Biotechnology Reports 39 (2023) e00804

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Research Article

Isolation, identification, and screening of biosurfactant-producing and hydrocarbon-degrading bacteria from oil and gas industrial waste

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ARTICLE INFO

Keywords:
Biodegradation
Marinobacter sp, Biosurfactant
Crude oil
Hydrocarbons
16s rRNA

ABSTRACT

Qatar is one of the biggest oil and gas producers in the world, coupled with it is challenging environmental conditions (high average temperature: >40 °C, low annual rainfall: 46.71 mm, and high annual evaporation rate: 2200 mm) harbors diverse microbial communities that are novel and robust, with the potential to biodegrade hydrocarbons. In this study, we collected hydrocarbon contaminated sludge, wastewater and soil samples from oil and gas industries in Qatar. Twenty-six bacterial strains were isolated in the laboratory from these samples using high saline conditions and crude oil as the sole carbon source. A total of 15 different bacterial genera were identified in our study that have not been widely reported in the literature or studied for their usage in the biodegradation of hydrocarbons. Interestingly, some of the bacteria that were identified belonged to the same genus however, demonstrated variable growth rates and biosurfactant production. This indicates the possibility of niche specialization and specific evolution to acquire competitive traits for better survival. The most potent strain EXS14, identified as *Marinobacter* sp., showed the highest growth rate in the oil-containing medium as well as the highest biosurfactant production. When this strain was further tested for biodegradation of hydrocarbons, the results showed that it was able to degrade 90 to 100% of low and medium molecular weight hydrocarbons and 60 to 80% of high molecular weight (C35 to C50) hydrocarbons. This study offers many promising leads for future studies of microbial species and their application for the treatment of hydrocarbon contaminated wastewater and soil in the region and in other areas with similar environmental conditions.

1. Introduction

The oil and gas industry in Qatar has invested in a comprehensive wastewater reduction and reuse program to achieve key objectives in accordance with the State of Qatar National Vision 2030 (QNV 2030 [1]). The first objective of this program is to reduce produced water injection into deep well formations. The second objective is to recycle wastewater using advanced technologies – effectively treating wastewater for further internal process use, thereby reducing the intake of desalinated water for industrial operations. Thirdly, to reuse suitable streams of treated water for irrigation where possible. The overall environmental goal is for industries to be able to reuse 65 - 70 percent of their wastewater discharge as polished water for utilities reuse. However, industrial wastewater has high salinity, and this coupled with the harsh desert climatic conditions with an average temperature ranging from 35 °C to 55 °C, drastically reduces the treatment potential of existing chemical and biological technologies [2]. Consequently, various alternate and novel treatment technologies have been applied and studied for their efficiency in degrading hydrocarbon components of wastewater, wastewater solids, and waste soil in different environments and the mechanisms behind each treatment process [3,4,5].

The conventional methods for the treatment of oily wastewater include coagulation/flocculation, flotation, sorption, and filtration [6]. The coagulation/flocculation technique is a combination of physical and chemical technique in which appropriate chemicals are added to neutralize the negative charges of the oil particles and form large flocs which are then separated through precipitation [6]. Whereas the sorption involves the removal of contaminants through adsorption or absorption mechanisms and various types of sorbents are reported for the treatment of industrial wastewater. The filtration is done using various

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https://doi.org/10.1016/j.btre.2023.e00804
Received 12 February 2023; Received in revised form 23 May 2023; Accepted 10 June 2023
Available online 13 June 2023

types of membranes (ultrafiltration, nanofiltration and reverse osmosis) in which particles larger than the membrane pores are removed from the wastewater [7]. Nevertheless, wastewater treatment strategies involving the stimulation of indigenous microorganisms are preferred to conventional methods for several reasons, mainly due to the high costs involved in the latter [8]. For this purpose, the isolation, and characterization of such indigenous microorganisms are required in order to optimize the treatment strategy. Moreover, the isolation of microorganisms from polluted environments is an efficient way to obtain bacteria with high biodegradation potential due to their genetic and metabolic adaptations over time in those extreme environments. Several studies conducted in the region have identified bacteria in soil samples infiltrated by crude oil and found several genera that are able to degrade hydrocarbons efficiently such as Pseudomonas sp., Bacillus sp., Acinetobacter sp., Marinobacter sp., Micrococcus sp., Rhodococcus sp., and Enterobacter sp. [9,10]. As many as 79 genera have been shown to be capable of petroleum hydrocarbon degradation [11]. However, it has been noted that there are no bacteria that is capable of degrading the entire hydrocarbon fraction. Instead, some bacteria are capable of degrading *n*-alkanes, while others prefer aromatic hydrocarbons [12]. To solve this problem, several researchers have used bacterial consortiums comprising 3 or more different strains of bacteria which can biodegrade the complete fraction of hydrocarbons. For example, a field study was conducted using a bacterial consortium containing more than 8 bacterial species for the treatment of hydrocarbon-contaminated soil. The bacterial consortium was able to degrade 86% of the hydrocarbons after 365 days of treatment [13]. Nevertheless, the use of bacterial consortium may influence the growth of individual bacteria leading to the dominance of one bacterium over another and eventually resulting in incomplete or ineffective hydrocarbon biodegradation. Therefore, the isolation and identification of bacteria from different contaminated environments is an ongoing area of research and there is a need to obtain bacteria or bacterial consortiums capable of degrading a complete fraction of hydrocarbons within the accepted time duration. Moreover, the bacteria or bacterial consortium should have the capability to live in wide environmental conditions to achieve an effective treatment.

Another significant challenge that affects the biodegradation process is the low bioavailability of hydrocarbons to the microorganisms [14]. However, some bacteria have shown the capability to produce certain kinds of surface-active molecules (called biosurfactants) that can effectively dissolve hydrocarbons in the water phase making them available and susceptible to biodegradation [15]. Biosurfactants are amphiphilic compounds with hydrophobic tails and hydrophilic heads that are either present on the surface of the microorganisms or produced by microorganisms under certain conditions [16–18]. These biosurfactants decrease the interfacial tension between the two phases (oil and water), increase the emulsification and solubility of hydrophobic components such as hydrocarbons and therefore improve the bioavailability of hydrocarbons [19]. Recently, the idea of exploring microorganisms that can both biodegrade hydrocarbons and produce biosurfactants has been fast-tracked to target applications for biodegradation, wastewater treatment, and environmental management [20]. Hence, the isolation of biosurfactant-producing and hydrocarbon-degrading bacteria living in the contaminated environments (wastewater, waste soil) provides a unique opportunity for efficient biological treatment of these environments. This may lead to the discovery of novel bacterial strains which have unique capabilities to degrade and clean up intractable complex compounds.

The overall aim of this research is to obtain highly efficient biosurfactant-producing and hydrocarbon-degrading bacteria which can be used for the bioremediation of oil and gas industrial wastewater in Qatar. To serve this purpose, the isolation, identification, and characterization of bacteria present in such contaminated environmental samples is a must. Therefore, the objectives of this research were formulated as follows: (1) to conduct physical and chemical characterization of hydrocarbon-contaminated wastewater and sludge, as well as soil samples (collected from the Land treatment unit, LTU), (2) to isolate and identify bacteria from these contaminated samples which can use crude oil as a carbon and energy source, (3) to evaluate the capability of isolated bacteria to produce biosurfactants, and (4) to analyze the capabilities of the selected bacterial strain for the biodegradation of crude oil. Hence, this work aims to cover both the fundamental part of the research (characterization of wastewater and wastes) as well as applied research (application and demonstration of crude oil biodegradation at lab-scale by the selected strain). The hydrocarbon-contaminated wastewater, sludge, and soil samples were collected from the major oil and gas operations in Qatar. The results of this research will help to discover the unexplored resources of biosurfactant-producing and hydrocarbon-degrading bacteria present in the contaminated environments. Moreover, the capabilities shown by these indigenous bacteria can also be exploited for the treatment of heavily contaminated wastewater at the industrial level.

2. Materials and methods

2.1. Chemicals and culture media

All chemicals used in this study were of analytical grade and high purity. Crude oil was obtained from the local market. Artificial sea salt was obtained from Seachem and pure water was prepared using milliQ water filtration system. Two types of media were used for this experiment i.e., nutrient media (Luria-Bertani; LB) and mineral salt media (MSM). Both media were prepared with artificial seawater (0.4%); 40 g of artificial sea salt in 1 L of pure water, autoclaved at 15 psi for 15 mins in an autoclave machine (3870 ELVC – Tuttnauer / USA) and pH adjusted to 7.0 ± 0.2 with 1 M NaOH or HCl. The LB medium included 10 g of NaCl, 10 g of peptone, and 5 g of yeast extract in 1 L of artificial seawater [21,22]. MSM media was prepared with 1 L of artificial seawater containing 4 g NH_4NO_3; 2 g Na_2HPO_4; 0.53 g KH_2PO_4; 0.17 g K_2SO_4; 0.1 g $Mg.SO_4{\cdot}7H_2O$; and 1 ml of trace element containing per 100 ml; 0.1 g EDTA; 0.072 g $ZnSO_4{\cdot}7H_2O$; 0.29 g $MnSO_4.7H_2O$; 0.05 g H_3BO_3; and 0.1 g $NiCl_2$ [21].

2.2. Sampling site and collection

The samples of oily wastewater (10 L), chemical wastewater (10 L), hydrocarbon sludge (2 kg), and waste soil from LTU (2 kg, 1 m beneath the surface) were collected from an oil and gas plant site in Qatar. The sampling was done early morning and the temperature was recorded as 38 °C. It should be noted that these samples are representative of wastewater and hydrocarbon waste generated from oil and gas process operations. Samples were collected using a sterilized apparatus and placed in separate sterilized dark bags and bottles with appropriate labeling. All samples were transported to the laboratory in an icebox at 4 °C.

2.3. Physical and chemical characterization of collected samples

All samples were analyzed for physical and chemical characteristics including pH, conductivity (Hach HQ 440d multi, HACH / USA), TOC (total organic carbon) (Skalar TOC analyzer, Skalar / Netherlands), TDS (total dissolved solids) (Shel Lab, Sheldon / USA), TSS (total suspended solids), (Shel Lab, Sheldon / USA) oil and grease (Shel Lab, Sheldon / USA), chloride (argentometric titration) and sulfate (Hach DR. 2800, HACH / USA). Heavy metals were tested following the USEPA 3005A / 6010 C through the Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Optima 2000DV, PerkinElmer / USA) technique. Total petroleum hydrocarbons – TPH were measured following USEPA 8015 C, using the Gas Chromatography (GC) Mass Spectrometry Detection (MSD) (Agilent7890A GC, 5975 MSD, Agilent / USA) technique. For BTEX (benzene, toluene, ethylbenzene, and xylene), the standards of USEPA 5030C/8260 C were followed, and the extraction

was performed using Purge & Trap instrument (Teledyne Tekmar, USA) together with GC-MSD. The polycyclic aromatic hydrocarbons – PAH were measured following USEPA 3510C/8270 D standards using the GC-MSD instrument.

2.4. Isolation of hydrocarbon-degrading bacteria

For the isolation of hydrocarbon-degrading bacteria from soil and water samples, a methodology reported by AlKaabi et al. [23] was adopted with slight modifications. Sample (soil, sludge, and wastewater) were enriched by transferring 2 g for solids and 2 mL for liquids into 250 ml Erlenmeyer flask containing 25 mL of LB medium and incubated in a rotary shaker (KS 4000i control, IKA – USA) at 30 °C and 110 rpm for 3 days. An aliquot of 2 mL of the culture broth was used as inoculum in an Erlenmeyer flask of 250 mL MSM medium containing crude oil (300 ppm) as the sole carbon and energy source. The culture was then incubated in a rotary shaker at 30 °C and 110 rpm for 5 days (first adaptation). After 5 days, 2 mL inoculum of the first adaptation culture was added to fresh 250 mL of MSM medium containing crude oil (300 ppm) and incubated in a rotary shaker at 30 °C and 110 rpm for 5 days to decrease the load of non-efficient bacteria (second adaptation). After the second adaptation, the cultures were further diluted (10^{-3} – 10^{-7}) and 100 µL of each dilution was spread on LB agar plates and incubated at 30 °C for 48 h (Shell Lab – USA). After 48 h, colonies of different morphology were selected and individually transferred to the LB plate for further purification.

2.5. Molecular identification of the bacterial isolates

DNA was extracted from bacteria using DNeasy UltraClean Microbial Kit (Qiagen, Germany) as per manufacturer instructions. 16 s rRNA PCR was performed using the primers FW: AACMGGATTAGATACCCKG and Rev: GACGGGCGGTGWGTRCA amplifying the V5-V8 region. Polymerase chain reaction assay was carried out using the following protocol: 2 µL of extracted genomic DNA was combined with 10 µL of AmpliTaq Gold® PCR Master Mix (Thermofisher) along with 1.2 µL each of 5 µM forward and reverse primers and the mixture was made up to 20 µL volume with nuclease-free water. The PCR amplification was initiated at 95 °C for 10 min and followed by 40 amplification cycles (denaturation at 95 °C for 15 s, annealing at 53–58 °C for 30–60 s, and extension at 72 °C for 25–30 s). The resulting products were run on 2% agarose gel and 592 bp amplicons were cut from the gel and the DNA was purified using QIAquick Gel Extraction Kit (Qiagen, Germany) as per manufacturer instructions. For some strains, the primary PCR yields were extremely low and an additional round of PCR using 2 uL of the primary PCR product as the starting DNA was done to ensure enough yields to perform direct sequencing. Sanger sequencing using the primer: GACGGGCGGTGWGTRCA was performed at the Genomics core at Weill Cornell Medicine- Qatar on the ABI 3130xl instrument. The resulting sequences have been deposited in GenBank (accession numbers OL913922-OL913947). To maintain specificity, the sequences were trimmed to a length of 572 bp when exceeding the amplicon length. Strain identity was determined using BLASTN tool on the resulting sequences against the NCBI 16 s ribosomal RNA sequence (bacteria plus archaea) database. Top hit with > 90% query cover and the highest% identity was concluded as the final identity at the genus level.

2.6. Growth of isolates in oil-containing medium

To obtain growth curves of the isolated strains in oil-containing medium, the pure cultures of strains were inoculated into the MSM medium containing 300 mg/L of crude oil and incubated at 30 °C and 110 rpm for 7 days. The growth of strains was measured each day using UV/Vis. spectrometer (Lambda 25, PerkinElmer - Singapore) at OD600 nm as described by [24]. The sterile medium containing only 300 mg/L crude oil was employed as a control. To avoid interference, the samples were carefully taken from the culture medium only and the oil slick or oil emulsions were removed before analysis.

2.7. Screening of bacterial isolates for biosurfactant production

In order to screen bacteria for biosurfactant production, each isolate (2–3 colonies) from LB pure culture was inoculated in 50 mL of MSM medium with crude oil (300 ppm) and 1% glucose and incubated in a rotary shaker at 30 °C and 110 rpm for 7 days - 1% glucose was added to increase the yield of biosurfactants. After 7 days, the culture was centrifuged at 5000 rpm for 30 mins and the supernatant was used for biosurfactant screening tests. The biosurfactant-producing capacity was measured by the oil spreading method and emulsification stability test (E_{24}). The selection of biosurfactant-producing capacity was based on the ability of each strain to give positive results in both screening tests [25].

(a) Oil spreading test

The oil-spreading technique was carried out according to Morikawa et al. [26] and Youssef et al. [27]. In the beginning, 40 mL of distilled water was added to a petri dish followed by 50 µL of crude oil to the surface of the water. Then, 10 µL of the 7-day-old culture supernatant was pipetted on the crude oil, and the occurrence of a clear zone was observed on the oil surface. The diameter of the clear zone surrounding the oil was measured. Distilled water was used as a negative control and sodium dodecyl sulfate (SDS) was used as a positive control.

(a) Emulsification index test (E_{24})

The Emulsification index (E_{24}) was measured by adding equal volumes of crude oil and cell-free culture broth. This mixture was vortexed at a high speed for 2 mins and allowed to stand for 24 h [28]. The emulsification index after 24 h was quantified using Eq. (1):

$$E_{24} = \frac{H_e}{H_l} \times 100 \quad (1)$$

Where H_e and H_l denote the height of the emulsified layer and the height of the liquid layer, respectively. The emulsions formed by the isolates were compared with those formed by a 0.35% (w/v) solution of SDS in distilled water.

2.8. Extraction of biosurfactant from the bacterial culture

The isolate showing the best results for both screening tests was further studied for the biosurfactant characterization using previously published protocol with slight modifications [29]. Briefly, the biosurfactant extraction from the culture broth was first performed through centrifugation at 5000 rpm for 30 min at 5 °C. The supernatant was separated after centrifugation and the pH was adjusted to 2.0 using 6.0 M HCl. The supernatant was then stored at 4 °C for 24 h. The mixture of chloroform and methanol (2:1) was added to the supernatant in an equal volume and was mixed vigorously and then incubated overnight at 25 °C. The interfacial layer containing biosurfactants was then separated and centrifuged at 5000 rpm for 30 min at 5 °C. The pellet obtained at the end of the centrifugation was of biosurfactant, which was oven-dried overnight at 50 °C after adjusting its pH to 7.0 using 1 M NaOH solution. The dried extract of biosurfactant was then analyzed using Fourier transform infrared (FTIR) spectroscopy (Shimadzu, Germany). The spectra were recorded in the range of 400–4000 cm^{-1}.

2.9. Application of selected bacteria for crude oil biodegradation

The strain (EXS14) with the maximum growth and the highest biosurfactant-production capacity was used to apply and demonstrate the biodegradation of crude oil in laboratory conditions. The

experimental chamber containing 250 mL of MSM media was inoculated with 300 ppm of crude oil and 1 mL of EXS14 culture that contained approximately 1.5×10^8 cells/mL. Nitrogen (as ammonium nitrate) was added at 1400 ppm, salinity at 4%, and pH was kept at 7. Test bottles were agitated at 110 rpm at 30 °C for up to 14 days. The test bottles were closed by using sterile cotton which allowed the passage of air through them to maintain aerobic conditions inside the bottles. Experiments were performed in duplicates and individual flasks were analyzed at the beginning of the study (T0) and at 4 (T4), 7(T7), 10 (T10), and 14 days (T14). At the end of the experiments, the WAF (water-accommodated fraction of the oil) was separated from the rest of the crude oil using a separating funnel. 3 mL of methylene chloride (DCM) was then added to the WAF to extract the soluble fraction of hydrocarbons. Samples were then analyzed by Gas Chromatography-Mass Spectrometry (GC–MS) (Agilent – USA, Models: GC-7890B & MS-5977 B).

2.10. Analysis by GC-MS

The DCM extracted hydrocarbons were concentrated to 1 mL using DryVap concentrator system (Horizon Technology - USA. The Agilent GC–MS (GC-7890B & MS-5977 B) equipped with 30 $m \times 0.25$ mm id, 250 µm film Rxi-5HT analytical column (Restek). The GC injector temperature was 300 °C, and the column flow was 1.5 mL/min. The oven temperature was held at 50 °C and then ramped up to 320 °C. The MS source and quadrupole temperatures were 290 °C and 180 °C respectively. The chromatograms were manually integrated using Chemstation software to get the area of each peak of interest. Analytical standards from Accustandard and Restek were used to identify the aliphatic and aromatic hydrocarbon compounds' peaks and their respective retention time.

2.11. Statistical analysis

The data shown in the corresponding Figures and Tables represent the mean values and the standard deviations. Statistical analysis was performed using GraphPad Prism 8.3 software.

3. Results and discussion

3.1. Physical and chemical properties of collected samples

In this study, samples of sludge, waste soil from LTU, oily water (OW), and chemical water (CW) were collected from an oil and gas plant site in Qatar. Different parameters were analyzed for liquid samples (OW and CW) such as conductivity, TDS, chloride, sulfate, oil and grease, and TOC (Table 1). Additionally, all samples were analyzed for BTEX, PAHs, TPH (Table 2), and metals (Table 3). Initial analysis showed that the sludge and soil samples were neutral, the OW sample was basic, and the CW sample was acidic (Table 1). Comparing the two wastewater samples, CW was found to be more contaminated due to high conductivity, TDS, oil and grease, and TOC content as compared to the OW sample (Table 1).

BTEX and PAHs concentration were below the method detection limits except for CW where benzene, toluene, ethyl benzene, and xylenes concentrations were 116, 115, 43, and 584 ppm, respectively (Table 2). However, for TPHs including gasoline range, diesel range, and heavy fraction, the highest concentration was found in sludge samples followed by soil, CW, and then OW.

Table 1
Physical and chemical properties of the wastewater samples.

Parameters	OW	CW
pH	8.18	5.41
Conductivity (µS/cm)	80	2290
TDS (ppm)	37	1150
Chloride (ppm)	0.71	340
Sulfate (ppm)	–	18
Oil and grease (ppm)	1.4	21
TOC (ppm)	4.74	982

pH of the sludge and soil (LTU) samples were measured as 7.16 and 7.99, respectively.

Table 2
BTEX, PAHs, and TPHs in wastewater and waste soil samples.

Parameters	OW (ppm)	CW (ppm)	Sludge (ppm)	Soil (LTU) (ppm)
Benzene	< 0.1	116	< 0.1	< 0.1
Toluene	< 0.1	115	< 0.1	< 0.1
Ethyl Benzene	< 0.1	43	< 0.1	< 0.1
Xylenes	< 0.3	580	< 0.3	< 0.3
PAHs	< 0.1	< 0.1	< 0.1	< 0.1
Gasoline Range Hydrocarbons (C5-C10)	<0.1	240	4530	<1.0
Diesel Range Hydrocarbons (C10-C28)	270	15,750	1.2×10^7	2.0×10^6
Heavy Fractions (C29-C40)	250	3170	22,500	7140

Table 3
Concentration of metals in wastewater and waste soil samples.

Metals	OW (ppb)	CW (ppb)	Sludge (ppb)	Soil (LTU) (ppb)
Aluminum (Al)	0.33	0.254	472.2	8419
Barium (Ba)	–	0.638	37.11	633.7
Boron (B)	0.041	0.759	36.83	47.88
Chromium (Cr)	–	0.014	27.01	335
Cobalt (Co)	–	–	35.46	204
Copper (Cu)		–	35.27	383.7
Iron (Fe)	0.25	17.18	3251	193,600
Lead (Pb)	–		32.56	134.3
Lithium (Li)	–	0.082	4.381	17.58
Manganese (Mn)	–	0.878	69.75	1805
Molybdenum (Mo)	–	–	22.01	95.89
Nickel (Ni)	0.008	0.021	35.56	427.7
Zinc (Zn)	0.121	0.943	508.8	3851

Metal analysis showed soil samples to have the highest concentration of the total measured metals as shown in Table 3, followed by sludge, then CW and OW. Comparing metal analysis with the number of isolates, the maximum number of isolates were from soil samples, followed by OW and then CW. Previous studies have established those trace elements can affect the microbial community compositions, diversity, and activities and have deleterious effects on microorganisms at certain high concentrations [30–33]. Ding et al. [30] stated that the presence of certain concentrations of metals can positively impact the microbial community structure and diversity of carbon utilization. While Shuaib et al. [32] confirmed that heavy metal pollution caused intense changes in the microbial community composition, diversity, and activities. In this study, it was found that there was a strong relationship between the high concentration of certain metals and the number of isolates from each sample. The highest concentration of iron (Fe), manganese (Mn), copper (Cu), and cobalt (Co) was found in the soil sample followed by the sludge sample, the CW sample, and finally OW sample. These metals have been reported to act as electron acceptors in the soil where oxygen levels are limited. Electron acceptors increase bioremediation activity. In aerobic conditions, oxygen mainly acts as the electron acceptor, while, in an anaerobic environment, microorganisms use alternative electron acceptors such as Fe and Mn which were also found in the soil sample.

The physical and chemical characteristics of these samples were analyzed to understand their potential for biological treatment using microorganisms because it is important to understand the potential impact of these parameters on the efficiency of the biological treatment technology. The microorganisms produce biosurfactants that help them to interact with hydrocarbons and use them as a carbon source however, the ability of microorganisms to produce biosurfactants varies with the

medium composition. Gudina et al. [34] and Makkar and Cameotra [35], have demonstrated that biosurfactant production can be enhanced with metal-supplemented media. Makkar and Cameotra [35], have also shown that the addition of different metals such as iron, calcium, and magnesium can significantly affect the growth of bacteria and biosurfactant production. Similarly, Gudina et al. [34], found that the biosurfactant production of *Bacillus subtilis* increased when metal concentrations were increased.

Similarly, among physical parameters, pH is a very important parameter that affects bacterial growth and subsequently its biosurfactant production potential. The optimum pH for biosurfactant production is within the range of 5.7 – 7.8 [36]. Moreover, the presence of other nutrients like nitrogen, manganese, and sulfur and their ratio with respect to carbon have also been shown to affect biosurfactant production and microbial growth [37]. The presence and adaptability of the isolated strains in these wastewater samples showed their abilities to thrive under various conditions and demonstrated their potential for biosurfactant production. Thus, this study adds to the correlation between the physiochemical properties of waste soil, wastewater, and sludge samples, and their microbial community composition, diversity, and activities relating to hydrocarbon degradation and biosurfactant production.

3.2. Molecular identification of the bacterial isolates (16S rRNA gene sequencing)

A total of 26 bacterial isolates capable of hydrocarbon degradation were isolated from different contaminated samples i.e., LTU waste soil sample (n = 10), OW sample (n = 7), CW (n = 5), and hydrocarbon sludge sample (n = 4) (Table 4). All the isolated bacteria were successfully identified by 16 s rRNA sequencing. Several different genera of bacteria were identified, and the samples showed wide biodiversity. For instance, four types of bacteria isolated from the hydrocarbon sludge were identified as *Marinobacter* sp., *Halomonas* sp., *Ornithinibacillus* sp., and *Sinomicrobium sp* showing the diversity of bacteria surviving in the contaminated sludge (Table 4). Moreover, strains like *Halomonas* sp. were identified in more than one sample i.e., both in sludge and waste soil from LTU. This indicates that this type of bacteria dominates such hydrocarbon-contaminated extreme environments.

In regard to the strains isolated from LTU waste soil sample, the isolates were identified as *Marinobacter* sp., *Sinomicrobium* sp., *Salinicola* sp., and *Muricauda* sp. Most of the CW strains were identified as *Virgibacillus* sp., *Enterobacter sp*., *Ornithinibacillus sp,* and *Sinomicrobium* sp. In addition, strains from the OW sample were identified as *Bacillus* sp., *Lysinibacillus* sp., *Mesobacillus* sp., *Sinomicrobium* sp., and *Staphylococcus* sp. Interestingly, EXS09 and EXS14 from LTU waste soil samples were both identified as *Marinobacter* sp. Similarly, EXW01 and EXW04 from the CW sample were both identified as *Virgibacillus* sp. (Table 4).

Table 5 shows previous reports of the biodegradation and biosurfactant production capabilities of bacterial genera identified in this study. Some of the bacterial genera isolated and identified in this study have also been reported previously from marine environments and designated as efficient hydrocarbon degraders and biosurfactant producers. These include *Halomonas* sp., *Marinobacter* sp., *Ornithinibacillus* sp., *Chromohalobacter* sp., and *Idiomarina* sp. [38–43]. To the best of our knowledge, *Sinomicrobium* sp. have not been isolated before from waste soil from oil and gas operations and their hydrocarbon degradation and biosurfactant production capabilities have not been reported earlier.

Marinobacter was isolated from the hydrocarbon sludge and LTU waste soil samples in this study. This bacterium has been identified and studied widely for its ability to degrade a wide range of hydrocarbons and they are known as principal hydrocarbon degraders in the marine environment. Their ability to grow and tolerate different ranges of salinity has led to the frequent use of this bacteria in the biodegradation of hypersaline environments containing petroleum hydrocarbons [43, 69,70]. Furthermore, *M. hydrocarbonoclasticus,* belonging to *Marinobacter* genera, is also well-known for its ability to utilize a wide range of petroleum hydrocarbons and to produce biosurfactants. The obtained results are in line with previous surveys where these bacterial genera have been reported to be indigenous to marine environments.

3.3. Growth profile of isolated bacterial strains

The growth rate of the isolates in MSM containing crude oil (300 ppm) was determined by UV spectrometry at OD_{600}. The growth curve of the strains isolated from contaminated samples (sludge, LTU soil, OW, and CW) are depicted in Fig. 1. Among all the isolates, the strains isolated from LTU soil demonstrated the highest potential to grow in the oil

Table 4
Isolation and molecular identification of bacteria from wastewater and waste soil samples.

Sample Type	Strain	Bacterial identity (closest relative)	GenBank accession no.	Similarity (%)	Closest relative accession no.
Sludge (n = 4)	EXS01	*Halomonas shengliensis strain SL014B-85*	OL913922	99.11%	NR_044099.1
	EXS02	*Salinicoccus amylolyticus strain JC304*	OL913923	96.46%	NR_152,051.1
	EXS03	*Ornithinibacillus scapharcae strain TW25*	OL913924	98.42%	NR_117,927.1
	EXS04	*Marinobacter hydrocarbonoclasticus strain VT8*	OL913925	100.00%	NR_074783.1
Soil (LTU) (n = 10)	EXS05	*Chromohalobacter salexigens strain DSM 3043*	OL913926	98.15%	NR_074225.1
	EXS06	*Idiomarina xiamenensis strain 10-D-4*	OL913927	99.65%	NR_117,523.1
	EXS07	*Muricauda flavescens strain SW-62*	OL913928	98.24%	NR_042908.1
	EXS08	*Halomonas radicis strain EAR18*	OL913929	97.53%	NR_170,458.1
	EXS09	*Marinobacter hydrocarbonoclasticus strain VT8*	OL913930	99.65%	NR_074783.1
	EXS10	*Salinicola tamaricis strain F01*	OL913931	98.76%	NR_157,001.1
	EXS11	*Sinomicrobium pectinilyticum strain 5DNS001*	OL913932	94.54%	NR_134,174.1
	EXS12	*Bacillus safensis strain FO-36b*	OL913933	94.71%	NR_041794.1
	EXS13	*Pseudomonas stutzeri strain LMG 11,199*	OL913934	99.64%	NR_103,934.2
	EXS14	*Marinobacter hydrocarbonoclasticus strain VT8*	OL913935	99.64%	NR_074783.1
CW (n = 5)	EXW01	*Virgibacillus halodenitrificans strain ATCC 49,067*	OL913936	99.29%	NR_112,055.1
	EXW02	*Enterobacter asburiae strain JM-458*	OL913937	98.94%	NR_145,647.1
	EXW03	*Ornithinibacillus scapharcae strain TW25*	OL913938	98.59%	NR_117,927.1
	EXW04	*Virgibacillus halodenitrificans strain ATCC 49,067*	OL913939	99.47%	NR_112,055.1
	EXW05	*Sinomicrobium pectinilyticum strain 5DNS001*	OL913940	96.31%	NR_134,174.1
OW (n = 7)	EXW06	*Bacillus paralicheniformis strain KJ-16*	OL913941	99.82%	NR_137,421.1
	EXW07	*Lysinibacillus boronitolerans strain NBRC 103,108*	OL913942	98.06%	NR_114,207.1
	EXW08	*Bacillus vietnamensis strain NBRC 101,237*	OL913943	99.65%	NR_113,995.1
	EXW09	*Staphylococcus hominis* subsp. *novobiosepticus strain GTC 1228*	OL913944	98.24%	NR_041323.1
	EXW10	*Bacillus foraminis strain CV53*	OL913945	99.12%	NR_042274.1
	EXW11	*Sinomicrobium pectinilyticum strain 5DNS001*	OL913946	94.13%	NR_134,174.1
	EXW12	*Staphylococcus hominis* subsp. *novobiosepticus strain GTC 1228*	OL913947	99.82%	NR_041323.1

Table 5
Previous reports on the hydrocarbon biodegradation and biosurfactant production capabilities of the bacterial genera isolated in this research.

Bacteria	Hydrocarbon degradation capability	Biosurfactant production capability	References
Bacillus sp.	The genus of *bacillus* sp. is well-known for its ability to degrade a wide range of hydrocarbons including aromatic compounds.	Several *Bacillus* species have been identified as biosurfactant producers with high emulsification activity and surface tension reduction.	[44]; Al-38,45]
Chromohalobacter sp.	*Chromohalobacter* sp. has shown potential for aromatic hydrocarbon degradation.	*Chromohalobacter* sp. has been proved to produce biosurfactants using a variety of organic substrates including crude oil.	[38,39,46]
Enterobacter sp.	*Enterobacter sp*. has been reported previously for its ability to degrade various hydrocarbons such as PAHs.	The genus of *Enterobacter sp*. has been reported as biosurfactant producers with ability to increase the solubility of hydrophobic compounds.	[47,48]
Halomonas sp.	*Halomonas* sp. isolated from the marine saline environment is frequently reported as an efficient degrader of several hydrocarbons.	Biosurfactant production capability has been reported	[38, 40–42]
Idiomarina sp.	*Idiomarina sp*. shows potential capability for degradation of several hydrocarbons.	Member of *Idiomarina* sp. have shown emulsification activities and high reduction of surface tension.	[49–51]
Lysinibacillus sp.	*Lysinibacillus* sp. has been demonstrated to remediate complex petroleum hydrocarbon mixtures including crude oil.	Strains of *Lysinibacillus* sp. have been shown to produce biosurfactants which enhance biodegradation rate of hydrocarbons.	[52,53]
Marinobacter sp.	Members of the genera *Marinobacter* have been extensively described as hydrocarbon degrading bacteria in saline environments.	*Marinobacter* is known as a biosurfactant producer which enhances hydrocarbon solubilization in seawater.	[38,43,54]
Muricauda sp.	*Muricauda* sp. isolated from crude oil-contaminated seawater has shown to be capable of degrading crude oil.	*Muricauda* sp. has been identified as biosurfactant producer.	[55]
Ornithinibacillus sp.	*Ornithinibacillus* sp. has been described as effective degraders of several hydrocarbons such as benzene, toluene and ethylbenzene.	Not Reported	[56,57];
Pseudomonas sp.	Many of *Pseudomonas* species have been applied in bioremediation of	*Pseudomonas* species exhibit a remarkable potential to produce biosurfactants using	[58]; [59]; [50,60]
Salinicoccus sp.	Genomics study of *Salinicoccus* sp. identified 652 pathways for degradation of several environmental contaminants including hydrocarbons.	Not Reported	[61,62]
Salinicola sp.	*Salinicola* sp. isolated from hydrocarbon contaminated saline environment is shown to degrade hydrocarbons like naphthalene.	*Salinicola sp*. has been proved to produce biosurfactants with a variety of organic substrates including crude oil.	[46,63,64]
Staphylococcus sp.	*Staphylococcus* sp. has been isolated from oil-contaminated sites and demonstrated as efficient hydrocarbon degrading strain.	Species of *Staphylococcus* have been described as biosurfactant producers.	[12,58,65, 66]
Virgibacillus sp.	*Virgibacillus* sp. is known for its ability to degrade hydrocarbons including PAHs and immobilization of heavy metals.	Members of *Virgibacillus sp*. has been reported for its ability to produce biosurfactant and reduce surface tension.	[67,68]

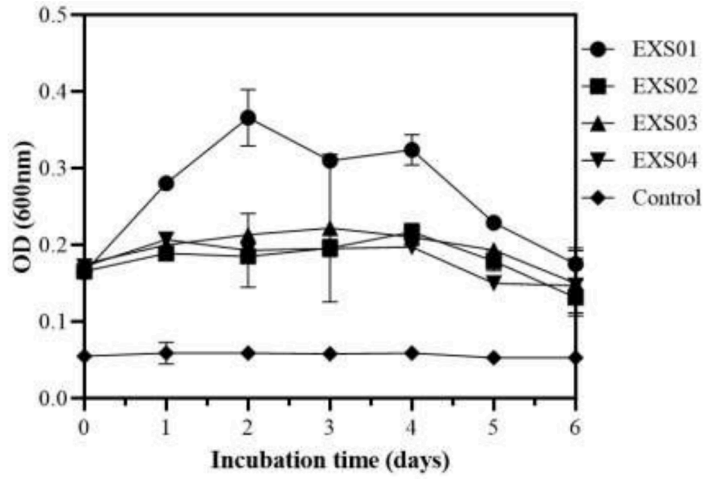
Table 5 (*continued*)

Bacteria	Hydrocarbon degradation capability	Biosurfactant production capability	References
	variety of hydrocarbons and their degradation pathways are well-studied.	various carbon sources.	

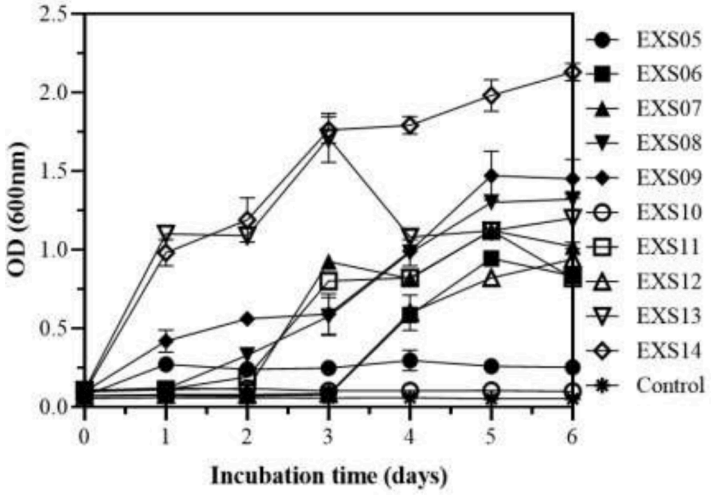
containing medium. As shown in Fig. 1b, the EXS14 (identified as *M. hydrocarbonoclasticus*) showed the highest growth, while EXS08 (*H. radicis*), and EXS09 (*M. hydrocarbonoclasticus*) demonstrated the second and third highest growth rates. Similarly, EXW07 (L. *boronitolerans*), and EXW10 (*B. foraminis*) isolated from OW samples also showed maximum growth. On the other hand, some other strains like EXS02, EXS10, EXW09, and EXW11 identified as *Salinicoccus amylolyticus, Salinicola tamaricis, Staphylococcus hominis,* and *Sinomicrobium pectinilyticum,* respectively demonstrated minimum growth rates in oil containing medium (Fig. 1a-d).

As noted above, different bacterial strains demonstrated different growth rates in the oil-containing medium showing differences in their potential to biodegrade hydrocarbons. These differences could be attributed to the physiological activity of the strains, the presence of hydrocarbon-degrading enzymes, and their expression and activity [71, 72]. Furthermore, this can also be attributed to the fact that different bacteria possess different catalytic enzymes which enable them to use different sources of carbon. For example, *Dietzia* sp. utilizes n-alkanes as a carbon source, while *Achromobacter xylosoxidan* can use both mono-aromatic and polyaromatic hydrocarbons [12]. This shows that bacteria employ different metabolic pathways for survival and growth which can be linked to their metabolic diversities and adaptation processes. That is why AlKaabi et al. [73] noticed that bacterial growth is not directly proportional to their hydrocarbon removal efficiency. Because one strain (Z3S1) isolated from contaminated soil produced 0.15×10^7 CFU per mL in an oil-containing medium but removed 38% of the TPH. In comparison, another strain (Z4D1) produced more biomass (i.e., 1.33×10^7 CFU per mL) but was able to remove only 19% of the TPH.

Similarly, it has also been noted that fast-growing bacteria struggle with changes in environmental conditions resulting in lag time in which



(a)



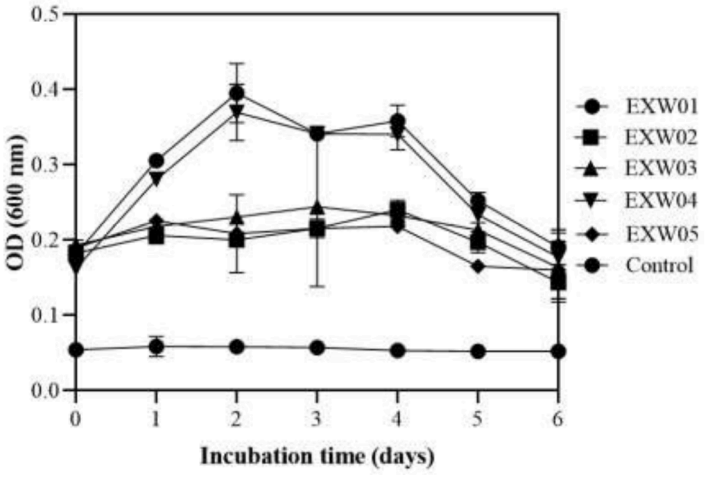
(b)

Fig. 1. The growth rate of strains isolated from (a) sludge, (b) LTU soil, (c) CW, and (d) OW samples. Error bars represent SD of the mean.

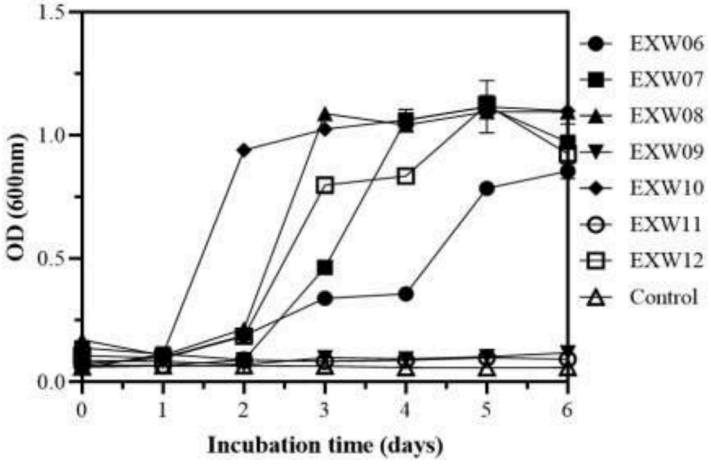
they were unable to grow for many hours until they adapt to the new conditions. In comparison, slow growers more easily adapt to changing conditions. This may explain why strains in this study isolated from the same environment showed different growth rates at day six and may have needed a longer time to adapt and grow. To support high growth rates, bacteria channel their gene expression and biosynthetic pathway to suit the environment in which they are growing. As a result, if the environment changes suddenly, significant gene expression and biosynthetic machinery changes need to occur to now enable the bacteria to grow in these new conditions [74,75].

3.4. Screening bacterial isolates for biosurfactant production

Crude oil generally sits on the surface of liquid media due to its hydrophobicity and low octanol-water partition coefficient (K_{ow}), reducing the interaction between bacteria (in water) and hydrocarbons (slick layer) which affects the biodegradation process. The biosurfactants produced by bacteria have a hydrophobic tail in their structure which enables them to attach to crude oil particles and get the crude oil available in the water column which is known as the water available fraction (WAF), and this allows the bacteria to utilize and degrade it. All bacterial isolates identified in this study were screened for the production of biosurfactants using two screening methods – an oil-spreading test and an emulsification index test. Table 6 shows the results of the oil spreading test and emulsification index for each isolate from the four studied samples. Out of the 10 isolates of the LTU waste soil sample, five isolates were positive for both screening tests. Among these positive isolates, the supernatant from the growing culture of isolate EXS14 formed the biggest clear zone diameter on the oil surface i.e., 4.6 cm, and showed the highest emulsification index (E24%) i.e., 68% (Fig. 2). On the other hand, the supernatant from the growing culture of strain EXS10 (isolated from the soil) formed the smallest clear zone diameter i.e., 0.2 cm, and showed the lowest emulsification index (E24%) i.e., 5%. The optical microscopic examination of the emulsified layer in Fig. 3 shows that the oil layer was broken into small oil droplets in the presence of EXS14. This is in consistent with the previous findings of Pi et al. [76] in which the oil emulsification in the presence of bacteria resulted in the breaking up of oil layer into the small oil droplets as it was visible under the microscope. Among the strains isolated from the hydrocarbon sludge sample, three out of four isolates were positive for both tests. The largest clear zone diameter on the oil surface was observed with isolate EXS04 (i.e., 3 cm). Regarding the strains isolated from the wastewater sample (OW), three out of seven isolates showed positive results for both tests. Isolates EXW07 and EXW08 gave the best



(c)



(d)

Fig. 1. (*continued*).

results in the oil spreading test and E24%.

Interestingly, when the correlation between the growth profiles of the strains with the biosurfactant production was made, it was observed that the EXS14 strain identified as *Marinobacter* sp. from the LTU had the highest growth in the oil-containing medium and produced the highest amount of biosurfactant (demonstrated by oil spreading and E24% tests). Although, another strain EXS09 isolated from the same LTU sample and was also identified as *Marinobacter* sp. did not demonstrate similar growth patterns and biosurfactant production potential. This suggests that in order to respond to environmental conditions, EXS14 may have been the result of a mutation to a more specialized and more robust variant to out-compete other strains such as EXS09. This has been demonstrated for many bacterial species that the combination of rapid growth rates and large population sizes results in the introduction of many unique mutations which are maintained by negative frequency-dependent selection. For example, static cultures of *Pseudomonas fluorescens* generate several niche-specialized variants (Bartell et al. 1988). One kind of variant overproduces extracellular polysaccharide (EPS), enabling the variant to float on the surface of the cultures, thus improving access to oxygen. However, this variant suffers if it becomes too dominant; the mats can become too thick to float, and then sink to the bottom of the culture. Therefore, it releases an enzyme to block other enzymes produced by other strains to slow their growth which enables it to survive longer in these conditions. The production of surface-active molecules from marine bacteria have been reported by several authors [77,78]. However, to the best of our knowledge, there have been no reports on the characterization of surface-active molecules produced by *Marinobacter,* although bacteria within this genus have often been retrieved from hydrocarbon-enriched marine communities.

FTIR analysis was done to identify the main functional groups of the biosurfactant produced by strain EXS14. Fig. 4 represents the spectrum of the extracted biosurfactant showing its characteristics peaks. The broad peak at 3440 cm^{-1} corresponds to the hydroxyl group (-OH) of the sugar component of the biosurfactant [79]. The two peaks at 2926 and 2857 cm^{-1} are corresponding to C-H stretching vibration of CH_2 and CH_3 groups of the aliphatic compounds in the lipid component of the biosurfactant [43]. The carbonyl stretching band of ester compounds is confirmed by the absorption peaks located at 1743 and 1155 cm^{-1} [80]. The absorption peak at 1450 cm^{-1} corresponds to C-H bending vibration indicating the presence of alkane in the produced biosurfactant. Additionally, the peak at 1492 cm^{-1} corresponds to N-O stretching band which represents the peptide group in the extracted biosurfactant. The peaks at 747 and 696 cm^{-1} attributed to C=C stretching band which indicates alkenes groups in the extracted biosurfactant [29]. Hence, as shown in the literature [43,81], the FTIR spectrum shows that the extracted biosurfactant contains sugars, and lipids indicating that it belongs to the glycolipid class of biosurfactants. However, for more accurate measurements, the nuclear magnetic resonance (NMR) spectroscopy and other characterization techniques (CMC determination) will be adopted to fully investigate the chemical structure and properties

Table 6
Biosurfactant screening for the isolated strains.

Sample Type	Strain	Zone of clearance (cm)	E24%	Bacterial identity (as per Table 4)
Sludge	EXS01	0.5	12	*Halomonas shengliensis strain SL014B-85*
	EXS02	NEG	NEG	*Salinicoccus amylolyticus strain JC304*
	EXS03	2	13	*Ornithinibacillus scapharcae TW25*
	EXS04	3	17	*Marinobacter hydrocarbonoclasticus VT8*
LTU	EXS05	NEG	NEG	*Chromohalobacter salexigens DSM 3043*
	EXS06	NEG	NEG	*Idiomarina xiamenensis strain 10-D-4*
	EXS07	2.8	13	*Muricauda flavescens strain SW-62*
	EXS08	0.2	NEG	*Halomonas radicis strain EAR18*
	EXS09	1.8	9	*Marinobacter hydrocarbonoclasticus VT8*
	EXS10	0.2	5	*Salinicola tamaricis strain F01*
	EXS11	2.5	14	*Sinomicrobium pectinilyticum strain 5DNS001*
	EXS12	NEG	NEG	*Bacillus safensis FO-36b*
	EXS13	NEG	17	*Pseudomonas stutzeri LMG 11,199*
	EXS14	4.6	68	*Marinobacter hydrocarbonoclasticus VT8*
CW	EXW01	NEG	5	*Virgibacillus halodenitrificans strain ATCC 49,067*
	EXW02	0.8	17	*Enterobacter asburiae strain JM-458*
	EXW03	3	18	*Ornithinibacillus scapharcae TW25*
	EXW04	2	9	*Virgibacillus halodenitrificans strain ATCC 49,067*
	EXW05	NEG	4	*Sinomicrobium pectinilyticum strain 5DNS001*
OW	EXW06	0.3	NEG	*Bacillus paralicheniformis strain KJ-16*
	EXW07	5	18	*Lysinibacillus boronitolerans strain NBRC 103,108*
	EXW08	6	17	*Bacillus vietnamensis strain NBRC 101,237*
	EXW09	0.6	NEG	*Staphylococcus hominis* subsp. *novobiosepticus strain GTC 1228*
	EXW10	2.3	14	*Bacillus foraminis strain CV53*
	EXW11	0.5	NEG	*Sinomicrobium pectinilyticum strain 5DNS001*
	EXW12	1.5	NEG	*Staphylococcus hominis* subsp. *novobiosepticus strain GTC 1228*

of the extracted biosurfactant.

3.5. Crude oil biodegradation by strain EXS14

To investigate the crude oil biodegradation potential of the isolated strains, further experiments were carried out using EXS14 as it demonstrated the maximum growth and highest biosurfactant production potential. EXS14 was identified as *Marinobacter hydrocarbonoclasticus* based on 16 s rRNA analysis as shown in Table 4. *Marinobacter* sp. has been reported previously to be a biosurfactant producing bacteria and has been extensively described as a hydrocarbon degrading bacteria in saline environments ([43,54,78]; and [38]). In this research, WAF was extracted and analyzed in order to explore the ability of *Marinobacter* sp. to biodegrade the available fraction of the crude oil. The concentration of WAF was measured on different days (T0, T4, T7, T10, and T14). In control samples, slow changes were observed in the WAF concentration which were found to slightly increase with time (Fig. 5). However, in samples inoculated with EXS14, there was a significant increase in the WAF fraction noted from T4 reaching its highest on T7 (Fig. 6). The total solubility of low-, medium-, high-molecular-weight hydrocarbon in inoculated samples increased significantly on T7 compared to T0 (Fig. 6). On the other hand, the solubility of these hydrocarbons increased very slightly in control sample (Fig. 5). This suggested that the biosurfactant produced by *Marinobacter* sp. enhanced the availability of hydrocarbons fraction of crude oil to the water phase.

The start of biodegradation was observed from T7 where significant reductions in the identified peaks were evident (Fig. 6). Therefore, the biodegradation ratio was calculated from peak intensities for each compound after it reached its maximum concentration on T7. The decrease in concentration of a compound (as shown by the peak intensity) after T7 was attributed to its biodegradation and the biodegradation ratio was calculated using the Eq. (2).

$$Biodegradation\ ratio = \frac{I_{T7} - I_{Ti}}{I_{T7}}\ x\ 100 \quad (2)$$

Where, I_{T7} is the peak intensity of the compound obtained at T7, and I_{Ti} is the peak intensity of the compound obtained on a given day.

Hence, it was noted that the biodegradation ratio for C12-C16, C17-C20, and C21-C31 *n*-alkanes reached 88%, 92.1% and 96% on T14 when their concentrations were compared with the T7 concentrations (Fig. 6). The concentration of *n*-alkanes decreased significantly within the 10 days of incubation demonstrating the ability of EXS14 to biodegrade these compounds (Fig. 6). The highest biodegradation ratio on T14 was for C27 *n*-alkane (99.2%), and the lowest was for C16 *n*-alkane (68.9%). This shows that the EXS14 strain biodegrades *n*-alkanes with relatively high carbon numbers more than with low carbon numbers. The ability of the *Marinobacter* sp. to biodegrade hydrocarbons is also reported previously [43,78]. Raddadi et al. [78], identified *Marinobacter* sp. isolated from marine sediments which was found to be capable of crude oil biodegradation in synthetic seawater. Similarly, *Marinobacter* sp. isolated from marine sediments was also shown to produce highly stable surface-active agents that could potentially be used in combatting marine oil spills. The surfactant production by this organism facilitated the substrate uptake and consequently its degradation. Gao et al. (2013) [82] also studied the degradation of polyaromatic hydrocarbons and differing length alkanes and its relation to biosurfactant production. They showed that the involved bacterium is a novel species of the genus *Marinobacter* for which the name *Marinobacter nanhaiticus* sp. nov. was proposed.

In this research, the ability of various bacteria isolated from contaminated environments to produce biosurfactants and to biodegrade hydrocarbons was demonstrated. Generally, biodegradation of such polluted environments is carried out either through bio-augmentation procedure (where the contaminated environment is supplemented with the bacteria or bacterial consortium that has high capabilities to degrade the target pollutant) or through bio-stimulation (in which the environmental conditions of the indigenous bacteria is optimized to enhance biodegradation process) [83]. Both approaches require preliminary laboratory work such as the identification of highly capable pollutant-degrading bacteria, their growth conditions, and their biodegradation rates as reported in this study. Additionally, the biological treatment of wastewater can also be carried out in engineered systems called bioreactors where the bacterial consortium is inoculated into the wastewater and the conditions of biodegradation are optimized in a closed reactor system. Each type of technique has its pros and cons in terms of cost, removal efficiency, level of expertise required, and others [84]. There are a few examples in the literature where the biodegradation process was implemented on the field scale such as Maddela et al. [85]; AlKaabi et al. [73]. In the research conducted by Maddela et al. [85], the ability of two isolated strains to biodegrade TPH was tested in oil-contaminated soil in a low-land area of the Amazon rainforest. A total of 87.45% removal of TPH was achieved after 90 days of treatment. Similarly, AlKaabi et al. [73] utilized a piles system to investigate the ability of two isolated strains for biodegradation of oil-contaminated soil. Two processes of bioremediation i.e.,

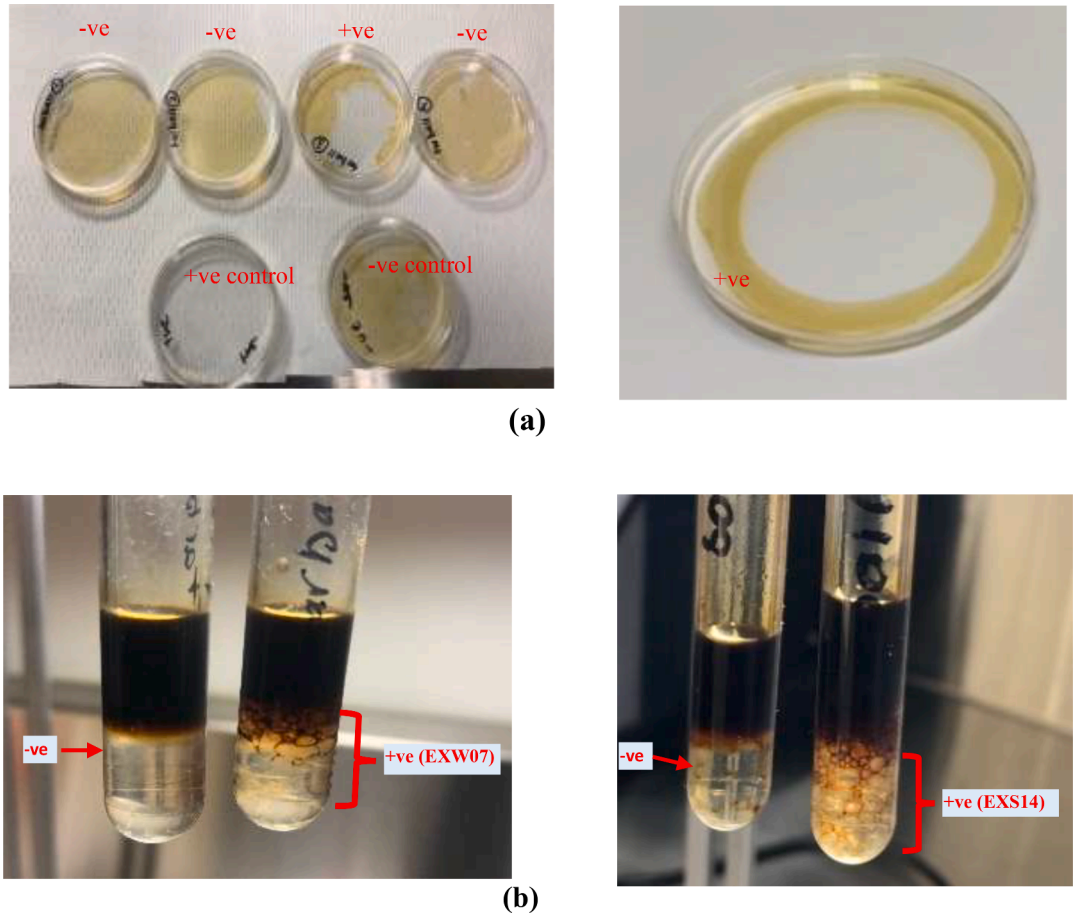


Fig. 2. Results of (a) Oil spreading test and (b) Emulsification index (E24%) test for strain EXW07 and EXS14.

bioaugmentation and biostimulation (where the isolated strains were introduced) and self-purification (natural attenuation) were investigated. Results showed that the bioaugmentation and biostimulation improved the rates of removal as 53% TPH removal efficiency was achieved after 90 days as compared to 30% achieved in the case of self-purification. Hence, it can be concluded that the bioremediation of hydrocarbons has great potential in the field and at the industrial scale and more work is needed to fill the knowledge gaps for translating laboratory work into the industrial level.

4. Conclusion

In this research, the wastewater, sludge, and waste soil samples from the major oil and gas industry in Qatar were characterized in terms of physical and chemical parameters. The results of the characterization showed that most of the samples are contaminated with hydrocarbons and heavy metals. The microbiological characterization of these samples helped to isolate a total of 26 bacterial strains which showed their potential to survive in these severely contaminated environments. The 16 s rRNA technique identified these strains as *Marinobacter* sp., *Halomonas* sp. *Bacillus* sp. and *Virgibacillus* sp. among others. Further laboratory research showed that these strains have variable growth rates in crude oil-containing growth medium and variable biosurfactant production potential. The isolate EXS14 identified as *Marinobacter* sp. showed the highest growth rate in the oil-containing medium as well as the highest biosurfactant production potential. When this strain was further tested for biodegradation of hydrocarbons, the results showed that it was able to degrade 90 to 100% of low and medium molecular weight hydrocarbons and 60 to 80% of high molecular weight (C35 to C50) hydrocarbons. Further research is guaranteed to perform whole genome sequencing of the isolate EXS14 to understand how this specie differ from other *Marinobacter* sp. isolated during this research as well as others reported in the literature.

This study, the first of its kind in Qatar, will pave the way for using green technologies to help address the environmental challenges in the State of Qatar. The new-found strains isolated from wastewater and waste soil can be used to treat industrial wastewaters. Applying these local microbes for industrial wastewater treatment represents a unique opportunity to use Qatar's bio-resources to develop technologies that can also potentially be applied in other regions with similar environmental conditions. The presence of these bacteria in the contaminated environments demonstrate their highly developed capabilities to survive in extreme conditions such as high salinity and high hydrocarbon, and heavy metal concentrations. In this regard, the real application of some of these isolated bacteria as consortium for the treatment of oil and gas industrial wastewater with high COD and pollutants concentration has already shown promising results during our ongoing studies. Moreover, the results of this research have also helped us to design a pilot-scale study for the biodegradation of hydrocarbon-contaminated wastewater which will help us to translate the laboratory-scale findings into the industrial applications.

Funding

This work was supported by ExxonMobil Research Qatar.

CRediT authorship contribution statement

S. Al-Marri: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **H.I. Eldos:**

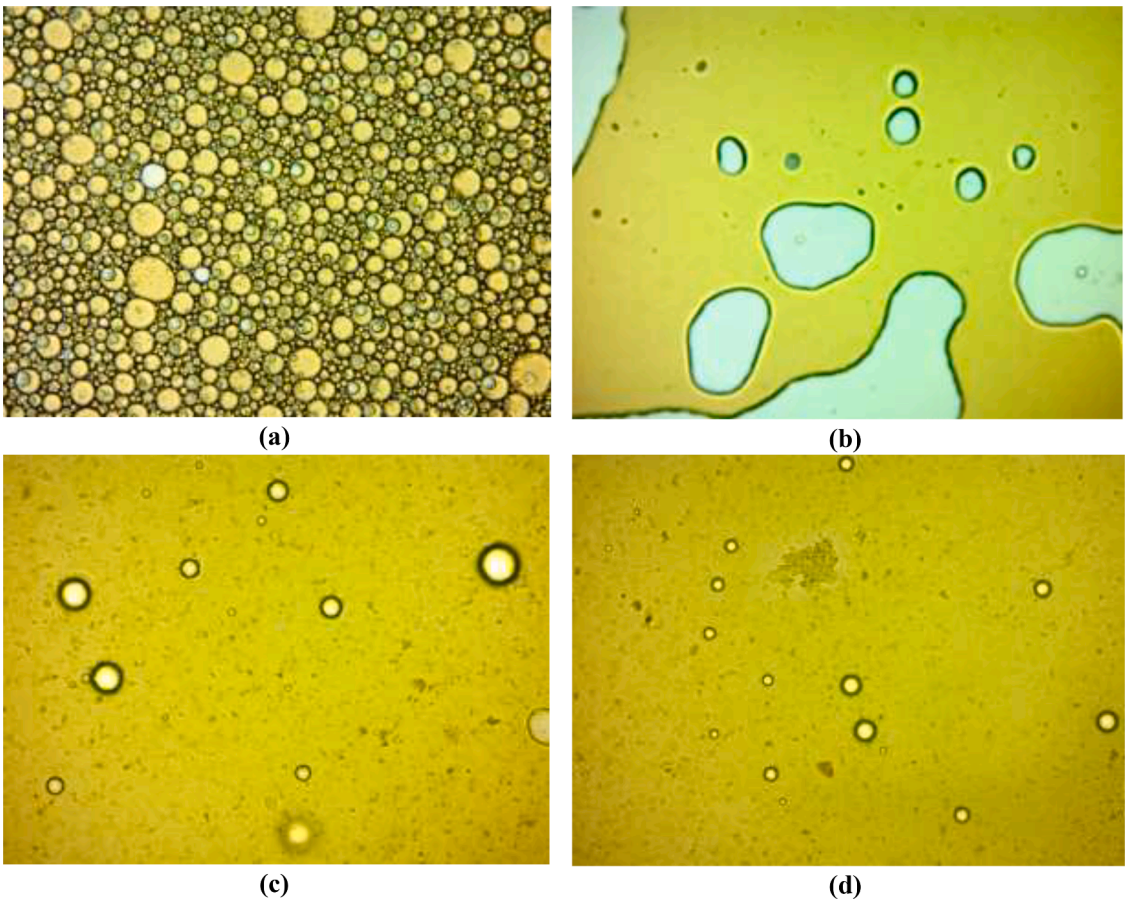


Fig. 3. Optical microscopic images (at 10x magnification) of oil emulsion formed after 24 hrs. during E24% measurements (a) Positive control (SDS); (b) Negative control (no oil emulsification); (c, d) Oil emulsions of crude oil formed by EXS14.

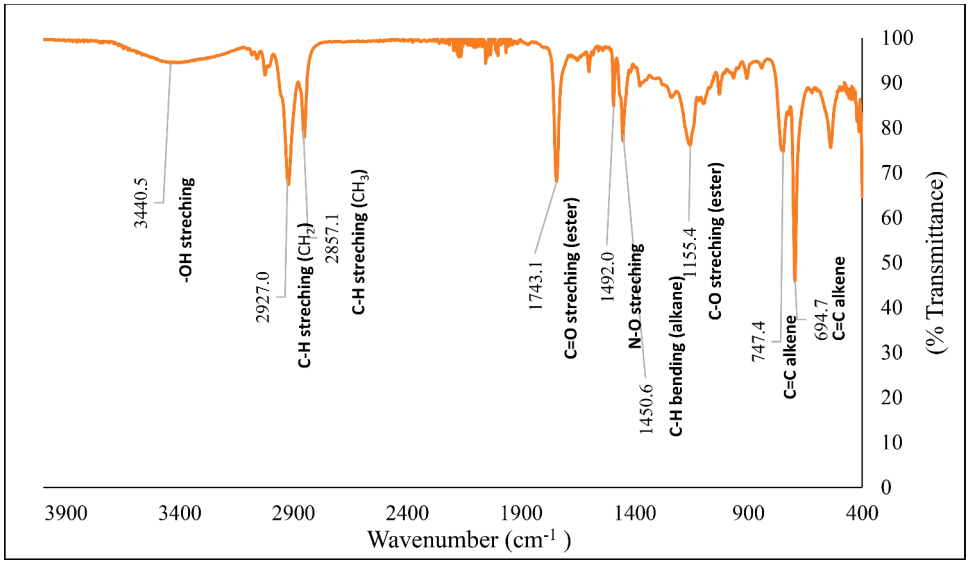


Fig. 4. FTIR spectrum of the biosurfactant produced by EXS14.

Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **M.Y. Ashfaq:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Supervision, Writing – review & editing. **S. Saeed:** Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **S. Skariah:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **L. Varghese:** Data curation, Formal analysis. **Y. A. Mohamoud:** Data curation, Formal analysis, Investigation. **A.A. Sultan:** Validation, Project administration, Supervision. **M.M. Raja:** Project administration, Resources, Writing – review & editing.

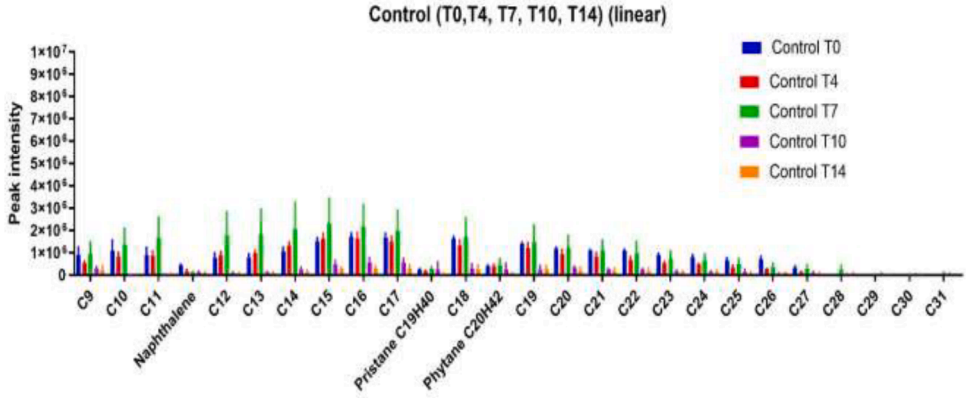


Fig. 5. Concentration of WAF in (MSM +300 ppm crude oil) control sample with no bacterial inoculation for T0, T4, T7, T10, and T14 days.

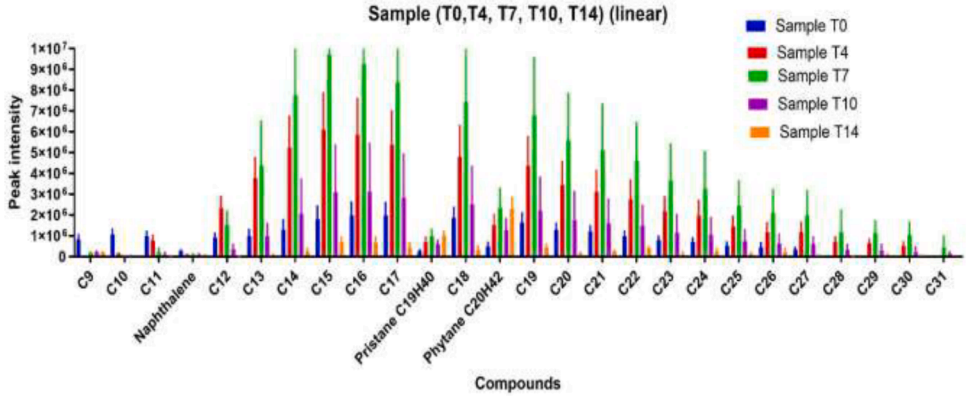


Fig. 6. Concentration of WAF in (MSM +300 ppm crude oil) with EXS14 strain for T0, T4, T7, T10, and T14 days.

Declaration of Competing Interest

Authors declare that they have no competing conflict of interest.

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