Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 98 (2007) 1339–1345
BIORESOURCE TECHNOLOGY

Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India

Kishore Das, Ashis K. Mukherjee *

ONGC-Centre for Petroleum Biotechnology, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784 028, India

Received 21 February 2006; received in revised form 19 May 2006; accepted 22 May 2006
Available online 7 July 2006

Abstract

The efficiency of *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM strains isolated from a petroleum contaminated soil sample from North-East India was compared for the biodegradation of crude petroleum-oil hydrocarbons in soil and shake flask study. These bacterial strains could utilize crude petroleum-oil hydrocarbons as sole source of carbon and energy. Bioaugmentation of TPH contaminated microcosm with *P. aeruginosa* M and NM consortia and *B. subtilis* strain showed a significant reduction of TPH levels in treated soil as compared to control soil at the end of experiment (120 d). *P. aeruginosa* strains were more efficient than *B. subtilis* strain in reducing the TPH content from the medium. The plate count technique indicated expressive growth and biosurfactant production by exogenously seeded bacteria in crude petroleum-oil rich soil. The results showed that *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains could be effective for in situ bioremediation.

Keywords: *Bacillus subtilis*; Biodegradation; Bioremediation; Biosurfactant; Polyaromatic hydrocarbon (PAH); *Pseudomonas aeruginosa*; Total petroleum hydrocarbon (TPH)

1. Introduction

Oil pollution accidents are nowadays become a common phenomenon and have caused ecological and social catastrophes (Burger, 1993; Burns et al., 1993; Shaw, 1992). Apart from accidental contamination of ecosystem, the vast amounts of oil sludge's generated in refineries from water oil separation systems and accumulation of waste oily materials in crude oil storage tank's bottoms pose severe problem because many of the standard treatment processes used to decontaminate soil and groundwater have been limited in their application, are prohibitively expensive, or may be only partially effective (Ferrari et al., 1996; Nicholas, 1987; Vasudevan and Rajaram, 2001). Therefore, despite decades of research, successful bioremediation of petroleum hydrocarbon contaminated soil remains a challenge (Rahman et al., 2003).

Both in situ and on-site treatment processes by involving the use of microorganisms to break down hazardous organic environmental contaminants avoid the economic and technical disadvantages (Ahlert and Kosson, 1983; Lee and Ward, 1985). Strains of *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM, isolated from a petroleum contaminated soil sample of North-East India (ONGC oil field) grew on a large number of hydrocarbon compounds as a source of carbon and energy demonstrating these strains might be efficient hydrocarbon degraders (Das and Mukherjee, 2005; Mukherjee and Das, 2005). These strains have been efficient producers of biosurfactants in hydrocarbons rich culture medium. There are reports describing the effect of exogenously added microbial biosurfactants in enhancing the bioremediation of

* Corresponding author. Tel.: +91 3712 267007/267008x5405; fax: +91 3712 267006.
E-mail address: akm@tezu.ernet.in (A.K. Mukherjee).

0960-8524/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2006.05.032

crude oil-polluted soils by indigenous microbes (Abalos et al., 2004; Cubitto et al., 2004).

The purpose of this study was to explore and compare the efficiency of thermophilic strains of *B. subtilis* DM-04 and *P. aeruginosa* M and NM indigenous to North-East India in biodegradation of crude petroleum-oil.

2. Methods

2.1. Chemicals

Aliphatic hydrocarbons (octane, dodecane, hexadecane, octadecane) and aromatic hydrocarbons [pyrene (PYR), phenanthrene (PHE) and anthracene (ANT)] were purchased from Merck–Schuchardt, Germany. Acetone and hexane were purchased from Merck India Ltd., India. Standard aliphatic hydrocarbons were procured from Sigma–Aldrich, USA. Crude petroleum-oil contaminated soil sample was collected from Oil and Natural Gas Commission of India (ONGC) oil field, Assam.

2.2. Microorganisms and maintenance

Strains of *B. subtilis* DM-04, *P. aeruginosa* M and NM strains were used and maintained as described earlier (Das and Mukherjee, 2005; Mukherjee and Das, 2005).

2.3. Isolation of biosurfactant

Crude biosurfactant from *B. subtilis* DM-04 and from *P. aeruginosa* M and NM strains were isolated by acid precipitation of cell free fermentative broth followed by its extraction with dichloromethane or methanol (Mukherjee and Das, 2005), and by acetone precipitation followed by acidification by 6.0 N HCl (Das and Mukherjee, 2005), respectively. The biosurfactants thus obtained were dried in vacuum, weighed and expressed in g/l of the cell free culture broth.

2.4. PAH solubilization effect of biosurfactant

PAH solubilization assay was done as described by Barkay et al. (1999). Briefly, any of the following 0.6 μg of ANT, or 6.0 μg of PHE or PYR (from 0.6 mg/ml stock in acetone) was distributed into glass test tube (10 mm × 170 mm) and kept open inside an operating chemical fume hood to remove the solvent, followed by addition of 3.0 ml of assay buffer (20 mM Tris–HCl, pH 7.0) and graded amounts of biosurfactant (125–500 μg/ml) obtained from respective bacterial strain used in this study. Tubes were capped with plastic closures and incubated in a vertical position overnight at 30 °C with shaking (200 rpm) in dark. Samples were filtered through 1.2 μm filters (Whatman) and 2.0 ml filtrate was removed in a clean tube to which 2.0 ml of hexane was added prior to extraction by vortexing for 2 min. This emulsion was centrifuged at 10,000 rpm for 10 min to separate the aqueous and hexane phases. PAH in the hexane extracts was measured spectrophotometrically (Hitachi U-2001) at 252, 250 or 273 nm for PHE, ANT or PYR, respectively. From a calibration curve of individual PAH (in hexane), the concentration of each PAH was determined. Assay buffer with biosurfactants but without PAH was extracted with hexane identically and served as blank. Control was also run in parallel where no biosurfactant was added to PAH before extraction with hexane.

2.5. Determination of bacterial growth in presence of crude petroleum-oil hydrocarbons

Replicates batch cultures ($n = 3$) were grown in 250 ml Erlenmeyer flasks containing 100 ml of mineral-salts medium (Das and Mukherjee, 2005) supplemented with crude petroleum-oil (measured and autoclaved) at a final concentration of 2.0% (v/v). Incubation was performed either at 45 °C temperature and pH 7.0 for *P. aeruginosa* M and NM strains (Das and Mukherjee, 2005) or at 55 °C temperature and pH 8.0 for *B. subtilis* DM-04 strain (Mukherjee and Das, 2005) with 200 rpm rotary shaking. Although these bacteria can grow on a wide range of temperature (25–55 °C) and pH (6.5–8.5), however the experiments were performed at the optimum growth temperature and pH of the respective strain. Un-inoculated flasks and flasks without crude petroleum-oil were served as control. Bacterial growth by utilizing petroleum hydrocarbons was assessed by measuring the bacterial cell population (viable count), protein concentration (Lowry et al., 1951), dry biomass (Makkar and Cameotra, 1998) and gravimetric measurement of residual crude oil remained in the culture medium (Okoh et al., 2001) post 120 h of inoculation.

2.6. Biodegradation of petroleum hydrocarbons in soil

The ability of the bacterial strains to remediate the petroleum crude-oil contaminated soil sample was investigated by carrying out the biodegradation experiment in soil for 120 d under indoor laboratory condition. Ten kilogram of crude petroleum-oil contaminated soil sample (level of TPH contamination was detected as describe below), was layered in rectangular trays of 30 cm × 45 cm × 10 cm (length × breadth × height) sizes. Prior to starting the biodegradation experiment, the water-holding capacity of the soil was determined and the soil samples were treated with aqueous solutions (1.0 mg/ml) of biosurfactants obtained from the respective bacterial strains at a dose of 10 ml biosurfactant solution per kg of soil and then left for 3 d (temperature ~37 °C and 80–89% humidity). The soil was then seeded with 1 l of mineral-salts media (Das and Mukherjee, 2005) containing 1×10^7 cfu of either *B. subtilis* DM-04 strain, or *P. aeruginosa* strains M and NM (mixed in a proportion of 1:1). Each experiment was run in triplicate. The soil was thoroughly trilled and M9 medium was added at every 2 weeks interval to maintain the moisture level in the soil. One litre of glucose solution (100 mg/l) was supple-

mented to each tray (ten kg of soil) at an interval of 30 d (decided on the basis of water-holding capacity of the soils) and continued up to 90 d. To determine the extent of biodegradation, the soil-phase TPH concentrations were analyzed after 120 d. A control was run in parallel where the soil was treated with un-inoculated medium.

Total microbial count (viable cell count) of the soil on the onset and after the end of the experiment was determined with standard dilution plating technique using sterile nutrient agar (Difco) growth medium. Survival of *B. subtilis* DM-04, *P. aeruginosa* M and NM strains in the petroleum hydrocarbon rich soil was determined by comparing the colony morphology and PCR-RFLP as described previously (Das and Mukherjee, 2005; Mukherjee and Das, 2005).

2.7. Chemical analysis of crude petroleum-oil from soil sample and moisture content of soil

Total petroleum hydrocarbon (TPH) was extracted from 10 g of soil by sequentially extraction with 100 ml of hexane, methylene chloride, and chloroform (Mishra et al., 2001). All the three extracts were pooled and dried at room temperature by evaporation of the solvents under a gentle nitrogen stream in a fume hood. After evaporation of the solvent, the amount of residual TPH was determined gravimetrically (Mishra et al., 2001). Further fractionation of TPH into aromatic, aliphatic, asphaltene and NSO (nitrogen, sulfur and oxygen-containing compounds) was done by silica gel column chromatography followed by gravimetric analysis (Walker et al., 1975). TPH extracts were dissolved in *n*-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on the top of a silica gel G (60–120 mesh) column (2 cm × 30 cm) and eluted with solvents of different polarities. The alkane fraction was eluted with 100 ml each of hexane followed by eluting the aromatic fraction with 100 ml of toluene. The NSO fraction was eluted with 100 ml of methanol and chloroform (Mishra et al., 2001).

For the determination of moisture content, 100 g of petroleum-oil contaminated soil samples were dried at 60 °C oven for overnight and then kept in a dessicator. The samples were then crushed in a mortar, sieved from a 36-mesh sieve, redried and weighed. The loss in weight was calculated as the moisture content of the soil.

2.8. Analysis of alkane fraction by gas chromatography

The alkane fraction was analyzed by gas chromatography (GC) using a FID detector (Varian Saturn 3800). The column was CPSil 8 low bleed (30 m × 0.25 mm × 0.25 μm) coupled with a CP-Sil 5 CB low bleed/MS (30 m × 0.25 mm × 0.25 μm) column with helium as a carrier gas. The column temperature was 80–240 °C for 30 min with 5 °C min^{-1} increment and hold at 240 °C for 30 min, the injector temperature was 240 °C and the transfer line temperature was 300 °C. Individual components present in the alkane fraction were determined by matching the retention time with authentic standards.

2.9. Statistical analysis

Statistical analysis was done by Student's "*t*" test.

3. Results

3.1. Growth characteristics of microbes and production of biosurfactants in crude petroleum-oil hydrocarbons

The bacterial strains utilized crude petroleum-oil hydrocarbons as sole source of carbon and energy, which was evident from the increase in bacterial dry biomass, protein content and cell density after 120 h of incubation (Table 1). *B. subtilis* DM-04, *P. aeruginosa* M and NM strains produced 0.65, 0.80 and 0.70 g/l biosurfactant, respectively (Table 1). Biomass was increased with incubation time in all cases, reaching maximal density that ranged from

Table 1
Growth of and biosurfactant production by *B. subtilis* DM-04, *P. aeruginosa* M and NM strains on crude petroleum-oil hydrocarbons

Growth time (h)	Dry biomass (g/l)	Cells/ml	Protein content (mg/ml)	Yield of biosurfactant (g/l)
Bacillus subtilis DM-04				
0	0.1	1.0×10^5	N.D.	0
24	0.2	2.0×10^5	0.2	0.11
72	0.71	5.0×10^5	0.6	0.42
120	0.90	6.6×10^5	1.0	0.65
Pseudomonas aeruginosa M				
0	0.1	1.0×10^5	N.D.	0
24	0.30	3.0×10^5	0.3	0.16
72	0.84	6.0×10^5	0.8	0.50
120	1.4	8.0×10^5	1.2	0.80
Pseudomonas aeruginosa NM				
0	0.1	1.0×10^5	N.D.	0
24	0.2	2.0×10^5	0.3	0.12
72	0.73	5.0×10^5	0.72	0.46
120	1.0	7.5×10^5	1.15	0.70

Results recorded at 0 h and 120 h of bacterial growth. Values represent the average of three experiments.

6.6×10^5 cell/ml to 8.0×10^5 cell/ml in 5 days. TPH degradation pattern showed that *B. subtilis* reduced 1.9 ± 0.2 mg (mean ± SD, $n = 3$) of TPH content, whereas *P. aeruginosa* M and NM strain reduced 2.7 ± 0.3 mg (mean ± SD, $n = 3$) and 2.3 ± 0.1 mg (mean ± SD, $n = 3$) of TPH contents, respectively. Given that carbon accounted for approximately 50% of cells dry weight (Vila et al., 2001) and considering the growth of bacteria in the absence of crude petroleum-oil (carbon source) was negligible (data not shown), it indicated an assimilation of approximately 24%, 26% and 22% of TPH as cellular carbon in *B. subtilis*, *P. aeruginosa* M and NM strains, respectively. No significant decline in pH of medium was noticed in all cases post 120 h of bacterial growth (data not shown).

3.2. PAH solubilization effect of biosurfactants

In general, the crude biosurfactants from all the three strains enhanced the apparent solubility of PAH in a dose dependent manner (Table 2). However, solubilization of pyrene by biosurfactants from all the three bacterial strains (~5 time higher apparent solubility compared to control) was significantly higher when compared with the phenanthrene or anthracene solubilization effect of biosurfactants (2–3 times higher compared to control). Moreover, the crude biosurfactant produced by *P. aeruginosa* NM strain displayed higher pyrene solubilization effect ($p < 0.05$) compared to crude biosurfactants produced by *P. aeruginosa* M and *B. subtilis* DM-04 strains (Table 2). On the other hand, crude biosurfactant from *B. subtilis* DM-04 strain at a dose of 500 μg/ml demonstrated significantly higher anthracene solubilization effect compared to the same property displayed by crude biosurfactants from *Pseudomonas* strains (Table 2).

Table 2
Dose dependent solubilization of PAHs by crude biosurfactants isolated from *B. subtilis* DM-04, *P. aeruginosa* M and NM strains

Source and concentration of crude biosurfactant (μg/ml)	Solubility of PAHs (ng/ml)		
	Pyrene	Phenanthrene	Anthracene
B. subtilis DM-04			
0	130.0 ± 30.0	500.0 ± 50.0	40.0 ± 10.0
125	520.0 ± 30.0	600.0 ± 50.0	60.0 ± 10.0
250	860.0 ± 31.0	700.0 ± 63.0	100.0 ± 19.0
500	1000.0 ± 29.0	900.0 ± 70.0	140.0 ± 35.0
P. aeruginosa M			
0	130.0 ± 30.0	500.0 ± 51.0	40.0 ± 10.0
125	500.0 ± 50.0	600.0 ± 100.0	50.0 ± 10.0
250	800.0 ± 50.0	800.0 ± 100.0	90.0 ± 17.0
500	900.0 ± 80.0	1000.0 ± 200.0	110.0 ± 20.0
P. aeruginosa NM			
0	130.0 ± 30.0	500.0 ± 50.0	40.0 ± 10.0
125	610.0 ± 30.0	700.0 ± 100.0	50.0 ± 11.0
250	800.0 ± 30.0	900.0 ± 100.0	90.0 ± 23.0
500	1200.0 ± 35.0	1100.0 ± 100.0	110.0 ± 21.0

Experiments were done as described in the text. Results represent mean ± SD of three independent experiments.

3.3. Analysis of TPH content and viable cell count of soil before bioremediation

The soil sample selected for bioremediation experiment was loamy and dark brown in color. The percent moisture content of the soil was determined as 18 ± 2 ($n = 3$). The pH of the soil was 6.8–7.0 before the biodegradation experiment (time zero) and did not change significantly at the end of experiment. The water-holding capacity of the soil increased from 55% to 68% at the end of experiment. The level of TPH contamination in soil was detected as 84.0 g kg^{-1} of soil and alkanes (55.0%) represented the largest constituent of the solvent extracted TPH, followed by aromatic fraction (20.0%), asphaltene fraction (16.0%) and then NSO (7%) fraction (Table 3). Indigenous bacterial population (viable cell count) of soil was determined as 1×10^4 cfu/g of soil.

3.4. Biodegradation of TPH and various fractions of crude petroleum-oil in soil

Bioaugmentation of TPH-contaminated microcosm with *P. aeruginosa* M and NM consortium and *B. subtilis* strain showed that TPH levels were reduced from 84 to 21

Table 3
Biodegradation of various fractions of crude petroleum-oil hydrocarbons in soil by *B. subtilis* DM-04 and *P. aeruginosa* M and NM consortia

Contaminants	Level of contamination in soil (g/kg of soil)						
	Before the experiment (0 d)	After the experiment					
		60 d post treatment			120 d post treatment		
		None (control)	*B. subtilis* DM-04	*P. aeruginosa* M and NM	None (control)	*B. subtilis* DM-04	*P. aeruginosa* M and NM
TPH	84.0 ± 0.5	83.0 ± 0.5	60.0 ± 1.2[a]	42.0 ± 1.1[a,b]	80.0 ± 0.1	39.0 ± 0.8[a]	21.0 ± 0.8[a,b]
Alkane fraction	47.0 ± 0.1	46.0 ± 0.2	35.0 ± 2.1[a]	24.0 ± 1.2[a,b]	45.0 ± 0.2	21.0 ± 1.1[a]	9.0 ± 0.6[a,b]
Aromatic fraction	17.0 ± 0.2	17.0 ± 0.2	12.0 ± 0.9[a]	10.0 ± 0.4[a,b]	16.0 ± 0.4	8.0 ± 0.4[a]	6.0 ± 0.3[a,b]
NSO and asphaltene fraction	19.0 ± 0.3	19.0 ± 0.2	16.0 ± 0.8[a]	10.0 ± 0.5[a,b]	18.0 ± 0.4	10.0 ± 0.4[a]	7.0 ± 0.4[a,b]

Experiment was done as described in the text. Each value represents mean ± SD of results obtained from three individual experimental trays.
Significance of difference with respect to control experiment [a] $p < 0.001$.
Significance of difference with respect to biodegradation by *B. subtilis* DM-04 [b] $p < 0.001$.

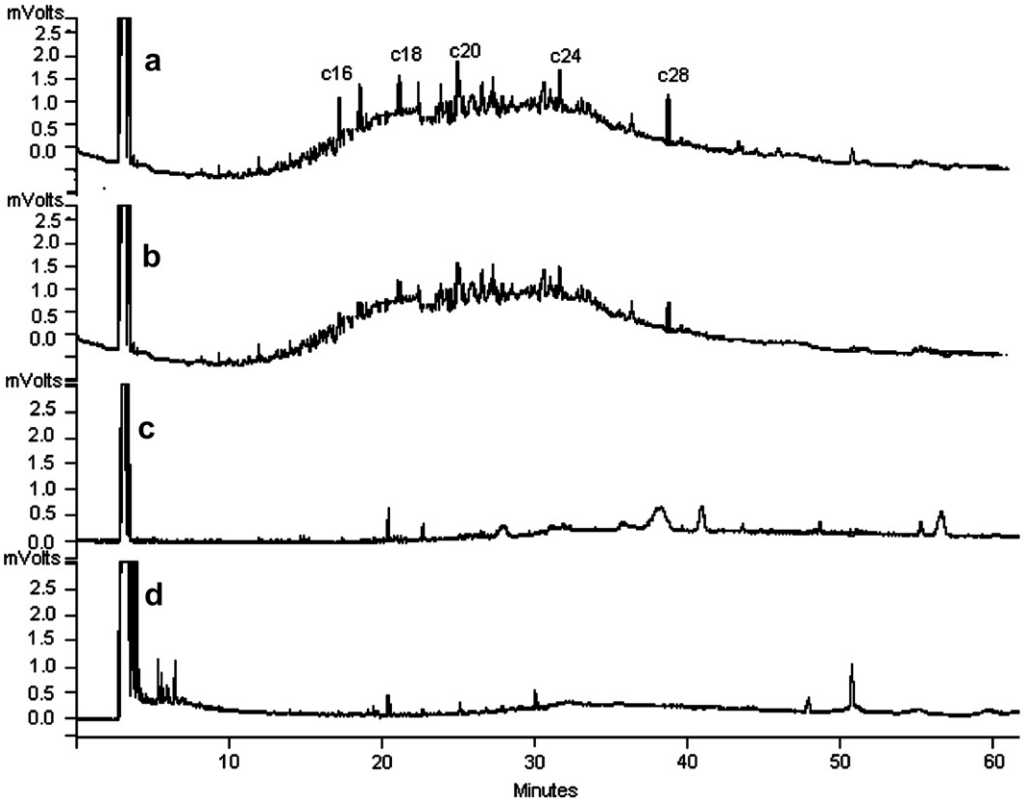

Fig. 1. GC fingerprinting of alkane fraction of soil seeded with *P. aeruginosa* M and NM strain consortia and *B. subtilis* DM-04 strain: (a) at 0 d (beginning of bioremediation experiment); (b) after 120 d without exogenously seed bacteria (control); (c) after 120 d with *B. subtilis* DM-04 strain; (d) after 120 d with *P. aeruginosa* M and NM strain.

and 39 g/kg of soil, respectively; in contrast, the TPH level was decreased to 80 g/kg in control soil (Table 3). Further, degradation of alkane, aromatic, NSO and asphaltene fractions by indigenous soil microbe (control soil) was insignificant compared to biodegradation of the same fractions by *B. subtilis* DM-04 and *Pseudomonas* consortia (Table 3). The plate count indicated expressive growth of exogenously added bacteria in petroleum-oil contaminated soil and their cell densities ranged from 1×10^{11} cfu/g soil (*B. subtilis* DM-04) to 2×10^{11} cfu/g soil (*P. aeuruginosa* M and NM). In contrast, biomass increased from 1×10^{4} cfu/g soil to 1×10^{6} cfu/g in control soil.

All the microbes in the present study exhibited higher biodegradation of *n*-alkanes fraction compared to aromatic or NSO fractions. As shown in Table 3, *Pseudomonas* consortia demonstrated significantly higher efficiency to biodegrade TPH as well as various other fractions of crude petroleum-oil hydrocarbons compared to *B. subtilis* DM-04 strain ($p < 0.001$).

3.5. Gas chromatographic analysis of alkane fraction

GC analyses of alkane fraction obtained from the petroleum-oil contaminated soil before (0 d) and at the end (120 d) of bioremediation experiment is depicted in Fig. 1. GC analyses showed that alkanes level was significantly reduced in petroleum-oil contaminated soil seeded with either *B. subtilis* DM-04 or *Pseudomonas* consortium.

4. Discussion

Survival of microorganisms in petroleum hydrocarbons medium after their inoculation is a key deciding factor in the rate of biodegradation of hydrocarbons either in soil or in liquid phase (Ramos et al., 1991). Since all the bacteria in the present study were isolated from a petroleum contaminated soil sample, they survived and adopted the oil-contaminated soil/liquid environment very easily as also reported by other authors (Rahman et al., 2003; Sugiura et al., 1997). This was evident from the significant increase ($p < 0.01$) in the population of *B. subtilis* and *P. aeruginosa* in soil as compared to control. However, higher growth rate ($p < 0.05$) of *Pseudomonas* strains compared to *Bacillus* strain might be related to higher breakdown and utilization of petroleum hydrocarbons by former strains. Besides, the necessity for seeding with hydrocarbon degrading bacteria might have arisen from the fact that indigenous microbes of soil were not efficient degraders of a wide range of complex compounds of crude petroleum-oil and therefore, introduction of efficient hydrocarbon degraders would be essential in order to effectively degrade all of the hydrocarbons in a complex petroleum mixture (Atlas, 1977). Since the supplementation of glucose as co-carbon source enhanced the rate

of biodegradation of PAH by bacterial strains used in this study (Das and Mukherjee, in press), glucose was added in the oil-contaminated soil after a regular interval to increase the rate of biodegradation of TPH by the seeded bacteria. However, present results were in contradiction to report of Chhatre et al. (1996) describing addition of nutrients in the soil was unlikely to have dramatic effect on the microbial degradation of crude oil.

TPH biodegradation potential of *B. subtilis* and *P. aeruginosa* strains from NE India were far higher than some previously reported values, even for mixed cultures (Chhatre et al., 1996; Del'Arco and de Franca, 2001; Obuekwe and Al-Zarban, 1998; Sugiura et al., 1997) and comparable to degradation of Nigerian crude-oil by *B. subtilis* and *P. aeruginosa* strains isolated from crude oil-polluted soil from Nigeria (Ilori and Amund, 2000). From the results on bacterial assimilation of about 26–22.0% of TPH carbon loss from growth, it could be concluded that all the TPH lost from the growth medium should not be considered as assimilated and same of it must have been catabolized and/or respired by bacteria. Further, in-contrast to the shake flask study, biodegradation experiment in soil was conducted at 37 °C temperature to ensure the capability of bacterial strain under study to degrade the TPH and its various fractions at an average outdoor temperature of soil in this region.

Biosurfactant secreted by bacteria are more effective than chemical surfactants in enhancing the solubility and biodegradation of petroleum hydrocarbons including PAH (Cybulski et al., 2003; Wong et al., 2004). Production of biosurfactant is related to the utilization of available hydrophobic substrates by the producing microbes from their natural habitat, presumably by increasing the surface area of substrates and increasing their apparent solubility (Ahimou et al., 2000; Maier, 2003; Mukherjee and Das, 2005; Ron and Rosenberg, 2001). However, there are only few reports of microorganisms producing surface-active compounds while growing on crude petroleum-oil hydrocarbons (Chhatre et al., 1996; MacElwee et al., 1990). In the present study, all the three bacteria were found to be efficient biosurfactant producers on petroleum hydrocarbons medium, as well as in soil (data not shown) which offered the advantage of a continuous supply of natural, non-toxic and biodegradable surfactants by bacteria at low cost for solubilizing the hydrophobic oil hydrocarbons prior to biodegradation. The differences in PAH solubilization effects of biosurfactant from different bacterial strains in this study could be related to the chemical nature as well as surface properties of biosurfactant. For example, major biosurfactants secreted by *B. subtilis* DM-04 were lipopeptide in nature containing higher amount of surfactins followed by iturins (Mukherjee and Das, 2005), whereas biosurfactant secreted by *P. aeruginosa* M and NM were a complex mixture of lipopeptides and glycoproteins (Das and Mukherjee, 2005). Moreover, the significantly higher PAH solubilization effect of biosurfactant from *P. aeruginosa* NM strain compared to *P. aeruginosa* M strain reinforces the hypothesis that a minor variation in biosurfactant isoforms between these two strains (Das and Mukherjee, 2005) might result in a large variations of the emulsification property and PAH solubilization effect of biosurfactant (Mukherjee and Das, 2005).

GC/FID analyses and other study demonstrated that *n*-alkanes (C_{14}–C_{30}) were preferentially degraded compared to PAHs present in crude petroleum-oil by all the bacteria used in this study. Crude petroleum-oil is a complex mixture of hydrophobic components like *n*-alkanes, aromatics, resins and asphalthenes and microorganisms are know to attack and degrade a specific component as compared with other components of oil. It has been observed that the same compounds in different crude oil samples were degraded to different extents by the same organisms (Sugiura et al., 1997), implying that the bioavailability of a particular compound in a crude oil sample and not it's chemical structure, may be a sole determining factor for effective biodegradation of the compound (Sugiura et al., 1997). Key factors such as presence of a specific and/or higher amount of inducible enzyme(s), substrate specificity of hydrocarbon degrading enzymes (Gibson and Subramanian, 1984; Kanaly and Harayama, 2000; Sharanagouda and Karegoudar, 2001) and presence of sphingolipids or other specific molecule(s) in the outer membrane structure of bacteria (Sugiura et al., 1997) may be responsible for higher metabolism of TPH by *Pseudomonas* strains compared to *B. subtilis* DM-04 strain.

In conclusion, the findings in this study showed that *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains could be useful in bioremediation of sites highly contaminated with crude petroleum-oil hydrocarbons. The thermophilic nature of these bacteria could add further advantage for their use in bioremediation of petroleum contaminated soils in tropical countries.

Acknowledgements

K. Das was the recipient of Senior Research Fellowship from ONGC-Centre for Petroleum Biotechnology, Tezpur University. This study was supported by financial grant from ONGC-Centre for Petroleum Biotechnology. Authors thank Prof. P.C. Deka, Chairman of ONGC Centre for Petroleum Biotechnology, and Vice-Chancellor, Tezpur University for his kind support to pursue this work.

References

Abalos, A., Vinas, M., Sabate, J., Manresa, M.A., Solanas, A.M., 2004. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT 10. Biodegradation 15, 249–260.

Ahimou, F., Jacques, P., Deleu, M., 2000. Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. Enzyme Microb. Technol. 27, 749–754.

Ahlert, R.C., Kosson, D.S., 1983. In situ and on-site biodegradation of industrial landfill leachate. Report to Dept. of the Interior, Washington, DC, on Contract No. 14-34-0001-1132. PB84-136787.

Atlas, R.M., 1977. Stimulated petroleum biodegradation. CRC Crit. Rev. Microbiol. 5, 371–386.

Barkay, T., Navon-Venezia, S., Ron, E.Z., Rosenberg, E., 1999. Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier Alasan. Appl. Environ. Microbiol. 65, 2697–2702.

Burger, A.E., 1993. Estimating the mortality of seabirds following oil-spills – effects of spill volume. Mar. Pollut. Bull. 26, 239–248.

Burns, K.A., Garrity, S.D., Levings, S.C., 1993. How many years until mangrove ecosystems recover from catastrophic oil-spills. Mar. Pollut. Bull. 26, 239–248.

Chhatre, S., Purohit, H., Shanker, R., Khanna, P., 1996. Bacterial consortia for crude oil spill remediation. Water Sci. Technol. 34, 187–193.

Cubitto, M.A., Moran, A.C., Commendatore, M., Chiarello, M.N., Baldini, M.D., Sineriz, F., 2004. Effects of *Bacillus subtilis* O9 biosurfactant on the bioremediation of crude oil-polluted soil. Biodegradation 15, 281–287.

Cybulski, Z., Dziurla, E., Kaczorek, E., Olszanowski, A., 2003. The influence of emulsifiers on hydrocarbon biodegradation by Pseudomondacea and Bacillacea strains. Spill Sci. Technol. Bull. 8, 503–507.

Das, K., Mukherjee, A.K., 2005. Characterization of biochemical properties and biological activities of biosurfactants produced by *Pseudomonas aeruginosa* mucoid and non-mucoid strains isolated from hydrocarbon-contaminated soil samples. Appl. Microbiol. Biotechnol. 69, 192–199.

Das, K., Mukherjee, A. K., in press. Differential utilization of pyrene as sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: Role of biosurfactants in enhancing the bioavailability. J. Appl. Microbiol.

Del'Arco, J.P., de Franca, F.P., 2001. Influence of oil contamination levels on hydrocarbon biodegradation in sandy sediment. Environ. Pollut. 112, 515–519.

Ferrari, M.D., Neirotti, E., Albornoz, C., Mostazo, M.R., Cozzo, M., 1996. Biotreatment of hydrocarbons from petroleum tank bottom sludge in soil slurries. Biotechnol. Lett. 18, 1241–1246.

Gibson, D.T., Subramanian, V., 1984. Microbial degradation of aromatic hydrocarbon. In: Gibson, D.T. (Ed.), Microbial Degradation of Organic Compounds, vol. 13. Marcel Dekker, Inc, New York, NY, pp. 181–242.

Ilori, M.O., Amund, D.I., 2000. Degradation of anthracene by bacteria isolated from oil polluted tropical soils. Z. Naturforsch. 55, 890–897.

Kanaly, R.A., Harayama, S., 2000. Biodegradation of high-molecular weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. 182, 2059–2067.

Lee, M.D., Ward, C.H., 1985. Environmental and biological methods for the restoration of contaminated aquifers. Environ. Toxicol. Chem. 4, 743–750.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

MacElwee, C.G., Lee, H., Trevors, J.T., 1990. Production of extracellular emulsifying agent by *Pseudomonas aeruginosa* UG1. J. Ind. Microbiol. 5, 25–32.

Maier, R.M., 2003. Biosurfactant: Evolution and diversity in bacteria. Adv. Appl. Microbiol. 52, 101–121.

Makkar, R.S., Cameotra, S.S., 1998. Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*. J. Ind. Microbiol. Biotechnol. 20, 48–52.

Mishra, S., Jyoti, J., Kuhad, R.C., Lal, B., 2001. In situ bioremediation potential of an oily sludge-degrading bacterial consortium. Curr. Microbiol. 43, 328–335.

Mukherjee, A.K., Das, K., 2005. Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. FEMS Microbiol. Ecol. 54, 479–489.

Nicholas, R.B., 1987. Biotechnology in hazardous-waste disposal: an unfulfilled promise. Am. Soc. Microbiol. News 53, 138–142.

Obuekwe, C.O., Al-Zarban, S.S., 1998. Bioremediation of crude oil pollution in Kuwaiti Dessert: The role of adherent microorganisms. Environ. Int. 24, 823–834.

Okoh, A., Ajisebutu, S., Babalola, G., Trejo-Hernandez, M.R., 2001. Potential of *Burkholderia cepacia* RQ1 in the biodegradation of heavy crude oil. Int. Microbiol. 4, 83–87.

Rahman, K.S.M., Thahira-Rahman, J., Kourkoutas, Y., Petsas, I., Marchant, R., Banat, I.M., 2003. Enhanced bioremediation of *n*-alkanes in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour. Technol. 90, 159–168.

Ramos, J.L., Duque, E., Ramos-Gonzalez, M.I., 1991. Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant TOL plasmid. Appl. Environ. Microbiol. 57, 260–266.

Ron, E.Z., Rosenberg, E., 2001. Natural roles of biosurfactants. Environ. Microbiol. 3, 229–236.

Sharanagouda, U., Karegoudar, T.B., 2001. Degradation of 2-methylnaphthalene by *Pseudomonas* sp. strain NGK 1. Curr. Microbiol. 43, 440–443.

Shaw, D.G., 1992. The Exxon-valdez oil-spill-ecology and social consequences. Environ. Conserv. 19, 253–258.

Sugiura, K., Ishihara, M., Shimauchi, T., Harayama, S., 1997. Physiochemical properties and biodegradability of crude oil. Environ. Sci. Technol. 31, 45–51.

Vasudevan, N., Rajaram, P., 2001. Bioremediation of oil sludge contaminated soil. Environ. Int. 26, 409–411.

Vila, J., Lopez, Z., Sabate, J., Minguillon, C., Solanas, A.M., Grifoll, M., 2001. Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: Actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 67, 5497–5505.

Walker, J.D., Colwell, R.R., Hamming, M.C., Ford, H.T., 1975. Extraction of petroleum hydrocarbons from oil-contaminated sediments. Bull. Environ. Contam. Toxicol. 13, 245–248.

Wong, J.W.C., Fang, M., Zhao, Z., Xing, B., 2004. Effect of surfactants on solubilization and degradation of phenenthrene under thermophilic conditions. J. Environ. Qual. 33, 2015–2025.