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Research article

Biostimulation of indigenous microorganisms for bioremediation of oily hypersaline microcosms from the Arabian Gulf Kuwaiti coasts

Dina M. Al-Mailem, Maha Al-Deieg, Mohamed Eliyas, Samir S. Radwan*

Microbiology Program, Department of Biological Sciences, Faculty of Science, Kuwait University, Kuwait

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ABSTRACT

Hypersaline soil and water samples were collected in summer and winter from the "sabkha" area at the Kuwaiti shore of the Arabian Gulf. Physicochemical parameters were analyzed, and found suitable for microbial oil-removal. Summer- and winter-microcosms were treated with individual cation (K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}) salts, and with animal blood and commercial yeast, as cost-effective vitamin sources. Those microcosms were exposed to the open environment for six winter and six summer months, and analyzed for their hydrocarbonoclastic microorganisms at time zero and in two month intervals. The hydrocarbonoclastic microbial communities in the microcosms consisted of halophilic bacteria and haloarchaea. The constituent bacterial species varied according to the season. Three species, *Dietzia kunjamensis*, *Marinobacter lacisalsi* and *Halomonas oxialensis* consistently occurred both in summer- and winter-samples, but the remaining species were different. On the other hand, the haloarchaeal communities in summer and winter were quite similar, and consisted mainly of *Haloferax* spp and *Halobacterium* spp. Treating the microcosms with cations and with vitamin-containing natural products enhanced microbial numbers and oil-removal. The effectiveness of the cations in oil-removal was in the order; Fe^{3+} (94%) > Ca^{2+} (89%) > Mg^{2+} (85%) > K^+ (82%). Thus, oily microcosms amended with trivalent and divalent cations lost most of the oil, and those amended with commercial yeast and with animal blood, as vitamin sources, lost 78% and 72% oil, respectively.

1. Introduction

Unlike studies on oil-bioremediation for nonextreme environments (Radwan, 2009), those for extreme environments are still rather few (Margesin and Schinner, 2001; Le Borgne et al., 2008; Martins and Peixoto, 2012; Jurelevicius et al., 2013; Fathepure, 2014; Ali et al., 2016a,b; Xiao et al., 2016; Edbeib et al., 2016; Corsellis et al., 2016). Hypersaline areas, namely those with salinities above that of seawater (Grant et al., 1998), are spread globally, and represent extreme environments, that become occasionally polluted with waste hydrocarbons (Lefebvre and Moletta, 2006; Dastgheib et al., 2012). Such areas may be bioremediated via the activities of halophilic/halotolerant hydrocarbonoclastic microorganisms (Oren, 2002). During the past few decades, interesting studies have been published on halophilic microorganisms with hydrocarbonoclastic potential from hypersaline environments (Oren et al., 1992; Emerson et al., 1994; Margesin and Schinner, 2001; Garcia et al., 2004; Nicholson and Fathepure, 2005; Zhao et al., 2009; Al-Mailem et al., 2010; Bonfa et al., 2011). Those microorganisms comprised, bacteria (e.g. *Marinobacter sedimentalis, Halomonas salina*, *Pseudomonas* sp.), archaea (e.g. *Halobacterium salinarum*, *Haloferax larsenii)* and fungi, which biodegraded oil and pure aliphatic and aromatic hydrocarbons at high salinities. Therefore, they may be useful in oil bioremediation for saline environments (Oren, 2002). Two bioremediation strategies are known (Bartha, 1986). The first involves "bioaugmentation" (i.e. inoculation) of the oily environment with exogenous, hydrocarbonoclastic microorganisms, and the second involves the "biostimulation" of hydrocarbonoclastic microorganisms already inhabiting the oily site. The latter approach can be achieved via fertilization with nutrients, e.g. N and P, addition of surfactants and by optimizing physical conditions, e.g. pH, aeration and others. Biostimulation, which depends on indigenous microorganisms well adapted to the sites' environmental conditions, is preferred by many authors over bioaugmentation (Radwan, 1991; Radwan et al., 1995; Nikolopoulou et al., 2013). More information about researchers'

* Corresponding author. Microbiology Program, Department of Biological Sciences, Faculty of Science, Kuwait University, PO Box 5969, Safat, 13060, Kuwait.
E-mail address: samir.radwan@ku.edu.kw (S.S. Radwan).

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experiences on biostimulation and bioaugmentation strategies is available in our earlier publications (e.g. Ali et al., 2016a,b).

Hypersaline environments in Kuwait are coastal areas locally called the supertidal "sabkhas". In earlier studies, our group recorded in those sabkhas, extremely halophilic bacteria and archaea, capable of hydrocarbon utilization at high salinities (Al-Mailem et al., 2010, 2012, 2014a). Growth and hydrocarbon consumption by such microorganisms were enhanced by specific amendments such is K- and Mg- salts as osmoregulators (Al-Mailem et al., 2013), and by pure vitamins (Al-Mailem et al., 2014b).

The major objective of this work was to elaborate on our earlier laboratory studies. Therefore, we considered the amendment-based (biostimulation) bioremediation of oily, hypersaline microcosms that were exposed to the open environment during the bioremediation process. We confirmed our earlier laboratory results and in addition, report on new cations and vitamin-rich, natural products (instead of the costly, pure vitamins) with more pronounced biostimulatory effects. Achieving microcosm biostimulation under open conditions is obviously an important transitional step between the *in vitro* study and the targeted field-bioremediation. In this context, Kuwait, is a small oil-producing country, that belongs to the semiarid region with harsh weather. In the dry summer, the maximum temperature frequently exceeds 50 °C and in the mild, rainy winter, the night temperature frequently drops to zero °C. Due to legal (e.g. oil production and transport) and illegal (e.g. deliberate disposal of oily wastes by vehicles) activities, the Arabian Gulf water body and shores are frequently polluted with hydrocarbons.

2. Materials and methods

2.1. Environmental samples

Hypersaline soil and water samples were collected from the sabkha area at the northern coast of Kuwait in sterile glass containers. Winter samples were collected in the first of October 2013, and summer samples in the first of April 2014. The samples were transported to the laboratory in an ice box, and started to be processed in the same day. Physicochemical parameters viz pH, temperature and dissolved oxygen contents were measured in the field using a water quality checker (WQC-24, Japan). Other chemical parameters were determined in the laboratory using an LECO-CHNS elemental analyzer (LECO-CHNS-932, St. Joseph, Michigan, USA). The mineral salts NO_2^-, NO_3^-, PO_4^{3-} and $Si(OH)_4$ were determined adopting standard colorimetric methods (Grasshoff et al., 1983) by flow injection analysis (FIA) using a LACHAT Instrument QuickChem 8000 autoanalyzer (Hales et al., 2004).

2.2. Experimental set-up

Soil heaps, 1 Kg, were dispensed in open glass dishes, and 1 L aliquots of the hypersaline water were distributed in open 5 L wide-mouth conical flasks. Those microcosms were treated as described below, and kept for six months in a protected area at the Botanical Garden, Kuwait University, exposed to the open condition, under which field bioremediation occurs. Winter samples were incubated from October to March, and summer samples from April to September. Soil samples were irrigated weekly with 150 mL aliquots of sterile tap water to keep them wet, especially during the hot, dry summer. The water microcosms were completed weekly to the volumes at time zero using sterile tap water. Hypersaline water was not used in order to avoid additional increases in salinity following excessive water evaporation.

2.3. Amendments for biostimulation

Two sets of microcosms were prepared, each comprising winter and summer samples. One set remained "pristine" (so designated through the text) as controls. The other set (designated oily) was artificially polluted with 1.5%, w/v light crude oil (from the Kuwaiti Oil Company). The oily soil was thoroughly mixed at time zero and in two month intervals, i.e. just before taking samples for analysis. The oily water microcosms were vigorously shaken. As expected, the oil remained immiscible at time zero. About two weeks later and through the experiment, the oil became miscible probably via extracellularly released biosurfactants.

For each microcosm sample, two groups of specific amendments were tested as biostimulators, one comprising cation salts as osmoregulators and the other group consisted of cost-effective natural products known to be rich in vitamins. As cations, we used separately K^+ as KCl (0.5 M), Mg^{2+} as $MgSO_4$ (1.5 M), Ca^{2+} as $CaCl_2$ (1.0 M) and Fe^{3+} as $Fe_2(SO_4)_3$ (0.25 M). As vitamin sources, animal blood (2%, wt/wt) and commercial yeast (2%, wt/wt) were used.

2.4. Analysis of hydrocarbonoclastic microorganisms

At time zero and in two month intervals, three replicate one-gram-portions were taken from each microcosm for microbial analysis. Hydrocarbonoclastic microorganisms were counted by the dilution-plating method, using mineral media with oil vapor as a sole source of carbon and energy. For analysis of bacteria, we used the mineral medium described by Sorkhoh et al. (1990), and for archaea, we used the medium suggested by Mevarech and Werczberger (1985). The NaCl concentrations in those media were adjusted to 1.5 and 2.0 M, respectively. Oil vapor volatilizing from plate-lid-fixed filter papers impregnated with 2 mL of crude oil served as the sole sources of carbon and energy. Triplicate plates were inoculated with 0.25 mL aliquots of common sample dilutions. The plates were sealed and incubated at 30 °C for bacteria, and 35 °C for archaea, for 12 d. The colony forming units (CFU) were counted, and the numbers per g microcosm were calculated. Judged by the morphological colony characteristics and staining and microscopic cell properties, representative colonies were isolated and purified following established procedures. The representative strains were characterized by comparing their 16S rRNA-gene sequences with those of standard strains in the GenBank database. The method was essentially as follows. To extract the total genomic DNA from individual microorganisms, a loopful of 48-h culture was homogenized in 200 μL of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, USA). The mixture was incubated in a water bath at 100 °C for 10 min, then cooled for 2 min and finally centrifuged at 14,000 × *g* for 3 min to collect the DNA-containing supernatant. The 16S rRNA-genes in the total genomic DNA were amplified by polymerase chain reaction (PCR). For bacteria, the mixture contained puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, UK), 1 μL (25 ng) of DNA template and 1 μL each of the universal primer combinations GM5F (5′-CCTACGGGAGGCAG CAG-3′) and 907R (5′-CCGTCAATTCM TTTGAGTTT-3′) (Santegoeds et al., 1998). For haloarchaeal isolates, 16S rRNA-genes were amplified using the archaeal primer combinations 0018F (5′-ATTCCGG TTGAGCC TGCC) and 1518R (5′-AGGAGGTGAGC CAGCCGC) (Cui et al., 2009). The reaction volume was completed to 25 μL with molecular water (Sigma, UK). Amplification was done in the Veriti Thermal Cycler (Applied Biosystems, USA) by a touch-down PCR in which the initial denaturation was at 95 °C for 5 min, and the annealing temperature started at 65 °C and decreased by 1 °C every cycle to 55 °C, at which additional 15 cycles were done. Denaturation was at 94 °C for 1 min, and primer extension at 72 °C for