Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 10	No. 3	pp. 369-376	2011

Original Research Paper

Optimization of Conditions for Naphthalene Biodegradation

Varsha K. Vaidya and Sharmila Kadam
Department of Microbiology, Institute of Science, 15 Madame Cama Road, Mumbai 400 032, Maharashtra, India

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 17/3/2011
Accepted: 13/4/2011

Key Words:
Polyaromatic hydrocarbons
Naphthalene
Biodegradation
Soil microcosm
Immobilization

ABSTRACT

Degradation of naphthalene by *Burkholderia glathei, Alcaligenes denitrificans* and *Pseudomonas putida* was optimized with respect to various environmental factors such as pH, temperature, salinity and addition of nutrients such as nitrogen and phosphorus. Maximum growth and biodegradation occurred at 1.0% of naphthalene at 30°C, pH 8.0 and 1.5% salinity. Addition of various nitrogen sources such as urea, ammonium nitrate and ammonium chloride as well as phosphorus sources such as KH_2PO_4 and Na_2HPO_4 resulted in an increased rate of biodegradation. A carbon to nitrogen to phosphorus ratio of 93:10:0.09 yielded maximum degradation of naphthalene. The application of the naphthalene degraders in abatement of pollution caused by naphthalene was studied in soil microcosm. The usefulness of immobilization technique for bioremediation showed encouraging results.

INTRODUCTION

The bane of industrial progress has been the generation and release into the environment of huge amounts of toxic compounds and xenobiotics like polyaromatic hydrocarbons (PAHs) which have caused widespread contamination of land and water. PAHs besides being persistent are toxic, possess low volatility and aqueous solubility, hydrophobic in nature, and have high solid water distribution ratios and high affinity for the sediments which stand against their ready microbial utilization (Johnsen et al. 2005). Because of the great number of petroleum contaminated sites requiring cleanup and the high cost involved with conventional approaches, there is an overriding need for research aiming at establishing cost-effective and environmentally acceptable mitigation technologies, primarily for the PAHs ranging in size from naphthalene (two rings, $C_{10}H_8$) to coronene (seven rings, $C_{24}H_{12}$) (Marta et al. 2006).

One such remedial technology is bioremediation that uses microorganisms to detoxify environmental pollutants and transform them into simpler, less toxic compounds. Microorganisms, especially bacteria, play an important role in the biodegradation of these hydrocarbons. It is now realized that microbial metabolism provides a safer, more efficient, and less expensive alternative to physico-chemical methods for pollution abatement. The high solubility of naphthalene compared to other PAHs and the fact that the naphthalene degradative genes are plasmid encoded has facilitated research on naphthalene degradation. It is often used as a model compound for *in situ* biodegradation studies of PAHs (Grimm & Harwood 1999). It has been shown that the acclimation of a microbial community to one substrate frequently results in the simultaneous acclimation to some, but not all structurally related molecules. Also, individual microbial species have the ability to act on several structurally similar substrates, and therefore, more easily act on their analogues after the first addition. These model organisms may be very useful for the study of complex aromatic hydrocarbon degradation and for bioremediation purposes (Nnamchi et al. 2006). Toluene and naphthalene degrading bacteria, obtained from contaminated lake sediment, were used to degrade both monoaromatics and polycyclic aromatic hydrocarbons (PAHs) (Horng et al. 2009). Tikilili & Chirwa (2009) successfully used naphthalene degrading bacterial species such as *Pseudomonas aeruginosa, Microbacterium esteraromaticum* and *Alcaligenes* sp. isolated from contaminated soil from a landfill site (Chloorkop, Johannnesburg, South Africa) in degrading naphthalene found in radioactive wastewater collected from radioisotope processing facility, South Africa. Thus, naphthalene degrading isolates could be very helpful in bioremediating PAH-contaminated sites.

Hence, the aim of the present study was to optimize the conditions for the degradation of naphthalene by the naphthalene degrading organisms, isolated from petroleum contaminated soils and to evaluate their potential for the bioremediation processes.

MATERIALS AND METHODS

All the media chemicals (AR grade) and reagents were purchased from Hi Media Laboratories, Mumbai, India.

Organisms: The present study was detailed on *Burkholderia glathei, Alcaligenes denitrificans* and *Pseudomonas putida* isolated by enrichment culture technique from petroleum contaminated soils in Mumbai, India. The isolates were preserved on nutrient agar slants at 4°C.

Optimization of conditions for naphthalene degradation: Conditions for the degradation of 1% naphthalene were optimized with respect to various environmental factors such as pH, temperature, salinity, and addition of nutrients such as nitrogen and phosphorus. The cultures for optimization were prepared as follows: 18 h old isolates grown on LB medium (g/L, Tryptone 10, Yeast extract 5, NaCl 5, D/W 1000 mL, pH 7.4) were transferred to Mineral Salt Medium (MSM) agar plate (g/L, NH_4Cl 2, KH_2PO_4 0.4, $CaCl_2$ 2, $FeSO_4$. $7H_2O$ 0.2, D/W 1000 mL, pH 7.4) with naphthalene supplied in the vapour form from the crystals placed in the lid of the plate. The plate was incubated at room temperature (28 ± 2°C) for 48 h. Inoculum was prepared by transferring one loopful of the growth from the MSM agar plate to MSM liquid medium containing 1% naphthalene dissolved in dimethyl formamide (DMF). Cells were allowed to grow overnight to an optical density of 1.0 at A_{600}. Ten percent of the 18 h old inoculum was added to 50 mL of sterile MSM medium containing 1% naphthalene as the sole source of carbon. The control flask was prepared by adding 1 mL of the solvent DMF alone (without naphthalene) and equivalent saline instead of the culture (Malakul et al. 1998). Flasks were incubated on shaker (120 rpm) at room temperature (28 ± 2°C) for 48 h. At the end of the incubation period, medium was centrifuged at 3000 rpm for 10 min. Supernatant was taken and residual naphthalene was estimated by measuring optical density using UV spectrophotometer at 276 nm. A standard curve of naphthalene was also plotted in the range of 100-1000 µg/mL. The residual naphthalene after 48 h of incubation for all the parameters used for optimization was estimated by UV spectrophotometer (Shimadzu UV1800 UV/VIS) at 276 nm.

Naphthalene degradation was studied over a pH range 6.0-9.0. The pH supporting the maximum degradation of naphthalene was used in further work. The isolates were further tested for their ability to grow and degrade naphthalene at 16°C at pH optimized in the earlier step. The impact of salinity on degradation was evaluated using various concentrations of NaCl (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) under the optimized conditions. The effect of different sources of nitrogen and phosphorus on the degradation of naphthalene was studied as follows: Three sets of six flasks each containing varying concentrations of ammonium chloride, ammonium nitrate and urea (5-25 and 50 mg nitrogen/100 mg naphthalene) in 50 mL of sterile MSM medium were used to study the effective nitrogen source for achieving the highest degradation of naphthalene. Two sets of six flasks each containing varying concentrations of K_2HPO_4 and Na_2HPO_4 (0.07-0.12 mg phosphorus/100 mg of naphthalene) in 50 mL of sterile MSM medium were used to evaluate the effect of phosphorus on degradation of naphthalene. The effect of Fenton's reagent (5 mL 2.8 M H_2O_2; 5 mL 0.1 M $FeSO_4$) on degradation was evaluated by adding 5 mL of Fenton's reagent to 50 mL of MSM broth inoculated with ten percent of inoculums under the optimized conditions.

Application of the naphthalene degrading organisms in soil: Degradation of the naphthalene in soil microcosm was determined as follows: Thirty g of moist soil was placed in 500 mL flask containing 100 mL of MSM broth with 1% naphthalene. The soil-naphthalene complex was mixed thoroughly and allowed to equilibrate for 72 h. 10 mL Fenton's reagent was added to naphthalene contaminated soil, the contents were gently mixed and inoculated with individual cultures and incubated at 28 ± 2°C. The residual concentration of naphthalene in soil was determined over a period of seven days. An aliquot of the soil slurry with an equal volume of hexane was mixed in a separator funnel, mixed for two minutes followed by extraction in cold conditions. The resulting organic phase was used for the spectrophotometric analysis of naphthalene (Marta et al. 2006).

Use of immobilized cells in degradation of naphthalene: The 18 h old cells of the isolates grown in MSM medium containing 1% naphthalene were harvested by centrifugation and washed twice in sterile distilled water. Alginate beads containing immobilized cells were prepared by dissolving 4 g of alginate in 100 mL of distilled water overnight and sterilized by autoclaving (121°C, 15 min). Ten percent (optical density of 0.1 at A_{540}) of inoculums was resuspended in the sodium alginate solution, stirring to complete homogenization. The mixture was extruded drop-wise through a hypodermic needle into a cold, sterile solution of $CaCl_2$ (0.2 M). The beads were left to harden in the same solution at room temperature with gentle stirring for 1 h. Finally, the beads were washed several times with 0.9% NaCl to remove excess calcium ions and untrapped cells. Sterile beads (without microorganisms) were used to monitor the abiotic loss of naphthalene (Feijoo-Siota et al. 2008). The concentration of residual naphthalene in the culture medium was determined over a period of seven days by measuring the optical density at a wavelength of 276 nm after cold extraction with hexane (Marta et al. 2006).

Effect of storage on the viability of immobilized cells: Cell viability of immobilized cells stored at 4°C was analysed after 15 days by suspending the beads in10 mL of a sterile 1% solution of sodium citrate. The suspension was vortexed to achieve a complete dissolution of the alginate. Serial dilutions were spread on LB agar. The colonies were counted on LB agar plates after incubation for 48 h at 28 ± 2°C (Feijoo-Siota et al. 2008).

RESULTS AND DISCUSSION

Microbial communities present in the contaminated soils can metabolize PAHs at greater rates than soil microbial communities found in uncontaminated soils. Bacteria exposed to pollutants can adapt to them by mutating or acquiring degradative genes. These bacteria can proliferate in the environment as a result of the selection pressures created by the pollutants. The positive outcome of selection pressure is that harmful compounds may eventually be broken down completely through biodegradation (Alquati et al. 2005, Van der Meer 2006).

As suggested by Feijoo-Siota et al. (2008), a slightly alkaline pH seems to be quite favourable for hydrocarbon degradation. In the present investigation, the range of pH for degradation of naphthalene was varied between 6.0 and 9.0. The extent of degradation for all the three bacterial isolates increased over a pH range of 6.0-8.0 with pH 8.0 showing the highest degradation for all the isolates. There was a drastic reduction in the degradation ranging between 60-68 % at pH 9.0. The degradation was the most adversely affected at the acidic pH of 6.0 for all the isolates, though the effect was pronounced in *Burkholderia glathei*, which showed a 88.05 % reduction in degradation compared to the degradation obtained at optimal pH (8.0) (Fig. 1). Dibble & Bartha (1979) observed maximum degradation rate at pH 7.8 in oil sludge samples, beyond which rate of degradation decreased. *Rhodococcus rhodochrous* KUCC 8801 and KUCC 8802 isolates showed their maximum growth at pH 8.0 in a crude oil containing medium kept at 40°C. Thavasi et al. (2007) also showed similar results, where maximum crude oil degradation occurred at pH 8.0, beyond that level a decreasing trend was observed. Pathak et al. (2008) showed that naphthalene degradation by *Pseudomonas* sp. HOB1 was augmented in the pH range of 7.5-8.5.

Temperature plays a very important role in the biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants and secondly by its effect on the physiology and diversity of the microbial milieu. A temperature increase decreases viscosity and increases diffusion rates of organic compounds. Therefore, higher reaction rates due to smaller boundary layers are expected at elevated temperatures. Increasing temperatures facilitate the solubility of PAHs and hence their bioavailability. Although microbial activity is generally reduced at low temperatures, many of the components in crude oil can actually be degraded in these conditions (Atlas 1981). Cold-tolerant isolates of *Pseudomonas* sp. from oil polluted Antarctic soils have been reported to utilize naphthalene, phenanthrene and fluorene, as well as BTEX, as the sole carbon and energy source (Aislabie et al. 2006). Jeon et al. (2004) reported *Polaromonas naphthalenivorans* sp. strain CJ2T, capable of growth on naphthalene at 4°C and 25°C (optimum, 20°C). In the present investigation, the biodegradation of naphthalene at 28 ± 2°C was compared to that at 16 ± 1°C in order to evaluate the potential of the isolates to be used as bioremediating agents in temperate conditions. The results indicated that at 16 ± 1°C the rate of degradation decreased for all the isolates viz. for *Burkholderia glathei* from 32.55% to 10.33% (a decrease of 68.27 %), for *Alcaligenes denitrifacans* from 36.25% to 12.93% (a decrease of 64.33%) and for *Pseudomonas putida* from 38.96% to 16.87% (a decrease of 56.69%). However, degradation did not cease completely indicating the ability of the isolates to grow and degrade naphthalene even under lower temperatures.

Salinity is a major factor which significantly affects biological activity in the marine environment. There are few published studies which deal with effects of salinity on the microbial degradation of hydrocarbons. In the study by Thavasi et al. (2007) *Pseudomonas aeruginosa* showed maximum growth and biodegradation activity at 3.5% salinity. In the present study, the MSM medium used in the earlier steps did not contain NaCl. When the medium was supplemented with NaCl in concentrations ranging from 0.5 % to 3% with an interval of 0.5%, it was observed that addition of 0.5% salt did not alter the rate of degradation for any of the isolate (Fig. 2). Except for a slight increase in degradation, shown by *Burkholderia glathei* at 1%, the degradation remained unchanged for the other two isolates. A sudden rise in degradation was seen at salinity of 1.5% with *Burkholderia glathei* showing a significant rise of 24.99%, while other two isolates showed an increase between 16 and 18 %. The results of the study indicate that the isolates used in the study would be less effective bioremediating agents in the marine environment. For further work, salinity was maintained at 1.5%.

Nutrients, especially nitrogen, phosphorus and in some cases iron are very important ingredients for successful biodegradation of hydrocarbon pollutants. Depending on the nature of the impacted environment, some of these nutrients could become limiting thus affecting the biodegradation processes. This is more pronounced in marine environments due to the low background levels of nitrogen and phosphorus in seawater unlike in freshwater systems, which regularly fluctuate in nutrients as a result of perturbations and receipt of industrial and domestic effluents and agricultural runoff. The addition of nitrogen and phosphorus fertilizers stimulates the biodegradation of hydrocarbons. It was reported that the biodegradation rate of hydrocarbons is directly proportional to the supplemented nitrogen (Atlas 1981, Cooney 1984). Leys et al. (2004) suggested a general theo-

retically calculated C/N/P ratio of 100/10/1 (expressed in moles) for hydrocarbon degradation. The minimal medium used in the study contained 0.2g NH_4Cl (54 mg nitrogen) as the source of nitrogen, which was replaced by three different sources of nitrogen viz., ammonium nitrate, urea and ammonium chloride, added to represent nitrogen concentration ranging from 5-50 mg N/100 mg naphthalene (93 mg C) i.e., C to N ratios of 18.6:1 to 1.86:1 (Table 1). The highest degradation was seen at C : N ratio of 9.3:1 with all the sources of nitrogen, with urea proving to be the best source of nitrogen showing an increase in degradation around 15%. The overall trend showed a decrease in the degradation below and above the optimum C : N ratio by all the isolates. Bastiaens et al. (2000) showed that the type of nitrogen supplement (i.e., ammonium versus nitrate) affected the degree of oil biodegradation observed. Supplementation with ammonium led to the progressive acidification of cultures, whereas nitrate amendment resulted in a neutral culture medium. In the present investigation, urea proved to be superior to both the sources of nitrogen.

A surprisingly large number of studies report no benefit or even deleterious effects when excessive levels of N are applied. Reports of specific inhibitory effects of excess N include an increased lag phase and preferential inhibition of aromatic degradation, although most indicate overall inhibition of microbial respiration and/or hydrocarbon degradation. These factors could have contributed to lowered rates of degradation at excessive concentrations of nitrogen in the medium. Although nitrogen is considered to be the major limiting nutrient, maximal hydrocarbon degradation occurs with supplementation of both N and P (Braddock et al. 1997). Feijoo-Siota et al. (2008) investigated the effect of availability of nitrogen and phosphorous on the uptake and degradation of naphthalene by free cells in seawater collected from a beach on the Galician coast (Lagos beach, Spain), (pH 8.20, and salinity 33 mg/L). When necessary, the seawater was supplemented with K_2HPO_4 as a source of phosphorous at a C : P ratio of 30:1, and NH_4NO_3 as a nitrogen source, at a C : N ratio of 10:1. The results at 30°C revealed that an initial naphthalene concentration of 25 mM was almost completely degraded (93%) within the first 6 days of incubation in samples supplemented with nitrogen and phosphorous, whereas in the non-supplemented samples, only 42% of the naphthalene was consumed after that time. In samples supplemented with nitrogen and phosphorous, 55% the naphthalene was degraded, whereas in the non-supplemented samples the degradation was only 34%. Thus, supplementing seawater with phosphorus and nitrogen was important in order to obtain the highest possible degree of biodegradation.

In the present work, two sources of phosphorus were employed viz., KH_2PO_4 and NaH_2PO_4 in ratios ranging from 0.07-0.12 mg P/100 mg naphthalene (C : P ratio ranging from 9.3:0.07 to 9.3:0.12) at C : N ratio (9.3:1) optimized in the earlier step. Both the sources of phosphorus showed an enhanced degradation at a C : P ratio of 9.3:0.09 with potassium di-hydrogen phosphate serving as the best source of phosphorus for all three isolates. Thus, the optimum ratio for the degradation was found to be 93/10/0.9. Thus, overall addition of sources of nitrogen and phosphorus to the medium resulted in an increase in the degradation of naphthalene for all the isolates viz., *Burkholderia glathei* (from 40.56% to 54.24% leading to an increase of 33.72% in degradation), *Alcaligenes denitrificans* (from 43.25% to 55.64% leading to an increase of 28.65% in degradation), and *Pseudomonas putida* (from 45.42% to 56.63% leading to an increase of 24.69% in degradation). The effect of nutrient supplementation on *Burkholderia glathei* was thus more pronounced.

PAHs present in the atmosphere or natural waters are believed to be degraded to a limited extent by indigenous hydroxyl radicals ($OH^{\cdot}$). The use of $OH^{\cdot}$ generated by mixing of H_2O_2 with $FeSO_4$ i.e., Fenton's reagent (5 mL 2.8M H_2O_2; 5 mL 0.1 M $FeSO_4$) has shown promise for the degradation of a wide range of environmental contaminants in aqueous systems as well as soil slurry bioreactors. Fenton's reagent brings about oxidation of PAH's to a more biodegradable compound for further degradation by microorganisms.

The application of Fenton's reagent to remediate matrices contaminated by polycyclic aromatic hydrocarbons (PAHs) shows that the matrix itself plays an important role, as the iron oxides seem to be able to decompose hydrogen peroxide, and thus initiate Fenton's reaction. The PAHs sorbed onto inner surface of the flask, and therefore not undergoing oxidation, or PAHs sorbed on sewage sludges, contaminated soils and sediments can be oxidized by the addition of Fenton's reagent (Martens & Frankenberger 1995). In the present study, the effect of addition of Fenton's reagent in the aqueous system (MSM medium) under the conditions optimized in the earlier steps was studied. Addition of Fenton's reagent enhanced the degradation of naphthalene for all the isolates. The pronounced effect was seen in case of *Burkholderia glathei* showing an increase in extent of degradation by 10.33 % (59.85% degradation), while *Alcaligenes denitrificans* and *Pseudomonas putida* showed an increase of 10.84 % (61.68% degradation) and 9.58 % (62.06% degradation) respectively.

Differences in the physical, chemical and microbial characteristics of ecosystems can affect the disposition and persistence of chemicals in the environment. Consideration of

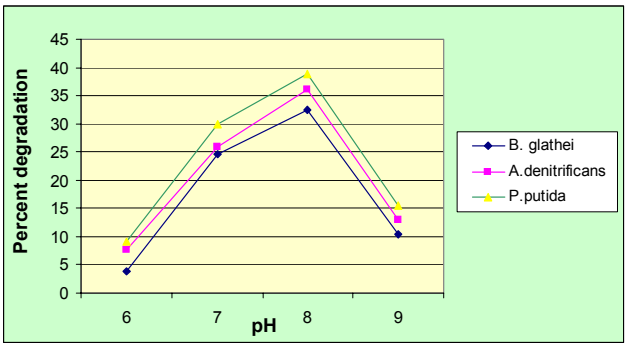

Fig. 1: Effect of pH on degradation of naphthalene.

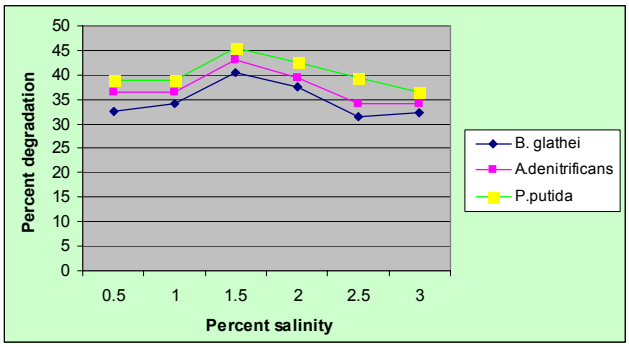

Fig 2: Effect of salinity on degradation of naphthalene.

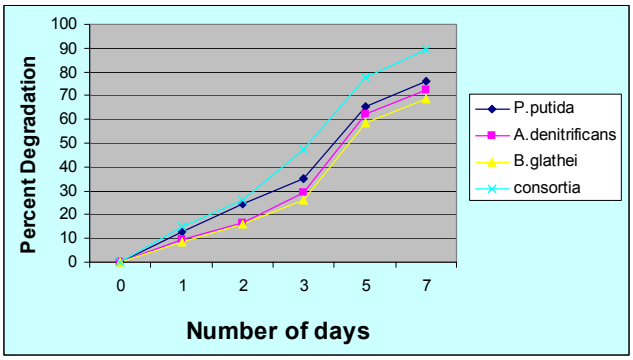

Fig. 3: Degradation of naphthalene in soil microcosm.

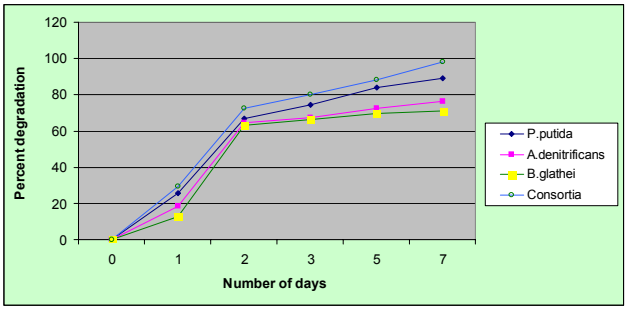

Fig 4: Effect of immobilization of cells on degradation of naphthalene.

these differences as well as the complexity of natural ecosystems and the physical and chemical properties of toxicants have led to the use of multicomponent microcosms as laboratory models to predict the toxic effects and degradation of chemicals in the environment (Giesy 1980). In the present investigation the individual isolates and the consortia consisting of 1:1:1 mixture of the isolates was tested for their ability to degrade naphthalene in soil microcosms under all the optimized conditions. The study was carried over a period of seven days in soil previously equilibrated with naphthalene. The results of the present investigation indicated the ability of all the isolates to establish themselves in the soil. Pathak et al. (2008) carried out simulated microcosm studies in the presence and absence of indigenous microflora confirmed its ability for naphthalene degradation and to colonize the soil. *Pseudomonas* sp. HOB1 was found to be highly potent in degrading higher concentrations of naphthalene under laboratory conditions as well as in simulated microcosms, similar to results found in this study. Heitkamp et al. (1987) studied naphthalene biodegradation in microcosms containing sediment and water collected from three ecosystems which varied in past exposure to anthropogenic and petrogenic chemicals. Mineralization half-lives for naphthalene in microcosms ranged from 2.4 weeks in sediment chronically exposed to petroleum hydrocarbons to 4.4 weeks in sediment from a pristine environment. Mixed microbial communities have the most powerful biodegradative potential because the genetic information of more than one organism is necessary to degrade the complex mixtures of organic compounds present in the contaminated areas (Pumphrey et al. 2007). This was reflected in the consortia proving to be superior to the individual isolates, showing 89.15% degradation at the end of seven days (Fig. 3). Amongst the individual isolates *Pseudomonas putida* degraded 76.27% of naphthalene at the end of seven days. The rate of degradation increased gradually over a period of seven days, though the degradation observed at the end of 48 h was lesser compared to the degradation in the liquid medium. The degradation observed at the end of 48 h in liquid medium was nearly equivalent to degradation observed on day five in the soil (58.56% for *Burkholderia glathei*, 62.18% for *Alcaligenes denitrificans* and 65.46% for *Pseudomonas putida*). The decreased degradation in soil can be contributed to the presence of organic matter in soil and sorption of naphthalene to the soil making it unavailable to the degrading organisms, despite the addition of Fenton's reagent. The degradation of chemicals in the environment can be affected by several factors which may differ among ecosystems, such as organic and inorganic nutrient levels, temperature, pH, previous chemical exposure, microbial adaptations and oxygen tension. In soil, PAHs are heterogeneously distributed

Table 1: Effect of addition of nitrogen source on naphthalene biodegradation.

Nitrogen source	mg ofnitrogen/100 mg of naphthalene (C:N)	Percent degradation		
		Burkholderia glathei	*Alcaligenes denitrificans*	*Pseudomonas putida*
NH_4NO_3	5 (18.6:1)	43.50 ± 1.10	45.00 ± 1.10	46.36 ± 1.12
	10 (9.3:1)	48.20 ± 1.18	49.10 ± 1.03	52.35 ± 1.16
	15 (6.2:1)	46.30 ± 1.05	47.80 ± 0.98	50.21 ± 1.21
	20 (4.65:1)	38.50 ± 1.00	39.10 ± 0.79	42.18 ± 1.12
	25 (3.72:1)	36.80 ± 0.95	37.00 ± 0.84	39.15 ± 0.93
	50 (1.86:1)	33.90 ± 0.88	35.80 ± 0.98	37.64 ± 0.84
Urea	5 (18.6:1)	45.60± 1.01	46.90 ± 1.06	48.50 ± 1.15
	10 (9.3:1)	49.70 ± 1.16	51.50 ± 1.22	54.00 ± 1.18
	15 (6.2:1)	47.30 ± 1.13	49.36 ± 0.93	52.10 ± 1.15
	20 (4.65:1)	46.10 ± 1.11	47.90 ± 1.08	48.20 ± 1.12
	25 (3.72:1)	45.23 ± 1.08	45.25 ± 1.09	46.90 ± 1.15
	50 (1.86:1)	41.16 ± 1.00	44.18 ± 1.10	45.20 ± 1.05
NH_4Cl	5 (18.6:1)	42.16 ± 0.97	44.20 ± 1.05	51.20 ± 1.07
	10 (9.3:1)	46.28 ± 1.02	48.12 ± 1.25	52.00 ± 1.12
	15 (6.2:1)	45.60 ± 1.01	46.82 ± 1.16	48.90 ± 1.02
	20 (4.65:1)	43.12 ± 1.01	44.25 ± 1.04	45.60 ± 1.00
	25 (3.72:1)	42.74 ± 0.88	43.26 ± 1.02	45.60 ± 1.01
	50 (1.86:1)	40.20 ± 1.00	43.25 ± 0.97	45.20 ± 1.13

Values in parentheses indicate C: N ratios.

Table 2: Effect of addition of phosphorus source on naphthalene biodegradation

Source of Phosphorus	mg of phosphorus/100 mg of naphthalene	Percent degradation		
		Burkholderia glathei	*Alcaligenes denitrificans*	*Pseudomonas putida*
KH_2PO_4	0.07 (93:0.7)	46.56 ± 1.10	48.12 ± 1.13	49.96 ± 1.04
	0.08 (93:0.8)	51.96 ± 1.28	53.45 ± 1.05	54.24 ± 1.17
	0.09 (93:0.9)	54.24 ± 1.17	55.64 ± 1.22	56.63 ± 1.12
	0.10 (93:1)	46.65 ± 1.00	47.96 ± 1.14	49.30 ± 1.09
	0.11 (93:1.1)	45.20 ± 1.09	46.12 ± 1.00	48.30 ± 1.00
	0.12 (93:1.2)	42.56 ± 1.01	43.21 ± 1.04	47.00 ± 1.05
Na_2HPO_4	0.07 (93:0.7)	45.56 ± 1.16	46.23 ± 1.16	47.56 ± 1.06
	0.08 (93:0.8)	51.96 ± 1.14	51.24 ± 1.18	52.13 ±1.23
	0.09 (93:0.9)	54.21 ± 1.21	53.14 ± 1.23	54.32 ± 1.18
	0.10 (93:1)	46.65 ± 1.01	46.20 ± 1.15	47.30 ± 1.15
	0.11 (93:1.1)	45.20 ± 1.06	44.30 ± 1.04	46.50 ± 1.07
	0.12 (93:1.2)	42.56 ± 1.02	42.30 ± 1.01	45.36 ± 1.03

Values in parentheses indicate C:P ratios.

and may be absorbed inside of organic particles, located in small pores that are inaccessible for bacteria, or otherwise occluded by the multitude of solid soil constituents (Heitkamp et al. 1987). The results obtained in the present study, however, showed between 8.46 % and 14.86 % degradation at the end of 24 h.

Treating large amounts of hydrocarbon contaminated liquid or soil in bioreactors is technically challenging and costly. Bioaugmentation (i.e. seeding the soil with hydrocarbon-degrading microorganisms such as fungal and/or bacterial cells) can circumvent many technical difficulties, but to date releasing free cells into a polluted environment has produced variable results. Failure of hydrocarbon degradation has often been attributed to the inability of the introduced bacteria to compete with the indigenous microorganisms that are better adapted to the rapidly changing environmental conditions (Alexander 1999). Therefore, it is suggested that immobilizing the bacteria in non-liquid inocula can achieve more homogenous conditions and better protection of the cells. An effective approach to finding a solution to continuous contamination is to concentrate these biodegraders on the natural or synthetic supports with high porosity thus allowing their use in different kinds of bioreactors (fluidized bed, packed bed). Immobilization presents many advantages such as the presence of high population density in a limited reactor volume, high conversion rates, limiting substrate inhibition and toxicity to micro-organisms by diffusional limitations, reusability and reduction in the overall cost (Trevor & Elsas 1992). Immobilization would also have the added benefit of

reducing the cells' exposure to the elements, thus preventing them from being washed away, particularly in an open water system. Immobilized materials, furthermore, have comparatively longer operating lifetimes due to an enhanced stability of the macromolecules or cells and, consequentially, protection from adverse conditions. Various methods have been described for the immobilization of bacteria and entrapment within polymeric gel matrices has often been successful. The most frequently used matrices are κ-carrageenan, alginate and polyacrylamide. Alginate, however, represents several advantages such as high porosity, chemical stability and low cost. It is a mild, fast and simple immobilization method. Diaz and co-workers (2001) reported that immobilization of bacterial cells significantly enhanced the biodegradation rate of crude oil compared to free-living cells over a wide range of culture medium salinity. Immobilizing agents offer cellular protection against adverse environmental conditions. In the present investigation, the three naphthalene degraders along with the consortia were immobilized and their ability to degrade naphthalene was followed over a period of seven days (1, 2, 3, 5 and 7 days). The rate of degradation increased steeply on day two followed by a gradual increase thereafter (Fig. 4). The immobilized cells degraded naphthalene to a greater extent compared to the free cells. Immobilized *Burkholderia glathei* showed an increase of 5.09 % in the rate of degradation, while *Alcaligenes denitrificans* and *Pseudomonas putida* showed an increase of 4.67 % and 7.46% respectively at the end of 48 h. Consortia again proved to be superior to the individual isolates showing 98% degradation of naphthalene at the end of seven days as compared to the individual isolates viz., *Burkholderia glathei* (71.16%), *Alcaligenes denitrificans* (76.25%) and *Pseudomonas putida* (89.12%). The effect of storage on the viability of the immobilized cells (initial inoculum 1×10^8 cells/mL) after storing for 15 days at 4°C was analysed and compared to the viability of freshly-made beads. The results showed no appreciable loss of viability indicating that storage at 4°C for 15 days did not affect the viability of the immobilized cells adversely. In a similar study Feijoo-Siota et al. (2008), showed that the immobilized cells can be stored at 4°C for at least one month with no appreciable loss of viability, whereas storage at -20°C was not as suitable. This is advantageous for practical use of the beads, because it shows that they do not require any special or expensive storage conditions.

The results of this work indicate that bioremediation technology has an immense potential for decontamination of polluted natural environments and concludes that successful application of bioremediation depends on hydrocarbon degrading microorganisms and *in situ* environmental conditions.

REFERENCES

Aislabie, J., Fraser, R., Duncan, S. and Farrell, R.L. 2006. Effects of oil spills on microbial heterotrophs in Antarctic soils: Potential for biodegradation of hydrocarbons in soil from the Ross Dependency, Antarctica. Polar Biol., 24: 308-313.

Alexander, M. 1991. Research needs in bioremediation. Environ. Sci. Technol., 25: 1972-1973.

Alquati, C., Papacchini, M., Riccardi, C. and Bestetti, G. 2005. Diversity of naphthalene degrading bacteria from petroleum contaminated soils. Annals Microbiol., 50(4): 237-242.

Atlas, R.M. 1981. Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiol. Rev., 45: 180-209.

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

Braddock, J. F., Ruth, M. L., Catterall, P. H., Walworth, J. L. and McCarthy, K.A. 1997. Enhancement and inhibition of microbial activity in hydrocarbon-contaminated Arctic soils: Implications for nutrient amended bioremediation. Environ. Sci. Technol., 31(7): 2078-2084.

Cooney, J.J. 1984. The fate of petroleum pollutants in freshwater ecosystems. In: Atlas, R.M. (ed.), Petroleum Microbiology. Macmillan Publishing Co., New York, pp. 399-434.

Diaz, E., Ferrandez, A., Prieto, M.A., and Garcia, J.L. 2001. Biodegradation of aromatic compounds by *Escherichia coli*. Microbiol. Mol. Biol. Rev., 65(4): 523-569.

Dibble, J.T. and Bartha, R. 1979. Effect of environmental parameters on biodegradation of oil sludge. Appl. Environ. Microbiol., 37: 729-739.

Feijoo-Siota. L., Rosa-Dos-Santos, F., de Miguel, T. and Villa, T.G. 2008. Biodegradation of naphthalene by *Pseudomonas stutzeri* in marine environments: Testing cells entrapment in calcium alginate for use in water detoxification. Biorem. J., 12(4):185-192.

Giesy, J.P. 1980. Isolation of bacteria degrading polycyclic aromatic hydrocarbons (PAHs) in a sub-Antarctic soil. J. Envir. Eng., 125(9): 874-890.

Grimm, A.C. and Harwood, C.S. 1999. NahY, a catabolic plasmid-encoded receptor required for chemotaxis of *Pseudomonas putida* to the aromatic hydrocarbon naphthalene. J. Bacteriol., 181(10): 3310-3316.

Heitkamp, H.A., Freeman, J.P., and Cerniglia, C.E. 1987. Naphthalene biodegradation in environmental microcosms: Estimates of degradation rates and characterization of metabolites. Appl. Environ. Microbiol., 53(1): 129-136.

Horng, R. S., Kuei, C. and Chen, W. 2009. Degradation of naphthalene in aquatic systems. J. Environ. Engg., 135(9): 854-860.

Jeon, C. W., Park, W., Ghiorse, W. C. and Madsen, E. L. 2004. *Polaromonas naphthalenivorans* sp. nov., a naphthalene-degrading bacterium from naphthalene-contaminated sediment. Int. J. Syst. Evol. Microbiol., 54: 93-97.

Johnsen, A.R., Wick, L.Y. and Harms, H. 2005. Principles of microbial PAH-degradation in soil. Environ. Pollut., 133: 71-84.

Leys, N.M., Bastiaens, L., Verstraete, W. and Springael, D. 2004. Influence of the carbon/nitrogen/phosphorus ratio on polycyclic aromatic hydrocarbon degradation by *Mycobacterium* and *Sphingomonas* in soil. Environ. Microbiol. Biotech., 66(6): 726-736.

Malakul, P., Srinivasan, K.R. and Wang, H.Y. 1998. Metal toxicity reduction in naphthalene biodegradation by use of metal-chelating adsorbents. Appl. Environ. Microbiol., 64(11): 4610-4613.

Marta, I., Alquati, C., Morgia, P., Mansi, A., Scotti, R., Nicolini, L. and Papacchini, M. 2006. Contaminated sites: Assessment of the metabolism, growth and genetic characterization of wild-type microbial strains able to degrade naphthalene. Prev. Today, 2(3-4): 35-50.

Martens, D.A. and Frankenberger, W.T. Jr. 1995. Enhanced degradation of polycyclic aromatic hydrocarbons in soil treated with an advanced oxidative process-Fenton's reagent. J. Soil Contam., 4(2): 175-190.

Nnamchi, C.I., Obeta, J.A.N. and Ezeogu, L.I. 2006. Isolation and characterization of some polycyclic aromatic hydrocarbon degrading bacteria from Nsukka soils in Nigeria. Int. J. Environ. Sci. Technol., 3(2): 181-190.

Pathak, H., Kantharia, D., Malpani, A. and Madamwar, D. 2008. Naphthalene degradation by *Pseudomonas* sp. HOB1: *In vitro* studies and assessment of naphthalene degradation efficiency in simulated microcosms. Environ. Poll., 103: 77-84.

Pumphrey, G.M. and Madsen, E.L. 2007. Naphthalene metabolism and growth inhibition by naphthalene in *Polaromonas naphthalenivorans* strain CJ2. Microbiol., 153: 3730-3738.

Thavasi, R., Jayalakshmi, S., Balasubramanian, T. and Banat, I.M. 2007. Effect of salinity, temperature, pH and crude oil concentration on biodegradation of crude oil by *Pseudomonas aeruginosa*. J. Biol. Environ. Sci., 1(2): 51-57.

Tikilili, P.V. and Chirwa, M.N. 2009. Microbial degradation of recalcitrant organics from radioactive waste using indigenous cultures of naphthalene degrading bacteria. Chem. Engg. Trans., 18: 845-850.

Trevor, J.T. and Elsas, J.D. 1992. Use of alginate and other carriers for encapsulation of microbial cells. Microb. Release, 1: 61-69.

Van der Meer, J.R. 2006. Environmental pollution promotes selection of microbial degradation pathways. Front. Ecol. Environ., 4(1): 35-42.