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Hydrocarbon degradation test among the microbial community in oil-contaminated soil of power generators in Kerbala city, Iraq

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Abstract

The present study was designed to isolate the microbial community from oil-contaminated sites and other non-oil-contaminated sites which served as control samples in Kerbala city. In addition to test the effect of hydrocarbons on the growth of some types of bacteria. Bacterial genera and species were identified based on their growth on nutrient agar and blood agar as well as biochemical tests. According to the high bacterial growth rate on crude oil, 5 bacterial isolates were selected for further study. Growth of some identified bacteria in Minimal salt medium amended with hydrocarbon as the sole carbon source was investigated. *Acinetobacter* sp., *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pantoea* sp., *Pasteurella pneumonia / haemolytica*, *Chryseobacterium meningosepticum*, *Bordetella* sp., and *Enterobacter cloacae* were the dominant phyla among all the soil samples. Although all tested bacteria were able to grow on mineral liquid media, *P. aeruginosa* had the most capacity for growth in this media.

The present study showed that selected bacteria were able to grow and utilize MSM that contains different concentrations of gasoline. From this data, it can be concluded that oil-degrading bacteria are abundant in soils contaminated with spent oil. Consequently, these data suggest that these microbes could be useful for their application in the biodegradation of contaminated soils.

Keywords: contamination, degradation, microbial community, oil, soil.

اختبار تحلل الهيدروكربونات بين المجتمع الميكروبي في التربة الملوثة بالنفط لمولدات الطاقة في مدينة كربلاء ، العراق

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الخلاصه

صممت الدراسة الحالية لعزل المجتمع الميكروبي من المواقع الملوثة بالنفط والمواقع الأخرى غير الملوثة بالنفط والتي كانت بمثابة عينات سيطرة في مدينة كربلاء. فضلا عن اختبار تأثير الهيدروكربونات على نمو بعض أنواع البكتريا . تم تحديد الأجناس والأنواع البكتيرية بناءً على نموها على وسط الاكار المغذي ووسط

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اكار الدم أجار المغذيات وأجار الدم بالإضافة الى اختبارات الكيمياء الحياتية. وفقا لمعدل النمو البكتيري العالي على النفط الخام ، تم اختيار 5 عزلات بكتيرية لدراستها اكثر. تم دراسة نمو بعض البكتريا المعزولة على وسط قليل الملح والمزود بالهيدروكربون كمصدر وحيد للكاربون. تم عزل وتشخيص سلالات بكتيرية من التربة وهي *Acinetobacter* sp., *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pantoea* sp., *Pasteurella pneumonia / haemolytica*, *Chryseobacterium meningosepticum*, *Bordetella* sp., *Enterobacter cloacae*. على الرغم من ان كل البكتريا المدروسة كانت قادرة على النمو في وسط السائل المعدني الا ان بكتريا الزائفة الزنجارية كان لها القدرة الكبيرة على النمو في ذلك الوسط. أظهرت الدراسة الحالية ان البكتريا المدروسة كان لها القدرة على النمو وتحليل وسط قليل الاملاح والذي يحتوي تراكيز مختلفة من الكازولين. وبالتالي ، تقترح هذه البيانات إلى أن هذه الميكروبات يمكن أن تكون مفيدة لتطبيقها في التحلل البيولوجي للتربة الملوثة.

1. Introduction

Crude oil is a complex mixture of a large number of hydrocarbons and other non-hydrocarbon compounds, and different chemical elements; subsequently the chemical composition of the oil varies qualitatively and quantitatively [1]. The problem of pollution of crude petrol and its derivatives has emerged through the increase in global demand for oil which is considered as the main source of energy. Attention to the role of microorganisms in the consumption and decomposition of oil waste and its derivatives has already begun. Many taxa of microbes have been isolated and diagnosed with the ability to degrade different types of hydrocarbons; most of these microbes belong to Gram-negative bacillus and cocci bacteria [2]. On other hand, other microorganisms have been reported to be hydrocarbons producer including bioethanol [3].

Such microbes that can be considered crude oil hydrocarbon degraders include bacteria, fungi, yeasts, and microalgae [2]. Many bacteria have the ability for emulsifying hydrocarbons in solution by way of producing surface active agents such as biosurfactants which lead to increase cell adhesion to the substrate [4]. Autochthonous microbial communities have an important role in the degradation of petrol pollution; the composition of microorganisms' communities will be changed when subjected to oil pollution. Microbial numbers and the biodiversity of oil contaminated soils decrease due to the toxicity of these oil compounds and the delay of the stimulation process to produce enzymes that oxidize these compounds [5]. Certain hydrocarbon-degrading bacteria predominate in oil- impacted environments due to natural selection resulting from continued contamination of soil with oil [6]. Other ecological factors such as; regional climate, soil type, characteristics, and vegetation have been found to influence the composition of the microbial community [4,7,8].

Balba *et al*., [9] have summarized the advantages of microbial soil analyses including providing general information on soil microbial activities, identifying indigenous microbial communities adapted to the contaminated soil conditions, and finally identifying the soil bioremediation bacteria. Soil organisms are responsible for performing vital functions in the soil ecosystem that interact directly with biological, air, and water systems [10]. They act as primary factors in nutrient cycling, regulation of soil organic matter dynamics, carbon sequestration, greenhouse gas emissions, changes in the physical structure of soil and water systems, and enhancement of plant nutrient efficiency [10].

The relationship between microorganisms, oil, and the environment is still not entirely clear, and additional experiments should be conducted to clarify the role of different species of microorganisms in petroleum decomposition for the purpose of reducing the environmental pollution.

Iraq has been suffering from a severe power supply shortage since the destruction of most power generation and processing plants during the first Gulf War, and therefore resort to using small generators owned by the people to generate electricity. It was the best solution at

that time, unfortunately, it has become a reality up to the present. However, the maintenance of these generators and the storage of fuel in tanks near the generators result in the contamination of the soil surrounding diesel generators. This led to soil contamination with a microbial community that differs in composition from the soil that is not contaminated with diesel. There is a distinct lack of information with regard to the analysis of the microbial community on oil contaminated soil. Therefore, the aim of this study was to provide basic information about the bacterial taxa on diesel-contaminated soil in Iraq.

2. Materials and methods

2.1. Sampling

Soil samples were collected from various locations in Kerbala, Iraq. Three oil-contaminated samples (5-10 cm depth) were collected from three power stations as follows: the first location was the generator of Science College in the new building of Kerbala University (station A); the second site was the generator of Tourism Science College in the old building of Kerbala University (station B); while the third site was from Al- kafeel fuel filling station which is located beside the new building of Kerbala University (station C). At the same time, other three samples not contaminated with oil which served as control samples were collected from at least 30 m around the contaminated areas (station D).

2.2. Isolation of Bacteria

Blood agar based medium was used for initial isolation, detecting the ability of bacteria for hemolysis of blood and detecting the type of blood hemolysis. Nutrient agar medium was used for initial isolation and to study the cultural and morphological characteristics of bacterial isolates. MacConkey agar medium was used for isolating Gram- negative bacteria and to test the ability of these bacteria in lactose fermentation. These media were purchased from Himedia/ India. Minimal salt medium (MSM) was used throughout the study to detect the ability of bacteria for consuming hydrocarbons as the sole carbon source and contained the following basal components: KHPO, 1 g, K_2HPO_4, 1 g, $(NH_4)_2SO_4$, 1g, $MgSO_4$, 0.2 g, $CaCl_2$, 0.02 g and $FeCl_3$, 0.05 g [11]. These components were successively added to 1L sterile distilled water. Initial pH of the medium was adjusted to 7.0, then afterward the medium was autoclaved.

2.3. Counting of bacteria

Total cultivable bacteria were counted by using the spread plate method on blood agar and nutrient agar [12]. Plate count of the soil bacterial population was performed as follows: sample of 1 g of soil was added to 9 mL of sterile distilled water and serial dilutions were prepared. After appropriate serial dilutions, 100 µL of the suspension was spread over the surface of triplicate Petri dishes and incubated for 24 h at 37ºC. After that, the counting of bacteria was conducted for all plates containing 30 to 300 colony forming units (CFU) to determine the number of total cultivable bacteria (CFU g^{-1}) present in the samples.

2.4. Identification of isolates

Morphologically different colonies were randomly isolated from plates containing 30 to 300 CFU. Pure cultures were obtained by repetitive streaking onto Blood agar, Nutrient agar, and MacConkey agar (as appropriate), and bacteria were maintained at 4 °C for further studies. The isolates were identified on the basis of morphology, biochemical tests such as Gram staining and tests for oxidation/fermentation, the production of acid from carbohydrates, oxidase enzyme production, and catalase enzyme production according to Bergey's Manual of Systematic Bacteriology (taxonomy) [13]. Further, API 20 System test was used according to the manufacturer's instructions for further identification of bacterial isolates.

2.5. Oil degrading test

In order to study the ability of tested bacteria for growth consuming hydrocarbons as carbon source, serial volumes were prepared in tubes, each one with 10 ml (6 tubes for each volume) as is shown in Table (1).

Table 1- Summary of the total components of tubes (MSM media and gasoline).

Tubes No.	Minimal salt medium (MSM)/ ml	Gasoline/ml	Total Number of tubes
1	9	1	6
2	8	2	6
3	7	3	6
4	6	4	6
5	5	5	6
6	4	6	6
7	3	7	6
8	2	8	6
9	1	9	6
10	0	10	6

Each independently was inoculated with an overnight grown culture of each identified bacterial genera including *Enterococcus faecalis*, *Streptococcus agalactiae*, *P. aeruginosa*, *P. pneumonia / haemolytica*, *C. meningosepticum* and consortium, tubes were then incubated at 35 °C for 48 h. Thereafter, the bacterial growth was measured spectrophotometrically at 420 nm.

2.6. Measurement of pH before and after bacterial growth

Medium with gasoline was used to determine the pH before and after bacterial growth. Each bacterial strain was inoculated in conical flasks containing 100 ml of MSM medium with 6 ml of gasoline. Values of pH were measured first for each flask. Bacterial isolates were incubated at 35 °C in a shaking water bath for 72 h. Values of pH were measured a second time after that. Each bacterial strain was cultured in triplicate. Controls without added cells were run to determine the abiotic oxidation of hydrocarbon [14].

3. Statistical analysis

Microbiology data obtained from the oil and non-oil contaminated soil samples were represented as the mean ± standard deviation (SD) of three replicates. Significant difference determinations were one-way and two-way ANOVA by means of Minitab statistical software version 16, IBM (Pennsylvania, USA).

4. Results and discussion

4.1. Counting of bacteria

The mean log counts of bacterial colonies isolated from soil samples which grew on blood agar (BA) and nutrient agar (NA) are presented in Figure 1. Mean log values for the bacterial population which grew in NA were 6.2 ± 0.3, 5.3 ± 0.4, 5.9 ± 0. 3 and 5.7 ± 0. 6 CFU g^{-1} while, mean log of 6.4 ± 0.4, 6.4 ± 0.5, 5.8 ± 0.5 and 5.5 ± 0. 5 CFU g^{-1} were recorded on BA media in the stations of A, B, C and D, respectively (Figure 1).

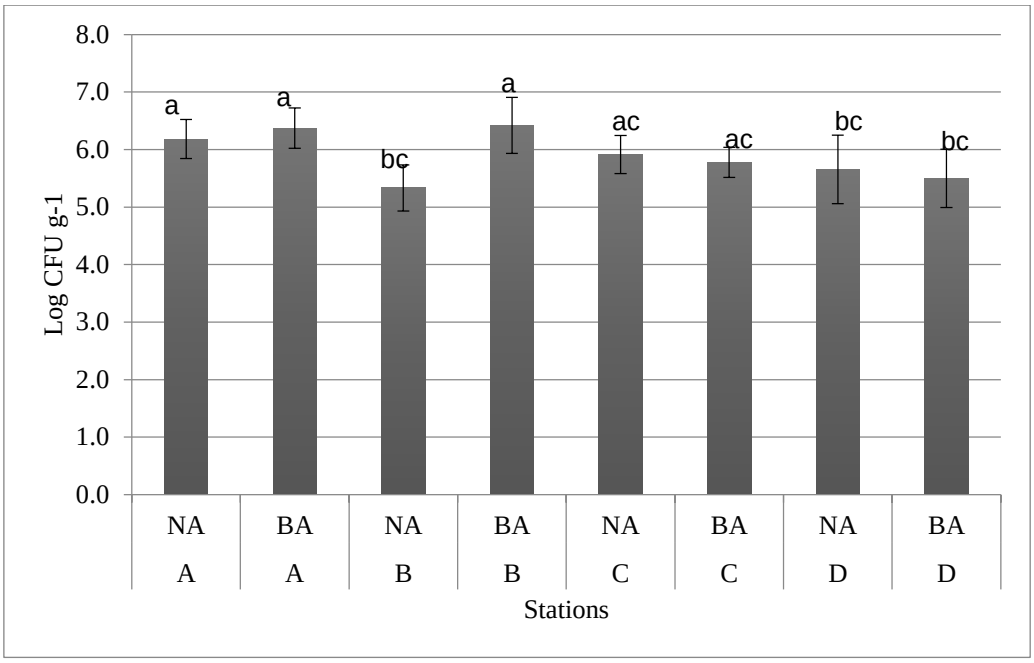

Figure 1- Number of bacterial colonies grown on nutrient agar (NA) and blood agar (BA) isolated from the stations.
Results are presented as mean log values ± SD in each station (n = 3). Columns having different letters are significantly different ($P < 0.05$).

A two-way ANOVA revealed significant differences between bacterial populations for media (P=0.004) and stations (P=0.02) (Figure 1). In station B, the bacterial population grown on NA were significantly lower compared to those grown on BA at the same station and of both media at station A. The current study demonstrated that the microbial community was detected through the oil-contaminated and non-oil-contaminated soils, where their numbers were observed in the stations at levels of log 5.3 – 6.4 CFU g^{-1}. Several prior publications have investigated the microbial community in the different areas of oil-contaminated and non-oil-contaminated soils by means of both conventional and molecular methods [14,15,16].
In agreement with the current results, data obtained from the study of Wolińska et al ., showed that the numbers of cultivable bacteria which could be isolated from contaminated and control samples were different but no significant differences were found [14]. Furthermore, different levels of heterotrophic and crude oil degrading bacteria were determined from three samples (mussels, seawater, and sediment) collected from two stations in Arab Gulf, using most probable number and cultivable viable count methods [17]. Sutton *et al*. demonstrated that the bacterial community structure and diversity could be significantly influenced by the presence of oil contamination and suggested that contaminated soil had lower diversity than clean samples [18]. These data are opposite to the present results that found the control soils (clean samples) had lower diversity than oil-contaminated soil. A slight increase in the microbial community in oil-contaminated soils compared to control soil could be attributed to the availability of hydrocarbons in soil which are considered a carbon source for bacterial growth [19].

4.2. Identification of bacterial isolates

Investigation of the total cultivable bacterial isolates by inoculation of samples on BA and NA media, and identification of pure colonies by biochemical tests, showed that 13 bacterial species were the dominant bacteria of each station (Table 2). *P. aeruginos*a, *P. pneumonia / haemolytica*, *C. meningosepticum* and *E. faecalis* were the dominant bacteria at the control station, while other bacteria were absent.

Table 2-Summary of some bacterial genera isolated from the stations in Kerbala city.

Bacterial isolates	Station A	Station B	Station C	Station D
Acinetobacter sp.	+	+	+	-
P. aeruginosa	+	+	+	+
P. fluorescens	+	+	+	-
Pantoea sp.	+	+	+	-
P. pneumonia / haemolytica	+	+	+	+
C. meningosepticum	+	+	+	+
Bordetella sp.	+	+	+	-
E. cloacae	+	+	+	-
S. agalactiae	+	+	+	-
Enterococcus sp.	+	+	+	-
Bacillus sp.	+	+	+	-
Staphylococcus sp.	+	+	+	-
E. faecalis	+	+	+	+

Representative soil isolates were classified into three phyla: Proteobacteria, Firmicutes, and Bacteroidetes; percentages of these phyla were 53.8%, 38.5%, and 7.7%, respectively (Figure 2).

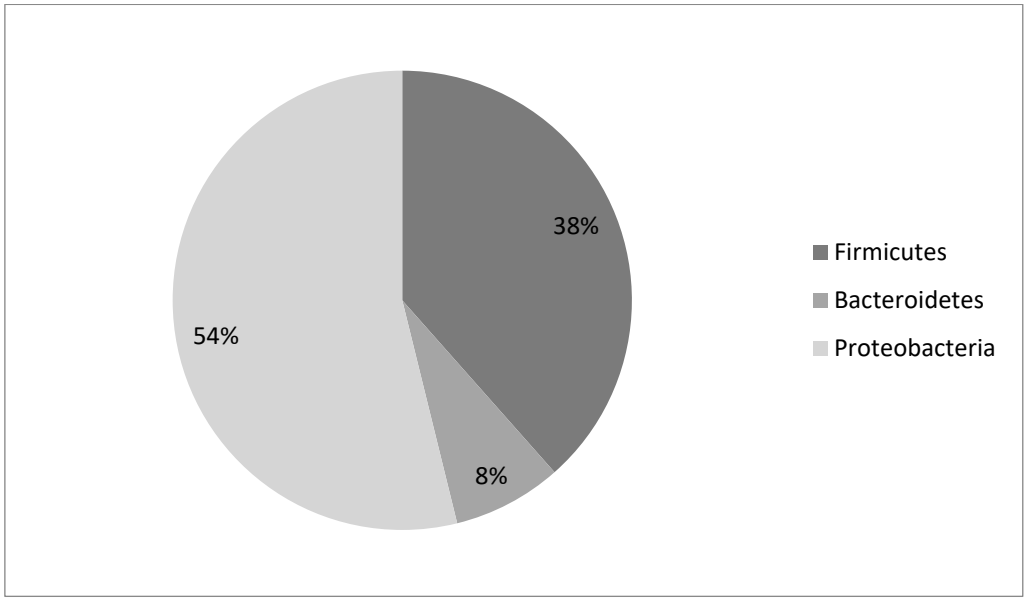

Figure 2-Proportion percentage (%) of cultivable bacterial phyla in the stations.

Based on the morphological results, oil contaminated soils were dominated by members of the phyla Proteobacteria, Firmicutes and Bacteroidetes, which are commonly reported to be the dominant communities in polluted soils [18,19,20,21]. In the present study, the most abundant phyla in polluted soils were related to Proteobacteria; this was in accordance to most previous studies which showed that Proteobacteria were the most predominant phyla [18,22]. *Acinetobacter* sp. have previously been reported to be present in polluted soil [19,23]. According to the literature, *Pseudomonas* sp. can be regarded as the dominant group of organic compound degrading bacteria in polluted samples [21,24,25,26,27]. *Pantoea* sp. have been previously reported to be present in the soil and water samples collected from different petroleum-contaminated sites in Kuwait [26].

C. meningosepticum was the only representative of *Bacteroidetes* in aerobic, mesophilic microbe with a temperature optimum of 28–30 ℃ [28]. *Chryseobacterium* sp. isolated from hydrocarbon-contaminated soil [28,29] were also among the most commonly identified cultivable isolates in all stations (including control station) in the present study. *Bacillus* and *E. faecalis* were isolated from hydrocarbon-contaminated soil in previous studies [20,26,29,30,31].

4.3. Oil degrading test

Table 3 showed that the results of the abilities of different bacteria in the analysis of hydrocarbons compounds. It is clear to see that *P. aeruginos*a (3) was significantly higher in light absorption which reflects the ability of this bacteria to grow on media containing hydrocarbon compounds compared with other microbes (P<0.05). On the other hand, the bacterial mixture (6) also had significant differences in light absorption. No significant differences were found between *P. aeruginos*a and the bacterial mixture (P>0.05). Additionally, there were no significant differences found between concentrations of gasoline, which represented hydrocarbon compounds (P>0.05).

Table 3- Summary of some bacterial genera isolated from the stations in Kerbela city.

No of tubes	*E. faecalis*	*S. agalactiae*	*P. aeruginosa*	*P.pneumonia / haemolytica*	*C. meningosepticum*	consortium	OD
	a*	a*	b*	a*	a*	b*	P=0.00
1	0.355	0.3	0.5	0.246	0.236	0.366	
2	0.425	0.35	0.521	0.362	0.16	0.581	
3	0.398	0.378	0.571	0.357	0.42	0.744	
4	0.46	0.485	0.495	0.395	0.444	0.376	
5	0.405	0.381	0.526	0.47	0.375	0.65	420 A
6	0.672	0.316	0.735	0.376	0.508	0.735	
7	0.21	0.392	0.781	0.334	0.387	1.047	
8	0.313	0.36	0.88	0.321	0.448	1.005	
9	0.245	0.31	0.85	0.24	0.42	1.003	
10	0.003	0.009	0.006	0.003	0.004	0.004	

* Column having different letters are significantly different (P < 0.05). Numbers (1-6) indicate the bacterial genera, while (1-10) indicate the total number of tubes which contain different volumes of MSM plus gasoline.

Significant differences between bacterial growth were found (P=0.000) (Figure 3). Tube (column no 6) which had 40% of MSM plus 60 % of gasoline was significantly higher (P < 0.000) in support of *E. faecalis* bacteria in comparison with components of other tubes which had the same concentration but different grown bacteria.

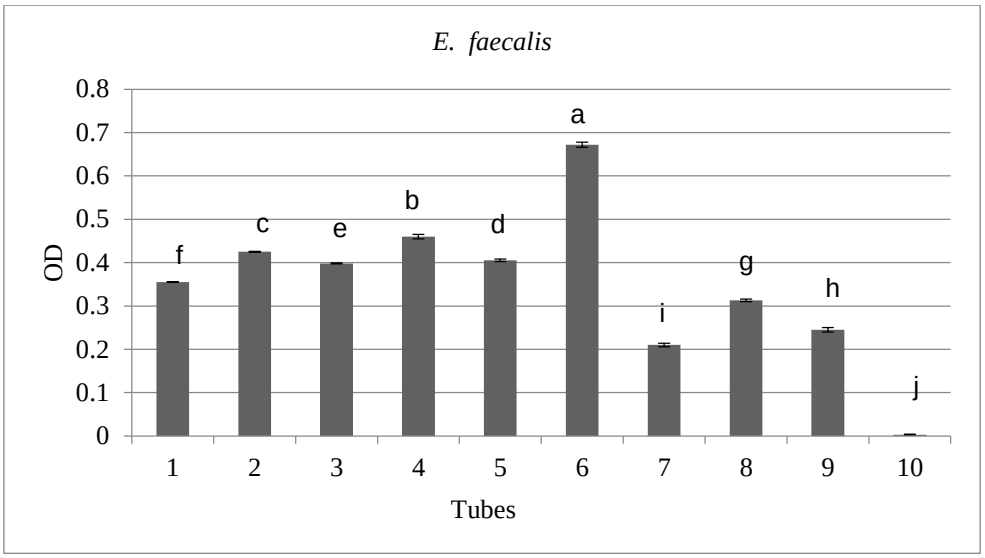

Figure 3-Growth of *E. faecalis* in the set of tubes (10 tubes) which contain different volumes of MSM media and gasoline. Columns having different letters are significantly different ($P < 0.05$).

Figure 4 showed that tube 6 revealed highly significant ($P < 0.05$) support the growth of *S. agalactiae* bacteria in comparison with components of the other tubes. Tube 10 which had only 10 ml of gasoline did not support the growth of this bacteria by means of lower significant differences compared with other tubes.

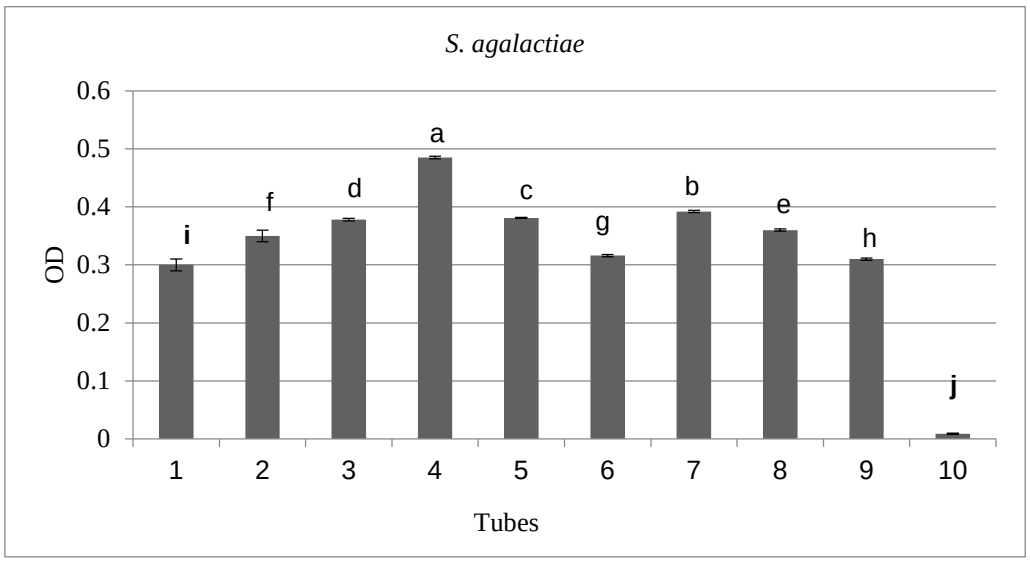

Figure 4-Growth of *S. agalactiae* in the set of tubes (10 tubes) which contain different volumes of MSM media and gasoline. Columns having different letters are significantly different ($P < 0.05$).

Significant differences were found between tubes (6, 7, 8, and 9), but these tubes were significantly higher ($P < 0.05$) in comparison to other tubes that encouraged the growth of bacteria *P. aeruginosa*. The same trend was shown in regard to tube number 10 (Figure 5).

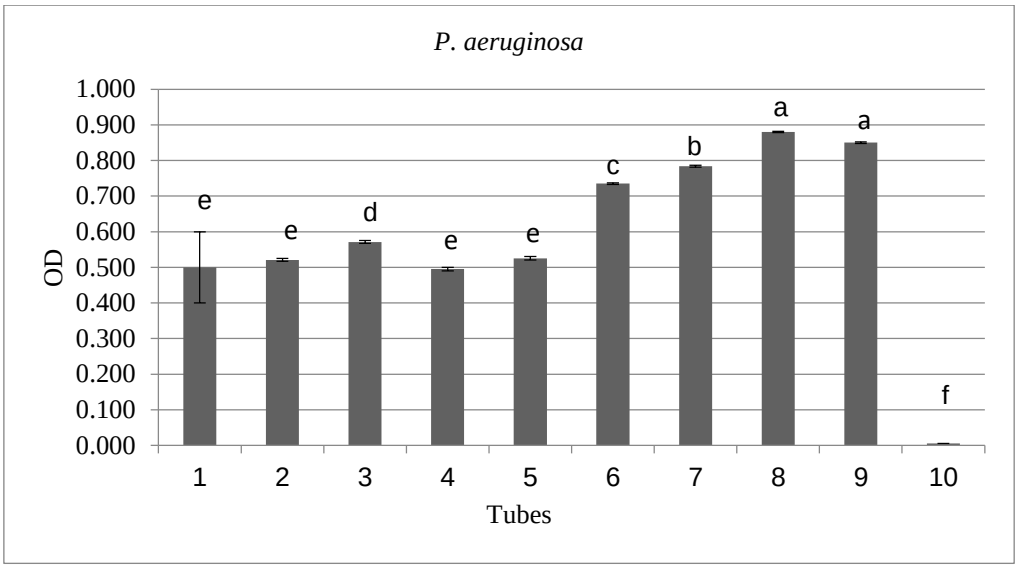

Figure 5-Growth of *P. aeruginosa* in the set of tubes (10 tubes) which contain different volumes of MSM media and gasoline. Columns having different letters are significantly different ($P < 0.05$).

Figure 6 showed that significant differences between all tubes were found; tubes 1 and 9 revealed significantly lower growth of *P. pneumonia / haemolytica*. On the other hand, tube 5 was significantly higher ($P < 0.05$) compared to other tubes supporting the growth of this microbe ($P < 0.05$). Again tube 10 exhibited the same tendency in inhibition of the growth.

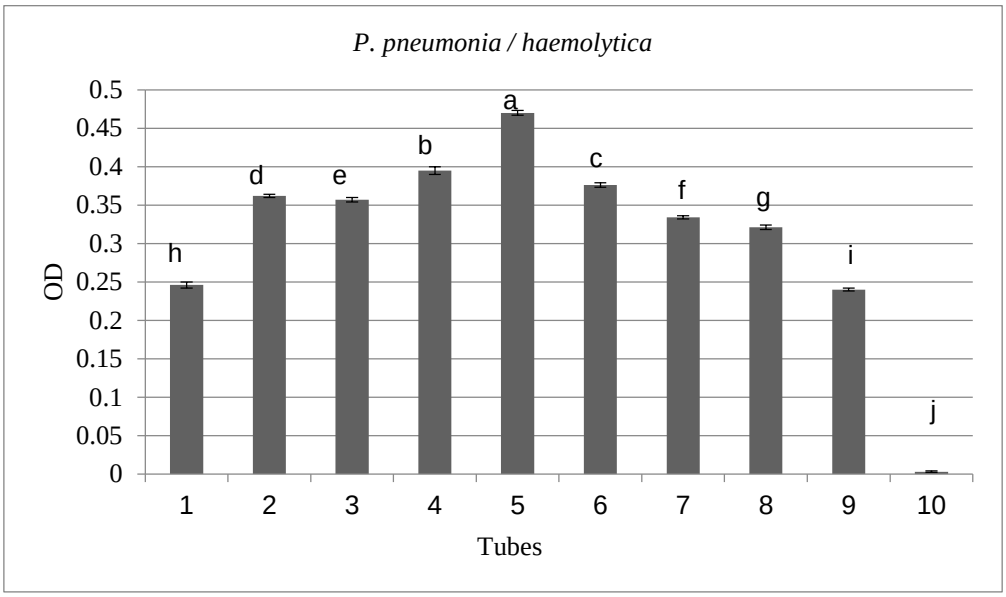

Figure 6-Growth of *P. pneumonia / haemolytica* in the set of tubes (10 tubes) which contain different volumes of MSM media and gasoline. Columns having different letters are significantly different ($P < 0.05$).

Tube 3 revealed significant differences in promoting the growth of *Chryseobacterium meningosepticum*, they had lower growth ($P < 0.05$) in comparison to other tubes. On the other hand, tube 6 was significantly higher ($P < 0.05$) compared to other tubes supporting the growth of this microbe ($P < 0.05$). Complete growth inhibition was found in tube 10 (Figure 7).

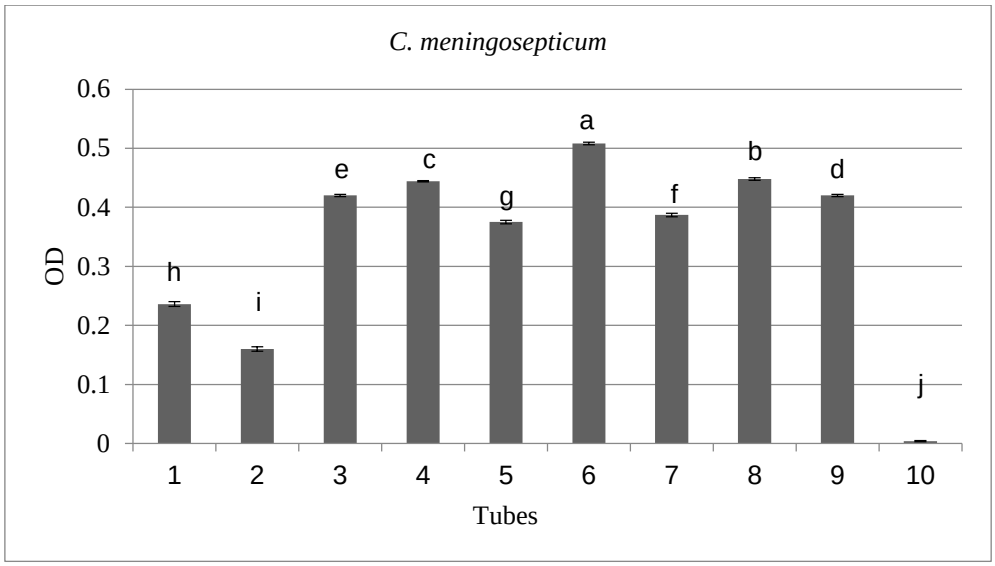

Figure 7- Growth of *C. meningosepticum* in the set of tubes (10 tubes) which contain different volumes of MSM media and gasoline. Columns having different letters are significantly different ($P < 0.05$).

Finally, lower growth of microbes was shown in tubes 1 and 4, at the same time significant differences in promotion or inhibition of growth were found between them. Tube 7 revealed significantly higher growth ($P < 0.05$) compared to other tubes. Inhibition of growth was found in tube 10 as well (Figure 8).

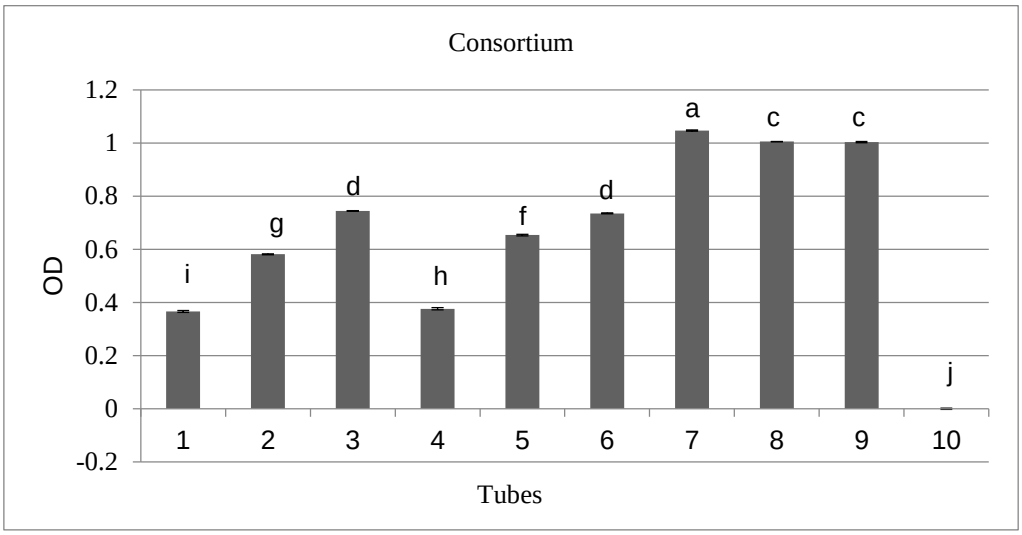

Figure 8- Growth of consortium in the set of tubes (10 tubes) which contain different volumes of MSM media and gasoline. Columns having different letters are significantly different ($P < 0.05$).

Some isolated genera produced a foam when they grew on hydrocarbons containing MSM medium (Figure. 9).

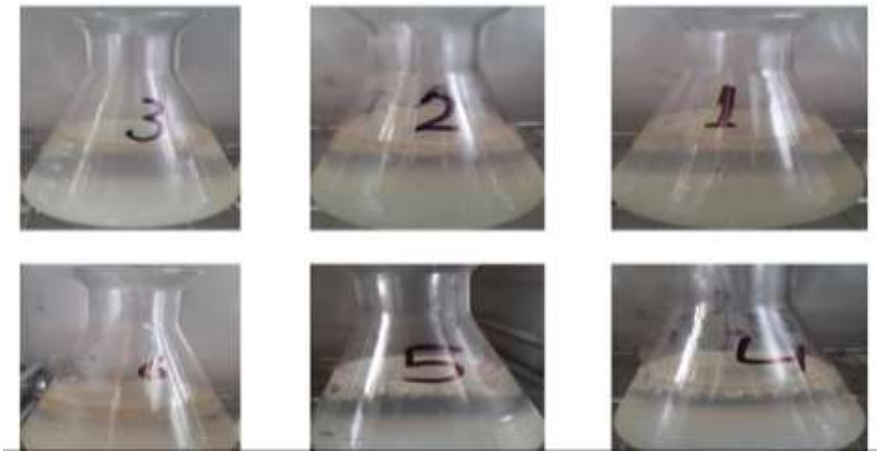

Figure 9-Excessive foam-forming properties of *different bacterial genera* grown on gasoline-containing MSM medium. Number codes *E. faecalis* (1), *S. agalactiae* (2), *P. aeruginosa,* (3), *P. pneumonia / haemolytica* (4), *C. meningosepticum* (5), and consortium (6).

After identification and diagnosis of isolated bacteria, some bacterial strains isolated from oil-contaminated samples were selected for further investigation due to their growth ability in the presence of hydrocarbons as the only source of carbon. Although all tested bacteria were able to grow on mineral liquid media amended with gasoline as the sole carbon and energy source, *P. aeruginosa* had the most ability in growing in this media. The activity of tested bacteria in oil degrading in the current study is in agreement with previous studies [31, 32, 33, 34, 35].
Biosurfactants can reduce surface tension by accumulating immiscible fluids at the interface, leading to an increase in the surface area of insoluble compounds, resulting in increased bioavailability and subsequent biodegradation of the hydrocarbon [36]. The agglomeration of used oil (diesel) in the form of a gel mass is called the phenomenon of mousse or pseudosolubilization. It is thought that the occurrence of this phenomenon in the environment is due to specific factors including weathering, the activity of microorganisms, decomposition of hydrocarbons, and the accumulation of emulsifying agents [37,38].
In spite of the fact that hydrocarbons can be affected by individual microbes or by a consortium of microbial strains belonging to either the same or different genera, previous studies have found that a consortium has more effect than individual cultures for metabolizing/degrading of hydrocarbons [39, 40].

4.4 Measurement of pH before and after bacterial growth

To determine the pH values before and after bacterial growth, conical flasks containing 100 ml of MSM medium with 6 ml of gasoline were used. Initial pH value was measured before bacteria inoculation and after 72 h incubation period at 30 °C, pH values were also measured (Table 4).

Table 4-Revealing the differences between pH values before and after the incubation period for each species of bacteria.

Bacterial isolates	Initial pH	pH after 72 h
E. faecalis	7.06	6.49
S. agalactiae	7.16	6.45
P. aeruginosa	7.14	6.41
P. pneumonia / haemolytica	7.11	6.43
C. meningosepticum	7.09	6.43
Consortium	7.00	6.31
Control	7.08	6.42

Varjani *et al*., [37] demonstrated that such factors including pollutant characteristics, physiology of microbes, environmental conditions (pH, temperature, etc.) and physicochemical factors of soil greatly affected the biodegradation rates. For instance, high temperatures have been recorded to increase the solubility of hydrocarbons, decreasing viscosity [41]. The research of Unimke et al., revealed that the hydrocarbon biodegradation rates increased with elevated of some physicochemical parameters including electrical conductivity and pH values [26]. Furthermore, media with pH of (7.4-9.0) was supportive of growth of Phenanthrene -degrading bacteria [42].

Hydrocarbon optimization studies using a bacterial consortium have found that 3%, crude oil or 1%, glucose, pH 7.2, 180 rpm incubation with 2% inoculum were optimal growth circumstances [38]. Furthermore, a local study showed that the environmental conditions of pH value (7), temperature (40 °) and 12 days of incubation beside mixed bacterial consortium were contributed to maximum sludge degradation [31].

The current study reports the identification of some bacterial species as components of oil-contaminated soil of local power generators in Karbala city, Iraq. Moreover, the identified bacterial isolates are considered to be local microbes with high efficiency for the decomposition of hydrocarbon components. A consortium was the best for the decomposition of hydrocarbon components at 37 °C and these data are similar somewhat to data obtained from the study of Nafal and Abdulhay [31].

4. Conclusions

The present study showed that selected strains characterized as *E. faecalis, S. agalactiae, P. aeruginosa*, *P. pneumonia / haemolytica* and *C. meningosepticum* were able to grow and utilize MSM containing different concentrations of gasoline. From the data, it can be concluded that oil-degrading bacteria are abundant in soils contaminated with spent oil. Consequently, these data suggest that these microbes could be useful for their application in the biodegradation of contaminated soils.

5. Ethical clearance

This research was ethically approved by the Research Ethical Committees of the Ministry of Environmental and Health and the Ministry of Higher Education and Scientific Research, Iraq.

6. Conflict of interest

The authors declare that they have no conflict of interest.

7. References

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