The Effects of Nutrients on the Biodegradation of Crude Oil

El Mutaz Nassir Hassan¹* and Ahmed Ali Mahdi²

1 Commission for Biotechnology and Genetic Engineering, National Centre for Research, Khartoum, Sudan
2 College of Agriculture, University of Khartoum, Khartoum, Sudan

Received February 2017, Accepted March 2017, Published Online April 2017

Abstract
The results of this study deal with addition of nutrient to the contaminated media with the hydrocarbon compounds (crude oil) on degradation of this compounds by bacteria, showed 14 isolates of bacteria exhibited good indications of ability to degrade crude oil, eight of which belongs to the genus Bacillus, two to Pseudomonas and four to Chromobacterium. Five of these promising strains were selected for further study, two of which (V10 and D35) belongs to the genus Bacillus and three (V16, D39 and W1) to Chromobacterium. The two Bacillus strains were further identified depending on biochemical tests as B. larvae (V10) and B. alcalophilus (D35). Strains D35, V10 and W1 were used as consortium for treatment. The addition of nitrogen, phosphorus or both fertilizers to the medium inoculated by a consortium of the bacterial strains D35, V10 and W1 resulted in improved crude oil degradation as revealed by chromatographic separation of the crude oil fractions.

Keywords: Bioremediation; Crude oil; biodegradation; Nutrients; Bacterial consortium

1 Introduction
Extensive petroleum hydrocarbon exploration activities often result in the pollution of the environment, which could lead to disastrous consequences for the biotic and abiotic components of the ecosystem if not restored. Remediation of a petroleum-contaminated system could be achieved by either physicochemical or biological methods. However, the attendant negative consequences of the physicochemical approach are currently directing greater attention to the exploitation of the biological alternatives (biodegradation) (Okoh, 2006). Many microbial species have been implicated in crude oil degradation leading to a successful bioremediation of contaminated environments. Improvement of microbial efficiency has been directed towards the utilization of microbial seeding as a means of controlling oil spills (Odu, 1972). Nutrient availability, especially of nitrogen and phosphorus, seems to be the most limiting factor. It was confirmed that these nutrients enhance growth of microorganisms, which leads to more rapid decomposition of contaminants (Chaineau et al., 2005; Coulon et al., 2005). The main objectives of this study are using locally-isolated bacteria to degrade crude oil hydrocarbons released in the local environment and enhance the efficiency of the bacteria that may participate in the degradation of these compounds.

2 Materials and Methods
2.1 Collection of samples
Soil samples contaminated by petroleum hydrocarbons and /or pesticides were collected from surface soil (0-10 cm depth), in addition to produced water samples from Heglieg oil-field. Crude oil (Nile Bland- Heglieg oil field) was brought from Central Petroleum Laboratory, Khartoum, Sudan.

2.2 Isolation and Screening for microorganisms having ability to degrade crude oil
Isolation was carried out within 24 h after samples collection. Ten grams of each soil sample (or 10 ml of produced water) were added to 90 ml of distilled water to give a 10⁻¹ dilution. One ml of each diluted soil sample and produced water was poured into a Petri dish, and about 15 ml of the Mineral Salt Medium (MSM) of Mills et al. (1978) as modified by Okpokwasili and Okorie (1988) was used with the following composition g/L: NaCl 10.0; MgSO₄.7H₂O 0.42; KCl 0.29; KH₂PO₄
0.53; \( \text{NH}_4 \text{NO}_3 \) 0.42; \( \text{Na}_2\text{HPO}_4 \) 1.25 and agar 15.0. The pH was adjusted to 7.0 and 10 ml of crude oil were added to one liter of sterilized medium were added and thoroughly mixed. Plates were left to solidify at room temperature. The plates were then incubated at 35°C for 12 days. Colonies appearing within 48-72 hours, in addition to those appearing at later stages but with large size were selected. The selected isolates were sub-cultured 2 -3 times, purified and preserved in Nutrient Agar slants at 4°C for further studies.

2.3 Identification of the selected bacterial isolates
Identification of the selected isolates was carried out according to Cowan and Steel (1974) and Sneath et al. (1986).

2.4 Preparation of a bacterial consortium
A loopful of overnight culture was inoculated into 100 ml sterile Nutrient Broth. The screw- cap bottles were kept on orbital shaker at 90 rpm for 12h at 38°C. (Equal volumes of approximately equal number of live cells) of the culture broths from the isolates (V10, D35 and W1) were mixed to prepare a bacterial consortium.

2.5 Treatments
The petroleum- amended Mineral Salt Medium Broth described above was prepared and distributed in 300 ml lots in screw- cap bottles. Treatments were set up as follows: No bacteria were added (control).
1. No bacteria were added (control).
2. \( \text{KH}_2\text{PO}_4 \) 1.06 g + the bacterial consortium.
3. \( \text{NH}_4\text{NO}_3 \) 0.84 g + the bacterial consortium.
4. \( \text{KH}_2\text{PO}_4 \) 1.06 g + \( \text{NH}_4\text{NO}_3 \) 0.84 g + the bacterial consortium.

The treatments were replicated 3 times and were incubated at 35°C for 14 days. The treatments were sampled at one and two weeks for determination of total petroleum hydrocarbons (TPH) using gas chromatographic analysis.

2.6 Oil extraction and chromatographic analysis
The medium of each treatment was transferred to a separation funnel and 3 ml chloroform were added and shaken vigorously. The separate layer of organic solvent was collected in a dried beaker. The process was repeated 2-3 times to ensure maximum extraction. Finally, the extract was evaporated in air and prepared for gas chromatographic analysis. Total petroleum hydrocarbons (TPH) were determined using a gas chromatograph. The instrument used was a VARIAN GC (model CP-3800) equipped with a split injector (split ratio 50/1) and a Flame Ionization Detector (FID) both set at 300°C; the carrier gas was nitrogen (1.50 ml min-1); the column was a fused silica capillary column (30.0 m x 0.32 mm, film thickness 0.25µm); temperature programming was 40- 280°C, 5°C min-1, injection volume 1 µl. Percentage of degradation was calculated by the following expression: % of oil degradation = \([\frac{(\text{TPH control} – \text{TPH treatment})}{\text{TPH control}}\) x 100.

3 Results and Discussion
A total of 96 isolates of bacteria were isolated from different petroleum- contaminated locations in Khartoum State which are shown in Table (1).

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Number of isolates obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas stations (S)</td>
<td>26</td>
</tr>
<tr>
<td>Khartoum industrial area (D)</td>
<td>43</td>
</tr>
<tr>
<td>Vegetables farms (V)</td>
<td>24</td>
</tr>
<tr>
<td>Heglig oil field produced water (W)</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
</tr>
</tbody>
</table>

Table 1 Sampling Source and Number of Samples Collected

The good (and sometimes excellent) growth of many of the present isolates in the petroleum-amended Mineral Salts Medium is a good indicator of the potential of bacteria to degrade crude oil. Riser-Roberts (1992) and Bundy et al., (2004) stated that petroleum hydrocarbons can be degraded by a wide variety of microorganisms such as bacteria, fungi, yeasts and microalgae. However, bacteria play the central role in hydrocarbon degradation. A large number of Pseudomonas strains capable of degrading PAHs have been isolated from soil and aquifers (Kiyohara et al., 1992; Johnson et al., 1996). Other petroleum hydrocarbon-degraders include Yokenella spp., Alcaligenes spp., Roseomonas spp., Stenotrophomonas spp., Acinetobacter spp., Flavobacter spp., Corynebacterium spp., Streptococcus spp., Providencia spp., Sphingobacterium spp., Capnocytophaga spp., Moraxella spp., and Bacillus spp. (Rusansky et al., 1987; Antai, 1990; Bhattacharya et al., 2003). Sudan soils are rich in microbial resources. Screening for oil-degrading microorganisms in the present study, using the Mineral Salt Medium amended with crude oil, revealed the potential of bacteria to use crude oil as a sole source of carbon as evidenced by the scattered growth of bacteria on the medium with no other microorganisms. This result agrees with Rahman et al. (2003) and Broojmans et al. (2009) who stated that bacteria are the most active agents in petroleum degradation, working as primary degraders of spilled oil in the environment. Yakimov et al. (2007) reported that several bacteria are even known to feed exclusively on hydrocarbons.
The isolates that showed excellent growth in the petroleum-amended Mineral Salts Medium Agar (MSMA), are D35 from gas station soil, V10 from Khartoum industrial area, and isolate W1 from Heglig oil field produced water and use in further investigations as they exhibited good indication of their ability to degrade petroleum hydrocarbons. In an investigation on crude oil degradation using a consortium of three of the selected isolates (V10, D35 and W1) in the Mineral Salt Medium Broth amended with 1% petroleum oil and receiving N, P or N + P, spectral output of crude oil was obtained using gas chromatographic separation of n-alkane fraction of the degraded oil after one and two weeks of treatment. Results of this investigation are displayed in Figs. 1 – 7. Figure 1 shows results of the gas chromatographic separation of the n-alkane fraction of crude oil after two weeks in the control treatment receiving no N or P fertilizers and not subjected to bacterial degradation. It can be seen that the absorbance of the nC13 - nC17 fraction was higher than 100 mVolts, and that of the fractions nC12, nC18 and nC19 was above 75 mVolts after two weeks. Figure 2 shows that with addition of 1.06g per liter phosphorus (as KH2PO4) to the Mineral Salt Medium amended with 1% crude oil and exposure to the consortium of bacteria, a decrease in the concentration of most of the fractions in the crude oil was observed compared to the control after one week. However, after two weeks of treatment, the reduction in concentration of crude oil fractions was highly remarkable, where some fractions were reduced to less than 50% of their concentration in the control treatment (Fig. 3). In the treatment fertilized with 0.84g nitrogen (as NH4NO3) per liter a highly remarkable reduction in the concentration of the fractions of crude oil was recorded after only one week of treatment (Fig. 4), with further reduction of these concentrations after an additional week of exposure to the bacterial consortium (Fig. 5). Figure 6 shows that addition of 1.06g phosphorus (as KH2PO4) and 0.84g nitrogen (as NH4NO3) per liter to the Mineral Salt Medium amended with 1% crude oil and exposure to the bacterial consortium resulted in no reduction or only a slight decrease in concentration of the oil components compared to the control after one week. However, after two weeks of treatment, a highly remarkable decrease in concentration of crude oil components compared to control was noticed, with complete disappearance of some of the components such as nC10 (Fig. 7).

Analysis of the oil extracts after one and two weeks revealed that the percentage of oil degradation increased with time. The addition of nitrogen, phosphorus or nitrogen + phosphorus to the Mineral Salt Medium Broth showed variation in the percentages of oil degradation. It can be noticed that, addition of nitrogen was the best treatment for oil degradation, followed by addition of both nitrogen and phosphorus, then phosphorus. Rosenberg et al. (1996) reported that nitrogen and phosphorus are limiting factors for oil biodegradation, and their availability to bacteria can affect their ability to consume oil products. Nitrogen and phosphorus act as fertilizers to increase the production of biodegrading bacteria, resulting in an increase of degradation rates. The microorganisms present in contaminated soil cannot necessarily be there in the numbers required for bioremediation of the site. Therefore, their growth and activity must be stimulated. Biostimulation usually involves the addition of nutrients and oxygen to help indigenous microorganisms. These nutrients are the basic building blocks of life and allow microbes to create the necessary enzymes to break down the contaminants.

4 Conclusion
The result of this study indicated that most of bacterial isolates had ability to degrade crude oil. The addition of nitrogen and phosphorus or both to the medium inoculated by consortium of the bacterial strain resulted in improved crude oil degradation as revealed by chromatographic separation of the crude oil fractions.

Author details
1 Commission for Biotechnology and Genetic Engineering, National Centre for Research, Khartoum, Sudan. 2 College of Agriculture, University of Khartoum, Khartoum, Sudan.

References


Figure 1 Crude oil not subjected to the consortium of the bacterial isolates in unfertilized Mineral Salt Medium (Control). X axis represents retention time (minutes) and Y axis represents response (mVolts).
Figure 2 Crude oil degradation after one week by a consortium of the bacterial isolates on Mineral Salt Medium containing 1.06g/L phosphorus (KH$_2$PO$_4$). X axis represents retention time (minutes) and Y axis represents response (mVolts).
Figure 3 Crude oil degradation after one week by a consortium of the bacterial isolates on Mineral Salt Medium containing 1.06g/L phosphorus (KH$_2$PO$_4$). X axis represents retention time (minutes) and Y axis represents response (mVolts).
Figure 4 Crude oil degradation after one week by a consortium of the bacterial isolates on Mineral Salt Medium containing 0.84 g/L nitrogen (NH$_4$NO$_3$). X axis represents retention time (minutes) and Y axis represents response (mVolts).
Figure 5 Crude oil degradation after two weeks by a consortium of the bacterial isolates on Mineral Salt Medium containing 0.84 g/L nitrogen (NH₄NO₃). X axis represents retention time (minutes) and Y axis represents response (mVolts).
Figure 6  Crude oil degradation after one week by a consortium of the bacterial isolates on Mineral Salt Medium containing 1.06g/L phosphorus (as KH$_2$PO$_4$) and 0.84 g nitrogen (as NH$_4$NO$_3$). X axis represents retention time (minutes) and Y axis represents response (mVolts).
Figure 7 Crude oil degradation after two weeks by a consortium of the bacterial isolates on Mineral Salt Medium containing 1.06 g/L phosphorus (as KH$_2$PO$_4$) and 0.84 g nitrogen (as NH$_4$NO$_3$). X axis represents retention time (minutes) and Y axis represents response (mVolts).

How to cite this article:

The “Journal of Nature and Natural Sciences (JNNSci)” is an international, peer-reviewed journal. Published articles are FREE to view, download and to print. The journal is a “Barkat Ali Firaq Trust for Education and Research (B.A.F.T.E.R)” publication. For more information, please visit www.bafter.org.

Your research contributions are invited at editor@jnnsci.com.