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Microbial-Enhanced Heavy Oil Recovery under Laboratory Conditions by *Bacillus firmus* BG4 and *Bacillus halodurans* BG5 Isolated from Heavy Oil Fields

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Abstract: Microbial Enhanced Oil Recovery (MEOR) is one of the tertiary recovery methods. The high viscosity and low flow characteristics of heavy oil makes it difficult for the extraction from oil reservoirs. Many spore-forming bacteria were isolated from Oman oil fields, which can biotransform heavy crude oil by changing its viscosity by converting heavier components into lighter ones. Two of the isolates, *Bacillus firmus* BG4 and *Bacillus halodurans* BG5, which showed maximum growth in higher concentrations of heavy crude oil were selected for the study. Gas chromatography analysis of the heavy crude oil treated with the isolates for nine days showed 81.4% biotransformation for *B. firmus* and 81.9% for *B. halodurans*. In both cases, it was found that the aromatic components in the heavy crude oil were utilized by the isolates, converting them to aliphatic species. Core flooding experiments conducted at 50 °C, mimicking reservoir conditions to prove the efficiency of the isolates in MEOR, resulted in 10.4% and 7.7% for *B. firmus* and *B. halodurans*, respectively, after the nine-day shut-in period. These investigations demonstrated the potential of *B. firmus* BG4 and *B. halodurans* BG5 as an environmentally attractive approach for heavy oil recovery.

Keywords: spore forming bacteria; *Bacillus firmus*; *Bacillus halodurans*; Microbial Enhanced Oil Recovery; biotransformation; heavy oil recovery

1. Introduction

Global energy requirements demand an increased production of crude oil. During conventional recovery methods, about 30–40% of crude oil is recovered while rest remains trapped in the reservoir [1–4]. Enhanced oil recovery (EOR) targets the trapped crude oil. Crude oil is a fossil fuel which is considered as non-renewable energy source. It is composed of a mixture of different hydrocarbons (including alkanes/paraffins, alkenes/olefins, cycloalkanes/naphthenes, and aromatics), complex hydrocarbons (such as polycyclic aromatic hydrocarbons), resins, asphaltenes, along with certain other hetero-species, containing nitrogen, oxygen and sulfur [5]. Heavy crude oil is characterized by high density or specific gravity, more resistant to flow with an American Petroleum Institute (API) gravity of less than 20°. Extraction of heavy crude oil needs higher energy input. Current methods of extraction include open-pit mining, steam stimulation, the addition of sand to the oil, and the injection of air into well to create subterranean fires that burn heavier hydrocarbons to

generate heat. Transportation of these types of crude oil through pipelines poses much difficulty and requires certain diluting agents. Sometimes heavy and light crude oils are mixed to facilitate transport through pipeline. This will result in contamination of the light crude and a reduction in its value [6].

Enhanced oil recovery (EOR) is a tertiary method of extracting residual oil from the reservoirs after the primary and secondary phases of production. EOR methods adopted will either modify the properties of reservoir fluids and/or the reservoir rock characteristics such as reducing the interfacial tension between oil and water, reducing oil viscosity, and displacing oil through porous rocks [7,8].

Microbial enhanced oil recovery (MEOR) has become an important, fast developing tertiary recovery method which uses microorganisms or their metabolites to enhance the recovery of residual oil [7–10]. MEOR is different from conventional EOR methods such as CO_2 injection, steam injection, chemical surfactant and polymer flooding, in that it involves injecting live microorganisms and nutrients into the reservoir so that bacteria and their metabolic products mobilize the residual oil. It is considered to be a more environmentally friendly method since it does not involve any toxic chemicals and it is easy to carry out in fields since it does not need any modifications of existing water-injection amenities [11–13]. MEOR takes place by different mechanisms, such as reduction of oil-water interfacial tension and alteration of wettability by surfactant production, selective plugging by microorganisms and their metabolites, oil viscosity reduction by gas production or degradation or biotransformation of long-chain saturated hydrocarbons, and production of acids which improves absolute permeability by dissolving minerals in the rock [14]. The microbial metabolic products include biosurfactants, biopolymers, acids, solvents, gases, and enzymes. The bacteria used in MEOR are usually hydrocarbon-utilizing, non-pathogenic, and are naturally occurring in petroleum reservoirs [15].

Biological processing of heavy oil is a cost-effective and eco-friendly approach which provides a higher selectivity to specific reactions to upgrade heavy oil. Microbial systems which are capable of biotransforming oil fractions are used in heavy oil reservoirs for increased oil recovery by reducing the oil viscosity [16]. Many microorganisms capable of biotransforming hydrocarbons using crude oil as the sole carbon source have been identified [16–22]. A successful field trial using oil biotransforming bacteria without injection of nutrients has been reported [3,23]. The role of spore-forming bacteria in crude oil biotransformation, and competent *Bacillus* strains existing in many oil-polluted sites have been widely studied [24–28]. The economy of countries, such as Oman, is highly dependent on revenues generated from crude oil production and a cost effective, environmentally friendly alternative method of upgrading and producing heavy crude oil will be a significant benefit. Also, the transportation of heavy oil through pipelines will be facilitated by biotransformation. The goal of this study was therefore to demonstrate the potential of *Bacillus halodurans* and *Bacillus firmus* for the biotransformation of heavy crude oil (4.57°API).

2. Materials and Methods

All chemicals and media were from Sigma-Aldrich Co. (St. Louis, MO, USA), Analytical Reagent (AR) grade.

2.1. Culture Media and Cultivation

Two different media were used for the isolation of bacterial cells for the biotransformation study, Bushnell-Haas (BH) [29] and mineral salt (Medium C) [30]. Medium C (pH = 7 ± 0.2) contained (g L^{-1}): NH_4NO_3 (4.002); KH_2PO_4 (4.083); Na_2HPO_4 (7.119); $MgSO_4 \cdot 7H_2O$ (0.197). To this was added, 1 mL of trace metal solution containing (g L^{-1}): $CaCl_2$ (0.00077); $FeSO_4.7H_2O$ (0.0011); $MnSO_4 \cdot 4H_2O$ (0.00067); Na-EDTA (0.00148). The BH medium (pH = 7 ± 0.2) consisted of (g L^{-1}): $MgSO_4$ (0.2); $CaCl_2$ (0.02); KH_2PO_4 (1.0); K_2HPO_4 (1.0); NH_4NO_3 (1.0); $FeCl_3$ (0.050). All media were sterilized by autoclaving at 121 °C at 15 psi for 15 min.

2.2. Characterization of Soil and Oil Samples

A total of 10 different soil samples were characterized for pH, mineralogy analysis using X-ray diffraction (XRD), extractable total petroleum hydrocarbons (eTPH) and moisture content as described previously [20]. Briefly, the heavy crude oil contaminated soil samples were mixed with anhydrous sodium sulfate to remove moisture in a capped conical flask. The eTPH was estimated by mixing 10 g of sample with 30 mL of dichloromethane (DCM, high pressure liquid chromatography (HPLC) grade, 99% pure), capped tightly, mixed well by inverting the flasks several times and then transferred to a mechanical shaker for 4–5 h and allowed the sediments to settle for 1 h. The solvent with the hydrocarbon was filtered through Whatman® qualitative filter paper, Grade 1 110 mm into a pre-weighted conical flask and allowed to concentrate overnight [31–33]. The moisture content of the soil samples were found to be in the range 0.018 to 0.024 m^3/m^3. The heavy crude oil viscosity was measured using a Rheolab QC rotational viscometer and API gravity with a DSA 5000 M density meter.

2.3. Isolation of Spore Forming Bacterial Strains Using Heavy Crude Oil as Carbon Source

Spore-forming bacteria were isolated from soil samples contaminated with heavy crude oil. The sampling site was a contaminated area near oil wells of one of the oil rigs in Oman. The subsurface soil samples (8 cm below surface) were aseptically collected from seven different regions in random manner around each well and mixed together. The soil samples were collected with pre-sterilized shovels into sterilized bags, properly labelled and transferred to the laboratory and stored at 4 °C until use. Heavy crude oil samples used in the study were collected from the oil field in sterile bottles and stored for further studies.

For the isolation of spore forming isolates, 1 g of soil sample mixed with 10 mL distilled water was vortexed thoroughly and the vegetative cells were killed by boiling the mixture in a water bath at 90 °C for 30 min [20,26]. 5 mL of the supernatant served as an inoculum for the first enrichment in both media in 250 mL conical flasks. 1% (w/v) heavy crude oil was added to the media used for the isolation as the sole carbon source. The flasks inoculated with the supernatant were incubated at 40 °C, 160 rpm for two weeks. A negative control flask without heavy crude oil was set up and incubated at the same conditions. A 1% (w/v) aliquot from the first enrichment served as the inoculum for second enrichment which was incubated at the same conditions for a further one week period. The enrichment technique for the isolation of bacteria has already been reported [34,35]. The dilutions from both the first and second enrichments were spread-plated on corresponding fresh agar plates and incubated at 40 °C for 24 h. Well-isolated single colonies were picked up carefully and by successive streaking in fresh agar plates resulted in pure isolates, which were stored in 60% (v/v) glycerol stock solution at −80 °C.

2.4. Identification of Bacillus firmus and Bacillus halodurans

Among the 40 isolates studied, the ones which showed maximum growth on agar plates were identified using a MALDI Biotyper (Bruker Daltonik GmbH, Bremen, Germany) [36] and 16S rDNA sequencing. For the Biotyper identification, a direct smearing method was used where 24 h-grown pure cultures were smeared on the target plate and layered with 1 µL sinapinic acid. The target plate was inserted in the Matrix Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometer (MALDI-TOF/MS) instrument and the protein fingerprints were generated. The integrated software generates an outcome list, by comparing the fingerprint of the reference sample with the reference spectra in the database, in which species with the most similar fingerprints are ordered according to their logarithmic score value (log (score value)) [37].

16S ribosomal DNA (rDNA) sequencing was performed using 27F and 1492R primers of the genomic DNA isolated using PowerSoil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), as reported before [20]. The amplification reaction (Polymerase Chain Reaction (PCR)), was performed using T100 thermal cycler. The amplification reaction was performed on a total volume

of 25 µL containing: 12.5 µL master mix (Taq polymerase and deoxynucleotide triphosphate (dNTP) mix), 9.5 µL double distilled water, 1 µL extracted DNA and 1 µL of each primer. PCR amplification was performed with an initial denaturation step at 94 °C for 3 min followed by 35 cycles of a 1 min denaturation step at 94 °C, a 2 min annealing step at 53 °C, and a 2 min elongation step at 72 °C, with a final extension step at 72 °C for 7 min using a 2720 thermal cycler. The PCR products were detected in 1.6% agarose gel electrophoresis. The PCR products were purified using QIAquick PCR purification kit (QIAGen, Carlsbad, CA, USA). The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Foster City, CA, USA) was used for de-novo sequencing. The sequencing was done using 3130 XL Genetic Analyzer (Applied Biosystem-Hitachi, Waltham, MA, USA). The sequences of the 16S ribosomal RNA (rRNA) genes identified in this study were submitted to the NCBI GenBank databases under the accession numbers KP119100 and KP119100.

2.5. Growth Characteristics during Biotransformation under Aerobic Conditions

The effect of heavy crude oil concentration on the growth of the isolates in BH medium was studied for a period of 10 days. The BH medium with 1% *w*/*v*, 3% *w*/*v*, 5% *w*/*v* and 7% *w*/*v* of heavy crude oil was inoculated with the two strains of bacteria, *B. firmus* and *B. halodurans* and incubated at 40 °C and 160 rpm. One-way ANOVA was conducted to determine if the heavy crude oil concentration had an effect on the growth of the isolate. A Kruskal-Wallis test was done to evaluate the effect of crude oil concentration in the pH of the culture medium.

2.6. Biotransformation Studies Using GC-MS

Isolates, *B. firmus* and *B. halodurans* were incubated in BH medium containing 1% heavy crude oil as the sole carbon source for a period of nine days to determine the biotransformation potential of the isolate under aerobic conditions. Seed cultures of the corresponding isolates were prepared from 24 h grown isolates in Luria-Bertani broth at 40 °C and 160 rpm. One percent (*v*/*v*) of the seed culture served as the inoculum for 100 mL BH medium with 1% heavy crude oil and incubated at the same conditions described. The contents of each flask for each isolate were extracted on the third, sixth, and ninth days of incubation for GC-MS analysis. All experiments were done in triplicate. The cell free extract was analyzed for the production of biosurfactant using Drop Shape Analyzing system-DSA 100 (Krüss GmbH, Hamburg, Germany) by measuring the surface tension (ST) and interfacial tension (IFT). IFT was measured against *n*-hexadecane.

The extraction of the biotransformed heavy crude oil at the third, sixth, and ninth days of incubation by the isolates were done by vigorously mixing the contents with 20 mL DCM in a separating funnel allowing the mixture to separate to different fractions. The DCM fraction with biotransformed heavy crude oil was collected carefully in a glass collection tube. The collected fraction was then purified by passing through silica G-60. The column was sequentially eluted with hexane to obtain the aliphatic fractions and then with hexane:DCM (1:1) to elute the aromatic fractions [38].

The fractions were analyzed by GC MS/MS with DB 5 capillary column (30 m × 0.32 mm internal diameter, 0.1 mm thickness) (Waters, Quattro Micro™ GC MS/MS, Micromass UK Ltd., Wilmslow, UK) following EPA Method 1655 [39]. Helium was used as a carrier gas and a constant flow rate of 2 mL/min was set. Injector and detector temperatures were 350 and 370 °C, respectively. The oven temperature program was: initial temperature 50 °C for 1 min, raised to 350 °C at a rate of 10 °C/min, and a hold at 370 °C for 1 min.

2.7. Core Flooding Experiments

The core flooding experiments were performed to study the ability of the isolates to degrade heavy crude oil under anoxic conditions and to evaluate the potential of the strain in heavy oil recovery. The heavy crude oil sample used in the core flood experiments was degassed and dehydrated. The brine was purged with nitrogen. The Berea sandstone cores (absolute permeability 350–360 × 10^{-2} µm^2) were cleaned in methanol using a Soxhlet apparatus. The cleaned cores after being dried at 80 °C for

24 h were saturated with filtered, sterilized formation water for 12 h in a desiccator under vacuum. The formation water was collected from one of the Oman heavy oil fields. The characteristics of brine was as reported before [20]. The cores were then placed in the core flood apparatus and heated in the oven provided in the system to 50 °C, mimicking the reservoir condition. The pore volume was calculated as the difference in the wet and dry weights of the core and was flooded with four pore volumes (PV) of brine at 0.4 cm^3/min to ensure 100% brine saturation and to degas the core. The cores were then injected with heavy crude oil until no more water was produced until it reached the irreducible water saturation (S_{wr}). The initial oil saturation was calculated volumetrically from the amount of injected oil and produced water. Secondary recovery of the heavy oil was done by flooding the core with brine at a rate of 0.4 cm^3/min, until no more oil was produced. The residual crude heavy oil in the core was measured from the volume of oil produced.

For the core flooding experiment, the mother inoculum was prepared by 24 h-grown isolates in Lysogeny broth (LB) medium (in Luria Bertani broth) (OD_{620} = 1.324; 1.06 × 10^9 CFU/mL for *B. firmus* and OD_{620} = 1.672; 1.34 × 10^9 CFU/mL for *B. halodurans*). Freshly prepared sterile BH medium was added to the mother inoculum in a ratio of 1:4. One PV of the mixture was injected into the core and the system was shut in for 9 days at 50 °C. For evaluating the potential of the strains in extra heavy oil recovery, after the shut in period, the extra recovered oil was collected in graduated tubes by flooding with brine, and then measured. A control experiment was performed at same conditions, but without the injection of the isolates. The effluent collected during the tertiary recovery was tested for the presence of the isolates by MALDI Biotyper. The extra recovered oil was analyzed by GC-MS for determining the biotransformation of heavy crude oil. Scanning electron microscopy (SEM; JEOL, JSM-7600F Field Emission SEM, Tokyo, Japan) analysis of the core specimen from the outlet, middle and inlet portions was done after fixation using glutaraldehyde and osmium, dehydration using ethanol and critical point drying. The specimens were then mounted on stubs and were coated with gold using sputter coater for SEM analysis [20].

2.8. Statistical Analysis

All data analyses were done using the statistical software MINITAB 14 (Minitab, Ltd., Coventry, UK) with a maximal Type 1 error rate of 0.05. Kruskal-Wallis non parametric test was used where the assumptions of analysis of variance (ANOVA) were not met.

3. Results

3.1. Characterization of Soil and Oil Samples

The heavy crude oil-contaminated soil samples were collected and stored appropriately. The pH of the 10 soil samples were measured as 8.5 ± 0.5. The eTPH of the soil samples were ~4.2% and the moisture content of ~0.024 m^3/m^3. The mineral compositions of the 10 soil samples measured by XRD showed that all of the soil samples contained calcite and quartz; albite and palygorskite were present in 8 soil samples out of 10 samples tested. Other minerals observed were anorthite, dolomite, gypsum, halite, microcline, muscovite, rutile, suhailite and takanelite (Table 1). The heavy crude oil sample viscosity was determined as 650,000 mPa·s and as 4.57° API.

3.2. Isolation and Identification of Oil-Oxidizing Bacteria, Bacillus firmus and Bacillus halodurans

The isolates that were capable of utilizing heavy crude oil as carbon source were isolated based on their morphology. The isolates which showed maximum growth on agar plates in short period of time were selected for the study. The isolates were identified initially by MALDI-Biotyper as *Bacillus firmus* and *Bacillus halodurans* with a score value above 1.8. Phylogenetic analysis of the 16S rRNA genes of the isolates BG4 and BG5 revealed >97% similarity to the sequences of *Bacillus firmus* and *Bacillus halodurans*, respectively.

Table 1. Minerology of soil samples from XRD analysis.

Mineral	Soil Samples									
	SA	**SB**	**SC**	**SD**	**SE**	**SF**	**SG**	**SH**	**SI**	**SJ**
albite	1	1	0	1	0	1	1	1	1	1
anorthite	1	0	0	0	0	0	0	1	0	0
calcite	1	1	1	1	1	1	1	1	1	1
dolomite	0	1	0	0	0	0	0	0	0	0
gypsum	1	0	1	1	0	1	1	0	1	1
halite	0	1	0	1	0	0	1	1	0	1
microcline	0	0	0	0	0	0	0	1	1	0
muscovite	0	0	0	0	0	1	0	0	0	0
palygorskite	1	1	1	1	1	1	1	0	1	0
quartz	1	1	1	1	1	1	1	1	1	1
rutile	0	0	1	0	0	0	0	0	0	0
suhailite	0	1	0	0	0	0	0	0	0	0
takanelite	0	0	0	0	1	0	0	0	0	0

1 = present; 0 = absent.

3.3. Growth Characteristics of Bacteria during Crude Oil Degradation under Aerobic Conditions

The growth characteristic study of the two isolates showed that heavy crude oil concentration up to 7% (*w*/*v*) had no significant effect on pH for both the isolates, where the pH increased from ~7.5 to ~9.5, at all crude oil concentrations. In contrast, the OD_{620} values showed significant effects for 1 and 3% (*w*/*v*) heavy crude oil concentrations for *B. firmus* in BH medium, while no significant effect was found for *B. halodurans*. Statistical analysis was performed using MINITAB 14 for determining the effect of heavy crude oil on the growth of the isolates. The ANOVA *p*-value for OD_{620} for *B. firmus* was $p = 0.004 < 0.05$ and the post hoc analysis, Tukey test showed that the growth rate at 1% and 3% (*w*/*v*) was significantly different from 5% and 7% (*w*/*v*) (Figures 1 and 2).

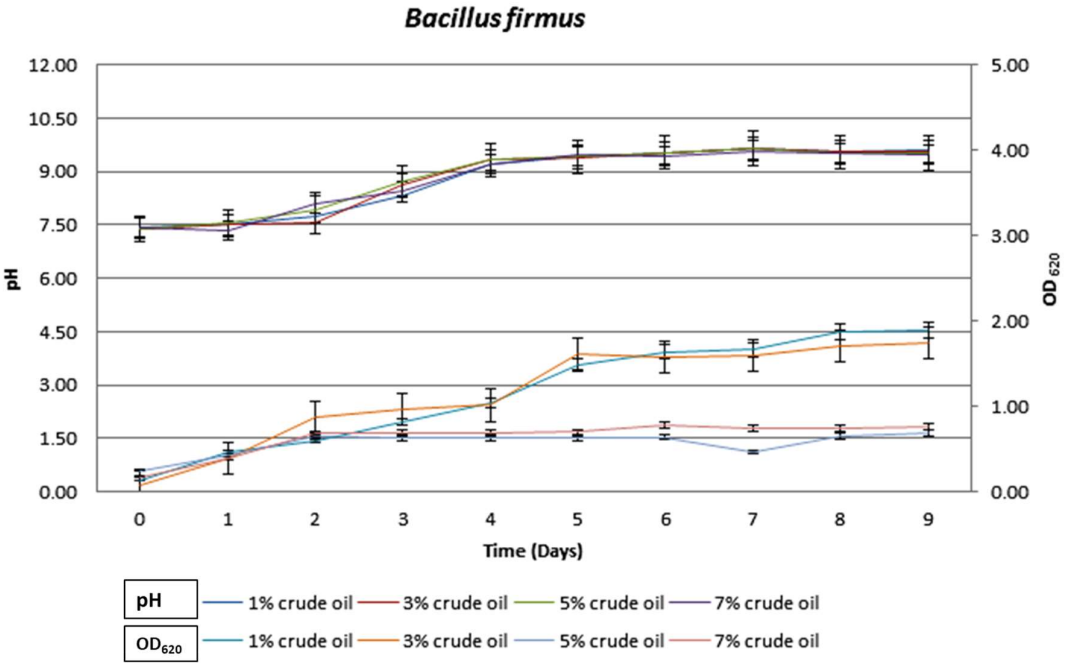


Figure 1. Growth profile of *B. firmus* in Bushnell-Haas (BH) medium. The isolate showed higher growth in presence of 1% and 3% heavy crude oil.

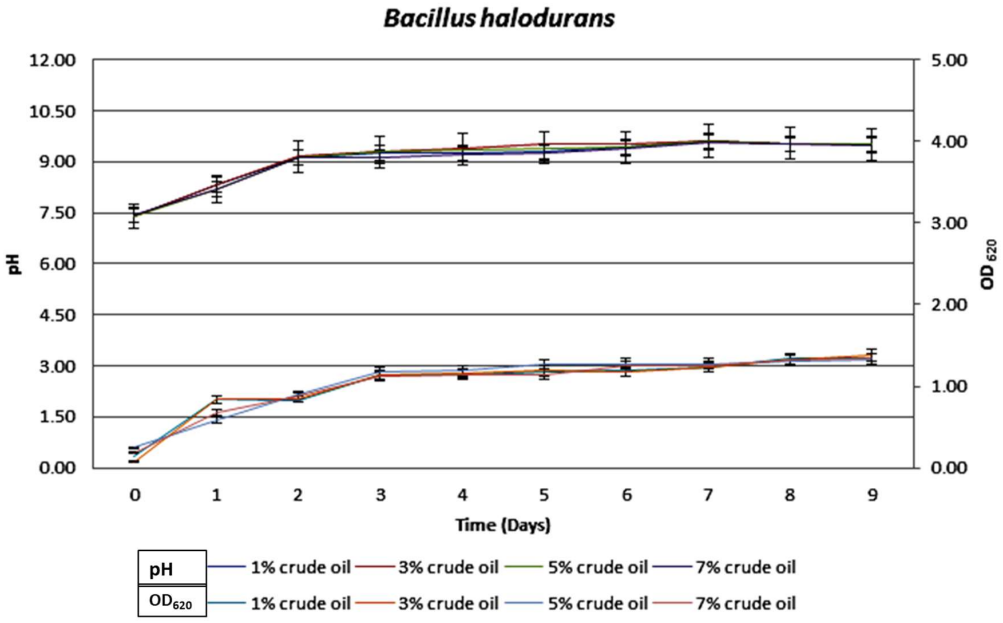


Figure 2. Growth profile of *B. halodurans* in BH medium. There was no effect of heavy crude oil concentrations up to 7% for the isolate.

3.4. Biotransformation Studies Using GC-MS

The biotransformed heavy crude oil incubated with *B. firmus* and *B. halodurans* for nine days in BH medium was extracted with DCM on the third, sixth and ninth days of incubation and was purified by passing through Silica G60 column. The fractions sequentially eluted with hexane and hexane:DCM (1:1) were analyzed using GC-MS. The analysis showed 81.36% biotransformation of heavy crude oil for *B. firmus* and 81.93% for *B. halodurans* compared to the abiogenic control. The total aromatic fractions reduced during the period of incubation were 70.80% for *B. firmus* and 47.77% for *B. halodurans* and aliphatics with 58.22 and 88.21% respectively. An increase in the concentration of aliphatic compounds was also observed (Figures 3–6).

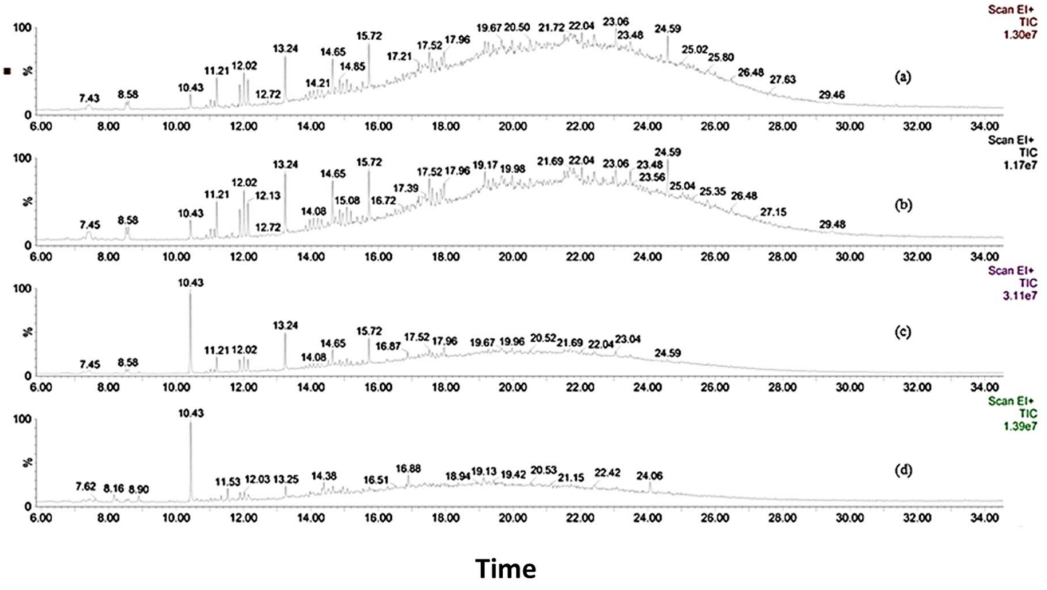


Figure 3. GC-MS chromatogram of heavy crude oil biotransformation by *B. firmus* on day 3 (**b**); day 6 (**c**); and day 9 (**d**); as compared to the control (**a**).

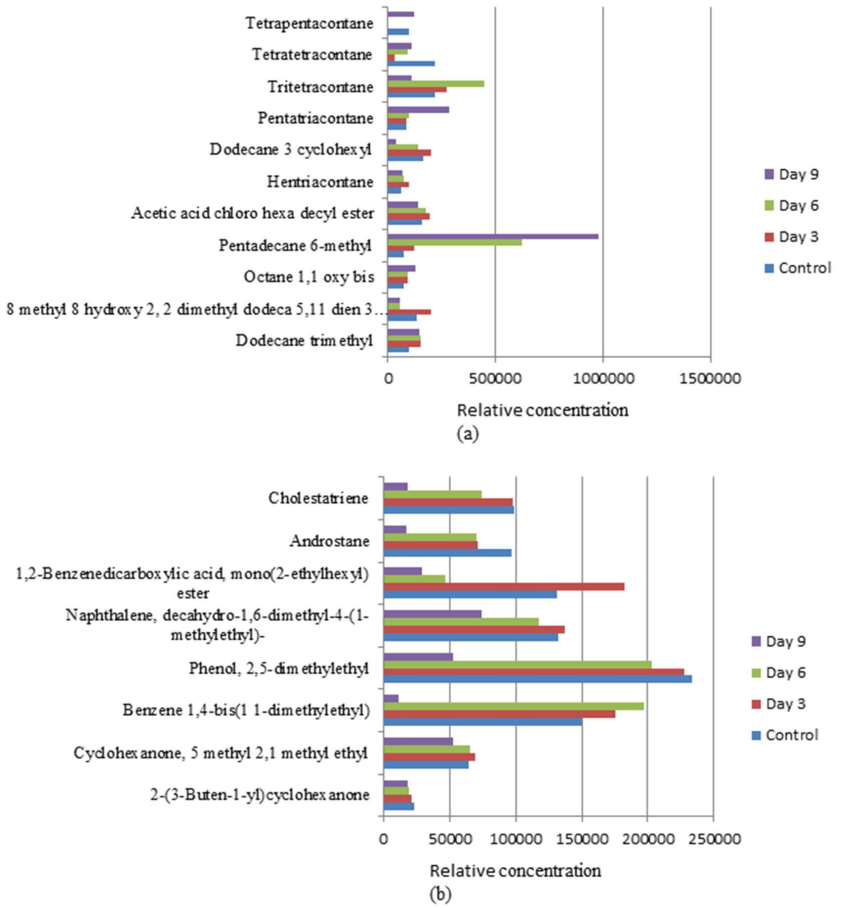


Figure 4. GC-MS analysis of bio-fractionated heavy crude oil by *B. firmus* (**a**) aliphatic compounds (**b**) aromatic compounds.

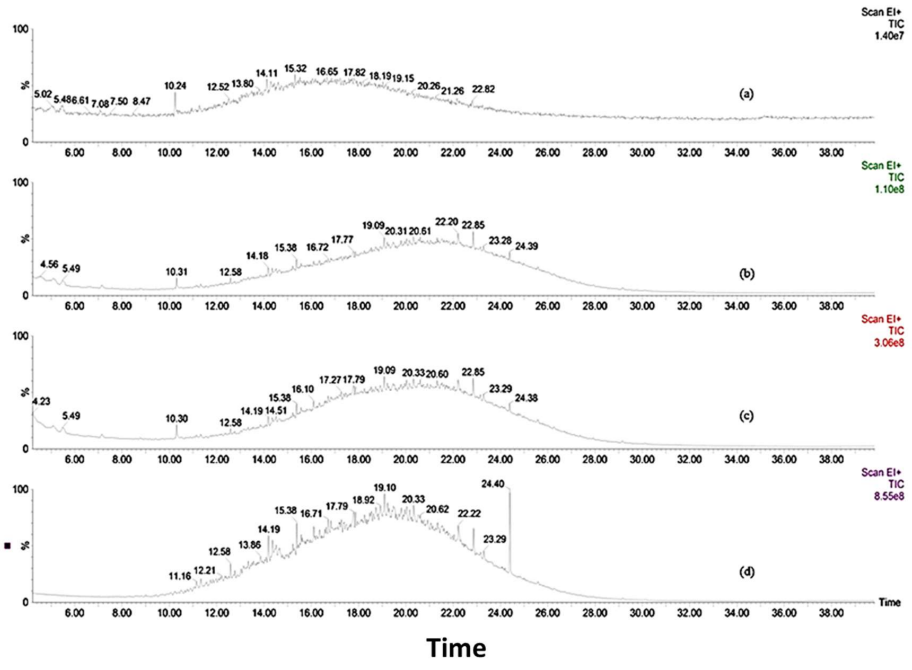


Figure 5. GC-MS chromatogram of heavy crude oil biotransformation by *B. halodurans* on day 3 (**c**); day 6 (**b**) and day 9 (**a**); as compared to the control (**d**).

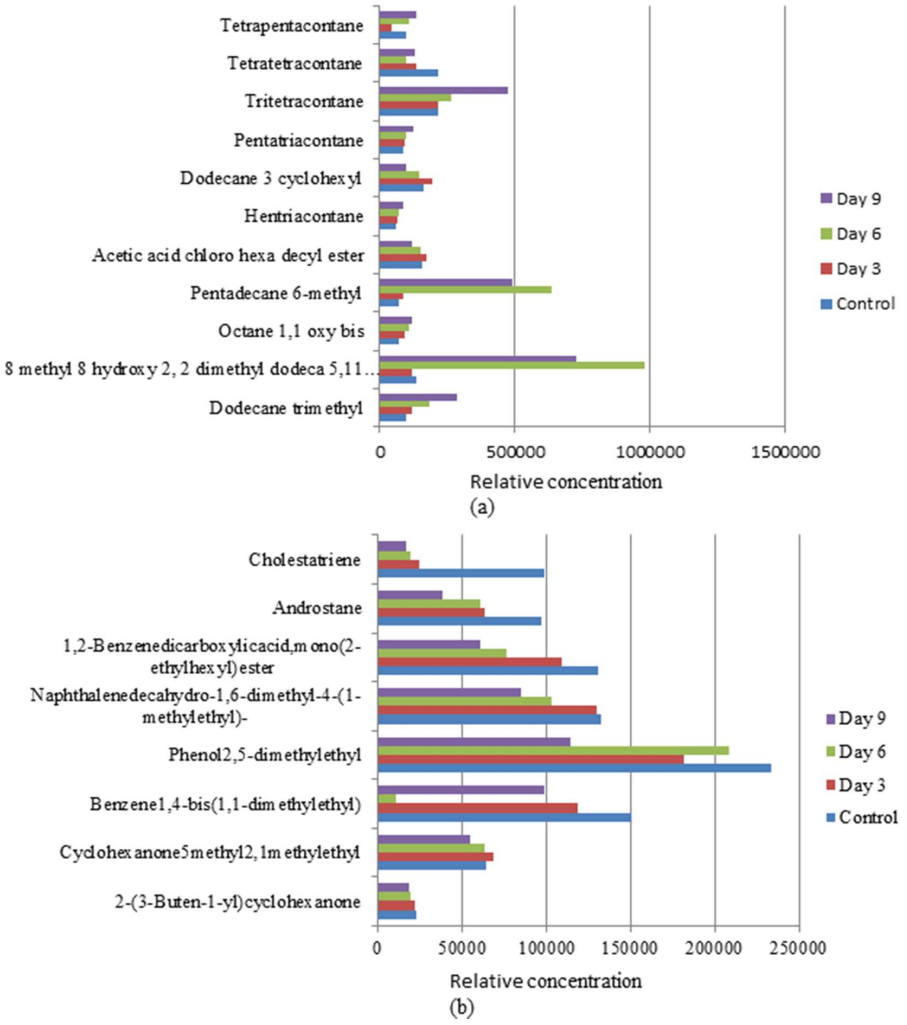
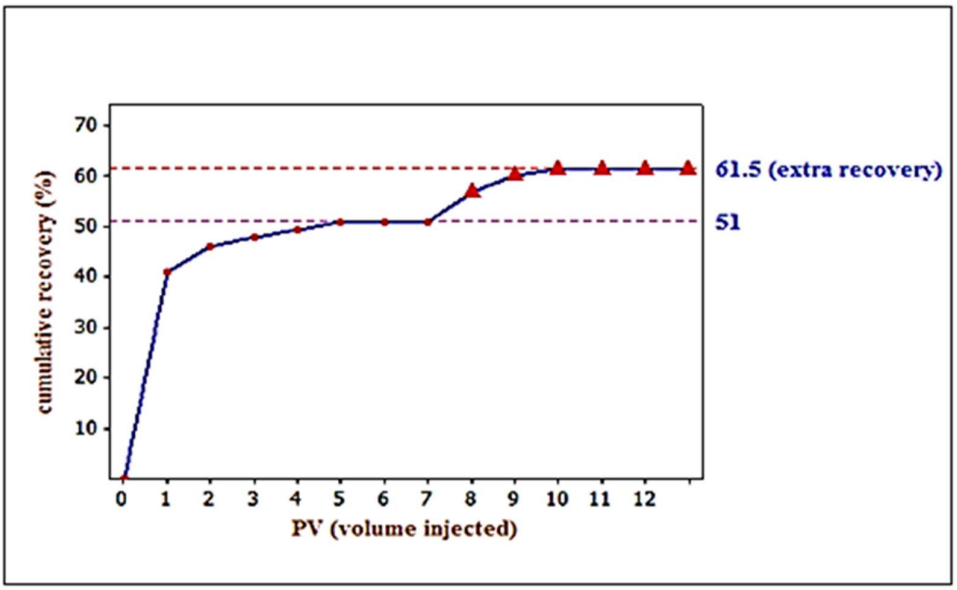


Figure 6. GC-MS analysis of bio-fractionated heavy crude oil by *B. halodurans* (**a**) aliphatic compounds (**b**) aromatic compounds.

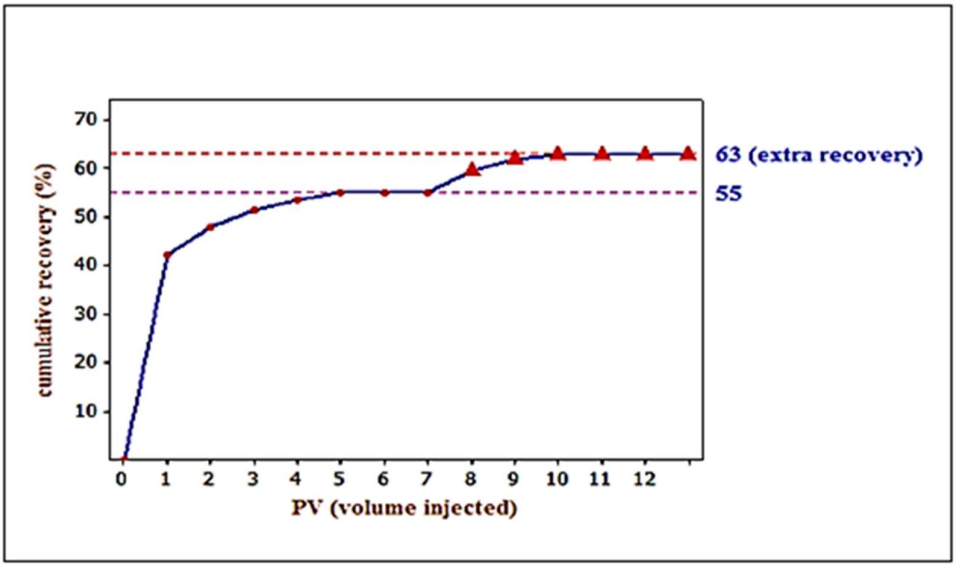
3.5. Core Flooding Experiments

Berea sandstone cores were used to evaluate the potential of isolates in heavy oil recovery. The experiment was conducted under anaerobic condition mimicking the oil field conditions. Throughout the experiment, the temperature was maintained at 50 °C and a pressure of 1000 psi. The oil initially in place (OIIP) in the core for *B. firmus* BG4 was 13.2 mL (S_{oi} = 79.6%). The water flooding (5 PV) resulted in recovery of 50.75% of initial oil (OI) corresponding to 6.7 mL of the initial oil present in the core. After nine days incubation with the isolate *B. firmus* BG4, the system was again injected with 5 PV of brine that resulted in a total recovery of 61.22% (7.18 mL) of initial oil present in the core, in which 10.46% (0.68 mL) was contributed by the action of the isolate.

The OIIP in the core for *B. halodurans* BG5 was 14.2 mL, S_{oi} = 84.02%. The water flooding resulted in recovery of 54.92% (7.8 mL) of OIIP for *B. halodurans*. The tertiary recovery by *B. halodurans* after nine days shut-in period resulted in 7.8% (0.5 mL) extra recovery of residual oil by the biotransformation of heavy crude oil compared to the control experiment. The extra recovery measurements were based on the residual oil (RO) present in the core (Figure 7a,b). No pressure changes were observed during bacterial flooding. The effluent analyzed using MALDI Biotyper revealed the presence of the isolates.



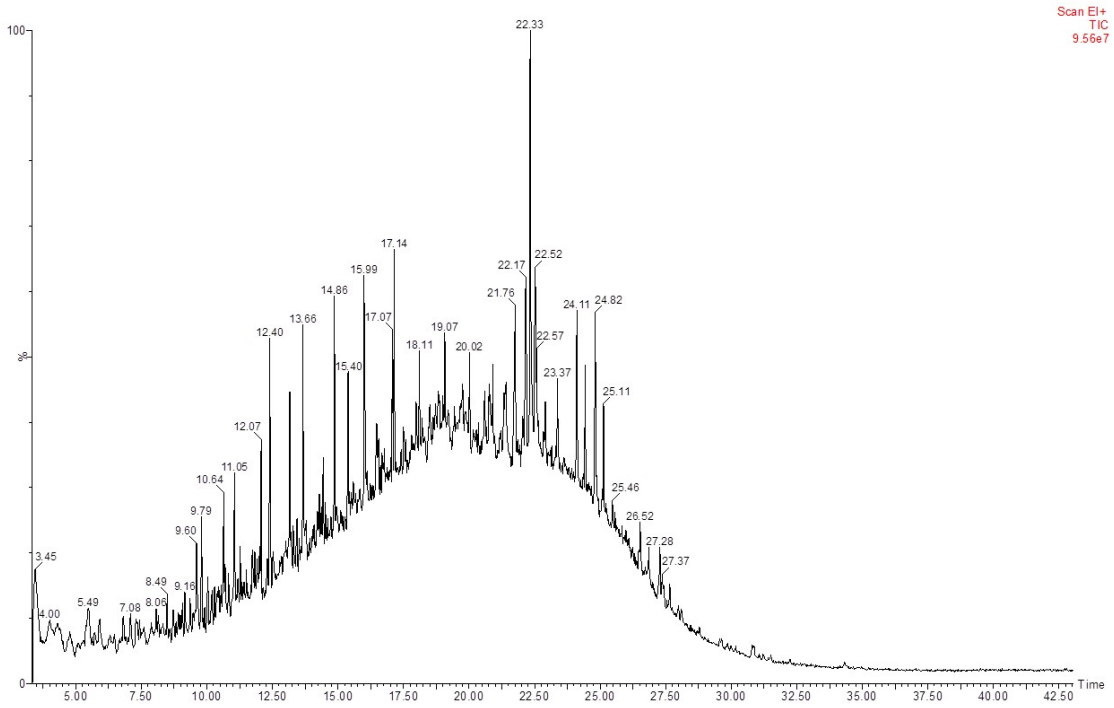
(a)



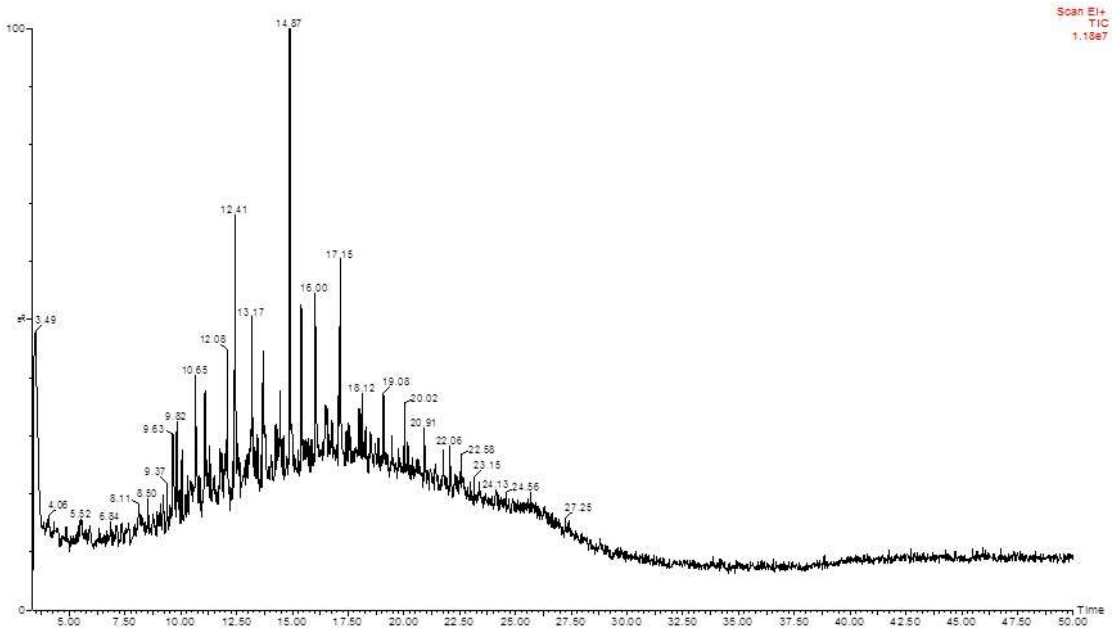
(b)

Figure 7. Cumulative oil recovery (**a**) by *B. firmus* and (**b**) by *B. halodurans*, after nine days shut-in period.

The migration of isolates inside the core was further determined by SEM analysis and the bioconversion of heavy crude oil was estimated by GC-MS analysis [3,40–42]. The extra recovered oil was analyzed using GC-MS which revealed that the biotransformation of heavy crude has occurred anaerobically. The percentage of aromatic compounds was reduced, and the concentration of lighter hydrocarbons has increased (Figure 8a–c; Tables 2 and 3). SEM analysis of the core indicated the presence of both isolates inside the core, which indicated their ability to grow anaerobically (Figures 9a,b and 10a,b).

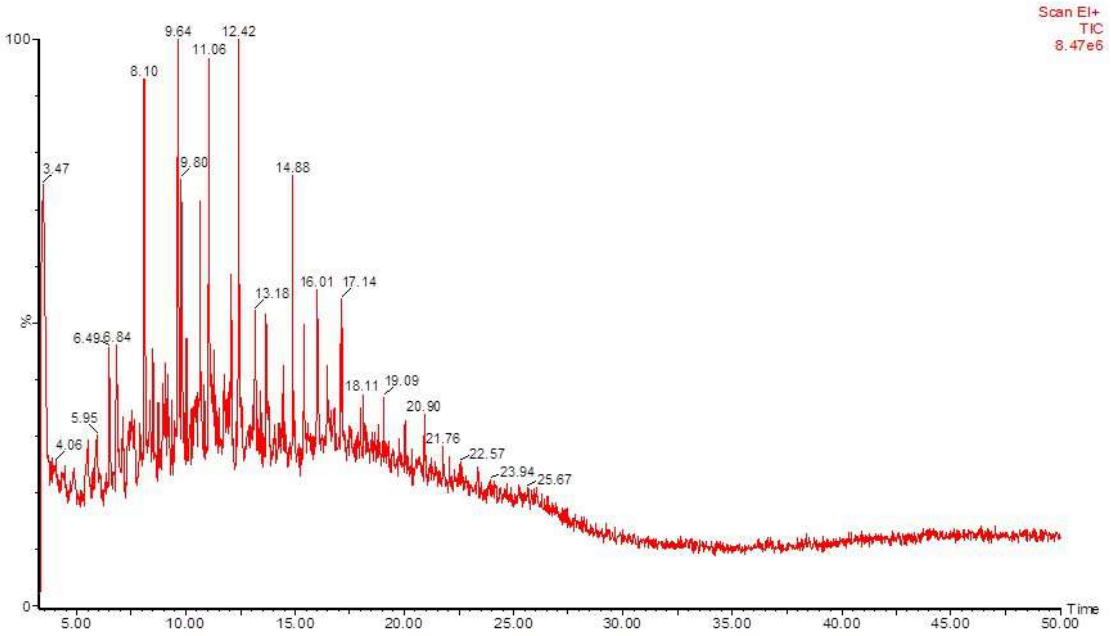


(**a**)



(**b**)

Figure 8. *Cont*.



(**c**)

Figure 8. GC-MS analysis of (**a**) control heavy crude oil used for core flooding experiment (**b**) extra recovered oil by the action of *B. firmus* during core flooding experiment (**c**) extra recovered oil by the action of *B. halodurans* during core flooding experiment.

Table 2. GC-MS chromatogram analysis for the extra recovered oil due to biotransformation by *B. firmus*.

RT	Identified Compound	Carbon No.
9.63	2-methyl-1-pentanol	C6
9.82	cycloheptanol	C7
10.06	1,2-dibromo-octane	C8
10.65	2,4,4-trimethyl-1-hexene	C9
11.08	1,2-dibromo-2-methyl-undecane	C12
12.41	1,2-dibromododecane	C12
13.17	3,7,11-trimethyl-1-dodecanol	C15
13.67	1-nonadecanol	C19
14.87	hexadecanoic acid, (3-bromoprop-2-ynyl) ester	C19
15.39	1-bromoeicosane	C20
17.15	5,15-dimethylnonadecane	C21
18.12	2-nitro-1,3-bis(octyloxy)benzene	C22
19.08	7-hexyldocosane	C28
20.02	11-decyldocosane	C32
21.76	tritriacontane	C33
22.06	1-hexadecylheptadecylcyclohexane	C39
22.58	tetratetracontane	C44

Table 3. GC-MS chromatogram analysis for the extra recovered oil due to biotransformation by *B. halodurans*.

RT	Identified Compound	Carbon No.
3.47	1,2-dibromo-2-methylundecane	C4
4.06	2-nitrocyclohexanone	C6
5.51	2,5-heptadecadione	C7
5.95	1,7-dichloroheptane	C7
6.49	2,2-dimethyl-3-pentanol	C7
6.84	1-chloro-heptane	C7
8.10	*N*-methylcyclohexanamine	C7
9.64	acetic acid, hexyl ester	C8
9.80	1,2-dibromo-octane	C8
11.06	1,2-dibromododecane	C12
12.42	1-chlorododecane	C12
13.18	1-nonadecanol	C19
14.88	1- eicosanol	C20
16.01	9-octadecenyl acetate	C20
17.14	dimethylnonadecane	C21
18.11	tetracosane	C24
19.09	7-hexyldocosane	C28
20.90	2-(1-decylundecyl)-1,4-dimethyl cyclohexane	C29
21.76	11-decyldocosane	C32
22.57	tritriacontane	C33

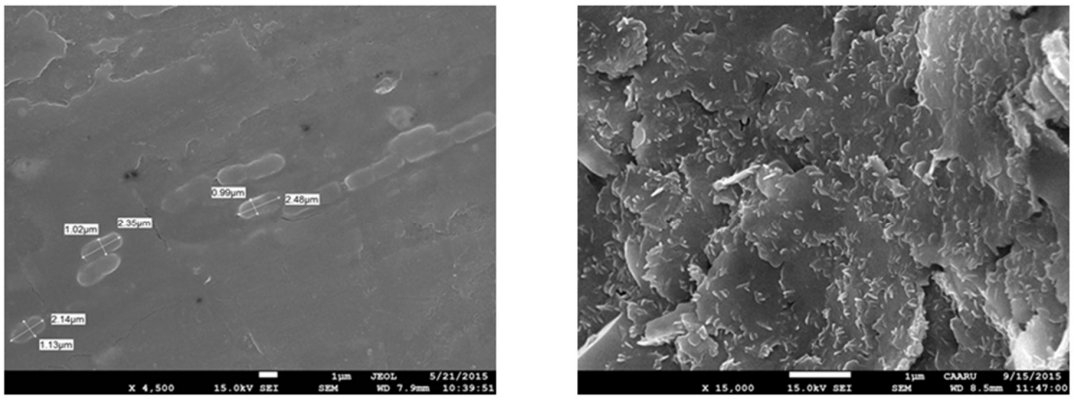


Figure 9. Scanning electron microscope (SEM) image (**a**) *B. firmus* in fresh BH medium; and (**b**) inside core.

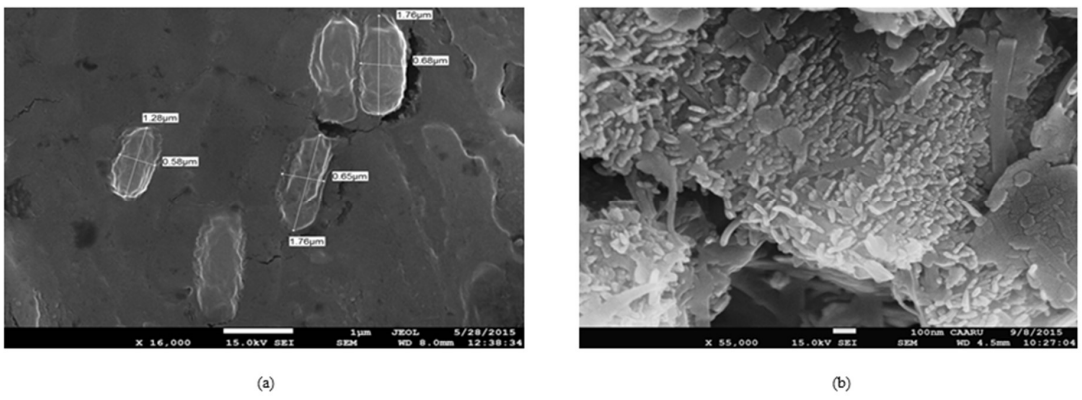


Figure 10. SEM image (**a**) *B. halodurans* in fresh BH medium; and (**b**) inside core.

4. Discussion

Heavy crude oil, of significant economic value, poses difficulty in recovery because of its high viscosity and low flow characteristics. EOR methods were employed to overcome the difficulty. MEOR is a tertiary recovery method which can enhance the recovery of crude oil [43]. The soil sample pH was found to be 8.5 ± 0.5, which was slightly alkaline in nature. It has already been reported that the fractionation of hydrocarbons is higher under slightly alkaline conditions [44–46]. The eTPH was found to be ~4.2%. The mineralogy study of the 10 soil samples showed that minerals such as calcite, quartz, albite, palygorskite, anorthite, dolomite, gypsum, halite, microcline, muscovite, rutile, suhailite and takanelite are present in the soil samples. The heavy crude oil sample viscosity and API gravity were determined as 650,000 mPa·s and 4.57°, respectively.

In this study, two indigenous strains, *B. firmus* and *B. halodurans* having the potential of biotransforming heavy crude oil were isolated from heavy crude oil contaminated soil samples collected from one of the Oman oil fields. There are reports for the ability of native bacteria to mineralize crude oil hydrocarbons in oil contaminated sites [47,48]. Identification of the isolates were done using protein profiling by Bruker's MALDI Biotyper [36] and by 16S rRNA gene analysis showing >97% sequence identity with respective genes in the National Center for Biotechnology Information (NCBI) database [49].

Heavy crude oil is a complex mixture of organic compounds [50] and is somewhat resistant to microbial action. Only a few microbes can act on crude oil, most of the strains identified being able to act on a narrow range of substrates [51]. The community composition of indigenous bacteria in Gulf beach sands indicated the abundance of members of the Gammaproteobacteria and Alphaproteobacteria as the major players in oil degradation [52]. Polycyclic Aromatic Hydrocarbons (PAH)-degrading capabilities of *Arthrobacter*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas* and *Rhodococcus* were studied extensively [53]. *B. stearothermophilus* was reported to utilize only hydrocarbons of C_{15} to C_{17} [54], whereas *A. borkumensis* AP1, SK2, and SK7 could act only on alkanes ranging from C_6 to C_{16} [55]. The isolates *B. firmus* and *B. halodurans* are the first reports to biotransform heavy crude oil of 4.57° API gravity.

The crude oil utilization capability of the isolates were determined by the study of growth characteristics in heavy crude as the sole carbon source, the technique has been used in several studies to determine the oil degradation potential of *Pseudomonas* and *Bacillus* sp. [47,56]. Higher concentrations of hydrocarbons might inhibit biodegradation by limiting nutrient or oxygen supply or by its toxic effects [57]. *B. firmus* and *B. halodurans* were shown to have significant growth in BH medium with up to 7% heavy crude oil, which implicates the isolates' tolerance to higher concentrations of heavy crude oil. The pH of the medium turned became more alkaline during the growth period. It was already reported that a slightly alkaline pH may enhance the rate of biodegradation [44–46]. The findings suggest the isolates as being potential candidates for biotransformation of heavy crude oil.

Using GC-MS analysis showed 81.36% biotransformation of heavy crude oil for *B. firmus* and 81.93% for *B. halodurans* compared to the abiogenic control, which was about 8–10%. Spore forming consortia isolated from Oman oil fields which could biotransform heavy oil after 12–21 days of treatment has already been reported [20,26]. The ability of mixed bacterial consortia to degrade 28–51% of saturates and 0–18% of aromatics present in crude oil or up to 60% crude oil was also reported [58,59]. *B. stearothermophilus* isolated from Kuwait oil fields was able to degrade pure hydrocarbons of a chain length of C_{15} to C_{17}, but were not able to degrade crude oil. *A. borkumensis* AP1, SK2, and SK7 was reported capable of utilizing only alkanes ranging from C_6 to C_{16} [55]. This study showed that isolates, *B. firmus* and *B. halodurans* were mostly utilizing aromatic fractions in the crude oil and fractionation of which led to increase in the amount of aliphatic compounds. Several enzymes such as oxidoreductase (laccases and cytochrome-P450 mono-oxygenase), xylene monooxygenase, catechol 2,3-dioxygenase, benzoyl-CoA reductase and others, are reported to play an important role in bacterial biodegradation of crude oil and polycyclic aromatic hydrocarbons [5]. We are further analyzing these

bacterial isolates for presence of genes encoding for such enzymes, which are responsible for heavy crude oil biotransformation.

Heavy oil that is trapped in oil reservoirs after primary and secondary recovery can be recovered by biotransforming the heavier fractions to lighter ones. *Bacillus* spp. that could degrade higher *n*-alkanes (>C_{27}) under anaerobic conditions were reported [60]. The most abundant compound present during the ninth day of incubation was hexadecanoic acid (RT 14.87) for *B. firmus* and dodecane (RT 12.42) for *B. halodurans* (Tables 2 and 3). Bacteria from the oil fields in Japan and China degrading *n*-alkane were reported [33,61]. *B. firmus* and *B. halodurans* in this study showed fractionation of higher *n*-alkanes having carbon numbers up to C_{54}. It was reported that *Thermus* sp. which was isolated from the reservoir of the Shengli oil field in East China, was capable of transforming crude oils [62]. MEOR studies using *Bacillus* spp. showed an extra recovery of 9.6% at 37 °C and 7.2% at 55 °C in core flood rig studies using crude oil of 26° API, due to the combined effect of biosurfactant and its biotransforming ability [63]. An extra recovery of 16% of 13.3° API crude oil was reported with *B. licheniformis* [64,65]. Youssef et al. [66] reported all the possibilities associated with microbial processes (both beneficial in EOR and detrimental) relevant to petroleum industry as in-depth analysis. In this study the extra recovered oil from tertiary recovery was 10.44% and 7.69%, respectively, for *B. firmus* and *B. halodurans*.

5. Conclusions

The ability of the isolates, *B. firmus* and *B. halodurans*, to grow at higher concentrations of heavy crude oil and their biotransformation ability by converting heavy fractions of crude oil to lighter ones, by utilizing mostly aromatic compounds indicated that the isolates showed promise for MEOR. The extra recovery of crude heavy oil in the core flood experiments and migration of bacteria in porous sand stone cores further confirms this. To the best of our knowledge, this is the first report of *B. firmus* and *B. halodurans* capable of biotransforming heavy crude oil of 4.57° API. All these findings have indicated that both isolates *B. firmus* and *B. halodurans* are promising candidates for MEOR applications and should be studied further.

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