

ARTICLE / INVESTIGACIÓN

Bioremediation by bacteria isolated from water contaminated with hydrocarbons

Khadija Fahim Muhsin*, Mohammed Fadhil AboKsour, Saba Hadi

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¹ Department of Microbiology, College of Science, Al-Mustansiriyah University, Iraq.
Corresponding author: khadija.f.muhsin@gmail.com

Abstract: Oil pollution is currently a global problem. However, an oil-contaminated ecology is rich in microorganisms that may utilize petroleum oil and hydrocarbons for growth, feeding, and metabolic processes. In the present study, fifty polluted water samples were collected from five stations (ten samples each) in the Al-Fahama oil refinery in eastern Baghdad. The water contamination parameters of these collected water samples were detected. Then, the percentage of water contamination with some heavy metals (zinc, lead, and cadmium) and radioactive elements (uranium, cesium and actinium) was measured. The proportions of these elements were compared within their limits permitted by the World Health Organization (WHO). Fifty-nine bacterial isolates were isolated from polluted water, and 24 isolates of them succeeded in analyzing crude oil. The results of the current study showed that seven isolates belong to the genus *Citrobacter amalonaticus* (29.16%), six isolates belong to *Enterobacter cloacae* (25%), three isolates belonged to both *Pseudomonas aeruginosa* (12.5%) and *Ochrobacterum anthropi* (12.5%), and human *Ochrobacterum*. With a percentage of 12.5%, two isolates of *Serratia marcescens* (8.3%) and one isolate of each *Pseudomonas fluorescens*, *Serratia fonticola*, and *Burkholderia pseudomallei* (4.16%) of each. The optimum of some conditions for the decomposition process was determined in terms of (pH, temperature and crude oil concentration) and the results showed that the optimum degradation conditions were 35°C at pH equal to 7.5 in the presence of 2% of crude oil. Several experiments were conducted to determine the most efficient isolates for oil analysis. *Burkholderia pseudomallei* and *Pseudomonas fluorescens* are the most active bacterial species in their oil degradation. Genes responsible for hydrocarbon analysis were revealed in twenty-four bacterial isolates using a polymerase chain reaction (PCR) assay. The results showed that the ALKB gene (alkane hydroxylase) was observed in all bacterial isolates that succeeded in analyzing crude oil with a percentage equal to 100%, NahAc gene (naphthalene dioxygenase) has been recorded in four isolates (16.7%), these four bacterial isolates were *Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, *Ochrobacterum anthropi*, and *Pseudomonas fluorescens*. Generally, the isolation rate of both *C. amalonaticus* and *E. cloacae* isolates was higher than in other studies, which may be due to the hydrocarbon pollution in isolation; both *B. pseudomallei* and *P. fluorescens* isolates were the highest active bacterial species in their oil degradation. Genetic results showed that the AlkB gene was the domain compared with other degradation genes used in the current study, followed by NahAc gene.

Key words: Bioremediation, heavy metal, *B. pseudomallei*, hydrocarbons, crude oil.

Introduction

Because of their energizing and carbon needs for development and reproduction, in addition to the need to relieve stress response caused by the presence of hydrocarbons in the microbial mass environment, most hydrocarbons encountered in the surroundings are eventually degraded or hydrolyzed by indigenous bacteria¹. Non-regulated oil sludge removal, oil extraction, refining, and leaks during storage and transport all resulted in considerable soil pollution and aliphatic and aromatic hydrocarbon buildup in the environment. Since many petroleum hydrocarbons may be detrimental to living creatures, the elimination of these organic contaminants has received a lot of attention². Hydrocarbon pollution is a big concern because of its wide-ranging effects on all forms of life. Pollution produced by increasing crude oil consumption is ubiquitous due to its extensive use and the attendant transportation and disposal concerns³; the composition of crude oil is a complex mixture of aliphatic, aromatic, and aromatic particles. Heavy elements

are naturally present in the soil, and their level rises due to human activities, including oil refining and insecticide use. Several places have such high quantities of metalloids and heavy metals that they have seriously affected the natural ecosystem. Heavy elements and metalloids reduced microbe activity, making them unsuitable for petroleum breakdown and hence less efficient⁴. More than 98% of natural raw rubber comprises hydrocarbon polymers, a chain-like structure of many atoms connected. The types of chemical bonds that bind the particles of the constituent molecules affect the chemical system of hydrocarbons⁵. Hydrocarbon contamination happens due to toxic organic compounds, petroleum, and pesticides, posing a severe environmental threat. Contamination produced by petroleum hydrocarbons is a source of concern because these are toxic to a variety of biological types⁶. Crude oil pollution is ubiquitous due to its widespread use, the associated disposal procedure, and unintentional spills. Petroleum is a complex combination

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of various hydrocarbons with high and low molecular weight. Petroleum combines alkanes, alkenes, and homo- and heterocyclic naphthenes, saturated and branched⁷. Heavy metal and other compounds like N, S, and O are aromatics made up of heteroatoms. Ethers, carboxylic acids, and significant aromatic compounds are all examples of resins, asphaltenes, and naphthene-aromatics are examples of hydrocarbons with diverse active groups^{8,10}. This study aimed to identify the bacterial isolates in the pollutant water of hydrocarbons and to determine their ability to degrade the hydrocarbons as well as the optimum conditions of the degradation process.

Materials and methods

Sample collection

Fifty water samples of polluted water were taken from five stations located in Al-Fahama east of Baghdad (Al-Quds station as a model); these samples were collected in sterilized one-litter containers (from 30 - 50 cm depth), Global positioning system parameters (GPS) of the isolation areas were illustrated in table (1).

Station	Longitude			Latitude		
1	33°	49'	13"	44°	34'	38"
2	33°	50'	09"	44°	32'	05"
3	33°	49'	05"	44°	34'	71"
4	33°	49'	13"	44°	34'	36"
5	33°	49'	10"	44°	34'	30"

Table 1. Global positioning system parameters (GPS) of the studied area.

Determination of the element levels in water samples

A: Determine some heavy elements levels in water samples

The levels of three selected heavy metals (cadmium, lead, and zinc) were examined and carried out in general company for foodstuff trade/food quality control division in Baghdad by using flame atomic absorption spectrometer in laboratories of the College of Science/ Al-Mustansiriyah University¹⁰.

B: Determine some radioactive element levels in water samples

Levels of uranium, cesium, and actinium were determined in collected water samples as radioactive elements to determine if there is any presence in the area. A high-purity germanium radiation detector (HPGe) was used to determine the radioactive levels in the general company for foodstuff trade/food quality control division in Baghdad in laboratories of examination and audit division of the Ministry of Commerce¹⁰.

Culture media

BH: Bushnell- Haas medium was prepared as illustrated in the previous study¹¹.

Bacterial identification

Identification of bacteria according to their culture, morphological, and biochemical test, which include (Gram stain, Catalase, Oxidase, Indole, Methyl red, Citrate, Urease, H₂S, motility, and VP test, as well as VITEK2 system¹⁰).

Determine the biodegradation ability of isolated bacteria

A. detection by using crude oil agar plates

A 0.1ml of crude oil was diffused by the glass spreader on BH media, which was prepared, then the bacterial isolates were cultured separately on a media, and the plates were incubated at 37 °C for five days.

B. Measure the dissolved oxygen values

Dissolved oxygen values (DO) of 20 ml of the BH broth media containing the isolated bacterial strains were measured by using DO meter daily for 15 days to determine the (COD) values in the culture¹².

C. DCPIP photosensitizer method

Bacterial isolates were inoculated into 100 mL of nutrient broth and cultured at 37°C in a shaker incubator with 150 rpm for 24 hours. The cultures were used as a source of hydrocarbon degradation inoculum. One hundred ml of BH media were made with one percent diesel as the sole carbon source. DCPIP (2,6-Dichlorophenolindophenol) was added at a concentration of 20 mg/l in the media. The media was sterilized for 15 minutes at 121°C, and each set of experiments received 1 ml, which was incubated at 150 rpm



Figure 1. Satellite image of the study area.

for 14 days; tubes without bacterial suspension and DCPIP solution were used and considered as negative and positive controls. Changing the color of the tubes from blue to colorless was a visual detection of the ability of bacterial isolates to degrade the hydrocarbons, and this color change can be measured spectrophotometrically at 630 nm by ELISA reader spectrophotometer¹².

D. Gravimetric analysis assay

Gravimetric analysis assay can be used to estimate the amount of hydrocarbons degrading biologically by bacterial isolates. 50 ml of BH broth culture of each strain was transferred separately in a 250 ml conical flask 1N HCl was added to the culture to stop the bacterial activity. Then 50 ml of petroleum ether and acetone (1:1) was added. After that, the mixture was transferred to the separating funnel and mixed well for 15 minutes. After another 15 minutes of sitting, the solution was divided into three phases: an organic phase on top, an aqueous phase in the middle, and a cell biomass phase on the bottom. The upper solvent, which contains the oil, was separated from the two other phases, and then it was transferred to a beaker; after measuring the weight of the empty beaker, and kept in the oven at 45-50° C till the solvent was evaporated, the final weight of the beaker was measured, and thus The ratio of degraded crude oil was calculated by using the formula below.

Crude oil degraded % = (weight of petrol degraded / weight of petrol present) X 100

Where the weight of petrol degraded = original weight of petrol - weight of residual petrol obtained after evaporating the solvent¹².

Determining the optimum parameters for the bacterial decomposition of hydrocarbons

A: Temperature degree

One hundred ml of BH broth was transferred into a 250 ml flask and then sterilized by autoclaving (121 ° C for 15 min). After that, 1% of the crude oil was added to the flask with one ml of the isolated bacterial suspension (0.5 McFarland solution), and the flask was incubated in a shaker incubator at different temperatures (25, 30, 35, and 40 °C). The results were recorded daily for five days to determine the optimum temperature for the hydrocarbon biodegradation¹³.

B. PH value

One hundred ml of BH broth (it was illustrated in 1.3.1.) was transferred into a 250 ml flask with different pH values (6, 7, and 8), and then it was sterilized by autoclaving (121 ° for 15 min). After that, 1% of the crude oil was added to the flask with 1 ml of bacterial suspension (0.5 McFarland solution), and the flask was incubated in a shaker incubator at 37 °C. The results were recorded daily for five days to determine the optimum temperature for the hydrocarbon biodegradation¹⁴.

C. Crude oil concentration

One hundred ml of BH broth was transferred into a 250 ml flask and then sterilized by autoclaving (121 ° for 15 min). After that, different concentrations of crude oil (1%, 2%, and 3%) were added to the flask with one ml of bacterial suspension (0.5 McFarland solution), and the flask was incubated in a shaker incubator at 37 °C. The results were recorded daily for five days to determine the optimum crude oil concentration¹⁴.

Detection of degradation genes in bacterial isolates

PCR was used to detect three catabolic genes that encode enzymes involved in degradation pathways in purified DNA. *alkB*, *C320*, *PAH*, *C120* and *Nah* genes were amplified using the primers illustrated in Table 2. The resulting products were tested on an agarose gel and stained with ethidium bromide to confirm gene amplification. PCR was programmed of three genes (*AlkB*, *Nah*, and *PAH*) into four stages: primary denaturation stage of one cycle of pre-denaturation at 95° for 5 minutes. This amplification stage of thirty-five cycles consists of 94° for 40 sec, 55° for 40 sec, 72° for 1 minute, followed by the final extension stage of one cycle as 72° for 10 minutes, while the PCR program of both *C230* and *C120* genes was designed as 5 min as initial denaturation at 95°C, thirty-five cycles at 94° for 20 sec, 63° for 30 sec, and 45s at 72°C, and final elongation for 10 min at 72°C. PCR products were then transferred and resolved separately by electrophoresis on agarose gel. The presence of a clear band in the product of any test using ultraviolet light referred to the presence of the required gene in the bacterial isolate¹³.

Primers	Sequence	(bp)	Reference
<i>AlkB-F</i>	TACGGGCACTTCGCGATTGA	100	11
<i>AlkB-R</i>	CGCCCAGTTCGAMACGATGTG		
<i>C230-F</i>	AAGAGGCATGGGGGCGCACCGGTTCGA	900	12
<i>C230-R</i>	TCACCAGCAAACACCTCGTTGCGGTTGCC		
<i>PAH-F</i>	CGGCGCCGACAAYTTYGTNGG	292	12
<i>PAH-R</i>	GGGGAACACGGTGCCRTGDATRAA		
<i>C120-F</i>	GCCAACGTCGACGTCTGGCAGCA	350	11
<i>C120-R</i>	CGCCTTCAAAGTTGATCTGCGTGGTTGGT		
<i>Nah-F</i>	TGGCGATGAAGAACTTTTCC	487	12
<i>Nah-R</i>	AACGTACGCTGAACCGAGTC		

Table 2. List of primers which are used in this current study.

Results

Results of water samples analysis

A: Results of heavy metals levels in water samples

The results of heavy metals (cadmium, lead, and zinc) estimations in water samples showed that zinc was recorded as a higher percentage compared with lead and cadmium, the zinc average was recorded as 1.168 mg l⁻¹, and the highest value was 1.41 mg l⁻¹, and the lowest value was 0.695 mg l⁻¹. The results showed that the lead element came in second place, with an average of 0.467 mg l⁻¹, where its concentrations ranged between 0.06 to 0.36 mg l⁻¹. Finally, cadmium concentrations were recorded as the lowest of the selected heavy metals, ranging from 0.002 to 0.0003 mg l⁻¹, with 0.001 as an average, as illustrated in Table (3).

B: Results of radioactive elements levels in the water sample

The energy of radioactive elements in water samples was zero in all the collected water samples, which indicates the absence of recent or previous radioactive contamination in the study area.

Results of bacterial isolation and identification

Fifty-nine bacterial samples were isolated from 60 water samples collected from oil refining wells in the Al-Fahama area; the percentage of the bacterial isolates from water samples was higher than the other previous studies; it was equal to 98.3 %, which was recorded as high value may due to the bacterial contamination in the study area, as well as the presence of many bacterial isolates that can withstand or degraded the hydrocarbons¹⁵. Out of the total isolates, twenty-four bacterial isolates succeeded in analyzing oil and using it as an energy source, where the percentage of isolation of oil-consuming bacteria was 40%. Bacterial isolates were identified by observing phenotypic characteristics on different used media, investigating microscopic characteristics with Gram stain, and performing biochemical and VITEK 2 tests. The results showed that the isolated bacteria represented several bacterial genomes. The current study results recorded that seven isolates (29.16 %) belonged to the genus *Citrobacter amaloonaticus*, six isolates (25 %)

belonged to *Enterobacter cloacae* bacteria as a second common collected isolates, three isolates of both *Pseudomonas aeruginosa* and *Ochrobacterum anthropic* bacteria, with percentage equal to 12.5 %. Moreover, the isolation results showed the presence of four other bacterial genera as one isolate of each, with a percentage equal to 4.16% for each; these bacterial isolates were (*Pseudomonas fluorescens*, *Serratia marcescens*, *Serratia fonticola* and *Burkholderia pseudomallei*), as shown figure (2).

Results of the optimum conditions of bacterial decomposition of hydrocarbon

A. Determine the optimum temperature

When biodegradation of crude oil is carried out on BH broth and solid media and incubated at four different temperatures (25, 30, 35 and 40) °C, the results showed that the optimum temperature for the decomposition of crude oil is 35 °C. The results we obtained agreed with the number of studies that showed that the biomass of oil-degrading *Pseudomonas aeruginosa* bacteria reaches a maximum of 35 °C. The biomass decreases at 25, 30 and 40 °C¹⁶ because the temperature affected hydrocarbon metabolism and the hydrocarbon's physical and chemical nature considered 35 °C the best temperature for analyzing hydrocarbon compounds¹⁷.

B. Determine the optimum pH value

When cultured bacteria in a BH broth media with crude oil, the pH was adjusted to find the best grade for crude oil breakdown; the optimal pH level was shown to be 7.5 according to the results of the current study. The pH of a bacterial cell influences its metabolism, which in turn influences hydrocarbon degradation, as well as biomass production¹⁸.

C. Determine the optimum crude oil concentration

To find out the appropriate concentration to increase the biodegradation process, this contains different concentrations of crude oil. After conducting several experiments, it was found that the best concentration of biodegradation of crude oil was 2%; when the experiment was performed at a concentration greater than 2%, we discovered that biodegradation was less efficient.

Sample	Cd	Zn	Pb
1	0.0003	1.230	0.233
2	0.0013	1.337	0.122
3	0.0008	1.410	0.059
4	0.0001	1.045	0.156
5	0.0004	1.239	0.151
6	0.0021	1.031	0.356
7	0.0023	0.695	0.113
8	0.0022	1.358	0.086
Average	0.0001	1.168	0.159
WHO	0.003	3.0	0.20

Table 3. Results of heavy metals levels in water samples.

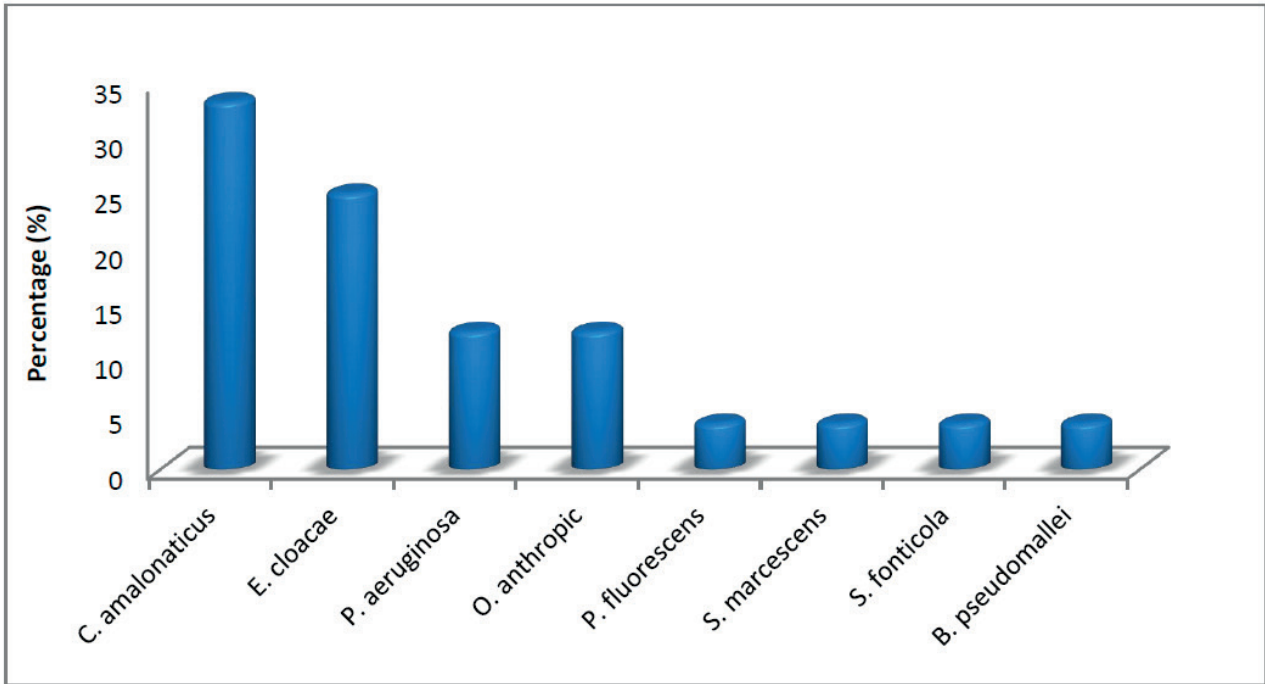


Figure 2. Isolation percentages of crude oil.

Quantitative results of the bacterial biodegradation to crude oil

The biodegradation rate of crude oil by the isolated and identified bacterial isolates was performed by using four different methods.

A. Result of measure of dissolved oxygen demanded method

It was noted that the *Burkholderia pseudomallei* bacteria achieved the most considerable significant difference from the Control, which reached 2.04, as it recorded the highest oxygen consumption. *Citrobacter alamoaticus* bacteria recorded the least significant difference from Control, which amounted to 4.13, where the percentage of moral error was 0.583, as these bacteria had the lowest oxygen consumption, see Figure 3.

Results of crude oil biodegradation by using agar plates

The results showed that the most efficient and fastest degrading isolates belong to the *Burkholderia pseudomallei* bacteria due to the presence of high significant differences between them and the Control, which amounted to 0.34, where the ratio of the standard line was 0.24, where the highest moral difference was achieved. *Serratia fonticola* bacteria showed the least significant difference, which amounted to 0.81, and the standard error ratio was 0.38.

C. Result of DCPIP electron acceptor method

Using the color reagent, *B. pseudomallei*, *E. Cloacae* and *P. aeruginosa* showed a noticeable color change after 120 hours of incubation. *C. alamoaticus*, *O. anthropic* and *P. fluorescens* noticeable discoloration appears after 168-192 hours. *S. fonticola*, *S. marcescens*, there is no noticea-

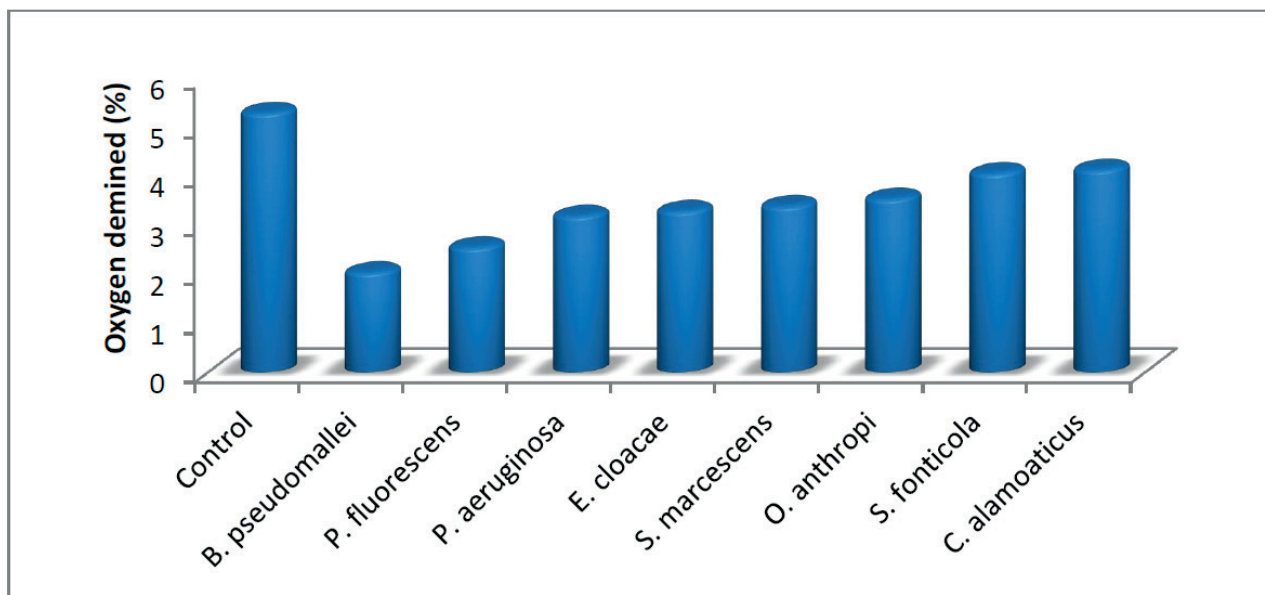


Figure 3. Results of bacterial dissolved oxygen consumption.

ble discoloration observed in the dye, as shown in Figure (4). The study used the color change of the dye Dichlorophenol Indophenol to determine the efficiency of bacteria in analyzing hydrocarbon compounds, Returns the dye's color disappearing as a result of absorbing an electron arising from the oxidation of hydrocarbons by bacteria¹⁹.

D. Result of gravimetric analysis assay

All the isolates could degrade the crude oil, and the degradation ranged from 1.14 to 2.43 gm. Moreover, the results showed that *P. aeruginosa* recorded the highest degradation compared with other tested bacteria, with 2.43 gm. The results also showed that the highest percent of degradation was recorded on the third day of incubation, as shown in Figure (5).

Detection of hydrocarbon degradation genes in the isolated bacteria

Detection of the presence of genes responsible for hydrocarbon analysis was revealed in twenty-four bacterial isolates that succeeded in analyzing crude oil by using polymerase chain reaction (PCR) assay. The results showed that *ALKB* gene (alkane hydroxylase) was observed in all bacterial isolates that succeeded in analyzing crude oil

with a percentage equal to 100%. *NahAc* gene (naphthalene dioxygenase) has been recorded in four isolates with a percentage equal to 16.7%; these four bacterial isolates were *Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, *Ochrobacterum anthropic*, and *Enterobacter cloacae*, see figure 6. On the other hand, *Todcl*, *C230*, and *Cat23* genes do not appear in all isolated bacteria. Table (4) demonstrates the appearance of the studding genes in isolated bacteria.

No.	Gene	Appearance
1	<i>AlkB</i>	100 %
2	<i>NahAc</i>	16.7%
3	<i>PHA</i>	0 %
4	<i>C230</i>	0%
5	<i>Cat23</i>	0 %

Table 4. Appearance of degradation genes in bacterial isolates.

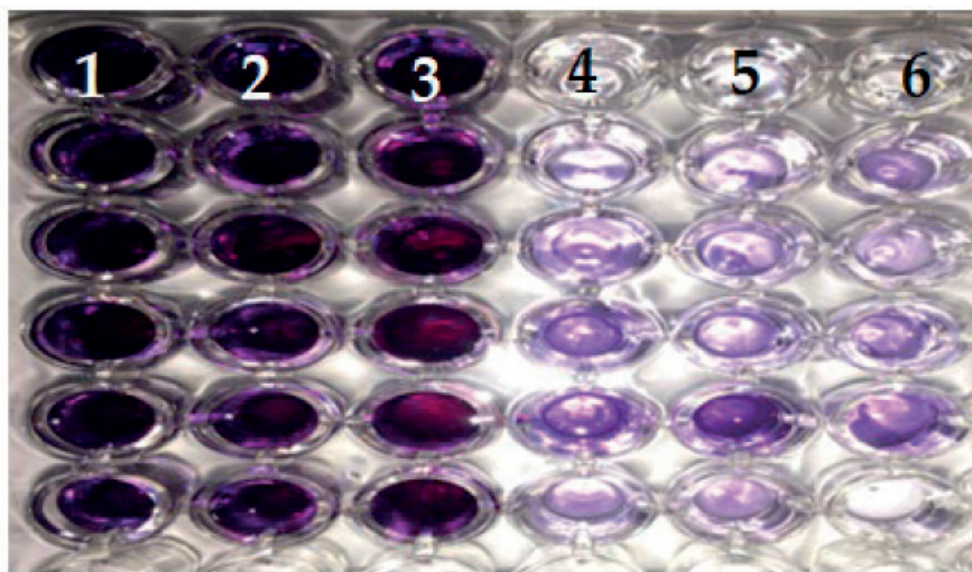


Figure 4. Result of DCPIP electron acceptor method. Column one (blue color) represents the Control, the second two columns recorded as negative results, while columns four to six (colorless) represent positive results.

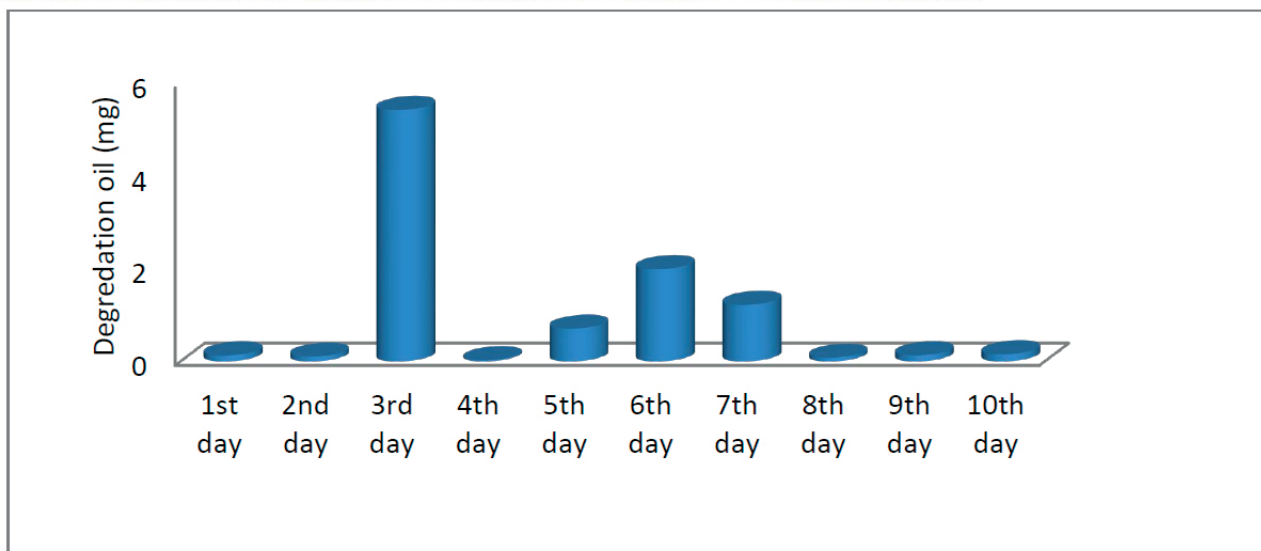


Figure 5. Results of crude oil biodegradation weight of the isolated bacteria.

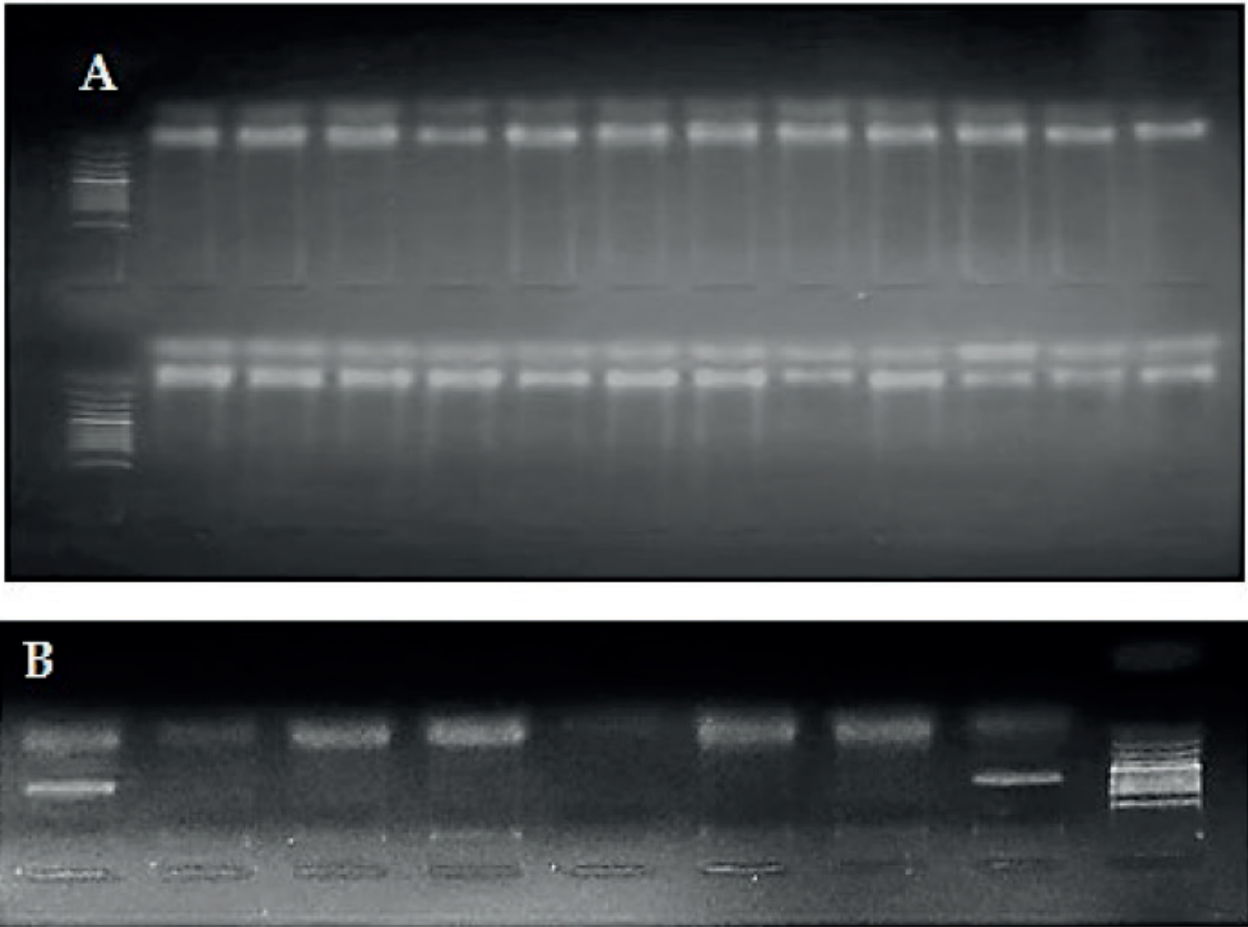


Figure 6. Occurrence of bacterial biodegradation results. Where A: ALKB gene, B: NahAC genes.

Discussion

The results showed that water samples collected in the current study were within the permissible limit of heavy metals that were chosen compared with acceptance levels of WHO²⁰. Although many local studies illustrated the high level of cadmium in the Najaf Sea in Iraq due to the association of its presence with the wastes presented for oil refining or places of extraction of fossil fuels²¹, another local study found that the concentration of cadmium and lead higher in Kirkuk irrigation project²². The results of the isolation of bacteria matched the isolation carried out in many previous studies²³, while the bacterial isolation percentage contrasted with the percentage reached by scientist²⁴, which was 23%. The difference in isolation rates is due to the difference in the size of the sample or the different conditions of isolation. Studies have shown that Gram-negative and specifically *Citrobacter amalonaticus*, ability to analyze hydrocarbon compounds is due to the presence of an enzymatic system capable of breaking down these compounds and using them as an energy source²⁵, its high analytical ability of hydrocarbons may be due to its possession of specific genes or plasmids, and it may contain in its cell walls²⁶. Bacteria can break down hydrocarbons because they have an enzyme that consists of the enzymes oxidase and peroxidase, which bind oxygen with these compounds, which helps degrade them²⁷.

According to a number of studies, the optimum temperature for oil degradation is 35-40 ° C, lower temperatures impact the physical state of crude oil by reducing alkane

volatilization, which leads to a rise in the viscosity of the oil and thus makes the biological analysis process more difficult²⁸, for different numbers of bacterial groups. At 20-30 °C, *Pseudomonas aeruginosa* bacteria destroyed 85 % of the crude oil²⁹.

Oxygen demanded is the amount of oxygen a microorganism uses to carry out the biodegradation process³⁰. The low percentage of oxygen is due to the ability of bacteria to consume it in the biodegradation process of hydrocarbons, as aerobic bacteria depend on the hydrogenase enzyme in the oxidation of hydrocarbons, and this explains the inverse relationship between the biodegradation process and the percentage of dissolved oxygen³¹. Many previous studies have shown that *Burkholderia sp* is one of the most suitable isolates for its ability to analyze toluene, naphthalene, kerosene, and alanine. The intermediate results of the analysis process were non-toxic. *Burkholderia* has oxygenase enzymes for the oxidation of substituted carbon³². Studies have observed the ability of both *Pseudomonas aeruginosa* and *Burkholderia pseudomallei* bacteria to decompose hydrocarbon compounds due to their possession of the enzyme 2,3-dioxygenase, which is responsible for inserting oxygen between the carbon chains, which leads to the splitting of benzene and the formation of intermediate compounds³³. Studies have shown that the *Burkholderia pseudomallei* bacteria contain a binary enzyme system capable of decomposing hydrocarbons with high efficiency (monoxygenases and dioxygenases), It includes a specific enzyme, Dihydroxycyclohexadienecarboxylate dehydrogenase, carbon, which makes it more efficient in decomposing benzene

and toluene and using it as a carbon source, A study was conducted to choose the best isolates for the hydrolyzed crude oil, as it showed that *P. aeruginosa* and *B. cereus* bacteria are among the best isolates of the oil that are attracted by the analyzes for their secretion into protein materials capable of cracking the chains between the carbon atoms²⁹. A study was conducted to analyze the rDNA of *Burkholderia pseudomallei* bacteria, and it was found that the ability of this bacteria to analyze complex compounds in the environment as a source of carbon is due to its possession of 2,2 dehalogenase (2,2-DCP)³⁴.

The gravimetric analysis assay method did not give tangible and accurate results to know the amount of residual hydrocarbons, and significant differences were observed between bacteria types during 10 days. Because of its properties, crude oil is one of the significant environmental pollutants capable of inflicting substantial harm to humans and the ecosystem. Prolonged exposure and high oil concentrations may result in liver or renal dysfunction, bone marrow damage, and an increased cancer risk³⁵.

Recent research suggests that bacteria that degrade hydrophobic chemicals like PAHs and alkanes have unique physiological characteristics. Sphingomonads have been shown to have a wide range of PAH metabolic capacities and to be engaged in the degradation of various refractory organic pollutants, including biphenyl and benzoate, pyrene. Furthermore, the breakdown of various PAH molecules by isolated bacteria appears through separate mechanisms³⁶. The catabolic genes can be used to track a bacterial strain's specific activities, The *AlkB* (alkane hydroxylase) gene encodes the enzyme monohydrogenase, The *NahAc* (naphthalene dioxygenase) gene encodes the enzyme dioxygenase, the enzymes monohydrogenase and dehydrogenase are responsible for the catalyze of aliphatic and PAHs compounds³². The majority of bacteria that degrade PAHs are Gram-negative, according to the study. The results of conventional features led to the identification of ASU-06 as *Sphingomonas sp.* The mono- and dioxygenases generated by bacteria were primarily responsible for the degradation of aliphatic and PAHs. As a result, six essential enzyme coding genes in *S. koreensis* strain ASU-06 were studied, including monooxygenase and dioxygenase. Monooxygenase (*alkB* and *alkB1*), dioxygenase (*NahAc*), and Catechol dioxygenase (C12O and C23O) genes have been discovered. One possible explanation is that the genes found are highly conserved among Gram-negative bacteria. However, no signal was seen because the PAH-RHD gene is conserved among Gram-positive bacteria. The naphthalene dioxygenase (*NahAc*) gene is fascinating as a PAHs degradation indicator since the enzyme produced by this gene destroys naphthalene and promotes the degradation of other PAHs compounds³⁶. Proteins or genes of 1-hydroxy-2-naphthoic acid dioxygenase have been found in Gram-negative and Gram-positive bacteria belonging to the genera *Pseudomonades*, *Nocardioidea*, and *Mycobacterium*, The reports showed the positive detection of the presence of the gene *NahAc* indicating that bacteria are on the analysis of polycyclic hydrocarbons, the study showed the presence of the *Nah* gene in many genera of negative bacteria, such as *Pseudomonas* and *Bolkhaderia*. Because genes conserved in Gram-negative bacteria are present in Gram-positive bacteria, the distribution of *NahAc* gene among PAH-degrading bacteria implies the influence of horizontal gene transfer (HGT)³⁷. Finally, the molecular technique recommended the identification of different area, including biology fields.

Conclusions

The results showed a high isolation rate of *C. amalonaticus* isolates compared with other results in the same area and the same thing for *E. cloacae* isolates. Also, the presence of crude oil should not be more than 2% as an optimum condition of biodegradation with pH close to natural and temperature more than room temperature, which differs from many other results that mentioned room temperature as an optimum temperature of crude oil degradation by environmental isolates. *B. pseudomallei* and *P. fluorescens* isolates were the most active bacterial species in their oil degradation. Genetic results showed that the *AlkB* gene was the domain compared with other degradation genes used in the current study, followed by the *NahAc* gene.

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Conflicts of Interest

The authors declare no conflict of interest.

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