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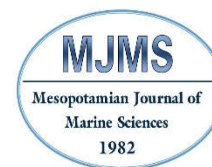

New GeneBank records of oil degrading bacteria isolated from polluted soil

iD **Fadhil N. Al-Kanany** *, iD **Satar A. Gmais**, iD **Raghad S. Jaafar**, iD **Omar A. AL-Badran**
Department of Biological Development of Shatt Al-Arab & N. Arabian Gulf, Marine Science Centre, University of Basrah, IRAQ
*Corresponding Author: e-mail: *alkanany170@gmail.com*

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Abstract -This work aim at isolation and identification of the dominant bacteria present in the oil polluted soil, as well as deposited the new isolates in the GeneBank. Some chemical parameters of contaminated soil were measured. Ten isolates were identified and characterized depending on the biochemical tests, as well as genetic characteristics using 16S rRNA gene comparing with reference isolates in the GeneBank, and through the detection of base pair sequence variations. These new local isolates were deposited in a GenBank under a new accession numbers; (OK576381.1, OK662612, OK662613, OK662614, OK662615, OK662616, OK662617, OK662618, OK662619, OK662620). Mineral salts medium (MSM) broth supported with crude oil as only source of carbon was adopted to isolate the bacteria. Culture media were supported with the same chemical parameters as the soil samples in order to simulate the physiochemical criteria of the environment. The successfully identified and recorded genera related to the above accession numbers were; Pseudomonas aeruginosa, Acinetobacter radioresistens, Pseudomonas oryzihabitans, Aeromonas hydrophila, Pseudomonas putida, Micrococcus luteus, Pseudomonas guariconensis, Achromobacter xylosoxidans, Bacillus foraminis, Pseudomonas stutzeri.

تسجيل جديد في بنك الجينات لبكتريا مكسرة للنفط معزولة من ترب ملوثة

فاضل نعمة الكنعان، ستار عزيز غميس، رغد شبر جعفر، عمر عبدالامير البدران
مركز علوم البحار، قسم التطور الاحيائي، جامعة البصرة، البصرة، العراق

المستخلص - يهدف هذا العمل إلى عزل وتشخيص البكتيريا السائدة الموجودة في التربة الملوثة بالنفط، وكذلك تسجيل العزلات الجديدة في بنك الجينات. تم قياس بعض المتغيرات الكيميائية للتربة الملوثة، وتم تحديد وتشخيص 10عزلات اعتماداً على الاختبارات البيوكيميائية ، وكذلك الخصائص الوراثية باستخدام S rRNA16. بعد مقارنتها بالعزلات المسجلة في بنك الجينات، ومن خلال الكشف عن الاختلافات في تتابعات القواعد النتروجينية ، تم تسجيل هذه العزلات المحلية الجديدة في بنك الجينات تحت أرقام انضمام جديدة ؛ (OK576381.1، OK662612، OK662613، OK662614، OK662615، OK662616، OK662617، OK662618، OK662619، OK662620). تم اعتماد مرق وسط أملاح معدنية (MSM) المدعم بالنفط الخام كمصدر وحيد للكربون لعزل البكتيريا. تم دعم الاوساط الزرعية بنفس المعلمات الكيميائية كما موجود في عينات التربة من أجل محاكاة المعايير الفيزيوكيميائية للبيئة. الأجناس التي تم تشخيصها وتسجيلها بنجاح والعائدة لارقام الانضمام المذكورة أعلاه كانت؛
Pseudomonas aeruginosa, Acinetobacter radioresistens, Pseudomonas oryzihabitans, Aeromonas hydrophila, Pseudomonas putida, Micrococcus luteus, Pseudomonas guariconensis, Achromobacter xylosoxidans, Bacillus foraminis, Pseudomonas stutzeri .

الكلمات المفتاحية: البكتريا المكسرة للنفط، الترب الملوثة، تسجيل جديد، عزلات محلية.

Introduction

Basrah city is the center of major oil companies operations in Iraq. (Republic of Iraq, 2016). Crude oil has properties that make it a substantial and effective pollutant of the ecosystem, causing severe harm to humanity. Chemical, physical, and biological treatments have all been employed

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to treat oil spills, but bioremediation is the most important of them (Yavari *et al*., 2015). In oil-contaminated soils, bacteria play an important role in environmental and biodegradable function processes (Peng *et al*., 2015). Petroleum components are released into the environment on a regular basis as a result of petroleum exploration, accidents, transportation, and leakage from waste disposal or storage sites, as well as from industrial sites(Ghoreishi *et al*., 2017). Petroleum can harm plants, animals, and humans since it includes hazardous compounds like benzene, toluene, ethyl benzene, xylene, and naphthalene (Sarkar *et al*., 2005). Petroleum (crude) oil is made up of a complex mixture of thousands of compounds. Petroleum hydrocarbons make up 50–98% of crude oil, making them a significant component depending on the source of the oil. Petroleum hydrocarbons could be biodegraded using a variety of microbes. Bacteria, on the other hand, are key biodegradable microorganisms that play an important role in hydrocarbon decomposition (Udgire *et al*., 2015). By far the most commonly housekeeping genetic marker has been the use of 16S rRNA gene sequences to investigate bacterial phylogeny and taxonomy. Universal PCR primers were employed to amplify the 16s rRNA gene, which is useful to diagnose bacterial species. The development of tools for analyzing the 16S rRNA gene sequence in a natural sample has gradually improved our ability to discover and identify bacteria (Azizan *et al*., 2018). The microbial population in the oil-contaminated environment of Basrah is predicted to change day by day as a result of extensive human activities related to the oil industry. The identification of bacterial strains reported in petroleum-contaminated locations will aid in understanding the dynamics of bacterial communities in oil-contaminated soils and provide helpful and necessary information for future petroleum-contaminated soil bioremediation efforts (Zhang *et al*., 2010). However, there are many more unknown petroleum-degrading bacteria in the environment than recognized isolates (Nie *et al*., 2014). Isolation of various bacteria from petroleum-contaminated soils has been accomplished utilizing culture-independent methods based on 16S rRNA genes (Jiao *et al*., 2016). The current study aimed to isolate oil-degrading bacteria from long-term polluted soils near oil companies in Basrah city, identifying and documenting of the new isolates in a GeneBank, and finally evaluate the distribution of new adapted bacteria due to prolonged oil waste exposure.

Materials and Methods

Soil sample collection

As shown in Table (1) and Figure (1), 250 gm of soil samples (15cm depth) were collected from six various oil-contaminated locations, all these sites were located in the west of Al- Qurna town, Basrah city, South of Iraq. The samples were collected in sterile plastic bags and brought to the laboratory, where they were maintained at laboratory temperature while the pH and salinity of the soil samples were evaluated.

Table 1: Coordination of the sampling stations

No.	Latitude	Longitude
1	30°45′01.29″N	47°19′35.93″E
2	30°48′49.43″N	47°19′30.73″E
3	30°48′46.99″N	47°22′14.13″E
4	30°49′17.04″N	47°19′31.78″E
5	30°52′16.97″N	47°20′06.56″E
6	30°52′40.86″N	47°19′38.22″E

Figure 1: Sampling stations (west of Al- Qurna town, Basrah city, South of Iraq)

Isolation of Bacteria

One gram of polluted soil was diluted to 10^{-4} with sterile D.W, grown in a 250 ml conical flask with 100 ml of mineral salts medium (MSM) consisting of (g/l): KCl (0.3), K_2HPO_4(1.03), KH_2PO_4(0.53), $FeSO_4.7H_2O$ (0.013), NaCl(1.5), $MnSO_4$.7H2O(0.53) and $CaCl_2$(0.23).

One hundred microliters of sterilized crude oil served as a sole carbon source (provided by Majnoon oil field) and trace elements stock solution of $FeCl_3.6H_2O$ (0.08 g/L), $ZnSO_4.H_2O$ (0.75 g/L), $COCl_2.6H_2O$ (0.08 g/L), $CuSO_4.5H_2O$ (0.075 g/L), $MgSO_4$. H_2O (0.75 g/L), H_3BO_3 (0.15 g/L) and 2ml of $Na_2MoO_4.2H_2O$ (0.05 g/L) were added. In a shaker incubator, they incubated for 7 days at 30°C with shaking at 150 rpm(Fujisawa and Murakami, 1980). The pH of the medium was set at the beginning while the salinity was set at 1.4mg/l. After the incubation period, one ml of the growth culture was diluted with normal saline to a concentration of 10^{-3}, and grown on a nutrient agar medium for 24 hours at 30°C. Then single colonies were selected from the agar to get pure colonies for the following steps.

Identification, biochemical characterisation, and molecular criteria

Pure bacterial colonies were cultured for 24 hours, morphological and biochemical criteria were carried out to identify the studied bacteria these include; Gram's staining, cell shape, catalase test, and oxidase test. The DNA was extracted and purified according to the instructions provided by the company (Qiagen/USA). Polymerase chain reaction (PCR) was used to amplify 1450 bp segment of the bacterial 16S rRNA gene using a universal 16S rRNA primers 27F-5'AGAGTTTGATCCTGGCTCAG3', 1492R-5'GGTTACCTTGTTACGACTT3' (Miyoshi *et al*., 2005), 2µl of pure DNA (50 ng/l), 3µl from each forward and reverse primers (62.5 mol/l), 25 µl Bioneer master mix (Bioneer, Korea) and deionized H2O were added to bring the total volume to 50 µl.

Thermocycler (3Prime, UK) with the following thermal profile; a gene amplification was used to incubate reaction through an initial denaturation at 96 ºC for 3 min. , followed by 27 cycles of amplification, denaturation at 96 ºC for 30 s, primer annealing at 56 ºC for 25 sec., primer extension was at 72 ºC for 15 sec. and finally at 72 ºC for 10 min. (Miyoshi *et al*., 2005). The PCR amplification result was evaluated using a 1.0 % agarose gel and a 100 bp DNA ladder using 1X

TBE buffer for 40 minutes at 120mA and 65V, gel was stained with ethidium bromide solution (0.5 µg/ml) for 20 minutes (Au - Lee *et al.,* 2012). A computerized UV transilluminator was used to observe the amplified nucleic acid (SYNGENE-GBOX F3, UK).

The NCBI web site was performed to analyse the degree of similarity between the PCR products and the sequences of other genes via data base queries (National Centre for Biotechnology Information, 2020) .

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Results and Discussion

Physiochemical parameters

The pH and salinity of the collected samples were measured and listed in Table (2). Salinity and pH of the collected soil samples were identified to simulate environmental characteristics that are reliant on culture media formulation. Samples of soil were collected from various polluted sites with diverse crude oil materials to obtain different bacterial types. Because the enzyme systems found in oil degrading bacteria differ with different available substrates, the diversity of soil contaminated sources leads to the diversity of different bacterial species. Mineral salts and trace elements were primarily used to make MSM media. Only oil degrading bacteria can grow in this media since crude oil is the only source of energy, which could be due to a lack of necessary enzymes to utilise this medium and benefit from hydrocarbons.

Table 2: pH and salinity of the soil samples

Sampling sites	pH	Salinity (ppt)
1	8.2	1.2
2	8.4	1.3
3	8.2	1.6
4	8.2	1.4
5	8.1	1.6
6	8.3	1.4

Morphological and biochemical criteria

Some morphological and biochemical characteristics of bacteria isolated from polluted soils must be determined prior to genetic identification. Table (3) depicts morphological and biochemical tests of studied bacteria to complete the subsequent genetic identification.

Table 3: Morphological and biochemical test results

Codes of purified bacterium	Gram's stain	Cell shape	Catalase test	Oxidase test
1	-ve	Coccobacillus	+	-
2	-ve	Rod	+	-
3	-ve	Rod	+	+
4	-ve	Rod	+	+
5	+ve	Coccus	+	+
6	-ve	Rod	+	+
7	-ve	Rod	+	+
8	-ve	Rod	+	+
9	-ve	Rod	+	-
10	-ve	Rod	+	+

Analysis and recording of DNA Sequences

Fifteen PCR products of targeted gene were sent to the MACROGEN/Korea "http://dna.macrogen.com" to get the gene sequencing. Using the Geneious Prime 2019 software version 1.1 (https://www.geneious.com). The raw sequences were visually reviewed and edited. The sequences were analysed by searching the National Center for Biotechnology Information Database (NCBI) for a similar sequence using the basic local alignment search tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The microbial population in these locations were the most diversified than expected, based on these overview data. *Pseudomonas* spp. is clearly the most dominant bacteria among the isolates (50 %), which may be owing to its own unique enzymes and metabolic pathways required to breakdown crude oil components (Das and Chandran, 2011). Furthermore, it is widely recognized that *pseudomonas* is one of the bacteria with a high hydrocarbon remediation potential (Stamenov *et al.*, 2015). The bacteria isolated in this study were suspected hydrocarbon tolerance and can grow in MSM medium containing 1% crude oil. Ten bacterial species were isolated, characterized, and deposited in a GenBank under a new accession numbers (Table 4).

Table (4): The identification results of 10 isolates of oil degrading bacteria by 16S rRNA sequences.

Sample no.	Bacterial type	Accession numbers (Submission from current study)
1	*Acinetobacter radioresistens* strain FadSat-1	OK662612
2	*Pseudomonas oryzihabitans* strain FadSat-5	OK662613
3	*Aeromonas hydrophila* strain FadSat-10	OK662614
4	*Pseudomonas putida* strain FadSat-12	OK662615
5	*Micrococcus luteus* strain FadSat-4A	OK662616
6	*Pseudomonas aeruginosa* strain alkanany	OK576381
7	*Pseudomonas guariconensis* strain FadSat-14	OK662617
8	*Achromobacter xylosoxidans* strain FadSat-19	OK662618
9	*Mesobacillus foraminis* strain FadSat-26	OK662619
10	*Pseudomonas stutzeri* strain FadSat-32	OK662620

Generally, the discovery of prominent bacterial communities in oil-contaminated soils in Al-Basrah environment supports in work of biological treatments at a low cost using green technology.

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