0C in a rotary shaker incubator at 200 rpm for 7 days. Following visible growth, 5 ml of the culture from each flask was transferred to fresh MSM containing 0.01% of PAHs and again incubated as stated above. This step was repeated thrice so as to get consistent growth of the cultures.

Isolation of bacteria from enriched culture

After the enrichment cycles, 1 ml of the culture was serially diluted from 10^{-1} to 10^{-6} in 0.9% NaCl and aliquots (100 µl) from 10^{-4} to 10^{-6} were plated on nutrient agar plates for isolation of bacterial strains. The plates were incubated at 37^0C for 24–48h. A single colony showing different morphological characters from each plate for each soil sample was picked and each was streaked on separate nutrient agar plate. This step was repeated several times so as to get pure culture of the isolates.

Storage, maintenance and growth of PAH degrading bacterial strains

Each of the purified bacterial isolate was transferred to nutrient agar slants and given accession code indicating the site from where they have been isolated viz., AHSAVBK1 to AHSDVBK8 from Ahmednagar, AUSEVBK9 to AUSHVBK15 from Aurangabad,NASIVBK16 to NASJVBK18 from Nasik, PUSKVBK19 from Pune and NGSLVBK20 and NGSLVBK21 from Nagpur. Pure cultures were sub-cultured once in 15 days and the purity of the bacterial strains was checked periodically by streaking on nutrient agar plates and incubating at 37^0C for 24-48 h.

Biochemical characterization of the isolated bacteria

For morphology studies and biochemical characterization, the bacterial strains were grown either in nutrient broth (NB)tests or nutrient agar plates. Morphological, cultural and biochemical test like Grams staining, motility test, biochemical tests namely carbohydrate fermentation, starch hydrolysis, gelatine hydrolysis, casein hydrolysis, H_2S production, IMViC test, urease test, nitrate reduction test, cata-

Table 1. Initial soil microbiological analysis.

Soil sample	A	B	C	D	E	F	G	H	I	J	K	L
cfu/g of soil	8.8×10^5	7.6×10^5	8.2×10^5	1.2×10^6	6.2×10^5	2.8×10^6	1.4×10^6	3.2×10^5	$2{,}4\times10^7$	7.8×10^6	9.6×10^5	3.6×10^6

lase activity and oxidase test were performed for the characterization of the isolated bacterial strains using the standard protocols from Dubey and Maheshwari (2012). Taxonomic identification upto genus level was done using Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

Screening and selection of PAH degrading bacteria

Liquid medium : In order to screen for the PAH degrading bacteria, purified isolates were screened by subjecting the selected isolates to grow in MSM broth supplemented with 0.01% of either anthracene, phenanthrene or pyrene (Supaka et al. 2001) and incubated in a rotary shaker incubator at 30+/–2^{0}C for 32 days at 150 rpm and compared with control MSM broth without bacteria. Degradation capacity of bacteria was monitored by measuring the optical density of the MSM broth cultures at 600 nm using a double beam visible spectrophotometer at regular time intervals.

Solid medium : Further, the bacteria were screened on solid MSM agar plates supplemented with 0.01% of anthracene, phenanthrene or pyrene as the sole carbon and energy source by spray–plate technique (Survery et al. 2004). Initialty, the plates were kept uncovered for one minute in a laminar flow cabinet for evaporation of the solvent. Culture plates were wrapped with aluminium foil, placed inside plastic bags so as to retain the moisture and prevent the plates from drying and incubated in the dark at 30+/–2^{0}C for 21 days.

Results and Discussion

Soil microbial analyses

The fertility of soil depends not only on its chemical composition, but also on the qualitative and quantitative nature of microorganisms inhabiting it (Subba Rao 2004). According to Van Hamme et al. (2003), a large number of microorganisms belonging to a variety of genera are able to utilize hydrocarbons as the sole source of carbon and energy and these microorganisms are widely distributed in nature. Therefore initial microbial analysis of the soil samples was carried out, to estimate the bacterial load in the contaminated soil samples prior to the screening of hydrocarbon utilizing bacteria. The indigenous bacterial population (cfu/g) of soil samples collected from various petroleum hydrocarbon contaminated sites is given in Table 1.

In the present study, substantial number of bacteria were found to be present in the soil samples. The total heterotrophic bacterial count was in the range of 3.2×10^5 (SI) to 2.4×10^7 (SH) cfu/g of soil sample, which indicated the ability of the microorganisms to adapt and grow in soils contaminated with hydrocarbon. From the present study, it is apparent that hydrocarbon contaminated soils harbor diverse hydrocarbon utilizing–degrading bacteria. The bacteria lpopulation in the contaminated soil samples indicate that the indigenous soil bacteria were carrying out their metabolic activity by utilizing the hydrocarbons from the soil as their carbon and energy source. When natural environments are contaminated with pollutants, the indigenous microbial communities are likely to contain microbial populations of different taxonomic characteristics, which are capable of degrading the contaminating chemicals (Bartha and Atlas 1977).

While studying the baseline properties of soil Chikere et al. (2009) reported the total heterotrophic bacterial count to be $1.8^3 \times 10^6$ cfu/g of soil. Bahuguna et al. (2011) analyzed the bacterial load in automobile contaminated soil which ranged from 5×10^2 to 2.1×10^5 cfu/g of soil from various soil samples. Their

Table 2. Morphological characteristics of bacteria isolated from PAH Contaminated soils. NDP : Non Diffusible Pigment.

Bacterial isolate code	Color	Size (mm)	Shape	Elevation	Margin	Consistency texture	Opacity
AHSAVBK 1	White (dull)	2-3	Circular	Convex	Entire	Dry rough	Translucent
AHSAVBK 2	White	1-2	Circular	Slightly convex	Entire	Creamy sticky	Translucent
AHSBVBK 3	Red NDP	2-3	Circular	Convex	Entire	Smooth clossy	Translucent
AHSBVBK 4	Yellow NDP	1-2	Circular	Convex	Entire	Mucoid	Translucent
AHSCVBK 5	Creamy white	3-4	Irregular	Flat	Undulate	Dry rough	Opaque
AHSCVBK 6	White	1-2	Circular	Convex	Entire	Smooth glistening	Translucent
AHSDVBK 7	Pinkish white	1-2	Circular	Convex	Entire	Smooth	Opaque
AHSDVBK 8	Orange NDP	3-4	Circular	Slightly convex	Entire	Dry	Opaque
AUSEVBK 9	Off-white	1-2	Circular	Convex	Entire	Smooth	Opaque
AUSEVBK 10	Off-white	1-2	Circular	Convex	Entire	Smooth mucoid	Translucent
AUSFVBK 11	Yellow	2-3	Circular	Convex	Entire	Smooth shiny mucoid	Translucent
AUSGVBK 12	Creamy white	2-3	Circular	Raised	Undulate	Dry rough	Opaque
AUSGVBK 13	Pale yellow NDP	1-2	Circular	Convex	Entire	Smooth mucoid	Translucent
AUSHVBK 14	Orange	3-4	Circular	Slightly convex	Entire	Dry	Opaque
AUSHVBK 15	White	1-2	Circular	Convex	Entire	Smooth	Opaque
NASIVBK 16	Creamy white	2-3	Circular	Raised	Undulate	Dry rough	Opaque
NASIVBK 17	Pale brown	1-2	Circular	Convex	Entire	Dry rough	Translucent
NASJVBK 18	Yellowish	1-2	Circular	Convex	Entire	Smooth glistening	Translucent
PUSKVBK 19	Off-white	1-2	Circular	Conves	Entire	Smooth	Opaque
NGSLVBK 20	Creamy white	3-4	Irregular	Raised	Entire	Smooth moist	Opaque
NGSLVBK 21	Pale brown	1-2	Circular	Convex	Entire	Dry rough	Translucent

study demonstrated inverse relationship between the PAH content and the bacterial count in contaminated soils stating that higher the PAH content lower was the bacterial count and *vice versa.* Soil microbiology of PAH contaminated soil from Iran was studied by Arbabi et al. (2004) and they found reasonable populations of bacteria in the range of $6.5 \times 10^5 - 7 \times 10^6$ cfu/g in the soil samples. Sediment samples from various sampling sites from Japan was assessed by Doan et al. (2017) for the total bacterial count, which ranged from 6.5×10^4 to 5.7×10^5 cfu/g of the sample.

Enrichment of PAH degrading soil bacterial

To boost the hydrocarbon utilizing bacterial growth, enrichment culture technique was employed and the cultures were harvested at the end of the incubation period. Positive growth was determined as an increase in the turbidity of the flasks containing PAHs as the sole carbon and energy source. The turbidity was determined in terms of optical density which showed varying ability of the indigenous bacterial community from each sampling site to utilize the PAHs. Also the PAH utilizing ability varied depending upon the PAH (anthracene, phenanthrene, pyrene) used for enrichment culture (Data not shown here). It was observed that the bacterial density after enrichment was found to be maximum in culture supplemented with phenanthrene as compared to that of anthracene and pyrene.

Serial dilution from the enrichment cultures were plated on nutrient agar plates. Representative colonies showing distinct morphology from the final enrichment were repeatedly sub-cultured to obtain pure cultures. Twentyone bacterial isolates capable of utilizing anthracene, phenanthrene and /or pyrene, were isolated from hydrocarbon contaminated soils. Depending upon the soil sampling site and the sample, each bacterium was given a code. The morphological (colony) characteristics namely color, size, shape, elevation, consistency and opacity, are given in Table 2. The cell morphology, Gram nature, motility, spore forming ability and oxygen utilization by bacteria are as shown in Table 3. Out of the 21 bacterial isolates, 19 were rods and only 2 were cocci; 15 bacteria were Gram +ve, whereas, 6 were Gram –ve in nature. The spore forming ability was expressed by 5 bacteria,

Table 3. Microscopic feature of bacteria isolated from PAH Contaminated soils. + Gram positive, – Gram negative.

Bacterial isolate code	Cell morphology	Gram nature (+/-)	Endospore (Y/N)	Motility (V/N)	Aerobic
AHSAVBK1	Short rods in chains	+	Y	Y	Aerobic
AHSAVBK2	Rods	+	N	N	Obligatory aerobic
AHSBVBK3	Short rods in pairs	-	N	Y	Facultative anaeobe
AHSBVBK4	Coccobacilli short rods	+	N	N	Aerobic
AHSCVBK5	Rods in chain	+	Y	Y	Aerobic or facultative anaerobe
AHSCVBK6	Cocei in clusters	+	N	N	Facultative anaerobe
AHSDVBK7	Coccobacilli short rods	+	N	Y	Aerobic or facultative anaerobe
AHSDVBK8	Short rods	+	N	Y	Facultative anaerobe
AUSEVBK9	Slightly curved short rods	-	N	Y	Strictly aerobic
AUSEVBK10	Short rods	-	N	N	Aerobic
AUSFVBK11	Cocci in tetrads	+	N	N	Aerobic
AUSGVBK12	Rods	+	Y	N	Aerobic
AUSGVBK13	Rods	+	N	N	Aerobic or facultative anaerobe
AUSHVBK14	Short rods	+	N	Y	Facultative anaerobe
AUSHVBK15	Rods	+	N	Y	Acrobic
NASIVBK16	Rods	+	Y	N	Aerobic
NASIVBK17	Rods	-	N	Y	Aerobic
NASJVBK18	Short rods	+	N	Y	Aerobic
PUSKVBK19	Slightly curved short rods	-	N	Y	Strictly aerobic
NGSLVBK20	Rods	+	Y	Y	Facultative anaerobe
NGSLVBK21	Rods	-	N	Y	Aerobic

whereas, 16 bacteria were non-spore forming. As regards to the motility test, 13 bacteria were motile and 8 bacteria were non-motile. The oxygen utilization was variable; some of the bacteria were aerobic where as others were found to be facultative anaerobes.

Many researchers (Riccardi et al. 2005. Al-Thani et al. 2009, Vijayalakshmi et al. 2010, Naveenkumar et al. 2010, Khan and Rizvi 2011, Kafilzadeh et al. 2011. Joythi et al. 2012, Erdogan et al. 2012) have isolated and characterized bacteria from soils contaminated with petroleum hydrocarbons and identified them using morphological characteristics as Gram +ve/-ve rods and cocci, as belonging to various genera depending upon their biochemical characteristics.

Biochemical characterization of the isolated bacteria

The biochemical characteristics of the isolated bacteria are summarized in Table 4. Of the 21 bacteria isolates, 10 bacteria were Oxidase +ve, all of the

Table 4. Carbohydrate fermentation of bacteria isolated from PAH contaminated soils.

Bacterial isolate code	Glucose	Dextrose	Sucrose	Maltose	Lactose
AHSAVBK1	+	+	+W	+W	–
AHSAVBK2	–	–	–	–	–
AHSBVBK3	+	+	+	+	–
AHSBVBK4	+W	+	+W	+	–
AHSCVBK5	+	+	+	+	–
AHSCVBK6	+	+	+	+	–
AHSDVBK7	–	–	–	-	–
AHSDVBK8	+W	+W	+W	–	–
AUSEVBK9	–	–	–	–	–
AUSEVBK10	–	–	–	–	–
AUSFVBK11	–	–	–	–	+
AUSGVBK12	+	+	+	+	–
AUSGVBK13	+	+	+	+	+W
AUSHVBK14	+W	+W	+W	–	–
AUSHVBK15	–	+	+	–	–
NASIVBK16	+	+	+	+	–
NASIVBK17	+	+	–	–	–
NASJVBK18	+	+	+	+	–
PUSKVBK19	–	–	–	–	–
NGSLVBK20	+	+	+	–	+
NGSLVBK21	+	+	–	–	–

Table 5. Biochemical characterization of bacteria isolated from PAH Contaminated soils. A: Oxidase; B: Catalase; C: Indole; D: Methyl Red; E: Voges Proskau; F: Citrate Utilization; G: Starch Hydrolysis, H: Casein Hydrolysis; I:Gelatin Hydrolysis; J: H_2S Production, K: Urease Production; L: Nitrate Reduction; *after 11 days ; W: Weakly.

Bacterial isolate code	A	B	C	D	E	F	G	H	I	J	K	L
AHSAVBK1	–	+	–	–	+	+	+	–	+	–	–	–
AHSAVBK2	–	+	–	+	–	–	–	–	+*	–	–	–
AHSBVBK3	–	+	–	–	+	+	–	+	+	–	+	+
AHSBVBK4	–	+	–	+	+	–	–	+	+	–	-	–
AHSCVBK5	+	+	–	+	+	+	+	+	+	–	–	+
AHSCVBK6	–	+	+	+	+	–	–	–	–	–	–	+
AHSDVBK7	+	+	–	+	–	–	–	–	–	–	–	+
AHSDVBK8	–	+	–	+	–	–	–	–	–	+	+	–
AUSEVBK9	+	+	–	–	–	+	–	–	–	–	+	+
AUSEVBK10	+	+	–	–	–	–	–	–	–	–	+W	+
AUSFVBK11	–	+	–	–	–	+	–	–	–	–	+	+
AUSGVBK12	+	+	–	–	+	+	–	+	–	+	–	–
AUSGVBK13	–	+	+	+	–	–	+	+W	–	+	+	+
AUSHVBK14	–	+	–	+	–	–	–	–	–	+	+	–
AUSHVBK15	–	+	–	–	–	–	–	–	–	–	–	+
NASIVBK16	+	+	–	–	+	+	–	+	–	+	–	–
NASIVBK17	+	+	–	–	–	+	–	+	–	–	+	+
NASJVBK18	–	+	–	–	–	+	+	–	+	–	–	+
PUSKVBK19	+	+	–	–	–	+	–	–	–	–	+	+
NGSLVBK20	+	+	–	–	–	–	–	+	+	+	–	–
NGSLVBK21	+	+	–	–	–	+	–	+	–	–	+	+

isolates were Catalase+ve; 2 were Indole +ve, 8 were +ve for Methyl Red test, 7 were +ve for Voges Proskauer test, 11 were Citrate +ve, 4 were +ve for Starch hydrolysis, 9 showed +ve result for Casein hydrolysis, 7 were +ve for H_2S production, 10 were +ve for Urease production and 13 were +ve for Nitrate reduction. Whereas, 11 bacteria were Oxidase –ve, 19 were Indole negative, 13 were–ve for Methyl Red test, 14 were –ve for Voges Proskauer test, 10 were –ve for Citrate utilization test, 17 were –ve for Starch hydrolysis, 12 were –ve for Casein hydrolysis, 14 were–ve for H_2S production, 11 were –ve for Urease production and 8 were –ve for Nitrate reduction.

The carbohydrate fermentation ability of the bacterial isolates is shown in Table 5. It was observed that 14 bacteria were glucose fermenting, whereas 7 of them were non-glucose fermenting; 15 were dextrose fermenting, whereas 6 were non-dextrose fermenting; 13 were sucrose fermenting, whereas 8 were non-sucrose fermenting; 8 were maltose fermenting, whereas 13 were non-maltose fermenting and only 3 bacteria were lactose fermenting, whereas 19 of them were non-lactosc fermenting.

From the phenotypic and biochemical characterization; and comparison with the Bergey's manual, the isolates were identifiable, upto the generic level. It was observed that the bacteria belonged to 11 families namely Bacillaceae, Brucellaceae, Pseudomonadaceae, Micrococcaceae, Enterobacteriaceae,

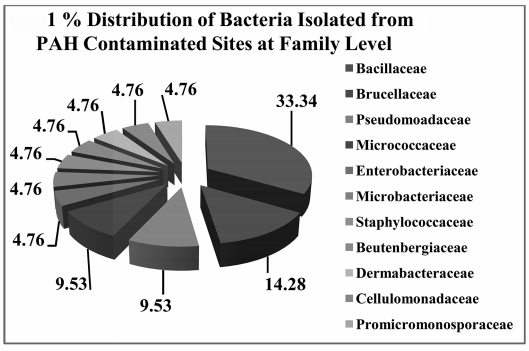

Fig. 1. Percent distribution of bacteria isolated from PAH contaminated sites at family level.

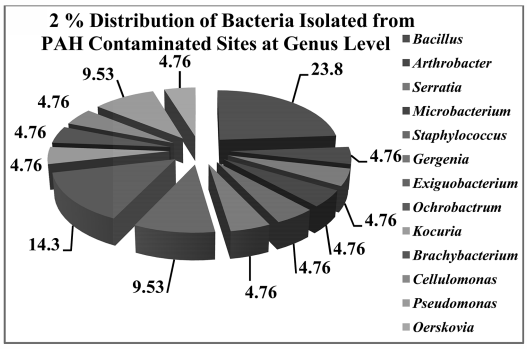

Fig. 2. Percent distribution of bacteria isolated from PAH contaminated sites at genus level.

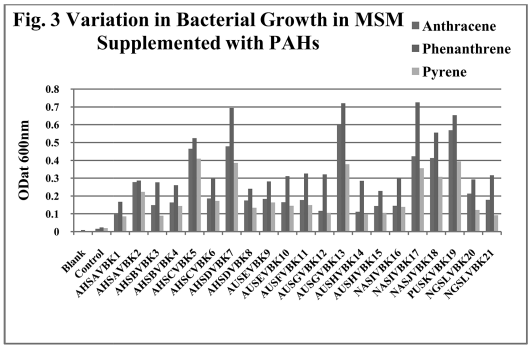

Fig. 3. Variation in bacterial growth in MSM supplemented with PAHs.

Microbacteriaceae, Staphylococcaceae, Beutenbergiaceae Dermabacteraccae, Cellulomonadaceae and Promicromonosporaceae.Familywise distribution of bacteria is shown in Fig. 1. Percentwise distribution of bacteria upto genus level is as shown in Fig. 2. It was observed that the isolated bacteria belonged to 13 genera viz., *Bacillus, Arthrobacter, Serratia, Microbacterium, Staphylococcus, Georgenia, Exiguobacterium, Ochrobactrum, Kocuria, Brachybacterium, Cellulomonas, Pseudomonas, Oerskovia*. In the present study, it was noticed that maximum number i.e. 5 were found to belong to *Bacillus* sp.and 3 to *Ochrobactrum* sp.The results bring to light that the indigenous bacteria coming from diverse families belong to different genera and are able to utilize the PAHs for their growth and metabolism.

Earlier works involved exploration of soils/sediments contaminated with petroleum hydrocarbons such petrol/diesel/engine oil/crude oil containing various PAHs for the indigenous bacterial population and their identification. Investigators isolated and identified bacterial populations belonging to various taxa as follows: Vargas et al. (1996) *Pseudomonas* sp., Riccardi et al. (2005) *Alcaligens* sp., *Bacillus megaterium, Bacillus firmus* and *Paenibacillys lautus.* Hilyard et al. (2008) sediments from several sites in the Elizabeth river Actinobacteria, Alphaproteobacteria. Betaproteobacteria, Gammaproteobacteria, Actinomycetes, Planctomycetes and the Cytophaga-Flexibacter–Bacteroides (CFB) group; Al-Thani et al. (2009) *Pseudomonas* and *Achromobacter* genus; Abd–Elsalam et al. (2009) *Escherichia coli,* soil bacterium, *Alcaligens* sp. and *Thiobacter subterrances;* Naveenkumar et al. (2010) *Micrococcus* sp., *Staphylococcus* sp. and *Pseudomonas* sp.; Ahamed et al. (2010), *Kocuria* sp.; Gomare (2011) genus *Pseudomonas* and *Bacillus*; Khan and Rizvi (2011) *Bacillus* sp.; Kafilzadeh et al. (2011) *Bacillus*, *Corynebacterium, Staphylococcus, Streptococcus,*

Table 6. Taxonomic distribution of bacteria isolated from contaminated sites.

Bacterial isolate code	Family	Genus
AHSAVBK1	Bacillaccae	*Bacillus*
AHSAVBK2	Micrococcaceae	*Arthrobacter*
AHSBVBK3	Enterobacteriaccae	*Serratia*
AHSBVBK4	Microbacteriaceae	*Microbacterium*
AHSCVBK5	Bacillaceae	*Bacillus*
AHSCVBK6	Staphylococcaceae	*Staphylococcus*
AHSDVBK7	Beutenbergiaceae	*Georgenia*
AHSDVBK8	Bacillaceae	*Exiguobacterium*
AUSEVBK9	Brucellaceae	*Ochrobactrum*
AUSEVBK10	Brucellaceae	*Ochrobactrum*
AUSFVBK11	Micrococcaceae	*Kocuria*
AUSGVBK12	Bacillaceae	*Bacillus*
AUSGVBK13	Dermabacteraceae	*Brachybacterium*
AUSHVBK14	Bacillaceae	*Exiguobacterium*
AUSHVBK15	Cellulomonadaceae	*Cellulomonas*
NASIVBK16	Bacillacea	*Bacillus*
NASIVBK17	Pseudomonadaceae	*Pseudomonas*
NASJVBK18	Promicromonosporaceae	*Oerskovia*
PUSKVBK19	Brucellaceae	*Ochrobactrum*
NGSLVBK20	Bacillaceae	*Bacillus*
NGSLVBK21	Pseudomonadaceae	*Pseudomonas*

Table 7. Growth of bacteria is MSM supplemented with PAHs.

Bacterial isolate code	Anthracene	Phenanthrene	Pyrene
Blank	0.001	0.009	0.003
Control	0.016	0.023	0.02
AHSAVBK1	0.096	0.167	0.086
AHSAVBK2	0.278	0.286	0.223
AHSBVBK3	0.149	0.277	0.089
AHSBVBK4	0.164	0.261	0.144
AHSCVBK5	0.465	0.525	0.409
AHSCVBK6	0.186	0.299	0.173
AHSDVBK7	0.479	0.695	0.386
AHSDVBK8	0.175	0.241	0.134
AUSEVBK9	0.183	0.281	0.164
AUSEVBK10	0.165	0.311	0.145
AUSFVBK11	0.177	0.326	0.149
AUSGVBK12	0.116	0.321	0.105
AUSGVBK13	0.603	0.721	0.378
AUSHVBK14	0.112	0.285	0.098
AUSHVBK15	0.144	0.228	0.106
NASIVBK16	0.145	0.3	0.139
NASIVBK17	0.423	0.725	0.356
NASJVBK18	0.413	0.556	0.308
PUSKVBK19	0.569	0.654	0.394
NGSLVBK20	0.213	0.293	0.122
NGSLVBK21	0.178	0.316	0.092

Table 8. Growth of bacteria on MSM agar plate supplemented with PAHs. (+++excellent growth, ++moderate growth, + weak growth. –No-growth).

Bacterial isolate gode	Anthracene	Phenanthrene	Pyrene
AHSAVBK1	+++	+++	++
AHSAVBK2	-	++	-
AHSBVBK3	++	+++	-
AHSBVBK4	++	+++	-
AHSCVBK5	+++	+++	+++
AHSCVBK6	+	+	+
AHSDVBK7	+++	+++	+++
AHSDVBK8	-	+	-
AUSEVBK9	++	+++	+
AUSEVBK10	++	++	-
AUSFVBK11	+	++	+
AUSGVBK12	+++	+++	+
AUSGVBK13	+++	+++	+++
AUSHVBK14	-	+	–
AUSHVBK15	++	++	+
NASIVBK16	+++	+++	+
NASIVBK17	+++	+++	+++
NASJVBK18	+++	+++	+++
PUSKVBK19	+++	+++	+++
NGSLVBK20	++	+++	-
NGSLVBK21	++	++	-

Klebsiella, Escherichia, Acinetobacter, Alcaligenes, Shigella and *Enterobacter* genera; Maiti et al. (2012) *Bacillus thuringiensis;* Erdogan et al. (2012) *Pseudomonas* sp., *Paucimonas* sp., *Stenotrophomonas* sp., *Escherichia* sp., *Enterobacter* sp., *Citrobacter* sp., *Acinetobacter* sp., *Aeromonas* sp., *Sphingobacterium* sp., *Klebsiella* sp., and *Pseudomonas* sp., Jyothi et al. (2012) *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Lactobacillus* sp., *Neisseria* sp. and *Corynebacterium* sp.; Neeraja et al. (2013) *Pseudomonas* sp.; Moghadam et al. (2013) *Roseovarius* sp.; Teli et al. (2013)*Micrococcus* sp. and *Pseudomonas* sp.

Screening for PAH degrading bacteria in liquid medium

Increase in the optical density of the MSM shows that the bacteria are able to utilise the PAHs as the sole carbon and energy source for their growth and metabolism. Table 6 and Fig. 3 show the variation in PAH utilizing abilities of the isolated bacteria. From the results it is clear that the ability of utilization of hydrocarbon differs from bacterium to bacterium and also on the PAHs being provided which is apt from the variation observed in their growth pattern. High number of certain hydrocarbon degrading microorganisms from an environment implies that those organisms are active degraders of that environment (Kebria D 2009). Joythi et al. (2012) isolated 7 bacteria from petroleum contaminated soil and studied their varying hydrocarbon utilizing ability by turbidometry and concluded that the isolates can be good petrol and diesel degraders.

Screening for PAH degrading bacteria on solid medium

The ability of bacterial isolates to grow on solid MSM supplemented with anthracene, phenanthrene or pyrene was examined. PAH utilization was indicated by zones of clearance surrounding the individual colonies. Bacteria showed varying PAH utilizing capacity with respect to authracene, phenanthrene and pyrene which were recorded as '+++' for excellent growth, '++' for moderate growth, '+' for weak growth, '–'' for no growth, which are summarised in Tables 7 and 8. Of the 21 isolates, 13 bacteria showed excellent growth on phenathrene, 09 showed excellent on an-

thracene whereas only 06 showed excellent growth on pyrene.There were 06 bacterial isolates which showed excellent growth on all the three PAHs namely anthracene, phenanthrene and pyrene.

It was observed that the isolates AHSCVBK5, AHSDVBK7, AUSGVBK13, NASIVBK17, NASJVBK18 and PUSKVBK19 were the bacteria which showed maximum growth in liquid as well as solid media which belonged to the genus namely, *Bacillus, Georgenia, Brachybacterium, Pseudomonas. Oerskovia* and *Ochrobactrum* respectively.

As observed in previous works, different genera of indigenous bacterial populations from PAH contaminated sites, varied from place to place. Therefore, the present study has future implications to harness indigenous bacteria from the study area and employ them for *in situ* bioremediation studies.

Conclusion

The results obtained from this work indicate that the soil samples collected from petroleum hydrocarbons contaminated sites from Maharashtra, India; harbor considerable populations of indigenous bacteria capable of PAHs degradation; and it is possible to use these isolated bacteria for degradation of PAHs from contaminated soils. For effective degradation and to facilitate bioremediation of petroleum hydrocarbon contaminated sites, further studies have to be made to elucidate the optimum conditions (viz., pH, temperature, concentration of PAHs, presence of surfactants) so as to bring about maximum degradation of PAHs.

References

Abd-Elsalam Hassan E, Elsayed E Hafez, Azhar A Hussain, Amany G Ali, Amr A El-Banafy (2009) Isolation and identification of three-rings polyaromatic hydrocarbons (Anthracene and phenanthrene) degrading bacteria. American-Eur J Agric and Environm Sci 5 (1): 31—38.

Adeline SY Ting, Carol HC Tan, Aw CS (2009) Hydrocarbon-degradation by isolate *Pseudomonas lundensis* UTAR FPE2. Malaysian J Microbiol 5 (2) : 104—108.

Ahamed Forkan, Hasibullah, Jannatul Ferdouse, Nural Anwar M (2010) Microbial degradation of petroleum hydrocarbon Bangladesh. J Microbiol 27 (1) : 10—13.

Ahmed Rifat Zubair, Nuzhat Ahmed, Geoffrey Michael Gadd (2010) Isolation of two Kocuria species capable of growing on various polycyclic aromatic hydrocarbons. Afr J Biotechnol 9 (24) : 3611—3617.

Al-Thani Roda F, Desouky AM Abd-El-Haleem, Mona Al-Shammri (2009) Isolation and characterization of polyaromatic hydrocarbons-degrading bacteria from different Qatari soils. Afr J Microbiol Res 3 (11) : 761—766.

Arbabi M, Nasseri S, Mesdaghinia AR, Rezaie S, Naddafi K, Omrani GH, Yunesian M (2004) Survey on physical, chemical and microbiological characteristics of PAH- contaminated soils in Iran. Iranian J Environ Hlth Sci Engg 1 (1) : 26—33.

Atlas RM, Bartha R (1998) Microbial ecology: Fundamentals and Applications. 4th edn. Benjamin/Cummings Publishing Company, Inc. California, USA, pp 511—530.

Bahuguna A, Lily MK, Munjal A, Singh RN, Dangwal K (2011) A study on the physico-chemical analysis of automobile contaminated soil of Uttarakhand, India. Int J Environm Sci 2 (2) : 380—388.

Bartha R, Atlas RM (1977) The microbiology of aquatic oil spills. Adv Appl Microb 22 : 225—266.

Bastiaens L, Springael D, Wattiau P, Harms H, de Wachter R, Verachtert H, Diels L (2000) Isolation of adherent polycyclic aromatic hydrocarbon (PAU)-degrading bacteria using PAH-sorbing carriers. Appl Environ Microbial 66 : 1834—1843.

Boldrin B, Tiehm A, Fritzsche C (1993) Degradation of phenan threne, fluorene, fluoranthene and pyrene by a *Mycobacterium* sp. Appl Environ Microbiol 59 : 1927—1930.

Boonchan S, Britz ML, Stanley GA (2000) Degradation and mineralization of high molecular weight polycyclic aromatic hydrocarbons by defined fungal-bacterial co-cultures. Appl Environ Microbiol 66 : 1007—1019.

Cerniglia CE (1993) Biodegradation of polycyclic aromatic hydrocarbons. Curr Opin Biotechnol 4 : 331—338.

Chikere CM, Okpokwasili GC, Chikere BO (2009) Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation. Afr J Biotechnol 8 (11) : 2535—2540.

Churchill SA, Harper JP, Churchill PF (1999) Isolation and characterization of a *Mycobacterium species* capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. Appl Environ Microbiol 65 : 549—552.

Daane LL, Harjono I, Zylstra GJ, Haggblom MM (2001) Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. Appl Environ Microbiol 67 : 2683—2691.

Dagher F, Deziel E, Lirette P, Paquette G, Bisaillon J, Villemur R (1996) Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soil. Cap J Microbiol 43 : 368—377.

Dagley S (1987) Lessons from biodegradation. Ann Rev Microbiol 41 : 1—24.

Doan Cong Dang Phi, Ayako Sano, Hisanori Tamaki, Hoang Nguyen Duc Pham, Xo Hoa Duong, Yoshie Terashima (2017) Identification and biodegradation characteristics of oil-degrading bacteria from subtropical Iriomote Island, Japan and tropical Con Dao Island, Vietnam. TROPICS 25 (4) : 147—159.

Doddamani HP, Ninnekar HZ (2000) Biodegradation of phenanthrene by a *Bacillus* species. Curr Microbiol 41 : 11—14.

Dubey RC, Maheshwari DK (2012) Practical Microbiology. 3rd Revised ed. S Chand & Company Ltd, New Delhi.

Erdogan Esin Eraydin, Fikrettin Sahin, Ayten Karaca (2012) Determination of petroleum-degrading bacteria isolated from crude oil-contaminated soil in Turkey. Afr J Biotechnol

11 (21) : 4853—4859.

Geiselbrecht AD, Hedlund BP, Tichi MA, Staley JT (1998) Isolation of marine polycyclic aromatic hydrocarbon (PAH) –degrading *Cycloclasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of Puget Sound *Cycloclasticus* strains. Appl Environ Microbiol 64 : 4703—4710.

Geiselbrecht AD, Herwig RP, Deming JW, Staley JT (1996) Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon degrading marine bacteria from Puget Sound sediments. Appl Environ Microbiol 62 : 3344—3349.

Gemma E, Tomas C, Maria T, Gumersindo F, Juan M (2006) Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. Chemosphere 64 : 408—414.

Gomare Lahane (2011) Degradation of polyaromatic hydrocarbons by isolated cultures from contaminated soils at petrol pump stations. Int J Recent Trends in Sci and Technol 1 (1): 9—13.

Grifoll M, Casellas M, Bayona JM, Solanas AM (1992) Isolation and characterization of a fluorene degrading bacterium: Identification of ring oxidation and ring fission products. Appl Environ Microbiol 58 : 2910.

Grifoll M, Selinov S, Gatlin CH, Chapman P (1995) Actions of a versatle fluorene-degrading bacterial isolate on polycyclic aromatic compounds. Appl Environ Microbiol 61 (10) : 3711—3723.

Hafez EE, Rashad M, Abd-Elsalam HE, EL-Hanafy AA (2008) The polyaromatic hydrocarbons as a serious environmental pollutants and the role of bioremediation to overcome this problem. In: Basu SK, Datta Banik S (eds). Environment, Health and Nutrition-Global Issues APH Publishing Corporation, New Delhi, India.

Harvey RG (1991) Polycyclic aromatic hydrocarbons: Chemistry and carcinogenicity, Cambridge University Press, Cambridge, England.

Hedlund BP, Geiselbrecht AD, Bair TJ, Staley JT (1999) Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. Appl Environ Microbial 65 : 251—259.

Hedlund BP, Staley JT (2006) Isolation and characterization of *Pseudoalteromonas* strains with divergent polycyclic aromatic hydrocarbon catabolic properties. Environ Microbiol 8 : 178—182.

Heitkamp M, Franklin W, Cerniglia C (1988) Microbial metabolism of polycyclic aromatic compounds: Isolation and characterization of a pyrene-degrading bacterium. Appl Environ Microbiol. 54 : 2549—2555.

Hilyard Edward J, Joanne M Jones-Meehan, Barry J Spargo, Russell T Hill (2008) Enrichment, isolation and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth River sediments. Appl Environ Microbiol 74 (4) : 1176—1182.

Holt JG, Krieg NR, Sneath PHA, Stalwy JT, Williams ST (1994) Bergey's Manual of determinative bacteriology, 9th edn. Williams and Wilkins, pp 71—561.

Johnsen AR, Wick LY, Harms L (2005) Principle of microbial PAH-degradation in soil. Environ Pollut 133 : 71—84.

Jyothi K, Surendra Babu K, Nancy Clara K, Amita Kashyap (2012) Identification and isolation of hydrocarbon degrading bacteria by molecular characterization. Helix 2 : 105—111.

Kafilzadeh Farshid, Parvaneh Sahragard, Hooshang Jamali, Yaghoob Tahery (2011) Isolation and identification of hydrocarbons degrading bacteria in soil around Shiraz Refinery. Afr J Microbiol Res 4 (19) : 3084—3089.

Kanaly RA, Harayama S (2000) Biodegradation of high-molecular weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182 : 2059—2067.

Kastner M, Breuer MJ, Mahro B (1998) Impact of inoculation protocols, salinity and pH on the degradation of PAHs and survival of PAH-degrading bacteria introduced in soil. Appl Environ Microbiol 64 (1) : 359—362.

Kastner M, Breuer-Jammali M, Mahro B (1994) Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). Appl Microbiol Biotechnol 41 : 267—273.

Kebria D Yousefi, Khodadadi A, Ganjidoust H, Badkoubi A, Amoozegar MA (2009) Isolation and characterization of a novel native *Bacillus* strain capable of degrading diesel fuel. Int J Environ Sci Tech 6 (3) : 435—442.

Khan Jahir Alam, Rizvi Syed Hasan Abbas (2011) isolation and characterization of microorganism from oil contaminated sites. Adv Appl Sci Res 2 (3) : 455—460.

Lawrence PW, Lynda BME (1999) Predicting biodegradation. Environ Microbiol 1 : 119—124.

Leahy JG, Colwell RR (1990) Microbial degradation of hydro carbons in the environment. Microbiol Mol Biol Rev 54 : 305—315.

Lin Y, Cai LX (2008) PAH degrading microbial consortium and its pyrene-degrading plasmids from mangrove sediment samples in Huian, China Mar Pollut Bull 57 : 703—706.

Maiti Arunkumar, Das Sudip, Bhattacharyya Nandan (2012) Isolation and characterization of a new bacterial strain from petroleum oil contaminated soil. Ind J Sci (JOS) 2 (2) : 103 108.

Meyer S, Moser R, Neef A, Stahl U, Kampfer P (1999) Differen tial detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. Microbiology 145 : 1731—1741.

Moghadam Mohsen Shahriari, GholAmhossein Ebrahimipour, Behrooz Abtahi, Alireza ghassempour (2013) Isolation, identification and optimization of phenanthrene degrading bacteria from the coastal sediments of Nayband Bay Jundishapur. J Microbiol 6 (9) : 13816.

Mueller J, Lantz S, Devereux R, Berg J, Pritchard P (1994) Studies on the microbial ecology of polycyclic aromatic hydrocarbon degradation. In : Hinchee R, Leeson A, Semprini L, Ong SI (eds). Bioremediation of chlorinated and polycyclic aromatic hydrocarbons, Lewis Publishers, Boca Raton, FL, pp 218—230.

Naveenkumar S, Manoharan N, Ganesan S, Manivannan SP, Velsamy G (2010) Isolation, screening and *in vitro* mutational assessment of indigenous soil bacteria for enhanced capability in petroleum degradation. Int J Environm Sci 1 (4) : 498—512.

Neeraja P, Madhusudhan NCH, Soma Shanker, Ravikumar M (2013) Isolation and characterization of anthracene degrading bacteria from soil contaminated with petroleum wastes. Int J Res Pharm Sci 4 (3): 380—385.

Pothuluri J, Cerniglia C (1994) Microbial metabolism of polycyclic

aromatic hydrocarbons. In : Chaudhary GR (ed). Biological Degradation and Bioremediation Toxic Chemicals, London : Chapman and Hall, pp 92—124.

Ramirez N, Vargas MC, Sanchez F (1996) Use of the sediment-chromotest for monitoring stimulated hydrocarbon biodegradation processes. Environm Toxicol and Water Quality 11 : 223—230.

Riccardi Camela, Maddalena Papacchini, Antonella Mansi, Alessandra Ciervo, Andrea Petrucca, Giuseppina La Rosa, Cinzia Marianelli, Michele Muscillo, Anna Maria Marcelon, Sergio Spicaglia (2005) Characterization of bacterial population coming from a soil contaminated by polycyclic aromatic hydrocarbons (PAHs) able to degrade pyrene in slurry phase. An Microbiol 55 (2) : 85—90.

Subba Rao (2004) Soil Microbiology. Oxford & IBH Publishing Co Pvt Ltd, New Delhi, pp 20—26.

Supaka N, Pinphanichakarn P, Pattaragulwanit S, Thaniyavarn \ S, Omori T, Juntongjin K (2001) Isolation and characterization of a phenanthrene degrading *sphingomonas* sp. strain P2 and its ability to degrade fluoranthene and pyrene via cometabolism. Sci Asia 27 : 21—28.

Survery S, Ahmad S, Subhan SA, Ajaz M, Rasool SA (2004) Hydrocarbon degrading bacteria from Pakistani soil : Isolation, identification, screening and genetical studies. Pak J Biol Sci 7 : 1518—1522.

Teli Nikhil, Verma Deepa, Gavankar Rohan, Bhalerao Satish (2013) Isolation, characterization and identification of diesel engine oil degrading bacteria from garage soil and comparison of their bioremediation potential. Int Res J Environ Sci 2 (2) : 48—52.

Uyttebroek M, Vermeir S, Wattiau P, Ryngaert A, Springael D (2007) Characterization of cultures enriched from acidic polycyclic aromatic hydrocarbon-contaminated soil for growth on pyrene at low pH. Appl Environ Microbiol 73 : 3159—3164.

Van Hamme J, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbial Mol Biol Rev 67 : 503—549.

Vargas MC, Ramirez NE, Rueda SM, Sanchez FN (1996) Isolation of microorganisms with capability to degrade polycyclic aromatic hydrocarbons (PAH'S) CT&F - Ciencia. Tecnologia Y Futuro 1 (2) : 45—53.

Vidali M (2001) Bioremediation:: An overview. Pure Appl Chem 73 (7) : 1163—1172..

Vijayalakshmi KA, Sudha Rao, Vedamurthy AB (2010) Evaluation of microflora in petrol contaminated soil. The Bioscan 5 (3): 423—426.

Watanbe Kazayu (2001) Microorganisms relevant to bioremediation. Curr Opinion in Biotechnol 12 : 237—241.

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T (2002) Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. Appl Microbiol Biotechnol 58 : 202—209.

Wrenn BA, Venson AD (1996) Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable number procedure. Can J Microbiol 42 : 252—258.

Zhong Y, Luan T, Wang X, Lan C, Tam NFY (2007) Influence of growth medium on co-metabolic degradation of polycyclic aromatic hydrocarbons by *Sphingomonas* sp. strain Phe B4. Appl Microbiol Biotechnol 75 : 175—186.