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Isolation and identification new bacterial strains isolated from different sources of Al-Rafidiyah oil field in Iraq

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ABSTRACT

In this study, twenty two pure bacterial strains isolates were isolated that can use crude oil as a carbon source. They occured at different frequency ratios for each of oil-contaminated soil samples, the produced water, and the crude oil of the Al-Rafidiyah oil field (Basrah south of Iraq). Bacterial isolates were characterized and identified based on phenotypic properties and molecular techniques. Fourteen strains, belonging to three genera of *Bacillus* sp., *Lysinibacillus* sp. and *Enterobacter* sp., were isolated from oil-contaminated soil and nine strains, belonging to *Bacillus* sp., *Lysinibacillus* sp., *Enterobacter* sp. and *Brevibacillus* sp., were isolated from produced water. Five strains, belonging to two genera, *Bacillus* sp. and *Pseudomonas* sp., were isolated from crude oil. New twelve strains were recorded as new strains and deposited in GenBank include *Bacillus cereus* strain ASWISA1, *Bacillus thuringiensis* strain ASWISA2, *Bacillus sonorensis* strain ASWISA3, Bacillus cereus strain ASWISA4, *Bacillus subtilis* strain ASWISA5, *Pseudomonas stutzeri* strain ASWISA6, *Bacillus subtilis* strain ASWISA7, *Bacillus cereu*s strain ASWISA8, Bacillus paramycoides strain ASWISA9, *Bacillus paramycoides* strain ASWISA10, *Brevibacillus brevis* strain ASWISA11, and *Enterobacter cloacae* strain ASWISA12.
Keywords: Oil filed bacteria, petroleum microbiology, Crude oil bacteria.

INTRODUCTION

Due to the high temperature, anaerobic conditions, high pressures, degree of salinity and different pH in the oil reservoirs, it has become as a new extreme environment for the growth of living organisms in these reservoirs (Elshafie *et al*., 2013; Cai *et al*., 2015). Although the extreme conditions support life, numerous studies over the past few years proved microbes are found in several oil reservoir environments. Divers groups of microbes detected in oil reservoirs. Microbial studies of such harsh environment have shown the presence of good different metabolic activities such as sulphate reducers, various hyperthermophilic fermentative microorganism, acetogens and methanogens from oil reservoirs worldwide (Magot *et al*., 2000; Orphan *et al*., 2003). By molecular techniques a wide diversity of bacteria depending in abiotic factors such as oxygen, temperature, pH, have been isolated from or have been detected in oil field samples (Telang *et al*., 1998; Al- Al-Tamimi 2015 Pannekens *et al*., 2019).

Oil reservoirs accommodate completely different phases wherever microorganisms will thrive, like formation water, and organic materials and crude oil (Kobayashi *et al*., 2012; Pannekens *et al*., 2019). Aerobic microorganisms have been found in oil reservoir with pH range (6.0 - 8.4) and temperatures ranging from (20 to 70°C). Some of these identified aerobic bacteria *Kocuria rosea*, *Rhodococcus ruber*, *Gordonia rubropertincta*, *Arthrobacter oxydans*, *Bacillus subtilis*, *B. cereus Cellulomonas cellulans*, *Pseudomonas fluorescens* and commonly bacteria of the genera *Clostridium*, *Bacteroides*, *Thermoanaerobacter*,*Thermotogales*, *Petrotoga*, *Thermotoga*, *Geosporobacter*, *Desulfotomaculum*, *Caminicella* represent anaerobic microflora were found in oil reservoirs (Pannekens *et al*.,2019).The current study aimed to isolate and identify some the bacterial strains from a variety of sources in the Al-Rafidia oil field.

MATERIALS AND METHOD

The bacterial strains were isolated from Al-Rafidiya oil field, Basra south of Iraq, 30.16°N 47.42°E. Twenty two samples of each oil contaminated soil, produced water and crude oil. The soil samples from the different sites of oil filed were collected in a sterile polythene bags at depth of 2-3 inches from the ground level using clean spatula, produced water samples were collected from separated tanks of water production. Meanwhile, the crude oil samples were collected from well head using 2.5 liter sterilized glass containers. All samples stored at 4°C in ice bag till transported to the laboratory. The daily reports of the oil field laboratories were used to for the physical and chemical properties of the samples, except the pH of soil which was measured in a 1:2 mixture of soil: water (0.01 M $CaCl_2$ solution) using pH electrode (Burghal, 2015).

Stimulation of indigenous microorganisms

To stimulate the indigenous microorganisms, soil samples were crushed and sieved through 2mm pore size (Fardoux *et al*., 2000 ; Dilmi *et al* ., 2017), then 5 ml soil suspension (5 g soil in 100 ml), 5 ml of produced water, and 5 ml of crude oil were add separately to 250 ml of Erlenmeyer flask contained 95 ml of mineral salt medium (MSM) composed of 2% of crude oil as a carbon source, 1g /l KH_2PO_4, 6 g/l $NaNO_3$, 1g/l K_2HPO_4, 0.02 g/l $FeSO_4$, 0.5 g/l $MgSO_4$, and 0.02 g/l Na_2MoO_4 at a pH of 7.0–7.2 (Zhao *et al*.,2017). The mixture was then shaken at 180 rpm at 35 °C for 48 hours using shaker incubator. Volume of 1 ml of the suspension was diluted serially and plated in triplicate on nutrient agar. The plates were incubated at

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37 °C. The various colonies obtained were taken and recultured by streaking methods on nutrient agar and MacConkey agar to obtain pure isolates which were kept on nutrient agar slants.

Identification of the pure isolates

(a) Morphological characterization

Pure isolated colonies that have grown on the nutrient agar plates were examined and recorded as colony morphological characteristics include color, size, shape, elevation, and margin. Micromorphology of bacterial isolates and their ability to Gram stain were recorded for all isolates via Gram stain protocol (Cheesbrough ,1991). Spore staining of bacterial cells, for all isolates, was carried out according to the method of Leboffe and Pierce (2015) .

(b) Molecular identification

Genomic DNA extraction

Genomic DNA was extracted from culture by using a commercial kit protocol (Promega Genomic DNA Purification Kit, USA).

Detection of genomic DNA

According to Lee (2012), 0.25g of agarose powder was dissolved in 25 ml of TBE buffer (1X) mixed gently, heated to near-boiling point, but avoid boiling, and added a very small amount of ethidium bromide dye using the micropipette tip and poured into casting tray with the comb inserted to make the appropriate special wells to add the DNA and left aside until it was solidified. Then the combs and the seal were removed gently from the tray and put down the gel gently in the electrophoresis chamber soaked by diluted TBE. Then 2 µl of bromophenol blue stain was mixed with 4 µl of extracted DNA on parafilm paper and loaded in wells of agarose by micropipette and electrophoresed by ban electric current from a power supply adjusted at 120 mA 60 V and for 35 min. UV light transmitter was used to recognize the migrated bands.

PCR amplification of 16S-rRNA gene

16S rRNA genes were amplified using polymerase chain reaction (PCR). Universal specific primers (27F AGAGTTTGATCMTGGCTCAG) and (1492RT ACGGYTACCTTGTTCGACTT) were used. For PCR, a mixture contained 5 µl of DNA, 2 µl of each primers solution, master mix 25 µl and 16 µl nuclease free water was applied. Amplification was carried out with thermal cycle machine after initial denaturation for 3 min at 95 °C, 30 cycles were performed, each cycle is consisting of 20 sec. at 95 °C, 20 sec. at 55°C and 30 min. at 72°C. Cycling was completed by a final elongation step at 72°C for 5 min. (AL-sheshtawy *et al.,* 2015; Al-Tamimi, 2015).

Analysis of the PCR products

After the amplification process, the PCR reaction products, with expected size of about 1.5 kb, electrophoresed with 100 bp DNA ladder of nucleic acid markers at 65V and 120 mA for 35 min. Agarose gel of 1.5% (w/v) was used in TBE buffer 1x. The gels were stained with 0.5mg/ml of ethidium bromide solution. The DNA was visualized using UV trans-illumintor (Watanabe *et al*., 2001) and the results were photographed and documented.

Sequence of PCR products

The approximately 1500 bp 16S rDNA of each isolates were purified and sequencing at Yang ling Tianrun aoke biotechnology company laboratories in China. The bacterial 16S rDNA obtained sequencing was then aligned with known 16S rDNA sequences Gen Bank using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

Main properties of samples

Data recorded for physical and chemical properties of produced water, crude oil used in this study were collected from the daily production reports of the oil field laboratories as listed in Table (1) and Table (2).

Isolation of bacterial strains from different collected samples

The best dilution to obtain single colonies showed 10^{-6} for soil samples, 10^{-2} for water samples, and 10^{-1} for crude oil samples. Screening the bacterial isolates for production of biosurfactant, twenty two pure bacterial strains were selected for their ability for bio-

Table (1): Properties of produced water sample taken from Al-Rafidiyah oil field.

Density (g/cm³)	Surface tension	pH	Salinity (ppm)	Temperature °C
0.854-0.862	55.8 -69.55	6.9 -7.5	18 -26	35-46

Table 2: Properties of crude oil of Al-Rafidiyah oil field.

Density (g/cm³)	Viscosity(cp)	API Gravity	Salt Content (ppm)	Water content %
0.852-0.861	5.8	29 -31	14-18	6.5- 7.3

surfactant production. The source of these bacteria strains were: eleven strains isolated from oil contaminated soil, seven strains isolated from produced water and four from crude oil and. The presence and distribution of bacterial strains obtained from different sampling sites indicates the ability of these microorganisms to utilize hydrocarbons as an energy source in these environments.

Identification and characterization of the pure bacterial isolates

(a)Morphological characterization

The phenotypic characteristics of the growing the colonies on nutrient agar medium, for all strains, were characterized. Based on Gram-staining, the results showed that 90.33% of the isolates were Gram-positive while 9.67 % of them were Gram-negative.

(b)Molecular identification

DNA extraction and 16S rRNA amplification analysis

The results of electrophoresis technique for genomic DNA extraction showed pure and clear isolated DNA for all isolates which proceed for 16S rRNA sequencing. Additionally, all isolates tested for the specific amplification of 16S rRNA gene sequences using a set of universal primers, 27 F and 1492R, yielded a single amplification of ~1500 bp for the entire isolates Figure (1). Based on partially sequenced of the sixty one obtained isolates, five genera were recognized and identified (Table 3). These genera were *Bacillus*, *Lysinibacillu*s, *Enterobacter, Brevibacillus* and *Pseud-omonas. Bacillus* species were the most dominant genera.

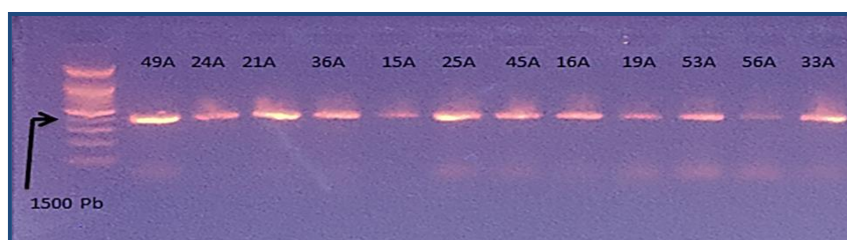

Figure (1): Agarose gel electrophoresis of 16S rRNA gene with 100 bp DNA ladder, showing . a single amplification band of ~1500 bp for selected isolates.

The frequency of each identified isolates revealed that the occurrence of the isolates obtained from contaminated soil were the highest and *Bacillus subtilis* IMG04 was the most frequented isolate (31%.). Meanwhile, *Bacillus cereus* was reported four times out of twenty nine isolates recovered from oil contaminated-soil and three times out of 21 isolates recovered from produced water (Table 3). Although the recovered isolates from crude oil reported was less in count the genus *Bacillus cereus* was recorded in high frequency (27% for both *Bacillus cereus strain US04* and *Bacillus cereus strain M2*). The second abundant genus was *Bacillus subtilis strain* followed by *Bacillus paramycoides strain*. Among identified genera, *Pseudomonas stutzeristrain* and *Brevi-bacillus brevis strain* were recoded once in crude oil and produced water, respectively.

According to the alignment and BLAST at NCBI the results of the new isolates 49A, 24A, 21A, 36A, 15A, 25A, 45A ,16A, 19A, 35A, 56A,and 33A are shown in Figure (2). These strains recorded a newt genetic identification depended on 16S rRNA and according to information available. After alignment with other 16S rDNA sequences in GenBank, they showed a high degree of similarity (99 - 99.93%) to reference strains.

The phylogenetic tree shown in Figure (2) reflects the relationships of the selected 22 different strains isolated from oil contaminated soil, produced water and crude oil samples. Among these 22 strains, 12 new strains were registered in the GenBank and were given the accession numbers as listed in Table (4).

Gram positive spore forming strains, belonging to the Bacillus genus, were the most dominant in the three sample sources. These results are in agreement with data reported by Al-Tamimi et al., (2019). In their study, they stated that Bacillus spp. was the most dominant bacterial species recovered from collected oil samples tested. In another study done by Hisham *et al*., (2019), they indicated the high diversity isolates obtained from petrol contaminated soil with the predominance of *Bacillus sp*.as biosurfactant producing in oil reservoir. Kumar *et al*., (2007) also, in their study, showed that *Bacillus sp*. isolated from oil contaminated soil produce biosurfactant over a wide range of pH, salinity and temperature.

It is difficult or even impossible to find a suitable classification and generalization for *Bacillus* spp. bacteria because they possesses a great diversity of physiological characteristic that allowed these species to colonize almost all natural environments including soil, air, lake sediments, water and soil. Contaminated hydrocarbons-soil and fodder as well as harsh environments such as acidic thermal waters, salt marshes and hot springs and the sub-Antarctic soil, also reportend the abundance of *Bacillus* species (Claus and Berkeley, 1986; Gopal *et al*.,2015).

Evans *et al*., (2004), in their study, pointed that *Bacillus* spp. are often the predominant species in environments exposed to various petroleum hydrocarbons. They also found *Bacillus licheniformis*, *B.* f*irmus*, *B. subtilis*, *B. foraminis*, *B.* from various soil samples and oil-contaminate soil.

Table (3): Identified bacterial strains isolated from different studied sources, oil contaminated soil, produced water and crude oil, and their frequency.

Source of isolation	Bacterial strain detected	No.of isolates	Frequency (%)
Oil contaminated soil	**Total Isolate No. 29**		
	Bacillus cereus strain US04	4	13.79
	Bacillus cereus strain ASWISA1	1	3.44
	Lysinibacillus boronitolerans strain SWIPB36	1	3.44
	Bacillus cereus strain A	2	6.89
	Bacillus cereus strain ASWISA4	1	3.44
	Bacillus subtilis strain HDZK- BYSB7	1	3.44
	Bacillus thuringiensis strain LU3	3	10.3
	Bacillus subtilis IMG04	9	31
	Bacillus subtilis strain ASWISA5	1	3.44
	Bacillus paramycoides strain SKA22	2	6.89
	Bacillus paramycoides strain ASWISA10	1	3.44
	Enterobacter aerogenes strain B19	1	3.44
	Bacillus paramycoides strain ASWISA9	1	3.44
	Bacillus licheniformis strain CC91	2	6.89
Produced water	**Total Isolate No. 21**		
	Bacillus thuringiensis strain ASWISA2	1	4.76
	Lysinibacillus macroides strain ZCGT05	4	19.04
	Bacillus sonorensis strain ASWISA3	1	4.76
	Bacillus cereus strain ASWISA8	2	9.52
	Enterobacter cloacae strain	3	14.28
	Brevibacillus brevis strain ASWISA11	1	4.76
	Enterobacter cloacae strain	1	4.76
	Bacillus cereus strain EC3 16S	4	19.04
	Bacillus cereus strain M2	4	19.04
Crude oil	**Total Isolate No. 11**		
	Bacillus cereus strain US04	3	27
	Bacillus subtilis strain ASWISA7	1	0.9
	Pseudomonas stutzeri strain ASWISA6	1	0.9
	Bacillus cereus strain M2	3	27
	Bacillus thuringiensis strain LU3	3	27

Morphological characterization of the selected twelve isolates (new record isolate) and their molecular identification showed their culture characteristic on nutrient agar and their ability for spore formation (Table 4). All isolates are rod shape and eight isolate out of twelve are spore former.

Identification based on molecular characterization was reported and revealed the presence of fourteen strains belonging to three genera of *Bacillus* sp., *Lysinibacillus* sp. and *Enterobacter* sp. were isolated from oil contaminated soil. Meanwhile, produced water showed recovery of nine strains belonging to *Bacillus* sp., *Lysinibacillus* sp., *Enterobacter* sp. and *Brevibacillus* sp., which reported only in this produced water sample (Table 4). Additional five strains belonging to two genera, *Bacillus* sp. and *Pseudomonas* sp., were isolated from crude oil.

The most common isolates recovered from the collected samples were belonging to *Bacillus cereus* and *Bacillus subtilis* and reported in a high frequency. *Enterobacter aerogenes* was recorded once in contaminated soil. *Enterobacter cloacae* was recorded twice in produced water sample.

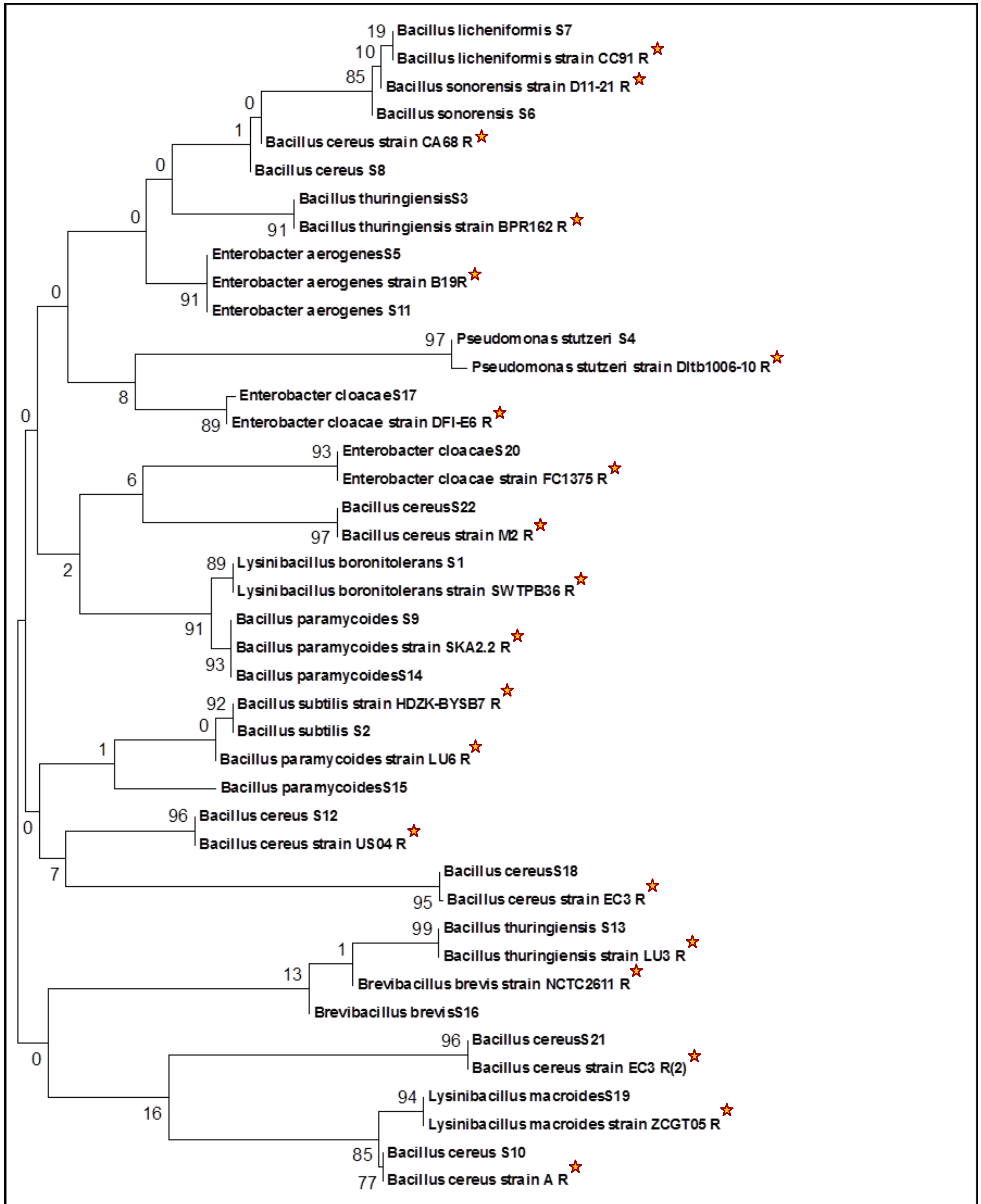

Figure (2): Rooted Neighbor Joining (N-J) phylogenetic tree constructed from concatenated sequences of 609 bp for each strain derived from an alignment of 16S rDNA gene sequences then produced from analysis conducted in MEGA 6 program. This N-J tree showing the distribution and phylogenetic relationships between 22 different strains (yellow asterisk) isolated from oil contaminated soil, produced water and crude oil samples in this study and 21 reference strains (R). All vertical branch lengths were drawn to scale Bootstrap values after 1000 repetitions are indicate.

Table (4): Morphological characterization and BLAST results of the16SrRNA gene sequences of the isolates.

Colony properties	Gram stain	Cell shape	Strain name	Accession number	Percentage identify	Name of the new strain in Gene Bank
Cream, raised, circular and undulate	+	Rode Spore forming	*Bacillus cereus* strain LDPE1	MN309828	99%	*Bacillus cereus* strain ASWISA1
Yellowish grey, .Large, opaque, , flat	+	Rode Spore forming	*Bacillus thuringiensis* strain BPR162	MN309829	99%	*Bacillus thuringiensis* strain ASWISA2
Brown yellowish , large ,irregular undulate	+	Rode Spore forming	*Bacillus sonorensis* strain D11-21	MN309830	99%	*Bacillus sonorensis* strain ASWISA3
Yellowish grey, Large, opaque, granular, flat colonies	+	Rode Spore forming	*Bacillus cereus* strain CA68	MN309831	99%	*Bacillus cereus* strain ASWISA4
white, large dull irregular, large , undulate, urbanite	+	Rode Spore forming	*Bacillus subtilis* IMG04	MN309832	99%	*Bacillus subtilis* strain ASWISA5
Yellowish grey, round, large, opaque, flat .irregular	-	Short rode	*Pseudomonas stutzeri* strain DItb1006-10	MN309833	99%	*Pseudomonas stutzeri* strain ASWISA6
Grey to yellow, round, large, opaque, flat .irregular	-	Short rode	*Bacillus subtilis* IMG04	MN309834	99%	*Bacillus subtilis* strain ASWISA7
Grey-yellow Large, opaque, , flat	+	Rode Spore forming	*Bacillus cereus* strain ISU-02	MN309835	99%	*Bacillus cereus* strain ASWISA8
White, dull irregular, large , undulate, ,dry	+	Rode Spore forming	*Bacillus paramycoides* strain LU	MN309836	99%	*Bacillus paramycoides* strain ASWISA9
White to cream, circular, opaque, waxy	+	Rode spore forming	*Bacillus paramycoides* strain SKA2.2	MN309837	99%	*Bacillus paramycoides* strain ASWISA10
White, circular, entire, smooth, flat, translucent	+	Rode Spore forming	*Brevibacillus brevis* strain NCTC2611	MN309838	98%	*Brevibacillus brevis* strain ASWISA11
Cream, medium, irregular, entire margin	-	Rod - non spore former	*Enterobacter cloacae* strain DFI-E6	MN309839	99%	*Enterobacter cloacae* strain ASWISA12

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عزل وتشخيص سلالات بكتيرية جديدة معزولة من مصادر مختلفة لحقل الرافضية النفطي في العراق

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الملخص العربي

خلال هذه الدراسة تم عزل اثنان وعشرون عزلة بكيرية تنقية التي لها القدرة على استخدام النفط الخام كمصدر للكاربون ، تواجدت وبنسب تردد مختلفة لكل من عينات التربة الملوثة بالنفط والمياه المنتجة والنفط الخام لحقل الرافضية النفطي (البصرة جنوب العراق) . تم وصف العزلات البكتيرية وتشخيصها بناء على الخواص المظهرية والتقنيات الجزيئية ، أربعة عشر سلالة تم عزلهما من التربة الملوثة بالزيت كانت تنتمي إلى ثلاثة أجناس هي *Bacillus* sp.، *Lysinibacillus* sp و *Enterobacter* sp. ، وتسعة سلالات تنتمي إلى *Bacillus* sp.، *Lysinibacillus* sp.، *Enterobacter* sp. و *Brevibacillus* sp. تم عزلها من المياه المنتجة. خمسة سلالات، تنتمي إلى جنسين، *Bacillus* sp. و *Pseudomonas* sp. تم عزلهما من النفط الخام . تم تسجيل اثني عشر سلالة كسلالات جديدة وإيداعها في بنك الجينات شملت كل من السلالات *Bacillus cereus* strain ASWISA1 و *Bacillus thuringiensis* strain ASWISA2 و *Bacillus sonorensis* strainASWISA3، *Bacillus cereus* strain ASWISA4، *Bacillus subtilis* strain ASWISA5 و *Pseudomonas stutzeri* strain ASWISA6، *Bacillus subtilis* strain ASWISA7، *Bacillus cereus* strain ASWISA8، *Bacillus paramycoides* strain ASWISA9، *Bacillus paramycoides* strain ASWISA10، *Brevibacillus brevis* strain ASWISA11 والسلالة *Enterobacter cloacae* strain ASWISA12.