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Research Article

Assessing the biodegradation efficiency of bacteria isolated and characterized from crude oil-contaminated soil

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Abstract

Oil pollution has serious effects on human health, and to remove or mitigate these negative effects, three bacterial strains were isolated from soil contaminated with crude oil collected from the Kasak refinery, west of Nineveh Governorate, at distances of (0, 20, 40, 60, 80) meters. The aim of the study is to evaluate the ability and efficiency of bacteria to analyze petroleum hydrocarbons to treat soil contaminated with crude oil. To be used in biodegradation experiments using crude oil, the bacterial isolates were identified according to their phenotypic characteristics and chemistry tests. The bacterial isolates were detected by genetic sequencing tests (16S rRNA), and they were registered with NCBI, which was registered under the special accession number for each bacterial isolate, the genus (BIAB1) with the number LC775009, the genus (BIAB2) with the number LC774612, while the genus (BIAB4) with the number LC774613. The use of two strains (BIAB1) and (BIAB2) in the study to evaluate the ability of bacteria to biodegrade using mineral salt media, by adding crude oil at specific concentrations (0.5%), (1%), (1.5%), (2%), and (3%) as a sole source of carbon. The results of the gas chromatography analysis showed that the best ability to analyze hydrocarbons by the two isolates BIAB1 and BIAB2 was at a concentration of (2%) with a rate of (96.42%, and 97.45%) respectively for a period of 12 days. The results revealed the extent of the efficiency of the bacteria in the Analysis of crude oil.

Keywords: Biodegradation, Crude oil, Hydrocarbon-degrading bacteria, Soil pollution

Introduction

The proliferation of oil pollution, arising from various sources such as refineries, accidental oil spills, leakage of oil and its byproducts, as well as the disposal of oil-based waste by human activities, has resulted in an alarming increase in sites contaminated with crude oil. Among the most hazardous constituents within these pollutants are polycyclic aromatic hydrocarbons (PAHs), a group of toxic and semi-volatile organic compounds of long-term environmental significance. PAHs exhibit relative persistence in soil and can enter the environment through the food chain, posing significant health and ecological concerns. They have the potential to infiltrate the environment, affecting the food chain and posing serious genetic toxicity to humans, along with mutagenic and carcinogenic properties. Furthermore, their deleterious effects extend to plants and animals, including a reduction in soil

fertility, which consequently results in diminished agricultural productivity (Baoune et al., 2019). The chemical classification of crude oil depends on its hydrocarbon composition, including aliphatic and aromatic constituents, asphaltenes, resins, alongside organic compounds featuring carbon, hydrogen, nitrogen, sulfur, and oxygen, and a plethora of compounds containing heavy metals (Al-Hawash et al., 2018). There are many ways to treat oil pollution: including physical and chemical treatment. However, as highlighted in a study by Khalid et al. (2017), physical technologies tend to be cost-intensive, beset with several disadvantages, requiring substantial energy inputs, and posing environmental harm, particularly due to the inability to decompose oil pollutants effectively, resulting in their persistence in the soil and ongoing contamination of the surrounding environment. Additionally, Ossai et al. (2020) noted the emission of toxic pollutants into the air and the generation of secondary pollution during the application of physical technologies,

coupled with elevated temperatures and the necessity to manage harmless background gases to avoid secondary pollution. According to a study by LV et al. (2022), this technology has been associated with a decrease in the solubility of various organic substances in water, along with challenges in completely removing oil pollutants that are bound to clay particles and residing within the soil and sediment matrices. Luo et al. (2019) found in their study that the introduction of chemicals can potentially give rise to secondary pollution within the soil environment or the atmosphere, such as oxidation residues. Consequently, the use of chemical technology necessitates the careful selection of appropriate chemicals and their precise quantities. In a related study, Zhao et al. (2020) highlighted that the technological maturity of chemical treatment using the plasma method is relatively insufficient and has high requirements for the accuracy of equipment manufacturing. Liao et al. (2019), in their research, corroborated that the quantity of chemicals employed significantly influences the efficacy of soil treatment. Excessive chemical usage can result in secondary pollution, while an inadequate quantity may lead to incomplete soil treatment. Biological treatment, employing microorganisms and their enzymes to eliminate hazardous pollutants or transform them into less toxic compounds, stands as a preferred and cost-effective method. It demonstrates high efficiency and rapidity while maintaining environmental friendliness. This approach has been well-documented in a study by Loyeh et al. (2020) and is thus regarded as an emerging and promising technology for treating environments contaminated with crude oil, particularly in cases involving polycyclic aromatic hydrocarbons (PAHs), as highlighted by Ławniczak et al. (2020). Given that bioremediation aims to achieve the safe, rapid, and efficient removal of oil pollutants from contaminated soil within a concise timeframe, it has emerged as a primary scientific research priority. Numerous researchers have presented studies addressing the challenges posed by oil pollution and exploring biodegradation as a viable solution. For instance, Al-Dhabaan (2019) demonstrated that *Bacillus* Sp bacteria exhibit a remarkable capacity for decomposing crude oil, achieving the highest recorded petroleum hydrocarbon decomposition rate of 68-70% during an incubation period spanning 21 to 28 days at a temperature of 30°C, based on gas chromatography analysis results. Furthermore, Abdulla et al. (2019) conducted a study in which they successfully isolated twenty-seven hydrocarbon-degrading bacterial strains from five distinct oil-contaminated areas. Their study showed the efficiency of bacteria for biodegrading petroleum hydrocarbons that were isolated from soil contaminated with crude oil, by testing the ability of the isolates to degrade hydrocarbons using a salt medium. Minerals were added to crude oil in different concentrations (2%), (3%), (5%), (7%), (10%) and for five different time periods (5, 10, 15, 20, 30) days. The study's findings indicated the capability of all isolates to analyze most hydrocarbons, with the exception of the *Bacillus cereus* isolate, which exhibited notably high efficiency in hydrocarbon analysis, achieving a rate of 98% degradation during a 15-day incubation period. In a separate study, Nafal (2020) elaborated

on the isolation of nineteen oil-degrading bacterial strains from contaminated soil. These strains were cultivated in a mineral salt medium amended with a 1% concentration of crude oil as a carbon source. Among these isolates, *K. rosea* and *B. amyloliquefaciens* demonstrated excellent growth and crude oil decomposition abilities, reaching efficiency levels of 68.9% and 93.8%, respectively. These optimal conditions were characterized by a pH of 7, a temperature of 40°C, and an incubation period of 12 days, as determined by gas chromatography (GC) analysis and compared to the control sample. Similarly, the study by Kridi et al. (2021) reported the isolation of nine bacterial strains from soil in three crude oil-contaminated sites, and that is to assess their hydrocarbon analysis capabilities using Pacto Bushnell Hans medium. The assessment involved monitoring changes in the medium's color after the addition of bacterial strains. In terms of petroleum hydrocarbon degradation, gas chromatography was employed to evaluate the efficiency of strains. Among the nine isolated strains, *Pseudomonas*, *Bacillus*, and *Micrococcus* exhibited noteworthy color changes, achieving degradation rates of 77%, 78%, and 78%, respectively, compared to the control sample. The results obtained through gas chromatography analysis revealed significant alterations in the composition of materials, which are present in crude oil, upon individual decomposition by the three bacterial strains, in contrast to the control sample (without inoculation). Additionally, Taher et al. (2022) conducted a study in which they isolated two bacterial strains from soil contaminated with crude oil. These strains were subjected to various concentrations (ranging from 0.5% to 3.0%) in a saline mineral medium to assess their hydrocarbon analysis capabilities. The gas chromatography results demonstrated that a 2.0% concentration of crude oil was optimal for hydrocarbon analysis by bacterial isolates AM-I-1 and AM-I-3, yielding efficiencies of 78.19% and 86.5%, respectively, over a 27-day incubation period. The aim of this study is to evaluate the ability and effectiveness of bacteria in the breakdown of crude oil particles, as well as in diminishing its toxicity. The research assesses the bacterial response to varying concentrations within Mineral Salt Medium (MSM), investigates the pH limitations, and examines the influence of temperature and incubation duration on bacterial isolates. Furthermore, the study aims to analyze the effectiveness of bioremediation in the treatment of soil contaminated with crude oil, with a specific focus on polycyclic aromatic hydrocarbons.

Materials and Methods

Collection of Samples

Soil samples were collected from soil sites contaminated with crude oil from the Kasak refinery according to longitude and latitude coordinates with specific distances of dimensions (0 Meter) (N 36° 27'28. 2636", E 42° 40'06.1932"), (20 Meter) (N 36° 27'27.5004" , E 42° 40'05.8116"), (40 Meter) (N 36° 27'26. 9568", E 42° 40'05.484"), (60 Meter) (N36° 27'27. 0792", E 42° 40'06.3264"), (80 Meter) (N 36° 27'27. 6984",

E 42° 40'06.7872"), From a depth of (15-30) cm, it was placed in polyethylene bags, then transported to the laboratory and stored at a temperature of (4°C) in the refrigerator until use.

Crude Oil Samples

The crude oil sample, obtained from the Kasak refinery, was meticulously prepared subsequent to its extraction from the wells and prior to undergoing any refining or treatment processes. This unaltered crude oil sample was carefully placed in opaque, sterile glass containers, with the required information recorded on them. Following transportation to the laboratory, it was appropriately stored in a refrigerated environment at a temperature of 4°C until it was ready for use.

Bacteria Isolation

The soil samples, collected from the specified distances, were meticulously homogenized and sieved through a 2 mm-diameter sieve to remove unwanted gravel and impurities. Bacterial isolation was carried out using the dilution method. Specifically, 1 gram of the contaminated soil sample was added to 9 millilitres of sterile distilled water within individual tubes. After thorough suspension homogenization, a series of dilutions were executed, ranging from (10^{-1} - 10^{-4}) utilizing the pour plate technique, where (1 ml) of each dilution was transferred to sterile Petri dishes, then Nutrient Agar was poured into it, three replicates for each dilution. After that, the dishes were incubated at a temperature of (37 °C) for a period of 24-48 hours (Perscott et al., 2005). Subsequently, the bacterial isolates were purified by selecting different colonies and transferring them from mixed culture plates to dedicated agar dishes suitable for each isolate. Incubation was performed at a temperature of 37°C for duration of 24 hours. The agar dishes containing the purified isolates were then stored in a refrigerator at 4°C until further use. The process of isolating pure strains was done based on the phenotypic characteristics of the colonies growing on Nutrient Agar medium, such as color, size, shape, texture, height, edge shape, and biochemical tests (Al-Jader et al., 2022).

Bacteria susceptibility test for crude oil analysis

The viability of the purified bacterial isolates was assessed using solid mineral salt medium (BHM) that had been supplemented with crude oil, which was diluted with diethyl ether (10% v/v). The diethyl ether was allowed to evaporate, leaving the oil layer concentrated in the center of the dish. The plates were subjected to incubation at a temperature of 37°C for a duration ranging from 1 to 7 days. The isolates that exhibited the largest circular diameter within the dishes were selected for inclusion in the study (Santhini et al., 2009).

Primary examination for the analysis of crude oil with bacteria

The bacterial isolates selected by sensitivity testing were inoculated into a beaker containing 50 ml of medium Liquid Minimal Salt Medium (MSM), which is prepared with a (1%) concentration from crude oil, was incubated in a shaker incubator set at 150 rpm for a duration of 14 days, maintaining a temperature of 37°C. Following incubation, the turbidity of the medium was quantified using a spectrophotometer, measuring the absorbance at a wavelength of 600 nm. (Kumar et al., 2006 and AlGhazawi et al., 2005).

Secondary examination of the analysis of crude oil with bacteria

A liquid mineral salt (MMS) medium was prepared. This medium composition included the following components per liter: CaCl₂.2H₂O (0.01 g/L), FeSO₄.7H₂O (0.01 g/L), MgSO₄.7H₂O (0.2 g/L), Na₂HPO₄ (6.0 g/L), NH₄NO₃ (4.0 g/L), KH₂PO₄ (4.0 g/L), and modified Na₂EDTA (0.014 g/L). The sterile liquid medium was dispensed into glass bottles (50 ml each) and autoclaved at 121°C for 15 minutes. The pH of the medium was adjusted to pH = 7 ±. Various concentrations of crude oil (0.5), (1), (1.5), (2), (3)% were introduced into the medium, with one bottle left without oil for comparison. Subsequently, the medium was inoculated with the bacterial inoculum. The inoculated medium was incubated in a shaker incubator operating at 150 rpm and maintained at a temperature of 37°C for a total incubation period of 288 hours. Following incubation, the turbidity of the medium was assessed using a spectrophotometer, with measurements taken at a wavelength of 600 nm (Venosa, 2003).

Bacterial identification and DNA isolation

The characteristics of bacterial colonies and the cellular composition of the isolated bacterial strains were studied and assessed. To perform a molecular diagnosis of the bacteria under investigation, a DNA extraction process based on the 16S rRNA region was carried out, utilizing an analysis kit provided by Psomagen. This molecular diagnosis facilitated the characterization of the isolated strains. In the molecular analysis, the bacterial strains were responsible for amplifying the 16S rRNA region within the genes extracted from the bacteria. This process involved the addition of 4 microliters (100 nanograms) of template DNA and 1 microliter (10 picomoles) of each gene-specific primer to the master mix. Multiplex reactions were conducted following a specialized program. The reactions occurred in individual tubes within a thermocycler, proceeding through several stages. These stages included an initial denaturation phase at 95°C for a single cycle lasting 6 minutes, followed by a denaturation stage at 95°C for 45 seconds for 35 cycles. Subsequently, an annealing stage took place at 65°C for one minute over 35 cycles, and finally, an extension phase ensued at 72°C,

lasting 1.5 minutes for one cycle, followed by a final extension at 72°C for 1 minute, also for one cycle.

PCR reaction

The PCR products were analyzed by loading the sample into an agarose gel that was prepared at a concentration of 2% with the addition of the DNA ladder, which contains ethidium bromide. After that, the samples were electrophoresed for a period of between (60-70) minutes. The gel is then photographed using a UV Trans illumination device.

The sequence of nitrogenous bases in the 16SrRNA gene

The bands resulting from the PCR reaction were extracted from the gel to conduct a nucleotide sequence test, after purifying them using the analysis kit prepared by Psomagen. The sequence of the nitrogenous bases of the SrRNA16 gene for the selected samples was diagnosed through gene sequencing using the Analyzer Genetic device model 3130 supplied by the Japanese company Hitachi. The PCR reaction products of the previously mentioned samples were sent with the primer for the resulting package to the United States of America, to match the sequences of the nitrogenous bases of the genes of the isolated samples with the gene sequences archived at the National Center for Biotechnology Information (NCBI). The analysis sought to identify similarities and matches through the utilization of the BLAST program, based on the analyzed sequencing results.

Nucleotide sequence detection

Three new bacterial strains were identified based on information available in the National Center for Biotechnology (NCBI) database. The findings from these samples were forwarded to Psomagen, a company in the United States of America, for further analysis. Then, the data was submitted to GenBank, where these strains were assigned new names and received unique accession sequences on the NCBI website Table (1).

Table 1. Specific sequencing of bacterial strains recorded in NCBI

Isolates	join sequence	NO.
<i>Serratia rubidaea</i> BIAB1	Lc775009	.1
Uncultured bacteria BIAB2	Lc774612	.2
<i>Paenibacillus terrae</i> BIAB4	Lc774613	.3

The phylogenetic tree was obtained using the MEGA 7 program according to (Chen et al., 2017), according to the figures (1 , 2 , 3), below, marked in yellow: (1) strain BIAB1 and (2) strain BIAB2 and (3) strain BIAB4. The genetic affinity tree showed similarity between BIAB2 and the bacteria pseudomonas aeruginosa 100% (Figure 2).

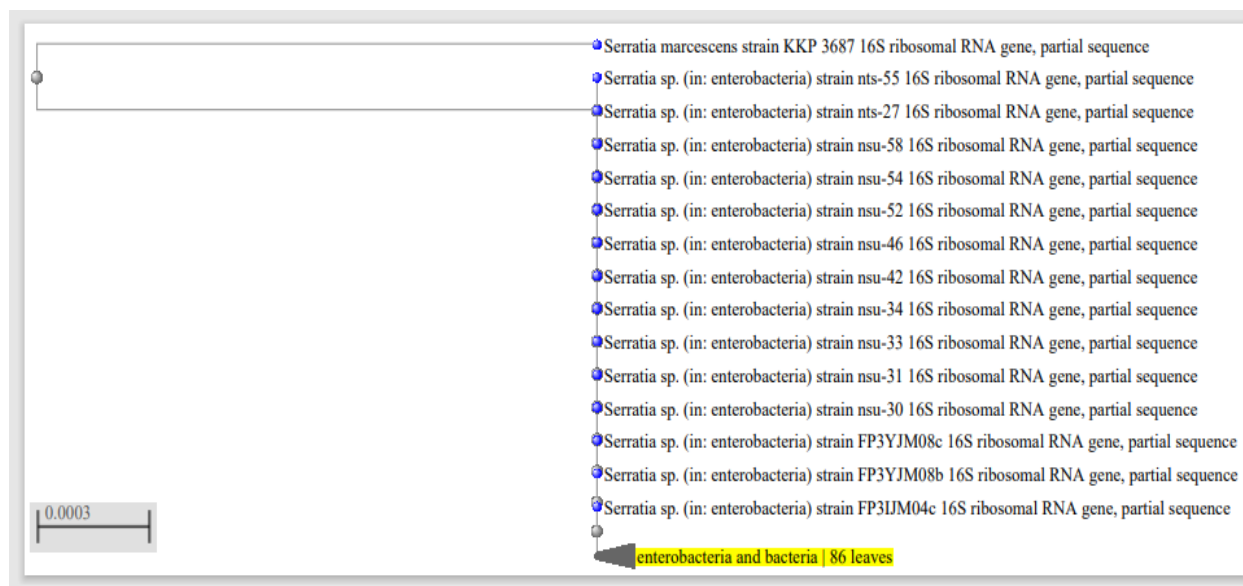


Fig 1. Phylogenetic tree for strain BIAB1 isolated from soil contaminated with crude oil

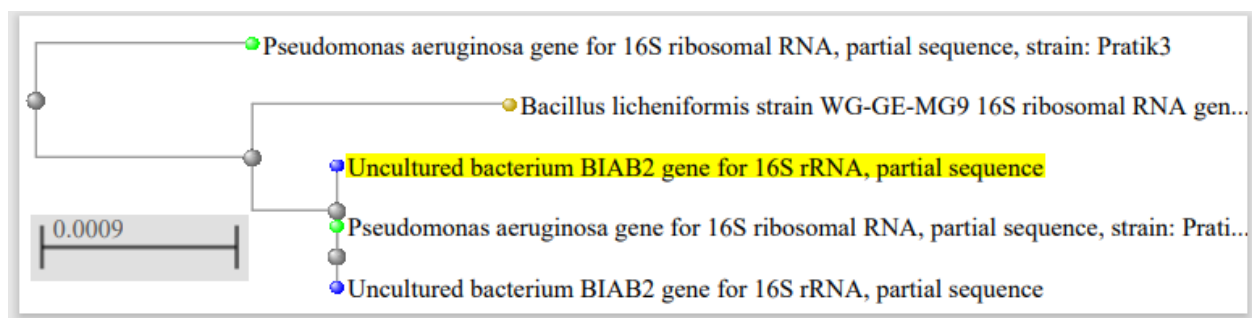


Fig 2. Phylogenetic tree for strain BIAB1 isolated from soil contaminated with crude oil

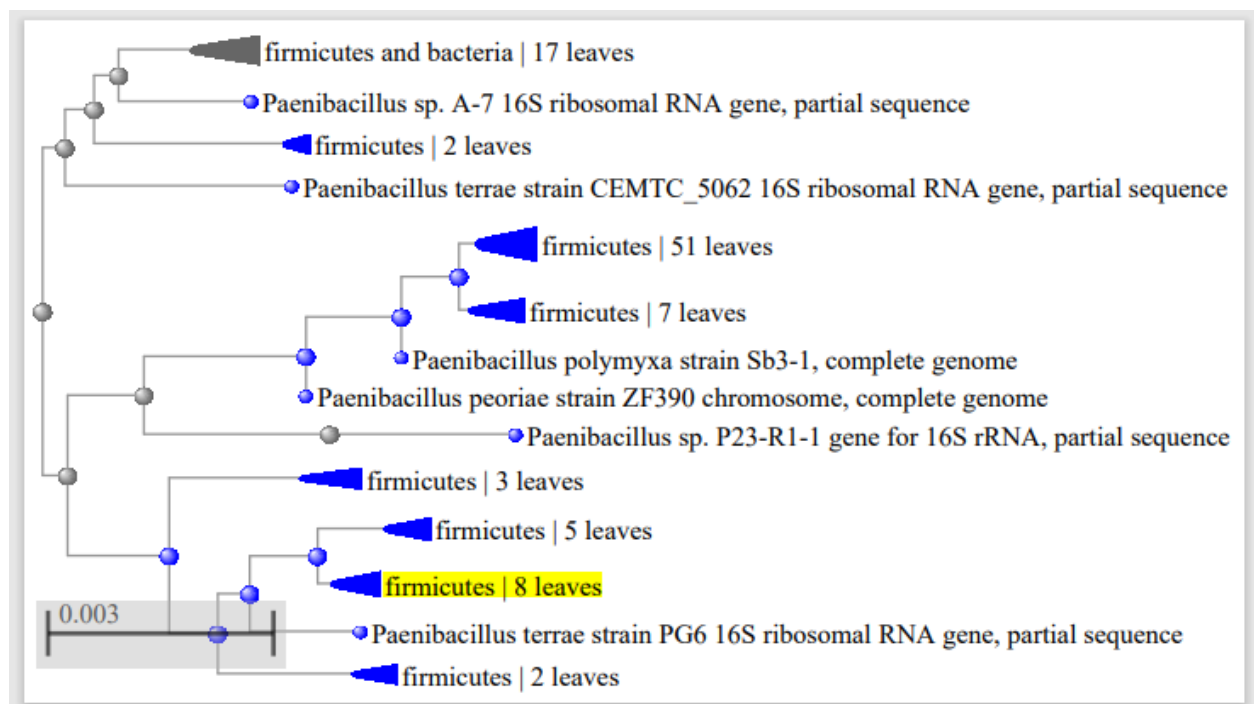


Fig 3. Phylogenetic tree for strain BIAB4 isolated from soil contaminated with crude oil

Gas chromatography (GC)

In the laboratories of the Ministry of Science and Technology in Baghdad, gas chromatography (GC) was utilized to determine the rate of decomposition of polycyclic aromatic hydrocarbons. The analysis was performed using a DB-5 column (30 m x 0.25 mm ID) coated with 5% diphenyl-poly dimethyl siloxane and a 0.25- μ m thick film, with the column temperature initially held at 40°C for one minute. Subsequently, the temperature was gradually increased to 120°C at a rate of 25°C/min, further raised to 160°C at a rate of 10°C/min, and finally elevated to 300°C at a rate of 5°C/min, where it was maintained for 15 minutes. Helium gas served as the carrier at a constant flow rate of 5 ml/min, while the Detector (FID) temperature was set at 330°C. Gas chromatography is a widely employed analytical method known for its selective and sensitive detection of various hydrocarbons, making it particularly suitable for analyzing

complex mixtures like those found in crude oil (Zhu *et al.*, 2010). % Of hydrocarbons biodegradation = $\frac{\text{total peak in control sample} - \text{total peak in test sample}}{\text{total peak in control sample}} \times 100$ According to Venosa *et al.*, 2003.

Analysis of polycyclic aromatic hydrocarbons

The Liquid Mineral Salt Medium (MMS) was dispensed into individual vials, each with a capacity of 100 ml. These vials were supplemented with various concentrations of crude oil, specifically 3%, 2%, 1%, 1.5%, and 0.5%. Subsequently, the medium was inoculated with the bacterial inoculum at a concentration of 1%. A control group was also prepared without inoculation. All vials were subjected to incubation in a shaker incubator set at 150 rpm, maintaining a temperature of 37°C. The incubation period lasted for 12 days.

Redox reactions using Dichloro pheno lindophenol

A bacterial suspension was prepared by mixing 1 ml of bacterial culture with 10 ml of Bushnell-Hass Medium (BHM). The BHM medium composition included the following components per liter: MgSO₄ (0.2 g), CaCl₂ (0.02 g), FeCl₃ (0.05 g), K₂HPO₄ (1.0 g), NH₄NO₃ (1.0 g), and KH₂PO₄ (1.0 g). The pH of the BHM medium was adjusted to approximately 7 and sterilized by autoclaving at 121°C for 15 minutes. To this mixture, 1 ml of DCPIP (2,6-dichlorophenolindophenol) and 1% crude oil were added. The incubation was carried out for a duration of 12 days at a temperature of 37°C. The assessment of bacterial activity was based on observing a color change in the medium at a wavelength of 660 nm. Bacteria capable of utilizing petroleum components generate electrons for oxidation-reduction reactions, which can cause the medium to change color to blue. Conversely, during reduction reactions, the medium remains colorless (Bidoia et al., 2010).

Statistical analysis

A completely randomized design method was employed to validate the presence of each bacterial type and to explore the effects of different concentrations and combinations thereof. The obtained data underwent statistical analysis and were compared using Duncan's multiple-range test at a significance level of $LSD \leq 0.05$ (Perscott et al., 2005).

Results and discussion**Identification and characterization of bacteria**

The bacterial strains BIAB1, BIAB2, and BIAB4 were diagnosed according to their phenotypic features and biochemical tests, as shown below in Table (2) and Table (3).

Table 2. Phenotypic Characteristics of Bacterial Isolates

S.No	Isolates	Characteristics of bacteria appearing on Nutrient Agar medium						Gram Stain	Cell Fusion	Creation of cells
		Shape	Color	Texture	Appearance	Hight	Edge			
1	BIAB1	Circular	Orange pink	Creamy	Bold	Plane	Steady	-	Single and binary	Stick
2	BIAB2	Circular	Brilliant white	Creamy	Bold	Plane	Steady	-	Single and binary	Stick
3	BIAB4	Circular	Brilliant white	Creamy	Bold	Plane	Wavy	+	Single and binary	Short rod

Table 3. Biochemical tests for bacterial isolates

NO.	Tests	BIAB1	BIAB2	BIAB4
.1	Oxidase	-	+	-
.2	Catalase	+	-	+
.3	Indole	-	-	-
.4	Urease	-	+	-
.5	TSI	A CO ₂ /A	K/K	A/A
.6	Simmon Citrate	+	+	-
.7	Methyl red	-	+	-
.8	Voges- Proskaoer	+	-	+
.9	Motility	-	-	-
.10	Mannitol salt agar	-	-	-
.11	EMB agar	+	-	-
.12	MacConkey agar	-	-	-
.13	Blood Hemolysis	B	γ	γ

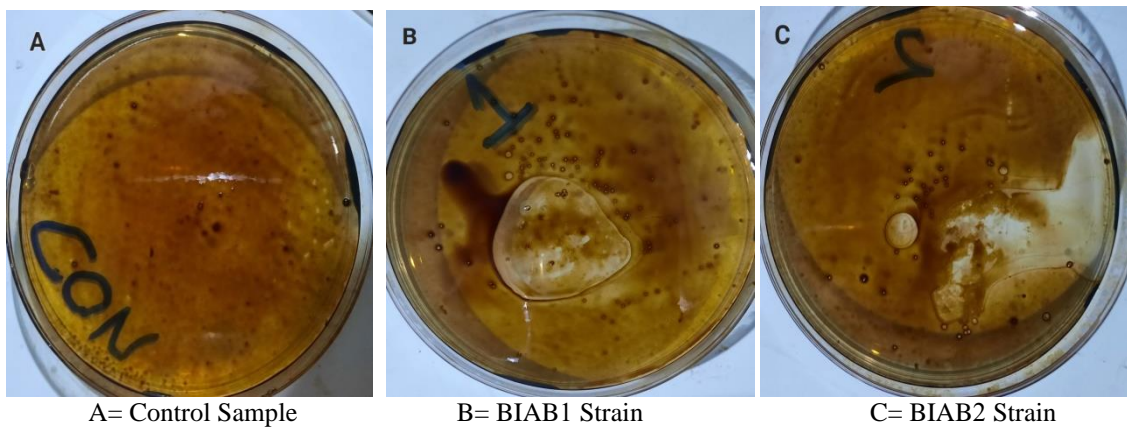


Fig 4. Sensitizing bacterial strains to crude oil (A= CONTROL, B= BIAB1, C= BIAB2)

Testing the ability of bacteria to degrade polycyclic aromatic hydrocarbons

The susceptibility of bacterial isolates to crude oil was assessed, and the extent of their decomposition activity was quantified within Bushnell-Hass Medium (BHM). Among the bacterial strains, BIAB2 exhibited a remarkable growth area of 2 cm, signifying strong decomposition capabilities, while strain BIAB1 displayed a substantial growth area exceeding 1 cm (Figure 4). The formation of transparent halos and growth zones around bacterial cells in the BHM medium is indicative of these strains' proficiency in degrading Total Petroleum Hydrocarbons (TPH), (Santhini et al. 2009). This

phenomenon suggests that the bacterial strains utilize crude oil as a source of carbon. Furthermore, the efficiency of these strains in degrading crude oil aligns with the results of Taher et al. (2022), confirming the ability and effectiveness of bacterial isolates in crude oil consumption. This was evidenced by the transparent growth zones surrounding bacterial colonies cultured on Bushnell-Hass Medium (BHM) agar supplemented with 1% crude oil, incubated at 37°C for 1-2 days. The continuous reduction of polycyclic aromatic hydrocarbons throughout the incubation process serves as a fundamental criterion for assessing the efficiency and capacity of these bacterial strains in the analysis and degradation of petroleum hydrocarbons.

Table 4. Different incubation periods for bacterial isolates

S. No	Isolates	Days				Average isolates
		12days	9days	7days	3days	
1	BIAB1	1.067ab	0.839bcd	0.613de	0.365f	0.721b
2	BIAB2	1.097a	0.843bcd	0.635cde	0.455e	0.757ab
	Average days	1.082a	0.841b	0.624c	0.41d	

*Similar letters mean there are no significant differences

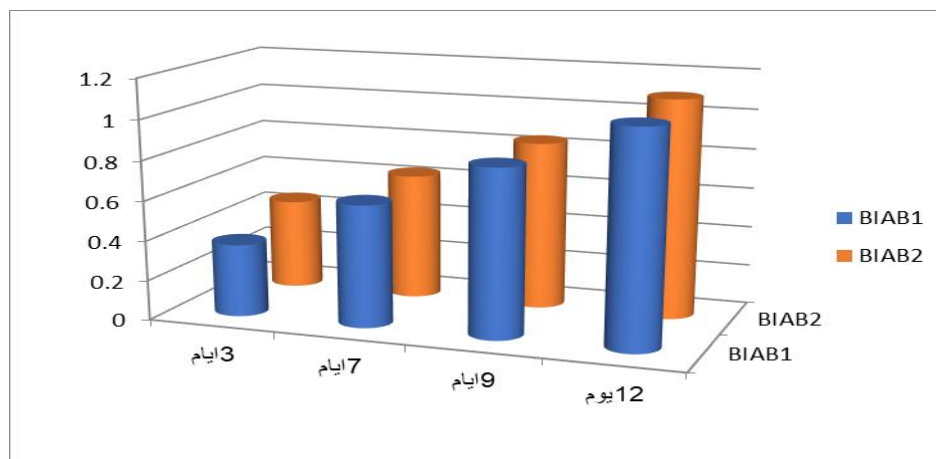


Fig 5. Shows the different incubation period

Table 5. Biodegradation of BIAB1 bacteria and percentage of PAHs removal

S. NO.	Compound	Control	0.5	1.0	1.5	2.0	3.0	Mean of Compound
1	Acenaphthene	256.9	88.9	72.6	55.6	20.2	29.9	53.44
2	Acenaphthylene	452.6	125.6	68.9	42.2	18.9	20.6	55.24
3	Anthracene	324.8	154.0	98.0	74.0	22.5	33.6	76.42
4	Benzo(A) Anthracene	205.6	44.9	32.5	12.6	5.9	10.6	21.3
5	Benzo(B) Fluoranthene	415.6	90.5	77.8	41.2	10.5	25.5	49.1
6	Benzo(K)Fluoranthene	254.6	112.	76.9	35.6	10.5	14.5	50.06
7	Benzo(G,H) Perylene	214.9	52.6	14.5	6.5	2.1	3.6	15.86
8	Benzo (A) Pyrenen	320.6	80.9	66.5	42.9	11.5	20.6	44.48
9	Chrysene	412.6	154.9	112.6	58.9	10.5	20.8	71.54
10	Dibenzo(G,H)Anthracene	236.9	55.9	40.5	21.5	5.9	8.9	26.54
11	Fluranthene	250.6	60.5	32.9	12.5	4.58	9.58	24.012
12	Fluroene	188.9	50.2	22.5	10.5	3.0	5.9	18.42
13	Indeno	145.6	12.5	10.5	6.9	2.4	3.6	7.18
14	Naphthalene	122.6	18.9	15.9	11.4	7.5	9.2	12.5
	Sum	3802.8	1103.1	742.6	432.3	135.98	216.88	
	Mean of Concentration	271.628	78.7929	53.0429	30.8786	15.4914	9.7129	
	Degradation %		70.99	80.47	88.63	96.42	94.29	

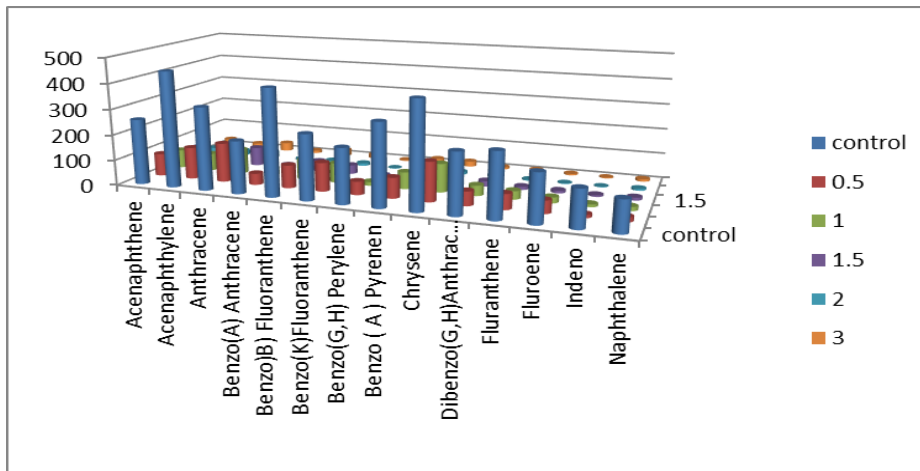


Fig 6. Diagram Showing the percentage of degradation of poly aromatic petroleum hydrocarbons for isolation BIAB1

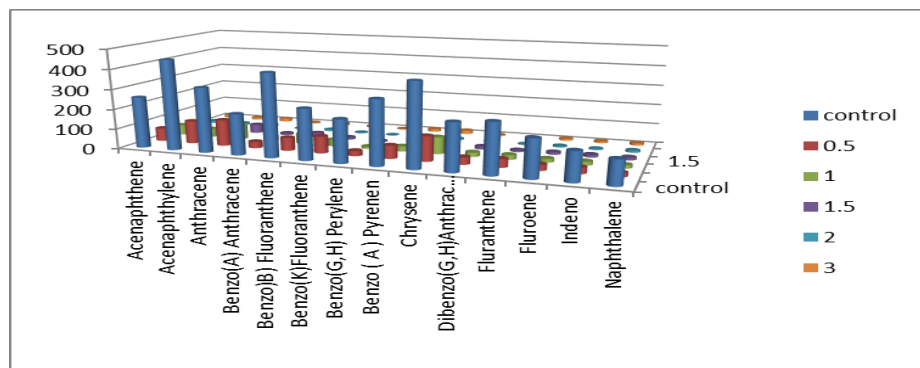
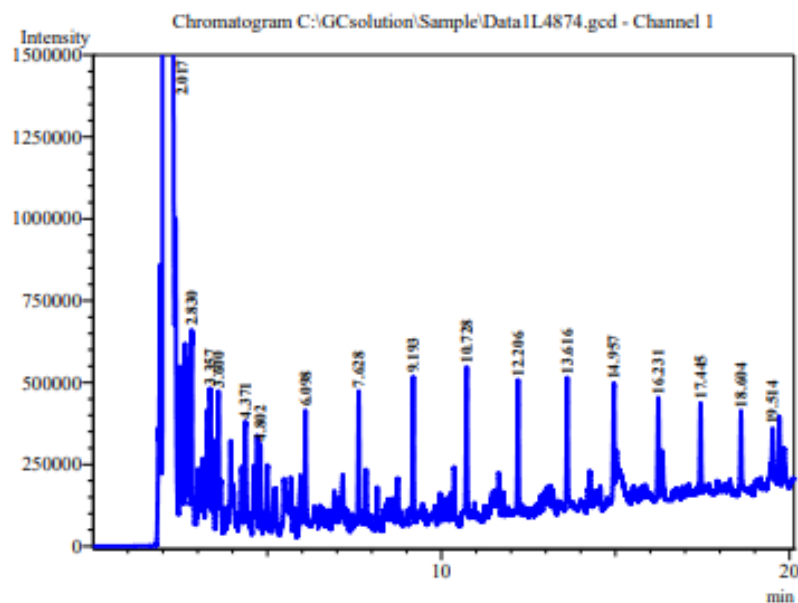


Fig 7. Diagram Showing the percentage of degradation of poly aromatic petroleum hydrocarbons for isolation BIAB2

Table 6. Biodegradation of BIAB2 bacteria and percentage of PAHs removal

NO.	Compound	Control	0.5	1.0	1.5	2.0	3.0	Mean of Compound
.1	Acenaphthene	256.9	65.9	50.9	40.6	11.5	15.9	36.96
.2	Acenaphthylene	452.6	115.9	42.6	24.6	8.9	10.9	40.58
.3	Anthracene	324.8	132.6	69.9	44.5	12.0	15.9	54.98
.4	Benzo(A) Anthracene	205.6	32.5	24.8	8.9	2.5	3.9	14.52
.5	Benzo(B) Fluoranthene	415.6	65.9	50.1	22.5	7.8	10.9	31.44
.6	Benzo(K)Fluoranthene	254.6	85.6	33.6	11.5	3.5	6.5	28.14
.7	Benzo(G,H) Perylene	214.9	25.6	10.5	3.6	1.8	2.5	8.80
.8	Benzo (A) Pyrenen	320.6	66.167	25.6	21.5	10.5	13.6	27.4733
.9	Chrysene	412.6	125.6	85.6	33.6	6.8	17.8	53.88
.10	Dibenzo(G,H)Anthracene	236.9	35.6	21.5	14.5	4.5	5.9	16.4
.11	Fluranthene	250.6	41.5	22.6	8.6	3.	5.1	16.20
.12	Fluroene	188.9	29.167	17.9	12.567	7.1	10.5	15.4467
.13	Indeno	145.6	30.8	18.9	13.267	5.8	9.5	15.6533
.14	Naphthalene	122.6	20.1	16.5	13.6	10.8	11.6	14.52
	Sum	3802.8	872.934	491	273.834	96.7	140.5	
	Mean of Concentration	271.628	62.3524	35.0714	19.5595	6.9071	10.0357	
	Degradation %		77.04	87.08	92.79	97.45	96.30	



Peak Table - Channel 1

Peak#	Ret. Time	Area	Area%	Height	Name
1	2.017	4104147616	99.6526	68422591	
2	2.830	837707	0.0203	489458	
3	3.357	1798454	0.0437	370451	
4	3.600	537424	0.0130	340787	
5	4.371	810878	0.0197	286129	
6	4.802	311735	0.0076	194431	
7	6.098	658312	0.0160	302943	
8	7.628	940888	0.0228	399457	
9	9.193	983015	0.0239	411369	
10	10.728	1461386	0.0355	455280	
11	12.206	1048249	0.0255	406787	
12	13.616	898922	0.0218	372297	
13	14.957	1456886	0.0354	337095	
14	16.231	613972	0.0149	268292	
15	17.445	666668	0.0162	263973	
16	18.604	638932	0.0155	244591	
17	19.514	644123	0.0156	157973	
Total		4118455167	100.0000	73723904	

Fig 8. The chemicals that make up the crude oil sample

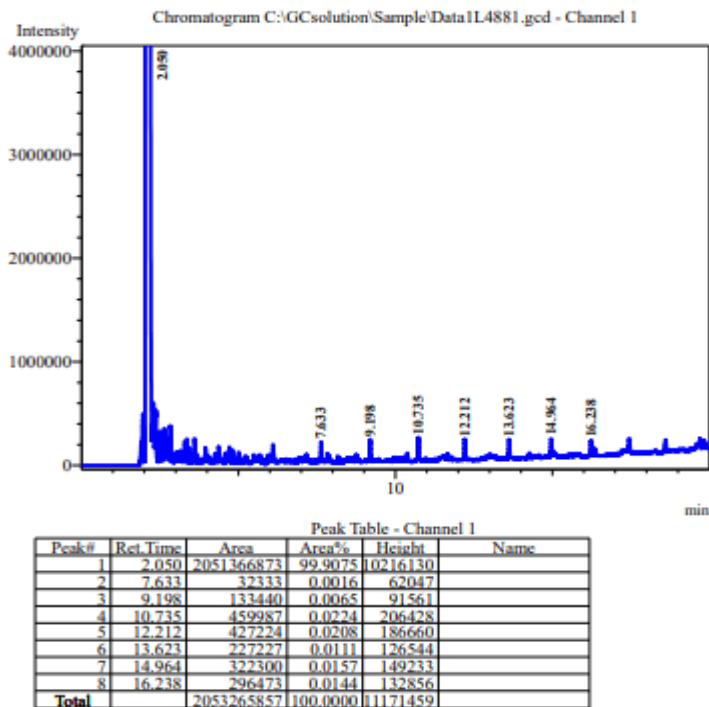


Fig 9. Chemicals that remained in the crude oil after its decomposition by bacteria for a 12-day incubation period at a concentration of 2% BIAB1

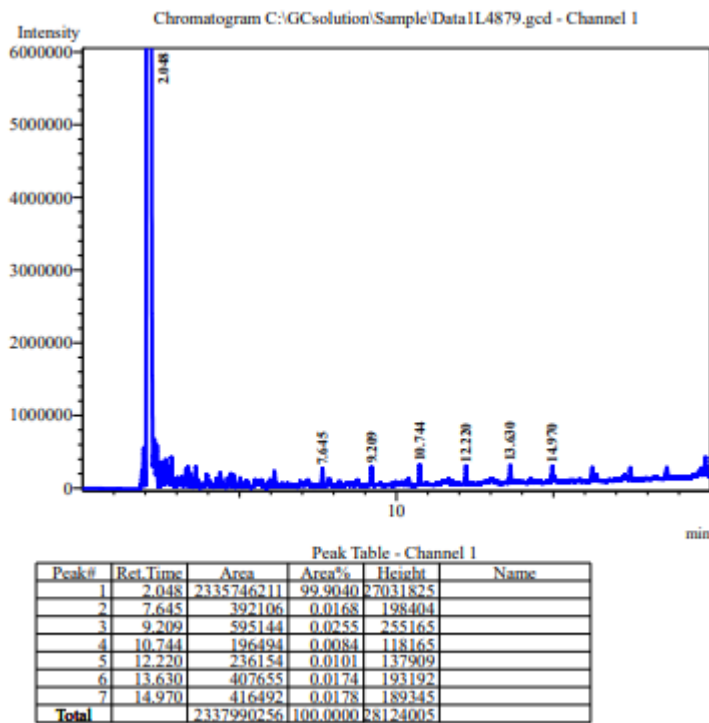


Fig 10. Chemicals that remained in the crude oil after its decomposition by bacteria for a 12-day incubation period at a concentration of 2% BIAB2

Incubation duration

Bacterial isolates were treated with different incubation periods (3, 7, 9, 12) days in mineral salt medium (MSM) by spraying the medium with crude oil at a concentration of 1%, and inoculating (0.5) bacterial isolates. Table 4 and Figure 5 show that the highest optical density for the isolate BIAB2 was on the third day (0.455), and it gradually increased with increasing incubation period until it reached the highest value on the twelfth day (1.097), bacteria adapt to live in polluted environments by increasing incubation periods through enzymes that they secrete to analyze crude oil, petroleum derivatives, and petroleum hydrocarbons to be used as a source of energy and thus the growth of bacteria (Roslee et al., 2020). As for isolate BIAB1, the lowest light density was recorded on the third day, amounting to (0.365), while the twelfth day was the lowest light density for the same isolate (1.097). These results are consistent with (Abdulla et al., 2019), and we find that isolate BIAB2 recorded the highest average optical density. (0.721), and isolate BIAB1 achieved the lowest light density for the average bacteria (0.757), and the lowest light density for the average days reached (0.41) on the third day. With increasing incubation periods, it reached the highest light density for the average days (1.082) on the twelfth day. The increase in bacterial growth was significant with increasing incubation times at $P \leq 0.05$. These results agree with (Al-Dhabaan, 2019) and (Nafal, 2020).

Table 5 and Figure 6 presents the gas chromatography (GC) results, which demonstrate the capacity of bacterial strains to degrade crude oil. The concentration of various hydrocarbons, including 3.0%, 2.0%, 1.5%, 1.0%, and 0.5%, was evaluated in the presence of the bacterial isolate BIAB1. Notably, at a concentration of 2%, BIAB1 exhibited a remarkable consumption rate, with an average remaining concentration of polycyclic aromatic hydrocarbons at 15.4914 mg/kg, corresponding to a consumption rate of 96.42% compared to the control sample without inoculation. This represents the highest percentage of polycyclic aromatic hydrocarbon consumption by the BIAB1 isolate. However, at lower concentrations (1% and 1.5%), the consumption rates of remaining aromatic hydrocarbons were comparatively lower, at 80.47% and 88.63%, respectively, with average concentrations of 53.0429 mg/kg and 30.8786 mg/kg, respectively, when compared to the control sample. This decrease in consumption rates at lower concentrations may be attributed to the limited carbon sources available to support bacterial growth, potentially impacting enzymatic activity and reducing biodegradation efficiency.

The lowest percentage of consumption of aromatic hydrocarbons from crude oil occurred at a concentration of 0.5%, with a rate of 70.99% compared to the control sample, and an average concentration of 78.7929 mg/kg. Temperature also has an effect on the biodegradation process. When the temperature decreases, the viscosity of crude oil increases, its solubility in water decreases, and the rate of enzymatic

activity decreases, which in turn affects the metabolic process of bacteria and thus reduces the ability of biodegradation of bacteria (Siles et al., 2018). The results also indicate a decrease in the consumption rate of petroleum hydrocarbons at a concentration of 3% (94.29%), with an average remaining hydrocarbon concentration of 97.129 mg/kg. In addition to the fact that high temperature reduces the viscosity and increases the solubility of the crude oil, and the bioavailability and enzymatic activities of the bacteria, the highest rate of hydrocarbon decomposition was obtained at temperatures ranging between 30 - 40 degrees Celsius, as indicated by (Al-Hawash et al., 2018). This decline in consumption efficiency at higher concentrations may be attributed to potential toxicity effects on living organisms, which could limit the bacteria's ability to biodegrade petroleum hydrocarbons. These findings align with previous studies (Abdulla et al., 2019; Kridi et al., 2021; Taher et al., 2022), which also noted significant increases in petroleum hydrocarbon consumption rates by bacterial isolates at lower concentrations.

The results presented in Table 6 and Figure 7 illustrate the proficiency of bacterial isolate BIAB2 in decomposing the aromatic hydrocarbons present in crude oil across various concentrations. The average concentrations of remaining polycyclic aromatic hydrocarbons at different concentrations (0.5%, 1%, 1.5%, 2%, 3%) were measured at 62.3524 mg/kg, 35.0714 mg/kg, 19.5595 mg/kg, 6.9071 mg/kg, and 6.9071 mg/kg, respectively. This corresponds to decomposition rates of compounds at 77.04%, 87.08%, 92.79%, 97.45%, and 96.30%, respectively, compared to the control sample. It's noteworthy that the decomposition rate of polycyclic aromatic hydrocarbons at a concentration of 0.5% was relatively lower. This is attributed to the fact that low concentrations of crude oil may not sufficiently stimulate the enzymatic systems of bacteria, which are involved in oxidation and reduction processes, as indicated in a previous study (Buswell et al., 1994). However, as the concentration increased, the decomposition rate gradually rose, reaching its peak at a concentration of 2%, as demonstrated in Table 5. The increase in the rate of aromatic hydrocarbon decomposition was statistically significant at a significance level of $p \leq 0.05$. Nevertheless, when the concentration further increased to 3%, the rate of decomposition declined. This decline can be attributed to the potential toxicity of high hydrocarbon concentrations on bacterial strains, which can adversely affect their ability to decompose hydrocarbons and their overall growth. Additionally, higher concentrations can lead to increased medium viscosity, impeding oxygen transfer and disrupting bacterial cellular activities, as suggested by Gupta et al. (2004). Remarkably, bacterial isolate BIAB2 exhibited greater efficiency and capability in decomposing polycyclic aromatic hydrocarbons when compared to bacterial isolate BIAB1, as evidenced by GC spectrophotometry. This difference in performance can be attributed to variations in the enzymatic systems of the bacterial strains and genetic diversity in the genes responsible for secreting enzymes that break down crude oil. These

results align with previous studies conducted by Al-Dhabaan (2019) and Taher et al. (2022). It is evident from these findings that both bacterial strains possess genes encoding enzymes that can break down complex components of crude oil into volatile or less complex compounds, which they utilize as a carbon source. This adaptive response of bacterial strains to their environmental conditions is essential for their survival. Most hydrocarbon-degrading bacteria prefer a neutral to slightly alkaline pH (Al-Hawash et al., 2018).

Conclusion

In this study, the ability and efficiency of bacterial strains isolated from soil contaminated with crude oil from the Kasak refinery were systematically evaluated. The study emphasizes the critical role of bacterial treatment in the removal or reduction of petroleum hydrocarbons' toxicity, as demonstrated by the considerable reduction in total petroleum hydrocarbons (TPH) across various concentrations employed in the research. The most remarkable biodegradation outcomes were achieved at the 2% concentration level, where both BIAB1 (96.42%) and BIAB2 (97.45%) exhibited exceptional biodegradation capabilities. Particularly, the BIAB2 isolate showed superior crude oil biodegradation when compared to the control sample without bacterial inoculation. These bacterial strains and their enzymatic system can be used in the biological treatment of soils contaminated with oil and its derivatives.

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Conflict of Interest

The author hereby declares no conflict of interest.

Consent for publication

The author declares that the work has consent for publication.

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