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Rhamnolipids and nutrients boost remediation of crude oil-contaminated soil by enhancing bacterial colonization and metabolic activities

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ABSTRACT

Bioaugmentation is a promising approach for the remediation of soil and water contaminated with petroleum hydrocarbons. Addition of rhamnolipids and nutrients in the soil enhance survival and metabolic activity of microbes and thus has the potential to restore the quality of soil to a greater extent. This study was carried out to evaluate the prospective role that these supplementary compounds can play in remediation of crude oil-contaminated soil by aiding bacterial strains to survive and perform better. A consortium of hydrocarbon degrading bacteria was inoculated in crude oil-contaminated soil and supplemented with different combinations of rhamnolipids and nutrients. Maximum crude oil-degradation of 77.6% was observed in the soil inoculated with hydrocarbon-degrading bacteria supplemented with rhamnolipids and nutrients. Moreover, addition of supplementary compounds enhanced bacterial survival as well as abundance and expression of alkane hydroxylase gene, *alkB*, in oil-contaminated soil. A strong positive relationship ($r = 0.94$) observed between gene expression and crude oil reduction indicates that catabolic gene expression is essential for hydrocarbon mineralization. All results from the investigation collectively confirm that addition of rhamnolipids and nutrients enhance bacterial colonization and metabolic activity thus improving remediation of crude oil-contaminated soil.

1. Introduction

Petroleum exploration, production, and transportation often lead to the discharge of hydrocarbon pollutants in soil and water (Aparna et al., 2011). The presence of hydrocarbons in the environment poses serious threat to living organisms due to their toxicity, mutagenicity, and carcinogenicity. Bioaugmentation, which is the addition of specialized and actively growing microbial strains to an indigenous microbial community, offers a promising approach for the elimination of hydrocarbon pollutants from contaminated environments (Pandey et al., 2009; Tyagi et al., 2011; Roy et al., 2013). However, microorganisms used for augmentation often show poor survival and activity (Afzal et al., 2013; Khan et al., 2013a; Okoro and Adoki, 2014). Apart from unfavorable environmental conditions in the new locality, low bioavailability of hydrocarbons and insufficient amount of nutrients are the main factors that influence survival and activity of the augmented bacteria (Gallego et al., 2001; Coulon et al., 2007; Ma et al., 2015). To overcome these constraints, the addition of biosurfactants and nutrients in the contaminated soil has been proposed (Arslan et al., 2014; Banat et al., 2014; Suja et al., 2014).

The use of biosurfactants has been reported to enhance hydrocarbon bioavailability in soil and consequently increase their microbial degradation (Marchant and Banat, 2012a; Kavitha et al., 2014; Qiao et al., 2014). This process is based upon the ability of rhamnolipids to promote hydrophilic/hydrophobic interactions leading to reduction in surface tension, blocking the formation of hydrogen bonds, enhancing oil mobilization, and creating micelles

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of oil in water (Perfumo et al., 2010; Marchant and Banat, 2012b; Banat et al., 2010, 2014). Nutrients supplementation of contaminated soil can also enhance crude oil degradation (Coulon et al., 2007; Arslan et al., 2014).

Soil microbes degrade hydrocarbons via different catabolic pathways. Aerobic metabolism of hydrocarbons begins with the terminal or sub-terminal integration of oxygen into the hydrocarbon by a hydroxylase enzyme. The best characterized system utilizes an integral membrane hydroxylase (encoded by *alkB*) to initiate metabolism of hydrocarbons. Once oxidized to a primary alcohol, consequent oxidation steps by alcohol and aldehyde dehydrogenases convert the compounds to fatty acids that may then be further metabolized via β-oxidation and the citric acid cycle (van Beilen and Funhoff, 2007). In anaerobic oxidation of hydrocarbons, sulfate or nitrate act as electron donors, and it has been proposed that alkane activation occurs by the addition of a C group (Callaghan et al., 2009).

Although extensive research has been carried out on evaluating remediation of crude oil-contaminated soil by augmentation with pollutant-degrading microbes, the capacity of rhamnolipids and nutrients to enhance the activity of hydrocarbon-degrading bacteria *in situ* and under natural field conditions by e.g. gene expression has not been addressed. Therefore, the aim of this study was to evaluate the effect of supplementing oil-contaminated soil with rhamnolipids and nutrients on the survival and metabolic activity of inoculated bacteria. Hydrocarbon degradation and nutrients utilization were monitored along with quantitative analysis of functional gene, *alkB*, which is a significant gene in hydrocarbon degradation. This will highlight the correlation between specific microbial populations and progress of oil-degradation (Andria et al., 2009; Afzal et al., 2011; Yousaf et al., 2011).

2. Materials and methods

2.1. Bacterial strains for soil augmentation

Four bacterial strains, which were previously isolated from crude oil contaminated soil were selected based on their high degradation potential of crude oil (tested in vitro). These strains included *Ochrobactrum intermedium* R2 (NCBI accession number: KM007085), *Microbacterium oryzae* R4 (NCBI accession number: KM257016), *Pseudomonas* sp. R7 (NCBI accession number: KM007088), and *Alcaligenes faecalis* R8 (NCBI accession number: KM007089), and carried alkane monooxygenase (*alkB*) gene. The strains were isolated by plating soil suspensions on Minimal Salt Basal Medium (Alef, 1994) amended with 1% (v/v) crude oil. Strains were evaluated regarding their degradation capacity of crude oil, *O. intermedium* R2, *M. oryzae* R4, *Pseudomonas* sp. R7 and *A. faecalis* R8 exhibited 68%, 67%, 57% and 55% crude oil degradation, respectively, in shake flask experiments. They were cultivated separately at 37 °C in Luria Bertani broth supplemented with 1% (w/v) crude oil for acclimatization. Cells of the 4 strains were harvested by centrifugation at 10,000 × *g* and re-suspended together at the ratio of 1:1:1:1 in sterile 0.9% NaCl solution. The amount of each pure culture cell suspension was adjusted by a turbidimetric method (Sutton, 2011) prior to addition to the mixture. Briefly, a series of dilutions (10^1–10^8) of each bacterial suspension were made and immediately absorption readings were taken in sequence with spectrophotometer at 600 nm. The dilutions were then immediately plated on LB agar plates to determine colony forming units (CFU ml^{-1}). The relationship between the absorbance and the number of bacterial colonies was graphed to obtain standard curves. The values in the linear range of these graphs were used to estimate the numbers of CFU in the suspension of each bacterial strain. The physiological state of each bacterium, and the nature and condition of the spectrophotometer were controlled during the estimation of CFU in a suspension.

2.2. Production of rhamnolipids

Three bacterial strains, *Pseudomonas aeruginosa* R25 (NCBI accession number: KM017986), *Pseudomonas aeruginosa* R21 (NCBI accession number: KC188783), and *Pseudomonas aeruginosa* R7 (NCBI accession number: KM007088), were used separately for the production of rhamnolipids. The bacterial strains were inoculated in nutrient broth amended with 4% glycerol and incubated in an orbital shaker at 37 °C. After 72 h, cells were separated and supernatant was used for the extraction of rhamnolipids. pH of the supernatant was adjusted to 2 and it was kept overnight at 4 °C. Rhamnolipids were extracted by vigorous shaking with equal volume of ethyl acetate. The extracted rhamnolipids were quantified by orcinol method with spectrophotometer (Wang et al., 2007). These bacterially-produced rhamnolipids were found to be 72% pure as compared to commercial rhamnolipids (95%, Sigma-Aldrich) by determining their critical micelle concentrations as described earlier (Abdel-Mawgoud et al., 2009).

2.3. Experimental site and setup

The effect of rhamnolipids and nutrients on the survival and metabolic activity of the bacteria used to augment remediation of crude oil-contaminated soil was investigated *in situ* within the premises of an oil production company, located in Chakwal district (32.55°N 72.51°E), Pakistan.

The crude oil-contaminated site was divided into seven plots and each plot was subdivided into three equal macrocosms/subplots (L = 0.7 m, W = 0.7 m, H = 0.7 m). Adjacent plots were separated with bricks and polyethylene sheeting to avoid leaching between the different blocks and ground (Fig. 1). Soil was collected from each macrocosm, allowed to air dry, sieved through a 2 mm mesh, and thoroughly mixed together. Physicochemical

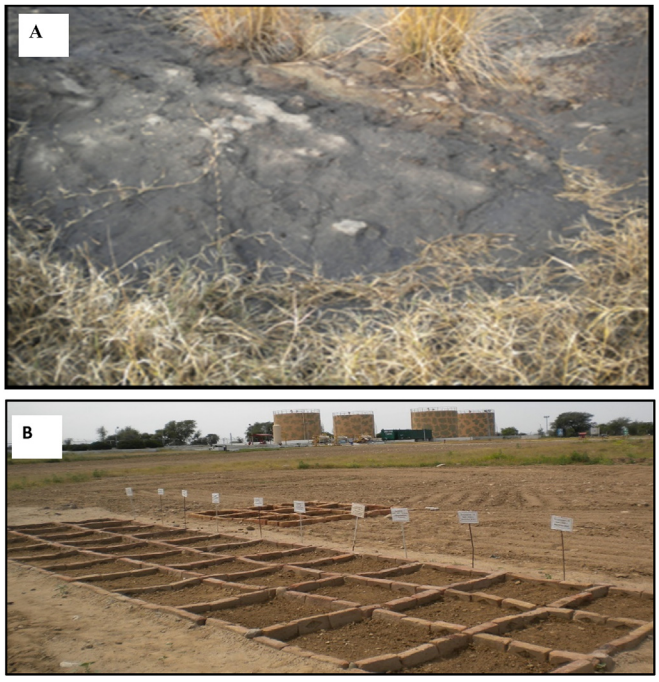

Fig. 1. Bioremediation of crude oil contaminated soil in the vicinity of an oil field. Crude oil polluted soil site (A), and bioremediation of the site (B).

characteristics of soil were measured and recorded as shown in Table 1. Equal amount (70 kg) of soil was added in each macrocosm.

Seven different treatments were setup in the plots. These treatments were:

Control (C): 0.9% NaCl solution
Treatment 1 (T1): Nutrients
Treatment 2 (T2): Bacterial consortium
Treatment 3 (T3): Bacterial consortium + nutrients
Treatment 4 (T4): Rhamnolipids
Treatment 5 (T5): Rhamnolipids + nutrients
Treatment 6 (T6): Rhamnolipids + bacterial consortium
Treatment 7 (T7): Rhamnolipids + nutrients + bacterial consortium

Wherever required in the treatments, 7 g of rhamnolipids (dissolved in 1 L deionized water) and 500 ml bacterial consortium (in sterile 0.9% NaCl solution) were applied until homogeneity was reached in each selected macrocosm. In the case of nutrients, 2.75 g kg^{-1} soil N, 0.38 g kg^{-1} soil P, and 0.4 g kg^{-1} soil K were added in the soil in the form of solution containing NH_4NO_3, KH_2PO_4 and K_2HPO_4 in 5 L deionized water. In this manner, N and P were supplemented to obtain a C:N:P weight ratio of 100:10:1.

Soil in all the plots was watered at intervals to maintain 20% (w/w) moisture content. Soil was thoroughly mixed in each macrocosm and 500 g soil was collected from each macrocosm at regular time intervals of 30 days. Samples were analyzed for bacterial population, *alkB* gene abundance and expression, oil degradation, and utilization of nutrients (N, P, and K).

2.4. Quantification of crude oil-degrading bacteria

Four grams of treated soil were suspended in 10 ml of 0.9% (w/v) NaCl solution and shaken at 180 rpm for 30 min. When soil particles settled, the aqueous phase was plated on minimal salt medium agar containing K_2HPO_4 (7.0 g l^{-1}), KH_2PO_4 (2.0 g l^{-1}), $(NH_4)_2SO_4$ (1.0 g l^{-1}), $MgSO_4$ (0.1 g l^{-1}), $CaCl_2$ (0.02 g l^{-1}), agar (15.0 g l^{-1}) (Bushnell and Hass, 1941) and crude oil (10 g l^{-1}).

2.5. Quantitative analysis of the abundance and expression of alkB gene

The abundance and expression of *alkB* gene in the soil samples was quantified by real-time PCR using an iCycler (IQ) (Biorad) as described earlier (Andria et al., 2009; Afzal et al., 2012). Briefly, DNA and RNA were extracted from the soil using FastDNA Spin kit and FastRNA Pro Soil-Direct kit (Biomedical, USA), respectively. For expression analysis, cDNA was transcribed from the RNA using RevertAid First Strand cDNA Synthesis Kit. The a*lkB* gene copy numbers were quantified relative to a standard curve of positive control. Standards were prepared by serial dilution of stocks containing purified PpalkB PCR products from *Pseudomonas* sp. strain ITRI53 DNA (Andria et al., 2009).

Table 1
The physicochemical characteristics of the soil.

Parameters	Values
pH	7.4 (0.14)
Total petroleum hydrocarbons (g kg^{-1})	47.5 (2.3)
Total carbon (g kg^{-1})	50.2 (2.7)
Total nitrogen (g kg^{-1})	2.25 (0.04)
Available phosphorus (g kg^{-1})	0.12 (0.02)
Available potassium (g kg^{-1})	0.10 (0.01)
Available sodium (g kg^{-1})	1.2 (0.06)
Water content (%)	10.2 (0.1)

Each value is a mean of three replicates, the standard error of three replicates is presented in parentheses.

2.6. Crude oil analysis in soil samples

Residual crude oil in soil samples was estimated by the following method: soil sample (10 g) was acidified to pH 2 with concentrated HCl in a glass beaker. After the addition of $MgSO_4 \cdot H_2O$ (12.5 g), a slurry was prepared by stirring the contents vigorously, and was allowed to stand for 15–30 min. The solids were detached from the beaker, ground in a porcelain mortar and transferred to a paper extraction thimble. Residual oil was extracted in a pre-weighed round-bottom flask with petroleum ether (200 ml), using a Soxhlet apparatus, for 4 h. The solvent was separated from the residual oil with a rotary evaporator. After evaporation at 40°C overnight in an oven, the amount of residual oil was quantified gravimetrically (Shabir et al., 2008; Das et al., 2014; Varjani et al., 2015).

2.7. Determination of nutrients and moisture content

About 1 g air-dried fine soil was digested with 1.0 g of mixture (K_2SO_4 100 g, $CuSO_4$ 10 g and selenium metal 1 g) and 3 ml of concentrated H_2SO_4, followed by distillation with Kjeldahl distillation unit (Velp, Model, UDK126A), titration of distillate against 0.01 N H_2SO_4 and calculation of total nitrogen in the soil sample (Keeney and Nelson, 1982). For phosphorus and potassium estimation, 20 g soil was shaken with distilled water for 30 min and filtered through Whattman filter paper no. 42. Phosphorus in the water extract was determined by colorimetric method, and potassium by using flame photometry (Seac, Italy, Fp20) (Rump, 1999). For moisture content determination, triplicate samples, each with approximately 10 g of soil in a pre-weighed Petri dish, were dried at 75 °C overnight, then cooled in a desiccator to room temperature and weighed. Drying, cooling, desiccating and weighing were repeated until a constant weight was obtained and percentage moisture content of soil sample was calculated.

2.8. Statistical analysis

All statistical analyses were performed using SPSS software package (SPSS in., USA.). The data was subjected to analysis of variance (ANOVA) and significant differences between means were determined by Duncan's multiple range tests ($p < 0.05$).

3. Results and discussion

3.1. Crude oil-degradation

Very low reduction (12%) in crude oil was observed in the control soil (Fig. 2A). This degradation is attributable to indigenous microorganisms that use some of the limited nutrients available and also to naturally occurring physicochemical processes such as volatilization, photo-oxidation, sorption, and/or adsorption. In soil augmented with only nutrients (T1), 18.5% oil reduction was observed, indicating that the augmentation with nutrients can only slightly enhance the degradation of crude oil as was also reported earlier (Rahman et al., 2003; Saikia et al., 2012). The addition of bacterial consortium in treatment T2 resulted in significantly higher reduction in crude oil concentration than the reduction achieved by nutrient stimulation only (T1). This highlights the promising role of specialized bacteria with capability to degrade crude oil for the remediation of crude oil-contaminated soil. As would be expected, the combined use of bacteria and nutrient augmentation in treatment T3, further enhanced crude oil-

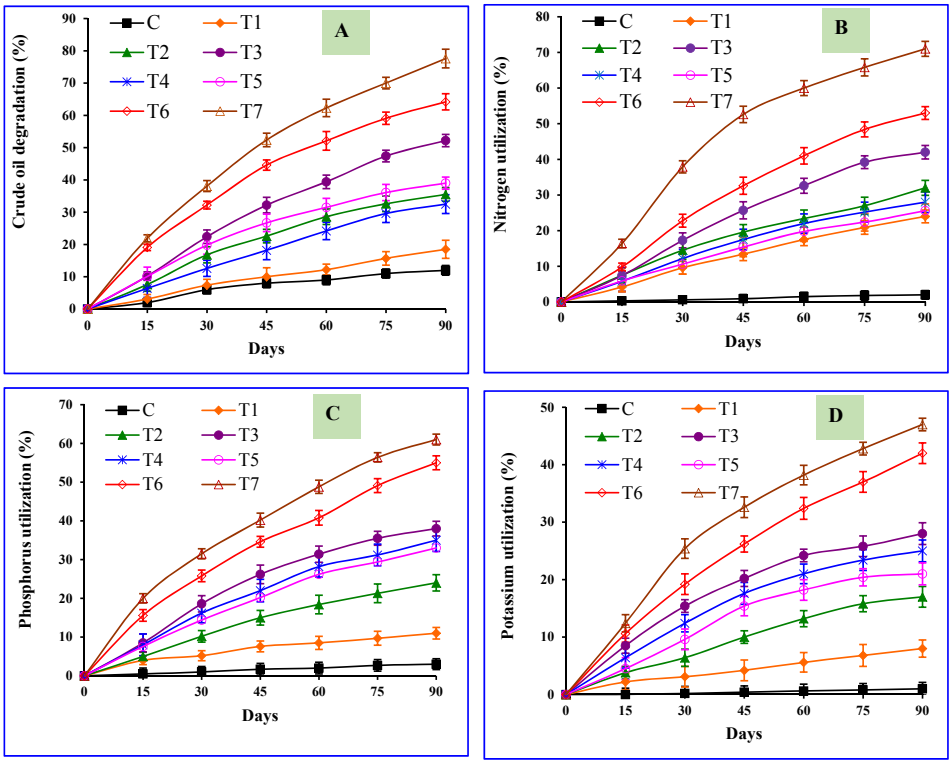

Fig. 2. Effect of nutrients, rhamnolipids and bacterial augmentation on biodegradation of crude oil (A), utilization of nitrogen (B), phosphorous (C) and potassium (D) during the remediation of crude oil contaminated soil. C = Control; T1 = nutrients (NPK); T2 = bacterial consortium; T3 = bacterial consortium + nutrients (NPK); T4 = rhamnolipids; T5 = rhamnolipids + nutrients (NPK); T6 = rhamnolipids + bacterial consortium; T7 = rhamnolipids + nutrients (NPK) + bacterial consortium, n = 3; bar indicates the standard error of three replicate.

degradation. These findings are in agreement with previous reports that the combined use of bacteria and nutrients is a better approach for the remediation of soil contaminated with petroleum hydrocarbons (Coulon et al., 2007; Shabir et al., 2008).

The addition of only rhamnolipids to the soil (T4) resulted in considerable crude oil-degradation (Fig. 2A). Previous investigation also reported that the addition of biosurfactants resulted in enhanced hydrocarbon degradation (Cameotra and Singh, 2008; Thavasi et al., 2011). Being biosurfactants, rhamnolipids enhance the bioavailability of hydrocarbons in the soil which improves hydrocarbon degradation activity of microbes (Perfumo et al., 2010; Marchant and Banat, 2012b; Banat et al., 2014). The augmentation of nutrients in addition to rhamnolipids (T5) led to a further increase in hydrocarbon reduction. Furthermore, when both rhamnolipids and hydrocarbon degrading bacterial strains were added to soil (T6), significantly higher degradation of crude oil was achieved than T1, T2, T3, T4, and T5. Maximum crude-oil degradation (77%) was achieved in the soil augmented with bacterial consortium, rhamnolipids, and nutrients (T7) (Fig. 2A). Recently, Okoro and Adoki (2014) also reported enhanced remediation of crude oil-contaminated soil with the use of microbes, nutrients, and surfactants. In this study, the oil concentration in soil decreased from 47.5 g kg^{-1} soil to 11.5 g kg^{-1} soil by the combined use of bacteria, nutrients and rhamnolipids within 3 months. According to the guidelines of the Railroad Commission of Texas (RRC), responsible authority for spills and discharges of oil contaminants, the application of bioremediation must decrease the concentration of total petroleum hydrocarbon to 10 g kg^{-1} soil within one year (USEPA , 2003).

3.2. Nutrients utilization

The utilization of nutrients was observed in all the treatments (Fig. 2B, C and D). The greatest consumption of nutrients occurred in experiment containing rhamnolipids, nutrients and bacteria where 71% of N, 61% of P and 47% of K present in the soil were taken up by the cells. Earlier studies corroborate that a large amount of nutrients are required for bacterial proliferation and their catabolic activity (Rosenberg et al., 1992; Arslan et al., 2014).

3.3. Bacterial population and alkB gene abundance and expression

The presence and activity of crude oil-degrading bacteria in the soil at different time intervals were assessed using cultivation and DNA-based methods (Fig. 3A, B, and C). Among the inoculated treatments, the bacterial population was significantly higher in those treatments supplemented with rhamnolipids and/or nutrients. Maximum bacterial population was observed in soil from T7 where oil contaminated-soil was augmented with consortium of oil-degrading bacteria and stimulated with both rhamnolipids and nutrients. The smallest numbers of bacteria were observed in T2 and T3.

Our results revealed that the abundance of all the inoculated bacterial strains involved in hydrocarbon degradation remained stable for about 1 month, then dropped by 1–2 orders of magnitude each 30 days (Fig. 3A, B and C). The fact that the abundance of inoculated bacteria decreased may result from insufficient nutrient availability and sub-optimal environmental conditions such as soil water activity, pH and ionic strength (Hai et al., 2009; Afzal et al., 2011; Yousaf et al., 2011). Secondly, the competition between inoculated bacteria and indigenous microbes for nutrients may have influenced inoculated bacteria survival (Olson et al., 2008; Bürgmann et al., 2005; Kaakinen et al., 2007). Thirdly, the decrease in hydrocarbon degraders may be due to a decline in easily biodegradable hydrocarbons (Andria et al., 2009; Yousaf

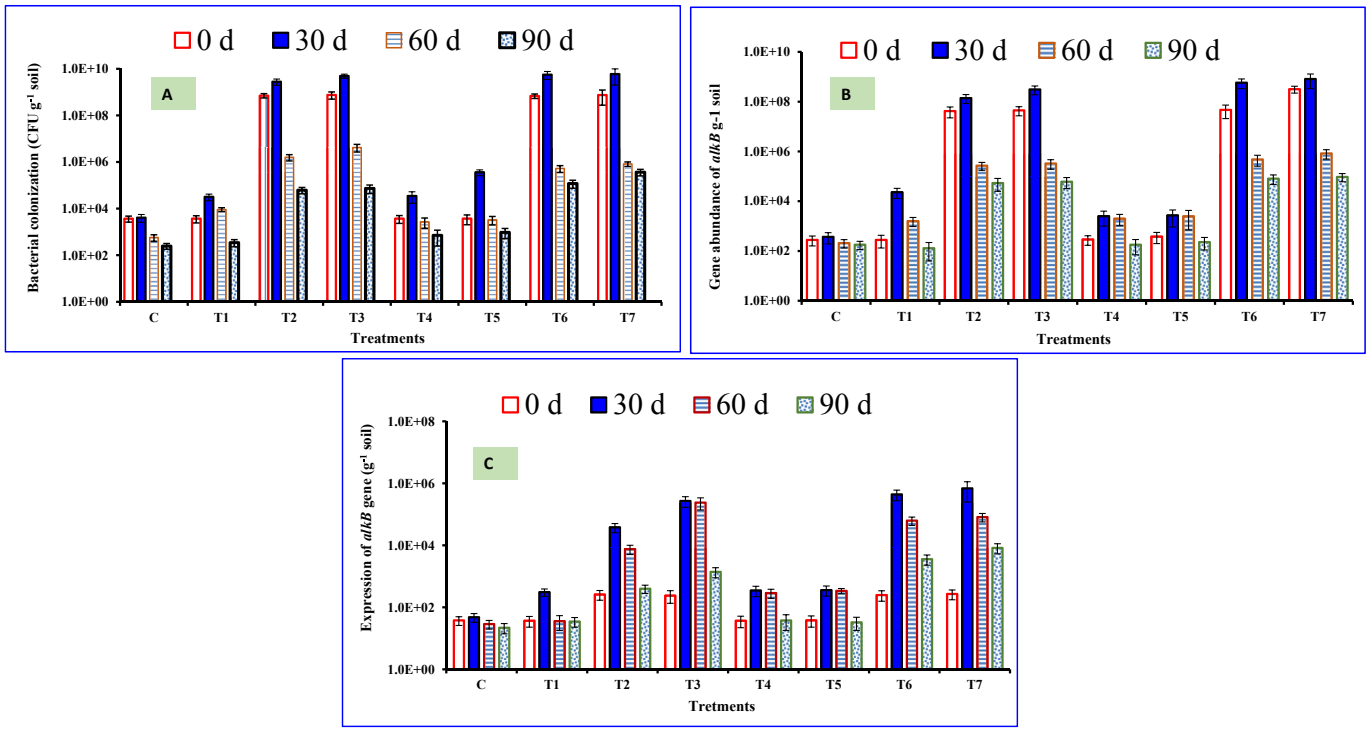

Fig. 3. Effect of nutrients, rhamnolipids and bacterial augmentation on colony forming unites (CFU) (A), the abundance of *alkB* gene (B), and numbers of *alkB* gene transcripts (c) during the remediation of crude oil contaminated soil. C = Control; T1 = nutrients (NPK); T2 = bacterial consortium; T3 = bacterial consortium + nutrients (NPK); T4 = rhamnolipids; T5 = rhamnolipids + nutrients (NPK); T6 = rhamnolipids + bacterial consortium; T7 = rhamnolipids + nutrients (NPK) + bacterial consortium, n = 3; bar indicates the standard error of three replicate. Values are shown on logarithmic scale along Y-axis.

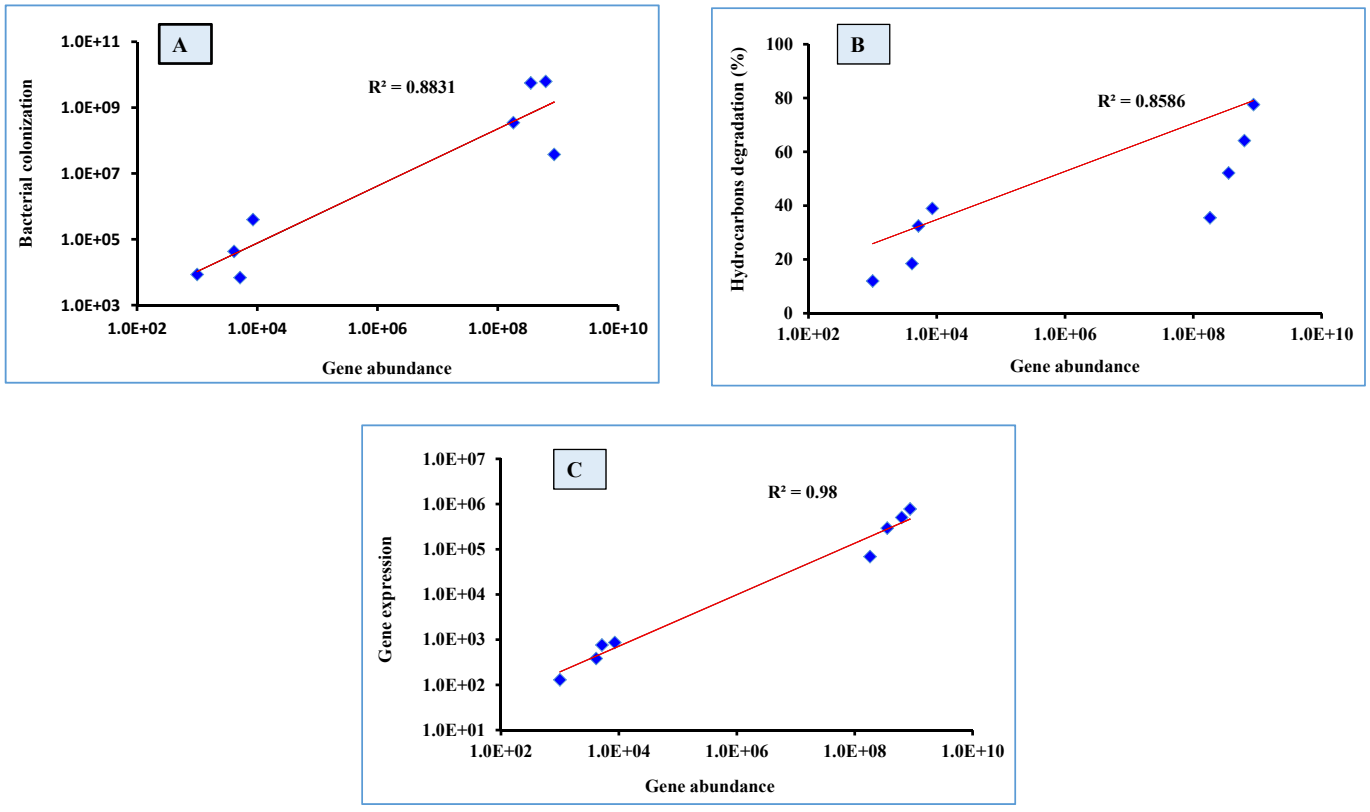

Fig. 4. Correlation between gene abundance (*alkB*) and bacterial colonization (A), gene abundance and hydrocarbons degradation (B), and abundance and expression of *alkB* (C).

et al., 2010; Afzal et al., 2012).

The copy numbers of *alkB* gene initially increased in the first month of experiment then decreased gradually. In the uninoculated soil, very low numbers of *alkB* gene were observed in comparison to the soil inoculated with crude oil-degrading bacteria (Fig. 3B). Among the inoculated treatments (T2, T3, T6 and T7), it was again noted that the overall number of gene copies was significantly higher in the treatments containing rhamnolipids: T6, and T7 in comparison to the remaining treatments and control. The gene expression (Fig. 3C) had a unique pattern with a distinct increase after 30 and 60 days followed by a decrease. Furthermore, the treatments with rhamnolipids also exhibited a slightly higher gene expression as compared to treatments without them: the highest gene expression levels being exhibited in T7 that contained both rhamnolipids and nutrients.

The abundance of degrading gene, *alkB*, showed positive correlation with gene expression ($r = 0.98$), hydrocarbon-degradation ($r = 0.87$) and bacterial numbers ($r = 0.88$) (Fig. 4A, B, and C). Catabolic genes may serve as markers of actual function: in the case of hydrocarbon degrading communities, strong positive correlations have previously been reported between gene copies and transcripts (Andria et al., 2009; Yousaf et al., 2011; Khan et al., 2013b) indicating that the presence of genes is related to their activity. As the average activities (*alkB* transcripts/numbers) were higher at the first sampling time (0.67) than the second (0.45) and third (0.22) sampling times, it revealed that a larger cell fraction of the inoculated bacteria was active during the first month of the treatment than the second and third months. This indicates that growth conditions such as more nutrients and/or activating substances might have been present in larger amounts during the initial month. The most interesting observation in this investigation is that the presence of rhamnolipids exhibited significant advantage in maintaining higher CFU counts as well as higher *alkB* numbers and expression in all the treatments where it was added in comparison to equivalent treatments that did not have rhamnolipids (Fig. 3A, B, and C and Supplementary Tables 2–4).

In conclusion we showed that inoculation with suitable bacterial strains has the potential to enhance degradation of crude oil, however, the process is strongly influenced by rhamnolipids and nutrients. The rhamnolipids and nutrients did not only affect microbial colonization and activities but also substantially influenced hydrocarbon degradation. The importance of such supplements should be considered in the design of bioremediation applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2016.08.010.

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