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Isolation, screening and characterization of hydrocarbon-degrading bacteria isolated from oil contaminated soil in Wasit province / Iraq

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Abstract

Eleven soil samples were collected from one of the oil sites in Wasit Governorate.65 bacterial isolates were isolated from soil samples by using BHM with 1% crude oil as carbon source. In primary and secondary screening (10) isolates of bacteria can remediate crude oil. Among the ten isolates, bacterial isolate named HD2 was most effective in bioremediation process. The results of biomass, optical density, and percentage of hydrocarbon remediation of HD2 were (0.63, 1.080 and 67 %) respectively. The results of morphological and biochemical tests showed that the bacterial isolate named HD2 belongs to the species *Pseudomonas putida*.

Key words: Biodegradation, crude oil, *Pseudomonas putida*.

1. Introduction

Soil pollution is a consequence of the accumulation of a wide range of chemical compounds generated either by natural or industrial processes [1]. Soil contamination has become a major global concern because of the threat to natural ecosystems and human health and with the accelerating pace of industrialization and urbanization [2]. Petroleum pollutants enter the soil environment through extraction, processing, and transportation [3]. The most dangerous and toxic aliphatic, cycloaliphatic, and aromatic hydrocarbons are the main contaminants in soil that has been contaminated with petroleum. The main source of hydrocarbon contamination in the soil is oil spills [4]. Petroleum hydrocarbons lessen the variety of plants and bacteria in the soil, reduce soil fertility, disturb the biological balance of the soil, and potentially endanger human health [5].

The most common traditional approach is excavation, followed by landfilling or incineration. Other methods such as bioremediation, soil vapor extraction, soil washing, thermal treatment and chemical oxidation used for remediating oil-contaminated soil [6]. Bioremediation, also called bioreclamation or biorestoration involves using organisms, primarily micro-organisms, to break down or detoxify hazardous waste into harmless substances such as carbon dioxide, water, and cellular biomass [7]. As a result, bioremediation seems superior to conventional treatment methods. Bioremediation is the process of fully mineralizing complicated organic pollutants into carbon dioxide, water, inorganic molecules, and cell proteins by using micro-organisms , or of converting harmful organic toxins into non-toxic compounds [8].

2. Materials and Methods

2.1 Collection of soil samples

The study area is in the southeastern part of Wasit province, near Tigris River, surrounded by agricultural areas and the Kardiya forest area, called Kardiya stores for petroleum products. Eleven soil samples were collected randomly from different locations at a depth of 5-10 cm as shown in (figure1).The soil sample used in this study was collected by using a soil auger, properly labeled, and transported in polythene bags to the lab. These soil samples contaminated oil were used to isolate the bacteria that degrade hydrocarbons.

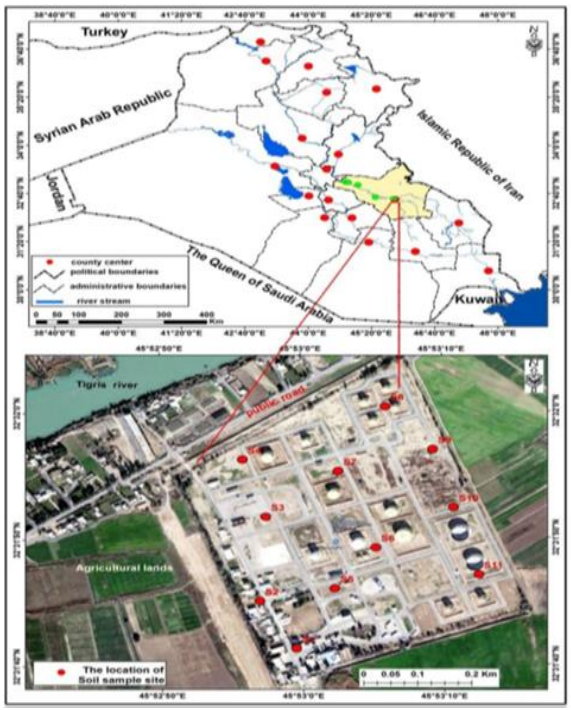

Figure 1: Location of the study area located in the southeastern part of Wasit province / Iraq.

2.2 Enhancement and isolation hydrocarbons degrading bacteria from soil samples

Bacteria isolated from soil samples contaminated with oil by technique for selective enrichment and using crude oil (was collected from the same study area) as the

sole source of carbon and energy. Fifty ml of Bushnell-Hass medium broth which is composed of KH_2PO_4 (1 g), K_2HPO_4 (1 g), $MgSO_4$ (0.2 g), $CaCl_2$ (0.02 g), $(NH_4)_2SO_4$ (1 g), and $FeCl_3$ (0.05 g). These components dissolve in (1 L) distilled water and pH of media was adjusted between (7-7.2) placed in 250 mL Erlenmeyer flasks and supplemented with (1 %) crude oil as hydrocarbon source sterilized by autoclave at 121 ºC for 15 minutes. After sterilization (1 g) from the original soil sample was added to each Erlenmeyer flask. The control flask contains BHM broth and crude oil without soil sample. All Erlenmeyer flasks were incubated in shaker incubator at 30 ˚C for 14 days at 150 rpm/min [9]. After incubation period optical density with spectrophotometer (600 nm) was measured for each flask and loop full of each flask (sample and control) cultured on plates of MSM agar composed from $NaNO_3$ (1.0 g), KH_2PO_4 (0.5 g), K_2HPO_4 (0.5 g), $MgSO_4$ (0.1 g), $CaCl_2$ (0.01 g), and $FeSO_4$ (0.001 g). These components were dissolved in (1 L) distilled water and the pH was changed to 7.2. Then, 2 % (v/v) agar - agar was added, by streaking method. Three replicates of plates were made for each flask and all plates were incubated at 30 ˚C for 48 hours [9]. The single colony of various isolates selected bacterial colony was transferred several times from mixed culture plates onto plates of Lauria agar by streaking method and incubated at 30 °C for 24 hours to obtain pure cultures of bacteria. Then Pure culture plates were kept in a refrigerator at 4 degrees Celsius for additional experiments [9].

2.3 Screening of bacterial isolates that degrade hydrocarbons.

There are several techniques employed as follows to choose the most effective bacterial isolate for Hydrocarbon degradative activity.

2.3.1 Preliminary screening for oil degradation

Pure bacterial isolates were reactivated on nutrient agar medium and incubated at 30 ˚C for 24 hours. Then bacterial inoculum of each isolate prepares using L broth and incubated at (30 ˚C for 18 hours). 250 mL Erlenmeyer flasks filled with (50 mL) of liquid BHM with the pH set at 7.0 and 1 % crude oil added as a substrate and these flasks were sterilized by autoclave, after sterilization bacterial inoculum (5 mL) from each reactivated bacterial isolates have been used to inoculate the Erlenmeyer flasks and the flasks incubated at 30 °C for 7 days

at 150 rpm in shaker incubator. After the end of the incubation period, optical density (600 nm), biomass and the percentage of hydrocarbon decomposition measured to determine the most efficient bacterial isolates in decomposition [10].

2.3.2. Secondary screening

Two ways were used to evaluate the degradation.

a-Formation of clearance zone

Another way to confirm the results obtained from the previous experiment was done by dilute the crude oil with ether and then the ether solution spread on the surface of solid MSM plates uniformly, the ether immediately vaporized, and a thin layer of oil remained on the agar surface. Loop full of pure bacterial isolates cultured by spreading technique over a 1 cm area on the middle of solid MSM plates, then the plates were incubated at 30 ˚C from 24 to 144 hours. All experiments were performed in a triplicate for each bacterial isolate and the diameter of clear zone around colonies were determined [11].

b-The growth on mineral salt medium agar plates

Pure isolates obtained were cultured on mineral salt medium agar supplemented with 1 % v/v crude oil at pH-7 ± 0.2, this medium sterilized by autoclave at 121 °C for 15 minutes. After sterilization this medium is inoculated with loop full of each bacterial isolate in the middle of the agar plate (with three replicates for each bacterial isolates) and placed in the incubator according to the condition 30 °C for 7 days. The degree of microbial growth strong, weak, and medium or no growth is used to determine which isolates can grow in this medium [12].

2.4 Identification of the bacterial isolates that degrade hydrocarbons.

The bacterial isolates with bioremediation abilities were identified according to the phenotypic identification and biochemical characteristics were examined by standard methods including methyl red test, Voges Proskauer test, indole test, starch hydrolysis, catalase, oxidase, urease test, gelatin utilization test , motility test and Simon citrate test.

3. Results and discussion

3.1 Isolation of crude oil degrading bacteria

Table 1 shows that sixty-five (65) bacterial isolates were found in soil that had been polluted with oil at (11) distinct sites of

the study area in Wasit province/ Iraq. Numerous studies have demonstrated that the most potent bacteria for petroleum bioremediation were isolated from oil-contaminated soil and water, these isolates included various species of bacteria.

Table 1: Enumeration of microbes in oil-contaminated soil.

Soil sample	Number of bacterial isolates
A1	5
A2	8
A3	6
A4	9
A5	7
A6	6
A7	4
A8	5
A9	4
A10	6
A11	5
The Total	65

Enrichment of microorganisms capable of breaking down crude oil was carried out in BH medium with (1 %) crude oil as the only carbon source and by the addition of the soil were incubated in shaker incubator at 30 ˚C for 14 days at 150 rpm/min. 65 bacterial isolates were obtained using spread plating on MSM agar. Microorganisms capable of breaking down different petroleum hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), naphthalene, monoaromatic hydrocarbons like toluene, or aliphatic hydrocarbons like the n-alkanes, can easily be isolated from the environment, especially from petroleum-contaminated sites [13, 14]. Reported that from soil and water samples polluted with hydrocarbons, 16 bacterial isolates were isolated and cultivated for 48 hours on nutrient agar with 1 % (vol/vol) crude oil. Also 19 diesel-degrading bacteria were found in enrichment cultures from various sampling sites [15]. Data showed that bitumen soil, which was predicted to have been exposed to hydrocarbon pollution, from garage sites from which also diesel-degrading bacteria was collected. These bacterial isolates (65) were subjected to a primary and secondary examination to determine the most effective isolate in bioremediation of pollutants.

3.2 Screening of bacterial isolates that degrade hydrocarbons.

3.2.1 Preliminary screening for oil degradation

The ability of bacteria that isolated from soil samples to degrade crude oil was accomplished through growth of bacterial isolates in liquid BHM with 1% of crude oil as only carbon source incubated at 30˚C for 7 days at 150 rpm in shaker incubator. Table (2) showing ten isolates from 65 hydrocarbon

degrading bacteria were shown to have the highest ability for crude oil bioremediation ,while the other bacterial isolates showed weak results in degradation. The capacity for decomposition of these isolates was noted based on optical density (600nm), biomass and the percentage of hydrocarbon decomposition.

Table 2: The variations between bacterial isolates in primary screening.

Code of isolates	Optical density 600 nm	Biomass g/L	Hydrocarbon degradation %
HD1	0.130	0.25	56 %
HD2	1.080	0.63	67 %
HD3	0.525	0.13	27 %
HD4	0.443	0.12	42 %
HD5	0.168	0.08	28 %
HD6	0.447	0.15	58 %
HD7	0.997	0.45	50 %
HD8	0.265	0.18	27 %
HD9	0.247	0.03	31 %
HD10	0.563	0.15	66 %
Control	0.000	0.45	15 %

Results in (table 2) revealed the ability of (10) bacterial isolates to degrade oil as carbon source and the bacterial isolates named as HD2, HD7 and HD10 were more effective, especially HD2 was extremely effective. The optical density, biomass, and hydrocarbon degradation % for bacterial isolate HD2 were 1.080, 0.63, and 67 % respectively. Local microorganisms that have been isolated from locations where crude oil has been contaminated are more effective at degrading oil [16]. Demonstrated that thirteen bacteria strains were found in samples of diesel-contaminated soil and seawater from the research location after 24 hours of incubation. The results of the screening test revealed that the bacteria with the codes EL and CT had the best resistance to diesel pollution and the highest growth rates. It was demonstrated that both may be more able than the others to use diesel as a carbon and energy source. Furthermore, various hydrocarbon-contaminated areas, seventeen (17) bacterial isolates that could flourish on crude oil were found [17]. The 0.5 % diesel in Bushnell-Hass medium was used to screen for bacterial oil degradation. Six isolates were further chosen for a consortium that demonstrated highest hydrocarbon usage based on their ability to breakdown hydrocarbons.

3.3 Result of clear zone formation

Experiments of clear zone formation have been done to 10 (HD1-HD10) isolates that showed highest degradation capacity in primary screening. The clear zone that formed on solid MSM plates served as a gauge for the effectiveness of crude oil degradation by 10 bacterial isolates as shown in (table 3).

Table 3: The ability of bacterial isolates to degrade crude oil on MSM agar (30˚C for 7 days) by using diameter of a clear zone (cm) as indicator.

Bacterial isolate	Diameter of a clear zone (cm) on MSM plates
HD1	2.3
HD2	8
HD3	6.5
HD4	3.9
HD5	2.3
HD6	6.4
HD7	7.5
HD8	5.8
HD9	5
HD10	6

The bacterial isolate HD2 having a clear zone with the largest diameter of (8 cm) and the isolates HD1 and HD5 having a clear zone with the lowest diameter of 2.3 cm for each isolate after 7 days of incubation. The organisms that developed clear zones around the colonies were regarded as crude oil degraders.

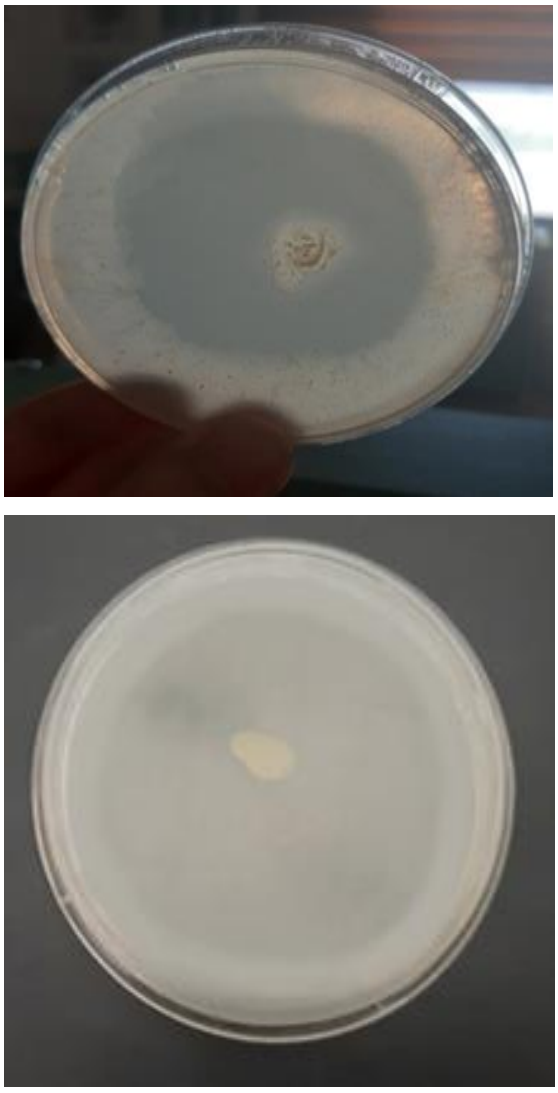

Figure 2: The formation of clear zone as result to degrade crude oil by bacterial isolate HD2 that appeared the highest diameter of clear zone (8 cm) on MSM agar [18].

Demonstrated that on mineral salt media, the largest clearance zone was formed by two bacterial isolates. On the seventh day of incubation, S2 (Bacillus subtilis) among these isolates displayed the highest growth

(0.85 mg/ml) and degradation, followed by S10 (Pseudomonas aeruginosa) (0.92 mg/ml). By using the plate assay method, the capacity of different bacterial strains to degrade crude oil was examined. Pseudomonas putida (8 mm in diameter) had the highest zone of exhibition in this investigation compared to Bacillus cereus (6 mm in diameter) [19].

3.4 The growth on mineral salt agar plates

The ability of pure bacterial isolates to grow in mineral salt agar supplemented with crude oil (1 % v/v) is used to determine which isolates can grow in this condition. The degree of microbial growth strong (+++), medium (++), weak (+) or no growth (-) serves as an indication for identifying isolates that can survive in such conditions. The growth efficiency on 1% crude oil was used to screen the degradability of crude oil by bacterial isolates (HD1-HD10).Crude oil disappeared from the medium, indicating its consumption by bacterial isolates.

Table 4: The ability of bacterial isolates (HD1-HD10) to grow in MSM agar supplemented with crude oil (1 % v/v).

Bacterial isolate	The degree of microbial growth
HD1	+
HD2	+++
HD3	++
HD4	-
HD5	-
HD6	++
HD7	+++
HD8	+
HD9	+
HD10	-

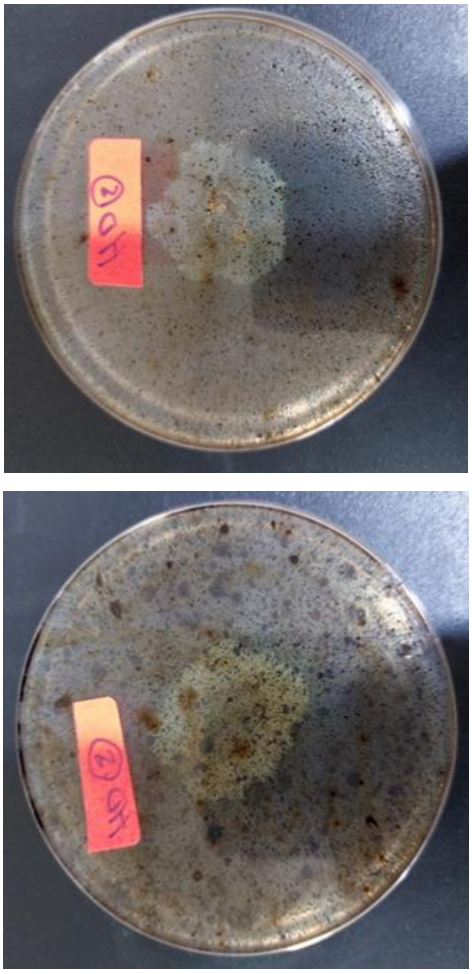

Figure 3: The growth of bacterial isolate HD2 on MSM agar supplemented with crude oil (1 % v/v).

As shown in (table 4) and (figure 3) bacterial isolate (HD2) showed the greatest oil degradation ability compared to other isolates and this result showed that HD2 could be used in bioremediation of soil contaminated with crude oil. It has been established that microorganisms that develop on soil that has been exposed to oil are far more capable of decomposing oil than those that are found on non-contaminated oil sites. This is an excellent illustration of adaptation. Sunar and et. al [20] Mentioned that the bacterial isolates are Pseudomonas sp., Acinetobacter sp., and Enterobacter sp. isolated from soil samples contaminated by petroleum hydrocarbon. These isolates can degrade diesel oil and all these isolates are able to grow on MS media that includes diesel oil and motor oil burnt as the only carbon source.

3.5 Morphological and biochemical characteristics of the bacterial isolates that degrade hydrocarbons pollutants

The bacterial isolates (HD 1-HD 10) with different susceptibility to bioremediation of hydrocarbon pollutants belong to various bacterial species such as Pseudomonas fluorescences and Pseudomonas aeruginosa and other bacterial species. The most effective bacterial isolate in bioremediation process of hydrocarbon pollutants as noticed from the results of primary and secondary screening was (HD 2). The bacteria isolate with bioremediation abilities were identified according to the gram stain, morphological identification of isolate (HD 2) cultured on lauria agar and the results of biochemical characteristics of (HD 2) presented in (table 5). Results of morphological and biochemical tests showed that HD 2 belongs to bacterial species Pseudomonas putida. The superior biodegradation of oil was done by Pseudomonas putida (76 %). The results of biomass and optical density for *P.putida* were (0.63, 1.080) respectively. This isolate was having high effectiveness in the main screening on mineral salts agar amended with oil also decomposed crude oil in liquid culture.

Table 5: Morphological and biochemical properties of the most effective bacterial isolate in bioremediation process (HD 2) Pseudomonas putida.

Morphological and biochemical properties of selected bacterial isolate (HD2)	Results
Gram reaction	Gram-negative
Colony shape	Circular
Colony color	Creamy
Texture	Mucous
Surface	Smooth
Colony elevation	Convex
Colony edge	zigzag edges
Methyl red	-
Voges-Proskauer test	-
Indole test	-
Gelatin test	-
Starch hydrolysis	-
Catalase test	+
Oxidase test	+
Urease test	-
Motility test	+
Simon citrate test	+

P. putida exists in a variety of environmental habitats due to its adaptable metabolism and minimal alimentary requirements. Additionally, it shows a very high sturdiness against harsh environmental circumstances, such as high temperatures, abnormal pH levels, or the presence of toxins or inhibitory solvents, Pseudomonas bacteria's enzymatic activity depends on the ecological environment it was isolated from [21]. A Gram-negative soil bacterium called Pseudomonas putida appears to be especially well-suited to produce natural products due to its excellent intrinsic metabolism and extraordinary resistance to numerous xenobiotics including PAHs [22]. The maximum biodegradation of oil was done by Pseudomonas sp. as degrading percentage was as 14.19 % and 11.97 % for diesel (4 % v/v) and burned engine oil (8 % v/v) after 7 days of incubation and the lowest degradation was done by *Acinetobacter sp* and *Enterobacter s*p [20]. Annie and co-workers [23] noticed the percent degradation reached was in the range of 31.6 % to 45.8 % for different TPHs when soil was supplemented with nutrients and mixed with coconut coir pith immobilized with *Pseudomonas putida*.

4 Conclusions

Sixty-five bacterial isolates were isolated from soil samples polluted with oil in Wasit Province, Iraq. Microorganisms in the soil would be able to utilize crude oil as their only source of carbon and energy. These organisms could then be used as possible bioremediation agents for soil that has been contaminated by petroleum. *Pseudomonus putida* was the most effective in bioremediation process.

5 References

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