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Rhizodegradation of Hydrocarbon from Oily Sludge

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Abstract

Rhizoremediation involves the use of plant growth promoting rhizobacteria (PGPR) to remove organic pollutants from terrestrial environment. The main objective of this study was to evaluate the efficiency of rhizoremediation through inoculation of hydrocarbon degrading bacterial strains with and without nutrients in the rhizosphere sporadically become contaminated with oily sludge. Bacterial strains viz. *Bacillus cereus (*Acc KF859972), *Bacillus altitudinis (*Acc KF859970)*, comamonas (*Acc KF859971) *and Stenotrophomonas maltophilia* (Acc KF859973) were isolated from various oily waste pits of oil fields. Autoclaved soil was treated with oily sludge at 7:3 ratio and Alfalfa plant was inoculated with individual plant growth promoting rhizobacteria (PGPR) with and without diammonium phosphate and ammonium nitrate. The different saturates and total hydrocarbon was extracted by Soxhelt extraction and was analyzed by GC-FID at 0, 5 and 10 d of incubation. The inoculation 30% sludge with *B. altitudinis* accelerated the rate of degradation of *n*-alkanes and some methyl branched than un-inoculated soils. Oily sludge inoculated with *B. altitudinis* indicated that more than 80% of hydrocarbons were degraded at day 5 of incubation. No further degradation was observed till the end of the incubation period. *B. altitudinis* is capable of degrading straight chain hydrocarbons rapidly than any other strains. Alfalfa is a N-symbiotic plant and increased nutrient content under the rhizosphere promote degradation of *n*-alkanes. All other bacterial strains also showed significant interaction with alfalfa for the biodegradation of oily sludge but the rate of degradation remained slow than with *B. altitudinis*. Inoculation with *B. altitudinis* accelerated the rehabilitation process and within 5 days the rehabilitation occurs.

Keywords: Rhizoremediation; PGPR; Bioremediation; *n*-Alkanes; Hydrocarbon; Oily sludge

Introduction

Over the last several decades, large quantity of oily sludge has been removed during drilling and stored in open pits within the vicinity of oil fields. The entry of such waste to the terrestrial or aquatic environment has threat to the agro-environmental ecosystems.

Oily sludge is composed of a wide range of organic compounds such as alkanes saturate, aromatics and aspaltenes [1]. Short chain hydrocarbons such as nC_{10} to nC_{12} are considered to be phytotoxic even at low concentration in the oily sludge. Moreover, such hydrocarbons are degraded rapidly however the rate of degradation primarily depends on the concentration of these hydrocarbons in oily sludge and secondarily on the length of the exposure of contamination to soil with such hydrocarbons [2]. Rahman et al. [3] reported that the addition of inoculation of bacterial strains, biosurfactant and fertilizers enhanced the degradation of *n*-alkanes. The *n*-Alkanes in the range of n-C_8-nC_{11} were completely degraded followed by nC_{12}-nC_{21}, nC_{22}-nC_{31} and nC_{33}-nC_{40} with % degradation of 100, 83-98, 80-85 and 57-73% respectively in 10 and 20% sludge at 56 days of incubation. Zand et al. [4] reported that 97% of total hydrocarbon was removed by planting Flax plant.

Oily sludge acts as a carbon substrate for diverse variety of microorganisms [5]. Siddiqui et al. [6] reported that bacterial population increased to 100 fold as compared to soil without previous history of hydrocarbon contamination. Fulekar [7] investigated two different soils and found that an increase in total microbial count was experienced with oily sludge contaminated soils, but clay soil showed no change after oil contamination.

Previous literature reported that there is a lag phase prior to utilize oily sludge as a carbon source by indigenous microorganisms [8-10]. These lag phases varies and depend on the type and concentration of hydrocarbons present in the oily sludge.

Numerous genera of bacteria such as *Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Bacillus, Brevibacterium, Geobacillus, Mycobacterium, Pseudomonas, Rhodococuss, Sphingomonas, Thermus and Xainthomonas* species has been isolated from oily sludge using PCR and DNA-DNA hyberdization [11,12]. Cerqueira et al. [13] isolated *Stenotrophomonasacidaminiphila, Bacillus megaterium and Bacillus cibi,* from petrochemical oily sludge and *Pseudomonas aeruginosa* and *B. cereus* capable of degradation of hydrocarbons from oily sludge of Brazil. Jiann-Hong et al. [14] isolated *B. altitudinis* from oily sludge and found that inoculation of such bacteria to soil become contaminated with benzene can accelerate the degradation of benzene.

Rhizoremediation includes both bioremediation and phytoremediation. Bioremediation means inoculation of oily sludge contaminated sites with hydrocarbon degrading bacterial consortium and nutrients more likely to reduce the lag phase required for indigenous bacterial population to degrade hydrocarbons into biomass, CO_2 and intermediate products. In addition to that phytoremediation means to use hydrocarbon tolerance plants such as alfalfa, soyabean, perennial ryegrass, fescue or kaller grasses or others to degrade hydrocarbons of oily sludge contaminated sites. Plants or rhizosphere will provide a unique environment for hydrocarbon degrading microorganisms to grow and increase in number and because of their combine effect of release of root exudates and microorganisms the rate of degradation of hydrocarbons is more rapid than in non rhizosphere environment of oily sludge contaminated sites. Through rhizoremediation natural

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rehabilitation process of degradation of oily sludge is more rapid than either through bioremediation or phytoremediation.

Pradhan et al. [15] reported that the degradation of poly aromatic hydrocarbons (PAHs) were greater in soil where switchgrass (*Panicumvirgatum*) and little bluestem grass (*Schizachyriumscoparium*) were grown. They found that around 57 and 47% of PAHs were degraded than uncontaminated soils. Omotayo et al. [16] reported that inoculation of 50 mg of crude oil per g of soil with consortium of hydrocarbon degrading bacteria such as *Arthrobacter* sp., *Bacillus pumilus*, *Bacillus sphaericus* and *Serratiamarcescens* and perennial rye grass has increased the rate of degradation of hydrocarbons and around 87.7% of total hydrocarbons were degraded from the soil at 45 days of incubation.

The study was aimed to enhance the natural rehabilitation process of oily sludge contaminated soils inoculated with plant growth promoting bacteria isolated from the host oily sludge and to understand the role of alfalfa on the degradation of hydrocarbons in oily sludge contaminated soils.

Materials and Method

Geology and samples description from potwar plateau

The Kohat-Potwar Plaeatu is situated in the south of the Himalaya and Karakorum Mountains. It is located between latitude 32° and 34°N and longitude 70° and 74°E. It is bounded by the Salt Range and Trans-Indus Range in the south, Kala chitta Range in the north. The Plateau opens up towards Jehlum River in the east. The west of the Plateau is bounded with Kurram-Parachinar Range [17]. Potwar plateau is an oil prone area. Up till now 40 structures have been drilled for oil exploration among which 10 structures have a potential of oil production [18]. The oily sludge from three oil fields within Potwar plateau were selected because of the difference in the reservoir depth, geological formation and age, API gravity, extent of degradation and crude oil composition (Table 1).

Sample collection

Soil samples were collected within the vicinity of Missa kasswal oil field of Potwar plateau. The root samples from Missa kasswal oil field were collected by the method of shaking and washing of root system as described by Berge et al. [19]. About 500 g of oily sludge was collected from oil waste pits (100×100 m) within oil fields and were brought to the laboratory for further analysis. Oily sludge was spread over plastic tray covered with aluminum foil. Around 50 mL of phosphate buffer saline solution was mixed thoroughly with glass spatula and the oily sludge was placed in glass bottles (250 mL). All glass bottles were incubated in the refrigerator at 4 °C for further analysis.

DNA extraction

Extraction of genomic DNA of bacterial strain was carried out by using the DNA GenElute Bacterial Genomic DNA Kit and DNA was visualized on agarose gel method as described by Kate [20].

PCR (polymerase chain reaction)

The genomic DNA of isolated strains was amplified by the method as described by Weisburg et al. [21]. The Polymerase chain reaction was performed by using two universal primers fd1 fD1=(AGAGTTTGATCCTGGCTCAG) and rd1=(AAGGAGGTGATCCAGCC).

Preparation of 30% sludge and treatment application

Soil was autoclaved at 121°C and sludge was mixed to soil. Sludge (30%) was prepared by mixing oily sludge with soil in 3:7 ratios. After mixing sludge with soil 1 kg samples of sludge treated soil was transferred to 1 kg plastic containers. The pots were covered with loosely fitted perforated lids and were incubated at room temperature for 60 days to allow sufficient time for most hydrocarbons to breakdown.

The nutrient broth (Nutrient Broth OXOID UK) was inoculated with pure culture of isolated PGPR strains and incubated in shaker incubator (EXCELLA E24 Germany) for 48-72 h at 150 rpm. Thereafter, the culture was centrifuged at 3000 rpm**.** The pellet was resuspended in autoclaved distilled water and the O.D was adjusted to 1 at 660 nm. The fertilizer solutions were made in distilled water by adding Ammonium nitrate and Diammonium phosphate (DAP). at the rate of N=70 µg/ml and P=7 µg/ml**.** Sludge (300 g) was filled in small pots measuring 3 cm × 3 cm^2 for single strain experiment in which case the single strain (20 ml) was added to the sludge and incubated at room temperature.

Nutrients such as N (NH_4NO_3) and $(NH_4)_2HPO_4$ were added at the rates of N and P i.e. N=70 µg/g and P=7 µg/g to each control and 30% sludge. The rates of N and P were N=70 µg/g and P=7 µg/g. Aqueous solutions (30 ml) of (NH_4NO_3) and $(NH_4)_2HPO_4$ solution were pipetted over the sludge and mixed well by hand several times. The PGPR inocula as single strain were added to the sludge. The Inocula was prepared by inoculating pure cultures of four PGPR of individual strain having O.D 1 at 660 nm. The samples were kept in large plastic containers and were covered with loosely fitted perforated lids and incubated at 22°C for 60 days.

Determination of total bacterial population

For viable count of aerobic heterotrophs, the pour plate method was used [22].

Breakdown of diesel hydrocarbons

Triplicates (5 g) were taken after 0, 5 and 10 days of incubation from the soil/treatments and were placed into cellulose thimble of known weight. These were placed into Soxhelt extraction apparatus. Anthracene (Sigma chemical company, Germany), was used as an internal standard. This was added to bulk sample of around 50 g of soil at the rate of 1 mg/g and mixed carefully before taking 10 g subsamples. Re-distilled dichloromethane (100 ml) was poured into a clean, pre-weight 250 ml round bottomed flask and were soxhelt extracted as described by Song and Bartha [22]. The extract was analysed by GC-FID (QC 20-Schmadzu) coupled to a flame ionization detector.

Sr. No.	Oil wells	Reservoir depth	Geological formation/age	API gravity	Extent of contamination (years)	Biodegradation level
1	ChakNaurang (CNG 1-A)	2687 m	Sakesar/chorgali/Khewra/Cambrian	18.4	27	Biodegraded (3)
2	Dhakni	4,844	Lockhart/shakesar/chorgali	39.31	24	Biodegraded (3)
3	MissaKeswal	2187m	Chorgali/lower cambrian	36.2	21	Non biodegraded (0)

Table 1: The difference in the depth, formation/age, API gravity and biodegradation of crude oil within Potwar plateau.

Crude oil fractionation into saturates (*n*-alkanes and isopreniods), aromatics and naphthlenes (isoprenoids)

Separation of saturates *(n-alkanes and isopreniods),* aromatics and polar *(risens and asphaltenes)* from crude oil was carried out by silica gel 60 column chromatorgraphy as described by Asif et al. [23] For column chromatography, a glass column (40 × 0.9 cm i.d.) with cotton wool at bottom was rinsed with 20 ml of dichloromethane. Around 200 g of silica gel was placed in 250 ml beaker and was activated at 120°C for 24 hours prior to be used for column chromatography. About 10 g of activated silica gel 60 with mesh size 35-70 mm (Fluka-Germany) was removed from the oven and was poured over the column with cotton wool at the bottom. The silica gel 60 was saturated with *n*-hexane (50 ml). Once the column bed is prepared around 50 mg of crude oil was transferred to the column from Pasteur pipette. The saturate fraction of crude oil was eluted with *n*-hexane (35 mL) and was collected in 50 ml of bottles. Once saturate fraction is obtained fractionation of aromatic compounds was carried out by adding a mixture of *n*-hexane:dichloromethane (35 mL, 7:3) which was collected in glass bottle (50 mL) prior to be analyzed by GCFID. Polar compounds were fractionated with 35 mL of methanol: dichloromethane (1:1). The solvent in each fraction was evaporated through roto-evaporator at 60°C temperature.

Gas chromatography-flame ionization detector (GC-FID)

GC-FID analysis was performed using a QC 2010 Shimadzu GCFID. A 30 m × 0.25 mm ID capillary column coated with a 0.25 µm 5% phenyl 95% methyl polysiloxane stationary phase (DB-5 MS, J & W scientific) was used for the analysis. 1 µL of the saturated or aromatic fractions (1 mg/mL in *n*-hexane) was introduced into the split/splitless injector using the QC 2010 auto-sampler. The injector was operated at 320° in pulsed splitless mode. Helium maintained at a constant flow rate of 1.1 mL/min was used as carrier gas. The GC oven was programmed from 75°C to 320°C at 10°C/min with initial and final hold times of 28.83 minutes, respectively.

The peak area of each standard on GC trace was converted to mg kg^{-1} of the hydrocarbon. The response factor of each external standard was calculated by taking the mean of response factor of 5 step dilutions calibration curve using following formulas.

The calibration curve is determined by the analysis of 3 calibration levels, i.e. 0.1, 0.25 and 0.5 mg kg^{-1}. Peak area was calculated and calibration curve was plotted for concentration vs response area or peak area. The calibration curves were best fitted to a linear curve. The correlation coefficients (R) were 0.9947. The quantification was performed from the mean of two calibration curves surrounding the samples.

Statistical analysis of DATA

The data of isolates were analyzed by complete randomized design using LSD and compare means by statistix 8.1.

Results and Discussion

Alignment of 16S rRNA sequence

For the isolate obtained from oily sludge of Missa kasswal the total length of sequence with 1497 nucleotide was obtained. The comparison of the nucleotide sequence with data nucleotide bank showed highest 99% sequence similarity for 1463/1466 nucleotide bases with that of *B. cereus* strain partial sequence (Acc KF859972).

For the isolate obtained from oily sludge of Missal kasswal the total length of sequence with 1535 nucleotide was obtained. The comparison of the nucleotide sequence with data nucleotide bank showed 99% sequence similarity for 1477/1485 nucleotide bases with that *Commamonas* bacterium clone EK_An354 16S ribosomal RNA gene, partial sequence (Acc KF859971).

For the isolate obtained from oily sludge of Chak Naurang the total length of sequence with 1501 nucleotide was obtained. The comparison of the nucleotide sequence with data nucleotide bank showed highest 99% sequence similarity for 1477/1482 nucleotide bases with that of *S. maltophilia* strain E56 16S ribosomal RNA gene, partial sequence (Acc KF859973)

For the isolate obtained from Dhakni oil field total length of sequence with 1506 nucleotide was obtained. The comparison of the nucleotide sequence with data nucleotide bank showed highest 99% sequence similarity for 1466/1475 nucleotide bases with that of *B. altitudinis* strain: 41KF2b 16S ribosomal RNA, partial sequence (Acc KF859970).

Several genera of bacteria were isolated from oily sludge such as *Pseudomonas, Bacillus*, *Proteobacteria* (with a predominance of *Alphaproteo bacteria* and *Gammaproteo bacteria*), *Actinobacteria, Cytophaga-Flavobacterium-Bacteroides, Firmicutes. Spirochaetes* [24-26] *S. maltophilia* and *B. cereus* were isolated from oily sludge (Chak Naurang) and (Missa Kasswal). Yoshida et al. [27], Erdoğan et al. [28], Saeideh Rajaei et al. [29] reported *S. maltophilia a* gram –ive bacteria causing human bacteria because it causes human infection which is very difficult to treat. However this is also common bacteria found in oily sludge. This is the first study reporting the presence of *Comamonadaceae sp* in oily sludge of Missa kasswal.

Bacterial Population during degradation

The bacterial count was lower in untreated sludge (30%). Inoculating sludge with PGPR increased the proliferation of the PGPR as evidenced by the log cfu/g sludge. The *B. cereus* being the most effective and showed 79% increase over that of untreated control. Addition of fertilizer decreased the cfu of *B. cereus* but had no significant effect on cfu of *S. maltophilia*. The cfu of *B. altitudines* was higher 67% in presence of fertilizer. At 10 d of inoculation the cfu was decreased even in untreated sludge but this decrease was 55% less in *B. cereus* and 32% in *S. maltophilia*. *B. altitudinies* showed 92% significant increase in presence of fertilizer when compared with untreated sludge (Figure 1a).

The cfu of *B. cereus* was 21% higher than untreated sludge in presence of alfalfa but it was 43% less than *B. cereus* used alone. The *commamonas* had 16% higher cfu in presence of alfalfa but the presence of alfalfa had decreased the cfu of *S. maltophilia* and *B. altitudinies* by 93 and 98% respectively. Addition of fertilizer had significantly increased the cfu of *B. cereus* in the presence of alfalfa. Even at 10 d after inoculation the cfu of *B. cereus* was higher 88% in presence of alfalfa.

Addition of fertilizer also increased the cfu of *S. maltophilia* and *B. altitudinies* over that of single inoculation in presence of alfalfa but the value was lower than that of *Commamonas* + alfalfa without fertilizer. However, this increase was of much lower magnitude than that of *B. cereus* alone. At 10 d of inoculation in presence of alfalfa variable responses observed being stimulatory for *S. maltophilia* and *B. cereus* in presence of fertilizer (Figure 1b).

Results revealed that all the PGPR bioinoculants isolated from oily sludge used to reinoculate the sludge showed increase in cfu of respective PGPR strain. Addition of fertilizer may be beneficial

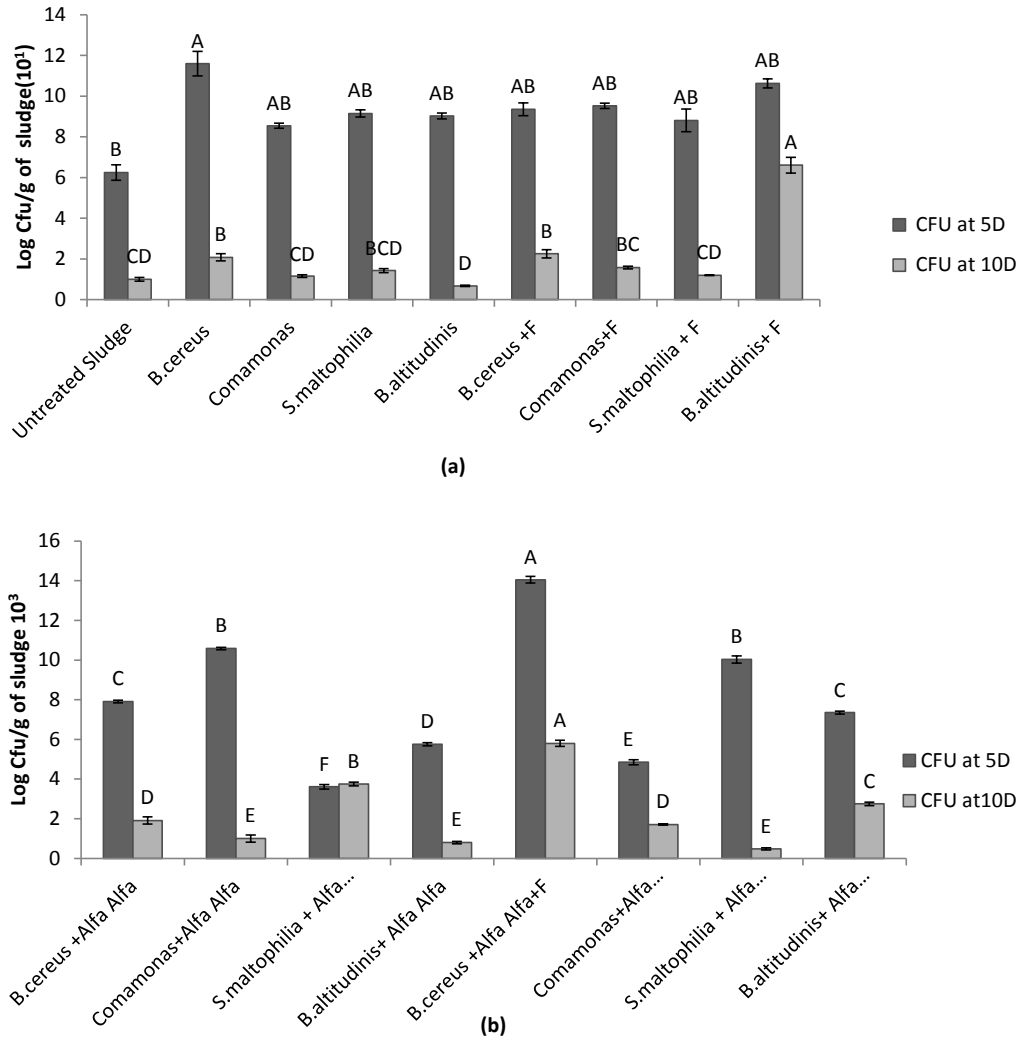

Figure 1: *S. maltophilia. B. altitudinies* showing 92% significant increase in presence of fertilizer when compared with untreated sludge.

for certain bacterial strains perhaps acting as C/N source eg in *B. altitudines* however, the addition of fertilizer and growing alfalfa may be beneficial for other strain e.g. *B. cereus* both for short term and long term incubation. The *B. cereus* can survive well in the oily sludge and show significant cfu even at 5 d and *B. altitudines* needed fertilizer to have higher cfu and maintained the increased cfu even at 10 d.

Planting alfalfa can assist *Commamonas* to proliferate in oily sludge whereas *B. cereus* needed fertilizer as C/N source along with alfalfa to have significant increase in cfu both in long term (10 d) and short term (5 d) of incubation in sludge (30%). Different species of bacteria exhibit different mechanism of survival in oily sludge. In presence of alfalfa the *S. maltophilia* exhibited lower cfu at 5 d which increased at 10 d possibly the microbe has to adjust with the root exudates of the plant at early stage but may outcompete at later stages. Whereas, *B. cereus* and *Commamonas* show inhibition in cfu when fertilizer was added along with the plant alfalfa. The existence of plants inspired microbial activities and bacteria populations and the microbial populations in soil were enhanced more greatly by strain inoculated without alfalfa and Indian mustard [30], the bacterial count was increased with all the strain when used in association of alfalfa and this increased in bacterial population is might be due to extra nutrition provided by root exudates of alfalfa as compared to strain used alone. This statement supports our result findings.

Degradation of *n*-alkanes

The fertilizer has significant effect on the degradation of (nC_{13}-nC_{16} series) when oily sludge was inoculated with *B. cereus*. Similar was the case with *commamonas* and *S. maltophilia* However B. *altitudines* decreased the % degradation by 20% but *B. cerus* has no significant difference in the degradation of *n*-alkanes in presence of alfalfa which showed 22% degradation in presence of alfalfa in contrast to 36% without alfalfa. At 10 d of inoculation 50% or greater degradation occurred with the addition of fertilizer even within 5 d of inoculation the % degradation was earlier and almost similar to that of 10 d of inoculation without fertilizer and no significant effect of further incubation period after inoculation was detected (Figure 2a and 2b).

Growing alfalfa has further improved the % degradation in *B. cereus* and *S. maltophilia* inoculated sludge but delayed and reduced the % degradation in *B. altitudinis*. At day 10 the effect was similar to that of single inoculation. Addition of both alfalfa and fertilizer enhanced

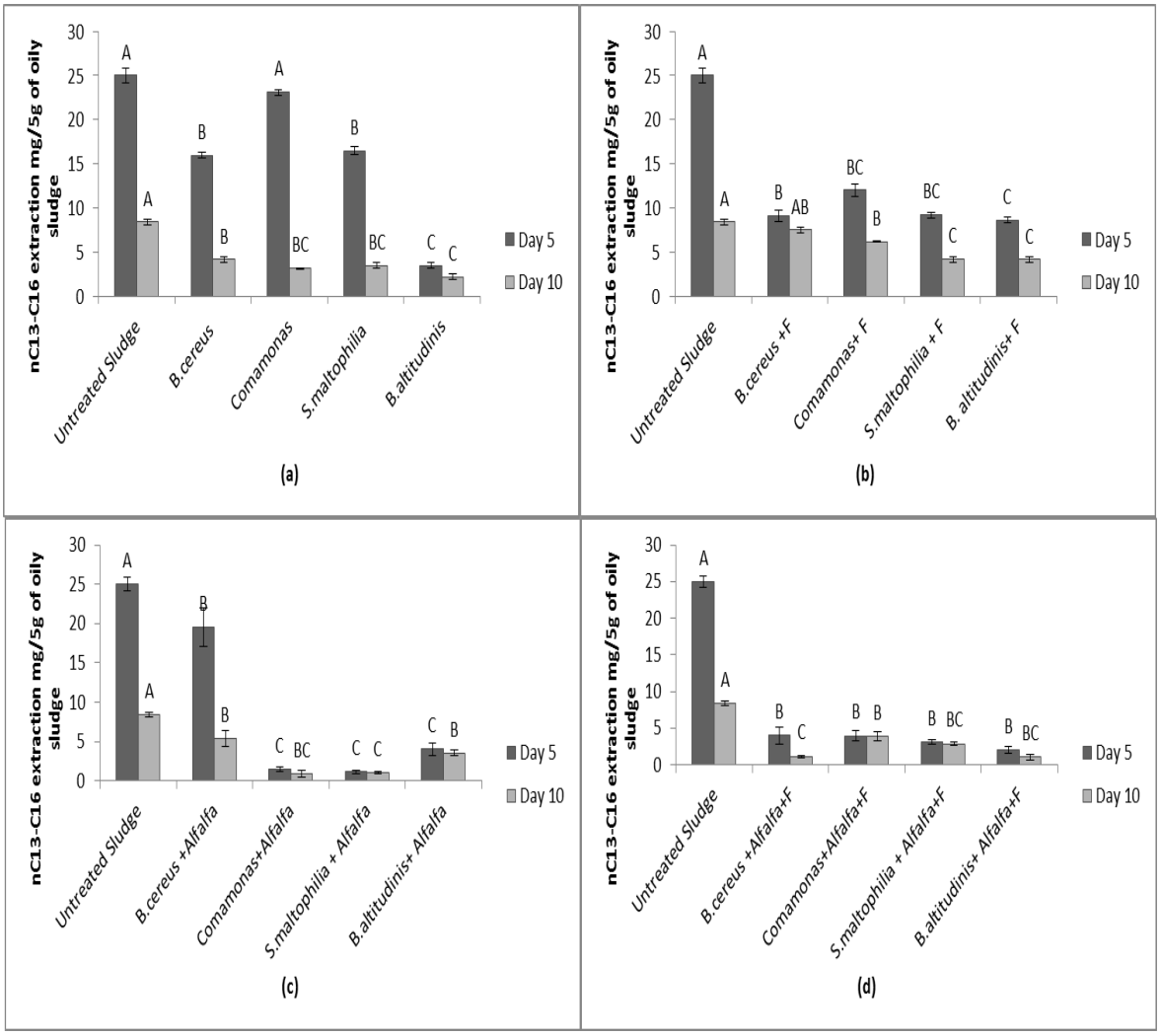

Figure 2: Effect of further incubation period after inoculation.

the degradation rate in *B. cereus*, *commamonas*, *S. maltophilia* and *B. altitudinis* (Figure 2c and 2d).

The concentration of nC_{19}-nC_{29} was 84 % higher at 5 d but was 64 %less in 30% oily sludge at 10 d of incubation as compared to nC_{17}-nC_{18}. *B. altitudinis* followed by *B. cereus* were most effective and showed 82 to 64% degradation at 5 d over that of untreated sludge (Figures 3 and 4).

Growing alfalfa significantly increased the rate and % degradation such that negligible amount of nC_{19}-nC_{29} were deleted in inoculated sludge. Addition of fertilizer to alfalfa + inoculation had no such marked effect except that in *S maltophilia* and *B. altitudines* which showed early degradation. In presence of both alfalfa and fertilizer the *S. maltophilia* inoculation resulted in maximum degradation over that of single inoculation (Figure 4c and 4d).

The results (Figure 5) revealed that degradation of pristane (isoprenoid) was enhanced by all the PGPR isolates., but the most effective were *B. altitudines* followed by *Commamonas* which enhanced the process of degradation, degrading about 50 to 80 % within 5 d of incubation. *B. cereus* took 10 d; alfalfa plantation was more effective for *B. cereus* and *S. maltophilia* than the fertilizer treatment. Whereas, combined treatment of fertilizer and alfalfa were not much effective to promote the effect of inoculation than that of alfalfa +inoculation. Addition of fertilizer significantly suppressed the process of biodegradation of long chain alkane's nC_{30}-nC_{33} both at 5 and 10 d of incubation. Instead growing alfalfa increased the rate of degradation in *B. cereus*, *Commamonas* and *S. maltophilia* but has suppressed the efficiency of *B. altitudinis* (Figure 6).

The degradation of nC_{13}-nC_{16} hydrocarbon (alkane series) was faster in presence of fertilizer and fertilizer +alfalfa but the rate and % degradation varied with the PGPR species. All the 4 PGPR used showed enhanced degradation in presence of fertilizer but planting alfalfa in oily sludge increased the % and rate of degradation in *Commamonas* and *S. maltophilia* because of the fact that alfalfa assist phytoremediation and degradation of hydrocarbon. The other fact might be due to indigenous microbes of oily sludge were not efficient enough to degrade multiple range of complex compounds of hydrocarbons and therefore, introduction of efficient hydrocarbon degrading bacteria can be useful to effectively degrade most of the hydrocarbons in a complex petroleum mixture [31] (Figure 7).

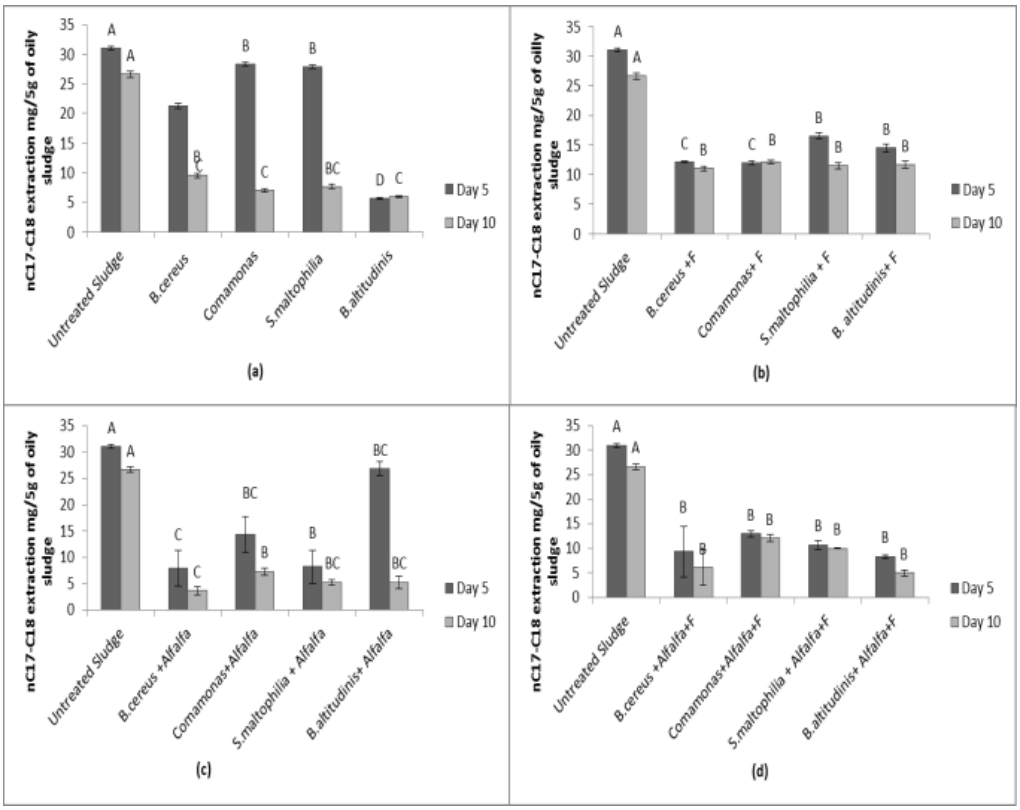

Figure 3: The concentration of nC_{19}-nC_{29} was 84 % higher at 5 d but was 64 % less in 30% oily sludge at 10 d of incubation as compared to nC_{17}-nC_{18}. *B. altitudinis.*

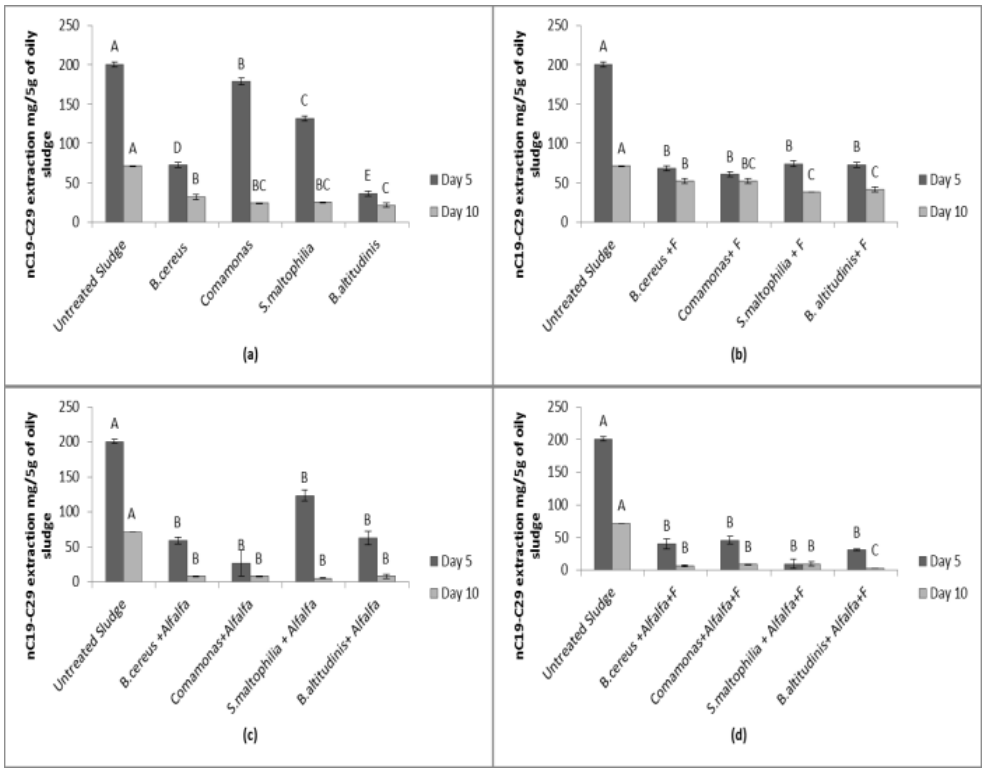

Figure 4: *B. cereus* were most effective and showed 82 to 64% degradation at 5 d over that of untreated sludge.

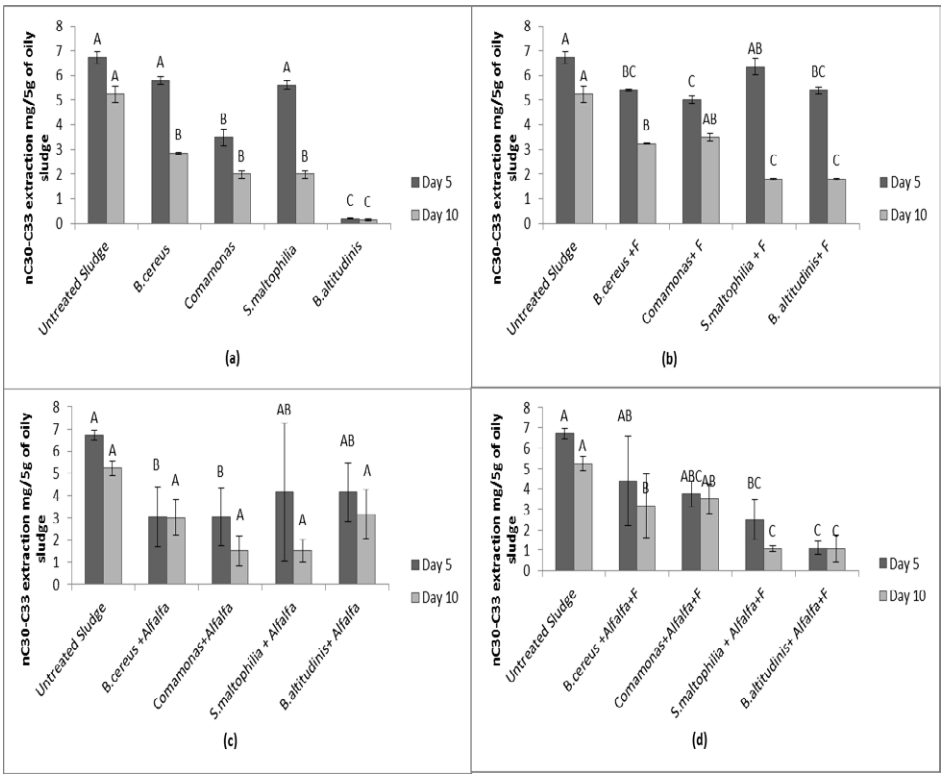

Figure 5: Growing alfalfa increased the rate of degradation in *B. cereus*, *Commamonas* and *S. maltophilia* but has suppressed the efficiency of *B. altitudinis*.

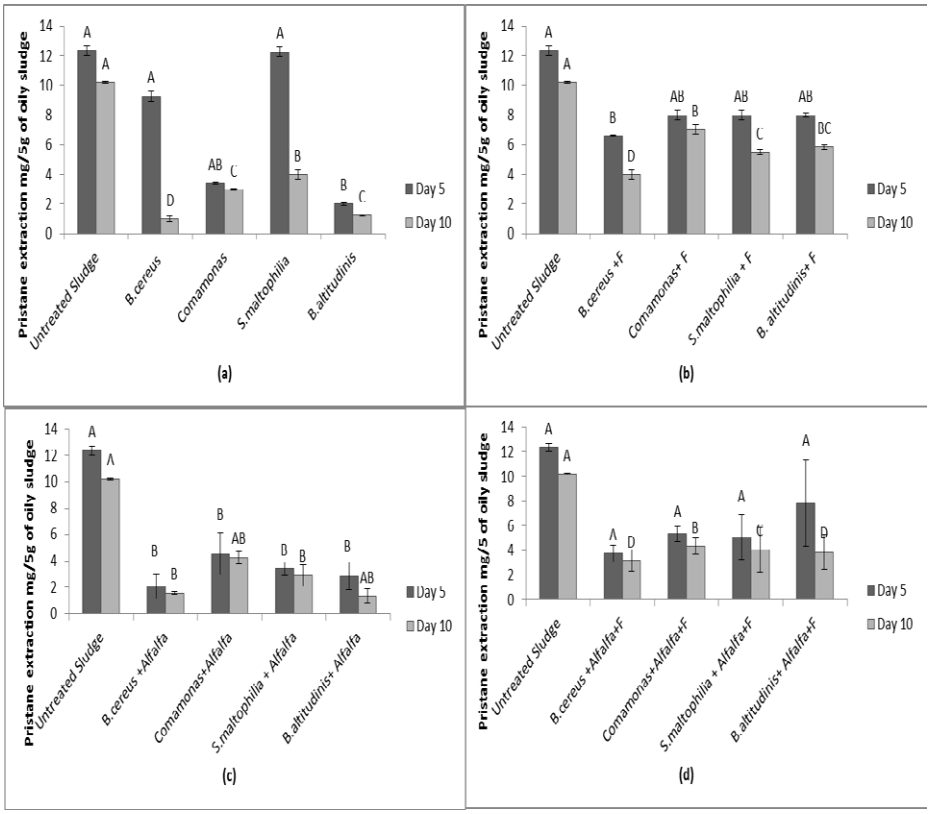

Figure 6: Degradation of pristane (isoprenoid) was enhanced by all the PGPR isolates.

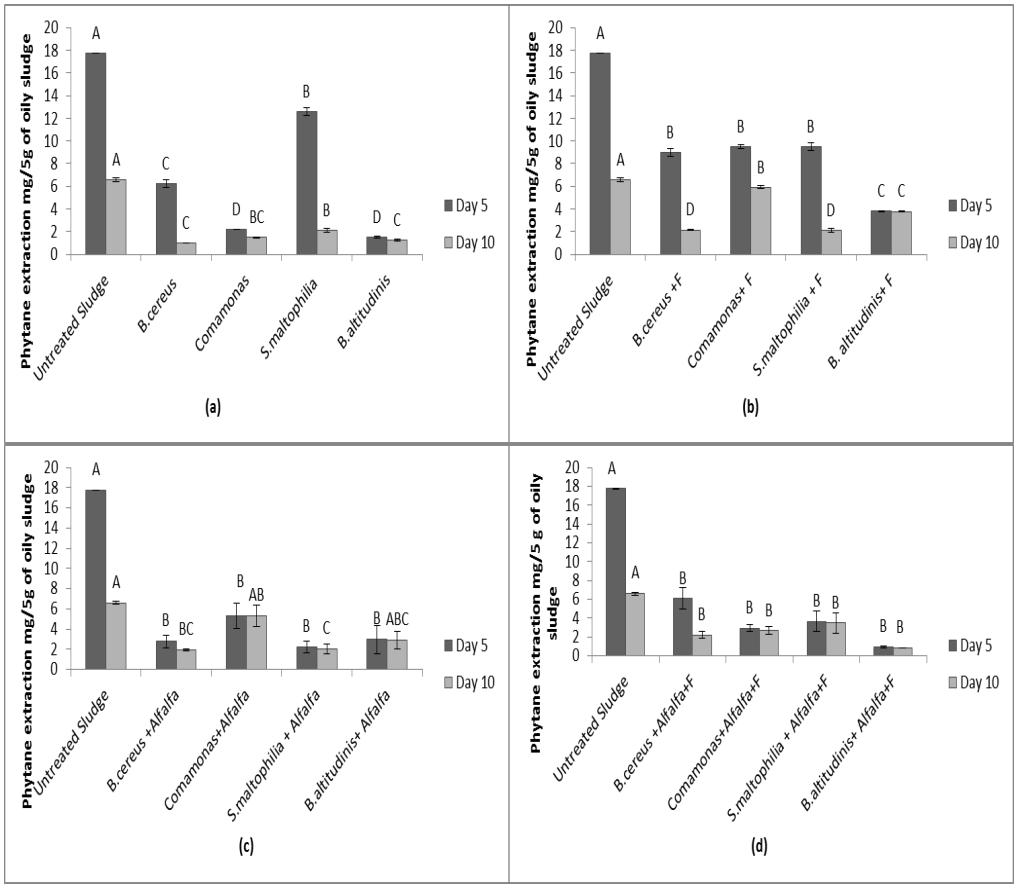

Figure 7: Phytane extraction mg/5 g of oily sludge.

For the degradation of nC_{17}-nC_{18} PGPR followed the similar pattern of response in oily sludge but the difference lies in the rate of degradation. The % degradation reached a maximum at 5 d and no further increased occurred at 10 d after addition of fertilizer and inocula. But the alfalfa plantation was not as effective as that for nC_{13}-nC_{16}. Neverthless *B. altitudines* inoculation has similar % and rate i. e. 80% of control (untreated sludge).

Noteworthy, the alfalfa plantation degraded the nC_{19}-nC_{29} nhydrocarbon series almost completely but took 10 d; further addition of fertilizer appeared effective for *S. maltophilia* even at 5 d and for *B. altitudines* at 10 d after inoculation. Also for degradation of long chain hydrocarbon nC_{30}-nC_{33} *B. altitudines* appeared most effective. The biodegradation potential of alfalfa was was assisted by the *B. cereus* and *Commamonas* further for the degradation of pristane *B. altitudines*>*Commamonas* were effective; the *B. cereus* took longer (10 d) incubation period.

Alfalfa augmented the degradation in conjunction with PGPR inocula. Alfalfa increased both the rate and % degradation of phytan, *B. altitudines* was exceptional and performed well alone and particularly in presence of alfalfa. Fertilizer addition further enhanced the rate of degradation by 5 d. The *B. altitudines* being most responsive to alfalfa as well to the fertilizer for total degradation of hydrocarbon. It is inferred from the present result that *B. cereus* and *B. altitudinis* can be the putative candidate for biodegradation of hydrocarbon, the latter was superior. The effects of *B. cereus* can be further augmented by fertilizer (N and P) and alfalfa. Bacterial population increases in soils contaminated with oily sludge than that of without the previous history of oily sludge contamination [5,6,32,33]. Natural rehabilitation of hydrocarbon contaminated soils is a slow process which can be accelerated when such soils were inoculated with plant growth promoting bacteria. This study revealed the contention that natural rehabilitation process of degradation of oily sludge when the contamination is only 30 % of sludge can be accelerated with *B. altitudinis.* Only 5 days are required to rehabilitate the soil with *B. altitudinis* alone and *n*-alkanes such as straight chain alkanes (nC_{13}-nC_{16}) and long chain alkanes (nC_{26}-nC_{33}) and disappeared from sludge after 5 days of incubation. The traces of isoprenoids and middle chain alkanes (nC_{17}-nC_{25}) were still present till the end of the incubation period. This suggested that *B. altitudinis* was best suitable bacterial strain for bioremediation of 30 % of oily sludge when *n*-alkanes (nC_{13} to nC_{33}) were dominant in contaminated soils. However bioremediation of oily sludge contaminated soils with *B. altitudinis* and nutrients were delayed and required 10 d to rehabilitate. The results of this study are in agreement with the findings of Muthuswamy et al. [34] who reported that *Bacillus* genera is capable of degrading short carbon chain length in hydrocarbon contaminated soils. Amund and Nwokoye [35] and Lal and Khanna [36], reported that *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus* were capable of degrading alkyl aromatic hydrocarbons (*Pseudomonas aeruginosa* strain WatGonce inoculated in hydrocarbon contaminated soils cannot degrade *n*-hexatriacontane (nC_{36}) or *n*-tetracontane (nC_{40}). *Acinetobacter calcoaceticus* S30 and *Alcaligenesodorans* P20 are able to degrade paraffins [36]. Sakai et al. [37] reported an *Acinetobacter strain*

can degrade very long chain n-alkanes (up to nC_{44}). Sakai et al. [37] demonstrated that *P. aeruginosa* K1 and *Rhodococcusequi* P1 were able to degrade alkanes of chain lengths from nC_7 to nC_{28} [38,39].

B. altitudinis was found most efficient alone and with alfalfa to degrade all the *n*-alkanes from nC_8-nC_{40}. However the degradation of *n*-alkanes was accelerated with the addition of fertilizer and in general these findings are in agreement with Mulligan et al. [40] who reported the enhanced degradation of *n*-alkanes by bioaugmentation. Thus study is the first to report *B. altitudinis* hydrocarbons degrading potential and are in close agreement with Jiann-Hong Liu et al. [14] who isolated *B. altitudinis* from oily sludge and found that inoculation of such bacteria to soil become contaminated with benzene can accelerate the degradation of benzene. It was found that the bioremediation of contaminated soils with *Commamonas*, *S. maltophilia and B. cereus* was not obvious at 5 d of incubation whereas the rehabilitation process was delayed till 10 d in soils treated with such bacterial strains.

Total Hydrocarbon degradation of oily sludge

The inoculation with *B. cereus* enhanced the process of degradation showing 56% total hydrocarbon by 5 d. The *B. altitudinis* degraded 80% of total hydrocarbon at 5 d and increased from 80-91% during 5 d and 10 d of incubation respectively. Inoculation of *Commamonas and S. maltophilia* resulted in the degradation of hydrocarbon from 22-86% and 16-85% respectively during 5 d to 10 d of incubations respectively (Figure 8a) With the addition of the fertilizer (Figure 8b) the %degradation of hydrocarbon increased by 58-63%, 22-63%, and 17-64% in *B. cereus*, *Comamonas* and *S. maltophilia* respectivly but was decreased in *B. altitudinis*. At 10 d of incubation all the strains showed significant difference in the %degradation of hydrocarbon. The %degradation increased from 63-81% in oily sludge treated with *B. cereus*, *Comamonas*, *S. maltophilia and B. altitudinis* respectively, when compared from 5 to 10 d of incubation. Planting alfalfa with and without inoculation of PGPR also showed degradation. The Figure 8c revealed that inoculation of alfalfa with *B. cereus* and *B. altitudinis* showed 78% and 79% greater degradation of total hydrocarbon respectively than untreated sludge at 5 d of incubation. The %degradation increased from 78-79%, 67-72% and 56-63% in oily sludge treated with *B. cereus*, *Comamonadas* and *S. maltophilia* respectively, when compared from 5 d to 10 of incubation. While with the addition of fertilizer and planting alfalfa inoculated with *B. cereus* showed almost similar% of degradation 78% at 5 d of incubation (Figure 8d) but was lesser in *B. altitudinis*. At 10 d of incubation all the strains showed significant difference in the %degradation of hydrocarbon. The %degradation increased from 78-85%, 69-90%, 68-75% and 57-74% in oily sludge treated with *B. cereus*,

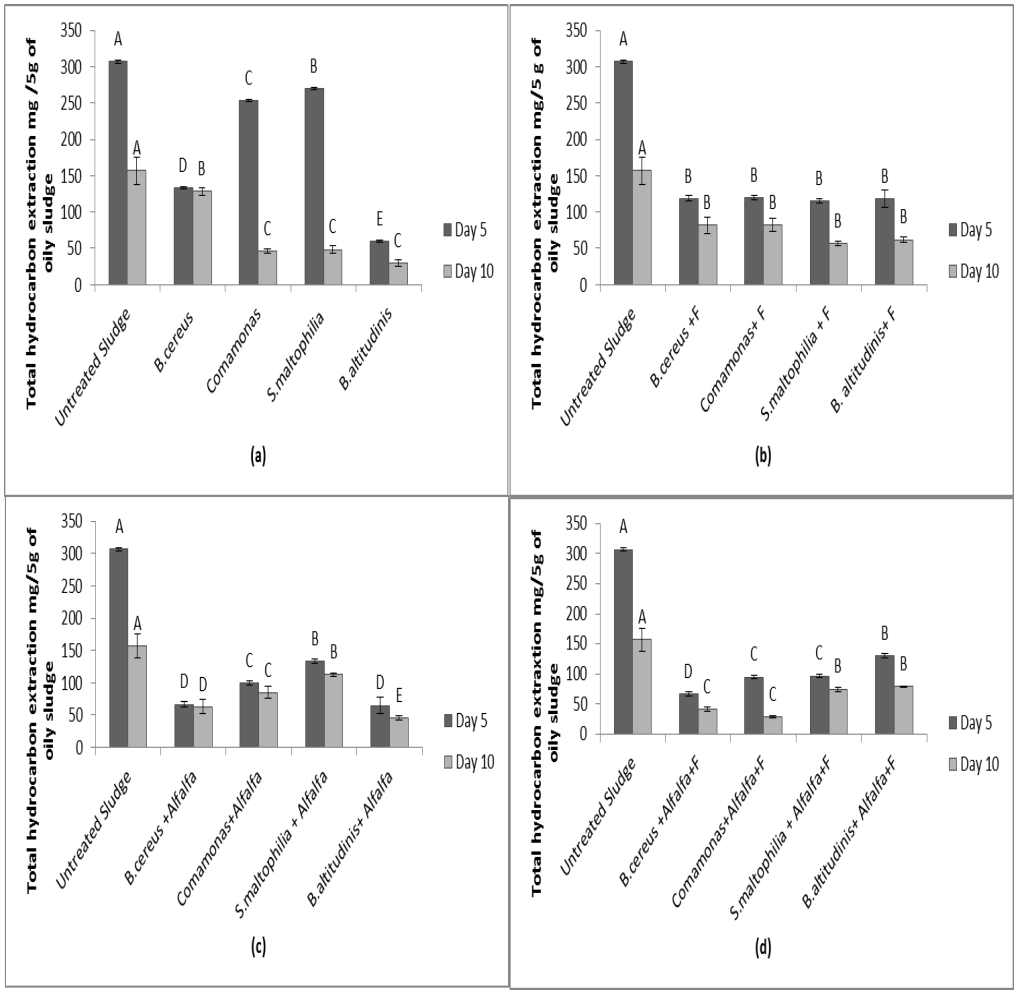

Figure 8: Inoculation of *Commamonas and S. maltophilia* resulted in the degradation of hydrocarbon from 22-86% and 16-85%.

Commamonadas and *S. maltophilia and B. altitudinis* respectively, when compared from 5 to 10 d of incubation.

The total hydrocarbon degradation potential of *B. altitudinis* strains at 5 d was higher from all other strains and present results findings are comparable to degradation of Nigerian crude-oil by *B. subtilis* and *P. aeruginosa* strains isolated from crude oil-polluted soil from Nigeria [41]. Since addition of some fertilizer as carbon source may enhance the rate of hydrocarbon degradation. Ammonium nitrate and Diammonium phosphate were added in oily sludge at 0 d of experiment to increase the rate of degradation of hydrocarbons by the seeded bacteria. The %degradation of hydrocarbons increased in all the strains except *B. altitudinis indicating* that fertilizer as N and P source is not required by this strain. However, these results were in close agreement with Choi et al. [42] and Kim et al. [43] who reported that the additions of nutrients were necessary to enhance the biodegradation of oil contaminants. Chaineau et al. [44], Carmichael and Pfaender [45] have reported the negative effects of high NPK levels on the biodegradation of hydrocarbons which also support our results that *B. altitudinis* showed decreased degradation with the addition of N and P.

Oily sludge is threat to soil for cultivation and it possess numerous micro floras capable for degrading such complex hydrocarbons. Treatment of such polluted site had been a common practice in oil refineries and yields good results. The treatment is carried out by enriching specific populations from the soil and by applying them back to the contaminated soil, where the population of oil-degrading microorganisms is low [46]. In the present study, the degradation of hydrocarbons from treated pots was much higher than that in the untreated pots. This observation is in agreement with those of Barbeau et al. [47] who reported that bioaugmentation of pentachlorophenol (PCP) contaminated soil by PCP-degrading microorganisms resulted in 99% reduction of PCP concentration. It was also observed that degradation of hydrocarbons was higher during first 5 d of inoculation which might be due greater bacterial population and sufficient nutrient availability for hydrocarbon degrading microbes. The degradation slow down later might be to degradation of some fraction take longer time to degrade in total hydrocarbons This shows that the indigenous population in the control plot was not adequate to stimulate degradation of the oily sludge. Bioaugmentation thus enhanced the process of bioremediation.

Growing alfalfa with and without fertilizer enhanced the removal of total hydrocarbon at 5-10 d. The result of this study is in agreement with the findings of Liu et al. [30] who reported that the degradation rate of diesel oil was accelerated up to 69% by planting alfalfa and Indian mustard plants to hydrocarbon contaminated soils. The increase in the rate of degradation because of growing alfalfa in hydrocarbon contaminated soils is more likely because of alfalfa fibrous root systems with large root surface areas and tap root systems which can penetrate deeper down the soil hence increased the mobility and the breakdown of contaminants [48-50] Plant exudates may also provide extra nutrients for the growth of bacteria and stimulated their growth. All of these reasons possibly stimulated higher microbial populations in rhizosphere and enhanced biodegradation of diesel oil. This suggested that microbial population and activity was enhanced under the hydrocarbon contaminated alfalfa rhizosphere. Nevertheless, Inoculation of alfalfa with oil-degrading bacteria was adequately maintaining their populations in the rhizosphere even with decreasing diesel content in the soil.

Rhizoremediation of oily sludge is a low cost technique which can enhance the rehabilitation process more likely because it is a combination of bioremediation and phytoremediation. The results of this study revealed that *Commamonas*, *S. maltophilia* were the plant growth promoting bacteria and enhanced degradation of 30% oily sludge was observed with alfalfa.

Conclusion

The present study was focused on the accelerated biodegradation of oil sludge by bacterial association with common and cost-effective plant, alfalfa. The results demonstrated that the accelerated degradation of oily sludge by fertilizer and plant-bacterial association. Different strains have different capabilities to enhance biodegradation of oily sludge. The differences in the bacterial diversity is related to the groups of hydrocarbons extracted from oily sludge. The natural rehabilitation process of hydrocarbon contaminated soil is slow however inoculation of such soil with *B. altitudinis* accelerated the rate of degradation of *n*-alkanes and some methyl branched than un-inoculated soils.

B. altitudinis is a strain which is capable of degrading straight chain hydrocarbons rapidly than any other strains. Nutrient addition to such soils had no effect on the rate of degradation of hydrocarbons demonstrate that the observation that enough nutrients were present in the soil initially on sludge. Alfalfa is an N-symbiotic plant and because of increased nutrient content under the rhizosphere promotes degradation of *n*-alkanes. Whereas all other bacterial strains showed significant interaction with alfalfa for the biodegradation of oily sludge but the rate of degradation remained slow than with *B. altitudinis*.

The natural rehabilitation process of degradation of toxic *n*-alkanes was enhanced in soil contaminated with 30% oily sludge under the alfalfa rhiosphere with sufficient nutrients are available for indigenous microbes to degrade such alkanes. Inoculation with *B. altitudinis* accelerated the rehabilitation process and within 5 days the rehabilitation occurs.

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References

1. Chakraborty R, Coates JD (2004) Anaerobic degradation of monoaromatic hydrocarbons. Appl Microbiol Biotechnol 64: 437-446.
2. Infante C, Morales F, Ehrmann EU, Hernández-Valencia I, Leon N (2010) Hydrocarbon bioremediation and phytoremediation in tropical soils: Venezuelan study case. Trends in Bioremediation and Phytoremediation. pp: 429-451.
3. Rahman KSM, Thahira-Rahman J, Kourkoutas Y, Petsas I, Marchant R, et al. (2003) Enhanced bioremediation of n-alkanes in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresource and Technology 90: 159-168.
4. Zand AD, Gholamreza N, Nasser M, Ravanbakhsh S, Alireza MT (2010). Total Petroleum Hydrocarbon (TPHs) Dissipation through Rhizoremediation by Plant Species. Polish Journal of Environmental Studies 19: 115-122.
5. Siddiqui S, Adams WA (2002) The fate of diesel hydrocarbons in soils and their effect on the germination of perennial ryegrass. Environ Toxicol 17: 49-62.
6. Siddiqui S, Adams WA, Schollion J (2001) The phytotoxicity and degradation of diesel hydrocarbons in soil. Plant Nutrition and Soil Science 164 : 631-635.
7. Fulekar MH (2010) Bioremediation Technology: Recent Advances. Springer 290.
8. Geetha, SJ, Joshi SJ, Kathrotiya S (2013) Isolation and characterization of hydrocarbon degrading bacteria isolate from oil contaminated sites. APCBEE Procedia 5: 237 241.
9. Palanisamy N, Jayaprakash R, Srilakshman K, Vasanthi NS, Preethy C, et al. (2014) Diesel biodegradation capacities of indigenous bacterial species

isolated from diesel contaminated soil. Journal of Environmental Health Science & Engineering 12:142.

10. Yousefi K, Khodadadi A, Ganjidoust H, Badkoubi A, Amoozegar MA (2009) Isolaton and characterization of a novel native Bacillus strain capable of degrading diesel fuel. International Journal of Environmental Science and Technology 6: 435-442.
11. Atlas RM, Bartha R (1993) Microbial interaction with Xenbiotic and inorganic pollutants. Fundamentals and Applications in Microbial Ecology 6: 544-600.
12. Sood N, Patle S, Lal B (2010) Bioremediation of acidic oily sludge-contaminated soil by the novel yeast strain Candida digboiensis TERI ASN6. Environ Sci Pollut Res Int 17: 603-610.
13. Cerqueira VS, Hollenbach EB, Maboni F, Vainstein MH, Camargo FA, et al. (2011) Biodegradation potential of oily sludge by pure and mixed bacterial cultures. Bioresour Technol 102: 11003-11010.
14. Jiann H, Liu J, Prakash M, Jiin-Shuh J, Chien-Yen C, et al. (2010) World Journal of Microbiology and Biotechnology.
15. Pradhan SP, Conrad JR, Paterek JR, Srivastava VJ (1998) Potential of Phytoremediation for Treatment of PAHs in Soil at MGP Sites. Soil Sediment Contamination 7: 467-480.
16. Omotayo AE, Shonubi OO, Towuru EG, Babalola SE, Ilori MO, et al. (2014) Rhizoremediation of hydrocarbon-contaminated soil by Paspalum vaginatum (Sw.) and its associated bacteria. International Research Journal of Microbiology (IRJM) 5: 1-7.
17. Asif M, Fazeelat T (2012) Petroleum geochemistry of the Potwar Basin, Pakistan: II - Oil classification based on heterocyclic and polycyclic aromatic hydrocarbons. Appllied Geochemistry 27: 1655-166.
18. Khan MA, Ahmed R, Raza HA, Kamal A (1986) Geology of petroleum in Kohat-Potwar Depression Pakistan. Am. Assoc. Petroleum Geochemical Bulletin 7: 396-414.
19. Berge KE, Ose L, Leren TP (2006) Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy. Arterioscler. Thromb. Vascular Biology 26: 1094-1194.
20. Kate W (1997) Preparation of Genomic DNA from Bacteria. Current Protocols in Molecular Biology, 2.4.1-2.4.5. John Wiley & Sons, Inc.
21. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697-703.
22. Song HG, Bartha R (1990) Effects of jet fuel spills on the microbial community of soil. Appl Environ Microbiol 56: 646-651.
23. Asif M, Grice K, Fazeelat T (2009) Assessment of petroleum biodegradation using stable hydrogen isotopes of individual saturated hydrocarbon and polycyclic aromatic hydrocarbon distribution in oils from the Upper Indus Basin, Pakistan. Org Geochem 40:301-311.
24. Wang C, Zöllner S, Rosenberg NA (2012) A quantitative comparison of the similarity between genes and geography in worldwide human populations. PLoS Genet 8: e1002886.
25. Zhang DC, Mörtelmaier C, Margesin R (2012) Characterization of the bacterial archaeal diversity in hydrocarbon-contaminated soil. Sci Total Environ 421-422: 184-96.
26. Ramadoss D, Lakkineni VK, Bose P, Ali S, Annapurna K (2013) Mitigation of salt stress in wheat seedlings by halotolerant bacteria isolated from saline habitats. Springerplus 2: 6.
27. Yoshida N, Yagi K, Sato D, Watanabe N, Kuroishi T, et al. (2005) Bacterial communities in petroleum oil in stockpiles. J Biosci Bioeng 99: 143-149.
28. Erdogan EE, Fikrettin S, Ayten K (2012) Determination of petroleum-degrading bacteria isolated from crude oil-contaminated soil in Turkey. African Journal of Biotechnology 11: 4853-4859.
29. Saeideh R, Syed MS, Fayez R, Behrouz S, Jamshid R (2013) Characterization and Potentials of Indigenous Oil-Degrading Bacteria Inhabiting the Rhizosphere of Wild Oat (Avena Fatua L.) in South West of Iran. Iranian Journal of Biotechnology 11: 32-40.
30. Liu CW, Liu HS (2011) Rhodococcus erythropolis strain NTU-1 efficiently degrades and traps diesel and crude oil in batch and fed-batch bioreactors. Process Biochemistry 46: 202-209.
31. Atlas RM (1977) Stimulated petroleum biodegradation. CRC Crit Rev Microbiol 5: 371-386.
32. Haichar FZ, Marol C, Berge O, Rangel CJI, Prosser JI, et al. (2008) Plant host habitat and root exudates shape soil bacterial community structure. International Society of Microbial Ecology 2: 1221-1230.
33. Supaphol S, Panichsakpatana S, Trakulnaleamsai S, Tungkananuruk N, Roughjanajirapa P, et al. (2006) The selection of mixed microbial inocula in environmental biotechnology: example using petroleum contaminated tropical soils. J Microbiol Methods 65: 432-441.
34. Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P (2008) Biosurfactants: properties, commercial production and application, Current Science 94: 736-747.
35. Amund OO, Nwokoye N (1993) Hydrocarbon potentials of yeast isolates from a pollute Lagoon. Journal of Scientific Research and Development 1: 65-68.
36. Lal B, Khanna S (1996) Degradation of crude oil by Acinetobacter calcoaceticus and Alcaligenes odorans. J Appl Bacteriol 81: 355-362.
37. Sakai Y, Maeng JH, Tani Y, Kato N (1994) Use of long-chain n-alkanes (C13-C44) by an isolate, Acinetobacter sp. M-1. Bioscience, Biotechnology and Biochemistry 58: 2128-2130.
38. Abalos A, Viñas M, Sabaté J, Manresa MA, Solanas AM (2004) Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by Pseudomonas aeruginosa AT10. Biodegradation 15: 249-260.
39. Norman RS, Frontera-Suau R, Morris PJ (2002) Variability in Pseudomonas aeruginosa lipopolysaccharide expression during crude oil degradation. Appl Environ Microbiol 68: 5096-5103.
40. Mulligan CN, Young RN, Gibbs BF (2001) Surfactant enhanced remediation of contaminated soil: a review. Eng Geol 60: 371-380.
41. Ilori MO, Amund DI (2000) Degradation of anthracene by bacteria isolated from oil polluted tropical soils. Z Naturforsch C 55: 890-897.
42. Choi SC, Kwon KK, Sohn JH, Kim SJ (2002) Evaluation of fertilizer additions to stimulate oil biodegradation in sand seashore mescocosms. Journal of Microbiology and Biotechnology 12: 431-436.
43. Kim SJ, Choi DH, Sim DS, Oh YS (2005) Evaluation of bioremediation effectiveness on crude oil-contaminated sand. Chemosphere 59: 845-852.
44. Chaineau CH, Rougeux G, Yepremian C, Oudot J (2005) Effects ofnutrient concentration on the biodegradation of crude oil and associated microbial populations in the soil. Soil. Biology and Biochemistry 37:1490-1497.
45. Carmichael LM, Pfaender FK (1997) The effect of inorganic and organic supplements on the microbial degradation of phenanthrene and pyrene in soils. Biodegradation 8: 1-13.
46. Forsyth JV, Tsao YM, Bleam RD (1995) Bioremediation: when is bioaugmentation needed? in Bioaugmentation for site remediation. edsHinchee R. E., Fredrickson J., Alleman B. C. (Battelle Press, Columbus, Ohio) 1-58.
47. Barbeau C, Deschênes L, Karamanev D, Comeau Y, Samson R (1997) Bioremediation of pentachlorophenol-contaminated soil by bioaugmentation using activated soil. Appl Microbiol Biotechnol 48: 745-752.
48. Ingham ER, Klein DA (1984) Soil fungi: relationships between hyphal activity and staining with fluorescein diacetate. Soil Biology and Biochemistyr 16: 273-278.
49. Shimp JF, Tracy JC, Davis LC, Huang W, Erickson LE, et al. (1993) Beneficial effects of plants in the remediation of soil and groundwater contaminated with organic materials. Critical Review on Environmental Science and Technology 23: 41-77.
50. Chaineau CH, Morel JL, Oudot J (2000) Biodegradation of fuel oil hydrocarbons in the rhizosphere of maize. Journal of Environmental Quality 29: 549-578.