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Molecular Detection of Hydrocarbon Degrading Bacteria from Petrochemical Polluted Soil

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Petroleum hydrocarbon pollution remains a major environmental concern, particularly in petroleum-impacted soils in Nigeria. Indigenous bacteria capable of utilizing crude oil offer a sustainable approach to bioremediation. This study investigated hydrocarbon-associated bacteria isolated from petrochemical-polluted soil using classical microbiological screening, 16S rRNA gene analysis and GC-MS based degradation assessment. Enrichment and biochemical testing suggested the presence of hydrocarbon-utilizing organisms. PCR amplification of the 16S rRNA gene produced ~1500bp fragments, and BLAST analysis showed 96% and 97% similarity to *Pseudomonas* and *Klebsiella* species respectively, indicating affiliation to these genera rather than confirmed species identity. GC-MS analysis of crude oil and before and after 5 weeks of incubation with the isolates showed a marked reduction in hydrocarbon peaks, demonstrating measurable biodegradation activity. It shows that *Pseudomonas* affiliated organism exhibited the highest degradation efficiency, achieving approximately 90% reduction in total petroleum hydrocarbon (TPHs). This isolate showed strong degradation of short and medium chain alkanes (C₁₁ - C₂₀), including complete removal of fractions such as C₁₆, C₁₇, C₂₀, and C₂₄. However long chain hydrocarbons above C₂₄ were less affected. The mixed consortium achieved 82% degradation suggesting moderate synergistic interactions, whereas the *Klebsiella* affiliated isolate showed the lowest activity with 47% TPH reduction. Overall, the findings demonstrate the superior biodegradation performance of the *Pseudomonas* species and highlight the importance of microbial selection when developing effective bioremediation strategies. The study underscores the relevance of combining molecular profiling with functional GC-MS validation to identify promising hydrocarbon-degrading bacteria for remediation of petrochemical polluted soils.

Keywords: Crude oil biodegradation, *Pseudomonas sp.*, *Klebsiella sp.*, Microbial consortium and GC-MS analysis.

1. Introduction

Traditional microbiological identification techniques including biochemical tests and morphology are often insufficient because many environmental organisms are difficult or impossible to culture. Studies show that more than 90% of microbes in the environment cannot be cultured using standard laboratory methods and traditional culture-based identification methods often fail to capture environmental microbial diversity and overlapping phenotypic traits that can result to misidentification (Das & Chandran, 2011). Molecular techniques particularly 16S rRNA gene sequencing overcome these limitations by enabling accurate taxonomic identification.

Hydrocarbon pollution, predominantly from crude oil and its derivatives, significantly impacts ecosystem by reducing soil fertility, altering microbial diversity,

and introducing toxic compounds into food chains (Abioye, 2011).

The contamination of terrestrial and aquatic ecosystems with petroleum hydrocarbons is a critical environmental issue due to their toxicity, persistence, and potential to bioaccumulate in living organisms. These pollutants originate from diverse anthropogenic sources such as oil spills, industrial discharges, and improper disposal of petroleum products (Ebakota *et al.*, 2017). Hydrocarbons, particularly alkanes and polycyclic aromatic hydrocarbons (PAHs) are chemically stable and resistant to degradation, thus posing long-term risks to both ecological and human health (Onwurah *et al.*, 2007).

Bioremediation, utilizing native or introduced microorganisms to detoxify environments, is gaining attraction due to its cost-effectiveness and

environmental compatibility (Perelo, 2010). Microorganisms play a pivotal role in the natural attenuation and engineered bioremediation of hydrocarbon-contaminated sites. Among the microbial community, certain bacterial taxa have evolved metabolic pathways that allow them to utilize hydrocarbons as a sole source of carbon and energy. These bacteria possess specific catabolic enzymes that initiate the oxidation of hydrocarbon chains, transforming them into less toxic intermediates through aerobic or anaerobic processes (Chailan *et al.*, 2004).

Pseudomonas species are well-documented hydrocarbon degraders, known for their ability to catabolize a broad range of aromatic and aliphatic hydrocarbons via oxidative pathways (Olowomofe *et al.*, 2018). They are gram-negative bacteria commonly found in soil and aquatic habitats. Additionally, they can form biofilms and biosurfactants, enhancing hydrocarbon emulsification and uptake (Souza *et al.*, 2014).

Klebsiella species though traditionally associated with clinical environments, has recently garnered attention for its environmental adaptability and metabolic plasticity. Strains of *K. pneumoniae* have demonstrated the ability to degrade hydrocarbons under aerobic and facultative anaerobic conditions, making them suitable candidates for bioremediation in both oxygen-rich and oxygen-poor environments (Rajkumari *et al.*, 2018).

Molecular techniques, particularly polymerase chain reaction (PCR), have transformed the study of microbial ecology by enabling the rapid and specific detection of microbial species in complex environments (Muthukumar *et al.*, 2022).

This study evaluates the hydrocarbon-degrading potential of *Pseudomonas* sp., *Klebsiella* sp. and a mixed bacterial consortium isolated from petrochemical polluted soil. We combined classical microbiology, molecular detection (16S rRNA sequencing), and quantitative GC-MS analysis to assess biodegradation efficiency and provide insights into the applicability of these isolates for bioremediation of crude oil contaminated environments.

2. Materials and Methods

2.1 Sample Collection

Soil samples were collected from a mechanical workshop in G. Allen area located in Sokoto South L.G.A at co-ordinates 13.0512° N and 5.2292250° E. Nine samples were collected from the subsoil of 10-15m depth using a sterile spatula. They were designated as ZB1, ZB2, ZB3, ZB4, ZB5, ZB6, ZB7, ZB8 and ZB9 which were 3 meters apart. The soil samples were put into sterile containers and transported to microbiology laboratory in Sokoto State University.

2.2 Physicochemical Analysis of Soil Sample

The physicochemical properties of the collected soil samples were determined using standard procedures. A composite soil sample was prepared by thoroughly combining subsamples collected from the same site and used for the analysis. Parameters assessed included pH, temperature, moisture content, electrical conductivity (EC), total petroleum hydrocarbons (TPH), and selected heavy metals which are Pb, Cd, Cr, Zn, Cu and Ni (Chikere *et al.*, 2015).

2.3 Serial Dilution

Sterile test tubes were label as 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} for each sample. Water (9ml) was added to each test tube, 1g of soil sample was added the first test tube and mixed all together. From 10^{-1} , 1ml was transferred to the second test tube containing 9ml of water and mixed. This was repeated to achieve serial dilution up to 10^{-4} (Nandhini and Josephine, 2013).

2.4 Preparation of Bushnell-Hass Medium

Bushnell-Hass medium containing KH_2PO_4 (1.0 g), NH_4NO_3 (1.0 g), K_2HPO_4 (1.0 g), MgSO_4 (0.2 g), CaCl_2 (0.02), FeCl_3 (0.05) and Agar (15.0g) was dissolved in 1litre of distilled water. The pH was adjusted to 7.0 ± 0.2 using 1M NaOH. The medium was sterilized by autoclaving at 121°C for 15 minutes. Soil samples were enriched in Bushnell-Haas medium supplemented with 1% (v/v) crude oil as the sole carbon source. The enrichment cultures were incubated at 30°C for 7 days on an orbital shaker at 150 rpm. After incubation, serial dilutions of the enrichment cultures were plated on the same medium solidified with agar for the isolation of hydrocarbon-degrading bacteria (Fatiha *et al.*, 2017).

2.5 Subculture

A single colony from previously cultured plate was transferred to a freshly prepared nutrient agar plate prepared according to manufacturer's instruction and incubated at 37°C for 24hrs (Jain *et al.*, 2020).

2.6 Morphological Characterization and Gram Staining

Morphological characterization was used to observe the colour, shape, size, texture and elevation of the colonies and recorded. Gram staining was carried out and the slides were then viewed under microscope for gram reaction and shape (Jawetz *et al.*, 2019).

2.7 Biochemical Tests

Biochemical tests were done such as catalase, urease, indole, simmons citrate, methyl red (MR), voges-proskauer (VP) and triple sugar iron (TSI)

test. These tests were conducted according to method described by (Forbes *et al.*, 2007).

2.8 DNA extraction

DNA was extracted from bacteria using Qiagen DNA extraction kit following standard protocol (Fatiha *et al.*, 2017).

2.9 Polymerase Chain Reaction (PCR)

The 16SrRNA sequence was amplified using the universal primer forward 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1492r (5'-TACGGCTACCTTACGACTT-3'). The PCR mix comprises of master mix 6.25µl, forward primer 0.5µl, reverse primer 0.5µl and nuclease free water 25µl. The DNA template (2µl) was added into 10.5µl of cocktail, mixed thoroughly and arranged in Bio rad thermal cycler (T100 model). The PCR profile used was initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 51°C for 1 minute, extension at 72°C for 1:30 seconds and final extension at 72°C for 5 minutes (Hanoon *et al.*, 2020).

2.10 Gel Electrophoresis

The resulting amplicons (PCR products) were checked by 1% agarose gel electrophoresis. Steps include preparation of agarous gel, gel casting and loading of samples with the loading die and set to run at 100V for 35 minutes (Magdeldin, 2012).

2.11 Purification of PCR Products

The amplicons were purified further using 2M sodium acetate washing techniques before sequencing. PCR product (10µl) was added to 2M sodium acetate (1µl) followed by absolute ethanol (20µl). This was kept for 1 hour, Spin at 13000rpm for 10 minutes. Then washed with 70% ethanol and air dried. There after it was re-suspended in 5µl sterile distilled water and kept at 4°C before sequencing (Kanakaraj, 2011).

2.12 DNA Sequencing Analysis

The amplified 16SrRNA gene products were sequenced using the Sanger sequencing method at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa.

2.13 Phylogenetic Analysis

The nucleotide sequences obtained were compared with other nucleotide sequences using BLASTn tools of the National Centre for Biotechnology Information (NCBI). The neighbour joining method was used to infer the evolutionary history. The software MEGA 11.0 was used to align all sequences and evolutionary analysis was conducted (Tamura *et al.*, 2021).

2.14 Microcosm set up

For this study, 30 g of soil was dispensed into sterile Petri dishes and amended with 1% (v/w) crude oil to simulate petroleum contamination. Each experimental microcosm was inoculated with either *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, or a mixed culture of both which were done in triplicate. A negative control consisted of sterile soil amended with crude oil but without bacterial inoculation, while crude oil was used as a positive control. All microcosms were incubated at 30 °C. After five weeks of incubation (T5), soil samples were collected from each microcosm and subjected to Gas Chromatography–Mass Spectrometry (GC-MS) analysis (Sharma *et al.*, 2022).

2.15 Sample Preparation and Gas Chromatography Mass Spectrometry (GC-MS) Analysis

At the end of the biodegradation experiment (week 5), soil samples from each microcosm were collected for residual hydrocarbon analysis using Gas Chromatography-Mass Spectrometry (GC-MS) which was carried out at Multi-User Research Laboratory, Ahmadu Bello University Zaria, Nigeria.

2.16 Calculation of Percentage Degradation

Percentage degradation of hydrocarbons was calculated by comparing the total peak area of the sample after 5 weeks to the initial peak area from Day 0 (Al-Hawash *et al.*, 2021; Ogidi *et al.*, 2023).

$$\text{Percentage Degradation} = \left(\frac{A_0 - A_t}{A_0} \right) \times 100$$

Where:

A_0 = Initial total peak area of hydrocarbons at Day 0

A_t = Residual total peak area of hydrocarbons at week 5

3. Results and Discussion

3.1 Physicochemical Analysis of Soil

This study provides comprehensive evidence that petrochemical-polluted soil from the study site supports both severe contamination and the presence of naturally adapted hydrocarbon-degrading bacteria. The physicochemical characteristics of the petrochemical polluted soils shown in Table 1 strongly influenced microbial survival and biodegradation potential. The near-neutral pH (6.91 ± 0.04), mesophilic temperature (28.6 ± 0.23 °C), and moderate moisture content ($3.21 \pm 0.08\%$) collectively created conditions favourable for enzymatic activity and microbial metabolism. This is significant because extreme pH, temperature, or moisture variations commonly reported in polluted environments often inhibit microbial activity and delay remediation.

The mean pH value of the petroleum-polluted soil reported in this study as shown in table 1 (6.91 ± 0.04) was within the range reported by Vidali (2010), who recorded a pH of 6.85 ± 0.06 in crude-oil-contaminated soils undergoing natural attenuation.

The mean temperature (28.6 ± 0.23 °C) obtained in this study within the range reported by Ismaila (2008), who recorded a temperature of $10^{\circ}\text{C} - 40^{\circ}\text{C}$ in hydrocarbon-polluted soils supporting biodegradation by mesophilic bacteria.

The mean moisture content (3.21 ± 0.08 %) in this study is in line with the findings of Ojukwu and Eboatu (2022) who reported that total moisture content of petroleum contaminated soils fell in the ranges 0.50-40.56%. However, it was within the range described by Less and Senior (1995), who observed successful biodegradation in soils with 3.0-3.5 % moisture.

The total petroleum hydrocarbon (TPH) concentration of 313.88 ± 1.32 mg/kg observed in this study confirms substantial petroleum contamination of the soil. However, this concentration did not completely suppress microbial life, as demonstrated by the successful isolation of active hydrocarbon-degrading bacteria. This suggests that the microbial community at the site has undergone long-term adaptation to petroleum stress, allowing persistence and metabolic specialization.

The mean electrical conductivity (EC) value (322.9 ± 0.64 $\mu\text{S}/\text{cm}$), organic carbon content (8.80 ± 0.05 %) in this study correlates to the findings of Ojukwu and Eboatu (2022) who reported that total organic carbon ranges from 7.29 -15.09 % in petrochemical polluted soil.

The elevated organic carbon ($8.80 \pm 0.12\%$), nitrogen ($1.47 \pm 0.20\%$), and phosphorus (3.15 ± 0.07 mg/kg) contents further indicate that the polluted soil was nutrient-enriched. This nutrient availability likely enhanced microbial growth and enzyme production, thereby supporting natural biodegradation processes without the need for external nutrient supplementation.

The mean nitrogen content was found to be (1.45 ± 0.11 %) and phosphorus (3.14 ± 0.04 mg/kg) which are higher than the requirement for arable soil. The overabundance of nitrogen decreases plant diversity in terrestrial ecosystems, and the combination of excess nitrogen and phosphorus in water bodies leads to algal blooms, eutrophication and biodiversity loss, as well as shifts in disease vectors such as malaria (Roberto 2016).

The concentration of total petroleum hydrocarbons (TPH) in this study (313.88 ± 1.32 mg/kg) was within the range reported by Ojukwu and Eboatu (2022), who recorded 107.5305-626.4060 mg/kg with that of

the non-petroleum contaminated soil being the least. This high TPH content supports the necessity for microbial remediation.

Table 1: Physicochemical Analysis of Petrochemical Polluted Soil

Parameters	Mean \pm SD
pH	6.91 ± 0.04
Temperature (°C)	28.6 ± 0.23
Moisture content	3.21 ± 0.08
C (%)	8.80 ± 0.12
N (%)	1.47 ± 0.20
P (mg/kg)	3.15 ± 0.07
TPH	313.88 ± 1.32
EC (mg/kg)	322.9 ± 0.64

Table 1 shows the physicochemical parameters of soil samples obtained from petrochemical polluted site. The values are mean \pm standard deviation as (SD) of triplicate determinations (n=3).

3.2 Heavy Metals Concentration of Soil Sample

Heavy metal analysis revealed high concentrations of chromium, copper, nickel, and lead, confirming co-contamination of the soil with petroleum hydrocarbons and toxic metals. However, the successful isolation of both *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* from this stressed environment demonstrates that these organisms possess strong tolerance mechanisms to both hydrocarbon and metal toxicity.

The mean concentration of lead (Pb) (0.84 ± 0.02 mg/kg), Cadmium (Cd) (0.12 ± 0.01 mg/kg), Iron (Fe) content (4.56 ± 0.07 mg/kg), Zinc (Zn) concentration (1.38 ± 0.03 mg/kg) and Copper (Cu) concentration (0.46 ± 0.02 mg/kg) as shown in Table 2 are all higher than permissible limits recommended by WHO (2001) and therefore confirms soil pollution.

Table 2: Heavy Metals Analysis of Petrochemical Polluted soil

Heavy Metal	Mean \pm SD
Cr (mg/kg)	136.73 ± 0.30
Ni (mg/kg)	15.15 ± 0.40
Cu (mg/kg)	88.31 ± 0.68
Cd (mg/kg)	0.12 ± 0.01
Pb (mg/kg)	13.71 ± 0.33
Zn (mg/kg)	1.38 ± 0.03
Fe (mg/kg)	4.56 ± 0.07

Table 2 shows heavy metals concentrations of the soil sample. The values are mean \pm standard deviation as (SD) of triplicate determinations (n=3).

3.3 Cultural, Morphological and Biochemical Characteristics of Isolates

In this study, *Pseudomonas* sp. produced small (2 mm), milky, mucoid colonies with a pulvinate elevation and circular margins as shown in table 3. The mucoid nature of *Pseudomonas* sp. observed in this study is in line with the findings of Ciofu *et al.* (2010) who reported that mucoid colonies result from alginate overproduction, enhancing biofilm stability and environmental persistence. *Klebsiella* sp. formed larger (6 mm), milky, watery colonies with an umbonate elevation and irregular margins as shown in Table 3.

Table 3: Cultural, Morphological and Microscopic Characteristics of Isolates

Isolate Code	Cultural Characteristics	Morphological Characteristics	Microscopic Characteristics
ZB1	Small, milky, raised	Mucoid, puctiform	Gram-negative rods
ZB5	Medium, milky, umbonate	Watery, irregular	Gram-negative rods

Table 3 shows the cultural and morphological features of the bacterial strains

The biochemical characterization of the isolates in this study revealed profiles consistent with the typical features of *Klebsiella* sp. as described in recent literature. Cultural, morphological, and biochemical characterization revealed that isolate ZB1 exhibited typical features of *Pseudomonas* sp. including Gram-negative rod shape, oxidase positivity, citrate utilization, and non-fermentative metabolism. Similarly, isolate ZB5 displayed classical characteristics of *Klebsiella* sp. including lactose fermentation, citrate utilization, urease activity, Voges-Proskauer positivity, and non-motility as shown in Table 4. The biochemical results obtained in this study are therefore in close agreement with those reported in previous works. Emerson *et al.* (2008) emphasized that while phenotypic identification remains useful, confirmatory testing using molecular methods such as PCR or MALDI-TOF MS targeting species-specific genes provides greater accuracy. These characteristics provide reliable phenotypic differentiation when molecular methods are not readily available.

Table 4: Biochemical Characteristics of the Isolates

Characteristics of isolate	ZB1	ZB5
Indole	-	-
Citrate	+	+
Urease	+	+
Methyl red	-	-
Voges Proskauer	-	+
Lactose	-	+
Glucose	-	+
Sucrose	-	+
Coagulase	-	+
H ₂ S Production	-	-
Gas Production	-	+
Bacteria Identified	sp. <i>Pseudomonas</i> sp.	<i>Klebsiella</i> sp.

Table 4 shows the biochemical characteristics of the isolates which were determined using standard microbiological methods to aid identification and classification.

3.4 Gel Electrophoresis

Gel electrophoresis of PCR-amplified products revealed distinct bands at approximately 1500 base pairs (bp) for both ZB1 and ZB5 isolates as shown in figure 1, consistent with the expected size for the 16S rRNA gene fragment amplified using universal bacterial primers. The presence of single, sharp bands in the corresponding lanes confirms the successful amplification of high-quality target DNA and supports the molecular identification of the isolates. The 16S rRNA gene is highly conserved across bacterial species but contains hyper variable regions that allow for species-level discrimination. Amplification of this gene is a standard approach for bacterial detection and taxonomy due to its universality and phylogenetic relevance. The observed ~1500bp band size is consistent with previous reports, where universal 16S primers (e.g., 27F and 1492R or other region-specific variants) are used to amplify regions ranging from 900 to 1500bp depending on primer pair and protocol (Lane, 1991; Janda & Abott, 2007).

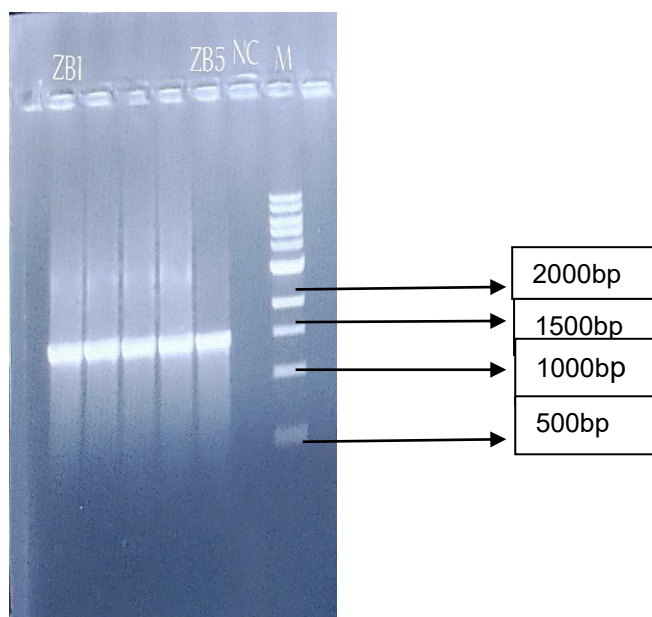


Figure 1: Gel image of isolated bacterial strains

Figure 1 shows the agarose gel electrophoresis image showing the amplified 16S rRNA gene products of the bacterial strains obtained from petrochemical polluted soil. Lane M represents 1 kb plus DNA ladder. Lane 1 corresponds to *Pseudomonas* sp. isolate, while Lane 5 represents *Klebsiella* sp. isolate.

3.5 16S rRNA Gene Sequencing

The sequencing analysis of the 16S rRNA gene fragment for the isolates that is ZB1 presumptively identified as *Pseudomonas* sp. revealed 96% similarity with *Pseudomonas aeruginosa* reference strains in the NCBI GenBank database as shown in table 5. While this level of sequence identity supports the classification of the isolate within the *Pseudomonas* genus, it falls below the commonly accepted species-level identification threshold of ≥ 98.7 – 99.0% , suggesting potential divergence or the presence of a closely related, but distinct, *Pseudomonas* species (Stackebrandt & Gobel, 1994; Kim *et al.*, 2014).

Recent studies have reported similar findings where 96% 16S rRNA identity in several *Pseudomonas*-like isolates from soil and wastewater, which were confirmed as novel strains through multi-locus sequence typing (MLST). Such results emphasize the limitations of single-gene analysis and the importance of combining phenotypic, biochemical, and genomic data for comprehensive identification (Parte *et al.*, 2020)

In light of these observations, while the biochemical and pigment production profiles support the identification of the isolate as *P. aeruginosa*, the 96% sequence identity suggests further molecular characterization is needed to confirm its taxonomy. This may indicate a novel environmental strain or

variant of *P. aeruginosa* with potential ecological or clinical significance.

The 16S rRNA gene sequence analysis of the isolate that is ZB5 presumptively identified as *Klebsiella* sp. revealed 97% similarity to reference sequences of *K. pneumoniae* in the NCBI GenBank database. While this level of identity confirms affiliation within the *Klebsiella* genus, it falls below the widely accepted threshold of $\geq 98.7\%$ required for confident species-level identification using 16S rRNA gene sequencing alone (Stackebrandt and Gobel, 1994; Kim *et al.*, 2014).

The observed sequence identity suggests that the isolate may represent either a divergent strain of *K. pneumoniae* or a closely related species, such as *Klebsiella variicola*, *Klebsiella quasipneumoniae*, or *Klebsiella africana*, which share substantial genetic overlap and have been frequently misidentified by conventional 16S rRNA sequencing. These species are part of the *Klebsiella pneumoniae* complex (KpSC), and 16S rRNA gene sequences often lack sufficient resolution to distinguish among them.

This finding also raises the possibility that the isolate may represent an environmental or emerging variant of clinical relevance. Environmental reservoirs of *Klebsiella* species have been increasingly reported in recent years, with several novel strains displaying reduced similarity to classical reference strains yet showing pathogenic potential (Rodrigues *et al.*, 2019).

Phylogenetic analysis based on the 16S rRNA gene sequence was performed to assess the evolutionary relationship of the isolate with other members of the *pseudomonas* genus. The resulting neighbour-joining tree placed the isolate in a well-supported clade containing reference strains of *Pseudomonas aeruginosa*, with high bootstrap values indicating strong phylogenetic relatedness. However, the isolate showed minor divergence from typical strains, suggesting either a unique environmental variant or strain-level diversity within the species.

Phylogenetic analysis further supported this observation by placing both isolates within the *P. aeruginosa* and *K. pneumoniae* species clusters, but with slight evolutionary separation from classical reference strains. This confirms that while the organisms belong to these species groups, they likely represent distinct environmental lineages shaped by ecological stress and geographic isolation. This explains why their 16S rRNA similarity values fall below conventional species-level thresholds and underscores the limitation of relying solely on single-gene sequencing for environmental isolates.

Overall, the phylogenetic tree confirms the isolate's classification as *Pseudomonas aeruginosa* and emphasizes the importance of combining sequence

3.6 Biodegradation of crude oil using indigenous organisms isolated from petrochemical polluted soil

The GC-MS analysis revealed a significant overall reduction approximately 90% in the abundance of hydrocarbon compounds in soil treated with *Pseudomonas aeruginosa*, compared to the untreated control (negative control, C⁻). This underscores the strong hydrocarbon-degrading capacity of *P. aeruginosa*, aligning with previous findings that identified the species as one of the most effective crude oil degrading bacteria in polluted environments (Ghosal *et al.*, 2016; Olatunya *et al.*, 2022).

In the chromatographic profile, it was observed that heavier hydrocarbon chains such as C₁₆ to C₂₀ were broken down into lighter compounds, such as C₁₁. These transformations occurred at similar retention times, suggesting complete or partial biodegradation. Specific compounds identified include heptadecane, 2,6,10,14-tetramethylpentadecane to 2,6,11,15-tetramethylpentadecane. Additionally, hydrocarbons ranging from C₁₅ to C₁₆ and C₂₂ to C₂₃ were also partially degraded.

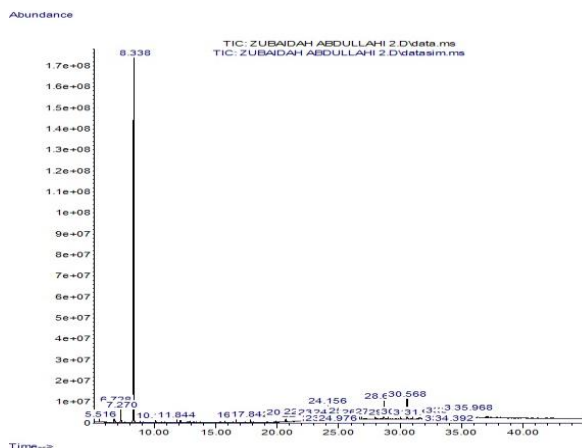


Figure 4: GC-MS chromatogram of soil sample treated with *pseudomonas aeruginosa* at week 5

Several hydrocarbon compounds were completely removed, indicating full degradation. These compounds, including C₁₆, C₁₇, C₂₀, and C₂₄, were present in the negative control with retention times of 20.628 min, 19.879 min, 20.790 min, and 20.189 min respectively, but were absent in the treated sample (T5).

The breakdown of heavier hydrocarbon fractions (C₁₆–C₂₀) into lighter chains, such as C₁₁, and the complete removal of certain compounds (e.g., C₁₆, C₁₇, C₂₀, and C₂₄) indicate robust catabolic activity and potential mineralization pathways. The absence of these peaks in treated samples suggests enzymatic mechanisms that cleave long-chain alkanes and convert them into simpler, less toxic molecules (Das & Chandran, 2011; Rahman *et al.*,

2020). The presence and transformation of compounds such as heptadecane and 2,6,10,14-tetramethylpentadecane further reinforce this degradation pattern.

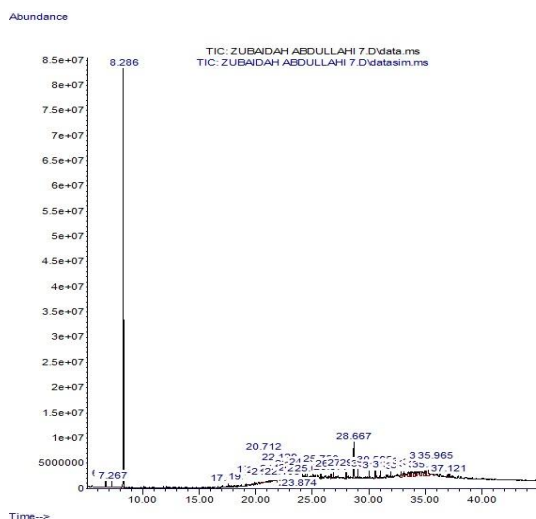
The observed degradation is likely facilitated by the production of extracellular enzymes such as oxygenase and hydrolases, which initiate alkane hydroxylation—a critical step in hydrocarbon degradation (Prince *et al.*, 2018; Yu *et al.*, 2021). The degradation of mid-range hydrocarbons (C₁₅–C₂₃) also supports the assertion that *P. aeruginosa* possesses versatile metabolic pathways capable of handling a broad range of hydrocarbon substrates.

Interestingly, while *P. aeruginosa* exhibited pronounced degradation of short- and mid-chain hydrocarbons, it was less effective against longer-chain compounds (>C₂₄), which remained relatively intact even after five weeks of treatment. This aligns with studies suggesting that long-chain hydrocarbons are more recalcitrant due to their lower solubility, higher hydrophobicity, and resistance to enzymatic attack (Varjani, 2017; Bello-Akinosho *et al.*, 2021). The limited bioavailability of such compounds may also reduce microbial access and uptake (Gogoi *et al.*, 2003).

These findings imply that while *P. aeruginosa* is a potent degrader of a wide range of hydrocarbons, its efficacy may be enhanced through synergistic interactions in microbial consortia, especially for high molecular weight hydrocarbons. Mixed cultures have been shown to possess broader enzymatic profiles and may overcome individual strain limitations through cooperative metabolism (Shin *et al.*, 2019; El Amrani *et al.*, 2022).

Overall, the ability of *P. aeruginosa* to degrade significant portions of crude oil components, including the complete elimination of several toxic hydrocarbons, highlights its suitability for application in bioremediation strategies, particularly in petrochemical-contaminated environments.

The GC-MS analysis of crude oil-contaminated soil treated with *Klebsiella pneumoniae* revealed appreciable hydrocarbon degradation efficiency, with approximately 47% reduction in total hydrocarbon abundance compared to the untreated control. This degradation rate highlights the organism's ability to utilize petroleum hydrocarbons as carbon and energy sources, confirming its potential role in bioremediation (Shao *et al.*, 2022; Suleiman *et al.*, 2021).



compounds, including C₁₃, C₁₄, C₁₇, C₁₈ and C₂₃ were present in the negative control with retention times of 21.616min, 21.194min, 20.628min, 22.003min, and 37.373 min respectively, but were absent in the treated sample (T5).

The GC-MS analysis of crude oil-contaminated soil treated with a co-culture of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* revealed a significant reduction in hydrocarbon content (approximately 82%), demonstrating their combined potential for hydrocarbon biodegradation. However, this degradation percentage was lower than the 90% observed with *P. aeruginosa* alone, suggesting that the co-culture did not exhibit synergistic enhancement but rather a slightly reduced overall activity.

This unexpected result may be due to competitive interactions between the two strains. In microbial consortia, while synergism is often expected, competition for nutrients, space, or oxygen can negatively influence degradation efficiency (El Amrani et al., 2022; Rahman et al., 2020). *P. aeruginosa* is known to dominate in hydrocarbon-rich environments due to its robust metabolism, biosurfactant production, and rhamnolipid-mediated hydrocarbon emulsification, which enhance substrate availability (Prince et al., 2018; Yu et al., 2021). The presence of *K. pneumoniae* may have interfered with these mechanisms by altering metabolic priorities or quorum sensing signals, potentially reducing the overall degradation rate.

Despite the lower degradation percentage compared to *P. aeruginosa* alone, the co-culture still outperformed *K. pneumoniae* used in isolation (47%). This indicates that *P. aeruginosa* likely remained the primary contributor to degradation within the consortium, while *K. pneumoniae* may have played a supporting role by degrading specific intermediate compounds or stabilizing the environment (Onuoha et al., 2020; Shao et al., 2022).

The chromatographic data further confirmed that the co-culture effectively degraded hydrocarbons ranging from C₁₂ to C₃₀, with complete disappearance of several compounds (C₁₃, C₁₄, C₁₇, C₁₈, C₂₃). These findings support the notion that co-cultures can widen the spectrum of degradable hydrocarbons, even if the overall degradation efficiency is not always maximized (Varjani et al., 2019; Bello-Akinosho et al., 2021). However, compounds above C₃₀ remained largely undegraded, consistent with prior studies that report the recalcitrance of high-molecular-weight hydrocarbons (Das & Chandran, 2011).

In summary, while the co-culture demonstrated strong hydrocarbon-degrading capability, it did not surpass the degradation potential of *P. aeruginosa* used alone. This result emphasizes that microbial

interactions in co-cultures are complex and not always synergistic, and that strain compatibility, metabolic balance, and environmental factors must be optimized to achieve maximal bioremediation outcome.

4. Conclusion

This study successfully identified *Pseudomonas* sp. and *Klebsiella* sp. as hydrocarbon-degrading bacterial strains isolated from petrochemical-contaminated soil using a combination of biochemical tests and molecular techniques. PCR amplification of the 16S rRNA gene followed by sequencing and BLAST analysis confirmed the identities of the isolates with 96% and 97% similarity, respectively. The ability of these organisms to degrade petroleum hydrocarbons highlights their potential application in environmental bioremediation strategies. The integration of classical and molecular approaches proved effective for accurate detection and classification of hydrocarbon-degrading bacteria, offering a reliable framework for future studies aimed at mitigating oil pollution through microbial intervention. Overall, the results of this study demonstrate that the polluted soil environment supports naturally adapted, genetically distinct hydrocarbon-degrading bacteria capable of surviving under combined petroleum and heavy-metal stress. This adaptability explains why the findings differ from previous studies that reported higher molecular similarity to clinical strains or required artificial nutrient amendment for effective biodegradation. The present study therefore highlights the importance of investigating indigenous microbial communities in real contaminated environments rather than relying solely on laboratory-optimized systems.

Authors' Contributions

ZB conducted the experiment, MB supervised the experiment, MG supervised preparation of manuscript, HI and SY assisted in conducting the experiment.

Conflict of Interest

The authors declare no conflict of interest.

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