

Genetic identification of hydrocarbons degrading bacteria isolated from oily sludge and petroleum-contaminated soil in Basrah City, Iraq

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Abstract. About EM, Burghal AA, Laftah AH. 2021. Genetic identification of hydrocarbons degrading bacteria isolated from oily sludge and petroleum-contaminated soil in Basrah City, Iraq. *Biodiversitas* 22: 1934-1939. Petroleum is a major problem for environmental pollution due to the extensive use, which can reach all parts of the environment. Contaminated environments are required to identify bacterial diversity that has a remarkable ability to tolerate and biodegrade toxic compounds. The process of oil degradation by microorganisms in their natural environment is affected by certain chemical, physical and biological conditions. Bioremediation techniques are powerful for removing oil pollution, which reflects the ability of microorganisms to destroy organic pollutants. In the current study, six bacterial isolates (S1-S6) were isolated from oil-contaminated soils. mineral salt medium (MSM) was used to carry out biodegradation, crude oil degradation was an evaluation by the gravimetric method. After two weeks of incubation showed S5 isolate the greatest efficiency in oil degradation 53.6%, S1 and S6 also gave good capability degradation (47.31% and 44.9%) respectively, while isolates S2 and S3 gave the lowest percentage. All isolates were identified by 16S rDNA amplicons as *Pseudomonas stutzeri*, *Psychrobacter faecalis*, *Pseudomonas songnenensis*, *Bacillus cereus*, *Psychrobacter quanticus* strain E9R, and *Psychrobacter quanticus* strain EA422 according to molecular identification. The current study indicated that hydrocarbon-degrading bacteria specially *Psychrobacter* can be used to clean soil from crude oil and other industrial discharges.

Keywords: Bioremediation, crude oil, degrading bacterial, hydrocarbons, molecular identification

INTRODUCTION

Petroleum oil contamination is a major environmental pollution problem through the extraction, exploration, oil industries and other human activities (Ehis-Eriakha et al. 2020). Hydrocarbons are the most commonly used fuel in the world, they produce energy for every used (Litvinenko 2020). Clearly, the expanded use of petroleum products caused pollution in all parts of the environment, Therefore, studies are ongoing to diagnose microorganisms with catabolic potentials to degrade these pollutants (Chaillan et al. 2004). Crude oil comprises compounds and other organic compounds, mainly alkanes, cycloalkanes, and aromatic alkanes, which represent 50% to 80% of the oil structure. Large numbers of petroleum crude oil and oily sludge are mutagenic and toxic (Koshlaf and Ball 2017). Therefore using microorganisms is stopped the toxicity of oily components, microorganisms have the ability to degraded hydrocarbons such as bacteria, fungi, yeast and microalgae. The ability of microorganisms to degrade hydrocarbons in polluted areas with crude oil has been substantiated (Obuekwe et al. 2009; Allamin et al. 2020). In oil degradation, bacteria have an active part, where many bacteria can break down the oil-contaminated found in water and soil (Brinkrolf et al. 2006; Das and Chandran 2011). The process of crude oil bioremediation by

microorganisms in their natural environment is an economical, low-cost and green technology due to certain chemical, physical, and biological conditions and the composition and concentration of the oil pollutant (Deng et al. 2020). Additional factors, including oxygen, salinity pressure, temperature, and moisture, are important variables in the type of environment (Ezeonu et al. 2012; Kumari et al. 2013). Bioremediation processes are effective methods in the removal of oil pollution, which reflects the ability of microorganisms to survive in each area of the biosphere because their enzymatic activity is surprising, promote under a range of environmental condition, then destroy organic pollutants, expected to be an economical and safe way of treating oil pollution (Chandra et al. 2013; Abatenh et al. 2017). To recognize the microbial diversity in the ecosystem usually phylogenetic methods are used. These analyses depending on 16S rRNA genes have a great perception of the essential role in hydrocarbon degradation by microbial diversity and correlation of information between functional catabolic gene and 16S rRNA gene results for utilization of oil (Li et al. 2013). In this work, the aim was molecular identification of bacteria community and diversity in the soils exposed to the along-time of oil pollutants using the phylogenetic methodology. This study provides data to understand the catabolization of petroleum compounds by microorganisms in contaminated soils.

MATERIAL AND METHODS

Collection of soil samples

The crude oil contaminated soil and oil sludge samples were collected from five sites near Basrah Refineries Company in Basrah governorate, Iraq (the area is of latitude 30° 27' 49" N and longitude 47° 40' 27" E) as shown in Figure 1. This company was founded in 1969 through the establishment of the Basrah refinery, which actually began production in 1974. The soil samples were collated under depth 10-20 cm by sterile tool and put in sterile bags, while sludge samples were collected from sludge located in the same site in sterile containers. Samples were transferred to the laboratory immediately and stored at 4°C until tests were carried out. All samples were used for bacteria isolation.

Soil properties

Soil Texture was measured in the Center of Marine Sciences/University of Basrah depended on the method of Folk (1974), pH and electric conductivity (EC) were measured using pH electrode (Sartorius, Germany), moisture was measured as described by Eaton et al. (2005), total organic carbon (TOC), total carbon (TC) and NPK were measured using method of APHA (2005) and Baird (2017).

Bacterial strains isolation

The degrading bacterial strains were isolated on the nutrient agar medium by using spread plate technique, a serial dilution of samples was prepared including 10^{-1} - 10^{-6} dilutions by dissolving 1gm of oily soil and sludge with 100 mL of sterile saline solution into a flask and incubated in an orbital shaker at 120 rpm for 15 minutes. The mixture

was left to settle into the bottom, 1 mL of each suspension transferred into a test tube containing 9 mL of dilution solution to prepare serially diluted solution. 0.1 mL from the diluted sample was spread with a glass L shape rod onto plate with nutrient agar and incubated for 24-48 hours at $30 \pm 2^\circ\text{C}$ (Kannan et al. 2018). After incubation, the bacteria colonies were checked and pure colonies were selected, each isolate was labeled and maintained at 4°C for further studies on fresh sterile nutrient agar medium. The isolates were identified by gram staining and morphological (shape and color of the colony) and biochemical characteristics were performed, then the colonies were kept in slant test tubes and stored at 4°C until the following tests are carried out (Barathi and Vasudevan 2001).

Biodegradation of crude oil in liquid MSM

After the bacterial strains were isolated and named S1 to S6, the efficiency of oil biodegradation of these isolates were tested using the Mineral Salt Media (MSM), that consist of g/L NH_4NO_3 , 4; MgSO_4 , 0.5; CaCl_2 , 0.2; KH_2PO_4 , 0.5; K_2HPO_4 , 0.5 and 1 mL trace element solution containing per 100 mL: CuSO_4 , 0.1g; CaCl_2 , 0.05g; MnSO_4 , 0.178g; ZnSO_4 , 0.042g. All of these components were homogenized by a magnetic stirrer, pH 7 ± 0.2 adjusted and sterilized by autoclave. Isolates were activated in 10 mL nutrient broth medium. Erlenmeyer flasks of 250 mL were implied containing 100 mL of MSM were prepared, the isolates inoculum (5% v/v) were added to media in addition to the 2 mL of commercial light crude oil. Control samples containing MSM and crude oil without adding bacterial inoculum, all samples were incubated aerobically with shaker incubator at rotation speed 120 rpm at 32 °C for 2 weeks (Sarshad et al. 2015).

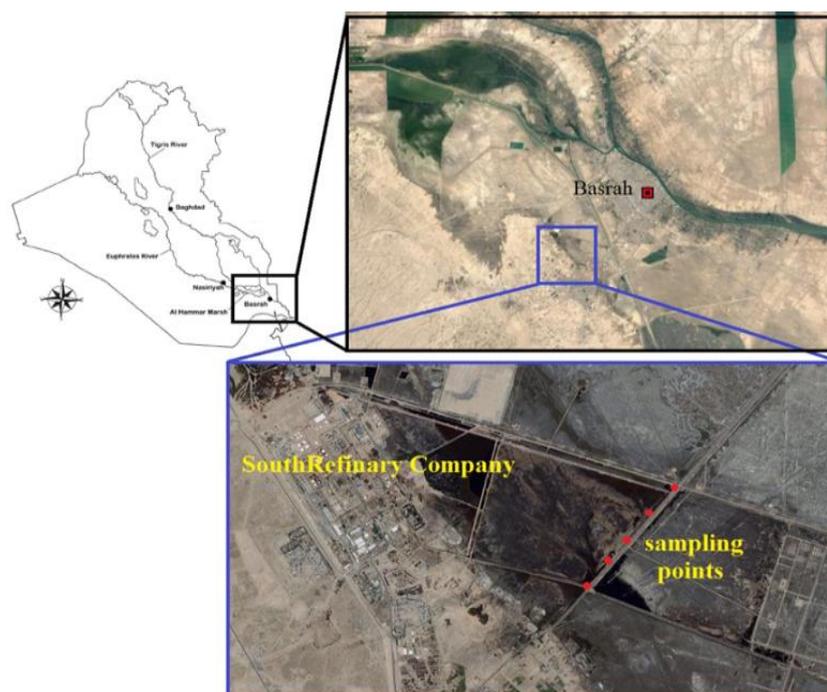


Figure 1. Map of samples collection site (red points represent mains location for sample collection)

Assessment of bacterial oil biodegradation

Crude oil degradation was evaluated by the gravimetric method. After two weeks of incubation, degrading processes appeared in the flasks at different proportions, after that the contents of the flask were placed in a separating funnel to extract the residual oil by adding 15 mL of chloroform to the separation funnel components three times to get all the remaining oil, the residual oil was fell down into Pre-weighed screw cap and then chloroform was vaporized on the hot plate under a fume hood, screws were cooled and weighed again to extract the weight difference. Oil in the flask control was also extracted in the same way (Bagherzadeh et al. 2008). The percentage of fractions was calculated and recorded. Gravimetric value for extracted oil was weighed for further calculation using the gravimetric method. The percent of Oil degraded was calculated as follows: (Allamin et al. 2020).

Weight of extracted crude oil = (weight of container after extraction oil – weight of the empty container).

Removal of crude oil% = (Control – sample of crude extracted) / control x 100

Molecular identification of isolates

Genomic DNA was extracted from six high-efficiency bacteria isolates by using Promega Genomic DNA isolation extraction kit according to manufacturer instructions (Promega, USA) bacteria were the following universal primers: 27F (AGAGTTTGATCMTGGCTCAG) (Lane 1991) and 1492R (TACGGYTACCTTG TTACGACTT) (Turner et al. 1999) to obtain products by thermal cycler (Bioneer, South Korea). The fragments of 16SrDNA genes amplification were performed by using a master mix of PCR (Promega, USA) contains a final volume of 50 µl, two primers (27F and 1492R), 2x master mix and dd. H₂O. Thermal cycler conditions including: first denaturation step at 94°C for 2 min followed by 35 cycles of second denaturation step at 94 °C for 1 min, step of annealing at 55 °C for 30s then extension step at 72 °C for 1 min and final extension for 10 min at 72°C (Liu et al. 2007). Amplified

16S rRNA gene products of approximately 1500 bp were confirmed by 2% (w/v) agarose gel and compared with 100 bp DNA ladder and visualized under ultraviolet radiation after staining with 0.05µl (0.5mg/mL) ethidium bromide (Thermo Fisher Scientific, USA). The products of 16S rRNA genes were transferred to Macrogen company in South Korea for sequencing. The 16S rDNA sequences were edited, analyzed and matched with sequences of the database in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST>) to evaluate the homology scores and detection of bacterial strains (Katoh et al. 2002).

RESULTS AND DISCUSSION

Soil properties

The results of the physical and chemical properties of the oil-contaminated soil were illustrated in Table (1). Which shows variation in parameters between soil collected from five sites. The pH value was range 7.5-8.66, percentage of soil moisture was 2.4-9.9%, EC of soil 1120-1616 µS/cm, the texture of soil according to the content type was belong to sandy loam, 10% clay (<0.002 mm), 77% silt (0.02-0.002mm), and 13% sand (0.02-2.0)mm. While the values of %TOC ranged from 1.35-2.45 and %TC ranged 1.039-1.886, N.P.K. levels in soil were 25.8-38 mg.kg⁻¹ (N), (2.3- 6.8) mg kg⁻¹(P), (0.45-1.4 3) mg/kg (K) respectively, the indigenous bacteria were (1.3×10⁴-4.2×10⁴) cfu/g.

After the primary isolation of bacteria from hydrocarbons contaminated samples and subculture on nutrient Agar, six colonies were selected from plates, three colonies were selected from soil samples plates, and three colonies were selected from sludge samples plates. The six colonies were labeled (S, S2, S3, S4, S5, and S6). Gram staining was used to identify the bacterial colonies showed five isolates were gram-negative, while one isolate (S4) was gram-positive spores forming (Table 2).

Table 1. Physical, chemical and microbiological features of experimental soil

Parameter	SITE 1	SITE 2	SITE 3	SITE 4	SITE 5
pH	7.8±0.15	7.5±0.1	8.66±0.55	8.52±0.04	8.4±0.3
Soil moisture (%)	3.4±	2.4±	1.5±	9.9±	2.8±
EC (µS/cm)	1145	1120	1547	1616	1134
Texture soil:	Sandy loamy				
Sand (%) 10; Silt (%) 77; Clay (%) 13					
TPHs g/kg	36.3	34.6	22.7	41.6	13.5
Organic TOC matter (%)	1.755	2.05	1.35	1.67	2.45
Organic C (TC)%	1.235	1.578	1.039	1.286	1.887
Total N (mg/kg)	38.5	24.3	35.3	28.6	25.8
P (mg/kg)	6.8	5.4	2.3	2.6	5.7
K (mg/g)	0.72	1.04	0.67	0.45	1.34
THBC cfu/g	3.2×10 ⁴	4.2×10 ⁴	1.2×10 ⁴	1.3×10 ⁴	2.3×10 ⁴

Assessment of bacterial biodegradation

The results of oil biodegradation by bacterial strains showed that six strains isolated from oil-contaminated sites had the ability for hydrocarbons degradation after ten days of incubation on mineral salt media. The extraction of remaining crude oil from media indicated the capability of oil-removing as showing in Figure 1, where the isolate S5 achieved the highest efficiency for oil degradation rate of 53.6%, S1 and S6 also gave good degradation rate of (47.3% and 44.9%) respectively, while isolates S2 and S3 gave the lowest percentage of oil degradation 14.6%.

Molecular identification of isolates

The isolates (S1, S2, S3, S4, S5, and S6) were identified according to 16SrDNA amplification. Based on the blasting results, isolates S1 and S3 belong to *Pseudomonas stutzeri* and *Pseudomonas songnenensis* species with 99% and 98% identity respectively. Isolates S2, S5 and S6 belong to *Psychrobacter* genus, These isolates detected as *Psychrobacter faecalis* 99%, *Psychrobacter quanticus* strain E9R 99%, and *Psychrobacter quanticus* strain EA422 100%, while isolate S4 closely related 99% with *Bacillus cereus* (Table 2).

In the current study Phylogenetic tree was constructed based on sequences of oil-degrading isolates from the NCBI- BLAST. The results of the phylogenetic tree placed three *Psychrobacter* isolates into two subgroups, The first subgroup included *Psychrobacter quanticus* while the second sub-group included *Psychrobacter faecalis*. two *Pseudomonas* isolates separated into two subgroups too included (*Pseudomonas stutzeri* and *Pseudomonas songnenensis*). In addition to one *Bacillus* isolate as explained in (Figure 2).

The physical and chemical parameters of the oil-contaminated sites are essential because they indicate the gradient of biological degradation and be used to assess treatment or weathering that occurs naturally in the environment (Devatha et al 2019). Microbes are acting as vital against when the contaminants have access to a diversity of substances to aid them to produce energy for the growth of cells. Many factors are affected on the bioremediation efficiency including the physicochemical properties of soil, the concentration of pollutants, and their accessibility to microbes (Truskewycz et al. 2019). Hydrocarbon pollutant treatment by bioremediation is an efficient strategy to remove soil contaminated, aiming to use microorganisms to solve this problem. This process has a lot of benefits to become very well-known in the environment and environmentally friendly by the possibility of using the materials resulting from the treatment as a source of energy, provide less toxic substances and less impact on the environment, the bioremediation technique has been used by many researchers and interested in these technologies (Mansur et al. 2016; Abatenh et al. 2017).

Grow on highly hydrocarbons concentration or refractory compounds its feature of *Pseudomonas* and *Bacillus* spp., this is due to their possession of a group of

enzymes that analyze chemical compounds. The possession of this natural bacterial capacity enables them to secrete enzymes to analyze when they need to do metabolic processes, leading to hydrocarbon biodegradation (Naik and Duraphe 2012). Several studies have reported the ability of various bacterial species to degrade hydrocarbons (Anaukwu et al. 2016; Wang et al. 2020; Perdigão et al. 2021). Ehis-Eriakha et al. (2020) report about the potential degrader's genes on a plasmid and chromosomal DNA in bacterial strains isolated from oil-polluted soil in Nigeria genetically identified, indicated 16 isolates have degradation activity in a liquid mineral medium. The highest potential for crude oil degradation by *Bacillus cereus* due to present various plasmids and chromosomes catabolic genes.

Table 2. Summarized the gram stain and morphological characteristics

Isolate	Gram stain	Cell shape	Colony color
S1	G-ve	Rod	Whitish(creamy)
S2	G-ve	Rod	Whitish(creamy)
S3	G-ve	Rod	Whitish(creamy)
S4	G+ve	Short rod	Whitish(creamy)
S5	G-ve	Rod	Whitish(creamy)
S6	G-ve	Rod	Whitish(creamy)

Table 3. List of oil-degrading strains identified according to 16SrDNA sequences

Isolates	Strains as in GenBank	Max. identity (%)
S1	<i>Pseudomonas stutzeri</i> strain DItb 1006-10R	99%
S2	<i>Psychrobacter faecalis</i> strain C18 16SR	99%
S3	<i>Pseudomonas songnenensis</i> ESF-139 7R	98%
S4	<i>Bacillus cereus</i> strain BY27	99%
S5	<i>Psychrobacter quanticus</i> strain E9R	99%
S6	<i>Psychrobacter quanticus</i> strain EA422	100%

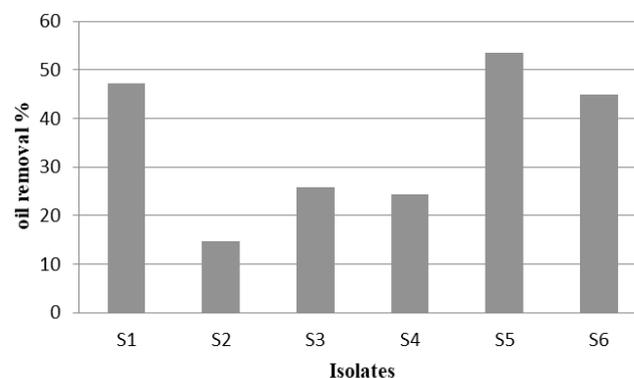


Figure 1. percentage of oil removing by bacterial isolates to TPHs after biodegradation through 14 days

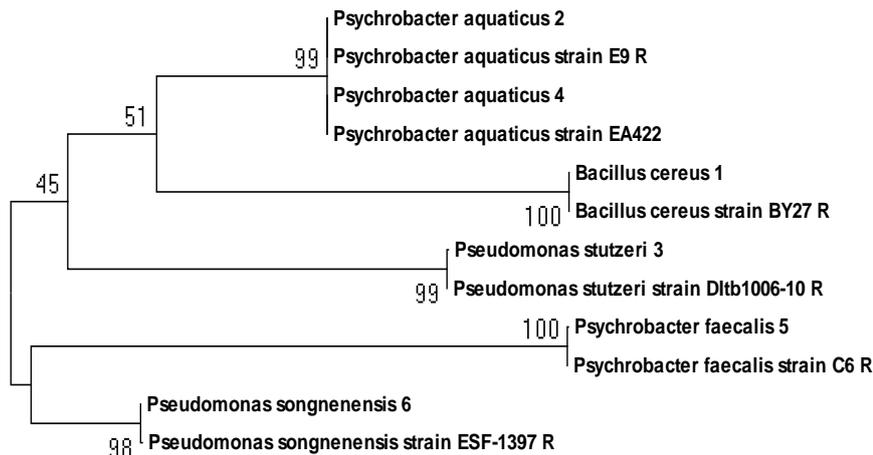


Figure 2. The neighbor-Joining phylogenetic tree created by MEGA 6 program, showed the relationships between 6 different isolated from (Soil and sludge contaminated with hydrocarbons) in this study and 6 reference strains (R).

Psychrobacter is one of the isolates, that it's not expected to show this biological activity because some of the species it is a pathogenic bacteria for humans such as *Psychrobacter immobilis* has been isolated from the eye, and other bacterial infections, these bacteria may be the cause of opportunistic infections. The clinical articulation of this species is practically unknown, although it has been isolated in patients with meningitis and other infections. In the current study, these bacteria gave the highest percentage removal of oil as shown in Figure 1. Subsequently indicated several studies, in Antarctic habitats, *Psychrobacter* is widely distributed in ornithogenic soils, anchor, grease ice, and salt lake (Maruyama et al. 2000; Ayala-del-Río et al. 2010; Kashi et al. 2020). *Psychrobacter* is a genus within the Gamma-Proteobacteria. The presence of some *Psychrobacter* in crude oil-contaminated soil and sludge has not been detected in scientific research therefore this study might the first time confirmed their ability to degrade hydrocarbons.

In the present study, this bacterium was isolated and identified from soils contaminated with hydrocarbons, confirms their ability to degrade hydrocarbons and gave the highest percentage in the removal and biodegradation of oil compared with other isolates. A study from Romaniuk et al. (2018) on *Psychrobacter* showed that it has tolerance to zinc, chromium, copper, and other heavy metals and resistance to arsenate. *Psychrobacter* cold-adapted indigenous microorganisms play a significant role in the *in situ* biodegradation of hydrocarbons in cold environments, where ambient temperatures often coincide with their growth temperature range (Margesin and Schinner 2001). However, since soil and sludge were collected from old petroleum contaminated sites, Oil concentration was high, the deterioration of oil has usually enhanced with changes in oil concentration, which is compatible with the results of Boshui et al. (2012).

Psychrobacter spp. prefer to grow at 20°C or down, but many strains may be tolerant salinity, where the ability to grow in concentrations of NaCl may reach more than 12%

(w/v), but these strains do not have the potential for hydrocarbon-degradation (Azevedo et al. 2013), the existence of several dangerous xenobiotics such as hydrocarbons, in some environments, increased occurrence of *Psychrobacter* spp, which can utilize these compounds. It has been observed in some species of *Psychrobacter*, a difference in biological activity, tends when the source of nitrogen and carbon is changed towards higher molecular weight hydrocarbons. The reason for its tolerance to change in the chemical composition of the medium and harsh conditions is its high adaptability to withstand abnormal conditions.

In conclusion, studying the diversity of bacteria that have the ability to biodegrade hydrocarbons is very important as the first stage in the treatment of petroleum hydrocarbons pollution. Six strains of bacteria were isolated from petroleum-contaminated soil. These strains are capable of using hydrocarbons in crude oil as the sole carbon source. The efficiency of hydrocarbons degradation after 10 days of incubation ranged from 14.63% to 53.6%. the isolates S1, S2, S3, S4, S5, and S6 were identified as *Pseudomonas stutzeri*, *Psychrobacter faecalis*, *Pseudomonas songnenensis*, *Bacillus cereus*, *Psychrobacter quanticus* strain E9R, *Psychrobacter quanticus* strain EA422 respectively. The ability to removing toxic substances from the environment, which is an effective advanced technology was successfully proved.

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