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Isolation, Identification Bacteria and Bioremediation of Soil Contaminate Crude Oil from Specific Area (Baiji_Iraq)

Maha Hamdei Hussein[1] and Ibrahim Omar Saeed[2]

[1]Department of Biology, College of Science, University of Tikrit, Tikrit, IRAQ.

[2]Department of Biology, College of Science, University of Tikrit, Tikrit, IRAQ.

[1]Corresponding Author: maha.h.hussein4408@st.tu.edu.iq

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ABSTRACT

In this paper new eight types of bacteria that degrading crude oil were isolated. The isolated were from Baiji, near Tikrit, the center of Salah al-Din Governorate, IRAQ. The nucleotide sequences of the 16rRNA gene revealed that these isolates belong to the genus *Bacillus, Cytobacillus* and *Staphylococcus*. These isolated bacteria proved their ability to degradation petroleum hydrocarbons with varying effectiveness, as *Cytobacillus firmus* IBMA1 bacteria proved 98% effective in degrading hydrocarbons among the mineral salts in petroleum, Bacillus *cereus* IBMA3 showed a capacity to crack oil compounds by 92% in the medium of SMS mineral salts agar and *Bacillus zhangzhouensis* IBMA4 showed a cracking ability of 90% for the compounds present in crude oil. The other bacteria degraded petroleum compound with different rates. Based on it, three species that are big destroyers were taken to bioremediate crude oil containment soil from the same polluted site.

The capacity of each basin is 10 kg. Each basin was treated with one type of bacteria over a period of two months. Then also, gas chromatography (GC) was used for soil samples after one and two months of treatment. The experiment was conducted from 17/2/2022 to 17/4/2022. The biological treatment was done using bacteria and contaminated soil. The results showed that IBMA1 bacteria have a great ability to consume PAHs with a consumption rate of 60.365% and that the lowest concentration of the remaining aromatic hydrocarbons was (6.5ppm), while IBMA3 bacteria showed their ability to consume PAHs with an average of 52.217% within two months. From the treatment, the lowest concentration of the remaining aromatic hydrocarbons was (9ppm) and IBMA4 bacteria showed the minimum ability to consume polycyclic aromatic hydrocarbons, with a consumption rate of 44.65% as an average consumption, and the lowest remaining concentrations of petroleum hydrocarbons were by (12ppm), while the average consumption of compounds during the two-month treatment period was 39.280%, and the results of the statistical analysis show the superiority of soil treatment with IBMA1 bacteria over IBMA3 bacteria and IBMA4 bacteria at the probability of $P \leq 0.01$.

Keywords- Bioremediation, Isolate Bacteria, Soil contaminations, Degradation.

I. INTRODUCTION

The oil spills have become one of the most significant problems facing the environment specially during the last few decades . There are now a significant number of oil spills occurring somewhere in the world. There are several examples of oil spills, such as the Taylor Energy disaster in the Gulf of Mexico (USA), which was triggered by a hurricane and the oil leakage still continues until now. Other examples of oil spills include the BP oil spill in the North Sea (UK) and the Exxon Valdez oil spill in the Another oil spill took place in Mumbai (India), and this one resulted in the release of 55 tons of crude oil into the Arabian Sea. A collision between two tanker cargo vessels triggered yet another oil spill in the Sundarbans not too long ago (Bangladesh). Accidents like these can take place at any time and have the potential to harm the surrounding ecosystem[1, 2].Because of the widespread contamination of the soil with crude oil and its byproducts, there is a huge environmental problem all over the world[3-5]. In addition, leaks and discharge of petroleum hydrocarbons sometimes occur as a consequence of blow-out accidents during oil field growth, oil pipelines and

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storage tank leakages, oil vessels and vessel leakage accidents, the well waxing of oil, and refinery and petroleum chemical manufacturing equipment[5, 6]. Hydrocarbons make their way into the soil during oil extraction, storage, and distribution, as well as during refining and processing. The ever-increasing demand for petroleum and oil products contributes to an increase in the amount of petroleum hydrocarbons that are found in the soil[7]. Therefore, marine and terrestrial ecosystems that are deteriorating as a result of contamination by crude oil need to be given attention[8]. Waste burial, incineration, washing and oxidation are now used to treat oil-polluted soils. These techniques can transform, mineralize, remove and extract petroleum pollutants in the polluted environment and transform them into a less harmful and stable form [9] .Despite the success of these treatments, they now have drawbacks. Dioxins, furans, polychlorinated biphenyls, and volatile heavy metals are released into the atmosphere as a result of incomplete combustion of petroleum [10, 11]. The process of using the metabolic processes of microorganisms in order to eliminate contaminants is known as bioremediation. The subfield of biotechnology known as bioremediation focuses on the various approaches that can be used to resolve issues pertaining to the environment. By utilizing the microbes and fungus, it also plays a significant part in the process of cleaning the environment of pollutants and contaminants. Bacteria play a significant part in this process since they are responsible for the transformation of dead materials into organic matter and nutrients. Bioremediation provides an effective and efficient technique to speed up the clean-up processes, which is why it is being investigated as a potential countermeasure to remediate soils that have been contaminated with oils[12]. There are different types of bioremediations, and they are all employed with the goal of removing harmful compounds and toxins from the natural environment. In the prior research, oil-degrading microorganisms from the soil sample were isolated and characterized after having been through the process of being isolated. In addition to this, the oil-degrading genes of non-oil-degrading bacteria were allowed to express themselves[13, 14]. Numerous bacterial genera, such as Acinetobacter Burkholderia, Gordonia, Dietzia, Brevibacterium, AeromicrobiCelerobacter, Mycobacterium, and Sphingomonas, have been isolated from petroleum-contaminated soil and have the potential to degrade hydrocarbons[2, 15, 16]. Some of these bacterial genera include Acinetobacter Burkholderia, Gordonia, Dietzia, Brevibacterium, and Brevibacterium Analysis of the 16S rRNA gene sequence, in conjunction with other bioinformatics techniques, is utilized in order to correctly identify these bacteria. In addition, a wide variety of approaches to the screening and evaluation of microorganisms that degrade hydrocarbons have been established[17].

Iraq is one of the oil countries, it has the fifth largest oil reserves in the world , and with the presence of oil refineries in the south and north of Iraq, this affects the soil pollution as a result of oil leaks and spills. The soil is polluted and unsuitable for cultivation or for animals to live in During the past thirty years, Iraq was subjected to many wars. In these wars, the infrastructure of Iraq was destroyed, including the bombing of oil refineries, causing pollution to air, water and soil, and the effects of this pollution continue to this day.

Baiji refinery, one of the biggest and important refineries in Iraq, The location of this refinery is in Salahddin Governorate/ Iraq, near the Tigris River, which causes, in addition to soil pollution, water pollution as well. The result of sabotage operations during the ISIS event causes many leaks occur from the transmission lines and from the refinery as well that lead to the containment a large area of land around the refinery. The methods of physical and chemical treatment are very expensive and not useful in eliminating pollution completely, so the use of biological treatment is more acceptable because it is less costly and more efficient than other methods. Therefore, such studies are needed for treated the confirmation areas. These studies are useful for organization concerned with the environment and environmental resources, as well as the Ministry of Oil to ensure work in low-pollution conditions.

In this paper the work done to isolate different types of bacteria from contamination area and prove their ability to break down petroleum hydrocarbons and clean the area under investigated from pollution.

II. MATERIAL AND METHODS

2.1 Sample Collection

Random soil samples from various parts of the investigated area were polluted with crude oil, and those samples were gathered from five distinct parts of the Baiji region that had been contaminated by oil spills. The samples were put in sterile polyethylene bags, and the necessary information (sample number, date, weight, and temperature in each location) was recorded on them before they were carried to the laboratory and stored in a refrigerator at a temperature of 4 C^{o} until they were used.

2.2 Isolate Bacteria

The collected soil samples were mixed homogeneously and passed through a 2 mm sieve in order to get rid of unwanted gravel and impurities. The method of dilution and plating was used to isolate the bacteria by adding 1 gram of soil samples to 9 ml of distilled water and after homogenization, A series of dilutions were prepared from 10-1 to 10-4then the pouring method was used, where 1 ml of each dilution was transferred to a sterile Petri dish and a nutrient agar was added to it with three replicates for each dilution. The dishes were incubated at 37°C for 24–48 hours[18].

2.3 Identification and Characterization of Bacteria

It was observed that the isolates differed in terms of their form, size, and organization, as well as whether or not they were gram negative or gram positive bacteria. Several other biochemical assays, such as the oxidase test,

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were used to describe the bacterial isolates. Catalase. Indole, MR, VP, Citrate etc[19].

2.4 Analysis of 16srRNA

A set of Jet gene was used to purify DNA in order to extract DNA according to the manufacturer's instructions. Bacterial isolates were detected by amplifying the region of 16 srRNA extracted from bacteria using primers 16 f27 and 16 R1525, and then the reaction tubes were inserted into the thermopolymerizer (PCR) programmer was set at 35 cycles and the amplification was carried out as follows ;95Cfor 45s .56C for 1min .72C for 1min[20]

The sequence of the nitrogen bases of the samples was determined if the PCR reaction products were sent to the samples with the primers of the resulting package. The sequence was read for the genes based on the Genetic Analyzer 3130 device supplied by the Japanese company Hitachi. The sequences of the genes were matched with the sequences of the reliable genes at the National Center for Biotechnology Information NCBI. on the BLAST program

III. MEASUREMENT OF DEGRADATION RATIO OF HYDROCARBONS

Using broth mineral salts medium SMS, the isolated bacteria, which numbered 8 types of bacteria, were activated and observed for 24 hours at a temperature of 37 °C Incubating Then Petri dishes were prepared, in which the agar mineral salts SMS were poured then 1 ml of crude oil was added, then 1 ml of the pre-activated bacterial samples were added. The dishes were incubated for two weeks at a temperature of 37°C[21]

IV. BIODEGRADATION OF CRUDE OIL BY GAS CHROMATOGRAPHY (GC) TECHNIQUE

Gas chromatography used in the detection of PAHs in the laboratories of the Ministry of Science and Technology, column separation was carried out in a (30 m × 0.25 mm i.d.) DB-5 column (J&W Scientific, Folsom, CA) coated with a 0.25-µmthick film of 5%diphenyl–polydimethylsiloxane. The samples were injected in the split mode at an injection temperature of 280 °C. The column temperature was initially held at 40 °C for 1 min, raised to 120 °C at the rate of 25 °C/min, then to 160 °C at the rate of 10 °C/min, and finally to 300 °C at the rate of 5 °C/min, held at final temperature for 15 min. Detector(FID) temperature was kept at 330 °C. Helium was used as a carrier gas at a constant flow rate of 5 mL/min[22].

V. BIOREMEDIATION OF SOIL OIL CONTAMINATED

Three basins were taken and in each basin 10 kg of soil contaminated with crude oil was placed. The treatment was done by adding a bacterial suspension activated 24 hours ago at the rate of 240 ml per 10Kg of soil, and the concentration was measured by means of a spectrophotometer Where the density per liter of bacterial suspension is 0.5 nanometers then addea240ml a bacterial suspension IBMA1 to the first basin, and a bacterial suspension with the same concentration as the previous one and from IBMA3 bacteria to the second basin. was added. A bacterial suspension with the same concentration of the first. bacteria IBMA4 was added to the third basin The treatment period was two months, and then a GC test was performed on the samples to determine the extent of the cracking of the oil compounds in them.[23]

VI. RESULTS & DISCUSSION

6.1 biochemical tests:

A soil sample from the polluted site in Baiji showed, after its diagnosis, the presence of eight new isolates according to NBCI, and they were as follows: six isolates of Bacillus type, which are IBMA3, IBMA4, IBMA5, IBMA6, IBMA7 and IBMA8. Its isolate from Cytobacillus is IBMA1 and its isolate from Staphylococcus is IBMA2. The isolates were subjected to Gram-reactions and many biochemical properties reactions. The isolates showed five Gram-negative isolates: IBMA3, IBMA4, IBMA6, IBMA7 and IBMA1 and three Gram-positive types: IBMA2, IBMA5 and IBMA8.The rest of the biochemical properties are explained in the following table (1) [24].

Table 1: Biochemical Test

	Gram	Morphology	Urease	Citrate	T.S.I	Indole	V.P	M,R	Catalase	Oxidase
IBMA1	-	Rod	+-	-	A/A	-	-	-	-	-
IBMA2	+	Cocci	+	-	A/A	-	-	-	+	-
IBMA3	-	Rod	+	+-	K/K	-	-	-	+	-
IBMA4	-	Rod	+-	-	A/A	-	-	-	-	-
IBMA5	+	Rod	+-	-	A/A	-	-	-	-	+
IBMA6	-	Rod	-	-	A/A	-	-	-	-	+
IBMA7	-	Rod	-	-	K/A	-	-	-	+	-
IBMA8	+	Rod	+-	-	A/A	-	-	-	-	-

A=Acid K=Alkaline T.S.I= Triple Suguar Iron M.R=Methyl Red Regent V.P= Voges-Prpskauer Reagent

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6.1 16s RNA Gene Sequence Analysis

Eight bacterial strains were isolated from the contaminated site, diagnosed and biochemical tests were performed on them. After sequencing the 16s rRNA gene, the data was submitted to the genetic sequencing database at the National Center for Biotechnology Information NBCI website (https://www.ncbi.nlm.nih.gov) They were molecularly identified and showed that the species IBMA3, IBMA4, IBMA5, IBMA6, IBMA7 and IBMA8 of the genus Bacillus sp., type IBMA1 is of the genus Cytobacillus sp., and type lBMH2 is of the genus Staphylococcos sp. These strains showed a varying ability to break down or mutate the oil compounds[25].

6.2 Primary Screening of Crude Oil Degradation:

The results shown in the table (2) and Figure (1) indicate that the clearing area achieved by bacteria strains on MSM_agar medium prepared with 1% crude oil, while the IBMA3 isolate showed a very strong clearing area with a percentage of 98%, while the IBMA1 showed a good clearing area with 92%. IBMA4, and the isolate 90% offset areaWhile the clearing area for the rest of the bacterial isolates was weaker and less. The clearing area indicates the ability of bacteria to grow in the medium contaminated with crude oil and use it as a source of carbon, and according to the efficiency of each bacteria in analyzing hydrocarbons, we reached these results[26].

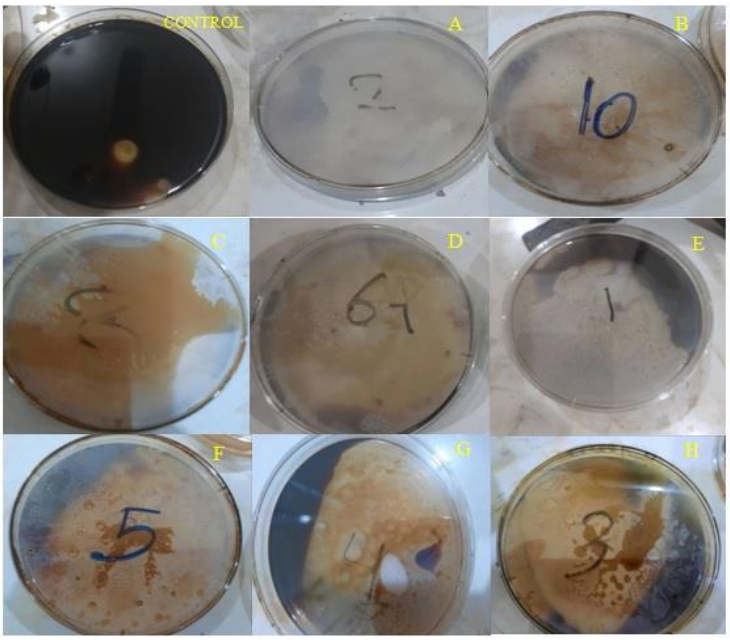

Fig. 1: Formation of the clear zone on MSM agar of crude oil for bacterial isolates (A)IBMA1 (B)IBMA3 (C)IBMA4 (D)IBMA7 (E)IBMA2 (F)IBMA6 (G)IBMA8 (H)IBMA5

Table 2: Degradation Percentage of Isolation Bacteria

No	Isolate bacteria	Degradation %
A	IBMA1	98
B	IBMA3	92
C	IBMA4	90
D	IBMA7	85
E	IBMA2	80
F	IBMA6	70
G	IBMA8	67
H	IBMA5	60

6.3 Gas Chromatography (GC) Results And Biodegradation Detection

Appendix (1) show that IBMA1 bacteria have the ability to consume polycyclic aromatic hydrocarbons with a consumption rate of 60.365% during two months of treatment. The lowest concentration of the remaining aromatic hydrocarbons was (6.5ppm). Microbial decomposition of organic pollutants usually occurs as a result of the action of microorganisms on their metabolism pollutants and their cell wall components.The microorganisms use organic materials as a source of carbon and electrons in order to obtain energy, according to what was stated [27]. While IBMA3 bacteria showed their ability to consume PAHs as an average of 52.217%

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during two months of treatment.The lowest concentration of the remaining aromatic hydrocarbons was (9ppm), which is the average percentage of consumption and this is consistent with [28]. IBMA4 bacteria showed the minimum ability to consume polycyclic aromatic hydrocarbons with a rate of 44.65% as an average consumption.The lowest remaining concentrations of petroleum hydrocarbons were (12ppm). The average consumption compounds during the two-month treatment period was 39.280%, and the lowest average consumption was for the Pyrene compound with a percentage (17%) , this difference in the consumption of hydrocarbons and the percentage of biodegradation can be attributed to the enzymatic system of bacterial species and the method of analyzing petroleum hydrocarbons or predation or competition, allowing a section of prokaryotes to prey on other prokaryotes competing for the same food and energy source, and predatory bacteria may be less willing to degrade crude oil [29]. The results of the statistical analysis show the superiority of soil treatment with IBMA1 bacteria over IBMA3 bacteria and IBMA4 bacteria at a probability of $P \leq 0.01$.

VII. CONCLUSIONS

Iraq is one of the largest oil-exporting countries in the world and owns more than one crude oil refinery. Baiji is considered one of the important strategic areas for the presence of the largest oil refinery in it, but it is also vulnerable to contamination of its lands with crude oil, and this pollution poses a threat to humans and plants. This prompts us to find a ways to treat polluted lands. One of these methods is using bacteria in the biological treatment of contaminated soil. This study confirmed the emergence of crude oil-degrading bacteria in the Baiji soil and with high efficiency. The study was carried out by isolating and diagnosing bacteria from the contaminated site, and then the biological treatment of this contaminated site was carried out with the same bacteria that was isolated from it. This shows a great benefit to get rid of oil pollutants from soil, and it is a feasible and low-cost method. The isolates showed the superiority of Cytobacillus IBMA1 bacteria over the rest of the isolates in the ability to consume PAHs. Next comes to the bacteria Bacillus IBMA3 and IBMA4 with a varying ability of polycyclic aromatic carbons to consume hydrocarbons.This will accelerate the process of reforming the Iraqi environment from oil pollution.

Author contribution:

Maha H. Hussein has isolated , identified the bacteria and prepared the first draft of the manuscript including expermental work and data analysis . Ibrahim S Omer has supervised and reviewed the work carried out here in this paper to the best for the good understanding of the researchers.

Data Availability:

All data generated or analyzed during this study are included in this manuscript.

DECLARATIONS

Ethics approval and consent to participate:

Not applicable.

Competing interests:

The authors declare that they have no competing interests

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Appendix 1

The method of biological treatment of contaminated soils using only bacteria

No.	Period / Component	First month of treatment	Second month of treatment	Average Bioremediation	Treatment / Compound	Control	Soil treat with IBMA3	Soil treat with IBMA4	Soil treat with IBMA1
1.	Acridine	153.000c	110.250e	131.625d	Acridine	210.000cd	106.000l-o	117.500j-m	93.000m-s
2.	Acenaphthene	175.750b	126.750d	151.250c	Acenaphthene	248.000b	119.000jkl	138.500ij	99.500l-r
3.	Acenaphthylene	239.000a	179.750b	209.375a	Acenaphthylene	322.000a	170.000fgh	193.000de	152.500hi
4.	Anthracene	71.000h-k	37.750op	54.375ij	Anthracene	95.000m-s	43.000w-z	52.000vwx	27.000a-z

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5.	benzo(a) anthracene	66.000jkl	36.750op	51.375ij	benzo(a) anthracene	88.000efg	40.500w-z	47.000wxy	30.000a-z
6.	benzo(b) Fluoranthene	148.750 c	85.500gh	117.125ef	benzo(b) Fluoranthene	188.000rst	92.500o-t	108.500l-o	79.500q-t
7.	benzo(k) Fluoranthene	188.500b	105.750ef	147.125c	benzo(k) Fluoranthene	235.000b	119.000jkl	129.500jk	105.000l-p
8.	benzo (g , h) perylene	154.250c	94.500fh	124.375de	benzo (g , h) perylene	190.000ef	102.500m-q	112.500k-n	92.500q-t
9.	benzo (a) pyrenen	182.000b	153.000c	167.500b	benzo (a) pyrenen	215.000c	151.000i	165.000h	139.000ij
10.	Chrysene	67.000i-l	50.500mno	58.750i	Chrysene	99.000m-r	45.500wxy	51.500vwx	39.000w-z
11.	dibenzo (a , h) anthracene	127.750d	88.750g	108.250g	dibenzo (a , h) anthracene	166.000h	90.500n-t	99.500l-r	77.000rst
12.	Fluranthene	128.250d	81.500ghi	104.875g	Fluranthene	168.000h	82.000p-t	95.000m-s	74.500ut
13.	Fluroene	109.000e	80.000gj	94.500h	Fluroene	136.000ij	81.500p-t	89.000o-t	71.500tuv
14.	Indeno	64.250klm	39.750nop	52.000ij	Indeno	90.000n-t	37.500w-z	51.000vwx	29.500a-z
15.	Naphthalene	67.750i-l	39.750nop	53.750ij	Naphthalene	96.000m-r	41.000w-z	50.500v-y	27.500a-z
16.	Phenanthrene	53.500mn	37.250op	45.375ij	Phenanthrene	88.000o-t	31.500a-z	41.500w-z	20.500a-z
17.	Pyrene	24.000pq	17.000q	20.750k	Pyrene	55.000vxy	9.000bc	12.000abc	6.500c

** Similar letters mean there are no significant differences*