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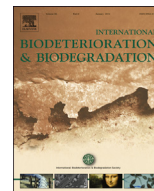

Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the Sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm experiments

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A B S T R A C T

Two oil-polluted sediments (PD and KH) were sampled from a coastal region in Oman for the isolation of hydrocarbon-degrading bacteria and for testing different bioremediation approaches. Fourty strains were isolated, eighteen were affiliated to *Marinobacter* whereas the rest belonged to *Pseudomonas*, *Halomonas*, *Hahella* and *Alcanivorax*. All strains grew well at 2–7% salinity and between 20 and 60 °C. The strains exhibited a better growth on long chain than on short chain alkanes. Biostimulation and bioaugmentation were compared in both sediments and oil biodegradation was followed by measuring CO_2 evolution and by gas chromatography (GC). The evolved CO_2 reached 0.45 ± 0.02 and 2.23 ± 0.07 mg CO_2 g^{-1} sediment after 88 days in the untreated PD and KH sediments, respectively. While the addition of inorganic nutrients resulted in 1.2–3.7 fold increase in CO_2 evolution in both sediments, the addition of the bacterial consortium was only effective in the PD sediment. The maximum CO_2 evolution was measured when both nutrients and bacteria were added and this corresponded to a total oil mineralization of 2.6 ± 0.12 and 1.49 ± 0.04% of the initial oil after 88 days in the PD and KH sediments, respectively. GC analysis confirmed the CO_2 data and showed that most of the degraded compounds belonged to alkanes. We conclude that the Omani polluted sediments contain halotolerant and thermotolerant bacteria and biostimulation is more efficient than bioaugmentation for their cleanup.
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1. Introduction

Oil production is the backbone of the economy in the Sultanate of Oman, and more than 60% of the oil transported from the Gulf region passes through its waters (Petrov, 2008). Enormous efforts are exerted to prevent oil contamination, however, small and large scale discrete spills can never be completely excluded. Contamination can occur by tanker accidents or tankers dumping ballast water along the coasts as well as during oil exploration and production. Oil spills, regardless of their cause, pose a serious threat for terrestrial and marine ecosystems and urgent remediation methods are always needed (Swannell et al., 1996).

Oil spill incidents have prompted the development of different physical, chemical, as well as biological techniques for dealing with oil pollution (Swannell et al., 1996; Smith, 1996). Bioremediation has been widely applied as a successful and cost-effective technique to treat different oil spills around the globe (Swannell et al., 1996; Vogel, 1996; Lee and Merlin, 1999; Timmis and Pieper, 1999). Two main bioremediation approaches are known; biostimulation and bioaugmentation. Biostimulation involves the addition of inorganic (mainly nitrogen and phosphorus) and organic fertilizers and was found to stimulate the growth of indigenous oil degraders and to increase biodegradation rates (Ward and Brock, 1978; Rivet et al., 1993; Bragg et al., 1994; Lee and Merlin, 1999). Bioaugmentation involves the addition of oil-degrading bacteria to supplement the existing microbial communities. This approach showed considerable success in bioremediating several polluted sites, although in some cases, bacteria isolated from other contaminated sites could not survive in the new environment (Vogel, 1996; Ko and Lebeault, 1999; Lee and Merlin, 1999; Timmis and Pieper, 1999). Bioaugmentation was beneficial in shortening the period required for the degradation of pollutants

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and in the degradation of compounds that are relatively recalcitrant to degradation such as chlorinated hydrocarbons (Vogel, 1996).

Although there is a substantial database concerning the types of bacteria capable of degrading oil components and ways to accelerate their degradation rates (Mearns, 1997; Jones, 1998; Head and Swannell, 1999), this knowledge cannot be simply extrapolated to polluted sites in Oman without keeping in mind the significant differences in environmental conditions. The soils of Oman are characterized by temperatures that can reach more than 55 °C. The coastal sediments experience salinities up to 10% due to high evaporation rates. Such features influence the diversity and activity of soil microorganisms as well as rates of natural degradation of pollutants, which are typically low at such extreme conditions (Margesin and Schinner, 2001). Bacteria isolated from elsewhere are unlikely to survive the harsh conditions of the Omani environment. Therefore, bioremediation strategies that rely on indigenous oil-degrading bacteria and their stimulation under field environmental conditions are required.

The present study was undertaken 1) to find out whether bacterial isolates from sediments of Oman with the ability to grow on petroleum compounds are similar or different than those isolated from other ecosystems and 2) to evaluate the effectiveness of biostimulation and bioaugmentation approaches in enhancing oil biodegradability in two heavily contaminated sediments. Indigenous bacteria were isolated from oil-polluted sediments, identified using 16S rRNA-based phylogeny and characterized for their growth at different salinities and temperatures, as well as on different alkanes. While biostimulation was achieved by the addition of nutrients (N and P), bioaugmentation was achieved by the addition of a bacterial consortium designed from our isolates. The extent of oil-degradation was studied by following the CO_2 evolution and was further verified by gas chromatography (GC) analysis of the residual oil fraction.

2. Materials and method

2.1. Physical and chemical characterization of the samples

Two sediment samples (PD and KH) were collected from the oil-contaminated land at Mina Al-Fahal (lat. 23° 37′ 49.92″N; long. 58° 31′ 23.84″E), a coastal area in Muscat, Oman. Oil contamination of the KH sediment was relatively more recent (six months ago) than that of the PD sediment (2 years ago). The shade temperature at the time of sampling was 32.1 °C. For salinity measurements, 10 g of sediment were mixed with 50 ml of deionized water and allowed to rest for 20 min. The pH and salinity of the filtrate were measured using calibrated YSI instruments. Total petroleum hydrocarbons (TPH) in the sediments were measured using GC as described before (Weisman, 1998). A standard soil hydrometer method was used to determine the percent sand, silt and clay in both samples (Klute, 1986), and this composition was used to identify the soil texture from a soil triangle (Brady, 1984). Nutrients were extracted and analyzed using Ion Chromatography (Jackson, 2000).

2.2. Isolation and characterization of hydrocarbon-degrading bacteria

2.2.1. Enrichment and isolation

The abundance of alkane-degrading bacteria in both samples was compared by estimating the most probable number (MPN) counts using hexadecane as a sole carbon source (Abed et al., 2007). Bacteria from both sediments were enriched on a defined artificial seawater medium supplemented with 1% (w/v) Omani crude oil as the sole carbon source as previously described (Abed et al., 2007). The medium contained $MgCl_2.6H_2O$ (5.6 gl^{-1}), $MgSO_4.7H_2O$ (6.8 gl^{-1}), $CaCl_2.2H_2O$ (1.47 gl^{-1}), KCl (0.66 gl^{-1}), KBr (0.09 gl^{-1}), KH_2PO_4 (0.15 gl^{-1}) and NH_4Cl (0.2 gl^{-1}) and was supplemented with trace elements mixtures (Widdel and Bak, 1992) and vitamins (Heijthuijsen and Hansen, 1986). Enrichments were performed using media of 2% and 7% salinity (w/v NaCl). All enrichments were performed under aerobic conditions at 30 °C. Axenic strains were obtained by plating on agar medium containing 20 mM acetate as the sole carbon source, and then re-tested for growth on oil.

2.2.2. Identification of the isolates

Nucleic acids from the isolates were extracted and 16S rRNA genes were amplified using polymerase chain reaction (PCR) as previously described (Muyzer et al., 1995; Abed and Garcia-Pichel, 2001). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Düsseldorf, Germany) and then sequenced with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster city, Calif.). Our sequences were aligned to sequences in the ARB database using the alignment tool of the ARB software package (Ludwig et al., 1998). These sequences were inserted into a maximum likelihood pre-established tree using the parsimony ARB tool. The 16S rRNA sequences of our isolates were submitted to the GenBank under the accession numbers JQ284260–JQ284299.

2.2.3. Growth of the isolates at different salinities and temperatures and on different alkanes

The growth of the obtained isolates at different salinities and temperatures was tested. The isolates were inoculated in tubes filled with 10 ml artificial seawater media with the salinities 2, 3, 5, 7, 10, 12 and 16% (w/v NaCl), amended with 20 mM acetate. The tubes were incubated on a rotary shaker at 100 rpm at 30 °C. The growth of the isolates was also measured at the temperatures 5, 20, 30, 40, 50 and 60 °C. Growth was monitored by following the changes of the optical densities of the cultures at 660 nm over a period of 4 weeks.

Twenty three strains, representing closely related species in different genera were tested for their growth on particular alkanes (Table 2), as a sole carbon source (Abed et al., 2007). The strains were inoculated in test tubes, each containing 10 ml of artificial seawater medium and 100 μl of a single alkane. The alkanes were filter-sterilized using solvent-resistant cellulose filters (0.2 μm pore size) prior to use. All incubations were done in triplicates at 30 °C in the dark with continuous shaking at 150 rpm. Growth was measured by following changes in optical density at 660 nm against biotic (without a substrate) and sterile (without bacteria) controls.

Table 1
Characteristics of the two studied sediments.

Parameter	Unit	PD	KH
TPH	mg g^{-1}	22 ± 0.7	55 ± 0.6
MPN on hexadecane		2.7×10^6	5.6×10^6
pH		7.46	7.43
Salinity	%	2	3.5
Soil texture			
Sand	%	30	43
Clay	%	8	6
Silt	%	62	51
Soil type		Silt loam	Silt loam
Nitrate	mg l^{-1}	21.13	2.18
Phosphate	mg l^{-1}	0.46	ND
Fluoride	mg l^{-1}	0.048	0.034
Bromide	mg l^{-1}	12.15	3.45
Sulfate	mg l^{-1}	451	27

TPH: Total petroleum hydrocarbons; ND: not detected.
MPN: Most probable number.

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