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Isolation and Identification of Microorganisms From Total Petroleum Hydrocarbon-Contaminated Soil Sites

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ABSTRACT

Crude petroleum oil is a rich source of hazardous materials that can cause soil and water pollution which can be treated by natural biodegradation processes. The aim of this study was to isolate and identify native oil degrading microorganisms from a petroleum-contaminated soil. The study was conducted at Changanassery, Kottayam district in Kerala, India. A total of six bacterial and four fungal strains were isolated from the samples. Isolated bacterial strains were *Pseudomonas aeruginosa, Bacillus subtilis, Pseudomonas* spp, *Bacillus* spp and *Staphylococcus aureus*. Fungal isolates were identified as *Aspergillus niger, Aspergillus spp, Aspergillus flavus, Pencillium* spp. It was observed that these organisms were able to utilize and degrade crude oil constituents.

Key words: crude oil, refined petroleum, biodegradation.

INTRODUCTION

Environmental pollution by petroleum hydrocarbons has become a serious problem all around the world. Petroleum oil pollution results from human activities such as drilling, manufacturing, storing, transporting, waste management of oil and vandalisation of oil pipe lines. The massive and extensive environmental pollution by petroleum industries constitutes socio-economic and public health hazards (Kobayashi and Rittman 1982). Petroleum oil pollution exerts adverse effects on plants indirectly by making toxic minerals in the soil more available to plants (Adams and Ellis 1960). Thus petroleum oil is a serious threat to ecology.

Petroleum is a complex mixture of different hydrocarbons including aliphatic (linear or branched), cycloalkanes, mono and polyaromatics, asphaltenes and resins and the majority of these compounds are stable, toxic and carcinogenic. High concentrations of these pollutants, due to their toxic and carcinogenic nature, can affect cell metabolism (Tanti *et al.* 2009). These hydrocarbons can slowly diffuse deep into the soil and cause pollution of bedrock and underground water resources. On the other hand, light hydrocarbons evaporate and form a layer of oil-contaminated dust leading to air pollution (De-qing *et al.* 2007).

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Bioremediation has become one of the most popular and promising technologies with growing demand to treat petroleum-contaminated soils because pollutants can be completely removed at low cost. Bioremediation uses beneficial microbes to degrade hydrocarbons in soil (Xu 2012). At present, various microorganisms have been detected in petroleum–contaminated soil or water. It has been found that individual microorganisms can mineralize only a limited range of hydrocarbon substrates. Therefore, a mixed microbial population with a broad range of enzymatic capacities is required to increase the rate and optimize petroleum biodegradation (Farinazleen *et al.* 2004).

Based on published reports, the most important hydrocarbon-degrading bacterial genera in soil environments include *Achromobacter, Arthrobacter, Bacillus, Nocardia, Nocardioides, Pseudomonas, Rhodococcus, Sphingomonas, Variovorax* and other unculturable bacterial clones (Chikere *et al*. 2009; Obayori and Salam 2010). Among the fungi, *Aspergillus, Candida, Cunninghamella, Fusarium, Mucor, Penicillium, Phanerochaete Rhodotorula, Sporobolomyces* and *Trichoderma* are hydrocarbon-degrading genera frequently isolated from soil (Chikere *et al*. 2011). The impact of these compounds on human health has stimulated great interest in the identification of microbial strains with specific hydrocarbon degrading activity (Mancera-López *et al.* 2007).The aim of this work is to isolate and identify bacterial and fungal isolates from total petroleum hydrocarbon contaminated soil (TPH sites).

MATERIALS AND METHODS

Collection of Soil Samples

Petroleum hydrocarbon-contaminated soil samples were collected from five different sites including petrol pumps and workshops at Changanacherry Municipality, Kottayam District, Kerala, India. Random soil sampling was preferred due to very small size of plots present in this region (Charan *et al*. 2013). Homogeneous samples were obtained in a simple random strategy at <10 cm in depth, following the procedures described by US-EPA (1996). Soils were mostly sandy and sandy loam to clay loam texture. All the soil samples were obtained during the month of September, 2015 and were dispensed into sterile containers and labeled (Osazee *et al.* 2013). Collected samples were gently sieved (<2mm fraction) and stored in sealed polythene bags at 4°C for 7 days prior to microbial and biochemical analyses (Sahoo *et al.* 2010).

Physiochemical Parameters of the Soil Samples

Physiological properties of the soil samples such as pH, organic carbon, calcium, magnesium, sulphur, iron, manganese, zinc, copper, and boron were determined. Soil samples were dried, homogenised, sieved with a 2-mm test sieve, and conserved at 4°C until physicochemical analyses were conducted (Zafra *et al.* 2014). The physico-chemical properties of soil samples were determined by following standard protocols with slight modifications. Parameters such as

calcium and magnesium were determined titrimetrically or estimated by flame photometer (model CL 26 D, make ELICO).

Heavy Metal Analysis

Heavy metal analysis in soil samples was carried out in triplicate following these procedures. One gram of soil was digested in a glass digestion tube of 250 ml along with 15 ml of nitric acid (HNO_3) at 140°C and the content was evaporated to dryness. The dried sample was further treated with 3 ml of perchloric acid ($HClO_4$) for oxidation from the sample solution for 30 min at 245°C. The content was cooled down after digestion, filtered and final volume was made up to 50 ml with distilled water. Heavy metal measurement was performed at Mancombu Rice Research Station with a Shi-madzu model AA 6300 Atomic Absorption Spectrophotometer. The radiation source was Hallow cathode lamps of metal.

Metals analysis by Atomic Absorption Spectrophotometer (AAS)

For the determination of heavy metals, the water samples were digested with 20 mL aqua-regia (HCl/HNO_3 3:1, volume ratio) in a beaker (open beaker digestion) on a thermostatically controlled hot plate. Then 5.0 mL hydrogen peroxide was added to the sample to complete the digestion and the resulting mixture was heated again to near dryness in a fume cupboard and filtered by Whatman no. 42 filter paper and the volume was made up to 50 mL by double distilled water. Metal contents of the water samples were analysed by AAS (Model: ECILTM AAS−4141) following the method of APHA.

Isolation and Screening of Petroleum Degrading Strains

One gram soil samples were weighed and dissolved into 99 ml of sterile prepared peptone water diluent under aseptic conditions (Harley and Prescott 2002). Serial fold dilutions were then made up to 10^{-6} and aliquots of each dilution were cultured on plates of Nutrient Agar for mean heterotrophic bacterial count and Sabouraud Dextrose Agar (SDA) for mean heterotrophic fungal count respectively by pour plate method (CFU/ml).

Identification of Bacterial Isolates

A number of physical and biochemical tests were performed for the identification of bacterial isolates with the help of the following standard methods (Barrow and Feltham 1993): (1) morphology observation by Olympus-CH40 microscope, (2) gram test, (3) indole test, (4) methyl red test, (5) Voges-Proskauer test, (6) citrate test, (7) catalase test, (8) oxidase test, (9) starch hydrolysis test, and (10) triple sugar iron agar test.

Determination of Bacterial Hydrocarbon Growth Capability

Growth of isolates on hydrocarbon incorporated in nutrient agar media was determined by culturing of bacteria with 5% of oil contaminated nutrient agar media (V/V of petrol) and a control nutrient agar media (without oil). Isolates

where streaked on the above prepared plates and incubated at 37°C for 24 h. After incubation, the control and oil incorporated plates were examined for the growth diameter.

Identification of Fungi

The fungal isolates were identified based on cultural and morphological characteristics. The cultural characteristics were determined by the physical appearance of the fungal colony isolates on the culture plates, while the morphological characteristics were determined by observing the mycelia of the isolates under the microscope in lactophenol cotton blue stain.

Determination of Fungi Hydrocarbon Growth Capability

Qualitative determination of fungi growing on hydrocarbon incorporated in SDA media was done by culturing of fungi. Three concentrations of oil contaminated SDA media (5%, 10% and 15% V/V of petrol) and one control SDA media (non oil contaminated) were prepared in 16 petri dishes and 4 isolated strains of fungi were inoculated in each. All the petri dishes where incubated at room temperature (28°C to 31°C) for 10 days. After 10 days, the control and radially growing colony were examined for the growth diameter.

RESULTS AND DISCUSSION

Physiochemical Parameters of the Soil Samples

The results of the physiochemical composition of the oil contaminated soil samples showed slightly acidic to neutral pH reading. These are similar to the results obtained by Atlas (1981), who reported that neutral pH enables biodegradation activity of bacteria in soil. Bacteria have limited tolerance for acid conditions and fungi are more tolerant. Since the pH in this study was low, it could be assumed that fungi were more involved in the degradation of oil due to its ability to adopt acidic condition and secretion of acidophilic enzymes. The number of culturable bacteria decreased gradually under acidic conditions, while the number of culturable fungi remained relatively constant over the acidic pH range. The ratios of culturable bacteria to culturable fungi were greater than one at pH 6; in contrast, the bacteria-to-fungi ratios were less than one at acid pH (Matthies *et al.* 1997).

The soils were found to contain the highest amounts of organic carbon. Sulphur, iron, manganese, zinc, and copper were higher in value. The lowest concentrations of calcium, was observed in the sulphur, iron, manganese, zinc, copper, boron, calcium, magnesium, pH and organic carbon in all contaminated soil samples (Table 1). Many studies reveal that the increasing total carbon content of the soil with increasing concentration of crude oil may be attributed to the high content of carbon in the oil. In soil, oil can rapidly decay and mineralise leading to the release of cations and trace elements (Nnaji *et al*. 2005). Oil products not only modify physicochemical and biological properties of the soil, but also contribute

TABLE 1
Physiochemical parameters of soil samples

Parameter	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
pH	5.60	6.15	5.89	6.24	7.09	6.194
Organic carbon	2.43	1.89	2.26	2.57	1.78	2.186
Calcium (p.p.m)	279	317	254	280	321	290.2
Magnesium (p.p.m)	85.40	95.20	88.40	73.30	83.20	85.1
Sulphur (p.p.m)	11.80	10.40	9.63	12.30	16.80	12.186
Iron (p.p.m)	236.80	289.60	243.40	255.50	274.30	259.92
Manganese (p.p.m)	37.37	6.35	18.68	29.92	26.14	23.692
Zinc (p.p.m)	59.11	4.78	49.29	49.22	49.20	42.32
Copper (p.p.m)	36.86	0.40	4.13	3.76	16.67	12.364
Boron (p.p.m)	0.16	0.07	0.15	0.13	0.07	0.116

to limiting the productive ability of crop (Wyszkowskwi and Wyszkowskwa 2005). Microorganisms possess mechanisms by which they degrade the crude oil compounds by utilizing them as carbon and energy sources.

Isolation of Bacteria and Fungi

A total 5 samples including one from a petrol pump soil and four from automobile workshop were collected from different sites. In the present study,six bacteria and four fungi were isolated from oil contaminated soil. Bacterial strains were isolated from the crude oil contaminated soil sample using nutrient agar medium and fungal strains were isolated using Sabouraud dextrose agar medium respectively (Tables 2 and 3). The total bacterial count ranged from 1.123×10^7 CFU/ml to 7.27×10^7 CFU/ml. Indeed, the presence of such a significant number of bacteria in the contaminated soils reflects their adaptive ability to survive even in the presence of various petroleum products. Previous studies show that oil pollution can change the composition and diversity of soil bacteria and this change of soil properties will greatly constrain the biodegradation rate of bioremediation of petroleum contaminated sites (Liu *et al.* 2005). The total fungal count ranged from 5.43×10^6 CFU/ml to 4.9×10^7 CFU/ml. In a related experiment, Onifade and Abubakar (2007) reported higher hydrocarbon utilizing fungi in crude oil polluted soil than in crude oil free soil. An increase in microbial load with depth of soil has been confirmed and attributed to migration of the oil downward. Adaptation of these hydrocarbons utilizing fungi increases with exposure (Head *et al*. 2006; Hamamura *et al*. 2006).

TABLE 2
Estimation of total bacterial population using CFU

Sample	No. of colonies	No of cells/ml
Sample 1	144	5.73×10^7 CFU/ml
Sample 2	345	7.24×10^7 CFU/ml
Sample 3	24	1.52×10^7 CFU/ml
Sample 4	67	1.123×10^7 CFU/ml
Sample 5	40	1.325×10^7 CFU/ml

TABLE 3
Estimation of total fungal population using CFU

Samples	No. of colonies	CFU/ml
Sample 1	13	4.9×10^7 CFU/ml
Sample 2	4	4.0×10^7 CFU/ml
Sample 3	4	5.43×10^6 CFU/ml
Sample 4	10	1.66×10^7 CFU/ml
Sample 5	5	1.3×10^7 CFU/ml

Identification of Bacterial Isolates

The six selected bacterial strains were labeled B1, B2, B3, B4, B5 and B6 based on their cultural characters. These organisms were identified based on their cultural, morphological and biochemical characteristics (Table 4). Based on these results, together with the morphological, physiological and biochemical characteristics, strain B1 was identified as *Bacillus cereus*; strain B2, as *Pseudomonas* sp., strain B3, as *Bacillus* sp., strain B4 as *Bacillus subtilis*, strain B5, as *Pseudomonas aeruginosa* and strain B6, as *Staphylococcus aureus*. However, the colony morphology and some physiological and biochemical properties of these *Pseudomonas* spp. and *Bacillus* spp. strains were quite similar. Therefore, further identification to the species level is needed. *Pseudomonas* was a common bacterium capable of degrading hydrocarbons (Kishore Das and Ashis Mukherjee 2007). Though *Pseudomonas aeruginosa* was a clinical strain, it can also grow on hydrocarbon as sole carbon source is a good degrader of oil (Szoboszlay *et al.* 2003). *Bacillus* sp could effectively biodegrade crude oil petroleum in liquid cultures as well as in polluted soil and sand (Salleh *et al.* 2003).In this study all the isolates were able to grow in mineral medium contaminated with crude oil. This results support the increased role of these bacterial genera in hydrocarbon biodegradation (Leahy and Colwell 1990).

Determination of Bacterial Hydrocarbon Growth Capability

In order to carry out the screening of the petroleum hydrocarbon degrading bacteria, each isolate was plated onto NA medium where crude oil served as the sole source of carbon and energy. The growth rate of each bacteria showed that *Pseudomonas aeruginosa* had the highest growth diameter in 5% petrol contaminated NA media culture and *Bacillus cereus* and *Pseudomonas* sp had the second highest growth diameter in 5% petrol while *Staphylococcus aureus* had the lowest growth rate at all the concentrations. Six isolated strains were capable of growing in polluted NA media and utilized petrol as sole carbon source (Table 5, Figure 1). The growth diameter of bacteria decreased with increasing petrol concentration except for *Pseudomonas aeruginosa* which saw an increase in growth diameter of colony with increasing kerosene concentration. The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalysed by oxygenases and peroxidases by many bacterial isolates. *Bacillus cereus* shows gradual decrease in growth diameter as compared to control while other species show slight changes in the diameter of colony growth, indicating higher degradation capacity due to different pathways that convert organic pollutants step by step into intermediates.

TABLE 4
Morphological features, physiological and biochemical characteristics of the strains

Characteristics	Strains					
	B1	B2	B3	B4	B5	B6
Morphological features						
Colony form	irregular	circular	circular	circular	circular	circular
Colony colour	white	Cream	cream	white	cream	yellow
Cell shape	rod	Rod	rod	rod	rod	cocci
Physiological and biochemical characteristics						
Gram staining	+	-	+	+	-	+
Catalase	+	+	+	+	+	+
Oxidase	-	+	+	-	+	-
Indole	-	-	-	-	-	-
M.R	-	-	-	-	-	+
V.P	+	-	+	+	-	-
Citrate	+	+	-	-	+	-
Starch hydrolysis	+	-	+	+	-	+
TSI	A/K(H_2S)	A/A	A/A	A/A	A/A	A/K

Note: "+" = positive; "-" = negative; A/A- acid butt and acid slant; A/K -acid butt and alkaline slant

TABLE 5
Growth diameters of bacterial strains in Oil- contaminated SDA media culture

Bacteria	Growth diameter/mm	
	Control	Concentration of petrol 5%
Bacillus cereus	7	4
	6	5
	5	4
Pseudomonas sp.	4	5
	3	4
	3	3
Bacillus sp.	5	5
	5	4
	4	2
Bacillus subtilis	6	4
	7	4
	5	3
Pseudomonas aeruginosa	7	6
	5	4
	1	4
Staphylococcus aureus	4	1
	2	1
	2	1

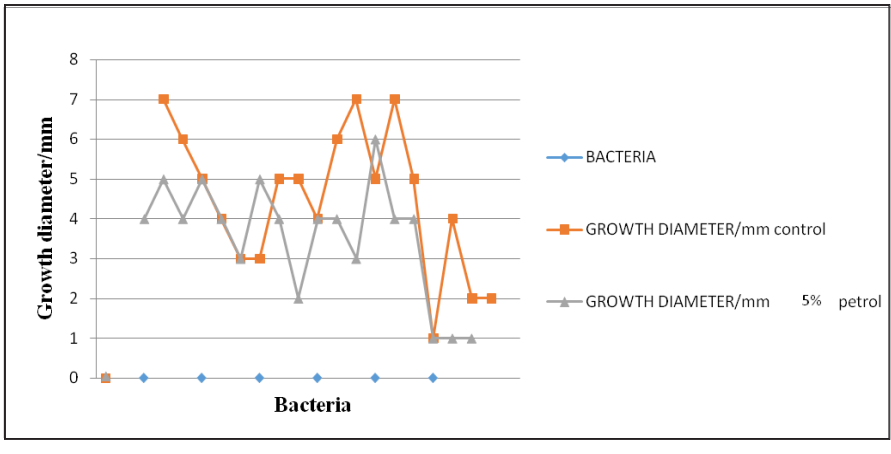

Figure 1. Growth diameters of bacterial strains in oil- contaminated SDA media culture

Identification of Fungi

The fungi exhibiting degradation of petroleum hydrocarbon were grown on SDA to examine morphology, viz size, mycelia and sporulation, and culture, viz color, texture, substrate color and colonial appearances. The identified funguses were *Aspergillus niger, Aspergillus flavus, Aspergillus* sp. and *Pencillium* sp.

Determination of Fungi Hydrocarbon Growth Capability

In order to carry out the screening of the petroleum hydrocarbon degrading fungi, each isolate was plated onto SDA medium where crude oil served as the sole source of carbon and energy. The growth rate of each fungus showed that *Aspergillus niger* had the highest growth diameter in all petrol contaminated SDA media culture and *Aspergillus flavus* had the second highest growth diameter in petrol contaminated SDA media culture while *Penicillium* sp had the lowest growth rate at all the concentrations. A similar observation was reported by Adekunle and Adebambo (2007) in which the isolated *Rhizopus* species from the seed of *Detarium senegalense* showed the highest ability to degrade kerosene compared to *Aspergillus flavus, Aspergillus niger, Mucor* and *Talaromyces.*

Four isolated strains were capable of growing in polluted SDA media, utilizing petrol as the sole carbon source (Table 6 & Figure 2). A study by Wemedo *et al.* (2002) also recorded that the genera of fungi such as *Penicillium, Aspergillus* and *Rhizopus* are associated with kerosene-polluted soil. As can be seen, the growth diameter of fungus decreased with increasing petrol concentration except for *Aspergillus niger* and *Aspergillus flavus* where the growth diameter of colony increased with increasing petrol concentration. *Pencillium* sp and *Aspergillus* sp showed a gradual decrease in growth diameter compared to the control while two other species showed slight changes in the diameter of colony growth, indicating a higher degradation capacity. Table 6 shows that at low and high concentrations of petrol contamination, *Aspergillus niger* had the highest bioremediation activity.

TABLE 6
Growth diameters of fungi strains in Oil-contaminated SDA media culture

Fungus	Growth diameter/cm			
	Control	Concentration of petrol		
		5%	10%	15%
Aspergillus niger	4	4	3.8	3.6
	4.5	3.8	3.5	3.5
Aspergillus flavus	3	2	1.8	1.2
	2.5	1.5	1.3	1
Aspergillus sp.	2.5	2.5	2.2	2
	2.2	2.2	2	1.5
Pencillium sp.	1	0.8	0.8	0.6
	0.9	0.8	0.7	0.5

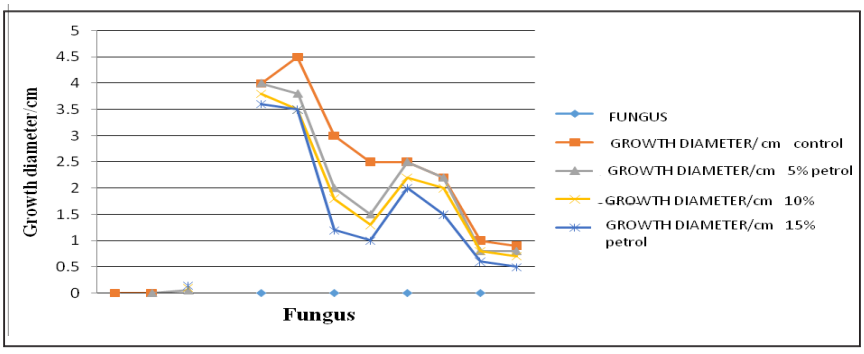

Figure 2. Growth diameters fungi strains in oil-contaminated SDA media culture

CONCLUSION

The microbiological study of petroleum polluted soil shows the presence of six bacteria: *Pseudomonas* sp., *Pseudomonas aeruginosa, Bacillus* sp., *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus and four fungi Aspergillus niger, Aspergillus flavus, Aspergillus* sp, *Pencillium* sp. The presence of heterotrophic bacteria is attributed to the tolerance of these microbes to wide variations of soil properties.

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