ENHANCED BIOREMEDIATION OF n-ALKANE IN PETROLEUM SLUDGE USING BACTERIAL CONSORTIUM AMENDED WITH RHAMNOLIPID AND MICRO-NUTRIENTS

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Abstract

The purpose of the present study was to investigate possible methods to enhance the rate of biodegradation of oil sludge from crude oil tank bottom, thus reducing the time usually required for bioremediation. Enhancement of biodegradation was achieved through bioaugmentation and biostimulation. 10% and 20% sludge contaminated sterile and non-sterile soil samples were treated with bacterial consortium, rhamnolipid biosurfactant and NPK (Nitrogen, Phosphorus and Potassium) solution. Maximum extent of n-alkane degradation occurred in the 10% sludge contaminated soil samples. The effects of treatment carried out with the non-sterile soil samples were more pronounced than in its sterile counterpart. Maximum degradation was achieved after the 56th day of treatment. n-alkanes in the range of nC8-nC11 were degraded completely followed by nC12-nC21, nC22-nC31 and nC32-nC40 with the percentage of degradation being 100%, 83-98%, 80-85% and 57-73% respectively. Statistical analysis using Analysis of Variance (ANOVA) and Duncan's Multiple Range test (DMRT) revealed that the level of amendments, incubation time and combination of amendments significantly influenced bacterial growth, protein concentration and surface tension at a 1% probability level. All tested additives bacterial consortium, NPK and Rhamnolipid biosurfactant had significant positive effects on the bioremediation of n-alkane in petroleum sludge.

Key words: Tank bottom sludge; Bacterial consortium; Rhamnolipid; Bioremediation; Bioaugmentation.

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 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 controlling 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, in-situ production may be stimulated at the site of contamination and can be recovered and recycled (Moran et al., 2000). There has 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 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., 2001a). 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 and enhance 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 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 hot air oven at 180^{o}C for 2 h and a part kept as normal condition (non-sterile). An oil degrading bacterial consortium containing five 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 with GS was isolated from gasoline station and DS from diesel station soils followed by its strain number were depicted in our strains. 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 % of 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 nonstrile 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). The treatments were all 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^{o}C. Triplicate sets of experimental samples were analysed at 0, 28, 56 and 84 days to enumerate total heterotrophic bacterial counts, protein content, percentage of n-alkane degradation, pH and surface tension (ST) were analysed.

2.3. Enumeration of bacterial population

Total heterotrophic bacteria were enumerated by using a pour plate technique on plate count agar (Merck, UK) after 24h incubation at 30^{o}C, which also allowed growth of all members of the added bacterial consortium.

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 a 3N NaOH solution and boiled for 3 min. After cooling at room temperature, 1 ml of a 1 M H_3PO_4 solution was added. 50 μL was taken and mixed with 950 μL Coomassie reagent and incubated at 30^{o}C for 10 min and the optical density was

measured at 595 nm using UV – visible spectrophotometer (Shimadzu model number UV – 2101PC). The total protein was estimated using a standard curve prepared with albumin (Bradford, 1976).

2.5. Surface tension analysis

The surface tension of the soil extract (soil: water ratio is 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.

2.6. Measurement of pH

The pH of the soil extract (soil:water ratio 1:10) was estimated using Microcomputer pH meter model 6171.

2.7. Hydrocarbon estimation

The hexane soluble n-alkanes (nC8-nC40) in the soil samples were determined using Gas chromatography. 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 was used for GC analysis. A capillary column (30 m Fused Silica column, Restek Corporation, USA) and GC (Perkin-Elmer 8310) 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 / 4min then increased at the rate of 10^{o}C / min to 330^{o}C and maintained at 330^{o}C for 20 minutes. Total recoverable petroleum hydrocarbon standard with purity of 99.9999% (to detect nC8-nC40) 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 $2.1 \pm 0.7 \times 10^3$ CFU/g was observed in non-sterile soil spiked 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). Two orders of magnitude increase

in the bacterial population were observed in soil samples spiked with 10% petroleum TBS after 56 days of incubation. The available nutrients were rapidly assimilated by soil microbes, thus depleting the nutrient reserves. So the objective of augmenting NPK solution to the soil samples was to restore the availability of essential nutrients. Several researchers have recently 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 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 denaturation of proteins present 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 spiked with 10% TBS (Table 3).

3.3 Biodegradation vs Surface tension

The indigenous microbial community of non-sterile and sterile soil caused a slight decrease in surface tension, evidencing that those microorganisms on their own were not able to produce a significant amounts of biosurfactants. 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 20% 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 has been obtained between reduction in surface tension of the fluid phase in a stirred soil bioreactor and the onset of biodegradation of hydrophobic petroleum hydrocarbons. It has also been previously reported about the rhamnolipid biosurfactant mediated reduction in surface tension (Banat et al., 2000; Noordman et al., 2000).

3.4. Effect of degradation on pH

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. Percentage of hydrocarbon degradation of about 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 spiked 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 (Fig 4, 5, 6).

The slowing tendency of utilization 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 literature 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) spiked with 10% TBS exhibited 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 bacterial degraders for them. Furthermore, the TBS spiked 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 preceding petroleum hydrocarbon mineralisation in sterile soil samples spiked 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 microflorae 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). Our results revealed complete degradation of nC8-nC11 and 73-98% of nC12 - nC40 with designed bacterial consortium amended with rhamnolipid and NPK solution in 10% TBS spiked soil samples at 56 days of incubation (Fig 3 and Fig 5), which was comparatively higher than all the earlier reports.

Dave et al. (1994) achieved a 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 contaminated environments is their strong adsorption even on coarse-grained and organic free soils by microporosity, so that they are no longer 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 offer the advantage of a continuous supply of a non-toxic and biodegradable surfactant at a low cost (Moran et al., 2000). However, the potential benefits of insitu application of surfactants must also be weighed against the possibility of increased ground water contamination caused by surfactant mediated enhanced mobility. Hence, the use of a repeated but smaller dosage schedule should be investigated as a 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 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 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 to boost the bioremediation of hydrocarbon polluted sites. We found that bioaugmentation with designed bacterial consortium followed by addition of rhamnolipid biosurfactant and NPK solution to soils contaminated with 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 the above combined treatment as a possible bioremediation technology for reclamation of oil sludge polluted soils. Statistical analyses using ANOVA and DMRT also showed that concentration, amendment and days of treatment at different factorial designs (C, A, D, C x A, C x D, A x D and C x A x D) were significant at 1% probability level for bacterial growth and protein concentration. Hence bioremediation of n-alkanes in 10% sludge amended soil can be achieved by treating with BC, NPK and rhamnolipid BS for 56 days.

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

Amendments	NS / 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.

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

S.No	Amendments /	Bacteria (CFU/g)							
		10% sludge				20% sludge			
	Days	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 ± 0.1 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 ± 0.01x 10^8a

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 with the same letter are not significantly different from each other at 5% probability level by Duncan's Multiple Range Test (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 /	Protein (mg/g)							
		10% sludge				20% sludge			
	Days	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 with the same letter are not significantly different from each other at 5% probability level by Duncan's Multiple Range Test (DMRT)

[B] Standard Error.

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 /	Surface tension (mN/m)							
		10% sludge				20% sludge			
	Days	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 with the same letter are not significantly different from each other at 5% probability level by Duncan's Multiple Range Test (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 /	pH							
		10% sludge				20% sludge			
	Days	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

[A]a, b, c, d, e: Arithmetic means with the same letter are not significantly different from each other at 5% probability level by Duncan's Multiple Range Test (DMRT)

[B] Standard Error.

Table 6. Significance level for the different parameters tested within our treatments computed by Duncan's Multiple Range Test (DMRT)

Parameter	Bacteria (x 10^3 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 * Significant at 5% probability level; ** Significant at 1% probability level; ns - not significant at 1% or 5% probability levels

Figure Captions

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

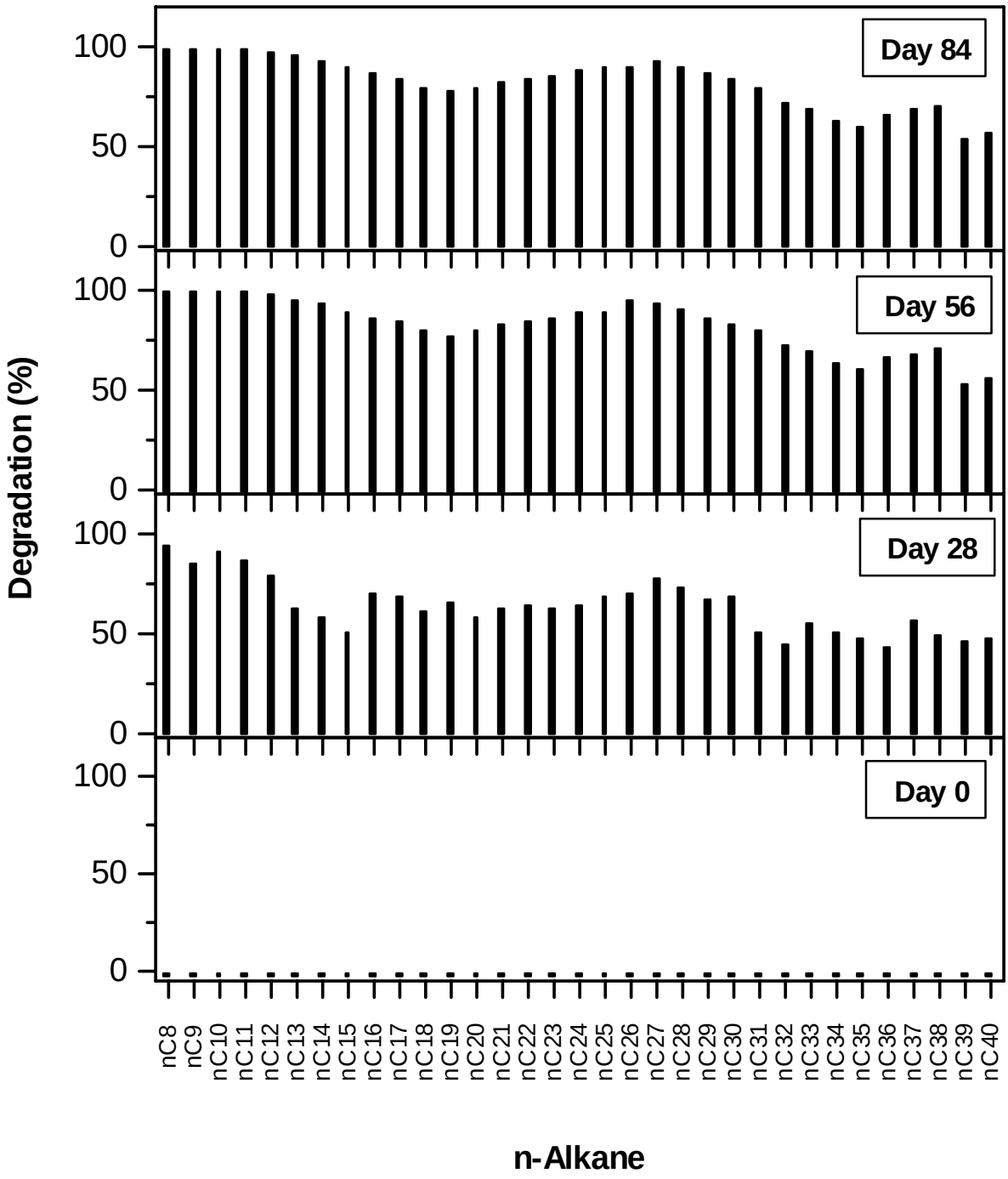
Fig. 2. n-Alkane degradation in nonsterile soil with 20% of tank bottom sludge and BC+NPK+RL at various time intervals

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

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

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

Fig 6. n-Alkane degradation in strile-sterile soil with 20% of tank bottom sludge and BC+NPK+RL on 56th day of treatment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Day 56
Day 28
Day 0
100
50
0
Degradation (%)
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nC9
nC10
nC11
nC12
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nC14
nC15
nC16
nC17
nC18
nC19
nC20
nC21
nC22
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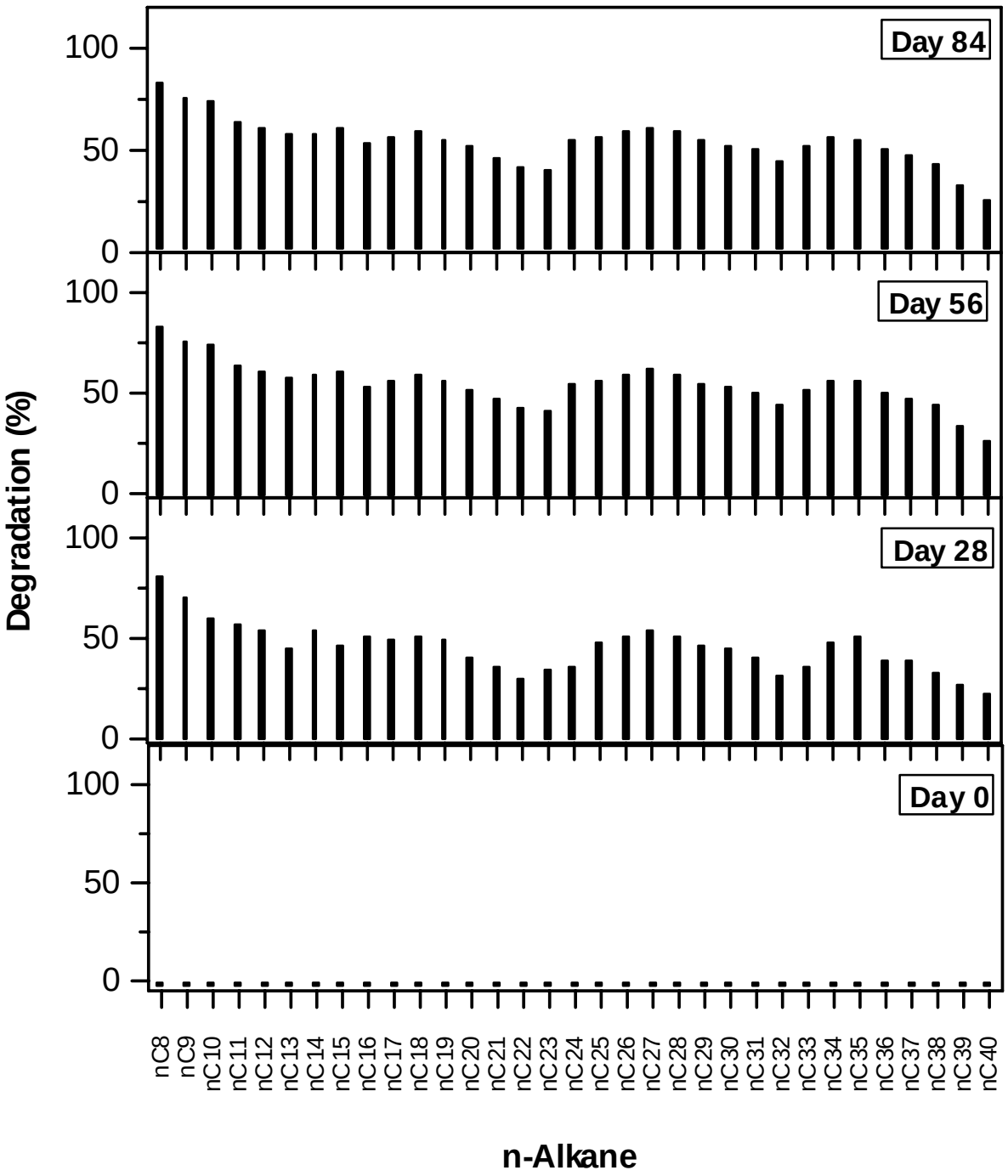
n-Alkane


Day 84
Day 56
Day 28
Day 0
100
50
0
Degradation (%)
nC8
nC9
nC10
nC11
nC12
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nC14
nC15
nC16
nC17
nC18
nC19
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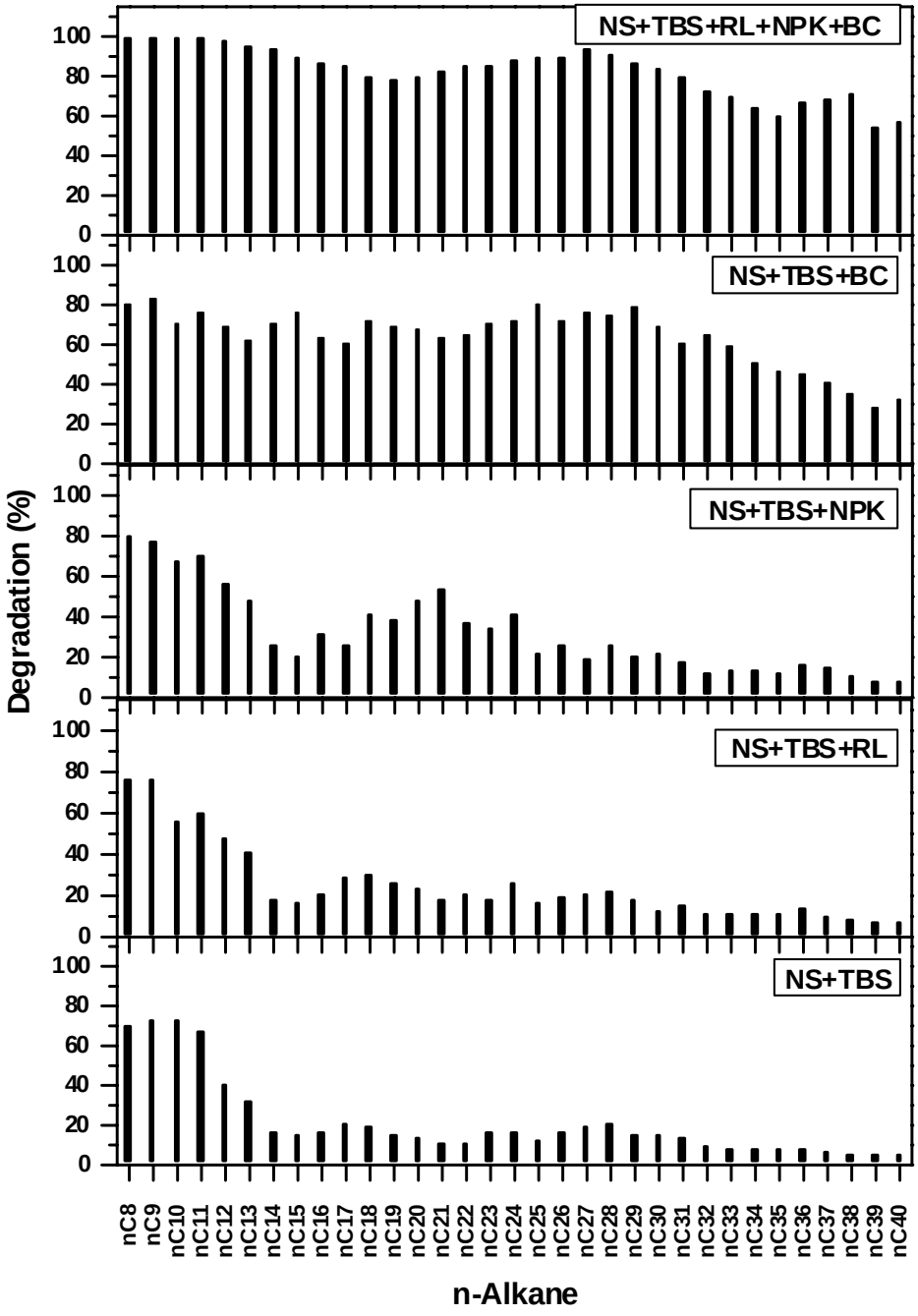
n-Alkane


NS+TBS+RL+NPK+BC
NS+TBS+BC
NS+TBS+NPK
NS+TBS+RL
NS+TBS
Degradation (%)
0
20
40
60
80
100
nC8
nC9
nC10
nC11
nC12
nC13
nC14
nC15
nC16
nC17
nC18
nC19
nC20
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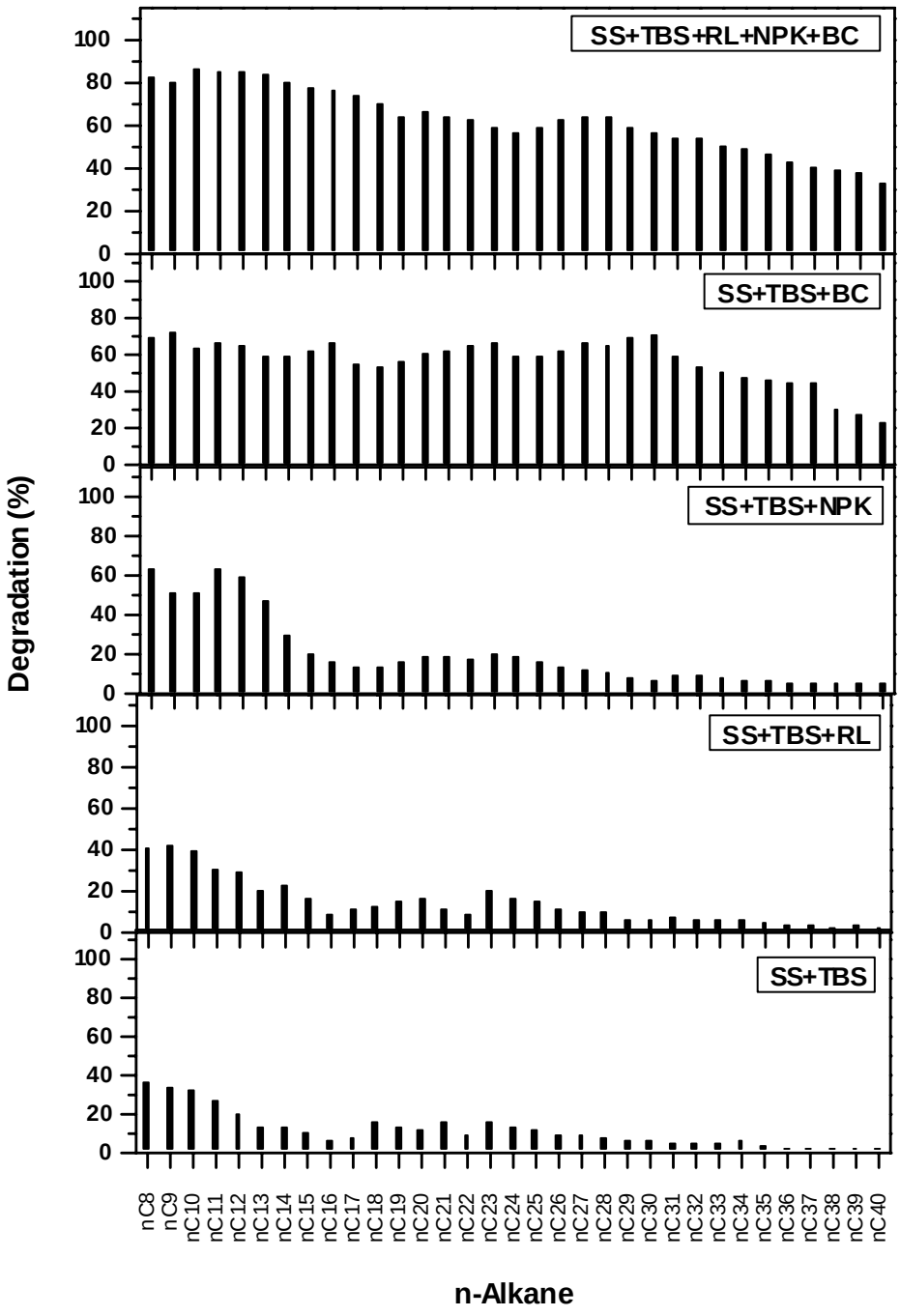
n-Alkane


SS+TBS+RL+NPK+BC
SS+TBS+BC
SS+TBS+NPK
SS+TBS+RL
SS+TBS
Degradation (%)
0
20
40
60
80
100
nC8
nC9
nC10
nC11
nC12
nC13
nC14
nC15
nC16
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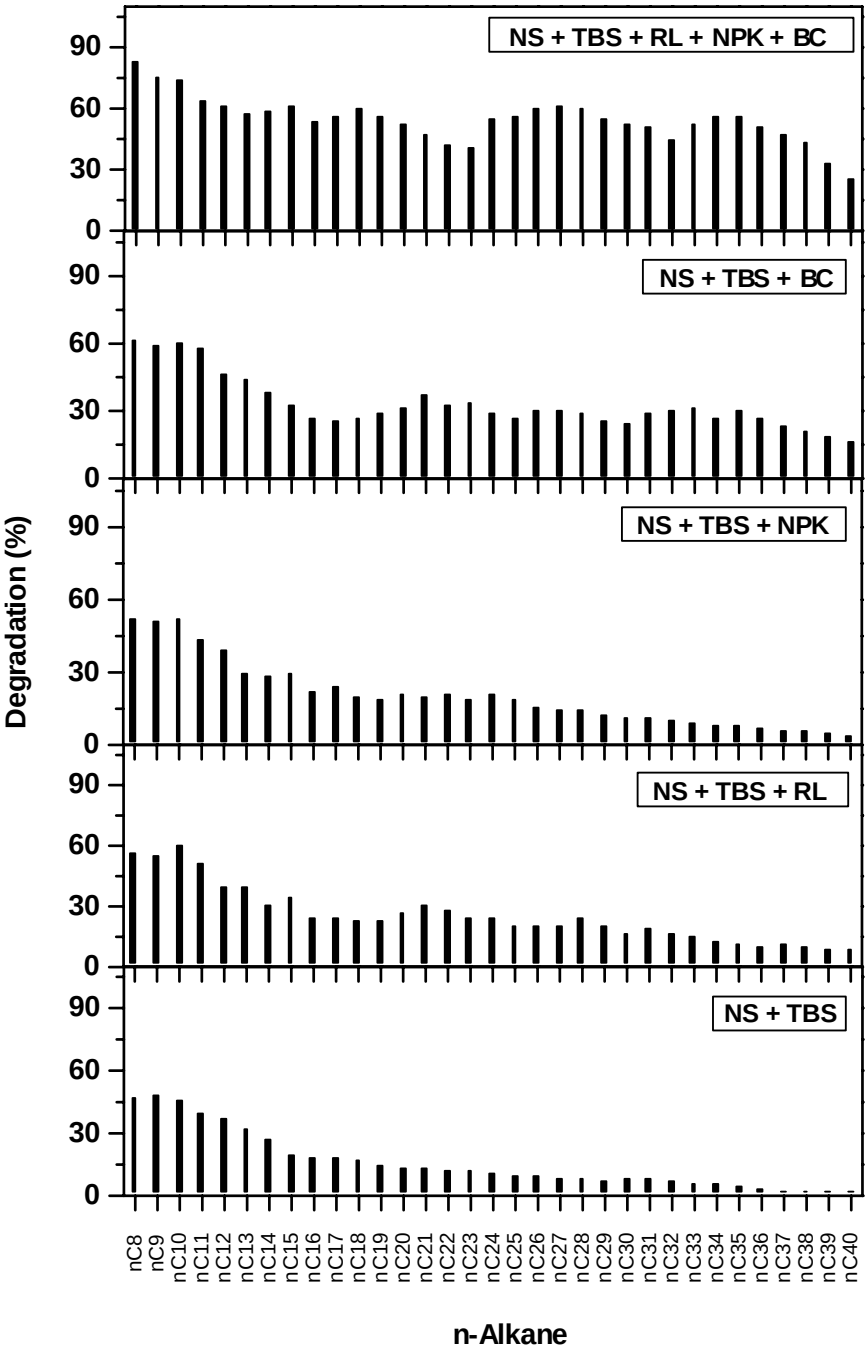
n-Alkane


NS + TBS + RL + NPK + BC
NS + TBS + BC
NS + TBS + NPK
NS + TBS + RL
NS + TBS
Degradation (%)
0
30
60
90
nC8
nC9
nC10
nC11
nC12
nC13
nC14
nC15
nC16
nC17
nC18
nC19
nC20
nC21
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nC25
nC26
nC27
nC28
nC29
nC30
nC31
nC32
nC33
nC34
nC35
nC36
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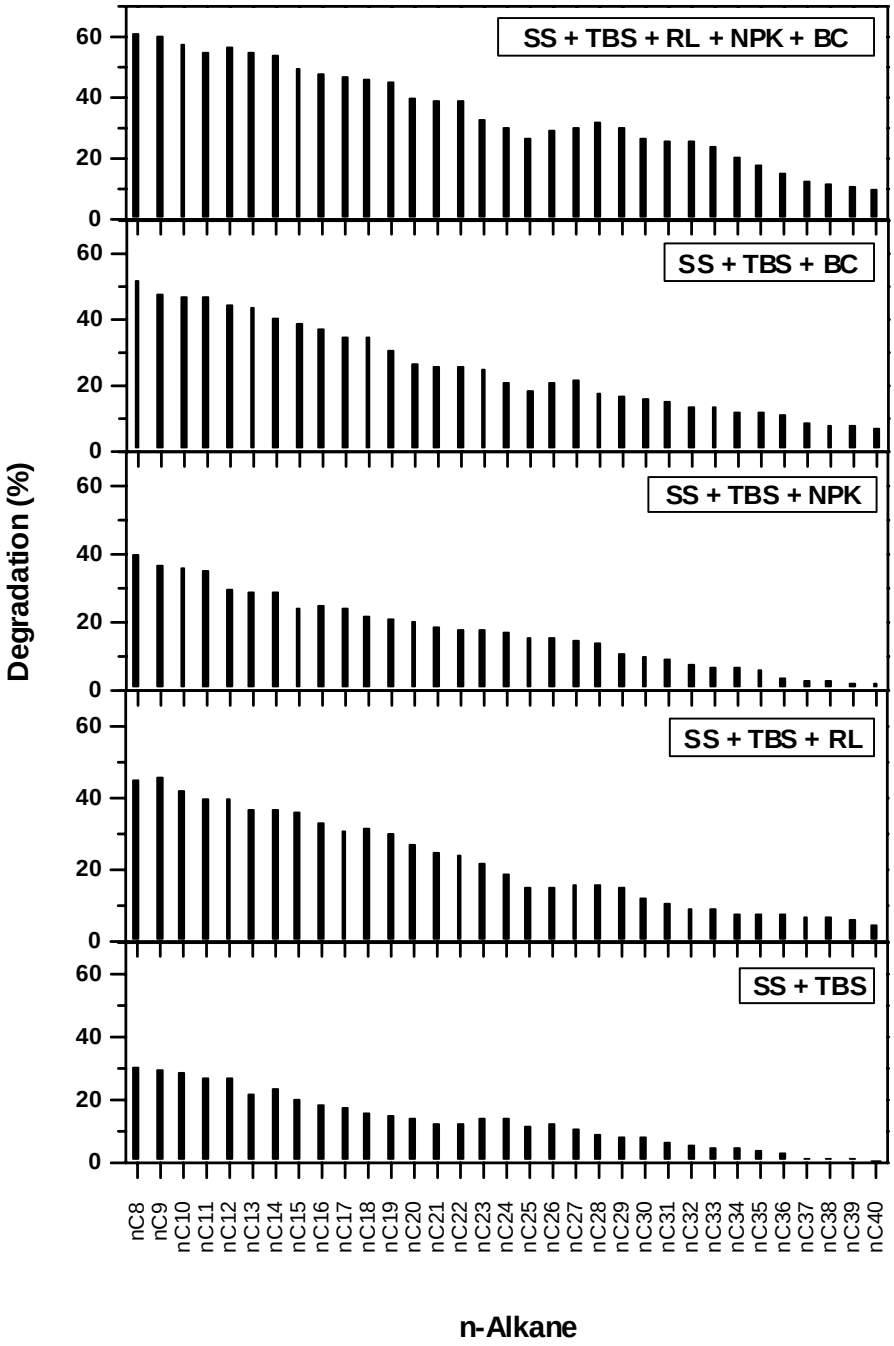
nC38
nC39
nC40
n-Alkane


SS + TBS + RL + NPK + BC
SS + TBS + BC
SS + TBS + NPK
SS + TBS + RL
SS + TBS
Degradation (%)
0
20
40
60
nC8
nC9
nC10
nC11
nC12
nC13
nC14
nC15
nC16
nC17
nC18
nC19
nC20
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nC40
n-Alkane