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BIOREMEDIATION OF TOTAL PETROLEUM HYDROCARBONS (TPH) IN HIGHLY CONTAMINATED SOILS

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Abstract

Restoring soils contaminated with petroleum oil via bioremediation is both environmentally friendly and economical. A new and sustainable approach, bioremediation can either occur naturally or can be improved by adding nutrients. Five bacterial isolates were isolated, and their biodegradation ability was initially tested. The results showed that isolate FB1 was the most effective in decomposing crude oil, with a percentage of 69%. It was diagnosed at the phenotypic, chemical, and molecular levels. It was discovered to belong to the genus *Bacillus* spp.

The effectiveness of natural attenuation with nutrients was assessed in this study for total petroleum hydrocarbons' deterioration (TPH) in heavily polluted soil in the Al-Faw area, south of Basra Governorate. the results showed that the addition of nutrients (NPK) led to an increase in the removal of TPH in the polluted soil sample by 80% during a treatment period of 60 days.

KEYWORDS: Petroleum hydro carbons, microcosm, bioremediation, Crude Oil

1. Introduction

Microorganisms are used in bioremediation, a highly appealing, innovative, economical, and ecologically benign approach, to eliminate petroleum hydrocarbon contaminants from water or soil (Wu *et al.*, 2016 ;Mrozik and Piotrowska -Seget, 2010). The majority of the microorganisms used in bioremediation are bacteria, fungus, and yeasts (Al-Hawash *et al.*, 2018). The microbial population reported to be most successful in degrading petroleum hydrocarbons is bacteria. (Hassanshahian *et al.*, 2012). Natural attenuation is a type of spontaneous bioremediation that exploits the soil's native microbial populations' inherent capacity for biodegradation, which allows them to extract and degrade pollutants (Bento et al., 2005). However, applying biostimulation techniques can improve the efficiency of naturally occurring bioremediation .To boost the potential of indigenous microbial communities to degrade petroleumThe addition of nutrients to contaminated soils is limited, including (N) nitrogen, (P) phosphorus, and (K) potassium (Suja et al., 2014; Li et al., 2016). Soil type, pollution, and environmental factors including soil pH, moisture content, temperature, and nutrient availability have a significant impact on the overall effectiveness of bioremediation (Admon et al., 2001). Therefore, it is essential to characterize and characterize a contaminated soil sample before starting a biodegradation experiment.

One of the five kinds of bacteria isolated from soil polluted with crude oil in our prior investigation demonstrated a strong propensity for breaking down the oil. This strain

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has a high capacity to break down crude oil in liquid medium and in soil that has been spurred with crude oil. However, More research is required to thoroughly assess and confirm the potential of these strains. A mini experiment was carried out on the basis of Contaminated soil samples from Al-Faw area, south of Basra Governorate in Iraq. The goals of this study were to: Examination of the efficiency of local microorganisms that degrade petroleum hydrocarbons in breaking down pollutants through natural attenuation by adding nutrients.

2. Materials and Methods

2.1. Soil sampling

Contaminated soil samples were collected from around the damaged conveyor pipe in Al-Faw neighborhood, south of Basra governorate. Samples were obtained from 0-15 cm below the soil surfaceThe soil sample has a lot of sticky black oil in it, which can leave a film of oil or soil on the glove.

The previously published techniques were used to determine the soil's temperature, pH, and moisture content. Roy *et al.*,(2014).Each soil sample that has been contaminated with TPH had its specific hydrocarbon components identified using gas chromatography-spectrometry. Prior to the experiment's setup, all of the aforementioned characteristics of the soil had been established.

2.2. Isolate bacteria from contaminated soil

Weigh 1 gram of soil, put it in a 250 ml beaker with 50 ml of MSM medium prepared in the laboratory containing 1% crude oil, followed by ten days of shaking incubation at 30 °C and 150 rpm. 0.1 mL medium was removed and distributed onto nutrient agar medium. The Petri dishes were then placed in an incubator for 24 hours at 37 °C. (Hasanshahian and Emtiazi, 2008).

2.2.1. Isolation and identification

On Nutrient agar, phenotypic parameters such as colony shape, color, texture, odor, and size were used to diagnosis samples at first. The cells were then stained with Grimm's dye, and a microscope was used to determine the type of dye, the form of the cells, and the method of assembly. Tests on biochemistry were conducted. To verify the diagnosis' findings (Almansoory *et al.*, 2019).

2.2.2. Molecular Identification

For taxonomic characterization of the isolated isolates, the 16S rRNA gene was analyzed. Using the CTAB technique, the bacterium's whole DNA was isolated (Winnepenninckx *et al.*, 1993The forward primer Bac27 F (5, -AGAGTTTGATCCTGGCTCAG-3,) and the reverse primer Uni 1392R were used to amplify the bacterial DNA. 16S rRNA loci (5, -GGTTACCTTGTTACGACTT -3,).A total of 50 l were used for the amplification process, of which 2 l were used for forward and reverse transcription as well as 2 l of DNA prepared in accordance with the procedure(Miyoshi *et al.* 2005).

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2.3. Testing the biodegradation potential of various bacterial species for crude oil

Mineral salt medium (MSM), containing 0.5 g NaCl, 0.025 g $MgSO_4$, 1.0 g KH_2PO_4, 0.1 g NH_4NO_3, 1.0 g $K_2HPO_4.3H_2O$, and 0.2 $(NH_4)2SO_4$, was used. The MSM pH was adjusted to 7 before being sterilized at 120 °C for 20 minutesUse 1% (v/v) crude oil as a carbon and energy source, and biodegradable MSM. 1 mL of purified culture of each bacterial isolate was added to MSM medium (100 mL/250 mL Erlenmeyer flask). For 10 days, all flasks were incubated at 30 °C with constant shaking at 150 rpm.. Control flasks were incubated without a living organism under the same conditions(Patil *et al.*,2012).

2.4. Gravimetric analysis

Weighing the amount of crude oil remaining after biodegradation to calculate the percentage of biological degradation (Luna *et al.*, 2013). The following formula was used to calculate the estimated crude oil deterioration efficiency:

$$\textbf{Degradation\%}=\frac{mg\ of\ crud\ oil\ control-mg\ of\ crud\ oil\ test}{mg\ of\ crud\ oil\ control}\times 100$$

2.5. Microcosm Experiment set up

TPH-contaminated soil samples were taken from the Al-Faw area (as previously mentioned) in order to conduct an experiment with a small amount of soil in a lab setting in order to determine the effect of extra nutrients (NPK) on the local microbial population's capacity for bioremediation. We took soil samples without first sterilizing them. Experiments with the microworld were conducted in aluminum containers without covers (25 cm in diameter and 10 cm high).All vessels first sterilized 75% ethanol. Before setting up microworld experiments. The weight of 2 kg of contaminated soil was placed inside the manufactured containers, The nutrients (NPK) were nitrogen (N), phosphorus (P) and potassium (K) added in different proportions, where three ratios were added (0.25, 0.5 and 0.75) g/kg for each sample of contaminated soil with three replications being made for each sample where they are added Nutrients every 20 days, taking into account the moistening of the soil and stirring the soil periodically using a sterile stainless steel laboratory spoon to ensure the uniform distribution of oxygen and nutrients in the sample, With a control copy, add contaminated soil without NPK for comparison.

Each sample's soil moisture was measured gravimetrically (Asadirad *et al.*, 2016). Distilled water is added to the microcosms to moisturize the soil in the form of different periods. To make sure that nutrients and oxygen were distributed uniformly throughout the sample, A sterile stainless steel laboratory spoon was sometimes used to stir the soil. TPH lysate levels and TPH lysates were calculated after 0, 20, 40 and 60 days of incubation.

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2.6. Analysis of the soil's total petroleum hydrocarbons (TPH)

The concentration of TPH in each soil sample on day 0 before the experiment started and in each microcosm treatment were calculated in order to assess the level of TPH degradation after 0, 20, 40, and 60 days of incubation. I took three grams of soil from each microcosm and placed it in the corresponding 50 milliliter beaker for each distinct circumstance (0, 20, 40, and 60 d). Following that, an oven preheated to 80 C was utilized for 24 hours.

Then, 1 g of dry soil, 2 g of Na2SO4, and 8 mL of carbon tetrachloride were added to a 10 mL centrifuge tube. (corresponding to each microcosm). After vigorously combining all tubes for one minute, all tubes were shaken for 30 minutes at a speed of 270 2. After shaking the tubes, they underwent a 15-minute, 4000 rpm centrifugation. A 250 mL aliquot of the extract was diluted with carbon tetrachloride into 25 mL volumetric flasks. The TPH concentration was then calculated using a GC on a diluted extract.

3. Results

3.1. soil texture

The rates of sand, clay and silt values varied in the study area, as sand was the largest component in the soil texture, in addition to the nature of the soil type, as its mixtures in the region were as shown in Table (1) as follows:

Table (1) Soil texture analysis of the study site

study area	sand %	silt %	mud %	Soil type
Faw	54.81	17.31	32.88	Mixed

3.2. Isolation and identification of bacteria

3.2.1. Culture Identification

Using soil samples from a hydrocarbon-contaminated site, we isolated 5 isolates of bacteria that biodegradation hydrocarbons from surface soil. Using morphological and biochemical methods, we identified the bacterial isolates in accordance with Bergey's taxonomy chart, providing evidence for specific bacteriology (Holt *et al.* 1994). Gram positive bacteria were used to examine the bacterial populations in all samples. Perhaps some cells. It does not produce colonies, and some colonies may merge beneath it if it becomes too crowded. Measurement mistakes could result from these actions. This necessitates diluting the samples. the accurate quantity of colonies. We sample the diluted solution in this line of work in batches of 10^{-3}. The diluted samples were transferred to nutritional agar using the spread-plate technique.

Colonies of the FB1 isolate were unequal, rod-shaped, and 4 mm in diameter. The FB2 isolates were symmetrically creamy colonies, with wavy borders and flat centres. FB3 colony isolates were 3 mm in diameter, and presented flat, adherent surfaces. Cream coloured, the colonies had uneven shaped borders. FB4 colonies were cream in color and were round. FB5 they had irregular rounded shapes with light colored flat edges that were also cream and translucent. Table 2 shows pictures of .

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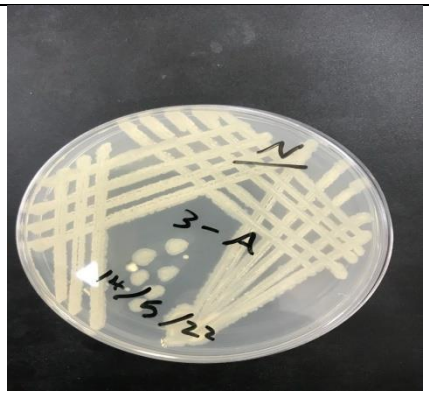
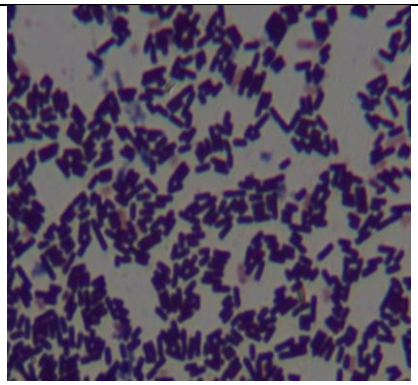
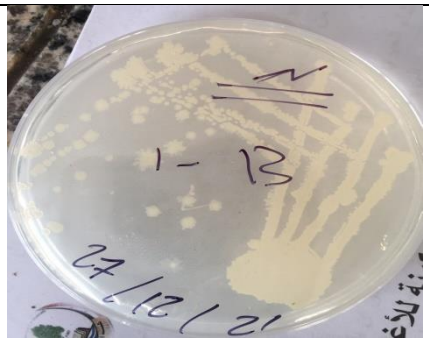
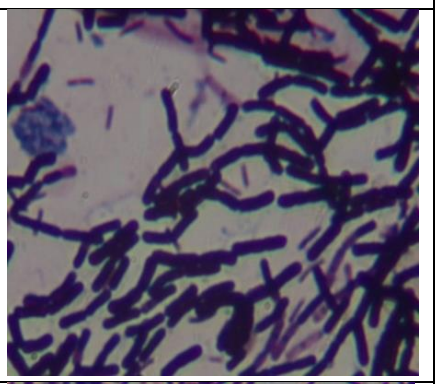
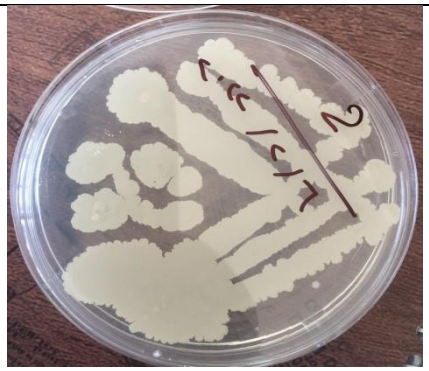
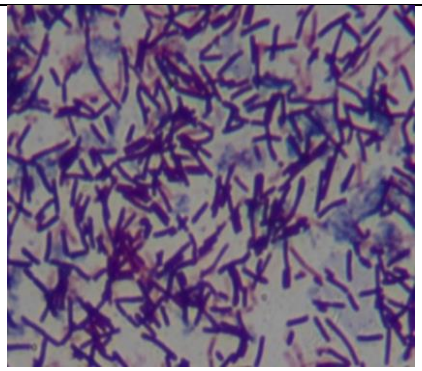
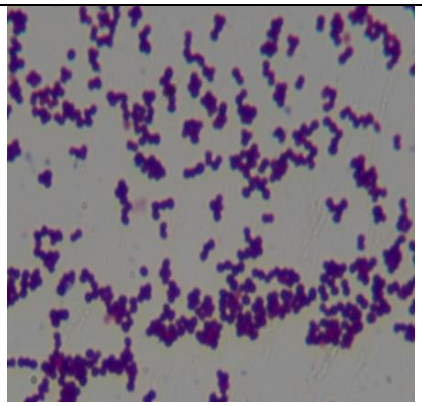
Table 2: Bacterial isolated from hydrocarbon-contaminated soil samples

Cod	Streak bacteria	Gram stain
FB1		
FB2		
FB3		
FB4		

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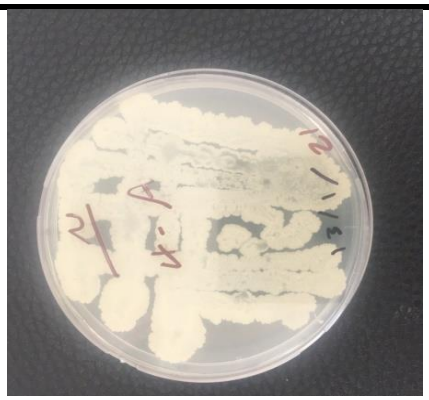
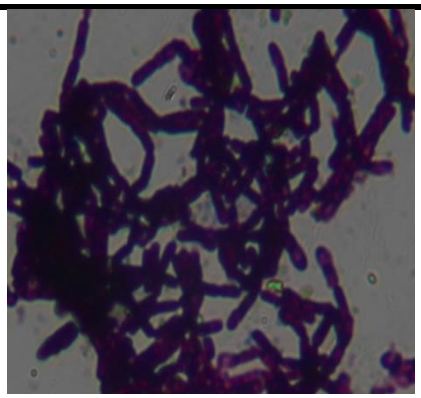
FB5		

3.2.2.Molecular identification

The results of DNA amplification of the bacterial isolates by polymerase chain reaction (PCR) showed that the size of the base pairs was 1500 base pairs (bp) when using genetic primers (F27 and R1392) compared to the DNA ladder. The Korean company was relied on to process the sequence of the nitrogenous bases of the 16S_ rRNA ribosomal gene, where the results of the bacterial isolation were presented after using the BLAST program to To compare the genetic sequences to find out the similarity and genetic compatibility with the genetic information in the NCBI Genome Bank in the table(3).

Table(3) Results of the analysis of the ribosomal gene sequences of the bacterial isolate

Isolate	Gr am Stain	Closed BLAST match	Strain	Accessio n No.	Identity
B	+	*Bacillus* sp.FB1	AIM ST LIP5	OP703527	99.45%

3.2. Average petroleum hydrocarbons in soil samples

Using a spectroflourometer, the total petroleum hydrocarbon concentrations in soil samples from the study region were determined, which is of type RF540 by Shimadzu and equipped with a DR3 by Shimadzu recorder, a xenon light source, and a quartz cell in which the sample is placed. The intensity emission spectra were fixed at a wavelength of 360 nm. excitation and irritation of 310 nanometers The recorded results showed that the total petroleum hydrocapone concentrations reached the highest value of 101.39 g/g. Gas chromatography (GC) was used to identify the total number and quality of the regular alkanes in the samples from the study area.

3.3. Crude oil biodegradation by bacteria strain

A graph was used to assess the crude oil's composition. Bacillus sp.FB1 treatment was given for 3, 7, and 10 days (Fig. 1). The findings demonstrated that crude oil could be used by Bacillus sp. FB1 as a carbon and energy source for growth. The bacterial strain used to digest crude oil in MSM was shown to be a potential degrader; by FB1, crude oil was degraded by 69% during the course of a 10-day incubation period.

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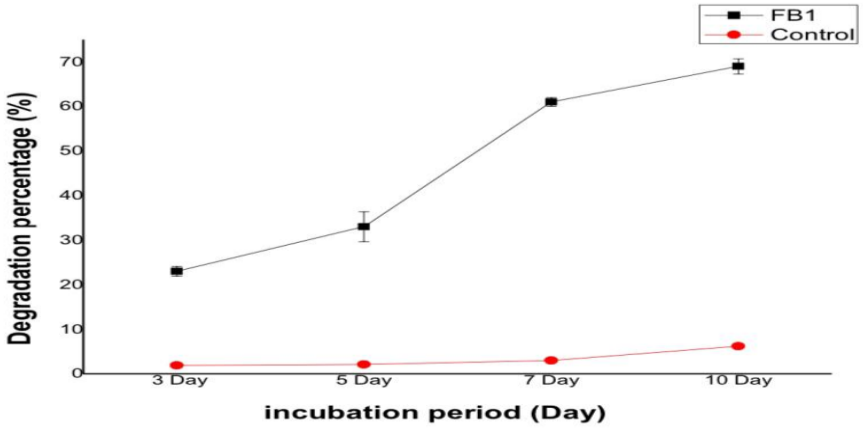

Fg.1.Biodegradation test under optimal conditions for growth

3.3. TPH degradation in soil microcosm under the addition of nutrients

The concentration of petroleum hydrocarbons in each replicate soil sample was estimated after 60, 50, 40, 30, 20, 10 days of treatment using a spectrophotometer of the Marine Science Center / University of Basrah, and the GC device was used at 0 days, 20 40 and 60 days to evaluate Extent of biodegradation of TPH The biodegradation of TPH significantly increased with incubation time in all replicate samples after the soil total hydrocarbon concentration reached 101.39 mg/g and the rate of fractionation was determined by soil microorganisms with the addition of NPK nutrients. Taking into account humidity, oxygen, pH and temperature. After an incubation period of 60 days, the biodegradation reached 80% at the addition of 0.5% of NPK while the percentage of degradation at the addition of 0.25% was 55% compared to 9% in the control sample, as shown in Figures 2, 3 and 4.

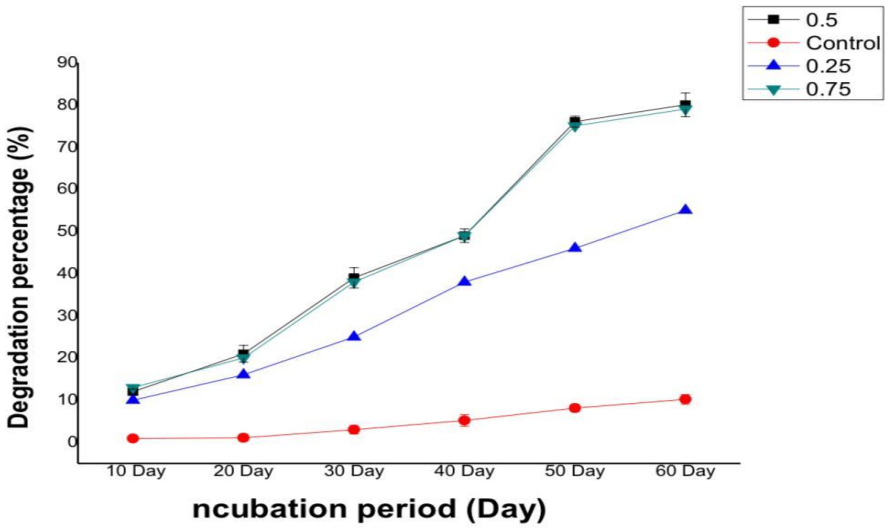

Figure (2) The effect of adding nutrients on the percentage of bio-fracturing in the soil during the 60-day treatment period, compared to the control sample without adding nutrients.

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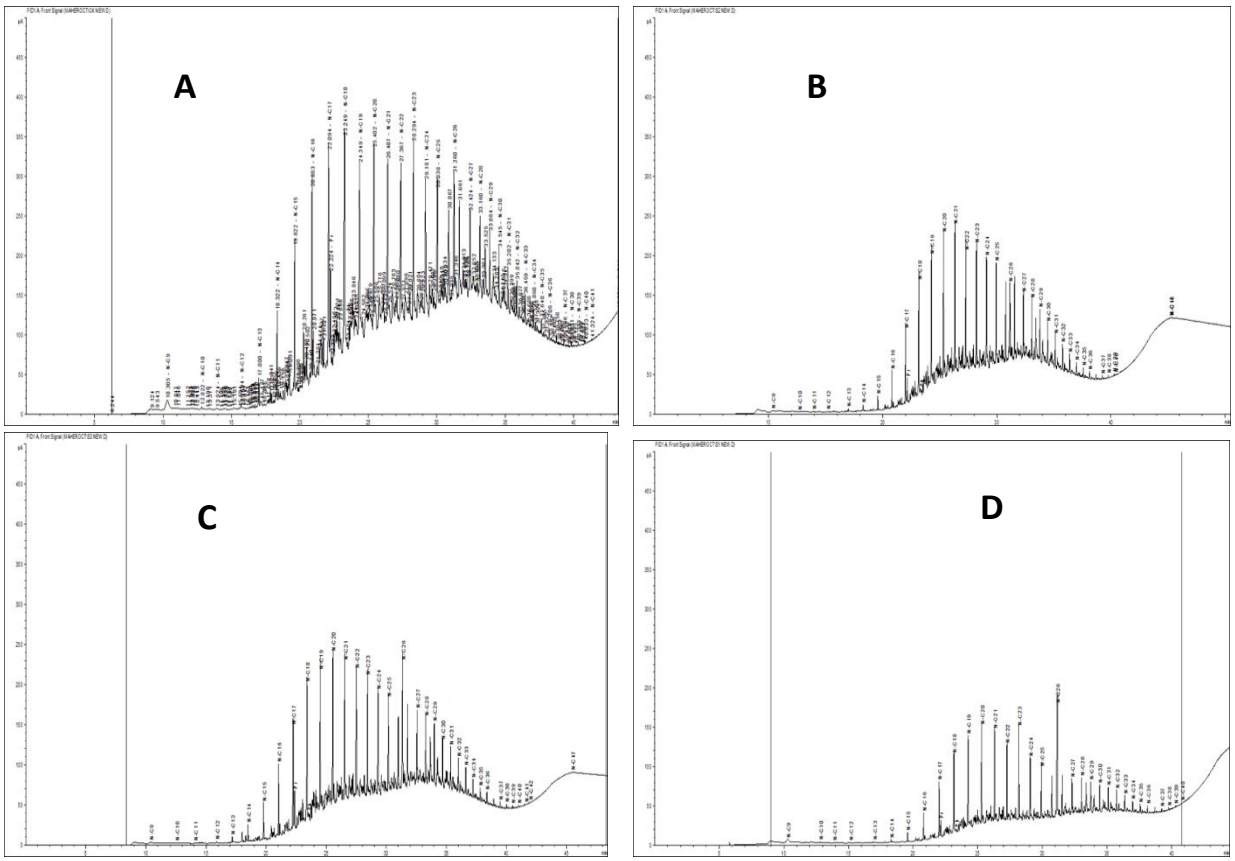
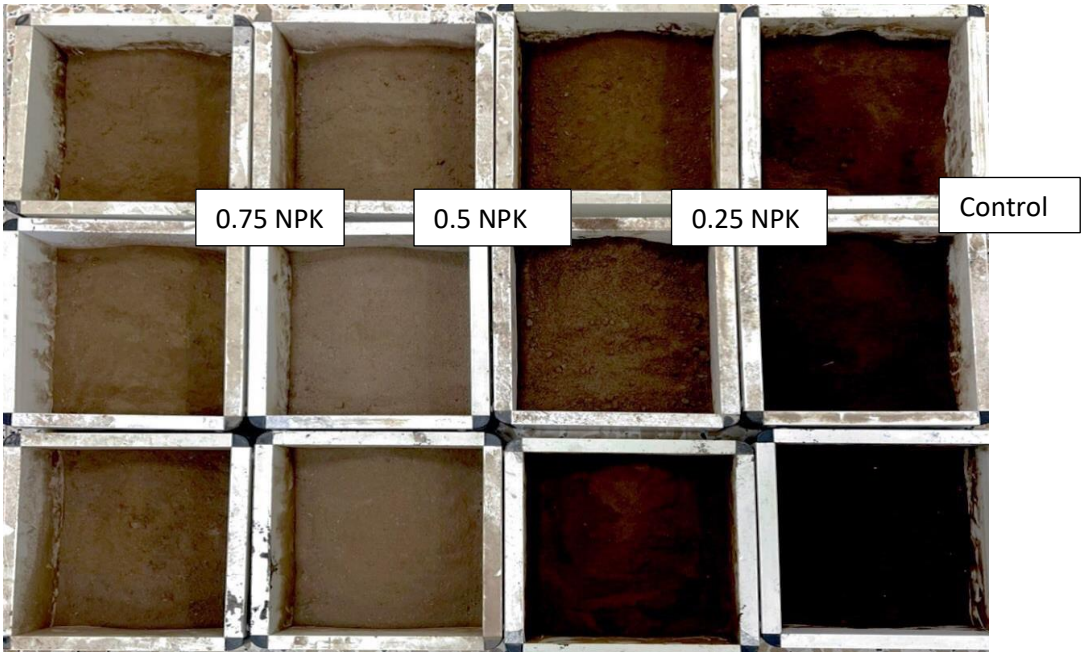


Figure (3) shows the gas chromatography trace of the remaining hydrocarbon concentrations in the soil during the treatment period A- the control sample without adding nutrients (NPK). B- Soil sample after 20 days of adding nutrients. C- Soil sample after 40 days of adding nutrients. D- Soil sample 60 days after adding nutrients.



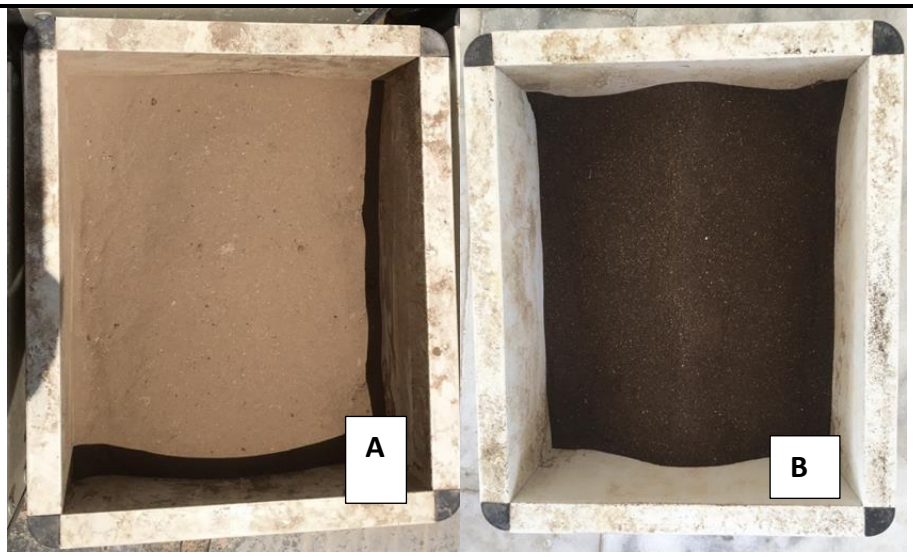


Figure (4).A- Soil bioremediation after 60 days B- control sample

4. Discussion

Since microorganisms are more environmentally friendly and cost-effective than physical and chemical methods for removing petroleum toxins, bioremediation has been the focus of extensive scientific research over the past 10 years. (Roy *et al*., 2014; Li *et al*., 2016). Bacteria including *Pseudomonas aeruginosa, Bacillus subtilis*, *Corynebacterium kutscheri*, and *Bacillus megaterium* have been shown in numerous studies to be capable of digesting a variety of harmful pollutants. (Thavasi *et al*., 2011).
A thumbnail of the research's The experiment was done on oil-contaminated soil from the Al-Faw region, south of Basra, to see if the native microbial population in the polluted soil has the ability to naturally attenuate TPH with nutrients.
The total observation showed after the experiment and during the incubation period (60) days Nutrients also play an important role Biodegradation of pollutants. . In this trial, nutrients were sporadically given out. Previous investigations have noted the effectiveness of natural bioremediation in both of the soil treatments. (Zhao *et al*., 2011; Suja *et al*., 2014; Wu *et al*., 2016). However, some research indicates that biostimulation gives the best results. (Yu *et al*., 2005 ;Mancera-Lopez *et al*., 2008 ; Hesnawi and Mogadami, 2013).

5. Conclusions

The amount of TPH degraders present at the outset of the bioremediation process, the TPH level, bioavailability, composition of the contaminated soils, and bioavailability all had a significant impact on its outcome. The biodegradation of hydrocarbon wastes was accelerated by the addition of various nutrients to the contaminated soil, and the greatest results were obtained when employing NPK.

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