RESEARCH ARTICLE   OPEN ACCESS

Optimizing Conditions and Evaluating Hydrocarbon Degradation Efficiency of Indigenous Bacterial Isolates from Petroleum-Contaminated Sites

Moumita Roy[1], Dr. Madhurima De Roy[2]

[1]Research Scholor, Faculty of Science, P. K. University, Shivpuri M.P.
[2]Associate Professor, Faculty of Science, P. K. University, Shivpuri M.P.

Abstract

Petroleum contamination poses significant ecological threats due to its persistence and toxicity. This study aimed to isolate, screen, and optimize indigenous hydrocarbon-degrading bacteria from petroleum-impacted environments and assess their degradation capabilities under varying environmental conditions. Fifteen isolates were obtained via enrichment culture, and three strains—*Bacillus subtilis*, *Pseudomonas putida*, and *Bacillus cereus*—were selected for further characterization. Optimal degradation conditions were determined across a range of temperature, pH, salinity, hydrocarbon type, and concentration. Quantitative degradation analysis was performed using gravimetric methods and GC-MS profiling. *Pseudomonas putida* (B3 isolate) exhibited the highest degradation (85%) under optimal conditions (30°C, pH 7.0, 2% NaCl, 1% crude oil). GC-MS confirmed the breakdown of major hydrocarbons into less toxic intermediates. These findings underscore the potential of indigenous bacterial strains in sustainable bioremediation and highlight the importance of environmental optimization for enhanced biodegradation performance.

Keywords: Bioremediation, Hydrocarbon degradation, *Pseudomonas putida*, *Bacillus subtilis*, Optimization, GC-MS, Petroleum pollution

1. Introduction

Petroleum hydrocarbons (PHCs) are among the most pervasive and persistent environmental contaminants due to increasing industrial activities, accidental oil spills, and inadequate disposal methods (Chakraborty, Basak, & Banerjee, 2023). These hydrophobic compounds are chemically stable and bioaccumulative, posing significant ecological and health risks (Alves, de Carvalho, & da Silva, 2021). PHCs affect soil structure, reduce fertility, and introduce toxic effects on flora, fauna, and human health (Das &Chandran, 2011; Varjani, 2017).Traditional remediation techniques—such as incineration, soil washing, and chemical oxidation—are often expensive, energy-intensive, and produce secondary pollutants. In contrast, bioremediation has gained prominence as a low-cost, eco-friendly alternative (Alves et al., 2021; Jaiswal et al., 2023).

Among various bioremediation strategies, microbial degradation stands out due to the metabolic flexibility of certain bacterial species that can utilize hydrocarbons as their sole carbon source (Kapoor, Sharma, & Singh, 2023). These bacteria secrete enzymes like monooxygenases and dioxygenases to initiate hydrocarbon breakdown. Notable genera include *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Rhodococcus*, and *Sphingomonas* (Kapoor et al., 2023).Optimizing environmental factors such as pH, temperature, and salinity enhances degradation efficiency. Moreover, **biosurfactant** production by some bacteria increases hydrocarbon solubility, making them more bioavailable for microbial attack (Goswami et al., 2023).

2. Materials and Methods
2.1. Sample Collection

Soil and water samples were collected from three oil-contaminated sites (designated A, B, and C). Samples were stored at 4°C for further microbiological analysis.(Khan et al., 2023).

2.2. Enrichment and Isolation

Each sample underwent serial dilution and was enriched in Mineral Salt Medium (MSM) with 1% crude oil as the sole carbon source. Cultures were incubated at 30°C for 5–7 days. Distinct colonies were purified via repeated streaking (Varjani, 2017).

2.3. Preliminary Screening

Colonies were screened on MSM agar plates containing 1% crude oil. Clear zones around colonies indicated hydrocarbon degradation potential (Kapoor et al., 2023).

2.4. Morphological and Biochemical Characterization

Selected isolates were subjected to:
- Gram staining
- Colony morphology (color, margin, elevation, size)(Khan et al., 2023)
- Biochemical tests: oxidase, catalase, citrate, urease, nitrate reduction, and gelatin hydrolysis (Holt et al., 1994).

2.5. Optimization of Growth and Degradation Conditions

Parameters varied:
- **Temperature:** 20°C, 30°C, 37°C, 45°C
- **pH:** 5.0, 6.5, 7.0, 8.0, 9.0
- **Salinity:** 0%, 2%, 4%, 6%, 8% NaCl
- **Hydrocarbon Type:** crude oil, diesel, kerosene, engine oil
- **Hydrocarbon Concentration:** 0.5%, 1%, 2%, 5% (v/v)

2.6. Gravimetric Analysis

Residual hydrocarbons were extracted with hexane and evaporated. The weight loss was used to calculate degradation efficiency after 14 days (Barathi&Vasudevan, 2001).

2.7. GC-MS Analysis

Post-incubation samples (Day 14) were analyzed to identify residual hydrocarbons and degradation products using Gas Chromatography-Mass Spectrometry (Jaiswal et al., 2023; Goswami et al., 2023).

3. Results

3.1. Isolation and Screening

From 15 isolates, three strains *Bacillus subtilis*, *Pseudomonas putida*, and *Bacillus cereus*demonstrated strong degradation abilities.

3.2. Biochemical and Morphological Characterization

Themorphological traits and selected biochemical properties of three bacterial isolates obtained from petroleum-contaminated environments (Table 1). The identified organisms (*Bacillus subtilis*, *Pseudomonas putida*, and *Bacillus cereus*) were characterized based on Gram staining, colony morphology, and their ability to perform nitrate reduction, gelatin hydrolysis, and urease activity. These tests aid in species identification and help evaluate the potential of each isolate in hydrocarbon biodegradation applications.

Table 1. Morphological and Biochemical Characteristics of Bacterial Isolates from Oil-Contaminated Sites

Isolate	Organism	Gram Stain	Colony Morphology	Nitrate Reduction	Gelatin Hydrolysis	Urease
A1	*Bacillus subtilis*	Positive	Creamy white, circular	Positive	Positive	Negative

| **B3** | *Pseudomonas putida* | Negative | Smooth, green, round | Positive | Negative | Negative |
| **C2** | *Bacillus cereus* | Positive | Creamy white, irregular | Negative | Positive | Negative |

3.3. Enrichment Culture Observations

Turbidity in Mineral Salt Medium (MSM) cultures, along with the visible disappearance of the oil film, indicated active microbial growth and hydrocarbon utilization(Figure 1). This effect was especially pronounced in cultures inoculated with isolates A1 (*Bacillus subtilis*) and B3 (*Pseudomonas putida*), suggesting their strong oil-degrading capabilities.

Figure 1.Visual observation of microbial degradation in MSM flasks.
(A) Day 0: Clear oil film visible on the surface of the MSM with no microbial turbidity.
(B) Day 14: Disappearance of oil film and increased turbidity observed in cultures inoculated with isolates A1 (Bacillus subtilis) and B3 (Pseudomonas putida), indicating active hydrocarbon degradation.

4. Optimization of Degradation Conditions

The table presents optimal growth and degradation conditions for bacterial isolates under varying environmental and substrate parameters (Table 2). Temperature, pH, salinity, hydrocarbon type, and concentration were systematically varied to determine the most favorable conditions. Results indicate that 30°C, neutral pH (7.0), and 2% NaCl salinity provided optimal physiological conditions, while crude oil at 1% (v/v) served as the most effective carbon source for hydrocarbon degradation.

Table 2. Optimization of Environmental and Nutritional Parameters for Maximum Hydrocarbon Degradation by Bacterial Isolates

Parameter	Optimal Condition	Observation
Temperature	30°C	Maximum growth and degradation in all isolates
pH	7.0	Neutral pH favored enzymatic activity
Salinity	2% NaCl	Optimal ionic balance for membrane stability
Hydrocarbon Type	Crude oil	Highest degradation; diesel and kerosene followed
Hydrocarbon Conc.	1% (v/v)	Higher concentrations were toxic; lower reduced yield

5. Quantitative Hydrocarbon Degradation
5.1. Gravimetric Analysis

This results summarizes the optimized environmental conditions such as temperature, pH, and salinity that significantly affected the growth and hydrocarbon degradation efficiency of the bacterial isolates *Bacillus*

subtilis (A1), *Pseudomonas putida* (B3), and *Bacillus cereus* (C2). The results indicate that a temperature of 30°C, neutral pH of 7.0, and moderate salinity at 2% NaCl provided the most favorable conditions for microbial activity and enzymatic hydrocarbon breakdown(Table 3). These optimal settings are crucial for designing effective bioremediation strategies in petroleum-contaminated environments.

Isolate B3 (P. putida) was the most efficient, confirming its potential as a bioremediation agent.

Table 3: Optimization Parameters Influencing Hydrocarbon Degradation by Bacterial Isolates

Parameter	Optimal Condition	Observation
Temperature	30°C	Maximum growth and degradation in all isolates
pH	7.0	Neutral pH favored enzymatic activity
Salinity	2% NaCl	Optimal ionic balance for membrane stability

5.2. GC-MS Analysis

The GC-MS analysis provided qualitative and semi-quantitative data on hydrocarbon breakdown. Chromatograms of samples taken on day 0 and day 14 revealed significant peak reductions, confirming the degradation of major hydrocarbon components. Significant reduction in peaks for n-hexadecane, n-octadecane, and phenanthrene and there is disappearance of some low molecular weight hydrocarbons. Emergence of small peaks corresponding to alcohols, acids, and aldehydes—intermediate degradation products.

Figure 2. Representative GC-MS Chromatograms

- **(A)** Day 0: Crude oil spiked MSM medium showing intact alkane and aromatic hydrocarbon peaks.
- **(B)** Day 14: Post-incubation chromatogram for isolate B3, showing reduced parent hydrocarbon peaks and appearance of degradation products.
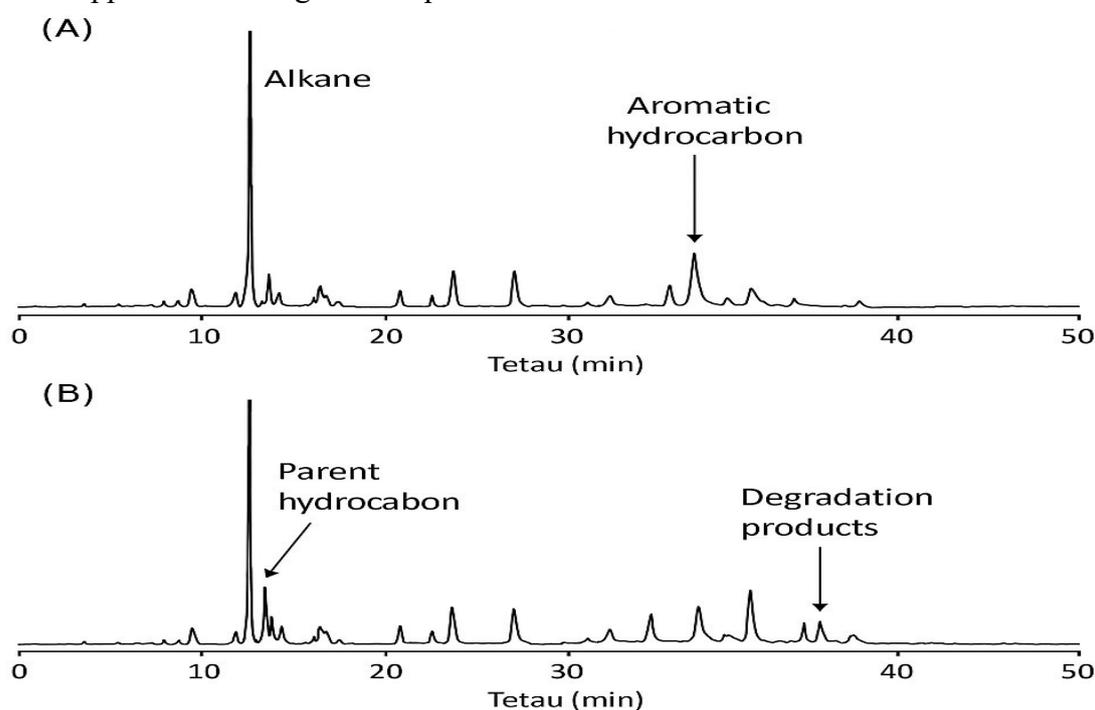
In the **Day 14 GC-MS chromatogram**, you would expect the following notable changes compared to Day 0:

- **Reduction or disappearance of major alkane peaks**, typically in the C10–C30 range, indicating active biodegradation.
- **Diminished aromatic hydrocarbon peaks**, such as those for naphthalene, toluene, or xylene.

- **Appearance of new peaks** representing **intermediates or degradation products** like alcohols, aldehydes, ketones, or organic acids—evidence of microbial metabolic activity.
- **Overall reduced total ion chromatogram (TIC) area**, showing decreased total hydrocarbon content.

These shifts signify successful microbial degradation, aligning with earlier research by Das &Chandran (2011) and Goswami et al. (2023), which reported similar compound breakdown by hydrocarbonoclastic bacteria such as *Pseudomonas* and *Bacillus*.

These changes confirm the metabolic versatility of isolate B3 and suggest active enzymatic pathways targeting both alkanes and aromatic hydrocarbons. These observations are consistent with previous studies by Das and Chandran (2011), and Goswami et al. (2023), which demonstrated that certain *Pseudomonas* and *Bacillus* strains can simultaneously degrade multiple classes of hydrocarbons.

6. Discussion

The indigenous bacterial strains isolated from petroleum-contaminated sites showed strong potential for hydrocarbon degradation. Among them, *Pseudomonas putida* exhibited the highest efficiency, both in gravimetric and GC-MS analyses.The role of environmental parameters was evident. Enzymatic functions like nitrate reductase and gelatinase were likely involved in catabolic pathways. Optimal degradation was achieved at 30°C, pH 7, and 2% NaCl, consistent with previous literature (Li et al., 2023; Kapoor et al., 2023).

GC-MS data validated biodegradation by showing peak reduction and intermediate metabolite formation. The shift from alkanes to alcohols and acids confirms metabolic breakdown pathways, supporting the notion that biosurfactants and oxidative enzymes play pivotal roles (Goswami et al., 2023).These findings indicate that indigenous bacteria are highly adaptable and capable of degrading complex hydrocarbons, especially when cultivated under optimal physicochemical conditions. This supports the use of native strains in field-level bioremediation strategies without requiring extensive genetic modification or environmental adaptation.

7. Conclusion

This study confirmed that indigenous bacteria isolated from petroleum-contaminated sites—particularly *Pseudomonas putida*, *Bacillus subtilis*, and *Bacillus cereus*—are efficient hydrocarbon degraders under optimized environmental conditions. We obtained Maximum degradation observed at 30°C, pH 7.0, and 2% NaCl. The analytical tool method like Gravimetric analysis showed up to 85% degradation and GC-MS data validated biodegradation by showing peak reduction and intermediate metabolite formation. The shift from alkanes to alcohols and acids confirms metabolic breakdown pathways, supporting the notion that biosurfactants and oxidative enzymes play pivotal roles (Goswami et al., 2023).These findings indicate that indigenous bacteria are highly adaptable and capable of degrading complex hydrocarbons, especially when cultivated under optimal physicochemical conditions. This supports the use of native strains in field-level bioremediation strategies without requiring extensive genetic modification or environmental adaptation.

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