INTRODUCTION

In oil-producing countries, in areas of oil extraction and oil processing, crude oil is one of the main soil and water contaminants. The leakage of crude oil from pipelines and refineries damages the environment. Various bacteria and fungi are able to use crude oil fractions as a sole carbon source and change them to non-toxic compounds such as CO₂ (Leahy and Colwell 1990; Cerniglia 1992; Hamamura et al. 2006). The aliphatic and some aromatic fractions in crude oil are the most biodegradable components, and resins and asphalts are believed to be resistant to biodegradation (Atlas 1981; Leahy and Colwell 1990). Some reports have shown that aliphatic and aromatic fractions of crude oil are efficiently biodegraded by oil-degrading bacteria in soil (Minai-Tehrani and Herfatmanesh 2007). In aerobic conditions oxygen plays an important role in the biodegradation of oil and its components (Odu 1978).

Water contamination by oil is harmful to humans. It can damage drinking water and also penetrate surface and subsurface water and damage cultivation areas and plants. Li et al. (2005) showed that about 32% of chemical oxygen demand (COD) was reduced in biodegradation of organic compounds in oil-contaminated waste water by bacterial treatment after 4 days. Some factors such as nutrients play an important role in bacterial growth and biodegradation of oil-contaminated water (Atlas 1992). The bacterial population can also enhance the efficiency of biodegradation (Obahiagbon and Akhabue 2009). The saturated fraction of crude oil-contaminated water was the main biodegradable part and the asphaltene fraction was hardly biodegraded (Thouand et al. 1999).

In this report the effect of oil-degrading bacteria isolated from contaminated soil near a refinery in Tehran on bioremediation of aliphatic and aromatic fractions of crude oil in liquid medium was studied. This study can be useful to clean up oil-contaminated water and promote a healthier environment.

MATERIALS AND METHODS

Table 1 Characteristics of oil-contaminated water used for microcosm study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC mV</td>
<td>25.5</td>
</tr>
<tr>
<td>DO mg/L</td>
<td>6.2</td>
</tr>
<tr>
<td>Chloride ppm</td>
<td>280</td>
</tr>
<tr>
<td>Alkalinity ppm</td>
<td>215</td>
</tr>
<tr>
<td>Hardness ppm</td>
<td>510</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
</tr>
<tr>
<td>TPH %</td>
<td>2.3</td>
</tr>
<tr>
<td>Aromatics %</td>
<td>0.6</td>
</tr>
<tr>
<td>Aliphatics %</td>
<td>1.5</td>
</tr>
<tr>
<td>Asphaltine %</td>
<td>0.11</td>
</tr>
</tbody>
</table>

TPH = total petroleum hydrocarbons

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for acceptable growth of microorganisms, hence some inorganic nutrients such as 1 g KH₂PO₄, 1 g Na₂HPO₄, and 0.25 g NH₄NO₃ were added to 1 L of water and sterilized to induce a suitable medium for their growth.

All chemicals were of reagent grade and obtained from Merck Co.

**Isolation of bacterial consortium**

The oil-degrading bacteria were isolated from the soil near the site where water was sampled according to the following procedure. 1 g of oil-contaminated soil was added to mineral salt liquid medium containing 2.5 g/L KH₂PO₄, 2.5 g/L Na₂HPO₄, 1 g/L NH₄NO₃, 0.2 g/L MgSO₄ and 0.01 g/L CaCO₃; pH was adjusted to 7 and 2% (v/v) sterilized crude oil was added to this medium as the only carbon source. The medium was cultured for a week in a reciprocal shaker, 150 rpm at 30°C. After 1 week, 2 ml of cultured medium suspension was transferred to new medium under the same conditions. This transfer was repeated twice and the bacteria in the last transfer were used for inoculating water microcosm. To ensure the presence of oil-degrading bacteria, the growing bacteria in the last medium culture were also transferred to a plate with solid medium, containing agar-agar enriched with 0.5% hexadecane as the carbon source and incubated for 72 h at 30°C. The growing colonies were considered to be oil-degrading bacteria.

**Oil extraction and analysis**

About 2 ml of cell culture (with 0.25 McFarland turbidity or about 7.5 ×10⁸ CFU/ml) of the last liquid medium were transferred to contaminated water obtained from the contaminated site. The dispersion of oil was observed in the medium which suggested the ability of bacteria to degrade oil. The bacteria were cultured for 96 h in a reciprocal shaker, 150 rpm at 30°C. After 96 h, the medium was centrifuged (5000 × g for 30 min) to precipitate the cells and the supernatant was separated for oil analysis. The supernatant was mixed with an equal volume of chloroform and transferred to a separating funnel. The suspension was shaken firmly to dissolve the oil fraction of supernatant in the solvent phase. The solvent phase was separated and evaporated in a vacuum chamber for 24 h. The residue was weighed to determine the amount of total crude oil. The total aliphatic and aromatic fractions of oil were extracted and analyzed according to Minoui and Minai-Tehrani (2009). In this method the residue was dissolved in n-hexane and filtered. 5 ml of filtered solution was loaded to a 1 × 25 cm glass column filled with silica gel (20 cm) and sodium sulfate (5 cm as a moisture capturing material). The column was pre-washed with n-hexane and 30 ml of n-hexane was used as the mobile phase to release the aliphatic fraction. The fraction was collected and the solvent was evaporated. The residue was weighed to determine the amount of total aliphatic fractions. The residue was dissolved in 150 μl n-hexane, and 1 μl was injected into a gas chromatograph (Hewlett-Packard GC 5890) column equipped with an FID detector and a fused silica capillary column. The carrier gas was H₂ with a flow rate of 60 cm/sec and the injection temperature was 300°C and that of the detector 330°C. The total peak areas of the hydrocarbons were taken as a quantitative amount of their concentrations and compared with time zero (T₀).

To release aromatic fractions from the column, 30 ml of n-hexane/dichloromethane (1:1, v/v) was used as the mobile phase. The aromatic fractions were collected and the solvent was evaporated. The residue was weighed to determine the amount of total aromatic fractions. The residue was dissolved in 5 ml acetonitrile and 20 μl was injected into a HPLC (Shimadzu LC 10A) column equipped with a UV detector at 234 nm with water/acetonitrile (1:2, v/v) as the mobile phase and a flow rate of 1 ml/min. Some polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene, anthracene, fluoranthene, pyrene and chrysene were prepared as standards and injected into the HPLC column. Their peaks were used to localize them in the main chromatogram.

The total peak area of each compound in the chromatogram was used to determine the reduction of PAHs and compare them with T₀. The above procedures were also done for the extraction of total aliphatic, aromatic and crude oil in the control sample containing the oil-contaminated water with inorganic nutrients but no bacteria. The control sample was also incubated in a reciprocal shaker for 96 h at 30°C. Each sample was prepared in triplicate.

**Statistical analysis**

Results were expressed as mean ± standard deviation (±SD) and the analysis of variance and statistical significant difference (P < 0.05) was performed by one-way ANOVA with mean separation using Tukey’s test. The statistical results were analyzed by GraphPad Prism 5 program.

**RESULTS**

**Oil reduction**

The reduction of total aliphatic, aromatic and crude oil after 96 h of incubation and its comparison with the control are shown in Fig. 1. For all cases, the difference between the treated samples (with bacteria) and the control (without bacteria) was significant. The total aliphatic fractions were decreased with a higher efficiency than the total aromatic fractions. The reduction of total crude oil was 30% in treated samples but only 7% in the control. The reduction of total aliphatic and aromatic fractions in the treated samples was 60 and 35%, respectively, in comparison with the control (20 and 10% reduction, respectively).

**Aliphatic reduction**

The GC analyses of aliphatic fractions are shown in Fig. 2. Fig. 2A shows the aliphatic fractions at T₀. In the control sample the aliphatic fractions decreased after 96 h of incubation in comparison with T₀ (Fig. 2B) while the aliphatic reduction was higher in the treated sample after 96 h (Fig. 2C). The aliphatic fractions were efficiently decreased in the treated sample in comparison with the control. Most aliphatic reduction occurred between C₁₄ and C₂₂. The highest aliphatic reduction was observed in C₁₇ (70%) and C₁₆ (67%), while in the control, the reduction was 40 and 30%, respectively.

The reduction in the ratios of normal C₁₇ and C₁₈ alkanes to their branched isomers, pristane and phytane, showed that these ratios were significantly reduced in the treated sample while their reduction in the control sample was not significant after 96 h of incubation (Fig. 3).
Aromatic reduction

HPLC analyses were done to determine the reduction of aromatic fractions in both control and treated samples. The reduction of some PAHs such as phenanthrene, anthracene, fluoranthene, pyrene and chrysene was analyzed. Fig. 4A shows the amount of aromatic fractions at T0. The comparison of the peaks in the control (Fig. 4B) and treated sample (Fig. 4C) after 96 h of incubation showed that the reduction of PAHs in the treated sample was higher than in the control. The highest reduction was observed for fluoranthene (43%) and phenanthrene (35%), while the reduction in the control was 23 and 7%, respectively (Fig. 5).

DISCUSSION

One of the most important risks of water contamination is oil leakage, which affects drinking or cultivation water. It can affect the health of humans, plants or other organisms. The bioremediation of oil-contaminated water has always been an important issue. Some bacteria have a high potential for biodegradation of crude oil (Belhaj et al. 1992; Marge-sin et al. 2003; Johnson and Hyman 2006). This report focuses on bioremediation of aliphatic and aromatic fractions of crude oil by oil-degrading bacteria. Our results show that the bacterial consortium isolated from soil had a high potential to biodegrade crude oil (30% of total petroleum hydrocarbons (TPH) was biodegraded in 4 days). Some reports indicated that the reduction of different crude oils in seawater was between 26-50% after 42 days of treatment (Atlas 1975) and a microbial consortium has reduced total hydrocarbon content of crude oil-contaminated water for about 99% after 9 weeks treatment (Ibhadode et al. 2009). Aromatic and aliphatic fractions of crude oil are the most biodegradable parts of the oil, and microorganisms prefer to use the aliphatic fractions as the primary carbon source. Biodegradation of diesel oil in liquid culture, which is mainly composed of aliphatic fractions, was about 90% after 13 days of incubation (Márquez-Rocha et al. 2001).

Biodegradation and volatilization are two main factors for reduction of aliphatic fractions in soil and water (Nicodem et al. 1997). The reduction ratios of normal C17 and C18 alkanes to their branched isomers, pristane and phytane, are usually used as indices of biodegradation and for monitoring the biological effect of microorganisms on the aliphatic fractions of oil (Atlas 1981; Seklemova et al. 2001). In the present work, the reduction of these ratios was highest in the treated sample, more than the control, suggesting that biodegradation plays the main role in treated samples in reducing the aliphatic fractions, while in the control sample, volatilization is primarily responsible for the reduction of aliphatic fractions. Most oil-degrading bacteria such as
Pseudomonas aeruginosa strains preferentially degrade middle to moderately long-chain alkanes (Ko and Lebeault 1999; Abalos et al. 2004).

Among the aromatic fractions biodegraded by the bacterial consortium, phenanthrene and anthracene were used more efficiently than fluoranthene, pyrene and chrysene. Fig. 5 shows that, compared with phenanthrene and anthracene, the reduction of fluoranthene, pyrene and chrysene was high in the control sample. This suggests that these PAHs were decreased in the medium by both volatilization and biodegradation, while in phenanthrene and anthracene, biodegradation was the main factor for their reduction. In other words, these two PAHs were biodegraded more efficiently than the others PAHs. This suggestion does not imply that the rate of volatilization of fluoranthene, pyrene and chrysene was higher than that of phenanthrene and anthracene, but rather, in our short experiment, the presence of aliphatic fractions and simple PAHs (like phenanthrene and anthracene) in the culture medium forced the bacteria to use these materials, and probably there was insufficient time to use the heaviest aliphatics and more complex PAHs. Previous reports have also indicated that low molecular weight PAHs with less than three rings disappeared rapidly from the contaminated water, while the PAHs with more than four rings remained for longer (Lee et al. 1978; Yamada et al. 2003).

In conclusion, our results showed that in the soil near the Tehran oil refinery there are potent oil-degrading bacteria which can biodegrade the oil from contaminated water with high efficiency in a short period of time. The addition of some inorganic nutrients to the contaminated water can produce a suitable medium for biodegradation (some of
these inorganic nutrients may penetrate the water from inorganic manure used in the cultivation lands. Nutrients play an important role in bacterial growth and oil bioremediation (Rodríguez-Blanco et al. 2010). Our findings also showed that the aliphatic fractions of crude oil were used up with a higher rate than the aromatic fractions and therefore, biodegradation can be used as an efficient and inexpensive method for removal of oil from the water around the oil refinery zone.

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