ISSN : 0974 - 7435 Volume 7 Issue 4

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 7(4), 2013 [131-136]

Bioremediation of crude oil contaminated soil by *Pseudomonas aeruginosa* strain-O2 and *Micrococcus varians* strain-X with different approaches

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ABSTRACT

The efficiency of two bacterial candidates namely; *Pseudomonas aeruginosa* strain-O2 and *Micrococcus varians* strain-X, in degradation of crude oil extracted from contaminated sediment samples was evaluated. GC-analysis of the degradation products indicated that both strains were able to use crude oil as carbon and energy source. Aliphatic hydrocarbon fraction of the sediment extract, especially long chain alkanes (n-C_{22}+), was degraded. For aromatic hydrocarbon fraction, namely methyl naphthalene compound, both strains were able to degrade 2,3,6- and 1,2,5-trimethyl naphthalenes to a comparable extent. Only *P. aeruginosa* strain-O2 was able to utilize 1,3,7- trimethyl naphthalene, while *M. varians* strain-X was able to use 1,2,4- trimethyl naphthalene as a sole C-source. Application of different strategies in bioremediation of sea sand contaminated with 10% crude oil indicated that the bioaugmentation with mixed culture of both strains was the optimal treatment strategy (approximately 93.45%). While, treatment applying either one of the two strains indicated that *M. varians* strain-X is more efficient in crude oil degradation. Comparable to that of natural conditions, biostimulation resulted in limited crude oil removal (approximately 36.42%). Results collectively indicated that the bacterial strains used in this study were suitable candidates for practical field application and in *situ* bioremediation, namely by bioaugmentation.

KEYWORDS

Bioremediation;
Biostimulation;
Bioaugmentation;
Pseudomonas sp.;
Micrococcus sp.;
Polluted sediments.

INTRODUCTION

As a result of twentieth-century industrialization, many harmful substances have been discharged into terrestrial and aquatic environments. The most widely distributed environmental pollution can be attributed to the spill of crude oil and various oil residues[1,2]. With increasing public attention regarding the preservation of the environment, the development of oil clean-up technologies has gained considerable interest. In recent years, a number of cost-effective techniques for remediation of oil-contaminated soil have been proposed[3,4]. Although physical methods of oil removal may cause more damage to soil than spilled oil itself, biological methods, such as bioremediation, may be more effective in removing oil without undue damage to the

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environment.

Generally, degradation of crude oil by mixed microbial populations is the ultimate fate of oil spills in nature[5-8]. Therefore, microbial clean-up can be advantageous, when compared to other remediation techniques[9-11]. Recently, bioremediation has proved to be a useful tool in removing oil[12,13] and growing interest in the use of several Pseudomonads and members of genus *Micrococcus* in degradation of crude oil have been reported[14-18].

Variety of bioremediation techniques have been developed to support and increase the degradation activities of native microbial populations (natural attenuation), thus allow for reduction in time and subsequent save in costs. Two main approaches are mostly commonly used in bioremediation technology namely; (a) environmental biostimulation involving the addition of mainly oxygen and/or mineral nutrients (usually combination of nitrogen, phosphorus and trace metals) and (b) bioaugmentation through the direct application of selected degrader microorganisms to the site[19-21]. In order to accelerate the natural biodegradation of affected sites, the density of hydrocarbon utilizers can be increased[1]. Bioemulsifiers was added to oil polluted soil for enhancement of oil availability[22].

The main objective of this study was to focus on the use of two potent crude oil degrading bacterial candidates for bioremediation of crude oil extracted from polluted sea sediment, obtained from the western harbor of Alexandria, Egypt. Special emphasis was given to the chemical analyses of its aliphatic and aromatic hydrocarbon fractions. Application of different bioremediation strategies of sea sand soil spiked with 10% (w/w) of crude oil namely; biostimulation, bioaugmentation in comparison to natural and weathering conditions was also conducted.

MATERIALS & METHODS

Wet sediments were collected from western harbor of Alexandria, Egypt as grab samples and were transported to the laboratory in coolers with ice. Upon receipt in the laboratory, sediments (wet) were sieved through a 2.0-mm sieve to remove debris. Samples were stored in glass jars at -20°C in deep Freezer. Crude oil was extracted from the sediment polluted sample by ultrasonic extraction in dichloromethane for several times. Typically 5 g of sediment sample was dried in oven at 40°C overnight. The sample was transferred to an ultrasonic device and extracted several times by 40 ml dichloromethane HPLC grade each time; the solvent was subsequently moved to a rotary evaporator operating at 39°C to concentrate the extracted oil to 2 mL volume.

Wet sea sand was collected from Eastern harbor of Alexandria, Egypt, as grab samples and transported to the laboratory under cold conditions. Upon receipt in the laboratory, sediments were dried and sieved through a 2.0-mm sieve to remove debris.

The two bacterial candidates used in this study, *Pseudomonas aeruginosa* strain-O2 and *Micrococcus varians* strain-X were isolated and identified as previously described[23]. Bacterial strains were grown on minimal salts medium (MSM), it was the modified medium of Ijah[24] with the following composition, (g L^{-1}): yeast extract, 0.5; NaCl, 0.5; $(NH_4)_2S0_4$, 2; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 5; KH_2PO_4, 2 and trace elements (with the following composition, (g l^{-1}): $FeSO_4$, 5; H_3BO_{-4}, 0.025; $CuSO_4.5H_2O$, 0.005; KI, 0.005; $CoSO_4$, 0.3; $MnSO_4.4H_2O$, 3; $ZnSO_4.7H_2O$, 5; $NaMoO_4$, 0.012, and distilled water up to 1 liter), 0.1 mL.

The amount of individual hydrocarbons of the oil samples was quantified by gas chromatography (GC)-FID-measurements. The sediment oil (0.5 µL) was injected into the GC (6890 Series, Agilent technology, USA), equipped with a programmable temperature vaporization inlet (PTV, Agilent Technology, USA) with a septumless head, working in split/splitless mode. The injector was held at a split ratio 1:50 and an initial temperature of 40°C. With injection, the injector was heated to 300°C at a programmed rate of 700°C min^{-1} and held at this temperature for the rest of the analysis time. Helium was set at a flow rate of 2 ml min^{-1}. Petroleum components were separated on a HP Ultra 1 fused silica capillary column (50 m× 0.32 mm i.d. × 0.52 µm film thickness, Agilent Technology, USA). The temperature of the GC oven was initially held at a rate of 40°C for 2 min, followed by a 3°C min^{-1} ramp to 140°C, then at a rate of 5°C min-*1* to 300 °C and held there for a further 25 min[25].

For simulation edxperiments, 200 g of sea sand

placed in a sterile plastic cell (20cm*20cm*7cm) were spiked with 10% crude oil to simulate crude oil contamination in a sandy area. The composition of the sieved sand was gravel (2%), sand (88%), and silt and clay (10%). The water holding capacity and the initial water content of the sand were 18.5% and 0.8% respectively, but the water content was adjusted to 60% of the water holding capacity, for the treatability study. The treatments evaluated were; (a) Biostimulation by addition of nutrient salts Nitrogen 0.2% (w/w), Potassium 0.5% (w/w) and Phosphorus 0.2% (w/w); (b) Bioaugmentation by specialized strain (either *Pseudomonas aeruginosa* strain-O2., *Micrococcus varians* strain-X.) or mixed culture of both candidates. Microorganisms were grown in nutrient broth medium for 24 hours prior to inoculation and 1.5% preculture was used as inoculum and aseptically added to the treatment cells; (c) Cell exposed to natural conditions; (d) Control cell contained sterilized sea sand spiked with crude oil, to determine the abiotic losses occur during the experiment. All prescribed treatments (TABLE 1) were incubated at 30°C and were conducted in triplicates and a mean value was taken.

Cold extraction was used for the extraction of crude oil from sea sand samples after 3 and 6 weeks for each of the treatment options. Sand samples were dried at the ambient temperature (ca. 25-30°C) to constant weight. Fifty grams of the sand placed in labeled, sterilized and chemically cleaned flasks. 100 mL of toluene was added to sand material, shaken and the residual crude oil was extracted using a separator funnel. The extraction procedure was repeated several times and each extract was filtered through cotton wool. The extracts were pooled together for further processing[26].

Crude oil degradation was measured colorimetrically and the method described by Udeme and Antai[27] was adopted. A standard curve of absorbance versus known concentrations of the spilled oil was drawn. Toluene (Aldrich chemicals) was used as the solvent for the crude oil and absorbance was read using a Corning colorimeter (model 253) set at 520 nm wavelength. The hydrocarbon concentration at week 3 and week 6 and the residual hydrocarbon concentrations in various treatment conditions were calculated from the standard curve after multiplying by the appropriate dilution factor[26].

TABLE 1 : Detailed description of different bioremediation strategies conducted.

Treatment	Nature of Treatment	Description of treatment
A	Bioaugmentation by *Pseudomonas* sp.	200g sea sand+90ml MSM+5ml crude oil+5% (v/v) inoculum.
B	Bioaugmentation by *Micrococcus* sp.	200g sea sand+90ml MSM+5ml crude oil+5% inoculum.
C	Bioaugmentation by mixed cultures.	200g sea sand+90ml MSM+5ml crude oil+5% inoculum.
D	Biostimulation	200g sea sand+90ml MSM+5ml crude oil+ 0.6g Peptone +1.5g K_2HPO_4 +0.6g KH_2PO_4.
E	Natural conditions	200g sea sand+90ml MSM+5ml crude oil.
F	Control	200g sterilized sea sand+90ml MSM+5ml crude oil.

RESULTS & DISCUSSION

In a trail to evaluate the efficiency of two oil-degrading bacterial isolates namely; *Pseudomonas aeruginosa* strain-O2 and *Micrococcus varians* strain-X, in degradation of crude oil extracted from polluted sediment samples, each bacterial strain was cultivated in MSM supplemented with extracted oil as a sole c-source. Chromatograms illustrated in Figure 1(left), indicated that the bacterial strains were able to use crude oil as carbon and energy source and were able to degrade aliphatic hydrocarbon fraction of the sediment extract to a great extent, especially long chain alkanes (n-C_{22}+). On the other hand, chromatograms illustrating the composition of the sediment extract aromatic hydrocarbon fraction of the control, *Pseudomonas aeruginosa* strain-O2 and *Micrococcus varians* strain-X samples are shown in Figure 1(right). Owing to the complexity of the chromatograms, concentrations were determined only for methyl naphthalene compound group (ion 170). The biodegradation potential of the two strains may be lower than that found in crude oil when comparing the concentrations of detected compounds. While, other compounds may be biodegraded but their peaks couldn't be resolved. Concerning the compounds of interest, both strains were able to degrade 2,3,6- and 1,2,5- timethyl naphthalenes to a com-

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parable extent, this may depend on the position of substitution on the compound. The capability of various Pseudomonads and members of *Micrococcus* sp. to degrade naphthalenes and other aromatic hydrocarbons was reported by several working groups[15,28-31]. However, both candidates lack the potential capability of degrading 1,3,6-, 1,4,6+1,3,5-, 1,2,7+1,6,7+1,2,6-timethyl naphthalenes, the results that could be explained by the dependence of the rate of biodegradation on the positions of alkyl substitution, that was found to be very much reduced with increasing number of methyl substitution[32,33]. On the other hand, this conclusion may conflict with that found by Fischer *et al.*[34] whom suggested that polymethylnaphthalenes with a 1,6-dimethyl substitution pattern are more susceptible to biodegradation than those isomers that lack this feature. Interestingly, *Pseudomonas aeruginosa* strain-O2 was able to degrade 1,3,7- trimethyl naphthalene while, *Micrococcus varians* strain-X was able to degrade 1,2,4-Trimethyl naphthalene. This led to suggestion that *Micrococcus varians* strain-X may have suitable enzyme system capable of metabolizing naphthalene and other aromatic compounds containing adjacent methyl substitution[15,29,30].

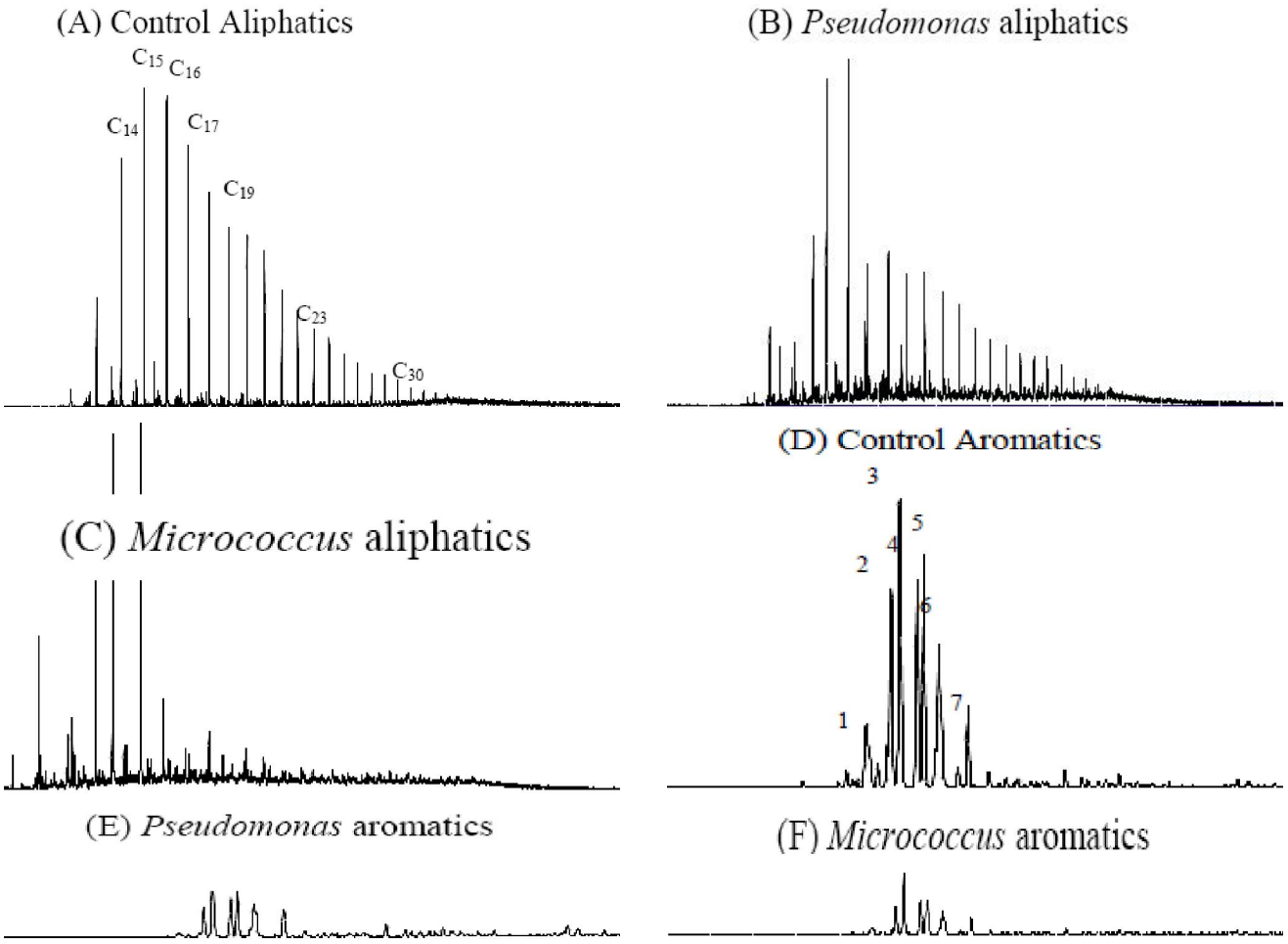

Figure 1 : GCprofiles of sediment extract (aliphatic and aromatic fractions) after 4 days incubation at 30°C: (A,D) uninoculated controls, (B,E) *Pseudomonas aeruginosa* strain-O2. (C,F) *Micrococcus varians* strain-X. Peak identifications: (1) 1,3,7-trimethylnaphthalene; (2) 1,3,6-trimethylnaphthalene; (3) 1,4,6+1,3,5-trimethylnaphthalene; (4) 2,3,6-trimethylnaphthalene; (5) 1,2,7+1,6,7+1,2,6-rimethylnaphthalene; (6) 1,2,4-trimethylnaphthalene; (7) 1,2,5-trimethylnaphthalene.

Owing to the degradation potential of the two bacterial strains, their use in bioremediation of sea sand contaminated with 10% crude oil applying different strategies was conducted according to plan described in TABLE 1. At the end of 3 and 6 weeks of incubation in each treatment option, the concentration of crude oil remained was determined and the per cent was calculated. Results shown in Figure 2 indicated that

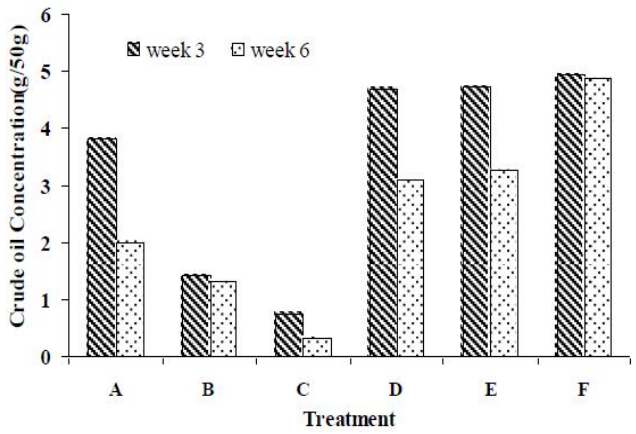

Figure 2 : Concentration of crude oil remaining in each treatment cell (g/50g sea sand) after 3 and 6 weeks of incubation during different bioremediation strategies. A) Bioaugmentation by *Pseudomonas* sp.; B) Bioaugmentation by *Micrococcus* sp.; C) Bioaugmentation by mixed cultures; D) Biostimulation; E) Natural conditions; F) Control.

bioaugmentation with mixed culture of both strains was the optimal treatment strategy recording approximately 93.45% reduction in crude oil in comparison to the control conditions due to degradation after 6 weeks incubation period. Moreover, it showed 1.59 - and 1.29-fold increase in crude oil degradation as compared to the amounts resulted from bioaugmentation experiments using either *Pseudomonas aeruginosa* strain-O2 or *Micrococcus varians* strain-X, respectively. These results are in concordance with that reported by other authors[21,26]. Also, treatment strategy applying either one of the two strains indicated that *Micrococcus varians* strain-X is more efficient in crude oil reduction than *Pseudomonas aeruginosa* strain-O2. Interestingly, more efficient degradation was recorded by the use of mixed culture; support the possibility that both strains have a complementary role in biodegradation of crude oil as reported in[23]. On the other hand, oil spills result in an imbalance in the carbon–nitrogen ratio at the spill site, because crude oil is essentially a mixture of carbon and hydrogen. This causes a nitrogen deficiency in an oil-soaked soil, which retards the growth of bacteria and the utilization of carbon source (s). In addition, certain nutrients like phosphorus may be growth-rate limiting. Therefore, addition of nutrients during biostimulation may provide suitable solution to nutrient limitations problems. In this work, treatment strategy resulted in limited crude oil removal (approxi. 36.42 %); this may be due to limited number of indigenous microbial community in sandy soil. For control sample, there was a little reduction in oil concentration after 6 weeks; this may be due to evaporation or disintegration of oil in the sand cells.

CONCLUSION

Our study indicated that strains of *Pseudomonas aeruginosa* strain-O2 and *Micrococcus varians* strain-X, isolated from oil polluted sediment, are capable of degrading crude oil extracted from soil sediment and to use it as a source of carbon and energy. Specific compound loss, due to bacterial degradation, was observed for both the saturated aliphatic hydrocarbon fraction and aromatic fraction. Therefore, it may be suggested that the bacteria used in this study are suitable candidates for practical field approaches for effective *in situ* bioremediation of polluted sites by a wide range of hydrocarbon pollutants. Bioaugmentation of polluted sites with microbes adapted to biodegradation of oil hydrocarbons could be an outstanding strategy for remediation of those sites, since in almost all cases; the indigenous bacteria lack the enzymatic capabilities for breakdown of oil heavy and toxic components. Biostimulation by addition of limiting nutrients was proved to be an effective tool in bioremediation of polluted sites in many previous studies, even though, it wasn't proved to be effective in this study. The transfer of laboratory experiments to large scale pilot studies is expected to be the next step in this study to provide an efficient strategy for bioremediation of crude oil in the field of environmental biotechnology.

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