



Determination of Hydrocarbons and Isolation of Inherent Bacteria from Crude Oil-Contaminated Soil for Ex-Situ Bioremediation

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Abstract: Bioremediation is environment-friendly, economic and effective method to deal with pollution of natural resources such as land and water. Crude oil-contaminated soil samples were collected and treated with the inherent community of bacteria after their isolation from it. The treatment efficiency was analyzed via capillary column gas chromatography in terms of hydrocarbon removal from the soil sample. The isolation of bacteria present in the crude oil contaminated soil sample was carried out via ten-fold dilution method. The qualitative analysis of the samples revealed that the soil sample consisted of various aromatic and aliphatic hydrocarbons containing carbon chains from C₁ to C₄₀ which comprised of chrysene, benzo (k) fluoranthene, benzo (a) pyrene, di-benzo (a, h) anthracenes, benzo (g, h, i) perylene, phenanthrene, anthracene, fluoranthene, pyrene, benzo (a) anthracene, benzo (k) fluoranthene, benzo (a) pyrene, di-benzo (a, h) anthracene along with different species of cocci and bacilli.

Keywords: Crude oil, contaminated soil, Hydrocarbons, Isolation, bioremediation

1. INTRODUCTION

Bioremediation is an effective technique for the restoration and cleanup of contaminated media including water, wastewater and soils. The ultimate aim of using this treatment method is to efficiently bioreduce the concentration of target compounds, which can be either organic or toxic, thus decreasing the contamination level of the media. Generally, soil is the most important environmental component as it presents itself not only as a geochemical sink for contaminants, but it also acts as a natural buffer in terms of controlling the transport of chemical compounds to either atmosphere or hydrosphere (Pendias, 2001). Oil contamination issue of both soils and water bodies has gathered momentum of late (Benyahia *et al.*, 2005). This is mainly happening either due to periodic oil spillages or via discharges of oily streams caused by three main reasons: a) worn-out aged pipelines, b) delayed maintenance, and c) secret poaching of oil-products from pipelines, blowouts from tanks, leakages and theft from the oils pipelines (Iturbe *et al.*, 2006). This usually results in high loading of petroleum hydrocarbons into soil that leads to qualitative decline of soils and render them unfit for their efficient utilization (Solano-Serena *et al.*, 2000). For such contaminated soils, bioremediation via inherent microbial community is an effective way to decrease the level of contamination since hydrocarbons present in the oily compounds are readily biodegradable depending on their concentrations along with consistent availability of both oxygen and nutrients (Khalid *et al.*, 2005, Margesin and Schinner, 2001 and Huesmann, 1997). In

this context, most of the soils, contaminated with petroleum hydrocarbons, are nowadays investigated for their treatment by means of bioremediation. In this regard, major issues that are dealt with include risk assessment, toxicity, physico-chemical properties of soils such as volatility, water solubility and degradability. In addition, biotreatment of soils contaminated by petroleum hydrocarbons is less expensive and highly effective method than the traditional pump-and-treat method.

The objective of this work was to analyze the soil sample in terms of its existing contamination level, isolation of the bacteria inherent to the soil followed by optimization of bioremediation potential of the isolated bacteria for the removal of oil contents from the contaminated soil.

2. MATERIALS AND METHODS

2.1 Sample location and collection

Soil samples from different affected areas of Tando Alam oil fields of Oil and Gas Development Company, Pakistan, were collected for this study. The texture of the soil was calcareous containing salts and saturated with crude oil fractions resulting in its contamination along with possible intermixing with water cycle as well. All the soil samples were collected from the given oil field waste pit W-13 randomly from its surface and at different depths of 6" and 12". All the samples were immediately refrigerated at 20°C after their collection and transportation to the laboratory until taken for their analyses.

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2.2 Chemicals

The analytical grade chemicals used with their respective ratios during this work include acetone, hexane, dichloromethane, silica, anhydrous sodium sulphate, alumina, activated copper, aliphatic and aromatic hydrocarbon standards. Nutrient agar, Peptone agar-agar, Beef extract, Sodium Chloride (NaCl) Grams iodine, Saffron, Acid alcohol (decolorizer 3 % HCl and 97 % ethyl alcohol), Crystal violet for identification of Hydrocarbons. The compositions of nutrient agar and nutrient broth media are shown in (Tables 1 and 2).

Table 1: Nutrient Agar Composition

Component	Value
Double Distilled Water	500 ml
Peptone	2.5 g
Beef Extract	1.5 g
NaCl	2.5 g
Agar	9 g

Table 2: Nutrient Broth Composition

Component	Value
Double Distilled Water	1000 ml
Peptone	5 g
Beef Extract	3 g
NaCl	5 g

Mixed in Magnetic stirrer for 10 minutes at pH value 6.8–7.2

2.3 Sample preparation

Sample preparation was carried out by standard method (Benyahia *et al.*, 2005). According to this method, soil sample was dried in a vacuum oven at 50 °C followed by its sieving up to 63 µm. Afterwards, 12 g of prepared soil sample was taken for extraction of oil via soxhlet extraction for a period of 8 hr using 300 ml hexane-acetone mixture with a ratio of 3:1. The solvent was separated by means of rotary vacuum evaporator at 25°C and purified by fractionation. Column chromatography of the samples was carried out using 6 g each silica and aluminum as well as 1g each anhydrous sodium sulphate and activated copper. All the analyses were performed thrice before taking the mean value. For identification of hydrocarbons, the prepared sample was detected by Clarus 500 chromatography (Perkin Elmer, Germany) system with FID detector operated at isothermal temperature of 300°C having 30 m capillary column with 0.53 mm diameter. The flow rate of nitrogen carrier gas was 40 ml / min and that of fuel gases hydrogen and oxygen (2%) was 450 ml /min (Blumer *et al.*, 1970).

2.4 Isolation of Bacteria

Bacterial species isolated from the oil contaminated soil by serial dilution agar plating method in which soil sample was diluted up to ten times and spread on respective sterile nutrient agar plates. These plates were incubated at 37°C for 24 hrs and observed for resultant growth after completion of the incubation period. Eight different bacterial isolates were selected for their colonial and morphological studies and were tentatively named as A1, A2, A4, S1, 2d, 3d, Sd and 2. (Fig. 1 and 2) show the growth of bacterial species on both nutrient agar and nutrient broth, respectively. 1g soil was added to 100 ml of sterilized media followed by its incubation for 24 hr. Different colonies were observed having different pigments, sizes and shapes. Then, these colonies were picked for pure culture isolation. Isolation was performed via dilution method (Ten-fold method) where six dilutions were prepared for screening of microbial colonies to isolate pure culture. Cultures were purified by different streaking techniques and purity was cross checked by gram staining procedure as done previously (Jahir *et al.*, 2011; Udeani *et al.*, 2009).

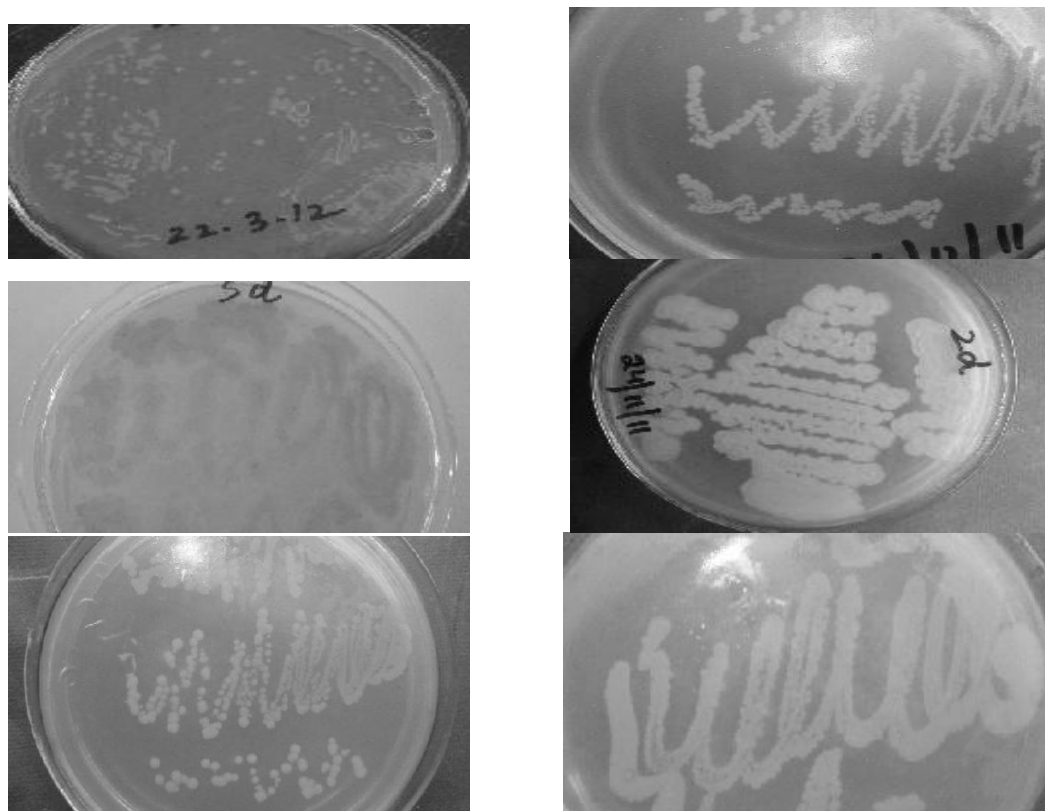


Fig. 1: Isolated bacterial species on nutrient agar



Fig. 2: Isolated bacterial species growth in nutrient broth medium

2.5 Experimental analyses

pH was determined using Jenway pH meter 3505(UK); electrical conductivity (EC) was carried out using Hach Conductivity meter (USA); macro and micro nutrients were assayed via Hach DR-2800 Spectrophotometer (USA); concentration of soluble salts was determined by Flame Photometer (Jenway , UK) and organic matter present in the soil were analyzed by crude titration method. Moisture % was determined by standard crude method in which 10 g of prepared soil sample were taken in pre-weighed petri plates and kept in an electric oven at 45°C for 12 hr before being cooled in desiccators to lower the temperature and weighed after. The same procedure was repeated until a constant weight was obtained and percentage moisture content present in the soil sample was calculated.

3. RESULTS AND DISCUSSIONS

Analysis of soil samples is shown in (Table 3), which shows soil characteristics in terms of pH, electrical conductivity, moisture, macro and micro nutrients, and concentration of soluble salts as well as organic matter present in them. The data in Table 3 implies that the presence of high concentration of salts has affected the soil profile changing its texture as calcareous. Soil moisture is deemed as the limiting factor in terms of having an effect on the soil nutrients. Moisture analysis of the three soil samples revealed that sample 1 had lesser moisture percentage than the other two samples as compared to the normal range for soil moisture. EC is on higher side in all samples but exceeds at high level in surface soil (Sample 1). Due to the presence of excess amount of soluble salts in the soil, essential nutrients are reduced to very low levels in all the samples, as reflected by Nitrogen proportion contained by all the three soil samples. Higher amount of soluble salts present in the samples also yielded the higher values for sodium contents and sodium adsorption ratio. The lower values obtained for macro and micro nutrients contained in the soil samples like Nitrogen, calcium, potassium and iron, were probably caused by biological activity as a result of their breakdown in the root cells via cation exchange capacity of the soil.

Three soil samples were analyzed via Gas Chromatography at PCSIR laboratories Karachi and the results are presented in (Fig. 3 and 4). The samples

Table 3. Soil samples analysis

Parameter	Sample 1	Sample 2	Sample 3	General Values
Moisture, %	5.2	15.3	17.2	9 %
Total Soluble Salt, ppm	62464	6963	6240	500-1000
Electrical Conductivity, dSm ⁻¹	95.7	10.88	9.75	2 - 8
pH	6.8	7.1	7.15	6.5 – 7.5
Sodium, ppm	25000	2300	2050	< 1000
Sodium Adsorption Ratio	80.5	23.8	24.7	< 13
Exchangeable Sodium, %	54	25.3	26.1	< 15
Potassium, ppm	81	50	33	150 – 200
Calcium, ppm	1150	92	56	500 – 1500
Nitrogen, %	0.034	0.061	0.05	> 3
Organic Matter, %	0.68	1.21	1.00	0.68 – 1.29
Iron, mg kg ⁻¹	0.251	0.133	0.56	2 – 4

analyses suggested that almost all components of aliphatic and aromatic hydrocarbons were present in the samples. In addition, surface soil contained fewer hydrocarbons with very low concentration as compared to the samples (samples 2 and 3) taken 6-12 in deep in the soil, which contained higher proportion of aliphatic hydrocarbons (mostly C-25) with concentration of 4000 µg/ml or higher. Considering that soil usually contains large amount of indigenous microorganisms that tend to consume hydrocarbons for their survival, this increased ratio of hydrocarbons present in the lower strata of the soil implies towards the penetration of hydrocarbons inside the soil and underground water, which can be a cause for concern for human health. These apprehensions were also expressed and thus vindicated by the people of the concerned localities.

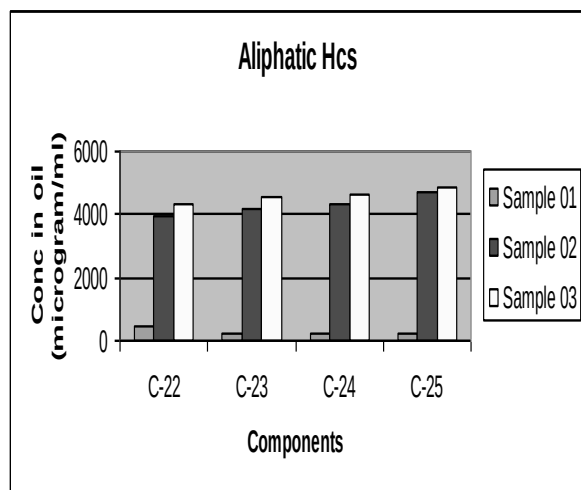
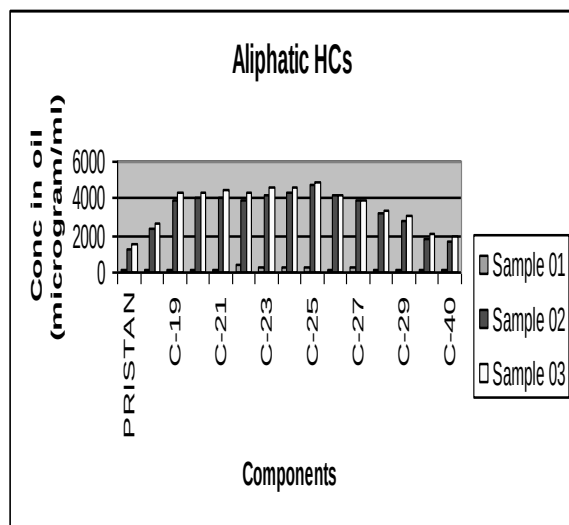


Fig. 3: Concentration of Aliphatic hydrocarbons up to C₄₀ in oil from the soil.

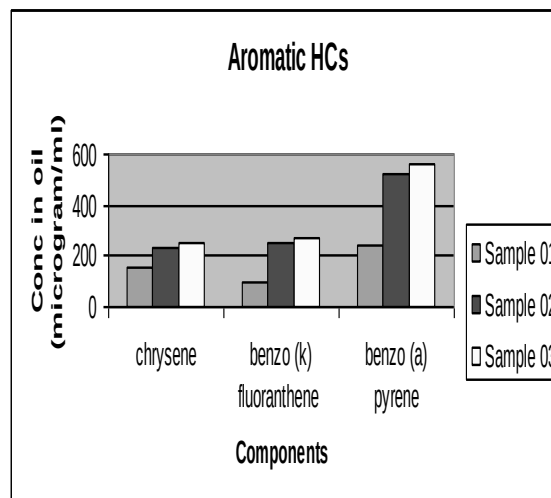
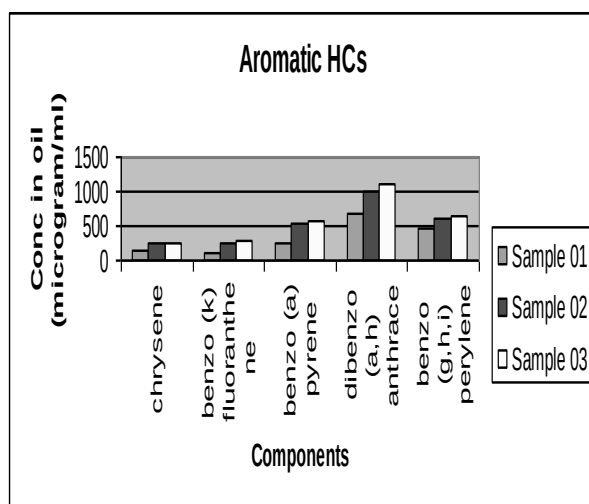


Fig. 4: Concentration of Aromatic hydrocarbons in oil from the soil.

Colonial characteristics of microorganisms are tabulated in **Table 4**, which shows the existence of variety of different bacterial genera along with their shape, size and arrangement. The colonial study of these species suggest that these are present in the soil samples in every possible shape, size and pigment profile varying from pink to yellow for most of the microorganisms. Morphological characteristics of the isolated species are given in **Table 5**, which indicates that most of the microorganisms were rod shaped and that they were always found in scattered positions.

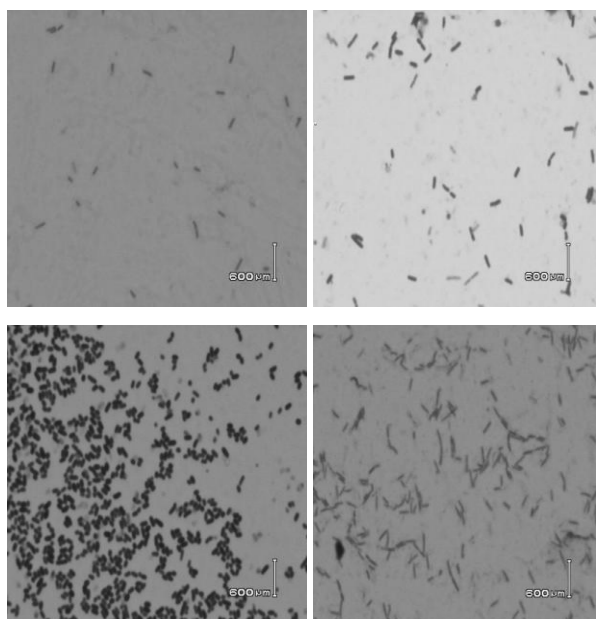
Table 4: Colonial Characteristics of microorganisms

ID	Colonial shape, size and arrangement	Pigment	Number of colonies
Sd	Elevated, viscous, small, scattered	pink	Uncountable
A4	Medium, Irregular, spreading with entire undulate or crenate to fimbriate edges	yellow and white	Lawn
S1	Large thick, Moist and glossy through granular to wrinkled. Shapes, irregular margin	pink	Lawn
3d	Small, Round, convex, scattered	yellow	Lawn
2d	Large, Moist and glossy through granular to wrinkled. Shapes, irregular margin	yellow	Lawn
2	Regular, small, convex, scattered	orange	Lawn

Table 5: Morphological Characteristics of microorganisms

ID	Gram Staining	Shape and arrangement	Specie Name
Sd	Gram positive	Rods, scattered	GPB
A4	Gram positive	Rods, scattered	GPB
S1	Gram positive	Rods, scattered	GPB
3d	Gram positive	Spherical, scattered	GPC
2d	Gram positive	Rods, scattered	GPB
2	Gram positive	Rods, scattered	GPB

Microscopic depiction of all the identified microorganisms is shown in **Figure 5**. Based on the observed characteristics of the biota, they have been identified to belong to bacillus and cocci families. The literature suggests that the given bacterial genera (bacilli and cocci species) are the most effective microorganisms of the hydrocarbons present in the oils (Khan and Rizvi, 2011).

**Fig. 5: Microscopic view of all identified microorganisms**

4.

CONCLUSIONS

It is concluded from this study that due to accumulation of hydrocarbons in excess in the soil samples such as phenanthrene, anthracene, pyrene, chrysene, etc, oil penetration occurs from 1 foot or higher below the surface of soil affecting the water cycle phenomenon, which may cause a serious impact on the human health. In order to prevent this from becoming a serious threat to the society, precautionary measures such as bioremediation of the soil samples need to be carried out on emergent basis, which is the next step of this research study.

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