Journal of Petroleum & Environmental Biotechnology - Open Access

Research Article
OPEN ACCESS Freely available online
doi:10.4172/2157-7463.1000102

Biodegradation of Petroleum and Aromatic Hydrocarbons by Bacteria Isolated from Petroleum-Contaminated Soil

Sayyed Hossein Mirdamadian[1], Giti Emtiazi[2], Mohammad H. Golabi[3] and Hossein Ghanavati[4]*

[1]*MSc of microbiology, Esfahan University, Esfahan, Iran*
[2]*Associate Professor of microbiology, Esfahan University, Esfahan, Iran*
[3]*Associate Professor of soil and environmental sciences, University of Guam, Mangilao, Guam, USA*
[4]*Research Associate at the analytical laboratory of the Esfahan Composting Factory, Esfahan, Iran*

Abstract

Five bacterial strains from genera *Pseudomonas*, *Rhodococcus*, *Micrococcus* and *Bacillus* isolated from petroleum-contaminated soils were selected for their capacity to grow in the presence of petroleum and some aromatic hydrocarbons. Their growth rates and biodegradation ability were investigated in mineral basic media supplemented with light oil, crude oil, aniline plus catechol, aniline, toluene and naphthalene. The results revealed the extents to which these strains could degrade different aromatic hydrocarbon that are toxic in the environment. The plasmid profiles of isolated bacteria were also determined and in some strains, biodegradation ability proved to be plasmid related.

Keywords: Aromatic Hydrocarbons; Biodegradation; Gas Chromatography; Growth Rate; Light; Crude Oil

Introduction

The contamination of soils and groundwater with petroleum compounds is among the most prevalent problems in environments worldwide [1]. In situ biodegradation is one of the primary mechanisms by which petroleum and other hydrocarbons are eliminated from the environment. Hydrocarbon-degrading bacteria are widely distributed in marine, freshwater, soil habitats and their use in bioremediation of hydrocarbon-contaminated soils, which exploits their ability to degrade and/or detoxify organic contaminants, has been established as an efficient, economical, versatile and environmentally sound treatment [2].

Petroleum compounds consist of four fractions: saturated hydrocarbons, aromatic hydrocarbons, nitrogen-sulphur-oxygen containing compounds and asphalthenes. Normally, of the saturated hydrocarbons, the straight-chain *n*-alkanes are most susceptible to biodegradation, whereas branched alkanes are less vulnerable to microbial attack. The aromatic fraction is more difficult to biodegrade and the susceptibility of its components decreases as the number of aromatic or alicyclic rings in the molecule increases [3]. Polycyclic (polynuclear) aromatic hydrocarbons occur extensively as pollutants in soil and water and are important environmental contaminants because of their recalcitrance. These compounds also constitute a potential risk to human health, as many of them are carcinogens [4]. The nitrogen-sulfur-oxygen containing and asphalthene fractions will not be addressed here.

Microorganisms that biodegrade the components of petroleum hydrocarbons are isolated from various environments, particularly from petroleum-contaminated sites [5]. Evaluations of indigenous microorganisms are needed so that bacterial community composition can be correlated with ability to degrade target pollutants [1].

Certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation, including the *alk* (C5 to C12 *n*-alkanes), *nah* (naphthalene) and *xyl* (toluene) pathways have been extensively characterized and are generally located on large catabolic plasmids [6].but many reports describe and characterize microorganisms that can catabolize both aliphatic and aromatic hydrocarbons [7]. Several environmental isolates of *Acinetobacter* sp. and *Alcaligenes* sp [8]. *Arthrobacter* sp [9] and two *Rhodococcus* strains [10] have been found to degrade both alkanes and naphthalene, although the genes and catabolic pathways responsible were not described.

In this study, we considered the potential of isolated bacteria for degradation of petroleum hydrocarbons, naphthalene, toluene, catechol and aniline. Candidate genera of bacteria were identified by biochemical characteristics and the role of plasmids in biodegradation of the compounds mentioned above was investigated.

Material and Methods

Sampling

Soil samples extending from the ground surface to a depth of 10–20 cm were collected from petroleum-contaminated areas near petroleum storage, distribution and refining areas. Samples were taken from areas near storage, areas near distribution facilities and areas near a refinery. Samples were then kept sterile and on ice and were transferred immediately to the laboratory where petroleum-degrading bacteria were isolated from them. Aseptic crude and light Persian oil were used in the assays for ability of the isolated bacteria to degrade petroleum.

Culture media

The basal culture medium contained (g/l) 1 KH_2PO_4, 1 K_2HPO_4, 1 $(NH_4)_2SO_4$, 0.04 $MgSO_4.7H_2O$, 0.004 $FeCl_3.6H_2O$ at pH 7. Inorganic

***Corresponding author:** Hossein Ghanavati, Research Associate,Analytical laboratory of the Esfahan Composting Factory, Esfahan, Iran, E-mail: ghanavatih@hotmail.com

Received August 21, 2010; **Accepted** October 19, 2010; **Published** October 21, 2010

Citation: Mirdamadian SH, Emtiazi G, Golabi MH, Ghanavati H (2010) Biodegradation of Petroleum and Aromatic Hydrocarbons by Bacteria Isolated from Petroleum-Contaminated Soil. J Pet Environ Biotechnol 1:102. doi:10.4172/2157-7463.1000102

iron and magnesium salts were added separately. For the isolation procedure, 2 ml/l petroleum, 5 mM catechol, 10 mM toluene, or 10 mM naphthalene were added to the basic mineral medium as the sole source of carbon. These media also contained designated oil, catechol, toluene and naphthalene broths, respectively. Aniline broth contained 10 mM aniline and the same basic mineral medium in the absence of ammonium sulfate. Aniline+catechol broth medium contained basic mineral medium (without ammonium sulfate) in addition to 10 mM aniline and 5 mM catechol. The hydrocarbons of these media were subjected to membrane filtration and were added to basal medium that had been sterilized by autoclaving for 20 min at 121°C. Media were solidified with 1.5% agar for plating growth. Nutrient broth and nutrient agar were used as enriched media.

Isolation and identification of hydrocarbon-degrading bacteria

Petroleum-degrading bacteria were isolated by an enrichment-culture technique in 250-ml Erlenmeyer flasks containing 100 ml oil Petroleum-degrading bacteria were isolated by an enrichment-culture technique in 250-broth medium and 1 g contaminated soil. This mixture was shaken at 100 rpm at 30°C on a rotary shaker for 1 week. Pure cultures were obtained by plating of culture from the third enrichment culture onto oil agar. Thirty-two strains were isolated that could grow on oil broth and agar media. Finally, the 5 of these 32 strains with the highest capacity for petroleum biodegradation were isolated and and in the second phase of the study, their abilities to degrade naphthalene, toluene, catechol and aniline were observed. The isolated bacteria were identified according to the criteria of [11] on the basis of colony morphology, pigmentation, Gram staining and biochemical characteristics [11].

Petroleum hydrocarbon biodegradation by isolated bacteria

Gas chromatography method.— Isolated bacteria were inoculated into 40-ml test tubes, each containing 20 ml basic mineral medium plus 2 ml light oil as source of carbon and were agitated at 180 rpm at 30°C for 3 days on a rotary shaker before being subjected to analysis by gas chromatography (GC). At various intervals the residual petroleum was extracted with acetone (1:1 v/v) and hydrocarbon degradation was monitored by capillary GC (Perkin Elmer Auto system equipped with 1022 GC plus. A 0.4-µl sample of the extracted petroleum sample was injected into a 25-ml CP-Sil-8Ch column with flame ionization detector. The carrier gas used was hydrogen at 4.5 psi. The injector and detector ports were maintained at 300°C. Oven temperature was programmed to climb from 100 to 130°C with a gradient of 30°C min^{-1}, then raised to 270°C at 10°C min^{-1} and finally held at this temperature for 5 min [12].Total petroleum biodegradation was determined by comparison of the total areas of the chromatograms containing isolated bacteria with those of the controls [13].

Growth rate determination by turbidometry

Isolated bacteria were inoculated into 250-ml Erlenmeyer flasks, each containing 100 ml light oil broth and were agitated at 100 rpm at 30°C on a rotary shaker for several days. Optical densities of inoculated and uninoculated control flasks were measured every day by spectrophotometry at 560 nm. The growth curves were recorded for comparison purposes.

Quantitative comparison method

Isolated bacteria were inoculated into 40-ml tubes, each containing 20 ml basic mineral medium plus 2 ml crude oil and were agitated at 180 rpm at 30°C on a rotary shaker for 20 days. Shaking was terminated after separation of oil and water occurred. The heights of crude oil in tubes were compared with those of controls and degradation was measured.

Biodegradation of aromatic hydrocarbons by isolated bacteria

Isolated bacteria were inoculated into 250-ml Erlenmeyer flasks, each containing 100 ml naphthalene, toluene, catechol, aniline, or aniline+catechol broth and were agitated at 100 rpm at 30°C on a rotary shaker for several days. Samples without inoculation were prepared as controls in all experiments.

Naphthalene, toluene and catechol biodegradation: Biodegradations of naphthalene, toluene and catechol were determined by turbidometry. Bacterial strains were grown in naphthalene, toluene, or catechol broth and optical density was measured as described above. For construction of growth curves, 1 ml of each bacterial strain grown in each medium was centrifuged (4000 rpm, 15 min) and re suspended in 1 ml sterile phosphate buffer (150 nm, pH 7) before optical-density measurement.

Biodegradation of aniline and aniline+catechol: Bacterial strains were grown in aniline or aniline+catechol broth and optical density was measured as described above. Their ammonium production was also assayed, by means of Nessler's reagent [14].

Investigation of plasmid presence and its effects on hydrocarbon degradation

Plasmid profile and curing: Isolated bacteria were inoculated into broth medium containing the hydrocarbon in which the bacteria had shown the highest growth rate. The media were used in late exponential phase as starter culture. For inoculation, 0.1 ml of this culture was added to 100 ml of nutrient broth plus Tween 80 (polyoxyethylene sorbitan monooleate) and was incubated at 30°C for 24 h. Serial dilution and pure plate techniques were used for growth of colonies on nutrient agar. Colonies were counted and 100 colonies were separated and patched out onto solid medium of the same composition or onto nutrient agar. The plates were incubated overnight at 30°C. Colonies able to grow on nutrient agar but not on solid medium were isolated and considered cured. The cured strains were sub-cultured in broth medium with the original hydrocarbon to confirm loss of degradation ability.

Days of incubation	Percentage of crude oil removed					
	Blank	*Pseud* L	*Rhodo.*	*Bacillus*	*Micro.*	*Pseud.* W
0	0	0	0	0	0	0
4	0.6	33	18	10	9	4
8	1.3	49	33	15	14	6
12	2.8	56	40	22	19	7
16	4.1	65	45	28	23	8
20	5	72	49	34	28	9

Table 1: Percentage of the crude oil biodegradation by isolated bacteria. *Pseud., Pseudomonas; Rhodo., Rhodococcus; Micro., Micrococcus.*

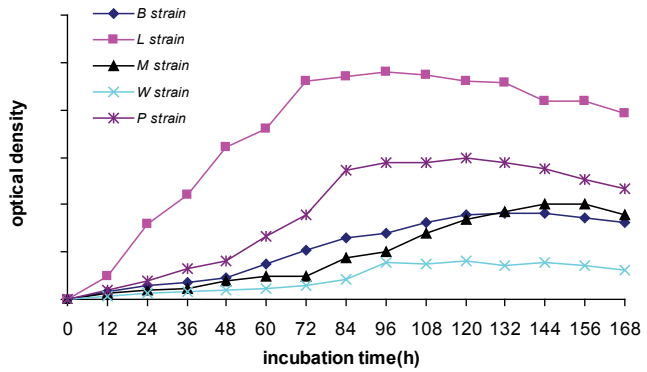

Figure 1: Growth curves in light oil medium of the five bacterial strains isolated from petroleum-contaminated soil.

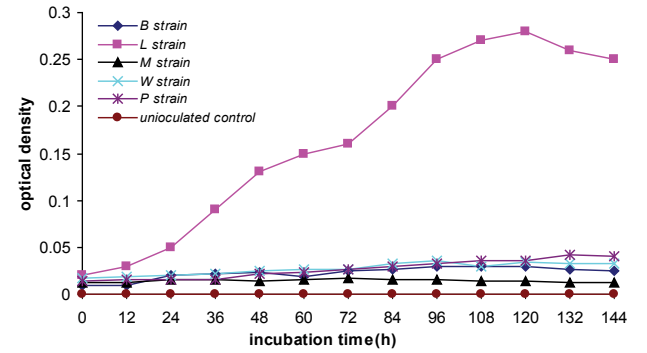

Figure 2: Chromatograms of the light oil medium after incubation for three days with the isolated bacterial strains. (A) Uninoculated control. (B) *Pseudomonas* strain L. (C) *Bacillus*. (D) *Pseudomonas* strain W.\ (E) *Rhodococcus*. (F) *Micrococcus*. Total biodegradations were obtained, respectively, at 53.4%, 15.5%, 22.4%, 52.1%, and 29.4%.

Plasmid extraction and electrophoresis: Plasmid DNA was extracted by a modified Kado and Liu procedure [15].After electrophoresis on a 0.7% horizontal agarose gel at 50 V for 3 h, the gels were stained with ethidium bromide and bands visualized with a UV transilluminator [16] Molecular-sized plasmids were determined by comparison with RP1 plasmid from *E. coli* strain JC3272 with 64-kb weight as a control.

Results and Discussion

Microbial strains

The five bacterial strains best able to degrade and use petroleum and aromatic compounds were selected and identified as two strains of *Pseudomonas* (designated L and W) and one strain each of *Rhodococcus*, *Micrococcus* and *Bacillus*.

Light and crude oil biodegradation

As (Figure 1) shows, *Pseudomonas* strain L showed the best growth in the presence of light and crude oil. The results of light-oil biodegradation, as revealed by GC are shown in (Figure 2). Thirteen peaks were found and the greatest degradation occurred in the higher, initial peaks.

Comparison of the total areas of the chromatograms shows that *Pseudomonas* strain L was also the best biodegrader of light oil.

The results of the quantitative comparison of residual crude oil with that in controls are presented in (Table 1). Again, *Pseudomonas* strain L best degraded the oil; it degraded 72% of crude oil in 20 days. The bacteria in our studies degraded crude oil more efficiently than did those investigated by [17] (63.87%) and [13] (57%).

Aromatic hydrocarbon biodegradation

(Figures 3–7) shows the biodegradation of toluene, naphthalene, aniline, catechol and aniline+catechol. The growth curves of toluene and naphthalene are shown in (Figure 3 and Figure 4). The greatest toluene degradations was produced by *Pseudomonas* strain L and the best naphthalene degradation by *Rhodococcus*. (Figure 5) shows

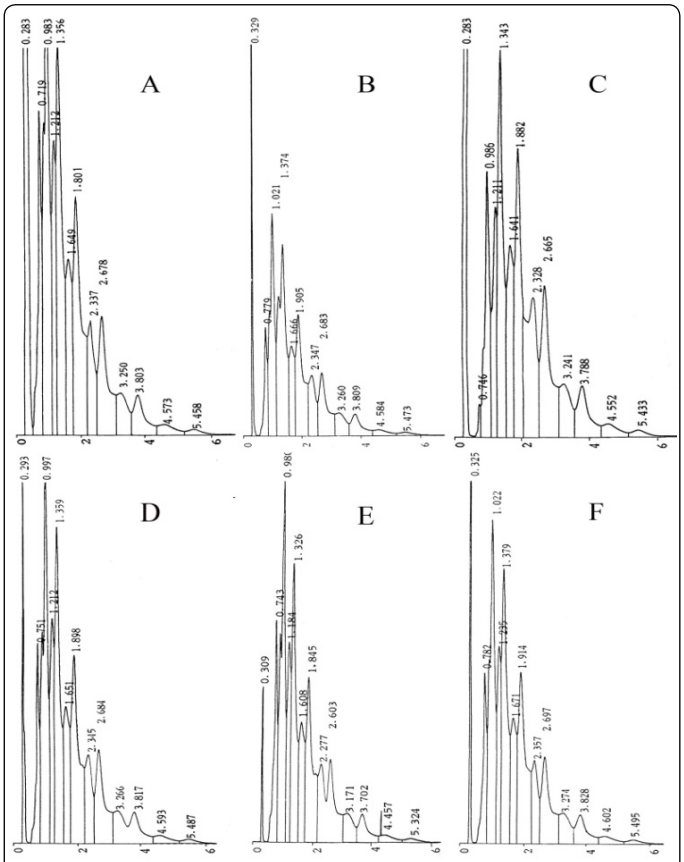

Figure 3: Growth curves in toluene broth of the five isolated bacterial strains.

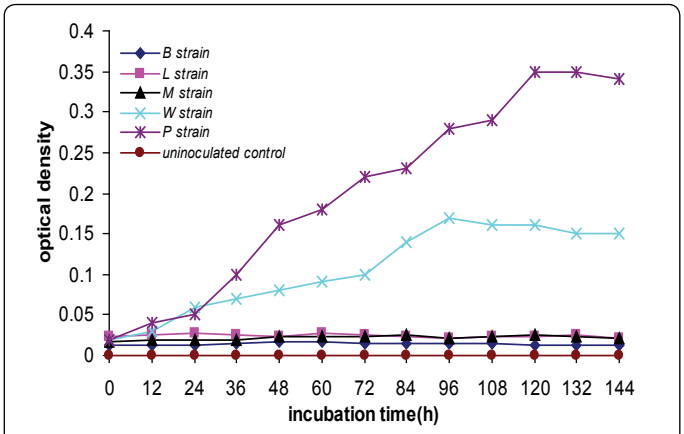

Figure 4: Growth curves in naphthalene broth of the five isolated bacterial strains.

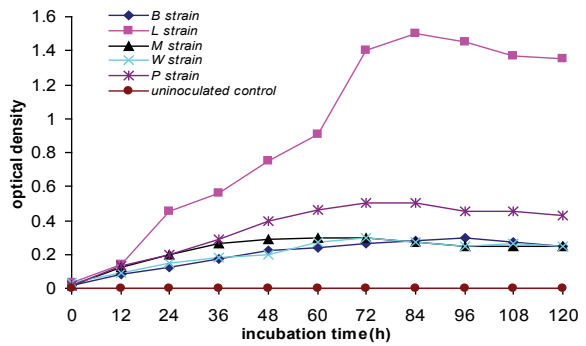

Figure 5: Growth curves in catechol broth of the five isolated bacterial strains.

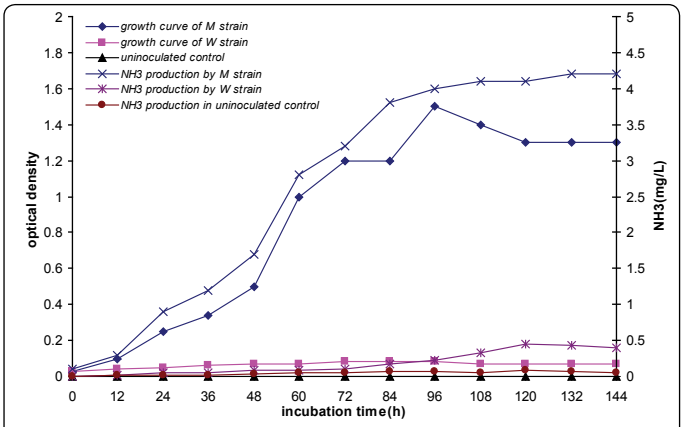

Figure 6: Growth curves and ammonium production in aniline broth of the five isolated bacterial strains.

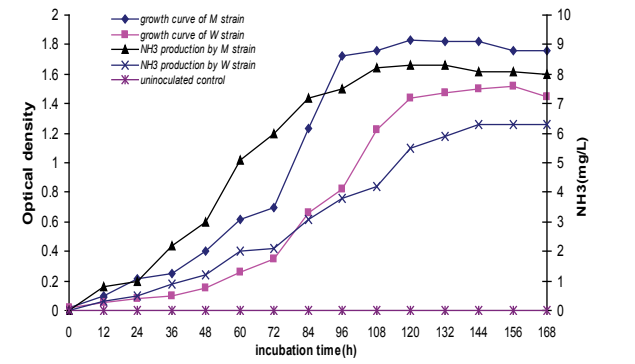

Figure 7: Growth curves and ammonium production in catechol-aniline broth of the five isolated bacterial strains.

catechol degradation. *Pseudomonas* strain L also degraded catechol significantly more rapidly than did other strains.

Ammonium production, shown in (Figure 6 and Figure 7), revealed that only *Pseudomonas* strain W and the *Micrococcus* strain could degrade aniline as a sole source of carbon and nitrogen. The results of the aniline+catechol biodegradation experiment suggest that presence of catechol can promote aniline biodegradation.

The five microorganisms used in this study differed in their ability to degrade aromatic hydrocarbons. Some of them, such as *Pseudomonas* strain W, could degrade all investigated hydrocarbon compounds, but the maximum degradation of aromatic hydrocarbons was shown by *Pseudomonas* strain L. Catechol and aniline best

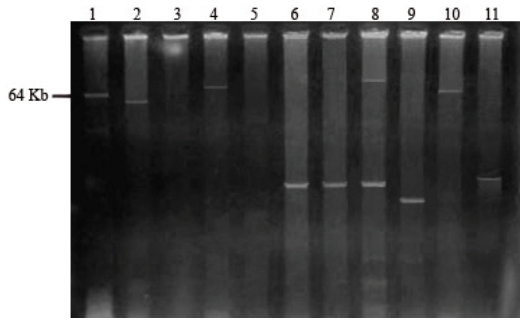

Figure 8: Electrophoretic separation profiles of plasmids from cured and non-cured strains. Lane 1, RP1 plasmid as DNA ladder. Lanes 2, 4, 6, 8, and 10 show the plasmid profiles from the non-cured strains *Pseudomonas* W, *Rhodococcus*, *Bacillus*, *Pseudomonas* L, and *Micrococcus* strains, respectively. Lanes 3, 5, 7, 9, and 11 show the plasmid profiles from cured cultures of the same five strains.

supported bacterial growth. [5] and [1] conducted similar experiments, studying biodegradation of naphthalene by bacteria isolated from oil-contaminated soils. [18] studied bacterial strains isolated from waste crude oil and their capacity for growth with naphthalene, phenanthrene, fluoranthene and pyrine as sole carbon sources. Our study differs from these, however, in that we studied growth rate of bacteria only in the presence of aromatic hydrocarbons.

Plasmid analysis

Catabolic pathways, which encode degradation routes of different aromatic and aliphatic hydrocarbons, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid [16].

In our study the cured *Pseudomonas* strain L grew successfully on toluene medium and the cured *Micrococcus* strains grew successfully on aniline media. Both *Rhodococcus* and *Pseudomonas* W cured strains grew on naphthalene medium. Cured *Bacillus* did not grow successfully on either crude oil or other hydrocarbon media. All strains that were successfully cured by serial sub-culturing in nutrient broth (i.e., all except *Bacillus*) lost the ability to degrade hydrocarbons.

(Figure 8) shows agarose gel electrophoretic separation profiles of plasmids from cured and uncured strains of the five bacteria used in our study. Curing experiments demonstrated that cured strains that lost their plasmids also lost their hydrocarbon biodegradation ability, so the capacity to degrade hydrocarbons is plasmid related in all the strains we studied except the *Bacillus* strain. Electrophoretic separation profiles of plasmidic DNA isolated from non-cured cultures showed that *Pseudomonas* strain L harbored two plasmids; curing deleted the large plasmid completely and removed a piece of the smaller one. Cured *Pseudomonas* strain L also lost the ability to degrade oil and toluene. In *Micrococcus, Pseudomonas* W and *Rhodococcus* strains, some deletions occurred. Despite several subculturings, no cured form the *Bacillus* strain was obtained, although in the wild strain, one plasmid was seen in agarose gel.

The plasmid mediation of ability to degrade phenanthrene was demonstrated by Coral and Karagöz [16] in curing experiments. [19] extracted plasmids from *Pseudomonas* and *Burkholderia* strains isolated from oil-contaminated soils that conferred ability to degrade naphthalene. [5] demonstrated the presence and also the role of two plasmids in catabolic pathways for alkane and naphthalene. Our study reveals that ability to degrade aromatic hydrocarbons is plasmid encoded.

References

1. Alquati C, Papacchini M, Riccardi, Spicaglia C, Bestetti G (2005) Diversity of naphthalene-degrading bacteria from a petroleum contaminated soil. Ann Microbiol 55: 237-242.
2. Margesin R, Schinner F (1997) Efficiency of indigenous and inoculated cold adapted soi microorganisms for biodegradation of diesel oil in Alpine soils. Appl Environ Microbiol 63: 2660-2664.
3. Barthakur HP (1997) Crude-degrading microorganisms: occurring in oil field of Assam. In Ecological Issues and Environmental Impact Assessment, ed. P. N. Cheremisrnoff, Houston, TX: Gulf Publishing Company 371-389.
4. Deziel, Paquette GR Villemur R, Lepine F, Bisaillon J (1996) Biosurfactant production by a soil Pseudomonas strain growing on polycyclic aromatic hydrocarbons. Appl Environ Microbiol 62: 1908-19125.
5. Whyte LG, Bourbonnière L, Greer CW (1997) Biodegradation of petroleum hydrocarbons by psychrotrophic Pseudomonas strains possessing both aAlkane (alk) and naphthalene (nah) catabolic pathways. Appl Environ Microbiol 63: 3719-3723.
6. Gary S, Sayler, Hooper SW, Layton AC, Henry King JM (1990) Catabolic plasmids of environmental and ecological significance. Microbial Ecol 19: 1-207.
7. Foght JM, Fedorak PM, Westlake DWS (1990) Mineralization of [^{14}C] hexadecane and [^{14}C] phenanthrene in crude oil: specificity among bacterial isolates. Can J Microbiol 36: 169-175.
8. Lal B, Khanna S (1996) Degradation of crude oil by Acinetobacter calcoaceticus and Alcaligenes odorans. J Appl Bacteriol 81: 355-362.
9. Efroymson RA, Alexander M (1991) Biodegradation by an Arthrobacter species of hydrocarbons partitioned into an organic solvent. Appl Environ Microbiol 57: 1441-1447.
10. Malachowsky K, Phelps TJ, Teboli AB, Minnikin DE, White DC (1994) Aerobic mineralization of trichloroethylene, vinyl chloride and aromatic compounds by Rhodococcus species. Appl Environ Microbiol 60: 542-548.
11. Krieg NR, Holt JG (1984) In Bergey's Manual of Systematic Bacteriology, ed. N. R. Baltimore, MD: Williams& Wilkins.
12. Kapley A, Purohit HJ, Chhatre S, Shanker R, Chakrabarti T, et al. (1999) Osmotolerance and hydrocarbon degradation by a genetically engineered microbial consortium. Bioresour Technol 61: 241-245.
13. Díaz MP, Steve JW, Grigson SJW, Peppiatt CJ, Burgess GJ (2000) Isolation and characterization of novel hydrocarbon-degrading euryhaline consortia from crude oil and mangrove sediments. Mar Biotechnol 2: 522-532.
14. Ghanavati H, Emtiazi G, Hasanshahian M (2008) Synergism effects of phenol degrading yeast and ammonia oxidizing bacteria for nitrification in coke wastewater of Esfahan Steel Company. Waste Manage Res 26: 203-208.
15. Woodford N, Johnson AP (1998) Molecular Bacteriology Protocols and Clinical Applications. Totowa, NJ: Humana Press.
16. Coral G, Karagöz S (2005) Isolation and characterization of phenanthrene-degrading bacteria from a petroleum refinery soil. Ann Microbiol 55: 255-259.
17. Palittapongarnpim M, Pokethitiyook P, Upatham ES, Tangbanluekal L (1998) Biodegradation of crude oil by soil microorganisms in the tropic. Biodegradation 9: 83-90.
18. Toledo FL, Calvo C , Rodelas B, González-López J (2006) Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst Appl Microbiol 29: 244-252.
19. Balashova, Kosheleva IA, Golovchenko NP, Boronin AM (1999) Phenanthrene metabolism by Pseudomonas and Burkholderia strains. Proc Biochem 35: 291-296.