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Impact of Bacterial Consortia on Biodegradation of Petroleum in the Contaminated Soils

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ABSTRACT

Rapid rate of industrialization, urbanization and automobile, cause severe contamination to air and soil. There has been exponential increase in numbers of automobiles in Indian subcontinent. In this study we used four native soil microorganisms for remediation of petroleum contaminated sites at Jaipur, India. The land areas of examination were exclusively selected for its use as a stand for heavy transport vehicles. Surface soil was collected from the areas within and surroundings of the Transport Nagar, Jaipur. Physiochemical studies, total petroleum hydrocarbon and effect of various bacterial consortia were investigated. Results show that pH, electrical conductivity, bicarbonate and water holding capacity in test samples (T1, T2 and T3) were substantially reduced. Measurement of chloride however, showed huge elevation ($P<0.05$) in all three test samples (T1, T2 and T3), which was calculated as 53.9 mg/100 g, 71 mg/100 g and 146.26 mg/100 g, respectively. Wide ranges of alkanes (C8 to C29) including both short chain and long chain alkanes were found in test samples. New peaks were observed following treatment with bacterial consortia. In test sample T1 intermediate compounds were observed. The best result was recorded for consortium D and E. Among these two consortia, consortium D (P. rhodesiae, C. henricii and P. koreense) showed exceptional growth from 3rd day onward. At 10th day of incubation growth of consortium D reached highest peak which was nearly 2-3 folds greater than other consortia. Our results showed that consortium D was a completely usable and functional bioremediation tool to improve soil physiochemical characteristics and with effective bio-degradation of petroleum hydrocarbon.

Keywords: Microbial bioremediation, Petroleum hydrocarbon, Soil contamination

INTRODUCTION

With exponential development of industry, automobiles, and aviation, the subsequent demand for petroleum has greatly increased. Nevertheless, plenty of petroleum was saturated to soil during the examination, translocation, and dispensation, and it resulted in significant environmental pollution [1,2].

Due to complexity involved in the transportation of soils and restrictive legislation for contaminated area makes on-site bioremediation as the sole method of treatment. Bioremediation make profitable by using its natural response to the pollutants. It is an effective, low-cost and environmental friendly tool to improve removal of contaminants from the environment [3,4].

Alkanes are saturated hydrocarbons, whereas, unsaturated hydrocarbon is a chemical compound that contains carbon-carbon double bonds and/or triple bonds, such as alkenes or alkynes. Bioremediation of petroleum hydrocarbon is not equal for both saturates and unsaturates. Rates of petroleum hydrocarbons biodegradation have been shown to be highest for saturates. Whereas, high-molecular-weight aromatics and polar compounds exhibit extremely low rates of degradation [5-7]. Although, this pattern is not universal, as Cooney et al. [8] reported greater degradation of naphthalene than of hexadecane in water-sediment mixtures from a freshwater lake. Similarly, Jones [9] observed extensive biodegradation of alkyl aromatics in marine sediments.

Biodegradation as well as bioremediation by selected bio-degradative microorganisms are the key factors in restoration of polluted environments [10]. Bioavailability of pollutants, and catabolic activity of introduced and/or inhabited microorganisms play important roles in bioremediation technologies [11]. Catabolic pathways operating in natural communities reflect interactions between microbial species under mixed culture conditions where extensive sharing of nutritional resources is common [12,13] and interaction of two or several strains is often a prerequisite for growth and biodegradation [14]. It has already been shown that a mixed culture of strains with different catabolic types overcomes incompatibilities in degradation of divergent substrate mixtures [12,15].

In this study we used four native soil microorganisms for remediation of petroleum contaminated sites at Jaipur, India. Test sites examined in this study were exclusively selected for its use as parking and/or servicing area for heavy transport vehicles.

MATERIALS AND METHODS

Sample collection

Surface soil (0-5 cm depth) was collected from the areas within and surroundings of the Transport Nagar, Jaipur (26.916040 N, 75.846296 E, 26.914338 N, 75.849720 E and 26.913570 N, 75.849307 E) from various automobile garages, fuel-storage tanks and at heavy vehicle station, these areas chronically exposed to small spills occurring during the day to day activity and uses of fuels, mainly of which are diesel (Table 1). Control sample was collected from site completely isolated from roadways and not accessible to vehicles. Samples were air dried and pass through 2 mm sieve. The processed samples were store in sterile poly beg at room temperature for future testing.

Table 1: Samples collected form following sites and their respective abbreviations

Sample Sites	Abbreviation
	Control
Surajpol Bazar Rd, Anaj Mandi	T1
Krishna Marketing, Lal Dungri	T2
Vijay Path, Lal Dungri	T3

Physiochemical characterization of soil

Determination of water holding capacity

WHC was determined according to Kneer-Rackzowski box, briefly, a small, perforated bottom circular box or cup is filled with soil. The soil filled box is then kept in a bigger container filled with water so that water enters from beneath. Excess water is then allowed to drain out and the moisture content of soil is determined [16].

Estimation of carbonate and bicarbonate in soil

Carbonate and bicarbonate, was estimated by simple acidimetric titration in the presence of phenolphthalein ($pH>8.5$) and later, in the presence of methyl orange ($pH<6.0$) indicators [17].

Estimation of chloride in soil

Chloride determination is based on the formation of nearly insoluble silver salt (white spongy precipitate of AgCl) with silver nitrate [17].

Estimation of calcium carbonate

The amount of HCl used in reacting with calcium carbonate was determined by titration with 1.0 N NaOH using phenolphthalein as an indicator [17].

Estimation of organic matter and organic carbon in soil

1. Organic matter and organic carbon in soil was determined by method designed by Walkley and Black [18]. Briefly, a weight of 0.25 g of soil was taken into a 500 ml conical flask and 10 ml of 1 N $K_2Cr_2O_7$ and 20 ml of

concentrated H_2SO_4 (containing 1.25% Ag_2SO_4) was added. Later, the flask was shaken well and allowed to stand for about 30 min. To this 200 ml of water was added, to dilute the suspension, furthermore, 10 ml of 85% phosphoric acid and 1 ml of diphenylamine indicator were also added. From this a 10 ml solution was titrated with 0.5 N ferrous ammonium sulfate till the color flashed from violet through blue to bright green. Control blank solution was treated similarly as above. Finally, the volume of ferrous ammonium sulfate was noted and calculated according to Walkley and Black [18].

Determination of pH

pH was determined according to the method explained in Practical and Field Manual by Pandey and Sharma [17].

Determination of available nitrogen and C/N ratio

Total nitrogen which can be converted into different forms (NO_3 or NH^+_4) available to plant is referred to as total available nitrogen. This constitute only 0.5-2.5% of the total nitrogen in soil at any given time. In the presence of excesses alkaline $KMnO_4$, the organic matter present in the soil gets oxidized. The ammonium thus release is distilled and absorbed in a known volume of standard acid, the excess of which is titrated with a standard alkali [19].

Degradation by bacterial consortia

Various bacterial consortia were prepared and grown in nutrient broth media (HiMedia, Mumbai, India). Euler sequence rotation of four different isolates labeled as I1 (Pseudomonas rhodesiae), I2 (Caulobacter henricii), I3 (Cupriavidus gilardii) and I4 (Phenylobacterium koreense) were used in these consortia. The 24 h culture of the bacterial consortia was inoculated in freshly prepared sterile MSM broth (HiMedia, Mumbai, India) supplemented with 2T engine oil (HiMedia, Mumbai, India). The inoculated flasks were incubated in shaker with controlled temperature at 150 rpm and 37°C for 10 days along with control blank. Optical densities of different consortia were observed daily at 600 nm on a UV-VIS spectrophotometer. Simultaneously, number of bacteria in every consortium was estimated daily (Table 2).

Table 2: Different consortia prepared

Consortium	Bacteria absent	Constituent bacteria
Consortium A	Nil	I1, I2, I3, I4
Consortium B	I1	I2, I3, I4
Consortium C	I4	I1, I2, I3
Consortium D	I3	I1, I2, I4
Consortium E	I2	I1, I3, I4

Evaluation of degradation by GC-MS

All samples were extracted using the Soxhlet extraction procedures as outlined in U.S. EPA method 3540 [20]. Each of the sample extracts was cleaned to remove moisture, polar hydrocarbons, colour interferences and any impurities during GC column analysis. In this work, dichloromethane (99.8%) was used as the extracting solvent (Sigma Aldrich, Germany). Toluene (99.8%), hexane/cyclohexane, methanol (99.9%), heptanes (99%), undecane, tetradecane, pentadecane were all HPLC reagent grades (Sigma Aldrich, Germany).

The determination of hydrocarbons in the soil was performed on the samples and standards using a Varian model BV CP 3800 GC-FID equipped with a split injection port and Combi PAL auto sampler. All samples were taken into 2 mL chromatographic vial, injected and separated on a Varian Chrompack capillary column CP 5860 with 95% methyl and 5% phenyl-polysiloxane phase. Carrier gas was helium 26 cm/s. Temperature profile during the chromatographic analysis was 50°C for 3 min; 8°C/min to 320°C hold 15 min and detector at 320°C.

Results of TPH following bacterial degradation on 15th day were compared with results of day one. New peaks and retention time were spotted for evaluation of intermediates formed by degradation of petroleum hydrocarbon.

Statistical evaluation

The mean values were compared using respective standard deviations followed by statistical comparisons between control and test samples for evaluation of significance changes in values by student's t-tests and Analysis of Variance test or ANOVA test. $P<0.05$ was considered as significant.

RESULTS

Physiochemical characterization

Samples were collected from three separate sites around Transport Nagar, Jaipur, India (Figure 1). Examined at various parameters viz. pH, electrical conductivity, available carbonate, bicarbonate, calcium carbonate, soil organic matter, soil organic carbon, total dissolved solids, chloride, water holding capacity, moisture content and C/N ratios. Table 3 shows comparative measurement of above parameters in samples (T1, T2 and T3) and control.

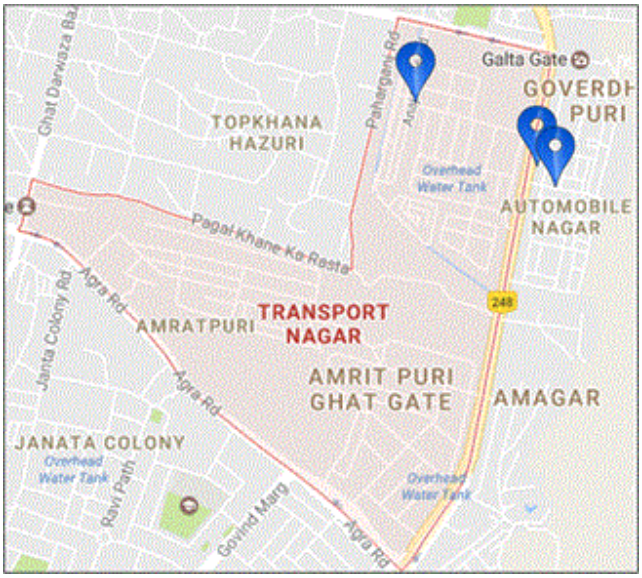

Figure 1: Site from where samples were collected

Result show that pH, electrical conductivity, bicarbonate and water holding capacity in test samples (T1, T2 and T3) were substantially reduced. In T2 the water holding capacity was reduced to about 50%, similarly, 30-40% reduction was observed in T1 and T3. Level of pH increased to almost 8 in all three test samples (T1, T2 and T3) comparing to 6.36 of control. In control sample bicarbonate was estimated as 610 mg/100 g, which was significantly higher ($P<0.05$) than T1, T2 and T3, estimated as 152.5 mg/100 g, 122 mg/100 g and 122 mg/100 g, respectively.

Soil organic matter, soil organic carbon, total dissolved solids and moisture content slightly increased in all three samples (T1, T2 and T3) comparing to control. Measurement of chloride however, showed huge elevation ($P<0.05$) in all three test samples (T1, T2 and T3), which was calculated as 53.9 mg/100 g, 71 mg/100 g and 146.26 mg/100 g, respectively. In control sample chloride was estimated as 4.2 mg/100 g, it was nearly 10-30 folds lower than what was observed in T1, T2 and T3. C/N ratios in test samples (T1, T2 and T3) also increase by 4-5 folds comparing to control (Table 3).

Table 3: Physiochemical characterization of control and test sample

Parameters	Unit	Control	T1	T2	T3
pH	-	6.36	7.95	7.85	7.82
Electrical Conductivity	ms	2.8	1.48	1.6	2.24
Available Carbonate	mg/100 g	-	-	-	-
Bicarbonate	mg/100 g	610	152.5	122	122

Calcium Carbonate	mg/100 g	1	1.5	1	0.5
Soil Organic Matter	%	0.103	0.413	0.31	0.413
Soil Organic Carbon	%	0.06	0.24	0.18	0.24
Total Dissolved Solids	ppt	0.251	0.888	0.954	1.33
Chloride	mg/100 g	4.2	53.9	71	146.26
Water Holding Capacity	%	24.66	18.98	14.24	16.98
Moisture Content	%	0.13	0.431	0.502	0.644
C/N Ration		4:01	21:01	17:01	16:01

Total petroleum hydrocarbon

Gas chromatography-mass spectrometer analyses of the residual hydrocarbon extracted from test samples (T1, T2 and T3) compared with control sample (Figure 2). The obtained compounds are presented in Tables 4-7. From the list of compounds, it was revealed that TPH is increased substantially in the test samples when compared with control sample.

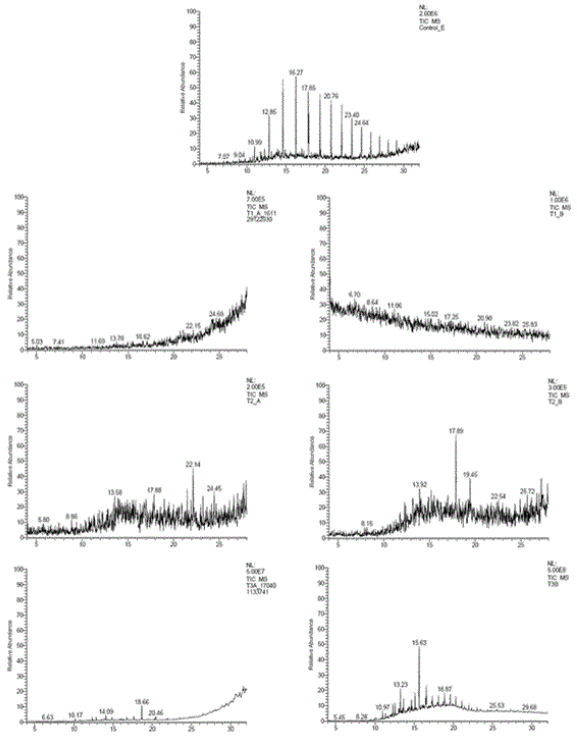

Figure 2: GC-MS chromatograms of control (a), T1A & T1B (b-c), T2A & T2B (d-e) and T3A & T3B (f-g). A=Sample collected on first day; B= Sample following treatment with bacterial consortium

Wide ranges of alkanes (C8 to C29) including both short chain and long chain alkanes namely, n-octane (C8), n-undecane (C11), 2 bromo-dodecan (C12), 2,6,10-Trimethyldodecane (C15), n-hexadecane (C16), pristane (C19), 3-methyl-nonadecane (C20), didecyl eicosane (C20), heneicosane (C21), 2 methyl -tetracosane (C24), 3-methyl-octacosane (C29) and n-nonacosane (C29) that were present in the control sample. In test samples various aromatic hydrocarbons, and PAHs were detected. From the various different PAHs detected in untreated sample, the consortium was able to partially degrade some of them (Tables 4-7).

Table 4: Compounds identified in control sample with its respective retention time

RT	Compounds
10.8	1,5-Diphenyl-hex-3-ene
11	Decane, 3-bromo
11.7	Benzenepropanol, propanoate
11.9	1-Methylamino-2-[(1-methyl-2-benzimidazolyl)methylamino]benzene
12.3	1,4,8-Metheno1Hcyclopent[f]azulene
12.9	Pentanoic acid, 1,1-dimethylpropyl ester
13.2	Methyl-2-(methoxycarbonyl)-3-methyl-5-phenylpent-4-enoate
13.7	Ethaneperoxoic acid, 1-cyano-2-methyl-1-phenylpropyl ester
13.8	1-Deoxydarabinitol tetrakis (trifluoroacetate)
13.9	Undecane, 4,7-dimethyl
14.1	2-Azetidinone, 3,3-dimethyl-4-phenyl-1(phenylethyl)
14.2	2,3,4-Trifluorobenzoic acid, 2-fluorophenyl ester
14.4	4-Hydroxy-5,6-epoxyáionone
14.6	10-Methylnonadecane
14.9	2,3,4-Trifluorobenzoic acid, 2-naphthyl ester
15.5	4-Butyl-6-ethyl-5-methyl-2-Hpyran-2-one
16.3	10-Methylnonadecane
17	2,2,5-Trimethylhexan-4-one
17.3	Heptanamide, N-(2-cyanoethyl) N-hexyl
17.9	6-Tetradecanesulfonic acid, butyl ester
17.9	5-Iodononane
18.2	Diphosphorous tetrafluoride
18.7	Methyl N-trifluoroacetyl-S-(prolyl-2-pyrrolidineacetate)
18.9	3,3-dimethylbutyric acid, 2,2,2-trifluoroethyl
19.3	Oxalic acid, 6-ethyloct-3-yl isobutyl ester
19.4	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)
20.8	Decane, 3-bromo
20.9	Pyridine, 3[(1,1-dimethylethyl)thio]
22.1	6-Tetradecanesulfonic acid, butyl ester
23.4	Sulfurous acid, decyl-2-propyl ester
24.5	Phosphonic acid, bicyclo[2.2.1]hept5en2yl, dimethyl ester
24.6	Decane, 3-bromo
25.8	Borane, diethyl(decyloxy)
27	1,1-Dimethylpropyl-2-ethylhexanoate
28	Sulfurous acid, butyl isohexyl ester
28.2	1(5'methylfurfuryl) pyrrolidine
29.1	Decane, 3-bromo
29.2	3-Buten-2-one, 4-(1,2-dihydroxy-2,6,6-trimethylcyclohexyl) [1à,1(E),2á]
29.4	Cis-1-methyl-3-nonylcyclohexane

29.4	3,4-Hexanedione, 2,2,5-trimethyl
30.1	Butyltertbutylisopropoxyborane
30.4	Sulfurous acid, cyclohexylmethyl nonyl ester
30.8	Butane, 2-azido-2,3,3-trimethyl
30.9	O-Butyl, O-1,2,2-trimethylpropyl methylphosphonate
31	2,6-Dodecadienoic acid, 10(bromoacetoxy)-11-methoxy-3,7,11-trimethyl, methyl ester
31.2	t-Butyl cyclopentaneperoxycarboxylate
31.3	DL-4,5-Octanediol
31.5	2-Aminooctadecane-1,3,4-triol-1,3:2,4-bismethaneboronate
31.7	Meso-2,5-Dimethyl-3,4-hexanediol
31.8	9-Phenanthrenemethyl-2,6-dimethylbenzoate

Table 5: Compounds identified in T1 with its respective retention time

RT	Compounds
19.29	Cyclohexane1carboxamide, 4-methyl-N-(4-tolyl)
20.75	3-Cyclohexene-1-ethanol, àethenylà, 3-dimethyl-6-(1-methylethylidene)
21.05	Trans-3-methylpent-3-ene-5-ol
21.23	Cyclohexanol, 2-bromo, trans
21.56	3,6-Epoxy2H, 8H-pyrimido[6,1b][1,3]oxazocine-8,10-(9H)dione,3,4,5,6-tetrahydro-11-methy-l4[(trimethylsilyl)oxy],[3R(à,4á,6à)]
22.15	N(7,7-Dimethyl-2-oxobicyclo[2.2.1]hept-1-yl)ethanesulfonamide
22.88	Succinic acid, di(2-ethylcyclohexyl) ester
23.52	Bis(2-ethylbutyl) diselenide 23.95 3,4Hexanedione, 2,2,5-trimethyl
24.11	Azetidine, 1-benzyl-2,2,3,3-tetramethy
24.18	2,2-Dimethylpropyl-2,2-dimethylpropanesulfiny-l-sulfone
24.22	1,4-Bis(methylsulfinyl)piperazine
24.28	1,3-Benzenediol, O-cyclobutanecarbonyl-O'(3-fluorobenzoyl)
24.36	1H-Pyrrole-2-carboxamide, 4-amino-N-(3-amino-3-iminopropyl) 1-methyl
24.55	Propane, 1,1,2,2-tetrakis(difluorophosphino)
24.69	1-Amino1, 3-bis-(4-amino-6-dimethylaminostriazin-2-yl)propane
24.96	3-Hexene, 1,1'[ethylidenebis(oxy)]bis, (Z,Z)
25.02	2,2Dimethylpropyl 2,2dimethylpropanethiosulfinate
25.12	4-Nonene, 2,3,3-trimethyl, (Z)
25.22	Cyclopropane, 1-bromo-1-chloro-2-fluoro
25.33	Ether, hexyl pentyl
25.38	10-Hydroxy-2,2,9,9-atetramethyl-1,2,5a, 6,7,8,9,9a-octahydro-1,4-methano-3-benzoxepin-5(4H)one
25.42	Acetic acid, trifluoro, lithium salt
25.49	1-Hexyl-2-nitrocyclohexane
25.54	2(1-Methylcyclopentyloxy) tetrahydropyran

25.69	1-Cyclohexyl-1-(4-ethylcyclohexyl) ethane
25.77	N(7,7-Dimethyl-2-oxobicyclo[2.2.1]hept1yl) methanesulfonamide
25.83	2(Cyclohexylimino)-3,3-dimethylbutane
25.96	Decane, 3-bromo
26.05	1-Hexyl-2-nitrocyclohexane
26.15	2-Pentene, 5-bromo-2,3-dimethyl
26.2	1-Hexyl-1-nitrocyclohexane
26.36	Bicyclo[3.2.0]heptan-3-one, 2-hydroxy-1,4,4-trimethyl, O-acetyloxime
26.43	Butanamide,2-(dimethylamino)N[7[(4-hydroxyphenyl)methyl]3(1-methylethyl)-5,8-dioxo-2-oxa-6,9-diazabicyclo[10.2.2]hexadeca-10,12,14,15-tetraen-4-yl]3-methyl
26.49	1-Cyclohexylnonene
26.55	Sulfurous acid, octadecyl-2-propylester
26.62	Cyclopropane, 1-(1-methylethyl)-2-nonyl
26.77	Butanoic acid, 2-methyl, 3-methylbutyl ester
26.88	trans-4-tertbutylcycloheptanol
27.07	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)
27.13	Cyclohexane,1,1'[1,2-bis(1,1-dimethylethyl)1,2-ethanediyl]bis,(R*,R*)(ñ)
27.29	1-Hexyl-2-nitrocyclohexane
27.38	Cyclohexanol, 4-ethyl-4-methyl-3-(1-methylethenyl),(1à,3à,4á)
27.47	O-Methyl,bis(O-1,2,2-trimethylpropyl)phosphate
27.57	Cyclohexane,1,1'[1,2-bis(1,1-dimethylethyl)1,2-ethanediyl]bis,(R*,R*)(ñ)
27.6	Pyrrolidine, 1-(1-oxo-1,4-methyl-8-hexadecenyl)
27.77	1-Hexyl-2-nitrocyclohexane
27.8	Cis-1-methyl-3-n-nonylcyclohexane
27.86	3-Methyl-1[(1H)-1,2,4-triazol1yl]butan-2-one
27.97	Pentyl glycolate

Table 6: Compounds identified in T2 with its respective retention time

RT	Compounds
12.17	5-(Indole-3-yl)methyl-2-thioxoimidazolidin-4-one
12.85	1,1,4,4,7,7,10,10,13,13,16,16-Dodecamethyl-1,4,7,10,13,16-hexasilacyclooctadeca-2,5,11,14-tetrayne
13.45	Bicyclo[2.2.2]oct-2-ene,1,4,5,5,6,6-hexafluoro
13.58	Benzene, [2(methylsulfonyl)1-propenyl]
13.92	2,2-Dimethylpropyl-2-dimethylpropanesulfinyl sulfone
14.03	2-Butanone, 3-chloro-4-hydroxy-1,4-diphenyl
14.15	4,5-Dichloro-N[(1,2,3,4-tetrahydroisoquinolin-2-yl) methyl]phthalimide
14.41	Ethanediamide, N,N'bis(1phenylethyl)
14.7	Octanamide, N(4-chlorophenyl)
14.78	Bis(trifluoromethylsulfonyl)methane

14.94	N,N-Dibutyl-3-coumarine carboxamide
15	1-Butene,1,1,2,3,3,4,4,4-octafluoro
15.22	2,4-Imidazolidinedione, 1,3,5-trimethyl-5-phenyl, (ñ)
15.5	1-Phenyl-2-methyloct-1-ene
15.64	Oxetane, 2,2,3,3-tetramethyl-4,4-diphenyl
16.36	Urea, N,N'di2-propenyl
16.64	Benzenehexanenitrile, á,ádimethylîoxo
16.99	Cis-2-Nitro-4-butylcyclohexanone
17.16	Diborane(4)tetramine, octamethyl
17.74	Benzeneacetamide, àethyl-N-formylàhydroxy
17.88	5-Hepten-3-one,5-ethyl-2-methyl
18.48	4-Butyl-5-methylpyrazalone
18.61	Butanoic acid, 2-methyl, 2-methylbutyl ester
18.99	2-Heptanone, 7-bromo1phenyl
19.52	1H-Imidazole,1(1-oxopentyl)
20.01	Methapyrilene metabolite
20.22	n-Butyl cyanoacetate
20.62	3-Oxobutan-2-yl 2-methylbutanoate
20.9	Propane, 2[(1,1-dimethylethyl)sulfonyl]2-methyl
21.5	2-Morpholino-4H-5,6-benzothiazin-4-one
21.92	7-Acetyl-2-hydroxy-2-methyl-5-isopropylbicyclo[4.3.0]nonane
22.14	1,3-Benzenediol,O,O'di(cyclohexanecarbonyl)
22.99	9-Phenanthrylmethyl-3-methoxybenzoate
23.22	Fumaric acid, 2-pentyl propyl ester
23.8	2-Thiophenecarboxylic acid, 4-isopropylphenyl ester
23.95	4H-Pyrido[1,2a] pyrimidine-3-acetic acid, 8-methyl-4-oxo, ethyl ester
24.17	2,5,5-Trimethyl-2-oxo1,2,3-dioxophosphorinane
24.45	Fumaric acid, di(3,5-dimethylcyclohexyl) ester
24.99	Carbonic acid, ethyl 3-(1-methylethoxy) phenyl ester
25.56	1,5-Bis(trifluoromethyl)pentasulfide
25.67	4,5-Dihydro-N-phenyl-3-furamide
26.13	Menadiane monopmethoxybenzoylhydrazone
26.62	Furan-2-carboxylic acid (cyanodimethylmethyl) amide
27	Cycloheptene, 5-bromo
27.04	(E)2-Hydroxyimino-3-oxobutyric acid, 1,1-dimethylethyl ester
27.41	(5aà,9aá,9bá)5,5a,6,7,8,9,9a,9b-octahydro-6,6,9a-trimethyl naphtho[1,2c]furan1(3H)one(drimenin)
27.58	Benzamide, N(4-cyanomethylphenyl) 4-fluoro
27.66	transCinnamyl tiglate
27.75	1,3-Bis(cyclopentyl)-1-cyclopentanone
27.93	2-(4-Bromobutyl) furan

Table 7: Compounds identified in T3 with its respective retention time

RT	Compounds
10.17	Sulfurous acid, 2-ethylhexyl tridecyl ester
10.77	Sulfurous acid, 2-ethylhexyl hexyl ester
12.36	Hexadecane, 2,6,10,14-tetramethyl
12.52	1-Methyl-4-isopropylcyclohexyl 2-hydroperfluorobutanoate
12.85	Sulfurous acid, butyl nonyl ester
13.58	Muurolane B
13.64	1-Hexyl-2-nitrocyclohexane
13.86	1,4-Methanonaphthalene-2,2,3,3-tetracarbonitrile, 1,4-dihydro-9-(1-methylethylidene)
13.93	4-Propionyloxypiperidine
14.09	Oxalic acid, isohexyl neopentyl ester
14.27	Decane, 3-bromo
14.85	10-Methylnonadecane
15.97	Pentanoic acid, 1,1-dimethylpropyl ester
16.06	6-Tetradecanesulfonic acid, butyl ester
16.77	Sulfurous acid, butyl nonyl ester
17.65	Pentadecane, 2,6,10-trimethyl
18.59	Sulfurous acid, butyl nonyl ester
18.66	Pentadecane, 2,6,10,14-tetramethyl
18.78	Decane, 3-ethyl-3-methyl
20.32	Sulfurous acid, butyl nonyl ester
20.46	Borane, diethyl(decyloxy)
21.97	Eicosane
23.54	Sulfurous acid, butyl nonyl ester
25.04	Oxalic acid, isohexyl neopentyl ester
26.48	17,21-Dimethylheptatriacontane
27.84	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)
28.06	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)
28.88	17,21-Dimethylheptatriacontane
28.96	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4methylpentyl)
29.2	6-Tetradecanesulfonic acid, butyl ester
29.25	17,21-Dimethylheptatriacontane
29.39	17,21-Dimethylheptatriacontane
29.45	Cyclohexane, 1(1,5-dimethylhexyl)-4-(4-methylpentyl)
29.5	17,21-Dimethylheptatriacontane
29.56	Cyclohexane, 1(1,5-dimethylhexyl)-4-(4-methylpentyl)

29.63	6-Tetradecanesulfonic acid, butyl ester
29.83	17,21-Dimethylheptatriacontane
30.04	17,21-Dimethylheptatriacontane
30.19	17,21-Dimethylheptatriacontane
30.39	Cyclohexane, 1(1,5-dimethylhexyl)-4-(4-methylpentyl)
30.46	17,21-Dimethylheptatriacontane
30.63	Meso-3,4-Dicyclohexyl-2,2,5,5-tetramethylhexane
30.72	Meso-3,4-Dicyclohexyl-2,2,5,5-tetramethylhexane
31	17,21-Dimethylheptatriacontane
31.06	6-Tetradecanesulfonic acid, butyl ester
31.17	17,21-Dimethylheptatriacontane
31.38	17,21-Dimethylheptatriacontane
31.54	6-Tetradecanesulfonic acid, butyl ester
31.63	9-Hexacosene
31.89	17,21-Dimethylheptatriacontane

Effect of bacterial consortia on soil

After 15 days treatment with various consortium new peaks were observed in the GC-MS chromatogram (Figure 2). These peaks were further compared with control to ensure change in petroleum contaminated sites. In test sample T1 intermediate compounds were observed such as; 4-Hydroxy-5,6-epoxyáionone; 2,6-Dodecadienoicacid, 10(bromoacetoxy)-11-methoxy-3,7,11-trimethyl, methyl ester and t-Butyl cyclopentane peroxy carboxylate. Likewise, in T2 one intermediate compound was found, i.e., 2,2,5-Trimethyl hexan. Similarly, in test sample T3, four new peaks were observed in chromatogram, these were further identified as Pentanoic acid, 1,1-dimethylpropyl ester; Undecane, 4,7-dimethyl; 6-Tetradecanesulfonic acid, butyl ester and Borane, diethyl(decyloxy) (Table 8).

Table 8: Compounds and intermediate compounds found in test samples also matched with control sample ; A=Sample collected on first day,B=Sample following treatment with bacterial consortium

Control to T1		**Control to T2**		**Control to T3**	
T1A	**T1B**	**T2A**	**T2B**	**T3A**	**T3B**
Decane, 3-bromo	Decane, 3-bromo	-	Decane, 3bromo	Decane, 3-bromo	Decane, 3-bromo
3,4-Hexanedione, 2,2,5-trimethy1	4-Hydroxy-5,6-epoxyáionone	-	2,2,5-Trimethylhexan	Pentanoic acid, 1,1-dimethylpropyl ester	Pentanoic acid, 1,1-dimethylpropyl ester
Cyclohexane, 1(1,5-dimethylhexyl)-4-(4-methylpentyl)	2,6-Dodecadienoicacid, 10-(bromoacetoxy)11-methoxy-3,7,11-trimethyl, methyl ester	-	-	10-Methylnonadecane	Undecane, 4,7-dimethyl
-	t-Butylcyclopentaneperoxycarboxylate	-	-	10-Methylnonadecane	6-Tetradecanesulfonic acid, butyl ester
-	-	-	-	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)	Borane, diethyl(decyloxy)

Degradation of petroleum hydrocarbon by bacterial consortia

A total of five different consortia were used to evaluate degradation of petroleum, contaminant from soil. These were consortia A-E, consortia A contained all four isolates, whereas, consortia B-E, and each lack one isolate, i.e., I1, I4, I3 and I2, respectively.

Result showed that following inoculation with various consortia significant changes in level of degradation was observed. The best result was recorded for consortium D which showed exceptional growth from 3rd day onward. At 10th day of incubation growth of consortium D reached highest peak which was nearly 2-3 folds greater than other consortia. Besides consortium D, consortium C showed a constant growth throughout the period of examination. Consortia B and C responded equally which grew constantly until 5th day of incubation, however, declined sharply following 6th day of incubation. Consortium A with highest number of isolates showed weakest response as the growth was only momentarily and sharply declined from 4th day onwards. However, it is to be noted that the peak of growth in consortium A was very close to that of consortium D (Figure 3).

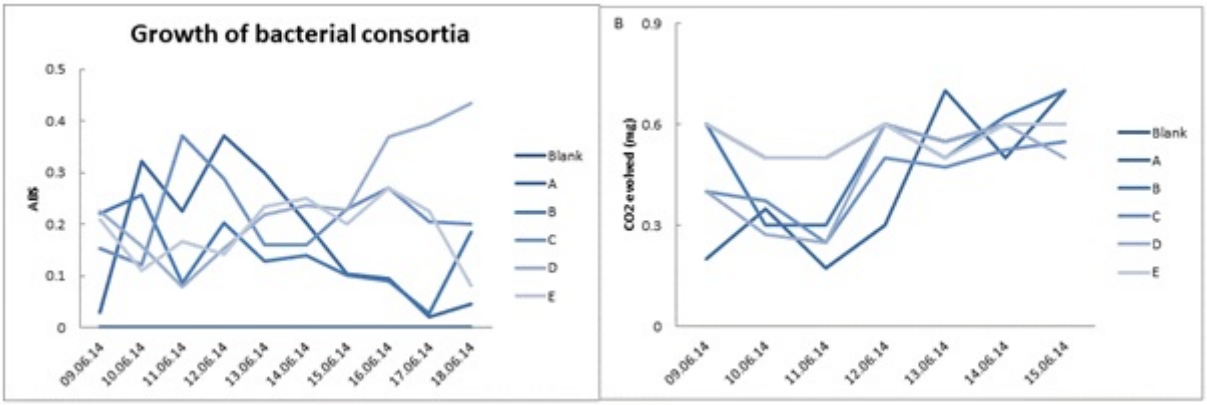

Figure 3: Degradation of petroleum hydrocarbon by bacterial consortia (A-E) (A) and their respective CO2 evolve (B)

DISCUSSION

The current rapid rate of industrialization and urbanization leads to contamination of environmental resources through huge amount of waste they generate. Automobiles cause severe contamination to air and soil. There has been exponential increase in numbers of automobiles in Indian subcontinent. Spillage of petroleum products at the stands and garages lead devastating effect on physio-chemistry of soil.

The present study shows that the physio-chemistry of sites from where samples were collected, are damaged. Among all test sites one at Vijay Path, Lal Dungri, was noted as most contaminated. Amount of total dissolved solids, chloride content, soil organic matter and soil organic carbon was among highest when compared to other two sites. Similarly, electrical conductivity was higher at this site. Although all test sites were found with higher TDS, which perhaps caused absence of crops and/or plants from all three sites of investigation. Previous studies show that over salinity of the soil is one of the main factors that limits the spread of plants in their natural habitats [21].

This study reported higher pH in all test sites which was close to 8, which may enhance mineralization of petroleum hydrocarbon. Earlier studies reported similar contest such as one by Tripathi et al., which state that the saline-alkaline soil exerts more effect on the biological process in the soil [22]. The effects of salinity on soil micro-organisms and microbial mediated processes have been investigated in the previous studies [22,23]. Mineralization of petroleum hydrocarbons can be carried out successfully at salt content of 20% [24]. However, a decrease in salinity would improve the accessibility of soil organic matter to the soil microbial community [25].

Chemicals that occur in TPH generally include hexane, benzene, propane, nonene, pentene, decane, etc. [26]. TPH is sum of VPH and EPH, hydrocarbons range between C2-C5 and C10-C28, respectively (ATSDR, CDC). Results of this study confirm presence of most of the EPHs and limited VPHs, which indicate heavy spillage of petroleum products at the sites. It was obvious from the results that higher numbers of identified VPHs compounds indicate greater assemblage of heavy vehicle at the site. In India most of the heavy vehicles run on diesel, therefore, comparing to control more petroleum hydrocarbon was present at the test sites. In samples collected from Krishna Marketing, Lal Dungri (T2), noted with presence of furan, which is considered toxic and hazardous in many countries. Furans are chlorinated, planar tricyclic aromatic hydrocarbons representing a class of compounds that includes 210 possible congeners [27]. Interestingly in test sample collected from Vijay Path, Lal Dungri (T3), noted with many peaks for single compound named as 17,21-Dimethylheptatriacontane. This compound has distinct appearance in egg-layers of ant *Harpegnathos saltator*, but not in infertile workers and queens [28]. Ant *Harpegnathos saltator* is an Indian ant mostly found in dry, open land. It was assumed that presence of 17,21-Dimethylheptatriacontane at T3 site might have *Harpegnathos saltator* hives under or around the sample site.

Presence of *Harpegnathos saltatori* was also noted in/on eggs of tobacco hornworm (*Manduca sexta* (L.) [29]. It means presence of *Manduca sexta* (L.) is also a possibility for detection of 17,21-Dimethylheptatriacontane at T3 site.

Microorganisms possess mechanisms by which they degrade the crude oil compounds by utilizing them as carbon and nitrogen sources. The pattern of degradation varies for different degrading microorganisms because different microorganisms possess different catabolizing enzymes [30]. Our study used selected bacteria to form five different consortia for evaluation of efficiency to degrade petroleum hydrocarbon. Our result showed that consortium D was best in degrading petroleum hydrocarbons as it has most stable and highest growth rate. This was composed of *Pseudomonas rhodesiae*, *Caulobacter henricii* and *Cupriavidus gilardii*. However, it was interesting to note that consortium E also consisted of *Pseudomonas rhodesiae* and *Cupriavidus gilardii* but the growth was limited and for short span. The only difference these two consortia had was *Caulobacter henricii* which was in consortium D and *Phenylobacterium koreense* which was in consortium E along with other two best performing bacteria i.e. *Pseudomonas rhodesiae* and *Cupriavidus gilardii*. It could be easily speculated that *Phenylobacterium koreense* was perhaps not symbiotic to *Pseudomonas rhodesiae* and *Cupriavidus gilardii*, therefore, a quick drop in growth rate. There was no reference found to relate this assumption, therefore; we assume that our interpretation exclusive to this study.

Our study also revealed that consortia A also contained both *Pseudomonas rhodesiae* and *Cupriavidus gilardii* but did not perform well. The growth elevated initially from 3rd day 5th day but declined sharply in following days. This consortium had two more members, i.e., *Caulobacter henricii* and *Phenylobacterium koreense*. We have already mentioned earlier that *Phenylobacterium koreense* needs lots of water to grow better, but the question is why did not grow while it supported by *Caulobacter henricii*? We think that there is perhaps a lack symbiosis between *Caulobacter henricii* and *Phenylobacterium koreense*. It is evident by growth of consortium B which growth was weakest among all. A strong candidate *Cupriavidus gilardii* could not manage to create some sort of symbiosis between these organisms. Since this study was exclusive no related information was found, more studies are required to ascertain the statement.

It was therefore according to our results it was concluded that *Pseudomonas rhodesiae* is a strong candidate in managing endosymbiosis between isolates as consortium C which despite presence of *Caulobacter henricii* and *Phenylobacterium koreense* grew substantially better than consortium B. Many previous studies claim *Pseudomonas* sp. superiority over other microorganism in degrading complex hydrocarbon. Microorganisms in soil are responsible for degradation, as they utilize hydrocarbons in the polymer backbone as the sole carbon source [31]. Usually, bacterial communities having mixed population are involved, of which the Pseudomonas is amongst the extensively found gram negative soil bacterium with the ability to degrade hydrocarbons and various organic molecules [32,33].

The degradability of petroleum hydrocarbon was estimated by applying these consortia to test samples. Following 15 days incubation GC-MS was performed to see if complex hydrocarbons break down to form intermediates. Our study show that various intermediates were formed such as; 4-Hydroxy-5,6-epoxyáionone, 2,6-Dodecadienoicacid, 10(bromoacetoxy)-11-methoxy-3,7,11-trimethyl, methyl ester and t-Butyl cyclopentan eperoxycarboxylate at Surajpol Bazar Road, Anaj Mandi. Similarly, 2,2,5-Trimethylhexan was observed as new peak at Krishna Marketing, Lal Dungri. Likewise, Undecane, 4,7-dimethyl, 6-Tetradecanesulfonic acid, butyl ester and Borane, diethyl(decyloxy) at Vijay Path, Lal Dungri. This is to be noted that these experiments were only for fifteen days; therefore the list of new compound might change in long-term incubation. Various heavy molecular weight (HMW) and low molecular weight (LMW) PAHs act differently when exposed microbial biodegradation. In addition to this, compared to HMW PAHs, LMW PAHs are reasonably more volatile and more soluble in water and consequently more susceptible to biodegradation [34].

Our results showed that consortium D was a completely usable and functional bioremediation tool to improve soil physiochemical characteristics and with effective bio-degradation of petroleum hydrocarbon. Our study evidently proves degradation of contaminants in test samples collected from all three sites.

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