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Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study

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ABSTRACT

Bioremediation of crude oil contaminated soil is an effective process to clean petroleum contaminant from the environment. In this study, we isolated 39 native crude oil degrading bacteria from different crude oil contaminated soils. From 16S rDNA sequences, we confirmed that the isolated bacteria belong to the genera *Lysinibacillus*, *Brevibacillus*, *Bacillus*, *Paenibacillus*, *Stenotrophomonas*, *Alcaligenes*, *Delftia*, *Achromobacter* and *Pseudomonas*. Four most effective strains (designated as AS03, N108, N002 and N78) were used for batch culture and microcosm evaluation. Gas chromatography analysis, further confirmed that the strain AS03, N108, N002 and N78 were able to degrade crude oil under both shake culture and microcosm study. Under microcosm, the soil quality was further improved significantly in the treatments of BF1-Mix (N108-AS03) and BF2-Mix (N002-N78). The improvement of soil quality was also confirmed by earthworm mortality bioassay and in plant test on rice (*Oryza sativa)* and mung (*Vigna radiata*). These findings demonstrated that the combine use of crude oil degrading bacteria along with nutrient supplements could revive crude oil contaminated soil effectively in large scale.

1. Introduction

Drilling activities in oil field areas in India is evident and also a century old operation including northeastern state of Assam. The major problem associated with crude oil excavation, is severe contamination of different landmass, water bodies and ground water reserves (Holliger et al., 1997; Ulrich, 2008). Abandonment of crude oil drilling sites, accidental spillage from crude oil production unit, refining, and distribution processes results in contamination of the environment. On the other hand, many of the components of crude oil including polar hydrocarbons, n-alkanes and unresolved complexes of branched and cyclo-alkanes, aromatics, resin and asphaltene residuals; many of which are persistent organic pollutants (Killops and Al-Juboori, 1990; Oudot et al., 1998). Due to the persistence nature of crude oil, it remains in the environment for long time, which affects the soil quality by changing the physical, physiological, biochemical properties and intrinsic heterogeneous microbial diversity (Margesin et al., 2003; Head et al., 2006). Plants are also susceptible to the oil exposure, due to phytotoxic nature of crude oil and immobilization of availability of plant nutrient in soil (De Jong, 1980; Udo and Fayemi, 1995). Inherent mutagenic properties of polycyclic aromatic hydrocarbons (PAHs), their difficult degradation results continued environmental threat and requires attention for remediation (Johnsen et al., 2007).

Physical, chemical and thermal methods have commonly been employed to clean up the oil-contaminated sites (Frick et al., 1999). However, these techniques are relatively expensive and also require site restoration (Lundstedt et al., 2003). Therefore, it is an urgent need for promotion of environment friendly techniques for reclamation of oil-polluted sites. Several studies have been reported on eco-friendly bioremediation (Glick, 2003; Zhuang et al., 2007; Gerhardt et al., 2009; Sarma Roy et al., 2013). These methods are relatively affordable and do not introduce any additional chemicals to the environment. The uses of biological materials like bacteria, fungi, algae with vermicompost, animal and plant compost, have been reported with good bioremediation potential (Riser-Robert,

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1992; Bundy et al., 2004). The use of beneficial free-living bacteria and rhizosphere associated plant growth promoting rhizobacteria (PGPR) for different bioremediation activity are gaining impetus. These bacterial strains have potential in recovering the oil contaminated sites as well as improving plant health (Glick, 2010). This is due to the simultaneous degradation of persistent petroleum hydrocarbon and plant growth promoting (PGP) activity of the beneficial microbes on plants (Yenn et al., 2014). The rate of crude oil degradation using beneficial microbes had also been augmented in the field by supplying limiting nutrients to the affected sites (Atlas, 1981). Das and Mukherjee (2007) have studied the comparative efficiency of two bacterial strains in crude petroleum oil contaminated soil from North-East India. Fernandez et al. (2011) reported microcosms study using micro-organisms, earthworms and plants assemblages to determine the effects of these organisms and their interaction on diesel degradation. However, the efficacy of bioremediation is mostly dependant on the nature of local edaphic and climatic condition of the contaminated sites (Gogoi et al., 2003).

Since, the crude oil production in Assam, India is a century old routine process, therefore, the environmental contamination due to crude oil exploration is highly alarming. As a consequence, it is important to assess the native bacterial strains for bioremediation of crude oil contaminated sites of Assam. The aim of present study was to isolate, identify bacterial strains from different crude oil polluted sites of Assam, India. Further we assessed the bacterial diversity and their crude oil biodegrading potential in *in vitro*. Emphasis was given to develop effective bacterial formulation to accelerate crude oil degradation using various nutrient treatments in microcosm experiment.

2. Materials and methods

2.1. Media and chemicals

All the media used in this study were purchased from Himedia Ltd., Mumbai, India. Hexadecane was procured from Supelco, USA. Fluoranthene, pyrene and anthracene were obtained from Aldrich, USA while dodecane, and phenanthrene were procured from Sigma–Aldrich, USA. Naphthalene was procured from Sisco, Mumbai, India. All chemicals used in this study were analytical grade. Crude oil was collected from GGS1 Geleky, ONGC, Sivasagar, Assam.

2.2. Soil sampling and characterization

All the crude oil contaminated soil samples were collected from five sites *viz.,* Lakuwa, Geleky, Amguri of Sivasagar district, Borhola and Jorhat urban area of Jorhat district, Assam, India (Table 1). From each location 10 samples were collected randomly by disturb sampling, up to a depth of 30 cm. To make a representative sample, bulk samples were prepared from each location and minimum of three samples were collected. The representative samples were immediately stored at 4 °C. Before chemical and biological analysis, soil samples were sieved to separate large particles like plant parts (roots, stem, and leave), cobbles, pebbles etc. Soil pH was estimated in soil:water suspension (1:2.5) using pH meter (Eutech, Malaysia), while moisture content were determined by drying the soil samples at 70 °C until a constant weight was obtained. Soil conductivity was determined in soil suspension using digital conductivity meter (IKON, India). Total soil organic carbon (SOC) was determined according to Walkley-Black, total soil nitrogen (N) by Kjeldahl digestion, while phosphorus (P) using phospho molybdic acid and potassium (K) by Flame photometry, respectively (Jackson, 1973).

2.3. Analysis of total petroleum hydrocarbon (TPH) composition

Total petroleum hydrocarbon (TPH) of the soil was determined according to Das and Mukherjee (2007). Briefly, TPH from 10 g soil was consecutively extracted with 100 ml of hexane, methylene chloride (CH_2Cl_2), and chloroform. All three extracts were pooled and dried in a fume hood at room temperature by evaporation of solvents under a gentle nitrogen stream over Na_2SO_4 and concentrated using a rotary evaporator to a final volume of 3.0 ml. After solvent evaporation, the amount of residual TPH was determined by taking the final weight of the sample.

2.4. Isolation of oil degrading bacteria

Isolation of bacterial strains was done using enrichment culture method from the collected soil samples. Contaminated soil samples were inoculated on a mineral medium M1 (g/L): 4.0, $NaNO_3$; 3.61, Na_2HPO_4; 1.75, KH_2PO_4; 0.2, $MgSO_4.7H_2O$; 0.01, $FeSO_4$; 0.05, $CaCl_2$; trace element solution 1 ml/L amended with 2% (v/v) crude oil and incubated for 72 h. From the grown enrichment culture, 1 ml was transferred to fresh M1 solid media (prepared by the addition of

Table 1
Geographical location, nature and composition of soil collected from different crude oil and spent engine oil contaminated sites used for isolation of crude oil degrading bacteria.

Sampling sites	Location	Nature of the sites	Number of isolates	TPH (%)	Organic C (g/Kg)	N (g/Kg)	P (g/Kg)	K (g/Kg)	pH	Moisture content (%)	Conductivity (mho m^{-1})
Lakuwa (50 samples)	Sibsagar, Assam (Longitude 27.1E, Latitude 94.49N)	Tea plantation sites and paddy cultivated site with oil contamination from 5 years	65	56.2 ± 1.2	2.8 ± 1.2	5.3 ± 0.3	1.6 ± 0.4	1.8 ± 0.8	7.65 ± 1.3	6.2 ± 1.3	1.0 ± 0.2
Geleky (35 samples)	Sibsagar, Assam (Longitude 94.70E, Latitude: 26.817N)	Paddy cultivation site with crude oil contamination	45	37.8 ± 0.2	4.1 ± 0.2	7.6 ± 0.7	1.4 ± 0.8	2.8 ± 0.5	8.9 ± 9.2	9.5 ± 1.3	2.1 ± 0.3
Amguri (20 samples)	Sibsagar, Assam (Longitude 94.53E, Latitude 26.83N)	Paddy cultivation site with crude oil contamination	18	15.2 ± 1.3	4.7 ± 0.9	7.6 ± 2.3	1.7 ± 0.5	7.6 ± 2.3	8.3 ± 0.2	18.2 ± 2.1	3.8 ± 0.2
Borhola (25 samples)	Jorhat, Assam (Longitude 94.12E, Latitude 26.36N)	Crude oil drilling sites	29	29.3 ± 1.1	1.3 ± 0.7	6.4 ± 0.3	1.9 ± 0.6	6.4 ± 0.3	8.2 ± 0.2	9.04 ± 0.2	1.9 ± 0.3
Jorhat Urban (40 samples)	Jorhat, Assam (Longitude 94.216E, Latitude 26.75N)	Spent engine oil contaminated sites from different automobile service centers.	43	33.2 ± 0.2	2.8 ± 2.6	8.2 ± 0.1	2.9 ± 0.7	8.2 ± 0.1	8.9 ± 0.3	7.5 ± 0.1	2.4 ± 0.2

Values are mean of three observations with 3 replications each, ±1.0: Standard error (SE) of observed value, TPH: Total petroleum hydrocarbon. A total of one hundred five numbers of oil contaminated soil samples were collected from Sivasagar and Jorhat district to isolate crude oil degrading bacteria. Most of the soil samples were basic in nature (pH as high as 8.9). The enrichment and isolation procedure resulted with 200 bacteria in M1 media, and these isolates could utilize crude oil as carbon source.

1.8% agar) amended with 2% (v/v) crude oil as sole carbon source and incubated for 72 h at 30 °C.

2.5. Screening of hydrocarbon degradation in shake flask

Screening of all bacterial isolates were done using liquid M1 medium with 2% (v/v) crude oil at 30 °C for 48 h by observing the visible breakdown of the crude oil layer along with the indication of biofilm formation. Similarly, the short-listed strains were further tested for their ability to grow in pure hydrocarbon which includes hexadecane, dodecane, fluroanthene, phenanthrene, anthracene, naphthalene etc. For this, pure hydrocarbon was added to M1 media to make a final concentration of 200 ppm. The growth of bacteria in pure compound was observed till 72 h at 30 °C and 150 rpm. Turbidity, biofilm and foam formation of cultural broth were considered as the indicator of the ability of bacteria to grow in crude oil and pure hydrocarbon. Biofilm formation was recorded on the basis of adherent aggregates of tested bacterial cells on the surface of culture flasks.

2.6. Identification and phylogenetic analysis of bacterial isolates

Morphological and biochemical characteristics of bacterial isolates were determined according to Bergy's manual. Molecular identification of bacterial isolates was confirmed by 16S rDNA PCR amplification. The PCR amplification was carried out using genomic DNA from each bacterium as template with 16S universal forward primer fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S universal reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) using Taq DNA Polymerase (Bangalore Genie, India) (Weisburg et al., 1991). The PCR reactions were denatured at 95 °C for 5 min, which was followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s and then final extension at 72 °C for 10 min. Purified DNA fragments were sequenced using the same sets of primers. The sequences were analyzed using BLAST (http://www.ncbi.nih.gov/BLAST/) to get a preliminary identification of the strains. The sequences were aligned using ClustalW program (http://www.ebi.ac.uk/clustalw/) of the European Bioinformatics Institute (EMBL-EBI) and Bio Edit Sequence Alignment Editor software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The cluster analysis was performed using Mega 5.

2.7. Nucleotide sequence accession numbers

The 16S rDNA gene sequences were deposited in Gene Bank under accession numbers JN410947, JN41094 and JQ900510–JQ900546 for different bacterial isolates.

2.8. Viscosity measurement of bacterial degraded crude oil

Viscosity of different bacterial mediated crude oil degradation was measured with Rheometers (Anton Paar Rheolab QC, India).

Table 2
Screening of hydrocarbon degrading bacteria.

Bacterial isolates	Different hydrocarbon used							
	Crude oil	Hexadecane	Dodecane	Fluroanthene	Naphthalene	Phenanthrene	Anthracene	Accession no.
*Lysinibacillusfusiformis*N169	++	++	++	−	−	−	−	JQ900510
*Pseudomonas aeruginosa*N72	++	+++	+++	++	++	+	+	JQ900511
*Brevibacilluslaterosporus*N35	++	++	+	+	+	−	−	JQ900512
Bacillus cereus N003	++	+	+	+	−	−	−	JQ900513
*Brevibacilluslaterosporus*N18	+	+	−	−	−	−	−	JQ900514
*Brevibacilluslaterosporus*N156	+	+	−	−	−	−	−	JQ900516
*Lysinibacillusfusiformis*N131	++	+	+	+	−	+	+	JQ900517
*Pseudomonas mendocina*N195	++	++	+	+	+	−	−	JQ900518
*Bacillus pumilus*N133	++	++	+	−	−	−	−	JQ900519
*Pseudomonas aeruginosa*N86	++	−	−	−	−	−	−	JQ900520
*Paenibacillusalvei*N184	++	−	−	−	−	−	−	JQ900521
*Pseudomonas aeruginosa*N146	++	++	−	−	−	−	−	JQ900522
*Bacillus pumilus*N50	+	+	−	−	−	−	+	JQ900523
*Stenotrophomonasmaltophilia*B9	++	++	+	−	−	−	−	JQ900524
*Lysinibacillussphaericus*N121	+	+	+	−	−	−	−	JQ900525
Bacillus cereus N158	+	+	++	−	−	−	−	JQ900526
*Pseudomonas aeruginosa*N144	+	+	++	−	−	−	−	JQ900527
*Pseudomonas aeruginosa*N141	++	++	++	−	−	−	−	JQ900528
*Alcaligenesfaecalis*N148	++	+++	++	−	−	−	−	JQ900529
*Lysinibacillussphaericus*N182	++	+	+	+	−	−	−	JQ900530
*Pseudomonas aeruginosa*N139 NA	++	++	++	++	+	+	−	JQ900531
*Pseudomonas mendocina*B8	+	+	++	++	+	−	−	JQ900532
DelftiatsuruhatensisB7	+	+	++	++	+	−	−	JQ900533
*Pseudomonas aeruginosa*N155	+++	+++	+++	++	++	++	+	JQ900534
*Pseudomonas aeruginosa*N151	+++	++	++	+	+	++	++	JQ900535
*Pseudomonas aeruginosa*B2	+++	++	++	+	+	+	+	JQ900536
*Pseudomonas aeruginosa*N153	++	+++	++	+	+	+	+	JQ900537
*Achromobacterxylosoxidans*N78	+++	++	+	+	+	−	−	JQ900538
*Pseudomonas aeruginosa*N152	+++	++	++	++	++	+	+	JQ900539
*Brevibacilluslaterosporus*N216	+++	++	+	+	+	+	+	JQ900540
*Lysinibacillusfusiformis*N190	++	+	+	+	+	+	−	JQ900541
*Bacillus pumilus*N191	+	−	−	−	−	−	−	JQ900542
*Pseudomonas aeruginosa*N83	++	++	+	+	−	−	+	JQ900543
*Lysinibacillusfusiformis*N43	++	++	+	+	+	+	+	JQ900544
*Pseudomonas aeruginosa*B10	++	+	+	−	−	−	−	JQ900545
*Lysinibacillusfusiformis*N139	+	+	++	+	+	+	−	JQ900546
*Pseudomonas aeruginosa*AS03	+++	++	+++	+++	+++	+++	+++	JN410947
*Pseudomonas aeruginosa*N108	++	+++	+++	++	++	++	++	JN41094
*Pseudomonas aeruginosa*N002	+++	++	++	++	++	+	+	JX035794

+: Only growth of bacteria occured, ++: good growth of bacteria occured (bacterial growth with flim or biosurfactatnt production); +++: Very good growth (high bacterial growth including flim and biosurfactant production), −: No growth or degradation.

For this, mineral media M1 with 2% (v/v) crude oil was inoculated with four bacterial isolates N002, N108, AS03, N78 and without inoculation of bacteria as control. It was then allowed to grow for 72 h at 30 °C under shaking condition. The 72 h old culture broth of each strain was directly used for viscosity measurement.

2.9. Microcosm study of crude oil contaminated soil

To test the efficacy of remediation of crude oil contaminated soil using the screened bacteria, simulated crude oil contaminated field plots as microcosm were created under green house condition. In brief, the soil collected from experimental garden of the institute was made to 8 × 3 m^2 microcosms with a depth of six inches. Each microcosm was then mixed with Assam light crude oil-soil ratio of 3:1 (w/w) and left for 30 days. Zero day reading of the soil was recorded at the end of 30 days and biological treatment was performed. A total of 7 different bioformulations, consisting nitrogen (N) as urea; potassium (K) as murate of potash; and phosphorous (P) as diammonium phosphate i.e., mixtures of nitrogen–phosphorus–potassium (NPK) was added in a ratio- 60:40:40 ha^{-1} (~150, 100, 100 mg $microcosm^{-1}$);

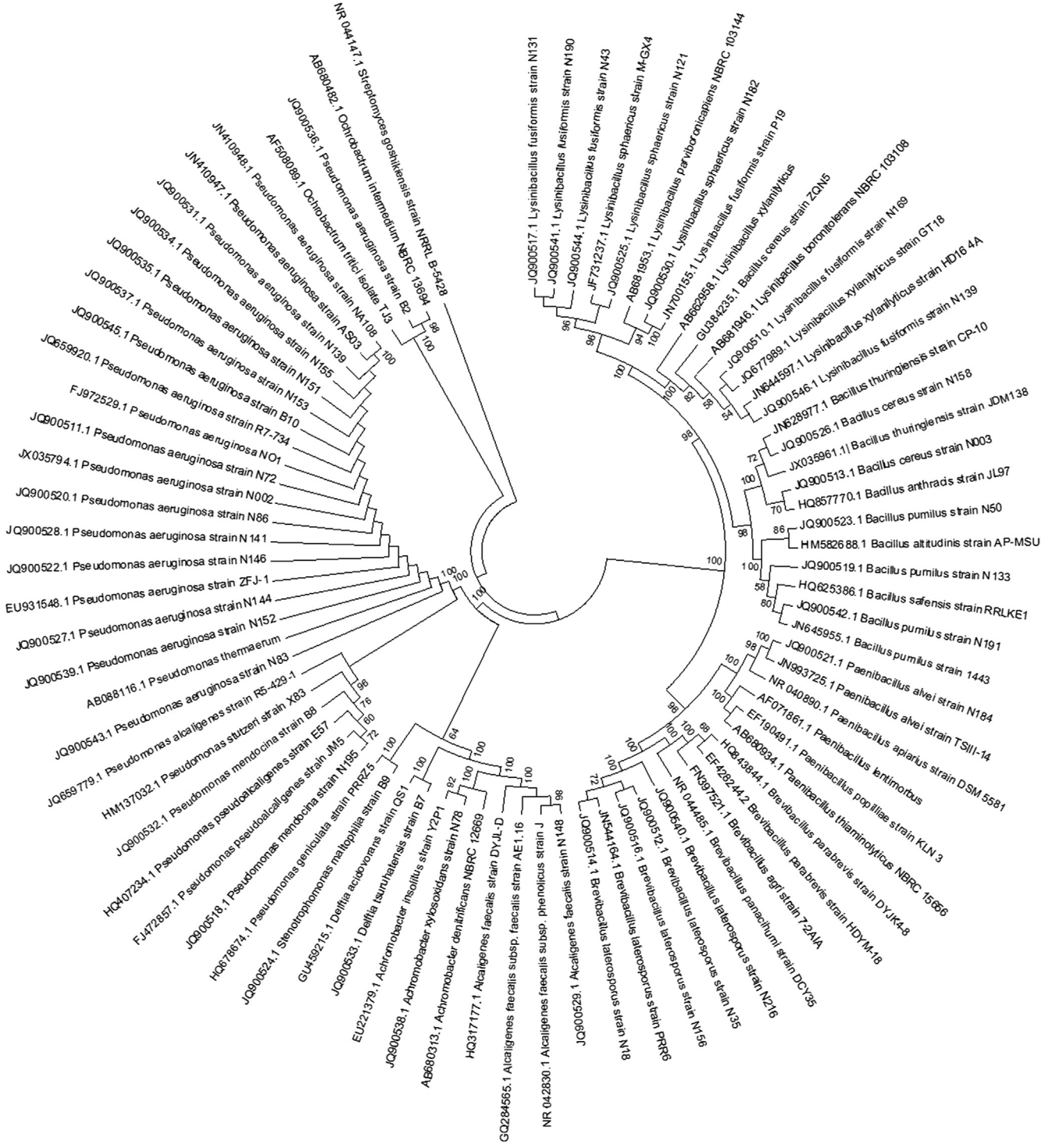

Fig. 1. Phylogenic tree of different crude oil degrading bacteria isolated from crude oil contaminated soil.

organic farmyard manure (OM), vermicompost (VC) at the rate of 180 kg ha^{-1} (~430 mg $microcosm^{-1}$). In BF1 a 72 h old culture mixture of N108-AS03 strains with 4.7×10^{13} CFU (Colony Forming Unit) mL^{-1} obtained from 48h old nutrient rich cultural broth (100 ml culture $microcosm^{-1}$), BF1-Mixed: (N108-AS03)-NPK-OM-VC each component added as given above, BF2: (N002-N78) strains 72 h old culture with 4.7×10^{13} CFU ml^{-1} (100 ml culture $microcosm^{-1}$), BF2-Mixed: (N002-N78)-NPK-OM-VC each component added as given above. It is important to note that the cfu 10^{13} ml^{-1} observed in these bacteria is indeed very high which suggest that these bacterial isolates can grow to a much higher density in growth medium. All the experiments were repeated 3 times with three replications in each treatment.

2.10. Sampling and soil analysis of microcosms

Triplicate samples ~100 g were taken from each microcosm at an interval of 4 weeks from the day of treatments. The soil samples from each microcosm were then analyzed for TPH, pH, moisture content as described above and different soil enzyme activities (dehydrogenase, phosphatase and urease) as per standard protocol (Bremner and Tabatabai, 1969; Camina et al., 1998; ; Smith and Chalk, 1980). Soil respiration, evaporation rate, CO_2 flux were analyzed by IRGA-CIRAS-2 (PP System, USA) attached with soil respiration chamber SRC-1. For measurement SRC-1 was attached to main system CIRAS-2. The main system was then pre-set to area (cm^2) at 78.5, CO_2 change at 60 ppm, the maximum amount of time from start of measurement to the end at 60 s. After successful completion of the set up the SRC-1 chamber was hold for ~15 s to flush out. After that the SRC-1 chamber was placed on the soil and allowed to measure. After completion of the measurement the data were recorded as soil- evaporation (gm^{-2}), respiration (gCO_2 m^{-2} h^{-1}) and temperature (°C), respectively.

2.11. Earthworm bioassay for crude oil toxicity

Soil toxicity test was performed using earthworm (*Eisenia foetidae*) to evaluate the improvement of soil health according to Dorn

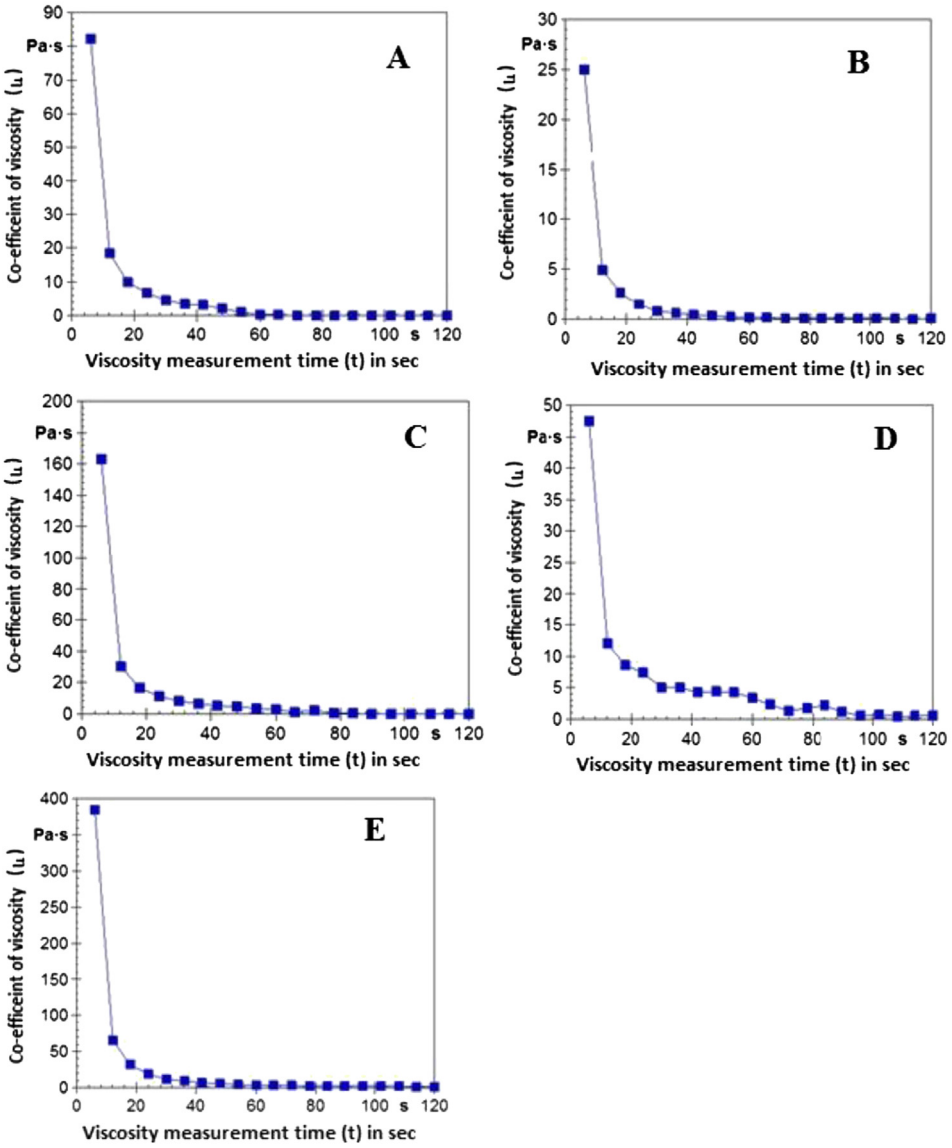

Fig. 2. Comparison of viscosity of crude oil after 72 h treatment of crude oil degrading bacteria under shake culture condition. In the control (the lowermost left hand side figure) the coefficient of viscosity is more than 300 after 72 h of treatment. In case of culture having N002, N108, AS03 and N72 the co-efficient of viscosity is below- 90, 30, 200 and 50, respectively. This suggests that the presence of these microbes have reduced the viscosity of the culture significantly in comparison to the control. Further, the exceptional uniform intrinsic viscosity deviation of N78 treated crude oil from the normal set data point due to distinct nature of crude oil breakdown and inhomogeneous floc formation. A = N002; B = N108; C = AS03, D = N78 and E = Control.

Table 3
Compounds possibly originating from crude oil degradation at the end of experiment with bacteria treated crude oil under shake flask condition.

Retention time	Peak area (in %)					Degraded products of crude oil
	Control	N108	AS 03	N002	N78	
11.16	121,564	0	33,149	0	0	Dodecane, 1-fluro
12.11	71,335	73359	0	60,535	64,292	Heptadecane 2
14.21	256,086	0	53,667	0	0	Heptadecane, 6,10 tetramethyl
15.67	1,102,872	381,849	252,575	322,951	385,871	Pentadecane 2,6,10,14 tetramethyl
21.71	1,286,408	87,556	76,906	0	14,705	1-dodecanol, 2- methyl
25.7	5,021,797	0	108,001	0	0	Sulfurous acid, butyl octadecyl ester
26.61	5,421,427	102,964	106,139	0	21,917	Sulfurous acide, butyl tetradecyl ester
27.5	1,518,873	134,963	108,400	29,019	52,593	Triacontane

and Salanitro (2000) with modification. The earthworms stock was reared in peat at an ambient temperature during the experiment. The earthworm bioassay was carried out in transparent polypropylene jars of 250 ml capacity with pores in the lid to facilitate gas exchange and easy monitoring. The jars were than filled with remediated and control soil from the microcosm experiment. To it 10 numbers of earthworms in each jar were allowed to grow and survival rate was recorded after 48 h. Adult worms weighing 250–500 mg each was only used in the experiment.

2.12. Plant germination and growth test

The bioremediation efficacy on oil-contaminated soil was also assessed by plant germination assay using seeds of mung (*Vigna radiata*) and rice (*Oryza sativa*). Soil samples were collected from each microcosm after 28 weeks of bioremediation and filled into small pots (15 cm width × 10 cm height × 10 cm depth) and seeds of mung and rice were sown. Regular watering was done to maintain the soil moisture content. The pots were kept in the growth chamber maintaining the photoperiod 12 h. After 15 days, the growth parameters (germination, dry weight, shoot height, and root length) of the seedlings were recorded.

2.13. Bacterial enumeration

Total CFU of the remediated soil was enumerated by serial dilution technique. The plates were incubated at 30 °C for 48 h and numbers of aerobic bacterial colonies were calculated on nutrient agar. On the other hand, total number of hydrocarbon utilizing bacterial (HUB) was enumerated by similar method on M1 media plate using crude oil as the sole carbon source. The plates were kept in inverse position with 1 ml of hexadecane on the lid and incubated at 30 °C for 48 h (Mills et al., 1978).

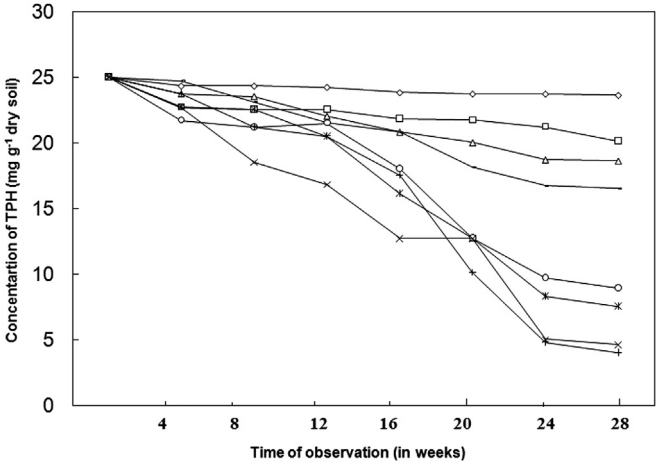

Fig. 3. Comparison of total petroleum hydrocarbon concentration in soils at the end of experiment under microcosm study. Date are mean of five individual observation; —◇— = open rhombus sign control; —□— = open square sign Nitrogen–phosphorus and potassium (NPK); —△— = open triangle sign organic manure (OM); —▬— = dash sign vermi compost (VC); —✕— = cross sign BF-1 (N108-AS03); —○— = open circular sign BF-2 (N002-N78); —✳— = cross sign mix –BF1 (N108-AS03-NPK-OM-VC); —+— = plus sign BF2-mix (N002-N78-NPK-OM-VC).

2.14. GC/MS analysis

For GC/MS analysis, the residual crude oil from the soil at the end of the experiment were extracted as mentioned above. Finally, the analysis of extracted crude oil along with breakdown product of crude oil due to treatment of bacterial formulations was done using Perkin Elmer Clarus 600 GC/MS equipped with Elite 5 MS column. The column and oven temperature was kept at 80–280 °C with an incremental column temperature at 8 °C min^{-1} and finally held at 280 °C for 10 min. The carrier gas was helium with a flow rate of 1 ml min^{-1}. The mass spectrometric data were acquired in electron ionization mode (70 eV). The interface temperature was 280 °C and mass range was 50–500 *m*/*z*. The individual components in the alkane and aromatic fractions were determined by matching the retention time with the authentic standards and with MS library TURBOMONAS, Version 5.40 PERKIN ELMER, CLARUS 600, USA.

2.15. Statistics

Analysis of variance (ANOVA) was done to compare significant differences among the treatments. Tukey's test was done to see the significant difference among the treatments at $p < 0.05$. All the analyses were performed using Origin pro and Prism III software.

3. Results

3.1. Isolation of oil degrading bacteria

The sampling sites and their nature have been described in Table 1. A total of one hundred five numbers of soil samples were collected from Sivasagar district and sixty five numbers from Jorhat district to isolate crude oil degrading bacteria. Most of the soil samples were basic in nature (pH as high as 8.9) along with low electrical conductivity, NPK level and soil moisture. The soil TPH was found in the range 15.2 to 56.2% for different sites. The enrichment and isolation procedure resulted with 200 bacteria in M1 media, and these isolates could utilize crude oil as carbon source. The highest number of isolates was obtained from Lakuwa (65 isolates) and the lowest from Amguri (18 isolates). A total 43 isolates were obtained from different spent engine oil contaminated sites from Jorhat urban areas.

3.2. Growth of bacterial isolates on various hydrocarbon sources

From the pool of 200 isolates, 39 (~20%) were screened for further study on the basis of their ability to grow in medium

containing crude oil as carbon source. For comparing the growth of the bacterial strains, in different hydrocarbons, the turbidity, biofilm and foam formation of cultural broth were considered. Cultural broth that showed high turbidity along with film and foam formation were indicated by very good growth (+++), both turbidity and biofilm or foam formation was indicated by good growth (++) and only turbidity were indicated by growth (+). It was found that 7 strains (17.9%) showed very good growth, 21 strains (53.8%) good growth while rest could show only (Table 2). In pure aliphatic hydrocarbons only 3 strains (N86, N184 and N191) could grow well. In polycyclic aromatic hydrocarbons (PAHs) *e.g.,* fluroanthene, naphthalene, phenanthrene and anthracene, very good growth was observed in AS03, 7–12% of the isolates showed good growth while 28–35.8% showed could only grow.

3.3. Identification of the bacterial isolates

Morphological and biochemical analyses followed by 16S rDNA gene sequencing confirmed that the isolated bacteria belong to the genera *Lysinibacillus*, *Brevibacillus*, *Bacillus*, *Paenibacillus*, *Stenotrophomonas*, *Alcaligenes*, *Delftia*, *Achromobacter*, and *Pseudomonas*. NCBI accession numbers were obtained for total 39 respective strains (Table 2). The relatedness of all 39 bacterial isolates with NCBI reference strain was shown in Fig. 1. Based on the primary screening, it was found that the strains *Pseudomonas aeruginosa* AS03, *P. aeruginosa* N108 and *P. aeruginosa* N002 and *Achromobacter xyloxidans* N78, showed consistently good degradation of crude oil and pure PAHs except N78. Hence, these four strains were further selected for shake culture and microcosm study.

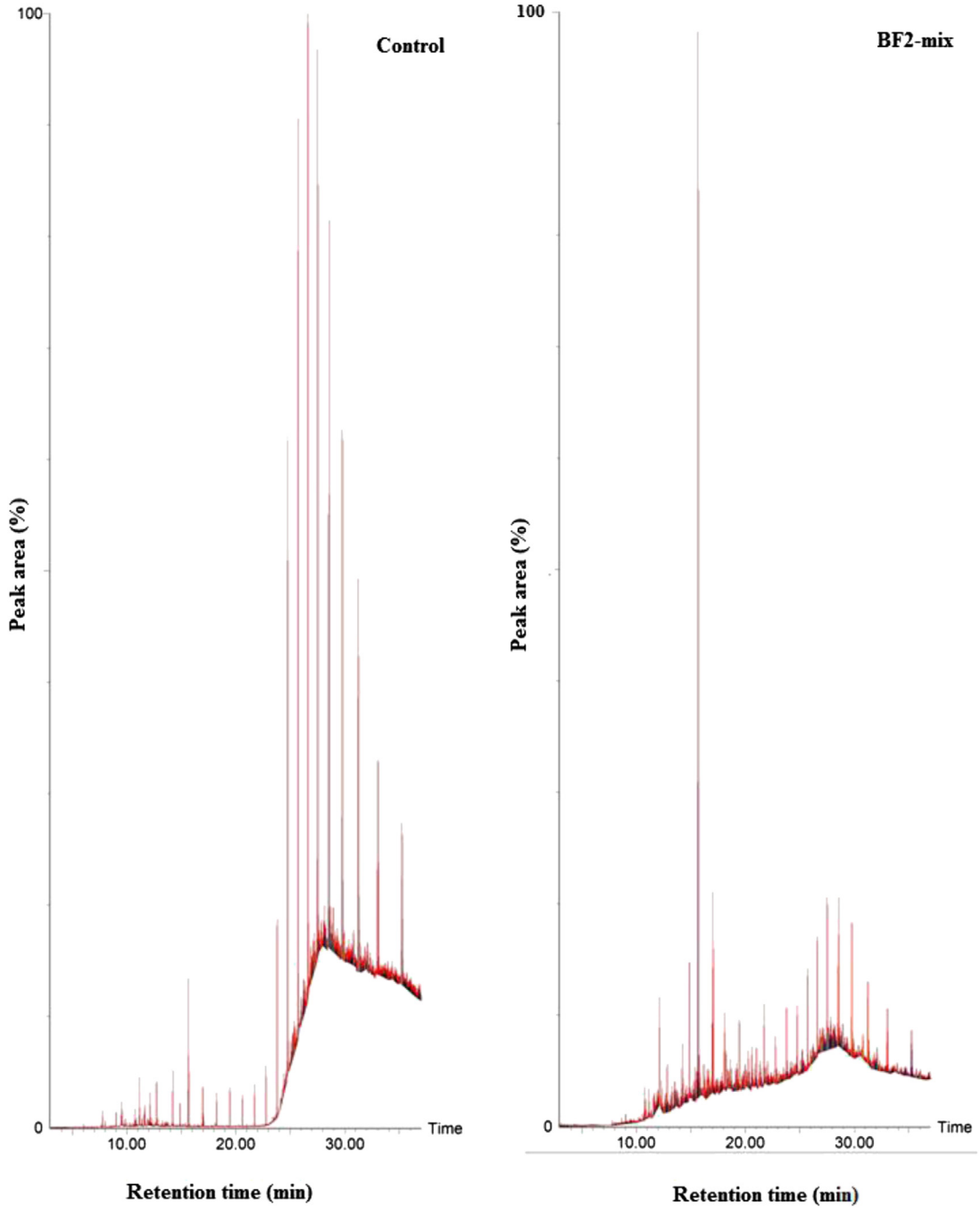

Fig. 4. Comparison of typical gas chromatogram of at the end of experiment with bacteria treated crude oil contaminated soils under microcosm study.

3.4. Crude oil degradation under shake culture condition

3.4.1. Decrease of crude oil viscosity

Among other parameters the effect of treatment of bacteria in crude oil degradation also inferred from changes in viscosity (Fig. 2). In the present study, it was found that the treatment of bacterial strains enhanced the fluidity of crude oil and the treatments followed the order N108 > N78 > N002 > AS03 > control or in reverse the viscosity followed opposite trend. However, the exceptional uniform intrinsic viscosity of N78 treated crude oil was observed from the normal set data point due to the nature of distinct crude oil degradation and inhomogeneous floc formation.

3.4.2. GC/MS analysis of crude oil degradation

The above observations such as the growth of bacteria in crude oil/hydrocarbon supplemented medium as well as the decrease in viscosity of the crude oil/hydrocarbon supplemented medium by the growth of the bacteria indicated the possibility of the degradation of the crude oil/hydrocarbon by these bacteria. To find out the degradation of the hydrocarbons we carried out GC/MS study. GC/MS study reveals the presence of certain hydrocarbon compounds (listed in Table 3) which might be mineralized by bacterial treatment. In case of crude oil degradation microcosm's experiments with bioformulations, it was observed that the cumulative decrease of TPH against 5% in control (which could be attributed to abiotic loss), 64–80% of TPH reduction was observed in bacterial treated microcosm (Table 6 and Fig. 3). Overall maximum reduction of TPH was found in BF2-Mix followed by BF1-Mix > BF1>BF2>VC > OM > NPK with respect to control. The GC analysis of microcosm samples confirmed that bacterial formulations were more effective in TPH reduction compared to other treatments (Table 7). For comparison, a chromatogram of GC analysis between control and one treatment is shown in Fig. 4. Like shake flask study, mineralization of crude oil degraded products due to treated bacteria was also evident in different microcosm's experiments. Some of the major compounds found at the end of the experiments in the GC/MS library search are listed along with their retention time and area percentage in Table 7.

3.5. Soil quality improvement

To assess the improved status of hydrocarbon remediated soil, several tests such as soil chemical, biochemical and bioassay were carried out (Table 4). Compared to control, NPK, OM and VC, the bacterial treated microcosm soil showed dropped in pH from basic to neutral along with marked improvement in soil moisture retention and soil enzyme activities.

The mortality percentage of earthworm for control soil was 63.3%, which decreased up to 30–36.7% for BF2-Mix (N002-N78) and BF1-Mix (N108-AS03) treatment, respectively. Similarly, maximum increase in seedling dry weight for both rice (54%) and mung (63%) was found for the treatments BF2-mix followed by BF1-Mix > BF2 > BF1 > NPK > VC > OM > control along with other growth parameters, such as seedling dry weight, root length and shoot length. Improvement of soil quality was also well correlated by the significant increase in soil CO_2 flux, soil respiration, and evaporation. The CFU count indicated a significant increase in indigenous and introduced bacterial population in the remediated petroleum oil contaminated soil. Highest CFU count was observed in BF2-Mix (2.1×10^3–1.2×10^7 CFU g^{-1} soil) followed by BF1-Mix (2.3×10^2–8.1×10^6 CFU g^{-1} soil) as compared to control soil (1.1×10^2–1.1×10^3 CFU g^{-1} soil). On the other hand, highest hydrocarbon utilizing bacteria (HUB) was seen in BF1-Mix, which ranged from ($2.1 \times 10^{10} - 5.2 \times 10^{10}$ CFU g^{-1} soil) as compared to the control (2.3×10^1–1.3×10^2 CFU g^{-1} soil) (Table 5).

4. Discussion

Crude oil contamination of soil and other ecosystems is an inherent global environmental problem associated with crude oil drilling, transportation, refining and related activities which demands immediate attention for restoration. Compared to chemical and physical methods of restoration of crude oil contaminated sites, eco-friendly bacterial mediated bioremediation have been gaining popularity and well reported (Glick, 2003; Zhuang et al., 2007). In the present study, crude oil utilizing bacteria were isolated from different level of crude oil contaminated soil of Assam for evaluating their efficacy in reclamation of crude oil contaminated soil. Soil analysis showed that Lakowa soil contains highest TPH with lower organic carbon and NPK content, but harbor higher numbers of total bacteria. Higher microbial population in Lakowa soil may be due to utilization of crude oil as carbon source by native bacteria. The lower pH and utilization of crude oil by microbes as energy source were reported earlier (Udo and Fayemi, 1995; Head et al., 2006). In the present study, altogether 200 morphologically distinct bacteria were isolated from crude oil contaminated soil of Assam and 39 different hydrocarbon-degrading bacteria were selected. Though there are reports on screening of large number hydrocarbon degrading bacterial strain from different ecological niches but reports are scanty on screening of hydrocarbon

Table 4
Comparison of soil physical and biological characters of remediated crude oil contaminated soil under microcosm experiment.

Treatments	Before remediation					After remediation				
	pH	Moisture content (%)	Enzyme activity (μg g^{-1} h^{-1})			pH	Moisture content (%)	Enzyme activity (μg g^{-1} h^{-1})		
			DHA	PEA	UEA			DHA	PEA	UEA
Control	9.2 ± 0.5	9.9 ± 0.2	60.7 ± 0.1	94.0 ± 0.2	70.0 ± 1.1	8.5 ± 0.2	9.9 ± 0.1	60.7 ± 0.3	94.0 ± 0.1	70.0 ± 0.1
NPK	8.7 ± 0.7	12.0 ± 0.1	75.3 ± 0.2	110.5 ± 0.3	94.7 ± 0.8	8.4 ± 0.1	14.7 ± 0.2	88.7 ± 0.1	119.0 ± 0.1	100.0 ± 0.1
OM	8.4 ± 0.3	13.6 ± 0.2	72.0 ± 0.1	126.0 ± 0.2	116.0 ± 0.7	8.1 ± 0.2	16.0 ± 0.1	80.0 ± 0.2	132.0 ± 0.2	121.5 ± 0.2
BF 1-Mix (N108-AS03)	7.2 ± 0.2	20.5 ± 0.5	170.6 ± 0.1	184.0 ± 0.7	156.0 ± 0.2	6.9 ± 0.2	25.0 ± 0.2	206.0 ± 0.1	209.6 ± 0.1	173.5 ± 0.1
BF 1	7.4 ± 0.2	12.6 ± 0.2	157.0 ± 0.5	158.0 ± 0.3	123.6 ± 0.3	7.1 ± 0.1	17.4 ± 0.3	168.0 ± 0.2	178.5 ± 0.1	133.0 ± 0.2
VC	8.0 ± 0.2	10.0 ± 0.7	87.8 ± 0.2	121.5 ± 0.2	117.0 ± 0.8	7.8 ± 0.1	13.0 ± 0.2	95.7 ± 4	130.0 ± 0.4	126.0 ± 0.1
BF 2- Mix (N002–N78)	7.5 ± 0.2	14.5 ± 0.3	117.5 ± 0.2	137.5 ± 0.2	134.0 ± 0.6	7.2 ± 0.2	20.3 ± 0.1	134.6 ± 0.3	150.0 ± 0.2	142.0 ± 0.2
BF 2	7.4 ± 0.5	10.2 ± 0.1	135.6 ± 0.3	143.1 ± 0.4	124.1 ± 0.3	7.4 ± 0.5	22.1 ± 0.2	126.4 ± 0.2	165.2 ± 0.4	132.8 ± 0.2

Values are mean of 3 observations with 3 replications each, ±1.0 = Standard error (SE) of observed value. NPK: Nitrogen–Phosphorus-Potassium, OM: Organic farmyard manure, BF1: N108-AS03, BF1-Mixed: N108-AS03 + NPK-OM-VC, VC: Vermicompost, BF2-Mixed: N002-N78 –NPK-OM-VC, BF2: N002-N78, DHA: Dehydrogenase enzyme activity, PEA: Phosphatase enzyme activity, UEA: Urease enzyme activity.

Table 5
Bioassay for determination of bioremediation efficacy of crude oil contaminated soil under microcosm experiment.

Parameters used			Treatments							LSD
			Control	NPK	OM	BF1-Mix (N108-AS03)	BF 1	VC	BF2	BF2-Mix (N002-N78)	
Earthworm toxicity test	Mortality percentage (after 48 h)		63.3 ± 0.2	46.6 ± 0.2	43.3 ± 0.2	36.7 ± 0.2	46.7 ± 0.2	50.0 ± 0.1	40.0 ± 0.3	30.0 ± 0.7	2.2
In planta test	*Vignaradiata*	Dry weight (gm)	1.4 ± 0.2	2.6 ± 0.2	2.3 ± 0.1	3.6 ± 0.2	3.1 ± 0.6	2.4 ± 0.2	3.3 ± 0.2	3.8 ± 0.2	2.1
		Shoot height (cm)	4.2 ± 0.2	7.2 ± 0.1	6.55 ± 0.2	12.1 ± 0.2	11.2 ± 0.9	6.2 ± 0.2	12.2 ± 0.3	14.1 ± 0.8	1.6
		Root length (cm)	1.2 ± 0.1	1.9 ± 0.2	2.1 ± 0.1	3.3 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	3.6 ± 0.23	0.9
	Oryza sativa	Dry weight (gm)	1.3 ± 0.1	2.2 ± 0.1	1.8 ± 0.2	3.8 ± 0.3	3.0 ± 0.2	2.1 ± 0.9	3.7 ± 0.2	4.2 ± 0.1	1.8
		Shoot height (cm)	4.4 ± 0.2	6.2 ± 0.2	5.8 ± 0.1	9.8 ± 0.3	7.2 ± 0.4	6.3 ± 0.2	8.8 ± 0.2	10.1 ± 0.3	1.9
		Root length (cm)	0.2 ± 0.1	0.9 ± 0.1	0.78 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	1.0 ± 0.123	1.3 ± 0.1	2.1 ± 0.2	1.9
Soil parameters	CO_2 flux (ppm)		1.9 ± 0.3	−86.5 ± 1.6	55.7 ± 1.1	8.3 ± 0.2	−12.51 ± 1.1	−38.7 ± 0.7	24.1 ± 1.3	8.3 ± 0.8	−6.9
	Evaporation ($gm^{-2}h^{-1}$)		19.3 ± 1.1	67.2 ± 1.1	0.8 ± 0.03	52.0 ± 0.4	−0.29 ± 0.02	−48.62 ± 0.2	2.5 ± 0.2	2.3 ± 0.3	5.2
	Soil respiration rate ($g\ CO_2 m^{-2}h^{-1}$)		−3.6 ± 0.3	23.8 ± 0.8	−4.5 ± 0.1	4.7 ± 0.2	−0.74 ± 0.1	−5.1 ± 0.2	−4.5 ± 0.3	−3.71 ± 0.1	0.6
	Temperature (°C)		30.3 ± 2.2	30.8 ± 0.7	30.9 ± 0.6	31.5 ± 1.7	31.86 ± 0.5	32.3 ± 0.6	31.6 ± 1.2	31.73 ± 0.4	2.0
Total CFU g^{-1} soil	0 week	Total CFU (gm^{-1}soil)	$1.1 \times 10^2 \pm 0.3$	$1.2 \times 10^2 \pm 0.2$	$2.2 \times 10^2 \pm 2.56$	$2.3 \times 10^2 \pm 2.2$	$2.7 \times 10^2 \pm 0.6$	$3.1 \times 10^2 \pm 2.3$	$4.1 \times 10^2 \pm 2.2$	$2.1 \times 10^3 \pm 0.3$	1.3
		HUB gm^{-1}Soil	$1.1 \times 10^1 \pm 1.2$	$1.2 \times 10^2 \pm 1.6$	$2.1 \times 10^2 \pm 3.6$	$2.1 \times 10^{10} \pm 1.9$	$1.3 \times 10^{10} \pm 0.4$	$2.1 \times 10^2 \pm 0.3$	$2.1 \times 10^{10} \pm 1.81$	$4.1 \times 10^{10} \pm 1.2$	0.9
	8 weeks	Total CFU (gm^{-1}soil)	$1.1 \times 10^3 \pm 0.4$	$3.2 \times 10^3 \pm 2.2$	$3.2 \times 10^4 \pm 2.7$	$4.2 \times 10^{10} \pm 3.2$	$3.1 \times 10^{10} \pm 0.6$	$8.2 \times 10^7 \pm 0.8$	$5.2 \times 10^{10} \pm 1.46$	$7.3 \times 10^7 \pm 1.2$	1.6
		HUB (gm^{-1}soil)	$1.1 \times 10^1 \pm 1.2$	$1.2 \times 10^2 \pm 1.6$	$2.1 \times 10^2 \pm 3.6$	$1.3 \times 10^{10} \pm 4.1$	$9.7 \times 10^{10} \pm 1.1$	$2.1 \times 10^2 \pm 0.3$	$8.3 \times 10^3 \pm 1.7$	$5.2 \times 10^4 \pm 1.4$	1.2
	16 weeks	Total CFU (gm^{-1}soil)	$4.1 \times 10^2 \pm 1.7$	$5.2 \times 10^2 \pm 2.3$	$6.1 \times 10^2 \pm 0.92$	$6.2 \times 10^5 \pm 1.2$	$5.3 \times 10^{10} \pm 1.8$	$7.2 \times 10^4 \pm 0.2$	$6.1 \times 10^{10} \pm 2.3$	$6.1 \times 10^6 \pm 1.3$	2.5
		HUB (gm^{-1}soil)	$1.2 \times 10^2 \pm 2.3$	$4.1 \times 10^2 \pm 3.21$	$5.1 \times 10^2 \pm 1.33$	$4.5 \times 10^{10} \pm 2.3$	$5.6 \times 10^{10} \pm 1.4$	$3.1 \times 10^3 \pm 0.1$	$3.1 \times 10^{10} \pm 2.5$	$9.1 \times 10^5 \pm 0.8$	1.6
	24 weeks	Total CFU (gm^{-1}soil)	$1.1 \times 10^3 \pm 2.5$	$6.2 \times 10^5 \pm 3.3$	$7.2 \times 10^5 \pm 1.5$	$8.1 \times 10^{10} \pm 3.1$	$8.2 \times 10^{10} \pm 1.5$	$8.1 \times 10^5 \pm 0.18$	$5.8 \times 10^6 \pm 0.9$	$1.2 \times 10^7 \pm 0.7$	2.2
		HUB (gm^{-1}soil)	$1.3 \times 10^2 \pm 4.2$	$9.2 \times 10^2 \pm 2.4$	$7.3 \times 10^2 \pm 1.8$	$8.2 \times 10^{10} \pm 1.6$	$2.1 \times 10^{10} \pm 1.35$	$6.1 \times 10^3 \pm 3.15$	$9.1 \times 10^{10} \pm 1.6$	$4.6 \times 10^6 \pm 0.6$	1.5

HUB: Hydrocarbon Utilizing Bacteria, Values are mean of 3 observations with 3 replications each, ±1.0 = Standard error (SE) of observed value; LSD: Lease significant different at $p < 0.05$ according to tukey's test. NPK: Nitrogen–Phosphorus–Potassium, OM: Organic farmyard manure, BF1: N108-AS03, BF1-Mixed: N108-AS03 + NPK-OM-VC, VC: Vermicompost, BF2-Mixed: N002-N78 -NPK-OM-VC, BF2: N002-N78, DHA: Dehydrogenase enzyme activity, PEA: Phosphatase enzyme activity, UEA: Urease enzyme activity.

degrading bacteria from varied environmental conditions of Assam. Finally 4 isolates (AS03, NA108, N002 and N78) were short listed and selected for further study. These four strains were selected on the basis of their best ability to degrade and utilize crude oil and pure aliphatic (*e.g.,* hexadecane and dodecane) and PAHs (like fluroanthene, naphthalene, phenanthrenen and anthracene).

Sequencing and subsequent phylogenetic analysis of the 16S rDNA gene identified the isolates as *Lysinibacillus*, *Brevibacillus*, *Bacillus*, *Paenibacillus*, *Stenotrophomonas*, *Alcaligenes*, *Delftia*, *Achromobacter* and *Pseudomonas* strains (Table 2). The occurenec of these bacteria in oil polluted soil and petroleum reservoirs were earlier reported by several workers (Lal and Khanna, 1996; Watanabe, 2001; Ganesh and Lin, 2009; Wang et al., 2010; Da Cruz et al., 2011; Manif et al., 2011). For first time, the abundance of hydrocarbon utilizing bacteria *Lysinibacillus*, *Brevibacillus*, *Paenibacillus*, *Stenotrophomonas*, *Alcaligenes*, *Delftia*, and *Achromobacter* in different crude oil contaminated soil of Assam has been reported.

Changes of viscosity of crude oil leading to high fluidity were identified as one of the indices of crude oil degradation (Glick, 2010). We found lower viscosity in bacterial treated crude oil (specifically N78) compared to control which is in conformity of earlier reported results (Banat, 1995; Desai and Banat, 1997; Shugla et al., 1999). Likewise, GC analysis of crude oil also confirms the degradation in shake flask like earlier reported by Omotayo et al. (2011).

The treatment of bacteria under microcosm condition also confirmed the degradation of complex hydrocarbons. Almost 80% decrease of total petroleum hydrocarbon (TPH) was obtained in 24 weeks in bioformulation BF2-NPK-VC-OF (Fig. 3). This treatment has significantly increased the rate of degradation as the number of potential hydrocarbon utilizing bacteria (native to the soil) was artificially inducted. Researchers have already reported such enhanced degradation attributed to the addition of laboratory-grown native hydrocarbon utilizing microbes (Erikson et al., 1995; Lal and Khanna, 1996).

To test the quality improvement of bioremediated soil, the soil pH, moisture content and different soil enzyme activities (like dehydrogenase, phosphatase and urease *etc.*) and bioassay were extensively and regularly monitored. The overall drop in soil pH, changes in soil moisture and soil enzyme activities during the course of biodegradation was confirmed in this study. Other researchers, have also been reported the alteration of soil- pH, moisture and soil enzyme activities during crude oil degradation (Sims et al., 1992; Kastner et al., 1998; Gogoi et al., 2012). Similarly, germination studies are considered short-term and primary assay for acute toxicity of pollutants. The present findings are also consistent and in confirmity with the previous reports of improved germination and plant growth after bioremediation (Wang and Bartha, 1990; Saterbak et al., 2000). Our data indicates that rice and mung plants treated with BF1-Mix and BF2-Mix promoted overall higher seedling growth. The decrease in phytotoxicity of microcosm remediated soil may be due to degradation of crude oil and the renewed activities of introduced bacteria.

The overall success in recovery of the crude oil contaminated soil to its original state was tested using earthworm toxicity assay. Several studies have reported the effect of crude oil on earthworm *e.g.*, mortality rate of *Eisenia fetida* increased in soils contaminated with 2% petroleum products (Geissen et al., 2008); diesel concentrations in soil exceeding 1% caused a dose-dependent weight loss in earthworms and increased mortality (Hanna and Weaver, 2002; Shin et al., 2005); and no lethal effect on earthworm with 0.1% TPH content (Schaefer, 2003). In this study, a reduction of 30–36% mortality rate of earthworm against 63% in control after 48 h of rearing remediated soil under microcosm were observed. To

Table 6
Comparison of total petroleum hydrocarbon concentration in soils at the end of experiment under microcosm study.

Time of observation (Weeks)	Name of the treatments								
	Control	NPK	OM	VC	BF1	BF2	Mix-1	Mix-2	LSD
0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	25.0 ± 0.0	0.00
4	24.3 ± 0.2	22.7 ± 1.0	23.7 ± 0.4	24.7 ± 0.4	22.7 ± 0.5	21.7 ± 0.9	22.7 ± 0.7	23.7 ± 0.1	0.98
8	24.3 ± 0.3	22.5 ± 1.1	23.5 ± 0.7	23.1 ± 0.5	22.5 ± 0.4	21.2 ± 1.2	18.5 ± 1.1	21.2 ± 0.7	0.69
12	24.2 ± 0.08	22.5 ± 1.1	22.0 ± 0.7	21.5 ± 1.5	20.5 ± 0.9	21.5 ± 2.3	16.8 ± 2.5	20.5 ± 1.1	0.61
16	23.8 ± 0.1	21.8 ± 0.8	20.8 ± 0.5	20.8 ± 0.8	16.1 ± 2.1	18.0 ± 0.8	12.7 ± 1.8	17.5 ± 1.1	0.42
20	23.7 ± 0.1	21.7 ± 1.0	20.0 ± 1.2	18.1 ± 1.2	12.7 ± 2.2	12.7 ± 1.7	12.7 ± 1.1	10.1 ± 2.7	0.30
24	23.7 ± 0.1	21.2 ± 1.9	18.7 ± 0.1	16.7 ± 1.4	8.3 ± 1.8	9.7 ± 1.5	5.0 ± 2.0	4.8 ± 1.4	0.21
28	23.6 ± 0.1	20.1 ± 1.0	18.6 ± 0.6	16.5 ± 1.0	7.5 ± 1.7	8.9 ± 1.2	4.6 ± 1.0	4.0 ± 0.6	0.19

Date are mean of five individual observation; ±1.0 = Standard error (SE) of observed value; LSD: Lease significant different at $p < 0.05$ according to tukey's test. NPK: Nitrogen–Phosphorus–Potassium, OM: Organic farmyard manure, BF1: N108-AS03, BF1-Mixed: N108-AS03 + NPK-OM-VC, VC: Vermicompost, BF2-Mixed: N002-N78 –NPK-OM-VC, BF2: N002-N78, DHA: Dehydrogenase enzyme activity, PEA: Phosphatase enzyme activity, UEA: Urease enzyme activity.

Table 7
Compounds found at the end of microcosm experiment with different bacterial treated crude oil.

Retention time	Peak area (in %)								Degraded products of crude oil
	Control	NPK	OM	BF1-Mixed (N108-AS03)	BF1	VC	BF2-Mixed (N002-N78)	BF2	
11.16	1,281,564	1,281,564	1,281,564	816,332	0	819,204	607,382	824,865	Dodecane, 1-fluro
12.11	73,359	60,535	64292	73,350	2,975,405	3,999,555	1283	3,960,775	Heptadecane 2
12.83	0	0	0	1,355,066	1,355,023	0	95	2103	Sulfurous acid, butyl heptadecyl ester
14.21	2,560,869	2,560,569	0	1,280,240	1,270,241	2,560,869	256,086	255,787	Heptadecane, 2,6,10,15 tetra methyl
15.67	2,102,872	322,951	2,102,872	381,849	252,575	1,220,987	1,102,872	385,871	Pentadecane 2,6,10,14 tetramethyl
16.96	0	0	0	1,109,881	0	0	921,061	0	Sulfurous acid, butyl tridecyl ester
18.09	3,869,314	3,869,314	3,869,314	0	0	3,869,314	2,646,432	2,646,531	2,2-Dimethyle-propyle 2,2- dimethyle – propanesulfinylsulfone
21.71	1,285,308	1,285,308	14,706	87,556	76,906	1,173,697	1,226,311	1,226,311	1- dodecanol, 2- methyls
25.76	866,914	866,914	866,917	0	3,119,345	1,695,597	5,021,797	412,178	Sulfurous acid, butyl octadecyl ester
26.6	1,228,507	2,844,308	21,917	102,964	106,139	1,228,507	5,421,427	2,039,256	Sulfurous acid, butyl tetradecyl ester
27.5	1,719,175	29,019	52,593	134,963	108,400	1,719,175	1,0494,247	3,050,159	Triacontane

NPK: Nitrogen–Phosphorus–Potassium, OM: Organic farmyard manure, BF1: N108-AS03, BF1-Mixed: N108-AS03 + NPK-OM-VC, VC: Vermicompost, BF2-Mixed: N002-N78 –NPK-OM-VC, BF2: N002-N78, DHA: Dehydrogenase enzyme activity, PEA: Phosphatase enzyme activity, UEA: Urease enzyme activity.

achieve the quality improvement of remediated crude oil contaminated soil, the survival of the introduced microorganisms is a deciding factor (Ramos et al., 1991). Gradual increase in microbial counts (total CFU/g soil and HUB/g soil) during the period of 28 weeks of soil bioremediation indicates the survival of introduced consortium, BF2-Mix and BF1-Mix, which were recorded in experimental findings very clearly.

5. Conclusion

From the above study it could be concluded that crude oil contaminated soil is a good habitat for potent hydrocarbon degraders of the genus *Lysinibacillus*, *Brevibacillus*, *Bacillus*, *Paenibacillus*, *Stenotrophomonas*, *Alcaligenes*, *Delftia*, *Achromobacter* and *Pseudomonas* strain. These bacteria singly and in consortia might have contributed to improve the quality of hydrocarbon-contaminated soil, which was supported by well-established bioassay carried out on plants and earthworm. Further, utilization and degradation of crude oil by the introduced bacteria was also traced from the GC analysis. The enhanced bioremediation of crude oil polluted soil was achieved in the combined treatment of bacterial consortia supplemented with nutrients.

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