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# Isolation, Screening, and Preliminary Evaluation of Crude Oil-Degrading Bacteria for Environmental Bioremediation

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**Abstract**

Petroleum pollution remains a major environmental concern due to the stubborn nature and harmful effects of hydrocarbons in contaminated ecosystems. In this study, bacteria native to three petroleum-polluted sites were isolated and evaluated for their ability to break down hydrocarbons. Using enrichment culture methods, a total of 15 bacterial strains were cultivated and subsequently tested for their degradative efficiency on mineral salt medium (MSM) enriched with crude oil. Out of these, three isolates (*Bacillus subtilis*, *Pseudomonas putida*, and *Bacillus cereus*) demonstrated considerable hydrocarbon degradation, evident from distinct clear zones formed around the colonies. Biochemical profiling revealed positive results for nitrate reduction and gelatin hydrolysis in some strains, whereas urease activity was not observed. Traditional staining methods and morphological assessments were employed to aid in the identification process. Initial degradation trials provided encouraging insights into the biodegradative capabilities of these isolates. Further research will involve optimizing environmental conditions for maximal breakdown and quantifying hydrocarbon degradation using advanced analytical techniques like GC-MS. Overall, this work highlights the usefulness of naturally occurring bacterial strains in developing eco-friendly approaches for the cleanup of petroleum-contaminated environments.

**Keywords:**

Bioremediation, hydrocarbon-degrading bacteria, petroleum pollution, *Pseudomonas putida*, *Bacillus subtilis*, microbial degradation.

## INTRODUCTION

The widespread release of petroleum hydrocarbons (PHCs) into the environment stemming from industrial activity, accidental spills, and improper disposal has created a pressing global pollution issue. These compounds are chemically stable, hydrophobic, and toxic, making them not only persistent in the environment but also capable of accumulating in living organisms and entering the food chain (Das & Chandran, 2011; Varjani, 2017). Both terrestrial and aquatic ecosystems face severe ecological and health consequences due to PHC contamination. Conventional remediation methods, such as incineration, excavation, chemical treatment, and physical recovery, though effective in certain scenarios, often involve high financial and environmental costs. These methods are energy-intensive and may leave behind harmful

residues or byproducts (Alves et al., 2021; Raghunandan et al., 2023). In contrast, the emphasis is increasingly being placed on environmentally friendly and cost-efficient alternatives like bioremediation (Das & Chandran, 2011; Jaiswal et al., 2023).

**Hydrocarbon-Degrading Bacteria**

At the core of bioremediation is the natural ability of certain microbes to metabolize petroleum-based pollutants. Indigenous bacteria, particularly those that have evolved in hydrocarbon-rich environments, possess specific enzymes that enable them to use hydrocarbons as energy and carbon sources (Kapoor et al., 2023; Subramaniam et al., 2023). Genera such as *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Alcaligenes*, *Rhodococcus*, and *Sphingomonas* are well-documented for their role in hydrocarbon degradation (Goswami et al., 2023; Khan et al., 2023). These microorganisms initiate the degradation process through the action of oxygenase enzymes, which add oxygen to hydrocarbon molecules, increasing their solubility and making them more accessible for further enzymatic breakdown. The final products of this metabolism typically include less harmful substances such as carbon dioxide, water, and fatty acids (Alves et al., 2021; Li et al., 2023).

**Microbial Bioremediation: An Eco-Friendly Strategy**

Microbial bioremediation represents a naturally occurring, yet highly adaptable, method for managing PHC pollution. This process can be enhanced through strategies like biostimulation—adding nutrients to boost indigenous microbial activity—or bioaugmentation, which involves introducing specialized strains into the contaminated site (Sivaram et al., 2023; Chakraborty et al., 2023). These approaches can be fine-tuned to match the environmental conditions of a specific site, enhancing degradation rates without the collateral impact of chemical or mechanical interventions. Unlike traditional remediation technologies, microbial remediation is less disruptive, generates minimal waste, and is often feasible directly at the contaminated site, making it a preferred method from both ecological and economic perspectives (Das & Chandran, 2011; Khan et al., 2023).

**Mechanisms of Microbial Degradation**

The degradation of hydrocarbons by bacteria may proceed under aerobic or anaerobic conditions. Aerobic degradation, which is more efficient and widely studied, relies on the availability of oxygen and the activity of enzymes like monooxygenases and dioxygenases, which catalyze the initial oxidation of hydrocarbons (Li et al., 2023). This pathway often leads

to complete mineralization, transforming harmful compounds into carbon dioxide and water. In oxygen-poor environments—such as subsurface soils or aquatic sediments—anaerobic degradation becomes crucial. Under these conditions, microbes utilize alternative electron acceptors like nitrate, sulfate, or ferric iron (Subramaniam et al., 2023). Remarkably, many hydrocarbon-degrading strains can adjust their metabolism to function in both aerobic and anaerobic conditions, maintaining degradation activity even when oxygen is scarce (Kapoor et al., 2023; Goswami et al., 2023).

**Role of Biosurfactants in Biodegradation**

An important aspect of microbial hydrocarbon degradation is the production of biosurfactants—surface-active molecules synthesized by bacteria. These compounds enhance the availability of hydrophobic hydrocarbons by emulsifying them into more accessible forms. In doing so, biosurfactants improve microbial uptake and degradation efficiency (Jaiswal et al., 2023). Species such as *Pseudomonas aeruginosa* and *Bacillus subtilis* are known to secrete potent biosurfactants like rhamnolipids and surfactin. These not only increase the solubility of hydrocarbons but also aid in bacterial motility and the formation of biofilms, which support microbial colonization of oil-contaminated environments (Sivaram et al., 2023; Chakraborty et al., 2023).

**Relevance of Indigenous Microbial Communities**

Working with bacteria native to contaminated sites has practical advantages. These microbes are already acclimated to local environmental conditions and may possess inherent resistance to the toxic effects of hydrocarbons (Khan et al., 2023). Additionally, their interactions with other microbial species in the environment can enhance overall degradation through cooperative metabolism (Alves et al., 2021). Harnessing such naturally adapted microbial communities minimizes the ecological risks associated with introducing foreign strains and increases the effectiveness of bioremediation strategies. Understanding their genetic diversity, enzyme systems, and adaptability is essential to developing site-specific, sustainable cleanup technologies (Goswami et al., 2023).

## MATERIALS AND METHODS

### Sample Collection

Soil and water samples were collected from three petroleum-contaminated sites (designated Site A, B, and C). Each site presented unique physicochemical properties to maximize microbial diversity (Mukherjee et al., 2017; Khan et al., 2023).

### Enrichment and Isolation

Serial dilutions were prepared and spread onto nutrient agar and MSM supplemented with 1% crude oil. Enrichment cultures were incubated to promote hydrocarbon-utilizing bacteria (Das & Chandran, 2011). A total of 15 morphologically distinct colonies were isolated (5 per site).

a) **Enrichment Culture**: Samples were incubated in mineral salt medium (MSM) supplemented with 1% crude oil to enrich hydrocarbon-degrading bacteria (Rahman et al., 2002).

b) **Serial Dilution and Plating**: Enriched cultures underwent serial dilution and were plated on nutrient agar and MSM agar containing crude oil as the sole carbon source.

c) **Incubation**: Plates were incubated at 30°C for 5–7 days, and distinct colonies were selected for further analysis (Kapoor et al., 2023).

### Preliminary Screening

Isolates were screened on crude oil-supplemented MSM agar for hydrocarbon degradation. Clear zones around colonies indicated degradation potential (Jaiswal et al., 2023).

### Morphological and Biochemical Characterization

a) **Gram Staining**: Determined the Gram reaction of isolates.

b) **Colony Morphology**: Assessed color, shape, size, elevation, and edge.

c) **Biochemical Tests**: Included oxidase, catalase, citrate utilization, urease activity, nitrate reduction, and gelatin hydrolysis tests (Holt et al., 1994; Cappuccino & Sherman, 2014). Selected isolates underwent Gram staining and colony morphology assessments.

### Determination of Optimal Degradation Conditions

To determine ideal conditions for degradation, bacterial isolates were tested under varying temperature (20°C–45°C), pH (5–9), salinity (0–8% NaCl), hydrocarbon type (crude oil, diesel, kerosene, engine oil), and concentration (0.5–5% v/v) (Alves et al., 2021; Li et al., 2023). These conditions collectively enabled the screening of optimal environmental parameters to enhance the bioremediation potential of selected bacterial strains.

**Table 1. Optimization of Environmental Parameters for Growth and Hydrocarbon Degradation by Bacterial Isolates**

| | | | | | |
|---|---|---|---|---|---|
| **Temperature**: | 20°C, | 30°C, | 37°C, | 45°C | |
| **Hydrocarbon Types**: | Crude | oil, | diesel, | kerosene, | |
| **Hydrocarbon Concentrations**: (v/v) | 0.5%, | 1%, | 2%, | 5% | |
| **pH**: | 5.0, | 6.5, | 7.0, | 8.0, | 9.0 |
| **Salinity**: NaCl | 0%, | 2%, | 4%, | 6%, | 8% |

### Quantitative Hydrocarbon Degradation

**Gravimetric Analysis**: Residual hydrocarbons were extracted using hexane and weighed post-evaporation (Barathi & Vasudevan, 2001).

## RESULTS AND DISCUSSION

### Isolation and Screening

A total of 15 morphologically distinct bacterial isolates were obtained, with five isolates from each site.. Among these, 3 were identified as *Bacillus subtilis*, *Pseudomonas putida*, and *Bacillus cereus* based on Gram staining and colony morphology. Preliminary screening revealed that four isolates exhibited significant hydrocarbon degradation, indicated by clear zones on MSM agar plates.

### Biochemical Characterization

Three potent hydrocarbon-degrading isolates were identified:

**Morphological and Biochemical Characterization**

1. **Isolate A**: *Bacillus subtilis*
    - Gram-positive
    - Creamy white, circular colonies
    - Positive for nitrate reduction and gelatin hydrolysis; negative for urease activity
2. **Isolate B**: *Pseudomonas putida*
    - Gram-negative
    - Smooth, round, green colonies
    - Positive for nitrate reduction; negative for gelatin hydrolysis and urease activity
3. **Isolate C**: *Bacillus cereus*
    - Gram-positive
    - Creamy white, circular colonies
    - Positive for gelatin hydrolysis; negative for nitrate reduction and urease activity

**Table 2: Biochemical Characterization of Selected Hydrocarbon-Degrading Bacterial Isolates**

| Isolate | Urease | Nitrate Reduction | Gelatin Hydrolysis |
|---|---|---|---|
| *B. subtilis* | Negative | Positive | Positive |
| *P. putida* | Negative | Positive | Negative |
| *B. cereus* | Negative | Negative | Positive |

This table summarizes the results of key biochemical tests—urease activity, nitrate reduction, and gelatin hydrolysis conducted on three hydrocarbon-degrading bacterial isolates (*Bacillus subtilis*, *Pseudomonas putida*, and *Bacillus cereus*). The positive outcomes for nitrate

reduction and gelatin hydrolysis in certain strains indicate the presence of enzymatic functions associated with hydrocarbon metabolism and biodegradation potential.

**Enrichment Observations**

Good enrichment cultures showed turbidity and oil depletion compared to sterile controls, validating microbial growth and hydrocarbon utilization.

**Optimization Parameters**

Preliminary studies have begun to assess optimal temperature, pH, and hydrocarbon concentration for degradation. Future experiments will include detailed GC-MS analysis to quantify degradation rates and metabolite formation.

**Optimization of Growth Conditions**

Optimal degradation activity for all bacterial isolates was observed at 30°C, indicating this as the most favorable temperature for hydrocarbon breakdown. The pH conditions also played a crucial role, with maximum degradation recorded at a neutral pH of 7.0. Regarding salinity, the isolates exhibited optimal growth and activity at a 2% NaCl concentration, suggesting moderate salt tolerance. Among the different hydrocarbon types tested, crude oil supported the highest degradation efficiency across all strains, followed by diesel, kerosene, and engine oil, respectively. When evaluating the impact of hydrocarbon concentration, the best degradation occurred at 1% (v/v) crude oil. However, increasing the concentration beyond this point resulted in reduced degradation efficiency, likely due to the toxic effects of excess hydrocarbons on microbial activity. The isolated strains exhibited robust hydrocarbon-degrading capabilities under various environmental conditions. The presence of specific enzymatic activities, such as nitrate reductase and gelatinase, suggests their potential role in the degradation pathways. The optimization studies highlight the importance of environmental parameters in enhancing biodegradation efficiency. These findings align with previous studies reporting the efficacy of *Pseudomonas* and *Bacillus* species in hydrocarbon degradation.

**Quantitative Analysis**

**Gravimetric Analysis of Hydrocarbon Degradation :** Gravimetric analysis was used to determine the residual hydrocarbon content post-incubation. The degradation efficiency was calculated as a percentage of the initial crude oil weight lost after 14 days. The results are shown in Table 1.

**Table 3. Gravimetric Analysis of Hydrocarbon Degradation after 14 Days**

| Isolate Code | Site | Initial Oil (g) | Residual Oil (g) | % Degradation |
|---|---|---|---|---|
| A1 | Site A | 1.000 | 0.520 | 48.0 |
| A3 | Site A | 1.000 | 0.455 | 54.5 |
| B3 | Site B | 1.000 | 0.150 | 85.0 |
| B5 | Site B | 1.000 | 0.240 | 76.0 |
| C2 | Site C | 1.000 | 0.320 | 68.0 |

*Note: Each experiment was conducted in triplicate. Values represent the mean of three replicates.*

These results confirm that the isolates from Site B, particularly **B3**, possess strong hydrocarbon-degrading ability, possibly due to site-specific adaptation or microbial consortia synergy.

## CONCLUSION

This study successfully isolated and characterized indigenous hydrocarbon-degrading bacteria from petroleum-contaminated environments. *Pseudomonas putida* and *Bacillus* species demonstrated efficient degradation, suggesting potential applications in bioremediation. Further work involving optimization and metabolite profiling will solidify their role in large-scale environmental remediation strategies. This study successfully isolated and characterized hydrocarbon-degrading bacteria from petroleum-contaminated sites. The identified strains, *Bacillus subtilis*, *Pseudomonas putida*, and *Bacillus cereus*, demonstrated significant potential in degrading various hydrocarbons under optimized conditions. The findings underscore the feasibility of employing indigenous microbial populations for bioremediation of hydrocarbon-contaminated environments. Future research should focus on scaling up these findings and exploring the genetic and enzymatic mechanisms underlying hydrocarbon degradation to enhance bioremediation strategies further**.**

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