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Bacterial Degradation of the Saturate Fraction of Arabian Light Crude oil: Biosurfactant Production and the Effect of ZnO Nanoparticles

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Abstract

The aim of this research was to study the influence of nanoparticles on the biodegradation of crude oil. Production of biosurfactants was also assessed. Crude oil-utilizing bacteria were isolated from oil fields via enrichment in chemically defined medium with crude oil as a sole carbon source. The isolates could be affiliated to the genera *Bacillus*, *Pseudomonas*, *Achromobacter*, and *Microbacterium* by 16S rRNA gene sequencing and phylogenetic analysis. GC/FID analysis revealed 52 to 98% degradation of the oil saturate fraction within one month. Nanoparticles of ZnO inhibited growth and crude oil biodegradation by one isolate (NBHCO4) in a concentration-dependent manner. Growth of the cultures containing ZnO nanoparticles was relatively slower than that of the nanoparticles-free cultures. Moreover, the oil degradation extent in the nanoparticles-containing cultures (22-50%) was less than that achieved in cultures lacking ZnO nanoparticles (80%). Two strains, NBHCO2 and NCEOW, emulsified and utilized water-in-oil emulsions (chocolate mousse). Biosurfactant production in only one crude oil culture (I-19) could be confirmed by the observed reduction in surface tension. Some isolates produced biosurfactants from water-soluble substrates such as glucose. The NBHCO2 strain produced a lipopeptide biosurfactant which reduced the surface tension of the growth medium from 72 to 27 mN/m. A gene of catechol dioxygenase was detected in the I-19, NBHCO4, and NCEOW isolates indicating the potential catabolic capabilities of these strains for degradation of the aromatic oil fraction. In conclusion, metal oxide nanoparticles can interfere with crude oil biodegradation. Biosurfactants are not necessarily a prerequisite for crude oil biodegradation. The isolates can be applied for bioaugmentation of petroleum-polluted soil and biosurfactants production.

Keywords: Bioremediation; Biosurfactant; Emulsification; Nanoparticles; *Bacillus*

Introduction

Petroleum hydrocarbons are among the most common environmental contaminants [1,2]. Petroleum and its hazardous products can be released into the environment through various production, processing, and consumption operations. Consequently, environmental pollution with petroleum and its products has become an issue of global concern, particularly with the anticipated boost in the production and consumption of fossil fuels [3-5].

Pollution with petroleum hydrocarbons in the Arabian Gulf region has become a serious environmental issue. In deed the Arabian Gulf is one of the most heavily polluted aquatic environments worldwide. It was estimated that ca 60% of the oil transported via the sea is shipped in the Arabian Gulf [5,6]. Furthermore, the Arabian Gulf region has witnessed the largest oil spill to date in terms of the amount of crude oil discharged. During the 1991 Gulf war the release of about 11 million barrels of crude oil has lead to the pollution of all environmental components in the region [7].

In Kuwait, where the situation was the worst, about 190 km of border trenches were filled with 3.5 million barrels of crude oil. Moreover, 400 km^2 of land were polluted because of the fallout from 800 burning wells which released 50 million m^3 of crude oil [8]. The marine environment and ground water reservoirs were also heavily impacted. Another devastating consequence is the formation of more than 300 oil lakes that covered over 49 km^2 of land with more than 40 million tons of crude oil [9,10]. In addition to land and water pollution with petroleum hydrocarbons, the 1991 Gulf war contributed massively to air pollution [11].

Although more than two decades have elapsed since the catastrophic Gulf war oil spill occurred, the consequences are still apparent today [10]. Following the 1991 Gulf war, some studies have been conducted to evaluate bioremediation as a cleanup technology in the Arabian Gulf territory [9,12,13]. Nonetheless, the work achieved to date is not up to the scale of the massive pollution problem. Most of the research done has focused on the marine environment of the Arabian Gulf. In contrast, a few studies have addressed the crude oil-degrading microbial populations in petroleum-impacted soil.

It is well known that one of the factors which often impede the biodegradation of hydrophobic substrates like hydrocarbons and crude oil is their low bioavailability [14,15]. This is because these substrates have poor aqueous solubility and therefore, are not readily accessible for microbial attack. To circumvent this problem, microbes, which exist predominantly in the aqueous phase, have evolved different mechanisms to get access to and utilize hydrophobic compounds. Some

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crude oil-degrading bacteria produce biosurfactants/bioemulsifiers that promote the aqueous solubility, and thus the bioavailability, of petroleum hydrocarbons by solubilization and emulsification [16,17]. Consequently, crude oil biodegradation can be enhanced by biosurfactants/bioemulsifiers production. However, this is not always the case because biosurfactants might also negatively impact the biodegradation process [18]. Therefore, further investigations are still needed to better understand biosurfactants production patterns and their role in crude oil biodegradation and action mechanisms. Noteworthy, studies on biosurfactants production by crude oil-degrading bacteria or bacteria inhabiting crude oil-polluted environments in the Arabian Gulf region are very scarce.

The presence of other environmental contaminants such as nanoparticles, can probably have an impact on crude oil biodegradation. Nanoparticles have gained tremendous interest due to their unique physicochemical properties compared to their bulk counterparts [19]. Potential impact of commercial nanoparticles on crude oil biodegradation has not been investigated so far. This is particularly demanding due to the increasing applications of engineered nanoparticles and nanotechnology which will inevitably lead to discharge of nanoparticles into the environment via various routes [19-21]. Accordingly, it is likely that nanoparticles may interfere somehow with the biodegradation of environmental pollutants since some of them are known to have antimicrobial activity [22].

This study was undertaken to isolate crude-oil degrading bacteria from petroleum-polluted soil collected from oil fields in Bahrain and Kuwait and to assess their biodegradation potential. Biosurfactants production by the isolated bacteria was also investigated. Furthermore, we tested the effect of commercially available ZnO nanoparticles on growth and oil biodegradability.

Materials and Methods

Chemicals, bacteria, soil, and oil samples

Chemicals and molecular biology reagents were obtained from Fluka (Switzerland), Sigma (USA) Promega and Qiagen (Germany), and the sterile filters (0.22 and 0.45 μm) were from Millipore (USA). Arab light crude oil was provided by Saudi Aramco (Kingdom of Saudi Arabia) and BAPCO (The Bahrain Petroleum Company). Nanoparticles (ZnO nanopowder < 100 nm) were purchased from Sigma-Aldrich (Germany). Petroleum-contaminated soil samples were collected from different locations in Bahrain (Awali oil field), and Kuwait (oil-impacted locations including those that date back to the 1991 Gulf War; Al Sabiriya field, and Al Moqawwa field). Moreover, crude oil samples mixed with water (production water) were collected from pipelines. All samples were collected in October 2010 in clean glass containers and kept at room temperature in the laboratory. These samples were used as a source of potential petroleum-degrading microorganisms. The I-19 strain is a *Pseudomonas* sp. isolated by a MSc student at the Arabian Gulf University [23]. The source of the I-19 strain is crude oil-contaminated soil collected from Al Sabiriya oil field in Kuwait. It was isolated from an enrichment culture in chemically defined medium with biphenyl as a sole carbon source. The I-19 strain could emulsify and grow on crude oil and it is included in the current study to assess its biodegradation potential. *Bacillus subtilis* DSM 3257 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). Genomic DNA from *B. subtilis* was used as a positive control for detection of the biosurfactants-related genes as described below.

Culture media and growth conditions

Enrichment of crude oil-degrading-bacteria and the biodegradation experiments (shake-flask cultures) were performed in mineral salts medium containing crude oil as a sole carbon source [24]. Filter-sterilized (0.22 μm Stericup filters, Millipore) crude oil was added to sterile mineral salts medium (2 or 5%, v/v). Biosurfactants production was also tested in 50 ml mineral salts medium containing 10 mM glucose from filter-sterilized stock solution as a sole carbon source. Isolation and purification of the bacteria was done via spread and streak plate techniques on Luria-Bertani (LB) agar. Inocula for the biodegradation experiments were prepared in 50 ml LB-broth (in 100 ml Erlenmeyer flasks). All liquid cultures were routinely incubated at 30°C with shaking at 200 rpm. LB-agar plates were incubated at 30°C for 48 hours. Growth of bacteria in liquid cultures was monitored by measuring the optical density at 580 nm (OD_{580}) with a Perkin Elmer lambda EZ210 spectrophotometer (1 cm optical path).

Enrichment and isolation of bacteria

Soil samples were suspended in distilled water (20 g/100 ml) and the suspension was shaken for 30 minutes. The soil suspension was then kept at room temperature and allowed to settle and aliquots of the soil slurry (1 ml) were inoculated in 100 ml mineral salts medium (in 250 ml Erlenmeyer flasks) containing 2% crude oil as a sole carbon source. Non-sterilized crude oil was used as inoculum in mineral salts medium (10%) without the addition of soil slurry to check for the presence of bacteria in the oil. Production water was inoculated (2%) into mineral salts medium containing 2% filter-sterilized crude oil as a carbon source. Enrichment cultures were incubated for one week. Samples from the various enrichments that showed signs of turbidity, emulsification/dispersion and/or change in color were serially diluted in sterile saline (0.9% NaCl). Aliquots from the culture dilutions (100 μl) were spread over LB-agar plates and incubated for 48 hours. Morphologically distinct single colonies from the spread plates were streaked across LB-agar plates three times consecutively for purification.

Preparation of inocula for the biodegradation experiments

To screen the isolated bacteria for crude oil degradation capacity, the various isolates were grown in LB-broth for 16-18 hours. The OD_{580} of the cultures was then measured and the cells were harvested by centrifugation (3500 rpm, 20 minutes) at 4°C. The cell pellet was washed once with basal medium (phosphate buffer+NH_4Cl) [24] and the washed cells were resuspended in 5 ml of basal medium. These cell suspensions were used as inocula. All manipulations of the cells were done on ice. Cells were harvested from 2 ml of the cell suspension by centrifugation (14,000 rpm, 5 minutes) and heated at 105°C for 15 hours in a drying oven for measuring the dry weight.

Crude oil biodegradation assays

Routinely, 2 ml of each cell suspension (0.04-0.2 gram dry cell weight/l) were inoculated into mineral salts medium containing 2% crude oil as a sole carbon source and incubated for 4 weeks. Cultures were inspected visually at intervals for turbidity, color change, and oil emulsification/dispersion. Cultures prepared for the growth curve experiments contained 5% crude oil. In all experiments, crude oil biodegradation was assessed by GC-FID after *n*-hexane extraction from the various cultures as described later. Uninoculated controls were included to monitor sterility and to compensate for abiotic loss of oil. Change in biomass was monitored by measuring the optical density (OD_{580}) at hourly intervals during the initial two days of incubation then once weekly.

Biosurfactants production

To test selected isolates for biosurfactants production, the bacteria were grown in mineral salts medium with crude oil (2%) as described earlier. Biosurfactants production was also assessed in cultures growing on water soluble substrates such as glucose (10 mM). Uninoculated medium was included as a control. After different time intervals (24 hours and 24 days), samples from the crude oil cultures (25 ml from the aqueous phase) and the glucose cultures (25 ml, after 4 days) were withdrawn and the cells were removed by centrifugation for 20 minutes at 3500 rpm. The supernatants were then filtered under vacuum through 0.22 µm membrane filters to remove residual cells. The surface tension of cell-free culture supernatants was measured with a Kruss K100MK3 tensiometer (Kruss, Germany) equipped with a platinum plate at room temperature via the Wilhelmy plate method [25].

Effect of ZnO nanoparticles on growth and biodegradation capacity

This experiment was conducted to assess the impact of commercially available ZnO nanoparticls on growth and crude oil biodegradation. Nanoparticles of ZnO were chosen because they are widely applied [26]. The isolate NBHCO4 was cultured in mineral salts medium containing crude oil in the presence of ZnO nanoparticles. One set of cultures contained 1 µg/ml and another set contained 10 µg/ml of ZnO nanoparticles. Control cultures without nanoparticles and uninoculated flasks were also included. All cultures were incubated for 28 days at 30°C. Growth was monitored by measuring the OD_{580} after time intervals and oil biodegradation was assessed by GC-FID.

Gas Chromatography (GC)

Gas chromatography was adopted to monitor the degradation of saturated hydrocarbons in the *n*-hexane-soluble fraction. The crude oil from all cultures was extracted once in *n*-hexane. The culture contents were mixed with 15 ml of *n*-hexane by vigorous shaking in a separating funnel for 5 minutes. After the two phases were resolved, the aqueous phase was allowed to drain and the organic phase was collected in clean glass flasks. A portion (1 ml) of the organic phase was then centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to a clean Eppendorf tube and diluted 50x in *n*-hexane. Samples (1 µl) from all dilutions were injected into an Agilent GC7890A-Markes Headspace System equipped with a FID (flam ionization detector). The column was a HP-5ms (5%-phenylmethylpolysiloxane, 30 m x 0.25 mm id, 0.25 µm film thickness) and the carrier gas helium was used at flow rate of 0.8 ml/min. The injector temperature was 250°C and the oven temperature was held at 70°C for 2 min then increased to 300°C at 20°C /min, thereafter held at 300°C for 8.5 min. The peak areas for some components of the *n*-hexane soluble fractions in the GC/FID chromatograms of the different experiments were compared. The peak area represents the % of the hydrocarbon constituent in the total amount of the hexane soluble fraction (relative abundance). To assess the extent of oil (hexane soluble fraction) biodegradation, the total area of all the peaks in each GC/FID chromatogram was taken as an indicator.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed to screen selected isolates for the presence of genes involved in the degradation of aromatic compounds and biosurfactants production. Genomic DNA was isolated from overnight LB-cultures of the different bacteria using the Wizard Genomic DNA Purification kit (Promga, USA) and was used as a template. The primers used are listed in Table 1. The PCR was run on a Biorad DYAD thermocycler (Biorad, USA) and the PCR mixture (20 µl) contained 0.2 µM from each of the forward and reverse primers, 7-200 ng of genomic DNA, and 2 µl of 20x Taq PCR master mix (Qiagen, Germany). The PCR conditions for each pair of primers were as mentioned in the respective reference.

Amplification, sequencing of 16S rRNA gene and phylogenetic analysis

A loopful of overnight grown cultures was transferred to 50 µl TE buffer (100 mM Tris-HCl–10 mM EDTA, pH 8.0) and boiled for 5 min. Then resulting cell extract was used as a template for PCR using Platinum Supermix (Invitrogen, USA). The 16S rRNA genes were amplified with eubacterial universal primers (Table 1). PCR mixture consisted of 25 µl Platinum Supermix, 1 µl of each primer (from 10 µM stock), 1 µl of cell extract, and was adjusted to 50 µl with distilled water. PCR was performed in Thermal Cycler (Applied Biosystem 2720, USA) and the PCR conditions were adjusted to 5 min for initial denaturation at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and finally 10 min at 72°C. The amplicons were subjected to electrophoresis using 1% agarose gel containing ethidium bromide (0.05%) with size marker (DNA ladder, Promega, USA).

Nucleotide sequences of amplified 16S rRNA were determined by automated fluorescent dye terminator sequencing method originally developed by Sanger et al. [32] using DYEynamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) with a model ABI 310 genetic sequence analyzer (Applied Biosystems, CA, USA) according to user manual. Obtained sequences were analyzed by Genetyx-Win MFC application software version 4.0. The reference 16S rRNA gene sequences were retrieved from the GenBank database (National Center for Biotechnology Information NCBI, National Library of Medicine, USA) [33]. Sequences were compared with their closest matches in GenBank with nucleotide-nucleotide BLAST to obtain the nearest phylogenetic neighbors (www.ncbi.nlm.nih.gov/BLAST). Multisequence alignments were performed by Clustal W1.83 XP software and phylogenetic trees were constructed with MEGA (The Biodesign Institute) [34] using evolutionary distance and neighbor-joining method [35].

Primer name	Primer sequence 5′-3′	Amplified gene or DNA fragment	Expected product size (bp)	Reference
23cat-f 23cat-r	CGACCTGATCTCCATGACCGA TCAGGTCAGCACGGTCA	2,3 catechol dioxygenase	238	[27]
sfp0-F sfp0-R srfA-F srfA-R	CTAGAATTCAGATTTACGGAATTTATATG GGGGAATTCAGGGTGTGCGGCGCATAC TCCGTTTTTCCTTGTTCACC TCTTTCTGCCACTGCATCAC	the genes of lipopeptide biosurfactants from *Bacillus subtilis*	642 707	[28] [28]
Kpd1 Kpd2	GCCCACGACCAGTTCGAC CATCCCCCTCCCTATGAC	the *rhlB* gene for rhamnolipid biosynthesis	226	[29]
27F 1500R	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACT	The 16S rRNA genes	1500	[30] [31]

Table 1: Primers used in this study.

Sequence accession numbers

The 16S rRNA gene sequences obtained in this study have been deposited in GenBank under accession numbers AB824289-AB824294 and AB836753 to AB836756

Results

Isolation and identification of bacteria

Various enrichment cultures revealed different patterns of color change, turbidity and dispersion of crude oil within one week of incubation. These changes indicated the ability of the organisms in the enrichments to utilize petroleum hydrocarbons as a carbon and energy source. Strong emulsification of the crude oil was also observed in the enrichment which contained only crude oil as an inoculum and carbon source. In total, 10 different bacterial strains could be isolated from the spread plates. Based on the results of the phylogenetic analysis, the isolated bacteria could be affiliated to the genera *Bacillus, Pseudomonas, Achromobacter,* and *Microbacterium* (Figure 1, Table 2). The majority of the isolates belong to the *Pseudomonas* and *Bacillus* genera. The phylogenetic analysis was based on full 16S rRNA gene sequence for the isolates NKCO2 and NKCO4 (isolated from Kuwaiti oil fields), NBHCO3 (isolated from Bahraini oil fields), and NCEOW (isolated from production water), whereas only partial sequence was considered for the remaining isolates (Table 2).

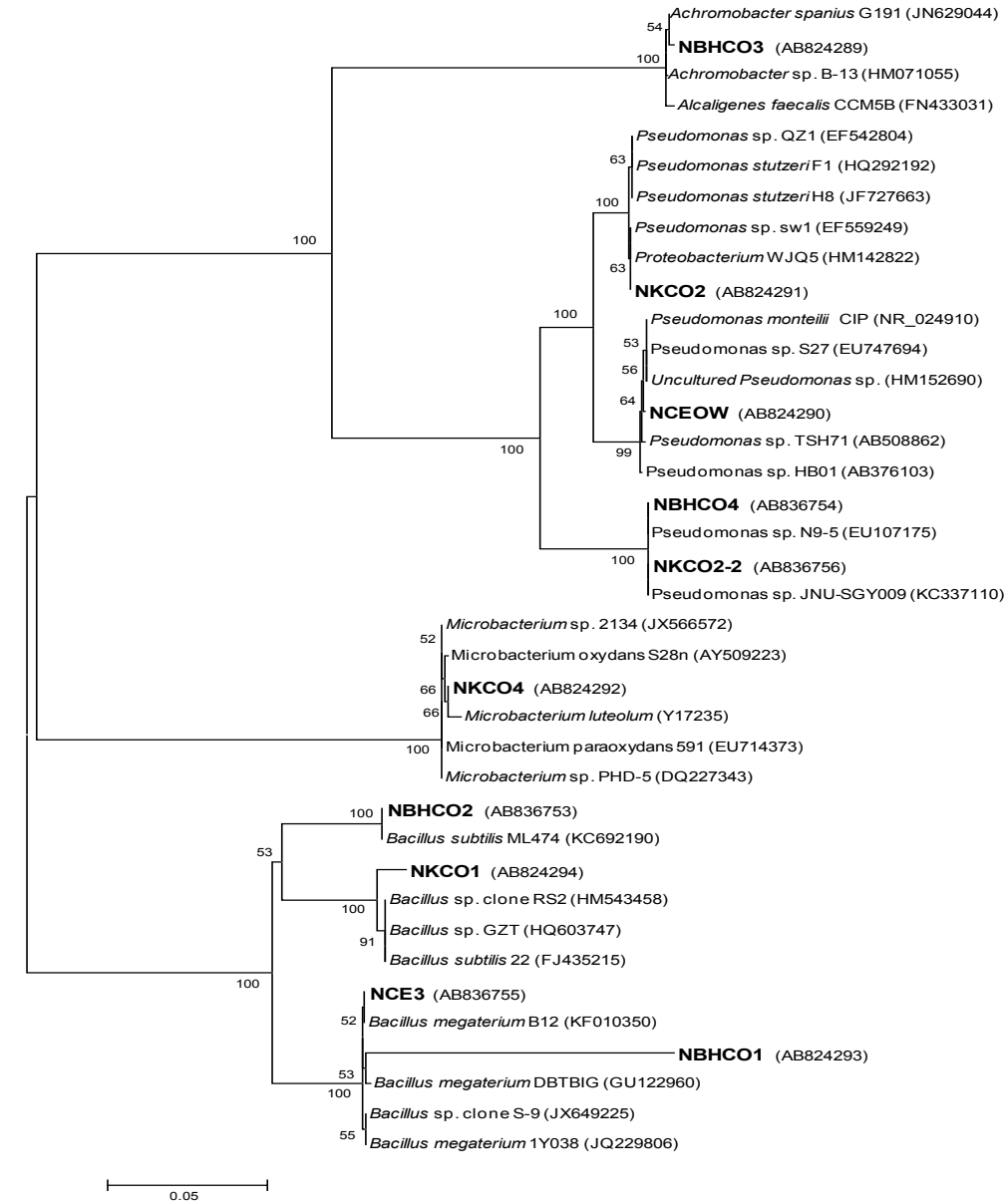

Figure 1: Consensus neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of isolated strains and related reference taxa. Numbers at nodes indicate bootstrap values of neighbor-joining analysis for 1000 replicates. The scale bar indicates substitutions per nucleotide. The GenBank accession numbers for the 16S rRNA gene sequences are given in parentheses after the strain name.

Strain	Source	Closest matches	Accession No.[a]	% of similarity	Accession No.[b]
NKCO2	Kuwait, Al Sabiriya Oil Field	*Pseudomonas* sp. sw1	EF559249	99	AB824291
NKCO4	Kuwait, Al Sabiriya Oil Field	*Microbacterium luteolum*	Y17235	99	AB824292
NBHCO1	Bahrain, Awali Oil Field	*Bacillus* sp. clone S-9	JX649225	98	AB824293
NKCO1	Kuwait, Al Sabiriya Oil Field	*Bacillus* sp. clone RS2	HM543458	99	AB824294
NBHCO3	Bahrain, Awali Oil Field	*Achromobacter spanius* G191	JN629044	99	AB824289
NCEOW	Bahrain, Production Water	*Pseudomonas* sp. TSH71	AB508862	99	AB824290
NBHCO2	Bahrain, Awali Oil Field	*Bacillus subtilis* ML474	KC692190	99	AB836753
NBHCO4	Bahrain, Awali Oil Field	*Pseudomonas* sp. N9-5	EU107175	99	AB836754
NCE3	Crude Oil	*Bacillus megaterium* B12	KF010350	99	AB836755
NKCO2-2	Kuwait, Al Sabiriya Oil Field	*Pseudomonas* sp. JNU-SGY009	KC337110	99	AB836756

[a]GeneBank sequence accession numbers of most closely related sequences
[b]GeneBank sequence accession numbers of isolated strain

Table 2: Bacteria isolated from oil fields.

Growth of the isolated bacteria on crude oil

Growth curves for the isolates NBHCO2 and NBHCO4 (isolated from Bahraini oil fields), NCE3 (isolated from crude oil), NCEOW (isolated from production water), NKCO2-2 and NKCO1 (isolated from Kuwaiti oil fields), during the initial 48 hours of incubation are shown in Figure 2. Fluctuations in the OD_{580} were observed in the NCE3 and NBHCO4 cultures. However, a net increase in turbidity was observed up to 26 hours of incubation. The OD_{580} then decreased and remained stable. The NBHCO2 culture was the only one which exhibited no fluctuations in the OD_{580}. After a 10-hour lag phase, the OD_{580} increased then became constant after 30 hours for 15 hours. The highest OD_{580} during the first two days was attained in the NBHCO4 and NCE3 cultures after 26 hours. After 28 days of incubation the OD_{580} of the NCE3 culture was 1.6 whereas that of the NBHCO4 was 2.4. Fluctuation in culture turbidity was also observed in the NKCO1 culture. An increase in the OD_{580} of the NKCO1 culture occurred up to 23 hours followed by sudden decrease which lasted for almost 8 hours. After 32 hours of incubation the OD_{580} increased again and remained almost constant. The culture turbidity of the NKCO2-2 strain increased steadily during the initial 48 hours. After 21 days of incubation the OD_{580} of the NKCO1 culture reached 5.5 whereas that of the NKCO2-2 was 1.25. The NCEOW culture turbidity decreased during the initial 6 hours then remained almost constant.

In some oil cultures, water-in-oil emulsions (the so called chocolate mousse) were produced within 24~72 hours of incubation. In those cultures no turbidity in the aqueous phase was observed even after prolonged incubation (4 weeks) except the NBHCO2 and NCEOW cultures. In both cultures, the chocolate mousse was dispersed and the aqueous phase became turbid after 3~4 weeks.

Biodegradation of crude oil by selected bacteria

As shown in Figure 3, the tested bacteria degraded saturated hydrocarbons in the *n*-hexane-soluble fraction of Arabian light crude oil within 28 days of incubation. This was confirmed by the detected decrease in the amounts of the saturate components as compared to uninoculated controls. To estimate the biodegradation extent achieved by the different isolates, the relative amount of the consumed saturate fraction was calculated in comparison with that remaining in the uninoculated controls. The NCE3 strain achieved the highest biodegradation extent (98%) followed by NBHCO4 (78%), then NKCO1 (75%) (Figure 4). Figure 5 displays the change in the relative amount of some saturated hydrocarbons after 28 days of incubation at 30°C. Both of the NKCO1 and NBHCO4 strains reduced the (%) of *n*-C12 (dodecane), *n*-C13 (tridecane), *n*-C18 (octadecane) and *n*-C19

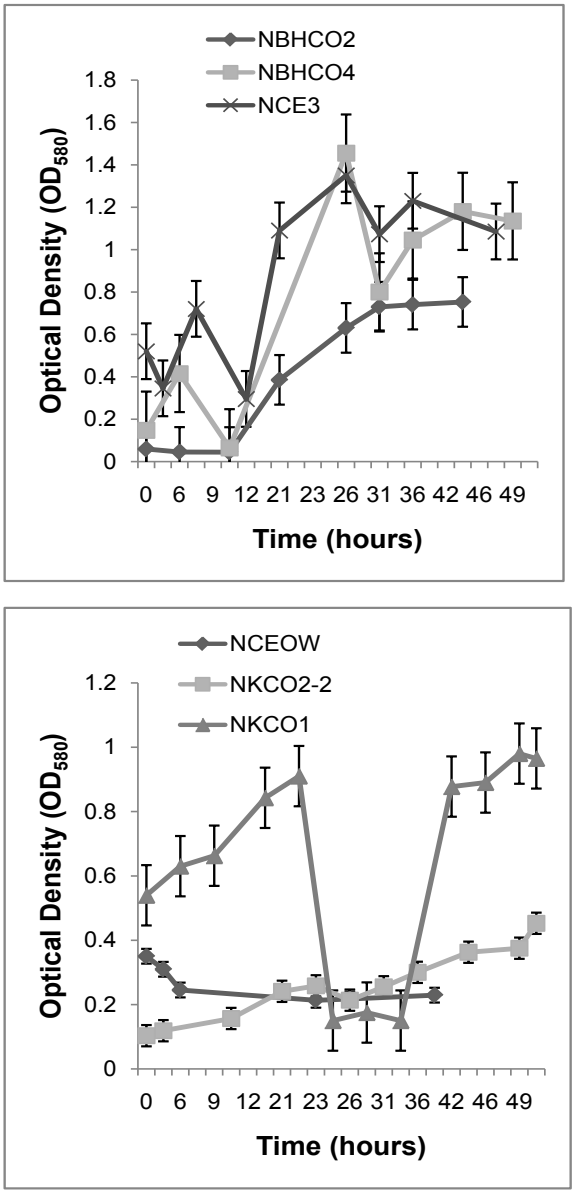

Figure 2: Growth of isolated bacteria in mineral salts medium containing crude oil (5% v/v) at 30ºC during the initial 48 hours of incubation. Results are means of duplicate measurements from two independent experiments. Error bars represent standard error.

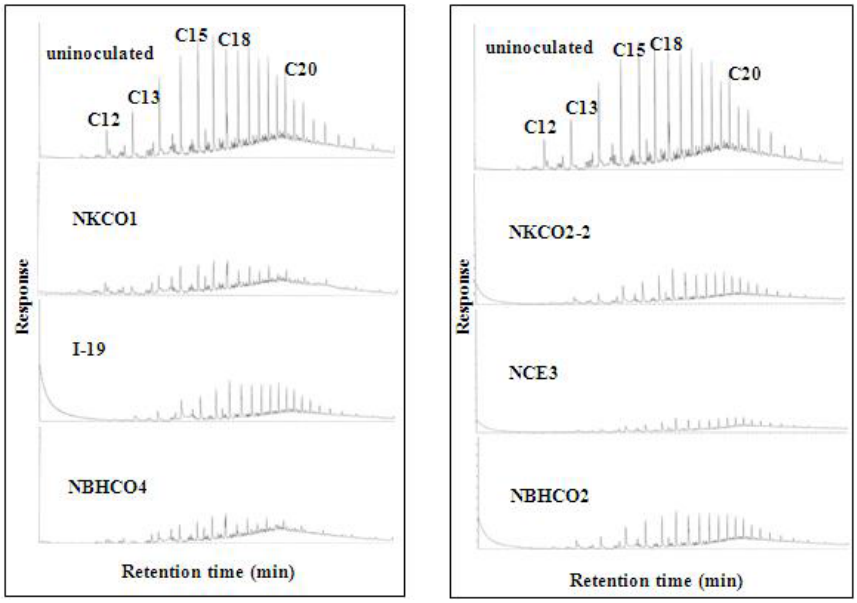

Figure 3: GC/FID chromatograms showing the biodegradation of the hexane-soluble fraction of Arabian light crude oil by some isolates at 30ºC for 28 days. The tested bacterial isolates are NBHCO2 and NBHCO4 (isolated from Bahraini oil fields), NCE3 (isolated from crude oil), I-19 (isolated from Kuwaiti oil field), NKCO-2-2 and NKCO1 (isolated from Kuwaiti oil fields).

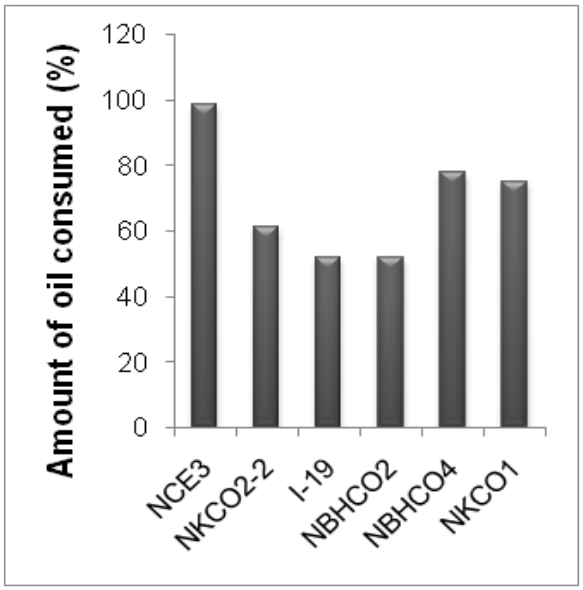

Figure 4: Amount of oil (%, hexane-soluble fraction) consumed after biodegradation of crude oil by the isolated bacteria at 30ºC for 28 days.

(nonadecane) hydrocarbons. The other tested isolates (I-19, NCE3, NKCO2-2) drastically reduced the *n*-C12 hydrocarbons % as compared to the uninoculated control. However, little increase in the *n*-C12% was observed in the NBHCO2 culture. The NCE3, NBHCO2, I-19 and NKCO2-2 isolates also reduced hydrocarbons in the range *n*-C13 and *n*-C18. The largest % reduction was achieved by the NCE3 strain. The degradation preference changed with the *n*-C15 (pentadecane) and *n*-C20 (eicosane) hydrocarbons. A relative increase in the % of the *n*-C15 constituents was observed in the NBHCO2, I-19 and NKCO2-2 cultures. On the contrary, the NCE3 strain achieved almost 50% reduction in the relative proportion of *n*-C15 hydrocarbons. The % proportion of the *n*-C20 hydrocarbons did not reveal significant change in most cultures. Exceptionally, the NCE3 strain caused ca 35% increase in the *n*-C20 hydrocarbon relative amount.

Effect of ZnO nanoparticles on growth and crude oil biodegradation

The ZnO nanoparticles were added to the NBHCO4 culture (1 µg/ml and 10 µg/ml) to test their effect on bacterial growth and oil biodegradation. The NBHCO4 strain grew in crude oil cultures containing ZnO nanoparticles within 28 days at 30°C. The color of the cultures turned brownish and the oil got dispersed. No apparent differences in color or oil dispersion pattern were observed between cultures having and those lacking nanoparticles. However, the growth of the nanoparticles-containing cultures was slower than that of the cultures containing no nanoparticles. The maximum OD_{580} was higher in the culture containing nanoparticles at 1 µg/ml (Figure 6).

The GC/FID chromatograms (Figure 7) revealed degradation of the hexane-soluble oil fraction in the nanoparticles–containing cultures. Interestingly, the amount of oil consumed by NBHCO4 was much less in nanoparticles–containing cultures. Cultures containing 1 and 10 µg/ml of ZnO nanoparticles consumed 50 and 22% of the hexane–soluble fraction, respectively, after 28 days of incubation. The nanoparticles-free culture consumed 80% of the hexane soluble fraction.

Biosurfactants production by the isolated bacteria

Dispersion/emulsification of crude oil in the growth medium by most of the tested bacteria occurred within the initial 2-3 days of incubation. In some cultures such as I-19, NBHCO4, and NCE3, oil dispersion was observed even a few hours after inoculation. The strongest dispersion occurred in the I-19 and NBHCO4 cultures. The cultures revealed different sizes of the oil droplets. These data indicate the presence of some sort of tensioactive agents (biosurfactants/bioemulsifiers) in the crude oil cultures. There was no significant

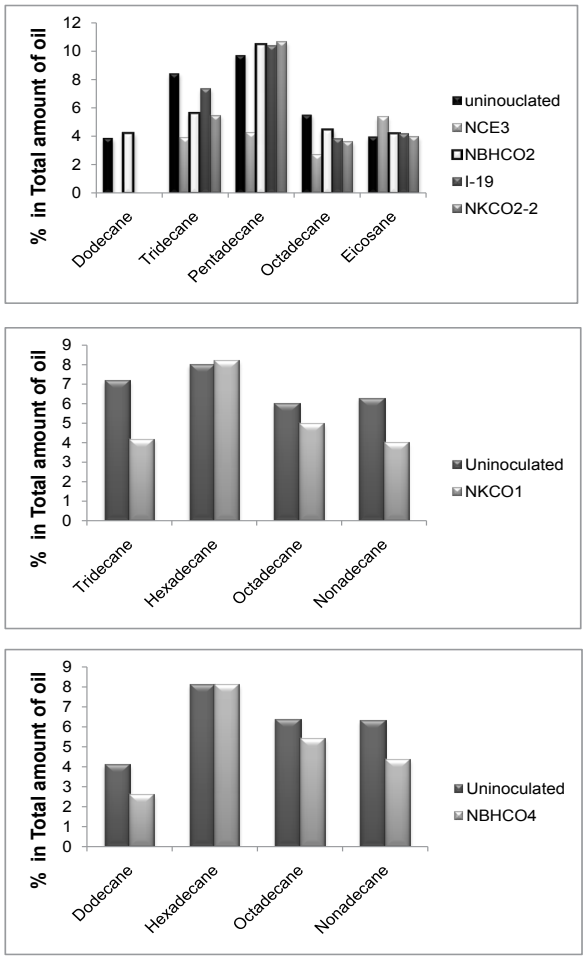

Figure 5: Changes in the relative proportions of hydrocarbon components in the hexane-soluble fraction of Arabian light crude oil after biodegradation by the isolated bacteria for 28 days at 30ºC.

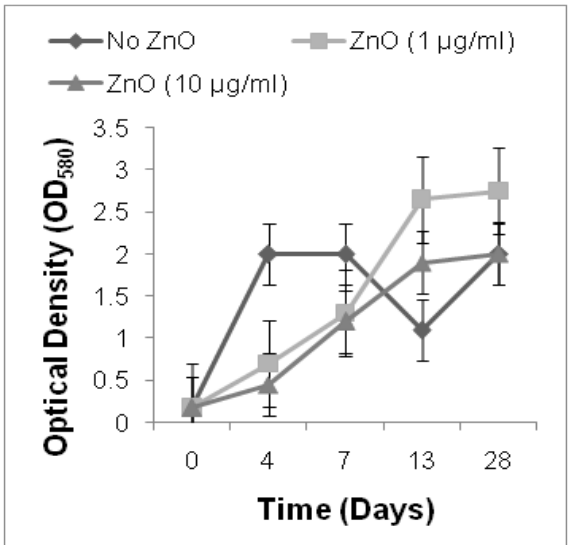

Figure 6: Growth of the NBHCO4 strain in mineral salts medium containing crude oil (5% v/v) at 30ºC in the presence or absence of ZnO nanoparticles.

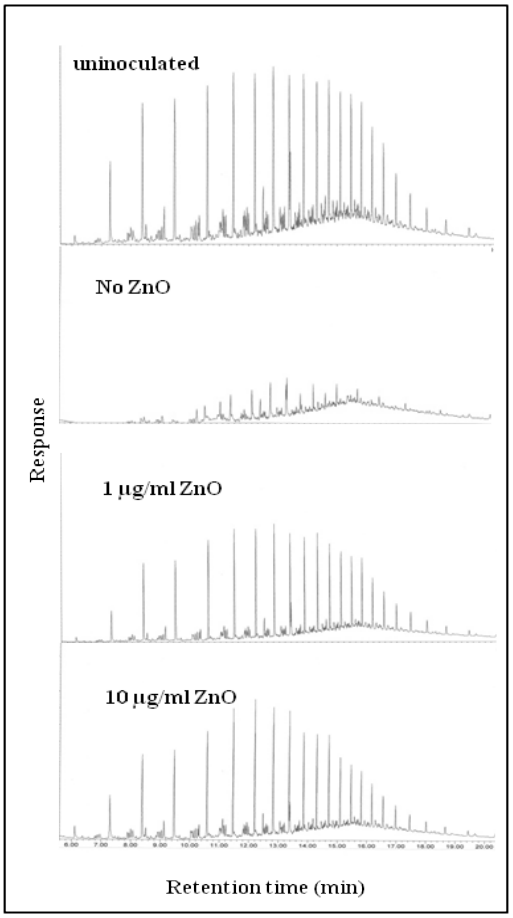

Figure 7: GC/FID chromatograms showing the influence of ZnO nanoparticles on the biodegradation of the hexane-soluble fraction of Arabian light crude oil by the NBHCO4 isolate at 30ºC for 28 days.

difference in surface tension between most of the crude oil cultures and the uninoculated medium (Figure 8). After 24 hours of incubation, the lowest surface tension was measured in the NKCO1 culture (54 mN/m). However, after 24 days, the surface tension in the I-19 culture decreased from 59.5 (after 24 hours) to 52.7 to record the lowest surface tension among the tested cultures. After two weeks of incubation, most of the oil droplets in the I-19 and NCEOW cultures coalesced. The tested bacteria produced biosurfactants using glucose as a sole carbon source. This was confirmed by measurement of surface tension (Figure 8). The differences in surface tension between the cultures and the uninoculated medium were much larger than those observed in the crude oil cultures. The lowest surface tension was measured in the NBHCO2 glucose culture (27 mN/m) after 4 days of incubation.

Detection of genes involved in aromatic hydrocarbons biodegradation and biosurfactants production

Gene of 2,3-catechol dioxygenase was detected only in NBHCO4, I-19, NCEOW. Genes of lipopeptide biosurfactants biosynthesis in *Bacillus subtilis* were detected only in the NBHCO2 strain (Figure 9). A rhamnosyl transferase-encoding gene involved in biosynthesis of rhamnolipids biosurfactants in *Pseudomonas aeruginosa* could not be detected in any of the tested isolates.

Discussion

As shown by phylogenetic analysis, the majority of the isolated bacteria belong to *Bacillus* and *Pseudomonas* genera. Literature search revealed that various species of *Pseudomonas* and *Bacillus* are common inhabitants of petroleum-polluted ecosystems. Furthermore, these organisms are well known for their capacity to degrade a range of petroleum hydrocarbons [36-38]. In contrast *Achromobacter* and *Microbacterium* spp. have been much less frequently isolated from crude oil-polluted niches [39,40]. This also agrees with the results of the phylogenetic analysis reported in this study. In the Arabian Gulf region, hydrocarbon-utilizing *Microbacterium* spp. were isolated from legume leaves [41] and along the Arabian Gulf coast [42,43]. Recently, Tanase et al. [44] isolated hydrocarbon-degrading *Achromobacter xylosoxidans* from oil-polluted soil. Also in line with our results, Minf et al. [16] isolated hydrocarbon-degrading *Achromobacter* sp. from Tunisian oil fields. To our knowledge, crude oil-degrading and emulsifying *Achromobacter* and *Microbacterium* species have not been reported before as isolates from oil-polluted soil in Kuwait.

Changes in culture turbidity, color, and oil dispersion/emulsification indicate the ability of the tested isolates to utilize petroleum hydrocarbons for growth and energy production. The fluctuation of the OD_{580} in most of the cultures during the initial two days could be attributed to attachment and detachment of the bacterial cells to and from the oil droplets. Bacterial adhesion to hydrocarbons is one strategy by which hydrocarbonoclastic bacteria overcome the extremely low aqueous solubility of hydrocarbons [45]. Similar results were reported by Bredholt et al. [46]. Moreover, Obuekwe et al. [47] investigated hydrocarbon degradation as a function of cell surface hydrophobicity and found that higher degradation extent is correlated with higher cell-surface hydrophobicity. Another plausible reason for the observed fluctuations in culture density could be the structural complexity and heterogeneous nature of the crude oil. Different types of interactions between the cells and the myriad of compounds in crude oil make the biodegradation process very dynamic. Petroleum hydrocarbons differ in their toxicity, accessibility, and recalcitrance. The turbidity profile of the NKCO1 culture mimics a diauxic growth curve. Transient decline in the turbidity of the NKCO1which remained for several hours could be due to growth inhibition by toxic oil components or oxygen deficiency. The recovery from this temporary stationary phase resulted in increase in culture turbidity again. However, other factors like the attachment to /detachment from the oil droplets may be involved.

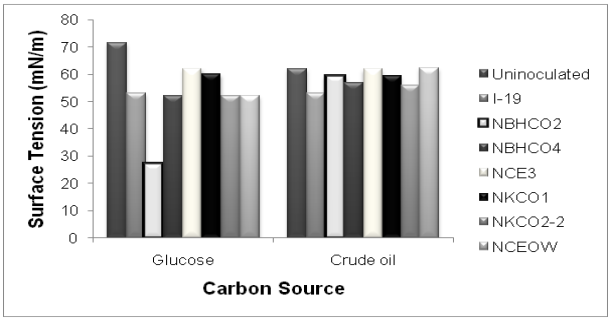

Figure 8: Change in surface tension of cell-free culture supernatants. Bacteria were grown at 30°C in mineral salts medium containing either glucose (10 mM) or crude oil (2%, v/v).

The isolated bacteria caused substantial degradation of the hexane-soluble (saturate) fraction of Arabian light crude oil within one month. Accordingly, these bacteria possess the catabolic machinery that enables them to utilize various aliphatic hydrocarbons of crude oil. This is common among bacteria isolated from crude oil-impacted ecosystems [44,48]. However the tested bacterial isolates achieved different degradation extents. Crude oil-utilizing microorganisms differ in their degradation capacities [47,49]. Although we do not have a direct evidence for the degradation of the aromatic fraction, the presence of a gene of catechol dioxygenase in some isolates suggests the ability of these bacteria to utilize aromatic substrates. Differences in biodegradation extent may be attributed to various factors such as cell surface hydrophobicity, enzyme activity and specificity, genes arrangement and regulation of gene expression, as well as biosurfactants/bioemulsifiers production. The highest degradation extent was recorded for NCE3 and NBHCO4. These two strains are among the isolates that caused strong dispersion of crude oil in the culture medium. This could be a reason for their higher biodegradation capacities [17].

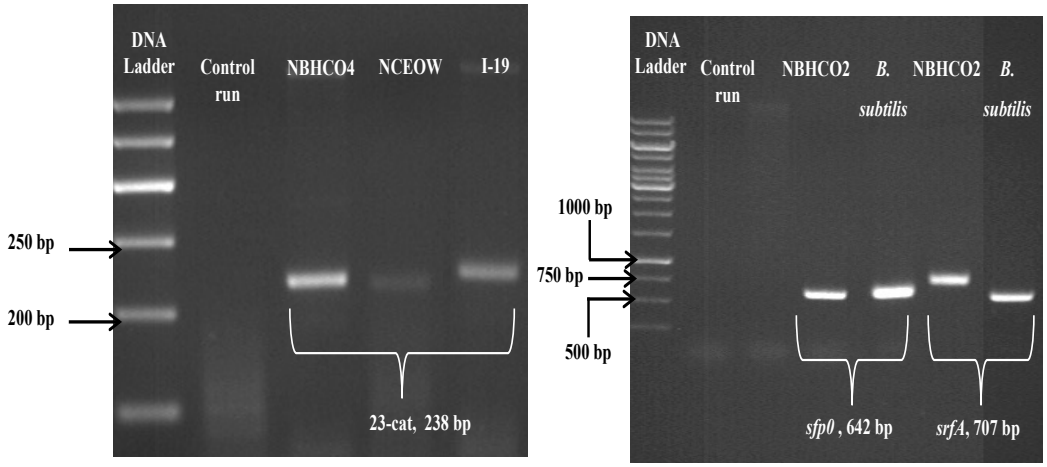

Figure 9: Images of agarose gels showing results of PCR for the detection of genes related to lipopeptide biosurfactants production (*sfp0* and *srfA*) and catechol dioxygenase in some isolates. Genomic DNA isolated from different bacteria was used as a template. Genomic DNA from B. subtilis was used a positive control for the *sfp0* and *srfA* genes. Control runs are PCR runs without template DNA.

It is worth mentioning that stronger oil dispersion does not always correlate with enhanced biodegradation efficiency [50]. The stability (decay constant) of the crude oil emulsion is another important factor. In line with this, NKCO1, which caused slight dispersion of the oil, achieved degradation extent comparable to that achieved by the stronger emulsifier NBHCO4. Interestingly, although the NCE3 isolate achieved degradation extent higher than that achieved by the NKCO1 strain, the NKCO1 culture reached OD_{580} more than 3 fold (5.5) higher than that of the NCE3 culture (1.6) after 3 weeks of incubation. It can be proposed that the NKCO1 strain utilized hydrocarbons from the other fractions such as aromatics, asphaltenes and resins and attained higher OD_{580}. Another explanation for this observation is that NKCO1 can achieve complete degradation of the hydrocarbons whereas NCE3 can only affect partial or incomplete degradation. This also might explain why the NKCO1 culture attained much higher biomass yield than that of the NBHCO4 culture although both achieved similar degradation extents for the saturate fraction. However, this needs further investigations.

The changes observed in the relative proportions of the various aliphatic hydrocarbons indicated that the tested bacteria did not utilize them equally. Preferential degradation of hydrocarbons by various bacteria has been reported by many authors [51,52]. The differential degradation of hydrocarbons could be due to toxicity and recalcitrance effects. Petroleum hydrocarbons are usually degraded in the following order *n*-alkanes > cycloalkanes and branched alkanes > aromatic hydrocarbons > resins and asphaltenes [51,53]. The differential utilization of the aliphatic hydrocarbons in the *n*-hexane-soluble fractions may reflect the different degradation extents.

The isolated bacteria appear not to be capable of utilizing the *n*-C16 and *n*-C20 hydrocarbons because no significant changes could be observed in the relative amounts of these compounds. The observed increase in the *n*-C20 hydrocarbons in the NCE3 culture can be attributed to recalcitrance or biotransformation of long-chain alkanes to *n*-C20. The same might apply for the observed increase in the *n*-C15 alkanes in the NBHCO2, I-19, and NKCO2-2 cultures. The reduction in the relative amounts of the *n*-C12, *n*-C13, and *n*-C18 hydrocarbons indicates utilization of the compounds by the tested bacterial isolates. The results reported by some authors are in a good agreement with the results reported here. Recently, Wang et al. [48] studied the degradation of crude oil by a novel *Dietzia* strain isolated from a deep oil reservoir. After 5 days of cultivation on crude oil, they observed a decrease in hydrocarbons with the chain length ≤ *n*-C25 and increase in the relative abundance of the ≥ *n*-C28 hydrocarbons. After 22 days of incubations, the ratios of ≤ *n*-C22 and ≥ *n*-C28 hydrocarbons decreased and there was an accumulation of hydrocarbons ranging from *n*-C23 to *n*-C27.

Palittapongarnpim et al. [54] reported that degradation of crude oil by some yeasts revealed decrease in the amount of long chain alkanes and accumulation of short chain fractions around *n*-C10. They explained these results by assuming that the long chain alkanes are degraded and transformed to short chain ones. Biosurfactants/bioemulsifiers interact variably with different hydrocarbons [48,55]. This differential behavior should preferentially enhance the aqueous solubility of certain hydrocarbons and consequently, promote their degradation over the less bio-available compounds.

Nanoparticles are gaining increasing interest due to their unique physicochemical properties and potential applications in the environmental and biological fields [19]. Very few studies have addressed the impact of nanoparticles on bioremediation and biodegradation of environmental pollutants. In this study, ZnO nanoparticles were chosen to test their effect on the growth of NBHCO4 and crude oil biodegradation. The results showed a negative impact of ZnO nanoparticles on the growth and crude oil degradation extent. The slower growth and lower degradation percentages of the ZnO–containing culture is probably due to some sort of inhibitory effect of ZnO nanoparticles. The observation that the effect of ZnO nanoparticles on the NBHCO4 culture was concentration-dependent further corroborates the inhibitory action. Some types of nanoparticles including ZnO, exhibit antimicrobial activity against several bacteria [22]. Recently, Tilston et al. [56] reported that nanoscale zerovalent iron inhibited microbial degradation of chloroaromatic compounds in soil. In contrast Fe/Ni nanoparticles promoted the biodegradation of phenol [57]. Obviously, the effect of nanoparticles on the biological degradation of environmental contaminants awaits further investigations.

Dispersion/emulsification of crude oil in the culture medium suggests the presence of tensioactive agents. Production of biosurfactants is known amongst crude oil and hydrocarbon-degrading bacteria [14,16]. This is another way by which hydrocarbonoclastic microbes circumvent the hydrophobicity and low aqueous solubility of the hydrocarbon substrates. In contrast to the proposed bacterial adherence to the oil droplets which was inferred from the observed fluctuations in culture density, the production of biosurfactants was confirmed by the observed reduction in surface tension in the I-19 crude oil culture. For the other crude oil cultures, the extent of surface tension reduction, as compared to the uninoculated control, was so small to conclude biosurfactants production. Probably, crude oil inhibited biosurfactants production or the tensioactive agent may be a bioemulsifier-type biosurfactant [58]. Alternatively, the bacterial cells themselves can act as biosurfactants/bioemulsifiers [59] or the produced biosurfactants are not excreted to the extracellular medium. This could be corroborated by the fact that the surface tension measurements were performed in cell-free culture supernatants. Moreover, dispersion of crude oil in most of the cultures was observed a few hours after the incubation. It is also worth mentioning that the presence of oils in the growth medium usually interferes with surface tension measurements [25]. Finally, the role of biosurfactants in biodegradation of hydrophobic compounds is still controversial [14].

The large reduction in surface tension observed in most of the glucose cultures may be due to biosurfactants that are qualitatively and/or quantitatively different from those produced in the crude oil cultures. Nayak et al. [60] reported that an efficient biosurfactant reduces the surface tension of the culture medium by more than 20 mN/m. This agrees with the surface tension measurements performed in the NBHCO2, I-19, NBHCO4, NBHCO2, NKCO2-2, and NCEOW cultures. The disability of these bacteria to reduce the surface tension in the crude oil cultures, similar to what they did in the glucose cultures, suggests that biosurfactants production might be prohibited. The carbon source is a significant determinant of the type, yield, and activity of the produced biosurfactants [61]. Glucose is widely used for biosurfactant production by many bacteria [61,62]. The biosurfactant produced by the NBHCO2 strain is a lipopeptide as confirmed by the detection of the relevant genes. Neither genes of lipopeptide nor those of rhamnolipid biosurfactants production could be detected in the other tested bacteria. This suggests that these bacteria may produce other types of biosurfactants/bioemulsifiers.

The absence of turbidity in most of the cultures containing water-in-oil emulsions (chocolate mousse) shows the inability of the tested bacteria to utilize these emulsions. Water-in-oil emulsions are known to be microbiologically recalcitrant [63]. This explains why most of the tested bacteria could not grow on them. Interestingly, two of the

isolated bacteria, NBHCO2 and NCEOW, transformed chocolate mousse into oil-in-water emulsions after prolonged incubation. This was concomitant with the appearance of turbidity in cultures of these two isolates. It can be concluded that both of these isolates are able to emulsify chocolate mousse and utilize the resulting oil-in-water emulsions after prolonged incubation. Similar results were reported for *Pseudomonas* sp. and *Rhodococcus* sp. [46,64].

Conclusions

From the results it can be concluded that oilfields in Kuwait and Bahrain represent a good source of petroleum-degrading bacteria. The multiple phenotypes (crude oil degradation and biosurfactants production) of the isolated bacteria make them promising for bioaugmentation of petroleum-polluted soil in the Arabian Gulf region as well as biosurfactant production. We showed that commercially available ZnO nanoparticles can interfere with the biodegradation process. However, it is important to conduct in-depth investigations of the impact of nanoparticles on the growth and biodegradation capacity of environmental microorganisms. Biodegradation of crude oil by some bacteria may occur without production of biosurfactants. However, the role of biosurfactants, in the bioremediation/biodegradation processes needs further investigations.

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