17

Bioremediation of Petroleum Sludge using Bacterial Consortium with Biosurfactant

K.S.M. Rahman[1], T.J. Rahman[2], I.M. Banat[2], R. Lord[1] and G. Street[1]

[1]Clean Environment Management Centre, School of Science and Technology, University of Teesside, Middlesbrough – TS13BA, Tees Valley, UK, Email: P.Rahman@tees.ac.uk; [2]Biotechnology Research Group, School of Biomedical Sciences, University of Ulster, Coleraine – BT52 1SA, Northern Ireland, UK

1. Introduction

Petroleum hydrocarbon continues to be used as the principle source of energy and hence an important global environmental pollutant. Apart from accidental contamination of the ecosystem, the vast amounts of oil sludge, generated in refineries from water oil separation systems and accumulation of waste oily materials in crude oil storage tank bottoms, pose great problems because of the expensive disposal methods (Ferrari et al. 1996; Vasudevan and Rajaram 2001). Despite decades of research, successful bioremediation of petroleum hydrocarbon contaminated soil remains a challenge. Petroleum is a complex mixture of non-aqueous and hydrophobic components like n-alkane, aromatics, resins and asphaltenes. Bioavailability might be the limiting factor in the biodegradation of such compounds.

Biosurfactants are amphiphilic compounds that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and a solid and increase the surface areas of insoluble compounds leading to increased mobility, bioavailability and subsequent biodegradation. They are produced by many bacterial strains that can degrade or transform the components of petroleum products. They are non-toxic, non-hazardous, biodegradable and environmentally friendly compounds (Banat et al. 2000), which may be produced cost effectively under *ex-situ* conditions, while *in-situ* production may be stimulated at the site of contamination and can be recovered and recycled (Moran et al. 2000). There have been recent successful reports on using them in enhanced oil recovery and in the release of bitumen from tar sands (Mulligan et al. 2001). Hence, reclamation of petroleum hydrocarbon polluted sites can be carried out by the bioremediation, which is an enhanced natural process of biodegradation, using biosurfactant producing and oil

degrading bacterial cultures. Bioremediation technologies generally aim at providing favourable conditions of aeration, temperature and nutrients to enhance biological hydrocarbon breakdown (Rahman et al. 2002a,b). In the present study, we investigated the effect of rhamnolipid biosurfactant (RL) produced by a *Pseudomanas aeruginosa* strain and addition of nutrients, such as nitrogen, phosphorus and potassium (NPK) and a bacterial consortium (BC) to augment natural fertility of the polluted site on the bioremediation of crude oil tank bottom sludge (TBS).

2. Methods

2.1 Soil and Microbial Cultures Preparation

Seashore sand samples from the Portrush coastal area of Northern Ireland and garden soil from the University of Ulster campus were collected. Both were sieved using a 1mm sieve and used at 1:1 ratio for the preparation of a composite soil sample. Part of the soil was sterilized in a hot air oven at 180^{o}C for 2 h and a part kept as normal condition (non-sterile). The sterility of the soil was confirmed by pour plate technique on plate count agar (Merck, UK). An oil degrading bacterial consortium containing five bacterial strains (*Micrococcus* sp. GS2-22 (21.7±1.4 x 10^5 CFU/ml), *Bacillus* sp. DS6-86 (30.3±0.9 x 10^5 CFU/ml), *Corynebacterium* sp. GS5-66 (27.4±4.7 x 10^5 CFU/ml, *Flavobacterium* sp. DS5-73 (18.9±3.6 x 10^5 CFU/ml), *Pseudomonas* sp. DS10-129 (32.6±0.8 x 10^5 CFU/ml) previously isolated on hydrocarbon containing medium were inoculated in 200 ml of nutrient broth and kept in a shaker for 24 h at room temperature. The strain name designated with GS was isolated from gasoline station and DS from diesel station soils, followed by its strain number, were depicted in our strains (Rahman et al. 2002a). Members of the bacterial consortium were selected depending on their efficiency of crude oil degradation (Rahman et al. 2002b). For the preparation of amendments, the rhamnolipid, produced by a *Pseudomonas aeruginosa* strain available at University of Ulster, was used.

2.2 Preparation of Amendments

To both sterile (sterilized in an oven at 180^{o}C for 3 h) and non-sterile soil samples, 10% and 20% of tank bottom sludge (TBS) with 87.4% oil and grease at pH 6.7 was added and mixed thoroughly. To find out the role of indigenous microbial populations present in soil and tank bottom sludge, controls were set up with sterile and non-sterile soil with no amendments. Other amendments containing bacterial consortium, NPK solution and rhamnolipid were set up to test the effects of these additives on biodegradation (Table 1).

Table 1. Preparation of the different treatments of sterile and non-sterile soil samples

Amendments	NS or SS (g)	TBS (%)	RL (mg)	NPK (mg)	BC (ml)	Moisture content (%)
NS +TBS	100	10 or 20				1.2
NS +TBS +RL	100	10 or 20	4			1.2
NS +TBS+NPK	100	10 or 20		0.1		1.2
NS +TBS+BC	100	10 or 20			1	1.2
NS +TBS+RL+NPK+BC	100	10 or 20	4	0.1	1	1.2
SS+TBS	100	10 or 20				1.2
SS+TBS +RL	100	10 or 20	4			1.2
SS+TBS+NPK	100	10 or 20		0.1		1.2
SS+TBS+BC	100	10 or 20			1	1.2
SS+TBS+RL+NPK+BC	100	10 or 20	4	0.1	1	1.2

NS - Non-sterile soil; SS - Sterile soil; TBS - Tank Bottom Sludge; BC - Bacterial Consortium; RL - Rhamnolipid; NPK - Nitrogen, Phosphorus and Potassium solution

The treatments were set up in sets of screw cap glass universal bottles as microcosms containing 10 g of soil samples and moisture content was adjusted at 12%. All microcosm tubes were incubated at 30°C. Triplicate sets of experimental samples were analysed at 0, 28, 56 and 84 days to enumerate total heterotrophic bacterial counts and to estimate protein content, percentage of n-alkane degradation, pH and surface tension (ST).

2.3 Enumeration of Bacterial Population

Total heterotrophic bacteria were enumerated by using a pour plate technique on plate count agar (Merck, UK) after 24 h incubation at 30°C, which also allowed growth of all members of the added bacterial consortium. Identity of the individual bacterial isolate was confirmed by biochemical test as described in our earlier report (Rahman et al. 2002a).

2.4 Total Protein Estimation

For the estimation of total protein, 1 ml supernatant without any soil particle was taken from soil: water mixture (1:10 ratio). It was centrifuged at 13000 rpm for 10 min and to the pellet obtained was added 1 ml of 3 N NaOH solution and boiled for 3 min. After cooling at room temperature, 1 ml of 1 M H_3PO_4 solution was added. A 50 µL aliquot was taken and mixed with 950 µL Coomassie protein assay reagent (Pierce, Rockford, USA) and incubated at 30°C for 10 min and the optical density was measured at 595 nm using UV-visible spectrophotometer (Shimadzu model no. UV – 2101PC, Shimadzu Europe Ltd., UK). The total protein was estimated using a standard curve prepared with albumin (Bradford 1976).

2.5 Characterization of Rhomnolipid using Mass Spectrometry

Rhamnolipid fraction from culture free supernatant was extracted by adding equal volume of Chloroform: Methanol (2:1) solvent mixture and mixed thoroughly. Then the organic layer was separated using separatory funnel, air dried and dissolved in methanol. Mass spectrometry characterization and detection of the rhamnolipid fractions under investigation were performed using an LCQ^{TM} quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA) with electrospray ionization (ESI). Standard solutions and samples under investigation were infused into the mass spectrometer at a flow rate of 10 μl/min. In the ESI, source nitrogen sheath and auxiliary gas flows were maintained at 50 and 5, respectively and referred to arbitrary values set by the software. The heated capillary temperature was 250^{o}C and the spray voltage set to 5 kV. Negative ion mode was used throughout and scans initiated over the 50-2000 m/z range.

2.6 Surface Tension Analysis and Measurement of pH

The surface tension of the soil extract (soil: water 1:10) was measured using a digital tensiometer (Kruss digital tensiometer model no. K9) equipped with a 6 cm De Nuoy platinum ring. To increase the accuracy, average of triplicates was used for the study. The pH of the soil extract (soil:water 1:10) was estimated using pH meter (Microcomputer pH meter model no. 6171, Jenco Instruments Inc., SanDiago, USA).

2.7 Hydrocarbon Estimation

The hexane soluble n-alkanes (nC8-nC40) in the soil samples were determined using gas chromatography (Perkin-Elmer GC model no. 8310). Soil and hexane (1:100 ratio) were mixed for 5 minutes in a vortex mixture and soil free hexane extract was separated using membrane filter and then used for GC analysis. A 30 m fused silica capillary column (Restek Corporation, USA) and GC with flame ionisation detector were used for analysis. The injection temperature was 250^{o}C; detector temperature 250^{o}C; column temperature was programmed as 50^{o}C/4 min, then increased at the rate of 10^{o}C/min to 330^{o}C and maintained at 330^{o}C for 20 min. Total recoverable petroleum hydrocarbon standard with purity of 99.9999% obtained from Restek Corporation, USA, was used to identify the n-alkanes. Degradation was estimated as the difference between the initial and final concentrations of the n-alkane fractions.

2.8 Statistical Analysis

The experiment was set up as a factorial design consisting of two concentrations they were 10% and 20% sludge contaminated soil x 10 treatments; 1) NS+TBS,

2) NS+TBS+RL, 3) NS+TBS+NPK, 4) NS+TBS+BC, 5) NS+TBS+RL+NPK+ BC, 6) SS+TBS, 7) SS+TBS+RL, 8) SS+TBS+NPK, 9) SS+TBS+BC, 10) SS+TBS+RL+NPK+BC x four time periods (0, 28, 56 & 84 days) x three replicates per treatment. Statistical analysis was carried out using Analysis of Variance (ANOVA). Mean of the various treatments were tested for level of significance at 1% and 5% probability by Duncan's multiple range test (DMRT) (Gomez and Gomez 1984).

3. Results and Discussion

3.1 Effect of Bacterial growth on Biodegradation

Sandy soil was used along with garden soil to increase the porosity and thus aeration for enhanced bioremediation. An initial bacterial population of about $2.1\pm0.7 \times 10^3$ CFU/g was observed in the non-sterile soil amended with 10% of tank bottom sludge. Low bacterial numbers may be because of the use of sandy soil with low nutrients and microflora. An increase in bacterial population was encountered in all amended soil samples particularly with rhamnolipid solution (Table 2). This may be due to the biosurfactant induced desorption of hydrocarbons from soil to the aqueous phase of soil slurries leading to increased microbial mineralization, either by increasing hydrocarbon solubility or by increasing the contact surface with hydrophobic compounds (Moran et al. 2000; Rahman et al. 2002d). Two orders of magnitude increase in the bacterial population were observed in soil samples amended with 10% petroleum TBS after 56 days of incubation. The available nutrients were rapidly assimilated by soil microbes, thus depleting the nutrient reserves. In fact the objective of augmenting NPK solution to the soil samples was to restore the availability of essential nutrients. Several researchers have also described an increase in microbial activity and rate of biodegradation following addition of inorganic nutrients (Radwan et al. 2000; Del 'Arco and de Franca 2001; Vasudevan and Rajaram 2001).

3.2 Change in Protein Concentration during Degradation

The protein estimation by Bradford's method was effective in monitoring the microbial population in the hydrocarbon contaminated soil sample. In the non-sterile control, the initial concentration of protein observed was 1.25 ± 0.16 mg/g of soil, whereas in sterile soil it was 0.001 ± 0.0 mg/g. This reduction may be due to the proteins destroyed in the soil during sterilization. The various amendments and mixed consortium caused proliferation of bacteria up to 56 days of incubation and resulted in an increased protein content in these treatments up to a value of 6.24 mg/g in soil samples amended with 10% TBS (Table 3).

Table 2. Bacterial growth during degradation of n-alkane in oil sludge treated with different amendments

S. No.	Amendments/ Days	Bacteria (CFU/g)							
		10% sludge				20% sludge			
		0	28	56	84	0	28	56	84
1	NS+TBS	2.1±0.7[B] x 10^3e[A]	6.1±0.3 x 10^3e	7.2±0.2 x 10^3e	2.4±0.4 x 10^3e	2.7±0.3 x 10^3e	4.1±0.2 x 10^3e	7.3±0.6 x 10^3e	6.7±0.6 x 10^3e
2	NS+TBS+RL	7.9±0.9 x 10^3c	8.1±0.5 x 10^3d	89.0±2.3 x 10^3d	59.0±1.2 x 10^3d	92.0±4.9 x 10^3c	31.0±1.8 x 10^3d	56.0±4.1 x 10^3d	39.0±0.1 x 10^3d
3	NS+TBS+NPK	2.8±0.4 x 10^3d	39.0±1.1 x 10^3c	660.0±15 x 10^3c	440.0±16 x 10^3c	6.4±2.3 x 10^3d	43.0±2.6 x 10^3c	91.0±6.3 x 10^3c	63.0±2.5 x 10^3c
4	NS+TBS+BC	240.0±11 x 10^3b	1.8±0.2 x 10^7b	4.3±0.1 x 10^8a	3.8±0.5 x 10^8b	220.0±16 x 10^3b	3.8±0.1 x 10^6b	5.6±0.2 x 10^7b	2.8±0.3 x 10^7b
5	NS+TBS+RL+NPK+BC	810.0±17 x 10^3a	6.8±0.4 x 10^8a	3.8±0.3 x 10^8b	4.1±0.5 x 10^{10}a	500.0±37 x 10^3a	1.7±0.1 x 10^7a	2.6±0.2 x 10^8a	2.1±0.1 x 10^8a
6	SS+TBS	0.12±0.01 x 10^3e	0.80±0.07 x 10^3c	0.97±0.8 x 10^3e	0.27±0.04 x 10^3e	0.14±0.02 x 10^3e	0.37±0.02 x 10^3d	0.68±0.04 x 10^3d	0.51±0.04 x 10^3c
7	SS+TBS+RL	0.18±0.01 x 10^3c	0.28±0.01 x 10^3e	2.50±0.3 x 10^3d	1.10±0.04 x 10^3d	0.19±0.01 x 10^3d	0.27±0.01 x 10^3e	0.99±0.01 x 10^3c	0.42±0.03 x 10^3d
8	SS+TBS+NPK	0.16±0.02 x 10^3d	0.56±0.04 x 10^3d	6.4±0.5 x 10^3c	5.2±0.6 x 10^3c	0.22±0.02 x 10^3c	0.84±0.08 x 10^3c	0.32±0.02 x 10^3e	0.12±0.01 x 10^3e
9	SS+TBS+BC	210.0±1.3 x 10^3b	640.0±49 x 10^3b	290.0±19 x 10^3b	170.0±14 x 10^3b	18.0±0.1 x 10^3b	6.7±0.04 x 10^6b	9.1±0.9 x 10^6b	8.9±0.7 x 10^6b
10	SS+TBS+RL+NPK+BC	370.0±55 x 10^3a	9.1±0.7 x 10^6a	3.0±0.1 x 10^7a	2.7±0.1 x 10^7a	270.0±16 x 10^3a	4.6±0.02 x 10^7a	3.9±0.2 x 10^8a	1.9±0.01 x 10^8a

NS - Non-sterile soil; SS - Sterile soil; TBS - Tank Bottom Sludge; BC - Bacterial Consortium; NPK - Nitrogen, Phosphorus and Potassium solution; RL - Rhamnolipid

[A]a, b, c, d, e: Arithmetic means within row with the same letter are not significantly different from each other at 5% probability level by DMRT; [B]Standard error

Table 3. Protein concentration during degradation of n-alkane in oil sludge treated with different amendments for a period of up to 84 days

S. No.	Amendments/ Days	Protein (mg/g)							
		10% sludge				20% sludge			
		0	28	56	84	0	28	56	84
1	NS+TBS	1.2e[A] ±0.16[B]	1.72d ±0.15	2.19d ±0.13	2.23d ±0.29	0.08d ±0.00	1.12e ±0.09	1.97e ±0.11	2.10e ±0.17
2	NS+TBS+RL	1.74c ±0.11	2.07c ±0.08	2.56c ±0.24	2.58c ±0.17	1.20c ±0.02	1.88c ±0.06	2.12d ±0.17	2.32d ±0.21
3	NS+TBS+NPK	1.29d ±0.07	1.58e ±0.04	1.58e ±0.08	2.25d ±0.09	0.08d ±0.01	1.24d ±0.10	2.30c ±0.20	2.40c ±0.28
4	NS+TBS+BC	2.15b ±0.19	3.99b ±0.24	4.24b ±0.21	4.83b ±0.16	1.70b ±0.11	3.10b ±0.17	3.70b ±0.24	3.98b ±0.11
5	NS+TBS+RL+NPK+ BC	2.41a ± 0.21	4.93a ±0.21	6.24a ±0.16	6.00a ±0.37	2.01a ±0.15	3.50a ±0.29	4.12a ±0.55	4.51a ±0.24
6	SS+TBS	0.01d ±0.00	0.05d ±0.01	0.07c ±0.00	0.08c ±0.00	0.02c ±0.00	0.06c ±0.00	0.09c ±0.01	0.09c ±0.01
7	SS+TBS+RL	0.01d ±0.00	0.05d ±0.00	0.07c ±0.00	0.09c ±0.01	0.02c ±0.00	0.06c ±0.00	0.07c ±0.00	0.08c ±0.00
8	SS+TBS+NPK	0.02c ±0.00	0.06c ± 0.00	0.07c ±0.00	0.07c ±0.00	0.03c ±0.00	0.05c ±0.00	0.06c ±0.00	0.07c ±0.00
9	SS+TBS+BC	1.87b ±0.06	3.20b ±0.24	3.50b ±0.27	3.59b ±0.27	1.70b ±0.08	2.70b ±0.15	3.05b ±0.09	3.21b ±0.24
10	SS+TBS+RL+NPK+BC	2.73a ±0.18	3.98a ±0.18	4.12a ±0.39	4.37a ±0.46	2.91a ±0.24	3.52a ±0.30	3.98a ±0.27	4.10a ±0.35

NS – Non sterile soil; SS – Sterile soil; TBS - Tank bottom sludge; BC – Bacterial consortium; NPK – Nitrogen, Phosphorus, Potassium solution; RL – Rhamnolipid biosurfactant solution

[A]a, b, c, d, e: Arithmetic means within row with the same letter are not significantly different from each other at 5% probability level by DMRT; [B]Standard error

3.3 Biodegradation versus Surface Tension

The indigenous microbial community of non-sterile and sterile soil caused a slight decrease in the surface tension, indicating that those microorganisms could not produce sufficient biosurfactant activities. Surface tension of the soil extract was 69.7±0.4 - 71.1±0.6 mN/m (milli-Newton/meter), which was reduced to 52.3±2.2 and 48.1±1.8 mN/m in NS+TBS+RL and SS+TBS+RL amended with 10% TBS respectively. A reduction in surface tension occurred because of the presence of rhamnolipid (RL) in NS+TBS+RL and SS+TBS+RL with 10% TBS amendment (Table 4). Furthermore, in soil samples augmented with a bacterial consortium and amended with rhamnolipid and NPK, a significant reduction in surface tension was noted after 56 days of incubation. A possible reason for this may be the rhamnolipid-mediated desorption of petroleum hydrocarbons, which increased their solubility and hence the biological activity of indigenous microflora or added hydrocarbon degrading bacterial consortium. In a study by Oberbremer and Muller-Hurtig (1989), a positive correlation was obtained between reduction in the surface tension of the fluid phase in a stirred soil bioreactor and the onset of biodegradation of hydrophobic petroleum hydrocarbons. It has also been reported that a rhamnolipid biosurfactant can mediate reduction in the surface tension (Banat et al. 2000; Noordman et al. 2000).

3.4 Effect of Degradation on pH

A range of pH 7.2 ± 0.3 to 7.2 ± 0.4 was estimated in the sterile and non-sterile soil samples. Alternatively, in soil samples amended with mixed consortium, rhamnolipid or NPK, an increase in pH was observed after 56 days of incubation suggesting the release of by-products during hydrocarbon degradation (Table 5).

3.5 Biodegradation of n-alkanes

Gas chromatographic analyses revealed all hexane soluble n-alkanes in the range of nC8–nC40, which were relatively abundant in tank bottom crude oil sludge. The degradation of the above was discussed in four different ranges, such as nC8–nC11, nC12–nC21, nC22–nC31 and nC32–nC40. The nC8–nC11 range consisted of volatile hydrocarbons. A percentage of hydrocarbon degradation of approximately 100% (nC8–nC11), 83-98% (nC12-nC21), 80-85% (nC22-nC31) and 57-73% (nC32-nC40) was noted in non-sterile soil samples with 10% TBS amended with RL+NPK+BC (Fig. 1). Among the different treatments, in NS+TBS+RL+NPK+BC amended with 10% TBS, all the hydrocarbons in the range of nC8- nC11 were degraded, whereas in SS+TBS+RL+NPK+BC with 10% TBS, NS+TBS+RL+NPK+BC and SS+TBS+RL+NPK+BC with 20% TBS, only 81-87%, 64-83% and 55-61% degradation was observed, respectively (Figs. 4-6).

Table 4. Surface tension of samples during degradation of n-alkane in oil sludge treated with different amendments for a period of up to 84 days

S. No.	Amendments/ Days	Surface tension (mN/m)							
		10% sludge				20% sludge			
		0	28	56	84	0	28	56	84
1	NS+TBS	69.7c[A] ±0.4[B]	70.3a ±0.9	65.5b ±2.7	67.7b ±0.9	70.1b ±0.5	67.1b ±0.4	63.1c ±1.9	70.5a ±0.4
2	NS+TBS+RL	52.3d ±2.2	69.8b ±0.4	69.7a ±3.1	65.1c ±1.1	57.1c ±2.1	69.1a ±0.2	66.8a ±0.3	69.9b ±1.0
3	NS+TBS+NPK	71.5a ±0.4	66.7d ±1.4	62.9d ±1.2	62.9d ±0.4	70.2b ±0.1	61.8e ±1.1	59.8e ±0.5	67.4e ±1.4
4	NS+TBS+BC	70.5b ±0.5	68.8c ±1.4	63.3c ±2.1	69.7a ±0.3	70.5a ±0.4	65.1c ±2.3	63.3b ±0.7	69.5c ±0.4
5	NS+TBS+RL+NPK+BC	32.1e ± 1.6	62.7e ±2.9	57.2e ±3.0	61.5e ±1.1	41.2d ±2.1	63.1d ±2.4	61.1d ±1.2	68.1d ±2.3
6	SS+TBS	70.1b ±1.5	70.6a ±0.2	69.4a ±0.6	69.2a ±0.9	71.1b ±0.6	69.2a ±1.3	68.9a ±2.0	67.5b ±0.7
7	SS+TBS+RL	48.1d ±1.8	61.1c ±3.1	62.9b ±2.4	57.4e ±2.3	67.1d ±1.2	64.5e ±3.4	64.7d ±3.4	65.5d ±1.5
8	SS+TBS+NPK	69.4c ±0.1	69.9b ±1.2	61.7c ±1.5	67.9b ±1.7	70.1c ±0.2	67.8b ±2.9	66.9b ±1.6	66.9c ±3.4
9	SS+TBS+BC	71.7a ±0.4	70.4a ±0.6	62.9b ±3.1	64.1c ±2.0	71.5a ±0.5	64.9d ±3.1	66.5c ±3.3	67.6a ±2.9
10	SS+TBS+RL+NPK+BC	40.1e ±2.6	59.3d ±1.7	61.9c ±0.4	62.4d ±1.6	47.2e ±2.1	65.5c ±4.0	61.3e ±0.9	58.9e ±3.7

NS – Non sterile soil; SS – Sterile soil; TBS - Tank bottom sludge; BC – Bacterial consortium; NPK – Nitrogen, Phosphorus, Potassium solution; RL – Rhamnolipid biosurfactant solution

[A]a, b, c, d, e: Arithmetic means within row with the same letter are not significantly different from each other at 5% probability level by DMRT; [B]Standard error.

Table 5. pH of the soil during degradation of n-alkane in oil sludge treated with different amendments for a period of up to 84 days

S. No.	Amendments/ Days	pH							
		10% sludge				20% sludge			
		0	28	56	84	0	28	56	84
1	NS+TBS	7.2a[A]±0.4[B]	7.1c ±0.4	6.9d ±0.6	6.9c ±0.4	7.2a ±0.1	7.1c ±0.5	6.7c ±0.2	6.9c ±0.4
2	NS+TBS+RL	6.9c ±0.2	7.0d ±0.1	7.0c ±0.2	7.0b ±0.3	6.9c ±0.5	7.0d ±0.1	7.1a ±0.4	6.9c ±0.6
3	NS+TBS+NPK	7.1b ±0.3	7.6a ±0.3	7.2b ±0.4	7.0b ±0.1	7.1b ±0.3	7.6a ±0.2	7.2a ±0.5	7.2a ±0.5
4	NS+TBS+BC	7.2a ±0.1	7.1c ±0.2	7.0c ±0.3	7.0b ±0.5	7.2a ±0.3	7.1c ±0.4	68b ±0.3	6.9c ±0.3
5	NS+TBS+RL+NPK+BC	6.9c ± 0.3	7.3b ±0.4	7.3a ±0.7	7.5a ±0.3	6.9c ±0.1	7.3b ±0.6	7.1a ±0.7	7.1b ±0.4
6	SS+TBS	7.2a ±0.3	7.1c ±0.4	6.9 ±0.6	7.0 ±0.4	7.2 ±0.5	7.0 ±0.5	6.8 ±0.4	7.0 ±0.7
7	SS+TBS+RL	6.8c ±0.2	7.2b ±0.3	7.1 ±0.3	6.9 ±0.5	6.7 ±0.6	7.1 ±0.6	7.2 ±0.5	7.1 ±0.6
8	SS+TBS+NPK	6.9b ±0.5	7.4a ± 0.4	7.2 ±0.2	7.3 ±0.1	6.9 ±0.4	7.3 ±0.3	7.8 ±0.3	7.1 ±0.4
9	SS+TBS+BC	6.9b ±0.1	7.2b ±0.5	7.0 ±0.4	6.9 ±0.3	6.9 ±0.3	7.2 ±0.4	7.0 ±0.2	7.0 ±0.3
10	SS+TBS+RL+NPK+BC	6.9b ±0.6	7.4a ±0.6	7.4 ±0.5	7.3 ±0.4	6.9 ±0.4	7.3 ±0.1	7.5 ±0.4	7.2 ±0.2

NS – Non sterile soil; SS – Sterile soil; TBS - Tank bottom sludge; BC – Bacterial consortium; NPK – Nitrogen, Phosphorus, Potassium solution; RL – Rhamnolipid biosurfactant solution

Aa, b, c, d, e: Arithmetic means within row with the same letter are not significantly different from each other at 5% probability level by DMRT; B Standard error.

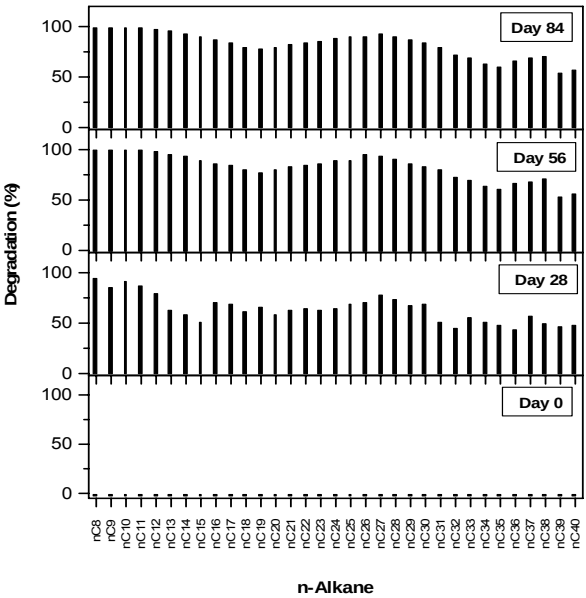

Fig. 1. n-Alkane degradation in non-sterile soil with 10% of tank bottom sludge and BC+NPK+RL at various time intervals

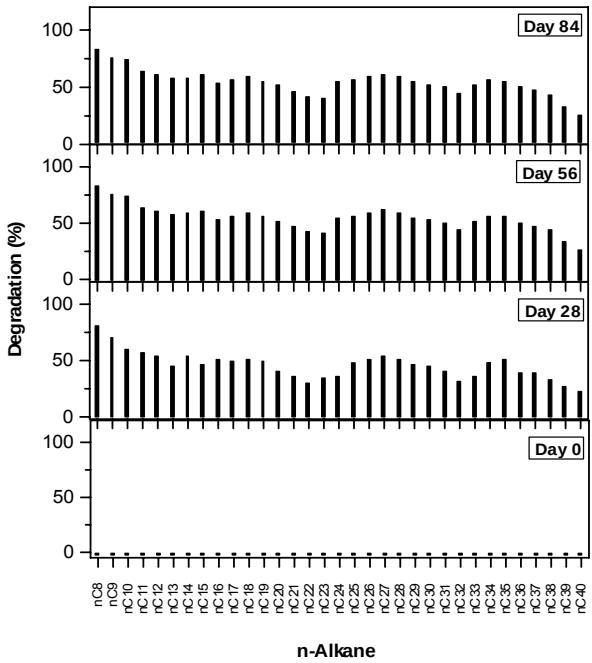

Fig. 2. n-Alkane degradation in non-sterile soil with 20% of tank bottom sludge and BC+NPK+RL at various time intervals

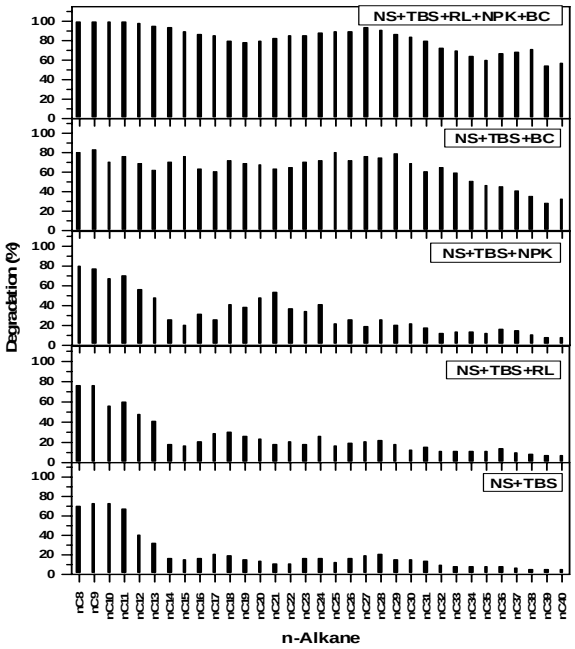

Fig. 3. n-Alkane degradation in non-sterile soil with 10% of tank bottom sludge and BC+NPK+RL on 56th day of treatment

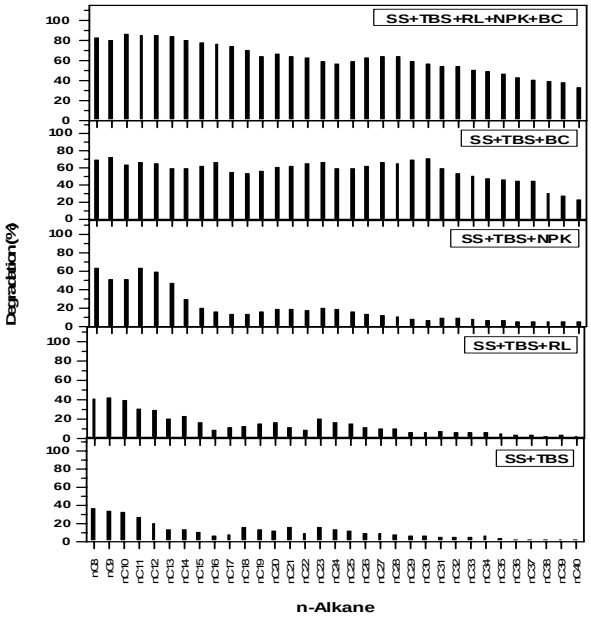

Fig. 4. n-Alkane degradation in sterile soil with 10% of tank bottom sludge and BC+NPK+RL on 56th day of treatment

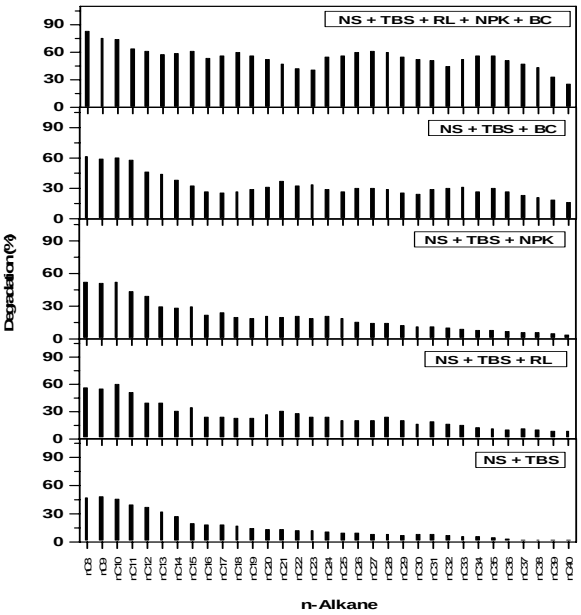

Fig. 5. n-Alkane degradation in non-sterile soil with 20% of tank bottom sludge and BC+NPK+RL on 56th day of treatment

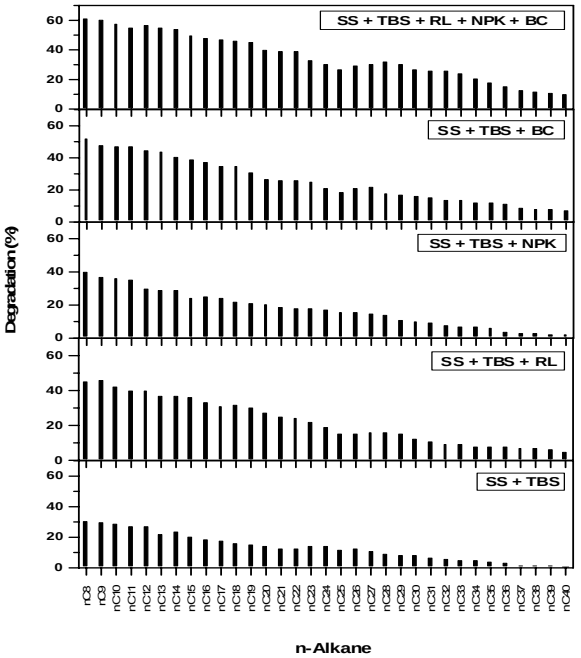

Fig. 6. n-Alkane degradation in sterile soil with 20% of tank bottom sludge and BC+NPK+RL on 56th day of treatment

The decreasing utilization trend after 56 days of incubation observed with soil samples amended with 10% TBS was not only due to the substrate depletion but also to the fact that the remaining hydrocarbons were relatively more resistant to biodegradation. The rate of petroleum biodegradation and quantity of hydrocarbon degraded depend on environmental conditions, chemical structure of the pollutant compounds, type and amount of oil present at the contaminated site (Del 'Arco and de Franca 2001). At 20% TBS concentration, the decrease in microbial degradation activity may be due to the toxicity caused by higher hydrocarbon contamination (Fig. 2).

The bacterial consortium enhanced the degradation of all the fractions of hydrocarbons from nC8-nC40 to various degrees in sterile and non-sterile samples supplemented with 10% and 20% TBS. This observation is in general agreement with the earlier report regarding the use of bioaugmentation (Mulligan et al. 2001). When compared to all the sets, different treatments of non-sterile soil (NS+TBS, NS+TBS+RL, NS+TBS+NPK, NS+TBS+BC and NS+TBS+RL+NPK+BC) amended with 10% TBS exhibited a higher percentage of hydrocarbon degradation (Fig. 3). The degree of degradation observed with SS+TBS was lower than that in the NS+TBS. These results indicated the ubiquitous distribution of diversified hydrocarbon structures, originating in particular from plants in the environment and consequently the presence of specific bacterial hydrocarbon degraders. Furthermore, the TBS amended soil samples treated with rhamnolipid or NPK lost substantially fewer hydrocarbons in the range of nC12–nC40 than those treated with bacterial consortium. In our study, no lag period was observed preceeding petroleum hydrocarbon mineralisation in sterile soil samples amended with TBS, suggesting the presence of an active hydrocarbon degrading population in the TBS. Addition of NPK solution alone had only a minor effect on hydrocarbon degradation compared to other soil amendments which may be due to a slight increase in biological activity of the microflora present in soil and sludge. The addition of rhamnolipid however, significantly enhanced the rate of biodegradation of hydrocarbon fractions by the bacterial consortium and the NPK solution in all the treatments.

When hydrocarbons are present in non-inhibitory concentration (available or desorbed form) in the soil, it may affect the rate of biodegradation by enhancing the biodegradation activity of the indigenous microbial population. Adding surfactants to soil contaminated with hydrophobic contaminants may increase the bioavailability of these compounds to hydrocarbon degrading microorganisms (Banat et al. 1991; Banat 1995). Complete degradation of nC8-nC11 and 73-98% of nC12 - nC40 was observed with the mixed bacterial consortium amended with rhamnolipid and NPK solution in 10% TBS amended soil samples at 56 days of incubation (Figs. 3 and 5), which was higher than all the earlier reports.

Dave et al. (1994) achieved 70% bioremediation of a slop oil contaminated soil using oil degrading cultures. One of the main reasons for the prolonged

persistence of hydrophobic hydrocarbons in the contaminated environments is their strong adsorption even on coarse-grained and organic free soils by microporosity, which makes them less available for hydrocarbon degrading microorganisms and remain even after bioremediation. Hence, for efficient and complete biodegradation, solubilization of these hydrocarbons with biosurfactants prior to bioaugmentation is advantageous. Moreover, use of biosurfactant producing hydrocarbon degrading microorganisms for bioaugmentation to enhance hydrocarbon degradation offers an advantage of a continuous supply of a non-toxic and biodegradable surfactant at a low cost (Moran et al. 2000; Rahman et al. 2002c). The biosurfactant used in this study is a dirhamnolipid type of surfactant. Mass spectrometry using electrospray ionization is an efficient method to characterize rhamnolipid biosurfactant and since *Pseudomonas* sp. DS10-129 had highest production, we analysed its fermentation broth (Rahman et al. 2002d). Daziel et al. (1999) reported about different rhamnolipid species produced by *Pseudomonas* sp. 57RP with mannitol and naphthalene as carbon source. We detected a presence of mono and dirhamnolipids the Rha-C10-C10 and the Rha-Rha-C10-C-10 (MW=504 and 650) (Fig. 7).

However, the potential benefits of *in situ* application of surfactants must be weighed against the possibility of increased ground water contamination due to surfactant-mediated enhanced mobility of contaminants. Hence, repeated use of smaller dose schedule should be investigated as means to control contaminant mobility together with careful monitoring of the rate and extent of hydrocarbon degradation.

All the results were statistically analyzed using ANOVA and DMRT procedures to determine significant parameters. The results presented in Table 6

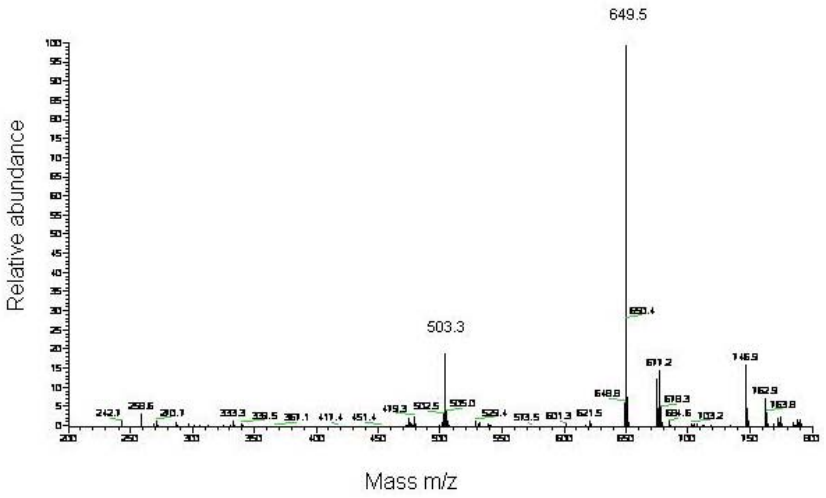

Fig. 7. Mass spectrum of rhamnolipids produced by *Pseudomonas aeruginosa* DS10-129 using soybean oil as substrate

Table 6. Significance level for the different parameters tested within our treatments computed by DMRT

Parameter	Bacteria (x10^3 CFU/g)			Protein (mg/g)			Surface tension (mN/m)			pH		
Factorial Effect	SE	CD	SL	SE	CD	SL	SE	CD	SL	SE	CD	SL
Concentration (C)	9.24	18.48	**	0.02	0.03	**	0.17	0.29	**	0.14	0.24	**
Amendment (A)	23.60	47.2	**	0.09	0.16	**	0.43	0.74	**	0.20	0.46	**
Days (D)	36.10	72.2	**	0.17	0.31	**	1.54	2.93	*	0.39	0.61	**
C x A	54.30	108.6	**	0.27	0.53	**	1.90	3.48	*	0.43	0.83	*
C x D	61.20	122.4	**	0.34	0.65	**	2.36	4.31	ns	0.35	0.67	ns
A x D	86.40	172.8	**	0.39	0.74	**	2.68	5.16	ns	0.67	1.24	ns
C x A x D	100.0	197.5	**	0.44	0.85	**	3.91	7.57	ns	0.62	1.29	ns

SE - Standard Error; CD -Cumulative Difference; SL - Significant level; ns - not significant at 1% or 5% probability levels.
* Significant at 5% probability level (within column); ** Significant at 1% probability level (within column)

revealed that all the above parameters were highly influenced by single factors (concentration (C), amendments (A), number of days (D) treated); two factor combinations (C x A, C x D and A x D) and three factor combinations (C x A x D) at a 1% probability level. However, the number of days treated (D), and the two factor combination C x A for surface tension and pH were significant at 5% probability level. Moreover, the two factor combinations C x D and A x D and the three factor combination C x A x D were not significant at 1% or 5% probability levels for surface tension and pH.

4. Conclusion

Several strategies have been attempted for bioremediation of hydrocarbon-polluted sites. Bioaugmentation with designed bacterial consortium, followed by the addition of rhamnolipid biosurfactant and NPK solution to soils contaminated with up to 10% tank bottom sludge, enhanced the rate of biodegradation over a period of 56 days. Pre-treatment of hydrocarbon contaminated soil with biosurfactants enhanced bioavailability of the hydrocarbons to microbial population. Furthermore, supplementation with inorganic nutrients like NPK solution enhanced the secondary successions of crude petroleum utilizers. For bioremediation, a single inoculation with the biosufactant-producing hydrocarbon degrading bacterial consortium at the beginning of the process would reduce the cost of inoculum preparation considerably. Hence we suggest a combined treatment as a possible bioremediation technology for the reclamation of oil sludge polluted soils.

Acknowledgements. We wish to thank Northern Ireland Environment and Heritage Service and European Regional Development Fund for financial supports.

References

Banat IM, Samarah N, Murad M, Horne R, Banerjee S (1991) Biosurfactant production and use in oil tank clean-up. World J Microbiol Biotechnol 7:80-84

Banat IM (1995) Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. Bioresource Technol 51:1-12

Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol 53:495-508

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protien utilizing the principle of protein-dye binding. Anal Biochem 72:248- 254

Dave H, Ramakrishna C, Bhatt BD, Desai JD (1994) Biodegradation of slope oil from petroleum Industry and bioreclamation of slop oil contaminated soil. World J Microbiol Biotechnol 10:653-656

Del ‘Arco JP, de Franca FP (2001) Influence of oil contamination levels on hydrocarbon biodegradation in sandy sediments. Environ Pollut 110:515-519

Ferrari MD, Neirotti E, Albornoz C, Mostazo MR, Cozzo M (1996) Biotreatment of hydrocarbons from petroleum tank bottom sludges in soil slurries. Biotechnol Lett 18:1241-1246

Gomez KA, Gomez AA (1984) Statistical procedure for agricultural research. John Wiley and Sons, New York, pp 680

Moran AC, Olivera N, Commendatore M, Esteves JL, Sineriz F (2000) Enhancement of hydrocarbon waste biodegardation by addition of a biosurfactant from *Bacillus subtilis* O9. Biodegradation 11:65-71

Mulligan CN, Yong RN, Gibbs BF (2001) Surfactant enhanced remediation of contaminated soil: a review. Eng Geol 60:371-380

Noordman WH, Brusseau ML, Janssen DB (2000) Adsorption of a multicomponent rhamnolipid surfactant to soil. Environ Sci Technol 34:832-838

Oberbremer A, Muller-Hurtig R (1989) Aerobic stepwise hydrocarbon degradation and formation of biosurfactants by an original soil population in a stirred reactor. Appl Microbiol Biotechnol 31:582-586

Radwan SS, Al-Mailem D, El-Nemr I, Salamah S (2000) Enhanced remediation of hydrocarbon contaminated desert soil fertilized with organic carbons. Int Biodeterior Biodegrad 46:129-132

Rahman KSM, Rahman TJ, Lakshmanaperumalsamy P, Banat IM (2002a) Occurrence of crude oil degrading bacteria in gasoline and diesel station soils. J Basic Microbiol 42:286-293

Rahman KSM, Banat IM, Rahman TJ, Thayumanavan T, Lakshmanaperumalsamy P (2002b) Bioremediation of gasoline contaminated soil by bacterial consortium amended with poultry litter, coir-pith and rhamnolipid biosurfactant. Bioresource Technol 81:25-32

Rahman KSM, Rahman TJ, Lakshmanaperumalsamy P, Banat IM (2002c) Towards efficient crude oil degradation by mixed bacterial consortium. Bioresource Technol 85:257-261

Rahman KSM, Rahman TJ, McClean S, Banat IM (2002d) Rhamnolipid biosurfactants production by strains of *Pseudomonas aeruginosa* using low cost raw materials. Biotechnol Prog 18:1277-1281

Vasudevan N, Rajaram P (2001) Bioremediation of oil sludge- contaminated soil. Environ Int 26:409-411