Characterization of crude oil contaminated soil bacteria and laboratory-scale biodegradation experiments

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ABSTRACT

In current study, bacterial flora of Chak Naurang oil field soils from Chakwal district of Punjab province was characterized and evaluated for crude oil biodegradation potential. Morphological and biochemical characterization revealed twenty-six bacterial strains belonging to nine different genera, such as Micrococcus, Mycobacterium, Corynebacterium, Bacillus, Citrobacter, Erwinia, Neisseria, Planococcus and Klebsiella. Three experimental samples (S1, S2 and S3) were designed by mixing 15 ml sterilized crude oil in 100 grams sterilized soil and incubated at 37˚C for a period of 15 days. In S1 samples, 10ml of individual bacterial suspension was applied separately and comparative findings demonstrated maximum degradation by Micrococcus (15-17.1%), followed by Corynebacterium (13.4-15.7%), Mycobacterium (13-15.3%), Bacillus (11.8-13%), Erwinia (7.9-9.8%), Citrobacter (6.8%), Neisseria (6.5%) and Klebsiella (6.0%) genera, respectively. Two Consortia, namely A (one member each of Bacillus, Corynebacterium, Planococcus and Micrococcus) and B (one member each of Bacillus, Corynebacterium, Planococcus and three members of Micrococcus) were designed and applied to S2 samples, biodegradation of 22% and 27.4% crude oil was noted by consortia A and B, respectively. S3 soil samples were amended with 1 gram of NKP-fertilizer (S3a), 1 gram of Banana peels (S3b) and 0.5 gram each of NKP-fertilizer and Banana peels (S3c), separately and consortia A and B were applied. Results demonstrated the degradation of 26.52%, 22.69%, 22.82% and 30.90%, 28.20%, 27.33% of hydrocarbon contents in S3a, S3b and S3c amended samples, by consortia A and B, respectively at 37˚C in 15 days. Current study shows diversified bacteria and highlights higher biodegradation rates of hydrocarbons in fertilizer amended soil samples by mixed bacterial assemblage.

Keywords: Hydrocarbon; Bacteria; Biodegradation; Consortium; Biostimulation

INTRODUCTION

During the last few decades, extraction of natural resources in order to fulfill growing human needs has resulted in throwing tremendous amounts of pollutants in plant’s natural ecosystems, where various natural phenomenon are being disturbed and only certain biotic factors (microbes, fungi and algae) are playing crucial role in mitigating the hazardous effects. One of such processes being played by bacteria in hydrocarbon contaminated environment is Biodegradation. Chances of accidental hydrocarbon contamination of environment start from the beginning of extraction process to the transportation of distilled fractions.

Crude oil being mixture of saturates, aromatics, condensed asphaltenes and resins provides challenging environment to microbial fate at spill site. Consequently, microbial community structure is shifted to the members with greater tolerance, high physiological compatibility with ecology and it is also influenced by physico-chemical parameters of soil (Hamamura et al., 2006).

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Biodegradation fate of contaminant depends upon its availability, chemical structure and particularly presence of microbial member capable of up taking and mineralizing it. Biodegradation studies have highlighted the significance of hydrocarbon degrading bacteria. Such bacterial strains have been extensively studied on genetic bases to understand mechanism of hydrocarbon utilization (Van Beilen & Funhoff, 2007). Furthermore, bacteria enhance uptake of organic pollutants from environment by the production of biosurfactants (Cameotra & Singh, 2008; Mahmound et al., 2008).

Diverse microbial strains belonging to bacteria, fungi and algae have been reported to utilize hydrocarbons as carbon and energy sources (Chaillan et al., 2004; Toledo et al., 2006). Consort effect of microbial members belonging to different genera have been documented previously in literature suggesting an efficient approach for environment rehabilitation (Ghazali et al., 2004; Gullotto et al., 2008; Shabir et al., 2008).

Use of biostimulants, such as poultry manure and inorganic minerals (Nitrates, Phosphates etc) to hydrocarbon spill site enhanced rate of biodegradation, such approach to efficiently mineralize organic pollutants is known as Biostimulation (Cheng et al., 2008; Nikolopoulou et al., 2009). Bioaugmentation refers to the use of exogenous microbial strains with greater potential to survive and remove pollutants at higher rates. It is being applied to cope accidental contamination of organic pollutants, which were once considered as recalcitrant, requiring longer time duration for removal (Ma et al., 2009).

This study aims to characterize bacterial fauna of oil field soil by investigating biodegradation activity of individual bacterial strain, in mixed assemblage and finding the effect of Nitrogen, Potassium and Phosphorus (NKP) fertilizer as well as banana peels on biodegradation of crude oil.

**MATERIALS AND METHODS**

**Soil Sampling**

The soil used in this study was collected from various localities of waste pit of oil fields of Chak Naurang, district Chakwal. Ten soil samples from each oil field were taken and transported in polythene zipper bags to Microbiology Laboratory of Department of Zoology, GC University Lahore for further studies.

**Isolation of Strains**

Microbial community from crude oil contaminated soil was enriched initially by preparing Mineral Salt (MS) medium enriched with 1% crude oil. MS medium was prepared by dissolving KNO₃, 2.0 g/L; KH₂PO₄, 1.5 g/L; Na₂HPO₄, 5.9g/L; NH₄Cl, 0.3 g/L; MgSO₄·7H₂O, 1.1 g/L; 5.0 ml of Trace element medium containing EDTA, 50g/L; ZnSO₄·7H₂O, 22.0 g/L; CaCl₂, 5.54 g/L; MnCl₂·4H₂O, 5.06 g/L; FeSO₄·4H₂O, 4.99 g/L; (NH₄)₆Mo₇O₂₄·4H₂O, 1.10 g/L; CuSO₄·5H₂O, 1.57 g/L and C₂Cl₂·6H₂O, 1.61 g/L in above solution. To 100 ml of 1% enriched MS medium 1.0 gram of oil field soil was added and incubated at 37°C for 96±02 hours at 100 rpm. From this enrichment suspension 5 ml of volume was transferred to 95 ml of 1% crude oil enriched MS medium and incubated at above mentioned conditions. Isolation of strains was carried by plating 100 µl of suspension on nutrient agar plates and subsequent incubation at 37°C for 24±02
hours. Isolated colonies were purified following standard method of streaking (Brown, 2002).

**Identification of Oil degrading bacteria**

Crude oil contaminated soil isolates were characterized to genus level by careful morphological examinations (Gram staining, spore staining acid fast staining and capsular staining) and biochemical (catalase, oxidase, nitrate reduction test, urease, indole, methyl red, voges proskaur, EMB, MacConkey agar, casein hydrolysis, blood hemolysis, carbohydrate fermentation, triple sugar fermentation and starch hydrolysis) examinations. Results of above mentioned phenotypic and biochemical characters of bacterial isolates were analyzed by Bergey’s manual of systematic bacteriology.

**Screening for Hydrocarbon biodegradation**

Screening for hydrocarbon biodegradation capabilities of bacterial isolates was carried out by streaking bacterial isolates on MS agar plates soaked with n-hexane, benzene and toluene separately.

**Soil preparations**

Garden soil from science block of GC University Lahore was collected and was sieved across 1.0 mm size mesh and dried at 60 °C for an hour. Garden soil was steam sterilized by autoclaving for an hour, after interval of 24 hours for three consecutive days. To this sterilized soil, 15 ml of autoclaved crude oil was added and thoroughly mixed in 16cm glass petriplates for crude oil biodegradation studies. Three sets of soil samples namely, S1, S2 and S3 were prepared by taking 100 grams of sterilized soil, artificially contaminated with 15 ml of crude oil, for individual, mixed assemblage/consortia and biostimulation based biodegradation studies, respectively.

**Inoculum and Consortium preparations**

Inocula were prepared by overnight incubation of pure cultures in nutrient broth at 37 °C at 100rpm. Two consortia, namely consortium A and B were designed by mixing equal volumes of overnight cultures of four (one strain each of *Micrococcus*, *Bacillus*, *Corynebacterium* and *Planococcus*) and six bacterial isolates belonging to four different genera (one strain each of *Micrococcus*, *Bacillus*, *Corynebacterium* and three strains of *Planococcus*), respectively.

**Determination of biodegradation activity**

From 26 bacterial isolates, 18 were selected to determine their biodegradation capabilities. To S1 soil samples, 10 ml of overnight individual inoculum were mixed separately, to S2 series consortia A and B were applied in triplicate and S3 samples were amended by adding 1.0 gram of NKP fertilizer, 1.0 gram dried banana peels and 0.5 gram each of NKP fertilizer and banana peels in separate samples in duplicate. *n*-Hexane extraction method was applied to find out residual oil concentrations as described by Urum *et al.* (2003).

**Microbial enumerations**

Standard method of serial dilution was applied for microbial enumeration during biodegradation studies. To a series of test tubes containing 9 ml sterilized water, 1 gram soil contents were diluted and by spread plate technique and further incubation at 37°C for 24±2 hours. Number of viable colonies were calculated to find colony forming units per gram of soil sample.
RESULTS AND DISCUSSIONS

Identification of Strains

Careful morphological and biochemical characterization revealed diverse microbial community belonging to 9 different genera, such as Micrococcus, Corynebacterium, Bacillus, Citrobacter, Erwinia, Neisseria, Planococcus, Klebsiella and Mycobacterium. Our findings are in agreement with already reported bacterial genera from hydrocarbon contaminated soils (Tam et al., 2002; Adebusoye et al., 2007; Singh & Lin, 2008). Various bacterial genera have been isolated from hydrocarbon contaminated soils, playing important ecological role in removal of hydrocarbons (Van Beinlen & Funhoff, 2007; Chaillan et al., 2004).

Biodegradation studies

In S1 samples, net 15 to 17.2% crude oil biodegradation was noted by Micrococcus, while 13.4 to 15.7% biodegradation was shown by Corynebacterium, followed by Mycobacterium 13 to 15.3%, Bacillus 11.8 to 13%, Erwinia 7.3 to 10.2%, Citrobacter 6.8%, Neisseria 6.5% and Klebsiella 6.0%, respectively in 15 days. Consortium A biodegraded 22% as compared to 27.4% of crude oil contents by consortium B, resulting 5.4% more effective biodegradation by consortium B in S2 samples. Abiotic weathering process resulted in 30% reduction in crude oil contents (Figure 1). Microbial potential of uptake as carbon and energy source have been studied extensively by individual as well as in mixed microbial assemblage (Ghazali et al., 2004; Ito et al., 2008) in order to understand their potential and role in environment rehabilitation processes.

![Fig. 1: Crude oil biodegradation by Individual](image1)

![Fig. 2: Crude oil biodegradation in amended soil and Mixed bacterial isolates samples by consortia](image2)

Consortium A, removed 26.52, 22.69 and 22.82% of crude oil in NPK, banana peels and mixed stimulants, respectively. While 30.90, 28.20 and 27.33% biodegradation of crude oil contents were shown by consortium B in NPK, banana peels and mixed stimulants, respectively in S3 samples. Use of stimulants to enhance microbial activity during treatment processes has opened a new horizon of key interest to environmental studies. Shabir et al. (2008) studied kerosene oil biodegradation by mixed bacterial culture under different nutrient conditions and found highest bacterial growth depicting higher biodegradation rates. Use of poultry and pig manure as biostimulants and various
strategies to enhance microbial activity to remove hydrocarbons as well as various organic contaminants have improved the understanding about behavior of microbial community in pollutant removal processes (Okolo et al., 2005; Cheng et al., 2008; Nikolopoulou & Kalogerakis, 2009). Current study revealed NKP fertilizer as better stimulant of bacterial biodegradation capabilities as compared to Banana peels (Figure 2). Enhanced biodegradation of crude oil from 22 to 32% was studied by the application of NKP fertilizer (Vyas & Dave, 2010).

### Table 1: Microbial enumeration (CFU/ml)

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Soil Set</th>
<th>Consortia</th>
<th>03 Day</th>
<th>06 Day</th>
<th>09 Day</th>
<th>12 Day</th>
<th>15 Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>S2</td>
<td>Consortium A1</td>
<td>1.2×10^2</td>
<td>2.6×10^2</td>
<td>5.5×10^2</td>
<td>4.0×10^2</td>
<td>1.8×10^2</td>
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<td>2.</td>
<td></td>
<td>Consortium A2</td>
<td>1.8×10^3</td>
<td>2.5×10^3</td>
<td>8.4×10^3</td>
<td>7.5×10^3</td>
<td>3.2×10^3</td>
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<tr>
<td>3.</td>
<td></td>
<td>Consortium A3</td>
<td>2.2×10^3</td>
<td>3.7×10^3</td>
<td>7.0×10^3</td>
<td>6.5×10^3</td>
<td>3.5×10^3</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>Consortium B1</td>
<td>1.5×10^3</td>
<td>2.0×10^3</td>
<td>9.0×10^3</td>
<td>7.2×10^3</td>
<td>5.0×10^3</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>Consortium B2</td>
<td>1.1×10^3</td>
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<td>5.3×10^3</td>
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<tr>
<td>6.</td>
<td></td>
<td>Consortium B3</td>
<td>1.4×10^3</td>
<td>3.6×10^3</td>
<td>5.7×10^3</td>
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<td>2.7×10^3</td>
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<td>7.</td>
<td>NKP</td>
<td>Consortium A</td>
<td>1.94×10^2</td>
<td>1.20×10^2</td>
<td>1.54×10^2</td>
<td>1.79×10^2</td>
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<td>8.</td>
<td></td>
<td>Consortium A</td>
<td>2.2×10^3</td>
<td>3.8×10^3</td>
<td>3.44×10^3</td>
<td>4.84×10^3</td>
<td>7.72×10^3</td>
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<tr>
<td>9.</td>
<td>Banana peels</td>
<td>Consortium A</td>
<td>2.34×10^2</td>
<td>1.72×10^2</td>
<td>3.16×10^2</td>
<td>4.76×10^2</td>
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<tr>
<td>10.</td>
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<td>Consortium A</td>
<td>2.93×10^2</td>
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<td>4.98×10^2</td>
<td>7.62×10^2</td>
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<td>11.</td>
<td>NKP+Ban.P</td>
<td>Consortium A</td>
<td>3.09×10^2</td>
<td>3.67×10^3</td>
<td>4.28×10^3</td>
<td>6.2×10^3</td>
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<td>12.</td>
<td></td>
<td>Consortium A</td>
<td>3.86×10^2</td>
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<td>14.</td>
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<td>Consortium B</td>
<td>3.74×10^2</td>
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<tr>
<td>15.</td>
<td>Banana peels</td>
<td>Consortium B</td>
<td>3.54×10^2</td>
<td>4.01×10^3</td>
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<td>7.4×10^3</td>
<td>7.29×10^3</td>
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<tr>
<td>16.</td>
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<td>Consortium B</td>
<td>3.72×10^2</td>
<td>3.96×10^3</td>
<td>4.20×10^3</td>
<td>5.26×10^3</td>
<td>7.80×10^3</td>
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<td>17.</td>
<td>NKP+Ban.P</td>
<td>Consortium B</td>
<td>4.0×10^2</td>
<td>4.29×10^3</td>
<td>5.04×10^3</td>
<td>6.19×10^3</td>
<td>8.98×10^3</td>
</tr>
<tr>
<td>18.</td>
<td></td>
<td>Consortium B</td>
<td>4.10×10^2</td>
<td>4.19×10^3</td>
<td>5.34×10^3</td>
<td>6.49×10^3</td>
<td>9.0×10^3</td>
</tr>
<tr>
<td>19.</td>
<td>Control</td>
<td></td>
<td>1.98×10^1</td>
<td>1.87×10^1</td>
<td>1.78×101</td>
<td>1.40×10^1</td>
<td>0.93×10^1</td>
</tr>
</tbody>
</table>

NKP = Nitrogen-Potassium-Phosphorus
Ban.P = Banana Peels
Reduction in weight of soil samples and microbial monitoring results supported decrease in residual oil contents. Microbial monitoring depicted increase in cell density up to day 09, followed by slight decrease till day 15 in S2 samples, while in biostimulation studies microbial density increased gradually till day 15, showing abundant availability of nutrients in S3 samples (Table 1).

Current study provides an explanation to the role of diverse indigenous bacteria individually, in consort form as well as enhanced biodegradation of crude oil contents in biostimulants supply. It also suggests the use of exogenous supplies of minerals along with organic sorbents to boost bacterial growth and hence their ecological role of removing pollutants.

REFERENCES


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