



Influence of Environmental Conditions on Bacterial Remediation of Oil-Contaminated Soil: A Case Study at Baiji Refinery

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ARTICLE INFO

Article history:

Received 01 October 2024
Revised, 01 October 2024
Accepted, 08 October 2024
Available online 09 October 2024

Keywords:

Total Petroleum Hydrocarbons (TPH),
Soil Contamination,
Environmental Pollution,
Bioremediation,
microbial Degradation

ABSTRACT


Total Petroleum Hydrocarbons (TPH) are a major environmental pollutant, posing serious risks to soil health and ecosystems. TPH contamination, often resulting from crude oil spills and industrial activities, significantly disrupts soil structure, reduces fertility, and hinders the growth of plants and soil-dwelling organisms. In the environment, TPH compounds persist due to their hydrophobic nature, leading to long-term ecological damage. They can infiltrate groundwater, compromising its purity and presenting health hazards to humans and wildlife. The existence of TPH in soil modifies the population of bacteria, reduces biodiversity, and impedes natural processes such as nutrient cycling. Effective bioremediation techniques, such as microbial degradation, are essential for mitigating the harmful impacts of TPH on soil and the broader environment. This study emphasizes the urgent need for sustainable remediation strategies to restore contaminated soils and protect ecological and human health.

This study investigates the influence of environmental circumstances on bacteria employed for the remediation of soil polluted with crude oil, focusing on bacterial strains sourced from the Baiji refinery in Iraq. Microbial rehabilitation is an economical and environmentally sustainable method for decomposing hydrocarbons into simpler molecules, making it an effective method for soil bioremediation. The study monitored biodegradation over seven months, assessing the degradation efficiency of total petroleum hydrocarbons (TPH) using gas chromatography-mass spectrometry (GC-MS). Environmental factors, including temperature, moisture levels, and nutrient availability, were closely monitored to assess their impact on bacterial activity. Ideal moisture range for bacterial growth and hydrocarbon degradation is often cited as 40% to 60% of the soil's WHC. Too much water can reduce oxygen availability in the soil, inhibiting aerobic bacterial growth, while too little water can limit microbial metabolism. Temperature affects bacterial metabolism and the rate of hydrocarbon degradation. Most hydrocarbon-degrading bacteria thrive in mesophilic conditions. The ideal temperature range for TPH degradation by bacteria is generally between 25°C and 35°C. At temperatures below 15°C, microbial activity and degradation rates slow significantly, while temperatures above 40°C can inhibit bacterial growth or even kill the microbes. The results revealed that the moisture: 40%–60% of soil's water-holding capacity and temperature 25°C–35°C maintaining these conditions promotes the optimal breakdown of hydrocarbons in bioremediation efforts. The results revealed significant degradation of crude oil, with a reduction of 40 initial compounds in untreated samples to 25-35 compounds in treated samples. The biodegradation rates increased steadily over time, with a removal efficiency reaching 72.38% by the end of the experiment. Additionally, the study demonstrated that environmental conditions, particularly temperature and moisture, play a pivotal role in optimizing bacterial degradation activity. These findings underscore the potential of bacteria in bioremediation efforts and the necessity of optimal environmental conditions, such as controlled temperature, moisture content, and nutrient levels, are essential for maximizing the efficiency of crude oil degradation in contaminated soils.

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<https://doi.org/10.61268/gc9va174>

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1. Introduction

Crude oil contamination presents a significant environmental challenge, especially in ecosystems where oil spills disrupt the balance of soil and water systems. [1]. These spills introduce toxic hydrocarbons, which can severely degrade soil health and cause long-term ecological damage. Conventional methods of cleanup, such as physical removal and chemical dispersants, have limitations in terms of efficiency, cost, and potential secondary environmental harm. As a result, biological remediation, specifically microbial degradation, has emerged as a sustainable alternative. [2].

Microbial remediation uses bacteria capable of breaking down hydrocarbons into less harmful substances. These bacteria utilize hydrocarbons as a carbon source, breaking down complex organic compounds into simpler substances such as carbon dioxide, water, and biomass. However, the effectiveness of this microbial degradation is significantly influenced by environmental factors, including temperature, pH, oxygen levels, and soil moisture significantly impact bacterial growth and activity, influencing the overall rate of bioremediation. Understanding how these factors interact with microbial populations is critical for optimizing bioremediation strategies for crude oil-contaminated soils. [3, 4]. This paper aims to explore the influence of various environmental factors on the effectiveness of bacteria in degrading crude oil-contaminated soil.

Impact of Environmental Conditions

Environmental conditions provide a vital function in the bioremediation process, as they directly influence the growth, survival, and metabolic processes of the bacteria involved. Some of the key environmental factors that affect bacterial degradation of crude oil include: [5].

- **Temperature:** Bacterial activity tends to increase with temperature, up to an optimal point. Most hydrocarbon-

degrading bacteria thrive in mesophilic temperatures (20-40°C), although some species are active at higher or lower temperatures. Temperature affects both the rate of microbial metabolism and the physical properties of hydrocarbons, such as their solubility and bioavailability.

- **Moisture:** Soil moisture content is another critical factor for bacterial growth. Adequate water availability is essential for microbial metabolism and nutrient transport. However, excessive moisture can lead to oxygen depletion, which in turn hampers aerobic microbial processes that are crucial for hydrocarbon degradation.
- **pH:** The acidity or alkalinity of the soil can significantly impact bacterial communities. Most hydrocarbon-degrading bacteria prefer neutral to slightly alkaline conditions (pH 6-8). Extremes of pH can inhibit microbial activity or alter the composition of bacterial populations in the soil.
- **Oxygen Levels:** Oxygen is a key electron acceptor in the aerobic degradation of hydrocarbons. In oxygen-limited environments, the efficiency of hydrocarbon degradation decreases, although certain anaerobic bacteria can still break down hydrocarbons under anoxic conditions. Ensuring adequate oxygenation of the contaminated site is therefore essential for promoting efficient bioremediation. [6, 7].

In this study, these environmental factors will be closely monitored to understand their influence on the breakdown of hydrocarbons by microorganisms in soil polluted with crude oil.

2. Methodology

2.1. Bacterial Isolation from Baiji Refinery Soil

Soil samples contaminated with crude oil were collected from various locations within the Baiji refinery, Iraq. These samples were

transported to the laboratory in sterile containers and stored at 4°C specify time 48 hours, to maintain microbiological integrity. Extended storage beyond this period could affect microbial viability and activity to preserve microbial integrity. Samples were sealed in sterile containers immediately after collection to prevent contamination. During storage and transportation, samples were kept under sterile conditions, with minimal handling, to ensure the accuracy of microbiological analyses. The soil selected based on visible contamination levels, proximity to known spill sites, or areas with a history of oil contamination. Soil samples were taken from a depth of 0–15 cm to capture surface and sub-surface contamination. A total of samples were collected using random sampling. The soil was analyzed for bacterial populations capable of hydrocarbon degradation. [8].

2.2. Extraction of Hydrocarbon-Degrading Microbes

1. Sample Preparation:

The contaminated soil samples were mixed with sterile water. Sterile deionized/distilled water was used to avoid introducing external contaminants or minerals that could affect microbial growth.” at a 1:1. The soil-to-water ratio of 1:1 was chosen based on optimizing the suspension of soil particles for microbial extraction. Samples were shaken at 200 RPM on a rotary shaker for 15 minutes to ensure uniform mixing and dislodgement of microorganisms from the soil particles. This suspension was allowed to settle, and the supernatant was used for bacterial isolation.

2. **Enrichment Culture:** Hydrocarbon-degrading bacteria were enriched by introducing 1 mL of the supernatant into 100 mL of minimal salt medium (MSM). The minimal salt medium (MSM) consisted of K_2HPO_4 and other relevant salts and trace elements. This formulation ensures the availability of essential

nutrients for microbial growth while restricting external carbon sources to encourage hydrocarbon degradation.” with 1% (v/v) crude oil serving as the sole carbon source. The 1% (v/v) crude oil was prepared by mixing with MSM and sterilized via autoclaving by phenol to prevent contamination by external microorganisms. The culture flasks were incubated at 30°C with continuous shaking at 150 rpm for 7 days. The incubation temperature of 30°C was selected based on previous studies that have shown it to be optimal for the growth and metabolic activity of hydrocarbon-degrading bacteria [1]. This temperature supports efficient enzyme function and degradation processes in mesophilic bacteria commonly found in oil-contaminated soils. Shaking at 150 rpm was used to ensure adequate oxygen transfer in the culture, as hydrocarbon-degrading bacteria are typically aerobic and require oxygen for metabolism. Shaking facilitates the oxygenation of the culture medium, improving bacterial growth and biodegradation efficiency [3]. The 7-day incubation period was chosen based on prior studies and preliminary tests, which demonstrated that this timeframe is sufficient for noticeable hydrocarbon degradation by the enriched bacterial culture. This period allows for an adequate assessment of bacterial adaptation and metabolic activity during hydrocarbon breakdown.

3. **Isolation and Purification:** After the incubation period, serial dilutions of the enriched culture were prepared and spread on nutrient agar plates supplemented with 1% crude oil. The plates were incubated at 30°C for 48 hours. Distinct bacterial colonies were isolated, purified by sub-culturing, and stored at -80°C in 20% glycerol for further use.

2.3. Characterization of Hydrocarbon-Degrading Bacteria

To identify and characterize the hydrocarbon-degrading bacterial strains, the following analyses were performed: [9].

1. **Morphological Characterization:** Bacterial isolates were observed under a microscope to study their colony morphology and cell structure.
2. **Biochemical Characterization:** Standard biochemical tests, including Gram staining, catalase, and oxidase tests, were conducted to identify the bacterial isolates. Gram staining was conducted to classify bacteria based on their cell wall structure, distinguishing between Gram-positive and Gram-negative species, which is crucial for further identification. The catalase test was used to assess the bacteria's ability to break down hydrogen peroxide, indicating aerobic metabolic pathways. Similarly, the oxidase test was employed to identify bacteria with cytochrome c oxidase, an enzyme involved in cellular respiration, aiding in the characterization of aerobic organisms
3. **Molecular Identification:** The selected bacterial strains were further identified genomic DNA was extracted from the bacterial isolates, and the 16S rRNA gene was amplified via PCR. The amplified gene products were then sequenced, and the resulting sequences were compared with those in the GenBank database to identify the bacterial species. Figure 1. Illustrated cultivation of a bacterial swab.

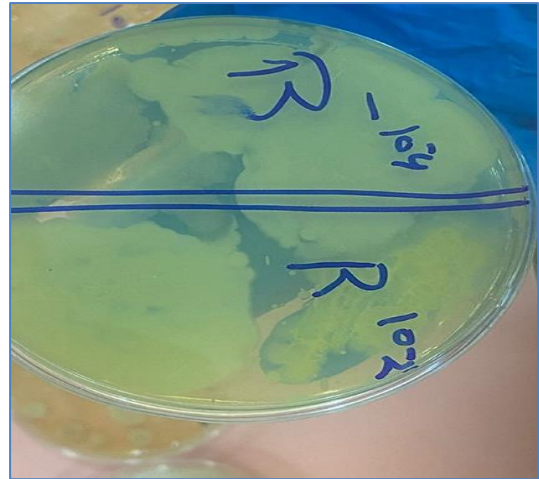


Figure 1. Cultivation of a Bacterial Swab

3. Soil Treatment with Bacteria

3.1 Experimental Setup: Soil from the Baiji refinery was used for bioremediation experiments. The soil was homogenized and divided into two main groups:

1. Control Group: Contaminated soil without bacterial treatment was used to monitor natural attenuation.
2. Treated Group: Contaminated soil was inoculated with a bacterial consortium consisting of isolated hydrocarbon-degrading strains. The bacterial suspension was prepared by growing the isolates in nutrient broth, followed by centrifugation to collect the cells. The bacterial pellet was re-suspended in sterile saline solution, and this inoculum was added to the soil at a

concentration of approximately 10^8 CFU/g of soil.

3.2. Biodegradation Monitoring

The treated soil samples They were left at thirty degrees Celsius for a duration of 7 months. Soil samples were collected monthly to evaluate hydrocarbon degradation. For chemical analysis, the hydrocarbon content in the soil was extracted with hexane and analyzed through Gas Chromatography-Mass Spectrometry (GC-MS). The total petroleum hydrocarbon (TPH) levels were assessed by tracking the decrease in hydrocarbon concentration over time. Microbial Activity Monitoring: The microbial population in the treated soil was periodically assessed by plate counting on crude-oil-enriched agar media to observe changes in bacterial colony-forming units (CFU) over the 7-month incubation period. The bacterial consortia used for hydrocarbon degradation primarily consisted of *Bacillus* species, which are well-known for their ability to degrade hydrocarbons. The consortium included multiple strains of *Bacillus*, identified through 16S rRNA gene sequencing. These strains have previously demonstrated high efficiency in breaking down petroleum hydrocarbons under various environmental conditions. Specific strains of *Bacillus* that were utilized are noted for their enzymatic capabilities in metabolizing alkanes and polycyclic aromatic hydrocarbons (PAHs). Incubation Conditions: The inoculum containing the *Bacillus* consortia was incubated with crude-oil-contaminated soil under controlled conditions. The incubation was carried out at 30°C, which is optimal for the growth and activity of *Bacillus* strains involved in hydrocarbon degradation. The inoculated soil was mixed thoroughly with the bacterial culture to ensure uniform distribution. The soil was incubated for a period of seven days, with constant shaking at 150 rpm to promote oxygenation, which is critical for aerobic hydrocarbon-degrading bacteria like *Bacillus*. The chosen temperature, duration, and mixing conditions were based on preliminary studies and existing literature that support these

settings as favorable for the biodegradation process.

4. Data Analysis

The effectiveness of bacterial treatment was evaluated by comparing the hydrocarbon degradation rates between the control and treated groups. Statistical analyses were performed to determine the significance of the differences in hydrocarbon degradation. The results were represented as a percentage of hydrocarbon reduction, and the efficiency of bioremediation was calculated accordingly.

Elimination of TPH Percentage Assessment
The removal percentage was calculated using the equation: Removal % = (total peak area of control - total peak area of test) / total peak area of control [10]. The calculation for determining TPH removal percentage is based on the total peak area obtained through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. GC-MS was employed to separate and identify the hydrocarbon components within the contaminated soil samples. The total peak area corresponds to the sum of the areas under the individual hydrocarbon peaks, as detected by the GC-MS.

For each sample, hydrocarbon extraction was carried out using hexane, and the extracts were injected into the GC-MS for analysis. The chromatograms generated by the GC-MS were used to quantify the hydrocarbons by integrating the area under the relevant peaks. This integration process involves calculating the total peak area for all detected hydrocarbons before and after the bioremediation process, allowing for an accurate determination of TPH reduction over time.

5. The findings:

The outcome of Bacterial Treatment on Spill Samples .The paper presents the findings on the remediation of crude oil spills using microorganisms, as seen in the figures.2. and tables 1. below (Control samples).

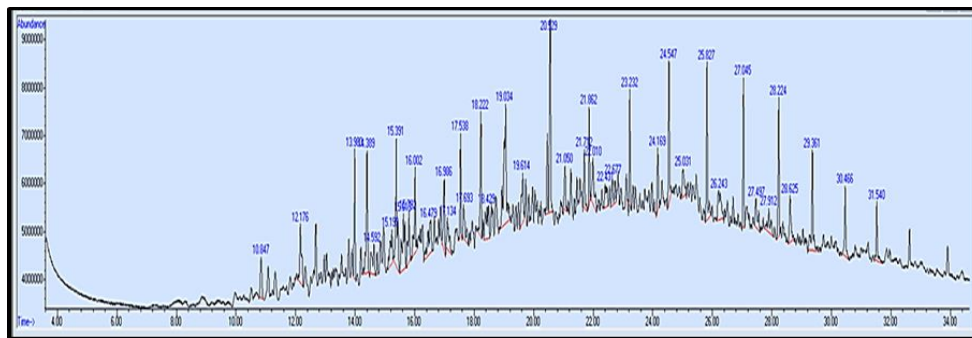


Figure 2. The control sample of spill samples

Bacterial remediation is a cost-effective method that breaks down organic contaminants into simpler substances, such as carbon dioxide, water, inorganic compounds, and cellular proteins. This process can also convert complex organic pollutants into more basic forms [11]. Microorganisms use these organic pollutants as their main carbon source, aiding in the degradation of toxins in the soil [12].

A Gas Chromatography-Mass Spectrometry (GC-MS) analysis of control samples (oil without microorganisms) identified various hydrocarbons. These results were compared to the GC-MS analysis of oil samples treated with bacteria. The biodegradation by the bacterial strain was tracked monthly over seven months. After each incubation period, the remaining oil hydrocarbons were extracted using hexane. The results showed that the biodegradation process produced new, lower molecular weight compounds, such as carboxylic acids and alcohols [13].

GC-MS analysis identified 40 chemical components in the control samples (refer to Figs. 1 and Tables 1). A number of these chemicals were lacking in the samples treated with bacteria, as shown by the absence of their peaks. In the treated samples, around 35 chemicals persisted, and the peak regions for several chemical compounds were markedly diminished relative to the untreated samples. This methodology yielded comprehensive insights into the deterioration of certain compounds and their decomposition in crude oil, as shown by [11, 13]. The monthly studies done over seven months proved that the rate of bacterial breakdown of total petroleum

hydrocarbons (TPH) in the spill samples was elevated, as evidenced by area measurements. In the control samples, 40 compounds were originally present, which diminished to 25-35 compounds following bacterial treatment. The area reduction and removal rates for the spill samples were 19.99%, 23.07%, 33.92%, 46.15%, 54.48%, 60.85%, and 72.38% throughout the seven-month treatment period.

The impact of environmental conditions on the efficiency of bacteria used for crude oil-contaminated soil remediation was observed through the biodegradation rates over the seven-month study. Several factors influenced the bacterial activity [14], including temperature, moisture content, nutrient availability, and oxygen levels. These factors played a crucial role in determining the rate and effectiveness of biodegradation. [15].

1. **Temperature:** The optimal temperature range for bacterial activity significantly enhanced the degradation process. Warmer conditions facilitated faster hydrocarbon breakdown, while cooler temperatures slowed bacterial metabolism and, consequently, the rate of degradation. Specify the equipment used to maintain temperature thermostats
2. **Moisture Content:** Adequate moisture was necessary to sustain microbial life and their metabolic processes. Soils with appropriate moisture levels showed faster degradation rates, while excessively dry conditions hindered microbial growth and activity. The

methods employed to measure soil moisture, where soil moisture probes. moisture levels were adjusted or maintained during the experiment by use regular watering schedules.

3. **Nutrient Availability:** The addition of nutrients like nitrogen and phosphorus positively affected the bacteria's ability to degrade hydrocarbons. The bacteria were more effective in environments with balanced nutrient levels, leading to higher degradation rates compared to nutrient-deficient soils.
4. **Oxygen Availability:** Oxygen levels were a critical factor, as the bacteria used in this study were primarily aerobic. Increased oxygen availability enhanced biodegradation, as aerobic bacteria could more efficiently break down hydrocarbons. In contrast, low oxygen levels reduced the rate of biodegradation.

The GC-MS analysis of the control (oil without bacteria) and bacterial-treated samples showed the influence of these environmental factors. The treated samples exhibited a progressive reduction in hydrocarbon compounds over time, indicating successful degradation. The removal rates increased steadily, with final reductions of 72.38% in the spill samples. These results suggest that environmental conditions significantly influence the bacterial remediation process, with optimal conditions leading to more efficient hydrocarbon breakdown. [16 , 17, 18].

6. Conclusion

This study highlights the effectiveness of microbial rehabilitation in degrading crude oil contaminants in soil. The bacterial strain isolated from the Baiji refinery demonstrated significant potential in breaking down complex hydrocarbons into simpler, less harmful compounds such as carboxylic acids and alcohols. Over the course of seven months, biodegradation was monitored using GC-MS analysis, which revealed a consistent reduction

in the number of hydrocarbons and their complexity.

In the control samples, 40 chemical compounds were initially identified, while the bacterial-treated samples exhibited a marked decrease in the number of compounds, reducing to 25-35 after treatment. The area of the compounds in the treated samples also showed a progressive decline, with removal rates of 19.99%, 23.07%, 33.92%, 46.15%, 54.48%, 60.85%, and 72.38% over the seven-month period.

This demonstrates the bacteria's increasing efficiency in degrading total petroleum hydrocarbons (TPH) over time.

The study confirms that microbial rehabilitation, using bacteria isolated from contaminated sites like the Baiji refinery, can be a cost-effective and efficient method for remediating crude oil-contaminated soils. The gradual reduction in hydrocarbons and the formation of simpler, less toxic compounds underscore the potential of this bioremediation approach in environmental cleanup efforts.

The study demonstrates that environmental conditions have a profound impact on the effectiveness of bacteria in remediating crude oil-contaminated soil. Factors such as temperature, moisture, nutrient availability, and oxygen levels were found to significantly influence bacterial activity and biodegradation rates.

Optimal temperature and moisture conditions accelerated hydrocarbon degradation, while nutrient-rich environments provided the necessary support for bacterial growth and metabolism. Additionally, sufficient oxygen availability facilitated faster breakdown of hydrocarbons, particularly for aerobic bacteria. The results clearly indicate that proper environmental management can enhance the efficiency of microbial rehabilitation efforts.

This study highlights the importance of tailoring environmental conditions to maximize the effectiveness of bacterial strains used for soil remediation. By optimizing these factors, microbial rehabilitation can be a highly

effective, environmentally sustainable solution for crude oil-contaminated sites.

Table (1). The control of spillage sample

Retention Time (R.T.)	Area (%)	Compound Name
9.809	2.50	Octadecanesulphonyl chloride
10.153	3.40	Hexadecane, 2,6,11,15-tetramethyl
10.42	3.53	Dodecane, 2,6,10-trimethyl-
12.506	4.43	1-Octadecanesulphonyl chloride
13.506	2.73	1-Decanol, 2-octyl-
14.222	2.74	1-Dodecanol, 2-octyl-
14.353	3.45	Heptadecane, 2,6,10,15-tetramethyl
14.638	2.46	3-Eicosene, (E)-
14.771	1.78	2-Methyltetracosane
14.968	1.48	1-Chloroeicosane
15.463	1.20	1-Decanol, 2-hexyl-
15.965	1.78	Tritetracontane
16.132	1.57	Heptadecyl heptafluorobutyrate
16.549	1.11	Hexacosane
16.650	1.68	Tetrapentacontane, 1,54-dibromo
17.223	2.20	Hentriacontane
17.430	1.57	n-Tetracosanol-1
18.136	5.28	1-Octadecanesulphonyl chloride
18.610	1.17	2-Methyltetracosane
19.528	5.14	1-Octadecanesulphonyl chloride
20.353	1.42	1-Decanol, 2-hexyl-
20.611	1.58	Nonahexacontanoic acid
20.859	1.96	Tritetracontane
21.111	1.16	Sulfurous acid, octadecyl 2-propyl ester
21.433	1.24	Tetrapentacontane, 1,54-dibromo
21.633	1.34	2,6,10,14-Tetramethyl-7-(3-methyl-ent-4-vinylidene) pentadecane
22.244	1.12	Hexacosane
23.167	1.67	Ethanol, 2-(octadecyl oxy)-
23.540	2.59	2-Methyltetracosane
24.335	1.49	Nonahexacontanoic acid
24.821	3.15	Heneicosane, 11-decyl-
25.246	2.13	Nonahexacontanoic acid
26.043	2.27	Eicosane
26.493	1.40	Nonahexacontanoic acid
26.908	1.46	Nonahexacontanoic acid
27.228	2.01	Tetracosane
27.627	1.68	2-Methylhexacosane
28.362	2.59	Tetracosane
29.470	1.97	Tetracosane
30.543	1.65	Tetracosane, 1-bromo-

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