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# Biodegradation of Total Petroleum Hydrocarbons and EPA-16 PAHs in Crude Oil by *Pseudomonas aeruginosa* KUD2

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## ABSTRACT

The complex mixture of hydrocarbons known as Speciated EPA-16 Polyaromatic Hydrocarbons (PAHs), which are frequently found in crude oil and are known to be extremely hazardous, mutagenic, carcinogenic, teratogenic, and immunotoxicogenic to different life forms, is included in the category of Extractable Total Petroleum Hydrocarbons (ETPHs), which also includes aliphatic compounds. Considering the environmental and public health hazards linked to ETPHs and PAHs, there is a compelling need to explore indigenous bacteria with the capability to efficiently degrade these contaminants. The present study investigated the biodegradation potential of bacterial isolates from oil-polluted soil, focusing on Extractable Total Petroleum Hydrocarbons (ETPHs) - Aliphatic and Speciated EPA-16 Poly Aromatic Hydrocarbons (PAHs) in crude oil samples. In this study, four predominant bacterial isolates, designated as KUD1, KUD2, KUD3, and KUD4, were isolated from oil-contaminated soil obtained from Okrika in Rivers State, Nigeria. These isolates exhibited positive outcomes in both the drop collapse and oil displacement tests, affirming their biosurfactant-producing, surface tension-reducing, and oil-displacing capabilities. Among them, KUD2 exhibited the highest emulsification index, highlighting its proficiency in forming stable oil-in-water emulsions. Consequently, KUD2, owing to its remarkable growth on agar plates containing 10% crude oil, was chosen for further investigation. The highly efficient isolate, KUD2 identified as Pseudomonas aeruginosa using 16S rDNA sequencing. The isolate exhibited enzymatic capabilities in the degradation of hydrocarbons, with catechol-1,2-dioxygenase enzymes detected with varying activity levels over time. Conversely, catechol-2,3-dioxygenase (C23O) remained consistently inactive throughout the experiment, suggesting alternative metabolic pathways or a lack of involvement in the studied biodegradation process. During the incubation period, the pH fluctuations correlated with the highest enzyme production, suggesting metabolic adaptations. Furthermore, P. aeruginosa KUD2 demonstrated significant increases in Alkane hydroxylase and Alcohol dehydrogenase secretion, reaching peak production after ten days of incubation signifying their active catalytic roles within the pathways associated with the investigated biodegradation process. Mass chromatograms revealed the presence of aromatic hydrocarbons in control crude oil samples, while KUD2 efficiently degraded ETPHs, reducing their concentration. Likewise, KUD2 demonstrated effective degradation of Speciated EPA-16 PAHs. The concentrations of aliphatic hydrocarbons in crude oil were significantly reduced after degradation, with some compounds being entirely degraded. Overall, this study underscores the potential of P. aeruginosa KUD2 for environmental bioremediation, as it efficiently degrades hydrocarbons and highlights the enzyme dynamics and pH influences in the biodegradation process.

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## INTRODUCTION

Environmental contamination resulting from crude oil and its derivatives has become a pressing global concern (Ureta Suelgaray *et al*., 2022). Various petroleum hydrocarbons are released into the environment by crude oil spills and leaks, including Extractable Total Petroleum Hydrocarbons (ETPHs) containing aliphatic compounds and Speciated EPA-16 Polyaromatic Hydrocarbons (PAHs) (Goveas *et al*., 2022). Due to their toxic, persistent, and bioaccumulative characteristics, these pollutants pose serious risks to both the environment and human health (Onyegeme-Okerenta *et al*., 2022; Priyadarshanee *et al*., 2022). The Environmental Protection Agency (EPA) has classified sixteen PAHs, out of the many that are known, as High Priority Pollutants. Due to their widespread distribution and long-term persistence in the environment, these 16 PAHs pose a risk to human health and other organisms. Several PAHs have been linked to cancer, either directly or indirectly (Hussar *et al*., 2012).

In addressing oil-contaminated environments, various techniques and remediation strategies have been employed, often accompanied by limitations and undesirable side effects. Among these methods, a promising, economical, and environmentally friendly method has emerged called "bioremediation," which uses microorganisms to break down pollutants. to mitigate the adverse effects of oil pollution (Aliko *et al*., 2022). Bioremediation, being a natural and sustainable approach, holds great promise in combating oil pollution, and the choice of appropriate microorganisms is crucial for its success (Patel *et al.*, 2022).

A number of studies have demonstrated that oil-rich environments, including oil spill sites and oil reservoirs, harbor a significant amount of bacteria that can break down hydrocarbons (Yang *et al*., 2015), among the diverse group of microorganisms capable of degrading oil, *P. aeruginosa* has garnered considerable attention due to its remarkable ability to metabolize hydrocarbons. This bacterium is renowned for its metabolic versatility, further highlighting its significance (Norat *et al*., 2022). Numerous studies have provided compelling evidence of *P. aeruginosa* 's proficiency in degrading both crude oil and PAHs ((Ivanova *et al*., 2022; Sharma *et al*., 2022; Baig *et al*., 2022; Liu *et al*., 2022). However, there exists a notable research gap concerning the utilization of Pseudomonas strains for the degradation of Extractable Total Petroleum Hydrocarbons (ETPHs) and Speciated EPA-16 Polyaromatic Hydrocarbons (PAHs).

While the potential of *P. aeruginosa* in biodegrading hydrocarbons is well-documented, there is a need to investigate specific strains capable of efficiently degrading crude oil. Additionally, the environmental concerns arising from the potential toxicity of the 16 PAHs to various life forms, coupled with their widespread presence and persistence in the environment, have underscored the necessity to degrade these PAHs in crude oil-contaminated soil.

This research aims to contribute to the expanding body of knowledge on microbial-based solutions for remediating oil-contaminated environments. Specifically, the study seeks to explore the biodegradation potential of *P. aeruginosa* strain KUD2. This strain represents a potential microbial-based remediation approach to effectively degrade Extractable Total Petroleum Hydrocarbons (ETPHs), including aliphatic compounds and Speciated EPA-16 Polyaromatic Hydrocarbons (PAHs).

## MATERIALS AND METHODS

### *Sample Collection*

Soil samples were collected from four different sites (A, B, C, and D) in a crude oil-polluted area of Okrika in Rivers State, Nigeria. The latitude and longitude coordinates of each sampling site were recorded for accurate geospatial identification. Soil samples were collected at each site using soil augers or soil corers, ensuring minimal surface contamination and capturing variations in soil properties. After collection, the soil samples were immediately placed in clean, airtight polyethylene bags to prevent moisture loss and minimize any potential contamination.

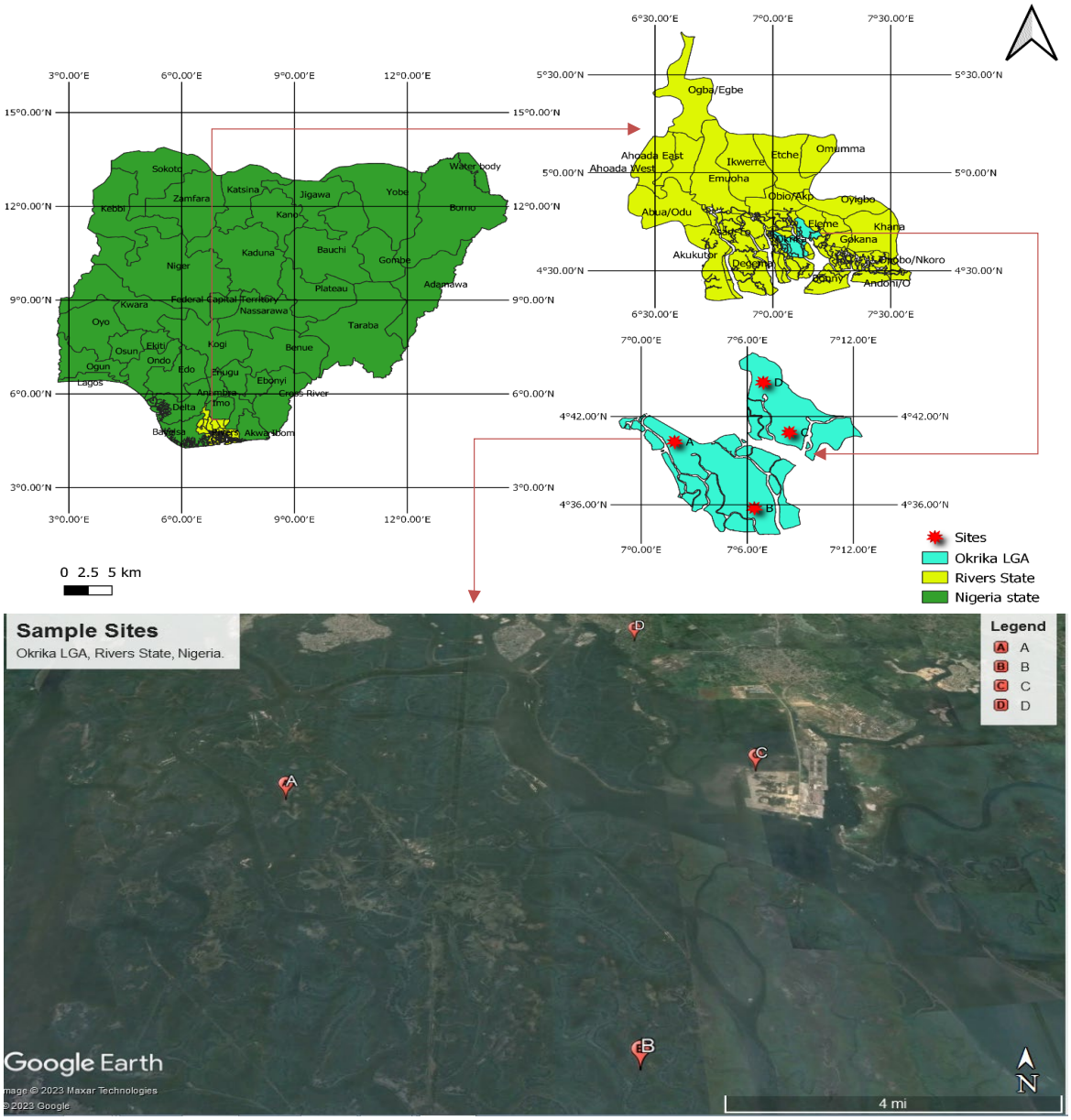


**Fig. 1.** Four Sampling Sites of severely Oil-Polluted soil locations in Okrika, Rivers State

The bags were then sealed tightly and stored in insulated cooler containing ice packs to maintain a low temperature during transportation to the laboratory. Upon arrival at the laboratory, the soil samples were stored at 4°C in a refrigerator until further analysis. The containers were stored under suitable conditions to maintain the soil sample as shown in Fig. 1.

*Isolation and selection of Bacterial strains Capable of Degrading Crude Oil*

The crude oil-degrading potential of the bacterial isolates was assessed using a selective enrichment process as outlined in the studies conducted by Diallo *et al*. (2020), Okoye *et al*. (2020), and Ejaz *et al*. (2021). Briefly, soil samples were serially diluted and cultured in Bushnell-Haas Medium (BHM) supplemented with crude oil as the sole carbon source to obtain isolated colonies. The cultures were incubated at 30°C for 72 hours. Distinct colonies that demonstrated the ability to degrade hydrocarbons and withstand the toxicity of crude oil compounds were

selected, purified, screened, and analyzed (Okoye *et al*., 2020).

*Screening for Biosurfactant Production*

The bacterial isolate was tested for biosurfactant production by inoculating a standardized pure culture (approximately $4.5 \times 10^8$ cells) into 30 mL of nutrient broth and incubating at 30°C on a rotary shaker at 150 rpm for 72 hours. After centrifugation at 3000 rpm for 30 minutes, the cell-free supernatant was collected for further assays.

**Oil Displacement Test:** Following Moussa *et al*. (2021), 10 µL of the supernatant was added to a thin diesel oil layer on a Petri dish. The diameter of the cleared zone indicated surface activity, with larger zones signifying higher activity.

**Drop Collapse Test:** A drop of the supernatant was added to a diesel oil drop on a glass slide. A collapse indicated a positive result (Youssef *et al.,* 2004).

**Hemolytic Assay:** Isolates were streaked on blood agar and incubated for 48 hours at 37°C. Zones of clearance were observed and recorded as α-hemolysis, β-hemolysis, or γ-hemolysis (Carrillo *et al*., 1996).

**Emulsification Stability Test (E24):** The emulsification index was measured by mixing 2 mL of kerosene with 2 mL of the supernatant, vortexing for 2 minutes, and measuring the emulsified layer after 24 hours (Balogun & Fagade, 2010).

*Identification and Characterization of Bacteria*

All the bacterial isolates were identified using 16S rDNA sequencing as outlined by Bhutia *et al*. (2021).Briefly, Quick-DNATM Fungal/Bacteria/Miniprep kit (Zymo Research, Catalogue No. D6005) was used for the extraction of the genomic DNA of the isolates. The primers listed in Table 1 were used to amplify the 16S target region of bacterial DNA using the polymerase chain reaction (PCR). The resulting PCR products were then separated by electrophoresis on a gel to visualize and confirm the presence of the amplified DNA fragments and purified (Zymo Research, ZR-96 DNA sequencing clean-up KitTM, catalog number D4050). Purified DNA fragments were analyzed using the Applied Biosystems' ABI 3500xl (Applied Biosystems, Thermofisher Scientific) Genetic Analyzer, which generated ab1 files containing sequencing data. These ab1 files were used in a BLAST search against the NCBI database to determine the identity of the bacterial isolates. A phylogenetic tree was constructed using software like MEGAX, to visualize the evolutionary relationships between the isolated bacteria and known species based on their genetic similarities.

*Biodegradation of Crude Oil*

The degradation of crude oil was carried out as described by Parthipan *et al*. (2017). A broth culture of *Pseudomonas aeruginosa* KUD2 at the exponential growth phase containing approximately $1.5 \times 10^8$ CFU $ml^{-1}$, estimated using 0.5 McFarland Standard were transferred into 250 mL of Erlenmeyer flask, each containing 100 mL of Bushnell-Haas Medium (BHM) supplemented with 1% (v/v) filter-sterilized crude oil as carbon source with respective other macro and micro nutrients sources. The flasks were incubated at optimum temperature for 7 days at 150 rpm. All experiments were performed in triplicate to ensure the reliability and reproducibility of the results. At everyday intervals during the biodegradation study the following parameters were evaluated: Total bacterial Counts (Fawole and Oso, 2004), To assess

**Table 1.** 16S Primer Sequences

| Name of Primer | Target | Sequence (5'-3') |
|---|---|---|
| 16S-27F | 16SrDNA Sequence | AGAGTTTGATCTGGCTCAG |
| 16S-1492R | 16SrDNA Sequence | CGGTTACCTTGTTACGACTT |

alkane hydroxylase activity, bacterial cells were washed, resuspended in 2 mL of 20 mM Tris–HCl buffer (pH 7.4), disrupted using a probe sonicator, and centrifuged at 8000 rpm for 10 minutes at 4°C. The cell-free supernatant was analyzed for activity by measuring the decrease in absorbance at 340 nm with a UV–Vis spectrophotometer. The reaction mixture included 20 mM Tris–HCl, 0.15% CHAPS buffer, 0.1 mM NADH, 10 µL of crude oil and 50 µL of crude extract, with activity expressed as 1 µmol of NADH oxidized per minute. For alcohol dehydrogenase activity, cells were washed, resuspended in 10 mM potassium phosphate buffer (pH 7.0), sonicated, and centrifuged. The supernatant was used to measure activity at 340 nm, with the reaction mixture containing 1 M Tris–HCl buffer (pH 8.8), 4 mM NAD+, 100 µL ethanol, and 50 µL of crude extract, and activity expressed as 1 µmol of NADH formed per minute. Additionally, the pH reduction in the medium during the degradation of crude oil (50 ppm) was measured using a pH meter (Jauhari *et al*., 2014), Catechol 1, 2 dioxygenase assay and Catechol-2,3-Dioxygenase Activity were determined as described by Tavakoli and Hamzah (2016). Briefly, For the degradation of crude oil, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were determined using spectrophotometric assays. Catechol 1,2-dioxygenase activity was measured by monitoring absorbance at 260 nm, corresponding to the formation of cis,cis-muconic acid. The reaction mixture included 1 mL of *P. aeruginosa* KUD2 cell-free extract, 1 mL of 0.8 mM catechol, 0.8 mL of 50 mM Tris-HCl buffer (pH 8.0), and 0.2 mL of 0.1 mM 2-mercaptoethanol. Catechol 2,3-dioxygenase activity was assessed by measuring absorbance at 375 nm. The reaction mixture contained 1 mL of *P. aeruginosa* KUD2 cell-free extract, 1 mL of 0.3 mM catechol, and 0.8 mL of 50 mM phosphate buffer (pH 7.0). All assays were performed in duplicate. After adding the cell-free extract, the mixtures were incubated at 30°C in a water bath, and aliquots were periodically removed to monitor the reaction progress using a spectrophotometer.

*Analysis of Control Crude Oil and the Degraded Sample [Liquid-Liquid Extraction Method]*

A total of 50 ml of the sample solution was carefully measured and transferred into a separatory funnel. Subsequently, 25 ml of dichloromethane (DCM) was accurately measured and added to the sample solution within the separatory funnel. The mixture was then vigorously swirled for approximately 3 minutes, with periodic venting to release any pressure build-up. Afterward, the funnel was clamped back in place to allow the solution to separate by standing. The organic layer, containing the extracted hydrocarbons, was collected and passed through 2 g of sodium sulfate in a filter paper. The filtered organic layer was then collected into a precleaned and well-labeled beaker.

For a second extraction, an additional 25 ml of DCM was added into the aqueous layer remaining in the separatory funnel. The extraction process was repeated, and the resulting emulsion part of the extract (about 25g) was sonicated using 15ml of DCM to facilitate separation. Next, the sonicated extract was combined with the previously filtered extract, and a third vigorous shaking was performed. The resulting mixture was then filtered again through 2g of sodium sulfate to ensure purity. The obtained extracts were allowed to concentrate to approximately 1.5ml, leading to higher analyte concentration for analysis.

To fractionate the concentrated extract into aliphatic and aromatic fractions, a column consisting of glass wool and activated silica gel was packed and conditioned with DCM. The fractionation was achieved using n-Hexane and DCM, respectively, as solvents. The resulting aliphatic and aromatic extracts were collected in clean vials, ready for subsequent hydrocarbon analysis.

*Preparation of the Crude Oil Extract*

Approximately 2ml of the crude oil sample was carefully measured and transferred into a cleaned beaker. The crude oil was then further transferred into a 25 ml volumetric flask using

dichloromethane (DCM) as the transferring solvent. To obtain an 80 ppm solution, the 25ml volumetric flask was filled up to the mark using DCM. Subsequently, the prepared solution was filtered through sodium sulfate to remove any impurities and transferred into a clean vial. The same dilution and filtration process was repeated using n-Hexane as the solvent to ensure the reliability of the results.

*Gas Chromatography – Mass Spectrometric Analysis*

PAHs analysis was performed using the Agilent Technologies 7890B GC system coupled with the 5977A MSD. The equipment was operated for the determination of Trace level 16 Priority PAHs as specified in Agilent Application Catalog 5991-5213EN, p-532.

*GCMS Operating Conditions*

Gas Chromatography Analysis was performed using an Agilent J&W HP-5ms UI column with dimensions of 30 m x 0.25 mm x 0.25 μm. Helium was used as the carrier gas, flowing constantly through the column.

The oven temperature program was set as follows: starting at 40°C for 1 minute, then ramping up to 320°C at a rate of 15°C per minute and holding at the final temperature for 12 minutes.

For the sample injection, the inlet injection port temperature was maintained at 290°C, and a pulsed splitless injection technique was employed. The Mass Spectrometric Detector (MSD) was operated with the following temperature settings: auxiliary temperature at 280 °C, source temperature at 300°C, and quad temperature at 180°C. The scan range for mass spectral data acquisition was set from m/z 40 to 600 Atomic Mass Unit.

## RESULTS AND DISCUSSION

In this study, four predominant bacterial isolates were selected from the oil-contaminated soil and designated as KUD 1, KUD 2, KUD 3, and KUD 4. All of these isolates exhibited positive outcomes in various assays, demonstrating their unique capabilities ranging from the biosurfactant production and their abilities to grow on various concentration of crude oil All isolates demonstrated positive results in the drop collapse test, indicating their biosurfactant-producing and surface tension-reducing abilities, This is a critical feature for microbes involved in bioremediation processes as it enhances their capacity to interact with hydrophobic substances like oil. Additionally, they showed positive results in the oil displacement test, signifying their capability to displace oil. Notably, KUD2 exhibited the highest emulsification index, highlighting its exceptional ability to form stable oil-in-water emulsions. Emulsification is a crucial step in the breakdown of oil, as it enables microorganisms to access and metabolize hydrocarbons more effectively.

Among these isolates, KUD2 was selected for further investigation due to its remarkable ability to grow on agar plates containing 10% crude oil. This distinctive trait makes KUD2 a promising candidate for bioremediation efforts targeting oil-contaminated environments.

The 16S rDNA sequences of the highly most degraded bacteria KUD4 revealed that the isolate showed 100% homology with the *P. aeruginosa* strain BRPO3, which is already documented in the NCBI database. This confirmed the isolate's identity as a *P. aeruginosa* bacterium. The phylogenetic analysis further illustrated the evolutionary relationship of the *Pseudomonas aeruginosa* isolate with other known bacterial species within the database as shown in Fig. 2.

This study revealed that the isolated *P. aeruginosa* KUD2 synthesized catechol-1, 2-dioxygenase with varying levels of activity over time, peaking at 90 units of time (2.08) and then gradually decreasing. It experiences a sharp decline between 120 and 150 units of time before partially recovering. This could suggest that C12O is sensitive to the time-dependent conditions and may be involved in a specific metabolic pathway or response, while **C23O**

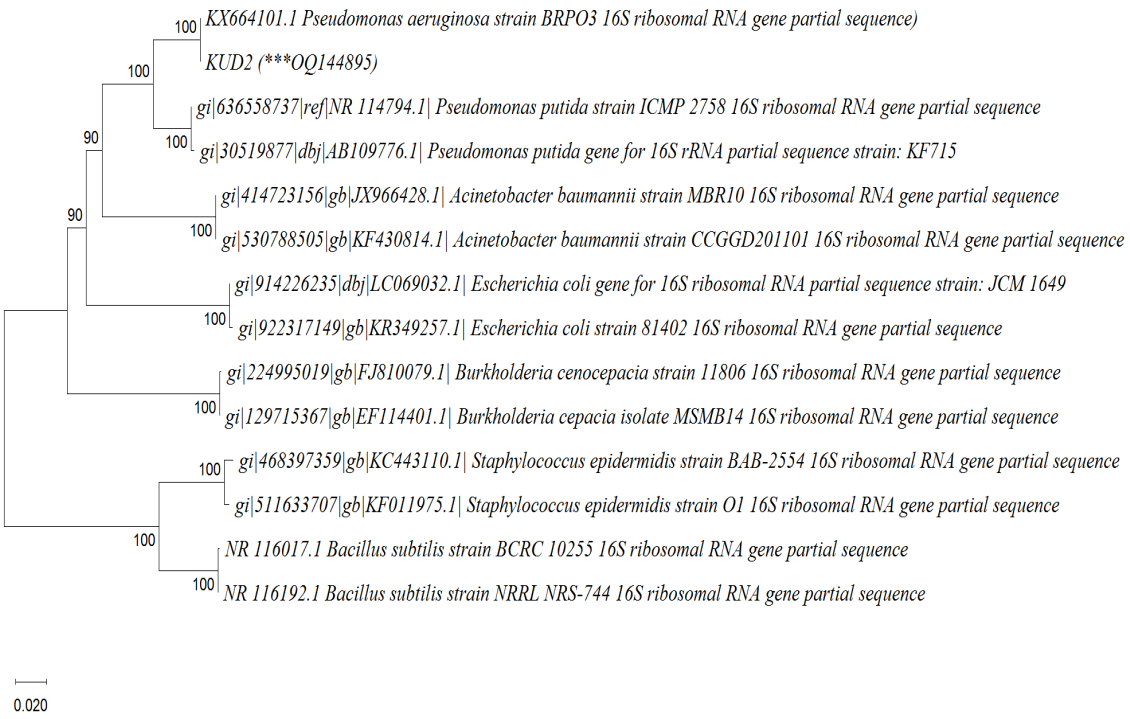


**Fig. 2.** Phylogenetic tree Oil degrading bacterial isolates and related sequences obtained from NCBI.

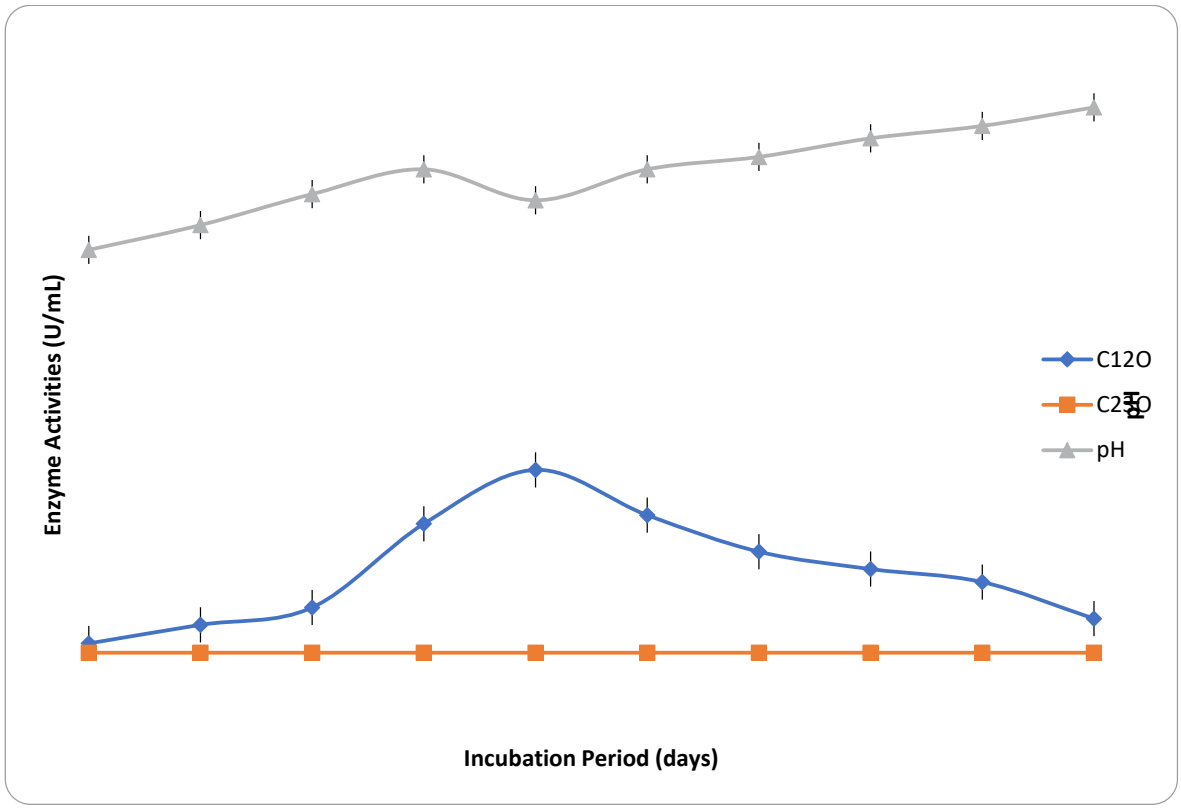


**Fig. 3.** Biosynthesis of Catechol-1,2-Oxygenase and Catechol-2,3-dioxygenase during biodegradation of Bonny light crude oil by *Pseudomonas aeruginosa* strain KUD2

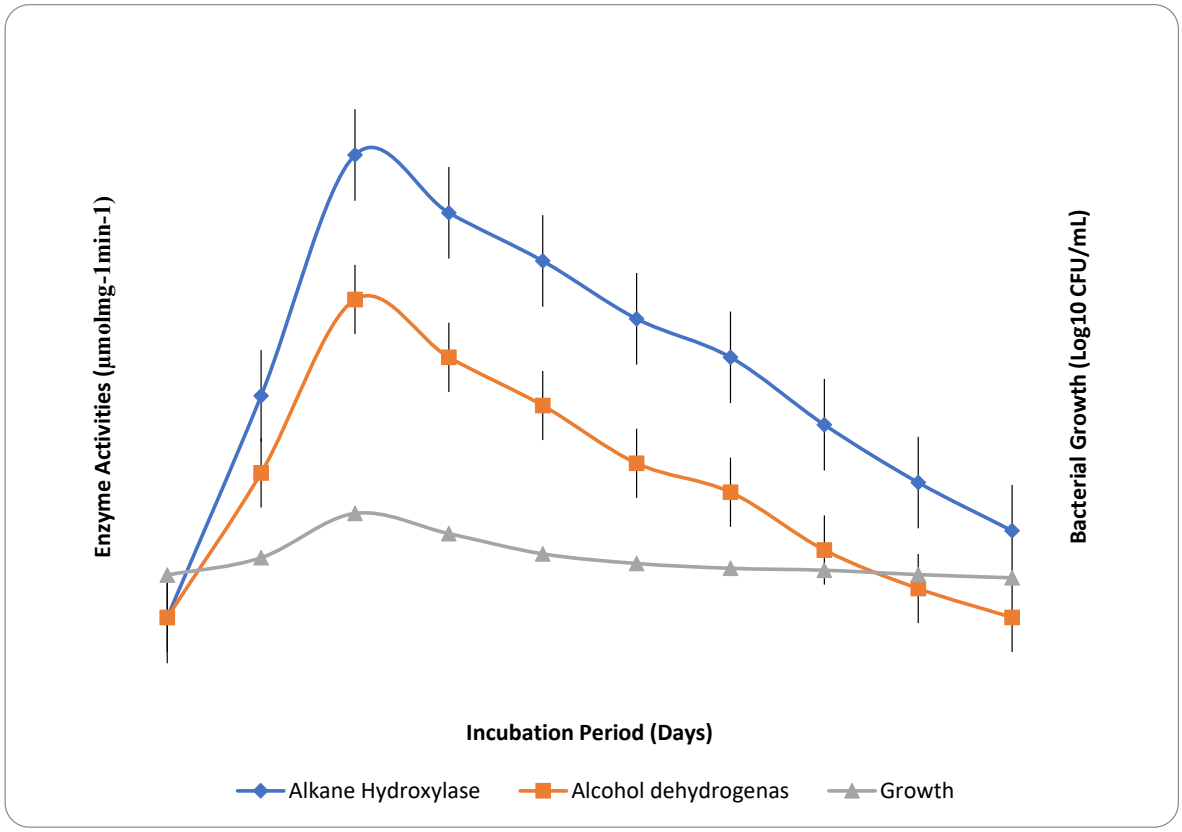


**Fig. 4.** Enzyme Activites of the Intracellular Alkane hydroxylase and Alcohol dehydrogenase during biodegradation of Bonnylight crude oil by *P. aeruginosa* strain KUD2

In contrast to C12O, C23O shows no activity throughout the entire experiment. It remains consistently inactive at all time points, suggesting that this enzyme may not be involved in the biodegradation process being studied or may have used alternative metabolic pathway. The pH values in the experiment appear to fluctuate slightly over time. Interestingly, during the incubation period, the pH of the medium steadily increased and then sharply declined, reaching a peak at pH 7.3, coinciding with the highest production of both enzymes as depict in Fig. 3. These pH changes could potentially be linked to the enzyme activities observed, as enzymes often have optimal pH ranges for their activity.

The study observed a significant increase in the secretion of Alkane hydroxylase and Alcohol dehydrogenase by *P. aeruginosa* strain KUD2 in an experimental flask. The production of Alkane hydroxylase rose from 1.0 µmolmg-1 to reach a peak of 50 µmolmg-1, while Alcohol dehydrogenase increased to 30 µmolmg-1, both after 10 days of incubation. Subsequently, Alkane hydroxylase decreased to 10 µmolmg-1, and Alcohol dehydrogenase was undetectable after the 10th day of incubation. A similar trend was observed in the growth of the isolate, as it steadily increased and then sharply declined after 3 days of incubation, coinciding with the peak production of both enzymes. After the peak production, the levels of Alkane hydroxylase and Alcohol dehydrogenase decreased steadily over time as shown in Fig. 5. As the biodegradation process progresses, the availability of easily degradable compounds decreases, leading to a decline in enzyme activities and microbial growth.

Fig. 5 displays the mass chromatogram of the extractable total petroleum hydrocarbons (ETPH) in the control crude oil samples. This chromatogram serves as a baseline representation of the various hydrocarbons present in the untreated crude oil. The peaks in the chromatogram correspond to different hydrocarbon compounds, with their heights indicating their relative

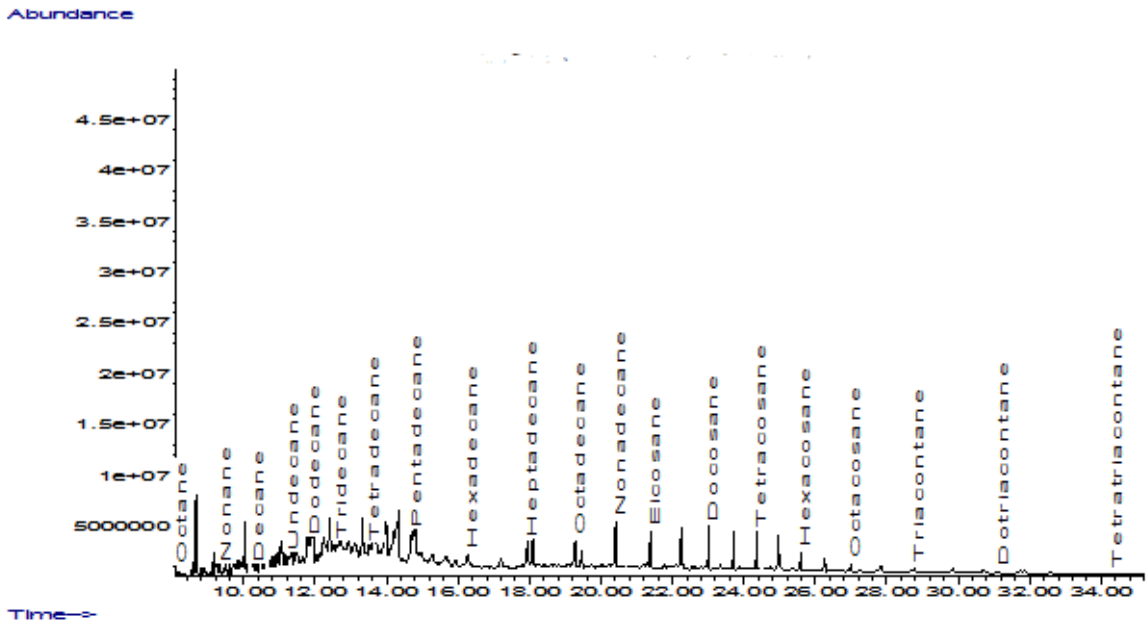


**Fig. 5.** Mass Chromatogram of the Extractable total Petroleum Hydrocarbon in the control crude oil samples


**Fig. 6.** Mass chromatogram of the degraded Extractable total Petroleum Hydrocarbon in the crude oil samples by *P. aeruginosa* strain KUD2

abundance. The ETPHs include a range of aliphatic hydrocarbons from n-C8 to n-C36. This figure provides a detailed overview of the hydrocarbon profile prior to any biodegradation treatment, showcasing the complexity and diversity of hydrocarbons present in the crude oil.

The biodegradation capabilities of *Pseudomonas aeruginosa* strain KUD2 were investigated concerning the Extractable Total Petroleum Hydrocarbons (ETPHs) comprising aliphatic hydrocarbons ranging from n-C8 to n-C36.

Fig. 6 illustrates the mass chromatogram of the degraded extractable total petroleum hydrocarbons (ETPH) in the crude oil samples treated with *P. aeruginosa* strain KUD2. This figure highlights the biodegradation capabilities of the bacterial strain, showing a significant

reduction in the concentration of ETPHs compared to the control sample (Figure 4). The degradation process is evident through the diminished peaks in the chromatogram, indicating the breakdown of aliphatic hydrocarbons ranging from n-C8 to n-C36. This reduction showcases the efficacy of *P. aeruginosa* KUD2 in bioremediation applications by effectively degrading various aliphatic hydrocarbons to minimal levels.

Fig. 7 presents the mass chromatogram of the poly aromatic hydrocarbons (PAHs) in the control crude oil sample. This chromatogram focuses specifically on the Speciated EPA-16 PAHs, providing a detailed analysis of the PAH compounds present before any treatment. The peaks in the chromatogram correspond to different PAH compounds, with their heights reflecting their relative abundance. This figure serves as a reference for the PAH content in untreated crude oil, highlighting the presence and abundance of toxic and environmentally persistent PAHs.

Figure 8 shows the mass chromatogram of the degraded poly aromatic hydrocarbons (PAHs) in the crude oil sample treated with P. aeruginosa strain KUD2. This chromatogram demonstrates the effectiveness of the bacterial strain in degrading PAHs, as indicated by the significant reduction in peak heights compared to the control sample (Figure 7). The decreased abundance of PAH compounds in this figure highlights the strain's capability to target and break down these toxic hydrocarbons, suggesting its potential use in bioremediation strategies for environments contaminated with PAHs.

The results provided in the Table 2 display the concentrations of Extractable Total Petroleum Hydrocarbons (ETPH) - Aliphatics before and after degradation by Pseudomonas aeruginosa strain KUD2. The values are presented in milligrams per liter (mg/L). Before degradation, the aliphatic hydrocarbons n-C8 to n-C36 were present in the crude sample, with varying concentrations. The initial concentrations of these aliphatic hydrocarbons ranged from 18.38 mg/L (n-C8) to 371.47 mg/L (n-C19). After the degradation process, the concentrations of the aliphatic hydrocarbons were significantly reduced, and some hydrocarbons were entirely degraded, leading to non-detection (ND). The extent of degradation varied among different hydrocarbons. For instance, n-C8 was not detected (ND) after degradation, indicating complete removal. Similarly, n-C36 was also not detected after degradation, suggesting complete

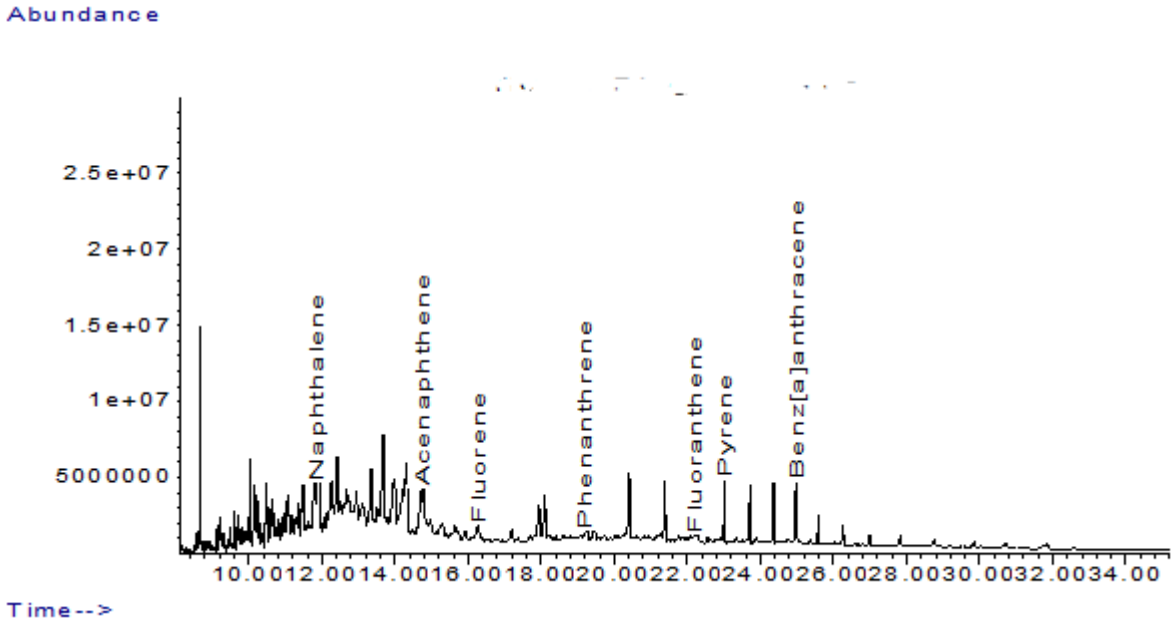


**Fig. 7.** Mass Chromatogram of the Poly Aromatic Hydrocarbons in the control crude oil sample

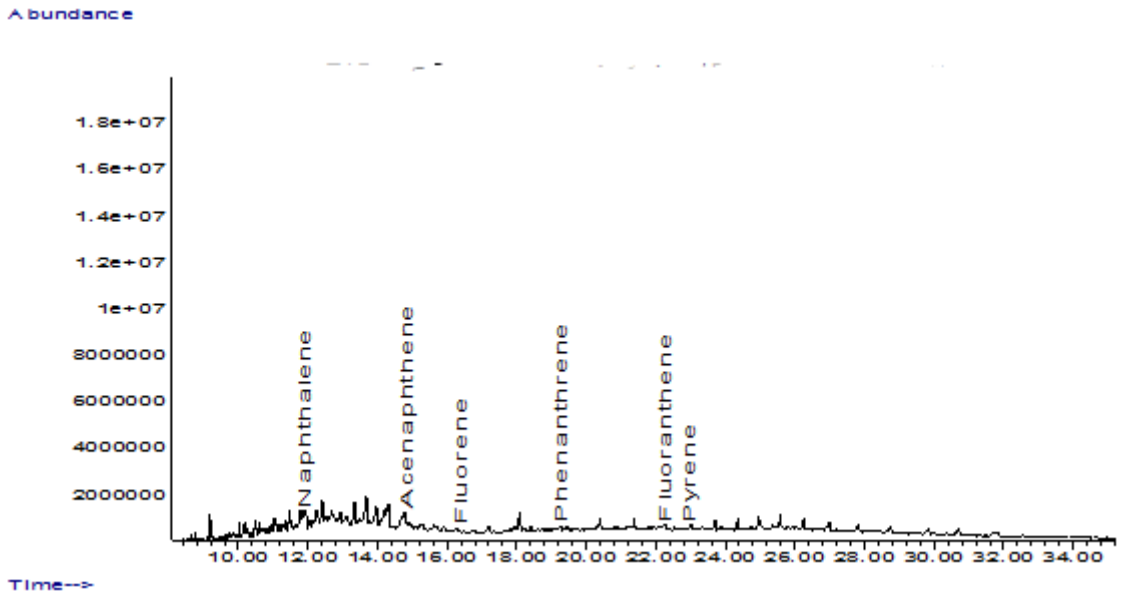


**Fig. 8.** Mass Chromatogram of the degraded Poly aromatic hydrocarbons in the crude oil sample by *P. aeruginosa* strain KUD2

degradation.

In contrast, some hydrocarbons showed traces of residual concentration after degradation, indicating partial degradation. For example, n-C9, n-C10, n-C11, and n-C12 were reduced to 0.06 mg/L, 0.09 mg/L, 0.10 mg/L, and 0.12 mg/L, respectively.

Table 2 Biodegradation of Speciated EPA-16 Poly Aromatic Hydrocarbons **(**PAHs) in crude oil sample by Pseudomonas aeruginosa KUD2 before and after a degradation process by *Pseudomonas aeruginosa* strain KUD 2. Before degradation, the PAHs Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz[a]anthracene, Chrysene, and Benzo[b]fluoranthene were present in the sample, with varying concentrations. After the degradation process, changes in the concentrations of the PAHs were observed. Some PAHs were completely degraded, leading to non-detection (ND) after degradation. These include Acenaphthylene, Anthracene, Benz[a]anthracene, Chrysene, and Benzo[b]fluoranthene.

For some PAHs, the concentrations were significantly reduced after degradation. For instance, Naphthalene was reduced from 35.31 mg/L to 0.11 mg/L, and Acenaphthene was reduced from 9.93 mg/L to 0.04 mg/L. Similarly, Fluorene decreased from 4.42 mg/L to 0.02 mg/L, and Phenanthrene decreased from 62.57 mg/L to 0.18 mg/L. While, Fluoranthene decreased from 18.77 mg/L to 0.07 mg/L, and Pyrene decreased from 20.61 mg/L to 0.08 mg/L.

Numerous investigations have demonstrated the presence of a substantial population of hydrocarbon-degrading bacteria in environments rich in oil, such as oil spill sites and oil reservoirs (Yang *et al*., 2015). Over time, bacteria in crude oil-contaminated soil undergo evolutionary adaptations as a response to prolonged exposure to pollutants. This adaptation process involves the upregulation of specific genes responsible for pollutant degradation, a phenomenon referred to as gene induction. Research has shown that bacteria can enhance their metabolic capabilities by activating genes that aid in the breakdown of contaminants, reflecting their adaptation to harsh environments (van der Meer *et al*., 2004; Singh *et al*., 2017). Concurrently, genes unrelated to pollutant degradation may be downregulated to conserve cellular energy and resources, optimizing the bacterial response to environmental

**Table 2.** Biodegradation of Extractable Total Petroleum Hydrocarbons-Aliphatic n-$C_8$, n-$C_{36}$ by *P. aeruginosa* KUD2.

| ETPH - Aliphatics | Before Degradation (mg/L) | After Degradation (mg/L) | Percentage Reduction (%) |
|---|---|---|---|
| n-C8 | 18.38±0.531 | ND | 100 |
| n-C9 | 65.37±1.888 | 0.06 ± 0.0017 | 99.91 |
| n-C10 | 44.43±1.283 | 0.09 ± 0.0026 | 99.80 |
| n-C11 | 79.54±2.297 | 0.10 ± 0.0029 | 99.87 |
| n-C12 | 62.76±1.812 | 0.12 ± 0.0035 | 99.81 |
| n-C13 | 55.21±1.594 | 0.09 ± 0.0026 | 99.84 |
| n-C14 | 88.17±2.545 | 0.09 ± 0.0026 | 99.90 |
| n-C15 | 212.21±6.124 | 0.15 ± 0.0043 | 99.93 |
| n-C16 | 203.04±5.857 | 0.09 ± 0.0026 | 99.96 |
| n-C17 | 291.03±8.400 | 0.14 ± 0.0040 | 99.95 |
| n-C18 | 287.65±8.307 | 0.22 ± 0.0064 | 99.92 |
| n-C19 | 371.47±10.726 | 0.30 ± 0.0087 | 99.92 |
| n-C20 | 308.12± 8.893 | 0.25 ± 0.0072 | 99.92 |
| n-C22 | 285.37± 8.240 | 0.19 ± 0.0055 | 99.93 |
| n-C24 | 311.23±8.987 | 0.24 ± 0.0069 | 99.92 |
| n-C26 | 226.76± 6.547 | 0.19 ± 0.0055 | 99.92 |
| n-C28 | 155.93±4.501 | 0.21 ± 0.0061 | 99.87 |
| n-C30 | 122.63± 3.540 | 0.13 ± 0.0038 | 99.89 |
| n-C32 | 89.92± 2.595 | 0.13 ± 0.0038 | 99.86 |
| n-C36 | 79.84±2.305 | ND | 100 |

Mean Percentage Reduction: 99.85%; Standard Deviation: 0.18%; ND-Not detected
Mean Values ± SEM; *n=3*

**Table 3.** Biodegradation of Speciated EPA-16 Poly Aromatic Hydrocarbons **(**PAHs) in crude oil sample by *P. aeruginosa* KUD2.

| PAHs | Before Degradation (mg/L) | After Degradation (mg/L) | Percentage Reduction (%) |
|---|---|---|---|
| Naphthalene | 35.31±1.0197 | 0.11±0.0032 | 99.69 |
| Acenaphthylene | ND | ND | - |
| Acenaphthene | 9.93±0.2867 | 0.04±0.0012 | 99.60 |
| Fluorene | 4.42±0.1276 | 0.02±0.0006 | 99.55 |
| Phenanthrene | 62.57±1.8064 | 0.18±0.0052 | 99.71 |
| Anthracene | ND | ND | ND |
| Fluoranthene | 18.77±0.5420 | 0.07±0.0020 | 99.63 |
| Pyrene | 20.61± 0.5950 | 0.08±0.0023 | 99.61 |
| Benz[a]anthracene | 14.70±0.4242 | ND | 100 |
| Chrysene | 22.50±0.6493 | ND | 100 |
| Benzo[b]fluoranthene | ND | ND | ND |
| Benzo[k]fluoranthene | ND | ND | ND |

ND; Not detected; - Not applicable; Mean
Values ± SEM; *n=3*

stress (Kumar *et al*., 2012). Bacteria exhibiting such capabilities are frequently identified and considered for deployment as environmental remediation agents, expediting the removal of petroleum hydrocarbon pollutants from the ecosystem (Kaczorek *et al*., 2012; Krasowska and Sigler, 2014). The biodegradation of Speciated EPA-16 PAHs and ETPHs—sixteen high-priority polycyclic aromatic hydrocarbons known for their toxicity and persistence—requires specialized metabolic pathways. These compounds demand sophisticated enzymatic systems for effective degradation due to their complex structures and environmental stability (Bollag *et al.,* 2000; Mrozik & Piotrowska-Seget, 2010). The ability to efficiently degrade such compounds underscores the significance of tailored bioremediation strategies. The observed capability of *P.*

*aeruginosa* KUD2 to form stable emulsions supports its potential as an effective bioremediation agent in oil-polluted environments. This ability to stabilize oil-water emulsions is indicative of the strain's proficiency in degrading hydrocarbons and its suitability for use in environmental clean-up efforts (Jain *et al*., 1991; Rahman & Gakpe, 2008).

This study provides novel insights, as existing literature does not document the biodegradation of Speciated EPA-16 PAHs and ETPHs in crude oil-contaminated soil prior to this work. Among the four prevalent isolates (KUD 1, KUD 2, KUD 3, KUD 4), all displayed positive results in the drop collapse test, indicating their ability to produce biosurfactants and reduce surface tension. This ability is crucial for enhancing oil degradation by making hydrocarbons more accessible to the microorganisms (Das and Mukherjee, 2020).

Furthermore, the isolates demonstrated positive results in the oil displacement test, indicating their capacity to displace oil, which is another important characteristic in oil degradation (Al-Saleh and Obuekwe, 2014). Among them, KUD2 exhibited the largest zone formation followed by KUD3 and KUD4. The results indicate that these isolates possess the potential for oil degradation, as they exhibit significant characteristics associated with biosurfactant production (Bento *et al.*, 2005). Remarkably, KUD2 displayed the highest emulsification index, indicating its strong capability to form stable oil-in-water emulsions. Emulsification is a crucial process in enhancing the bioavailability of hydrocarbons, facilitating their degradation by microorganisms (Kumar *et al*., 2022). The ability of KUD2 to form stable emulsions suggests its efficiency as a potential bioremediation agent for oil-polluted environments (Li *et al.,* 2021)

To confirm the identity of the highly efficient KUD2 isolate, 16S rDNA sequencing was performed. The sequence showed 100% homology with the *P. aeruginosa* strain BRPO3, a known hydrocarbon-degrading bacterium documented in the NCBI database. This finding confirms the isolate's identity as a *P.aeruginosa* bacterium. Phylogenetic analysis further supported the evolutionary relationship of the *P. aeruginosa* isolate with other known bacterial species (Fig. 1), reinforcing its potential as an effective biodegrading agent (Hamed and Elbendary, 2018).

Catechol-1,2-dioxygenase, alkane 1-monooxygenase, alcohol dehydrogenase etc play essential roles in the biodegradation of crude oil. This study shed light on the enzymatic capabilities of the isolated microorganism, *P.aeruginosa* strain KUD2, in the biodegradation of hydrocarbons. It was observed that the isolate synthesized only catechol-1,2-dioxygenase indicating its ability to metabolize catechol compounds commonly found in aromatic hydrocarbon degradation pathways via ortho cleavage pathway (Haritash and Kaushik, 2019).This finding aligns with previous studies that reported the prevalence of catechol-1,2-dioxygenase in certain hydrocarbon-degrading microorganisms (Wang *et al*., 2020).

Notably, during the incubation period, the pH of the medium exhibited fluctuations, increasing steadily and then sharply declining to reach a peak at pH 7.3. This observation suggests the possible involvement of metabolic processes and the production of acidic or alkaline metabolites during the degradation of hydrocarbons by *P. aeruginosa* strain KUD2 (Zhang *et al*., 2019). The pH fluctuations correlated with the highest production of catechol-1,2-dioxygenase suggesting that the microorganism might have adapted its enzymatic activity to the changing environmental conditions (Chandra *et al*., 2021).

Furthermore, the study demonstrated significant increases in the secretion of Alkane hydroxylase and Alcohol dehydrogenase enzymes by *P. aeruginosa* strain KUD2 during the experimental period. Alkane hydroxylase production peaked at 50 µmolmg-1 after 10 days of incubation, indicating the isolate's efficient hydrocarbon degradation capability (Wang *et al*., 2018). Similarly, Alcohol dehydrogenase increased to 30 µmolmg-1 after 10 days, further confirming the microorganism's potential to metabolize aliphatic hydrocarbons (Eroğlu and Yıldız, 2021).

The temporal trend in enzyme production and the growth of the isolate exhibited similar patterns. After reaching peak production, the levels of Alkane hydroxylase and Alcohol

dehydrogenase decreased steadily over time. This decline in enzyme production might be attributed to the depletion of readily available hydrocarbon substrates or the shift in metabolic pathways as hydrocarbon concentrations decrease (Qin *et al*., 2022). Moreover, the decrease in enzyme activity could indicate the onset of the stationary phase in the growth curve, with a decrease in cellular activity as nutrient availability declines (Kłosowska-Chomiczewska *et al*., 2020).

The results demonstrate the considerable enzymatic potential of *P. aeruginosa* strain KUD2 in degrading hydrocarbons, with a preference for catechol-1,2-dioxygenase pathway and efficient Alkane hydroxylase and Alcohol dehydrogenase production. The fluctuation in pH during incubation suggests possible metabolic adaptations, while the decline in enzyme activity over time may be attributed to changes in hydrocarbon availability or the bacterial growth phase. This study contributes to a better understanding of the enzymatic mechanisms employed by *P. aeruginosa* KUD2 in crude oil biodegradation, which has significant implications for potential bioremediation applications in oil-polluted environments.

The Mass Chromatogram presented in Fig. 4 reveals the presence of various aromatic hydrocarbons in the control crude oil samples, as indicated by the peaks. This indicates the complexity of the hydrocarbon composition in the crude oil, with different compounds detected at varying levels of abundance. The investigation focused on the biodegradation capabilities of *P. aeruginosa* strain KUD2 concerning the Extractable Total Petroleum Hydrocarbons (ETPHs) comprising aliphatic hydrocarbons ranging from n-C8 to n-C36.

Fig. 5 displays the Mass Chromatogram of the degraded ETPHs in the crude oil samples treated with *P. aeruginosa* strain KUD2. The results demonstrate the bacterium's remarkable efficacy in degrading all the aliphatic hydrocarbons present in the crude oil to a minimal level. This biodegradation process effectively reduced the concentration of ETPHs, illustrating the potential of *P. aeruginosa* KUD2 for environmental bioremediation applications. The significant reduction in aliphatic hydrocarbon content confirms the strain's ability to metabolize a wide range of aliphatics and contribute to the remediation of petroleum-contaminated environments (Zhao *et al*., 2021).

This study places significant emphasis on polycyclic aromatic hydrocarbons (PAHs) because these pollutants have been conclusively identified as posing high levels of toxicity and exhibiting mutagenic, carcinogenic, teratogenic, and immunotoxic effects on various forms of life (Patel *et al.,* 2020). Consequently, the Mass Chromatogram presented in Figure 6 focuses on the Speciated EPA-16 Poly Aromatic Hydrocarbons (PAHs) present in the crude oil. The peaks in the chromatogram correspond to different PAH compounds, and their heights indicate their relative abundance. After degradation by *P. aeruginosa* KUD2, the abundance of the hydrocarbons drastically reduced, suggesting the efficient degradation of PAHs by the bacterium. This is in line with previous studies that have reported the ability of *P. aeruginosa* to degrade PAHs (Song *et al*., 2020).

The concentration data presented in Table 1 further supports the effectiveness of *P. aeruginosa* KUD2 in degrading aliphatic hydrocarbons in the crude oil. Before degradation, the crude sample contained various aliphatic hydrocarbons with varying concentrations, ranging from 18.38 mg/L (n-C8) to 371.47 mg/L (n-C19). After the degradation process, the concentrations of the aliphatic hydrocarbons were significantly reduced, with some hydrocarbons being entirely degraded (non-detection). The extent of degradation varied among different hydrocarbons, with complete removal observed for n-C8 and n-C36, and partial degradation for others, such as n-C9, n-C10, n-C11, and n-C12. These results highlight the efficiency and substrate specificity of *P. aeruginosa* KUD2 in hydrocarbon biodegradation (Kumar *et al*., 2022).

## CONCLUSION

In conclusion, this study underscores the substantial enzymatic potential of *Pseudomonas aeruginosa* strain KUD2 in the biodegradation of hydrocarbons from crude oil. Previous research has established the presence of hydrocarbon-degrading bacteria in oil-rich environments, but this study is the first to document the specific biodegradation of Speciated EPA-16 PAHs and ETPHs in crude oil-contaminated soil. The strain exhibited a marked ability to produce biosurfactants and form stable oil-in-water emulsions, which is crucial for enhancing hydrocarbon bioavailability and degradation. Enzymatic analyses revealed that *P. aeruginosa* KUD2 efficiently produces catechol-1,2-dioxygenase, alkane hydroxylase, and alcohol dehydrogenase, supporting its capability in breaking down complex hydrocarbon structures. Observed pH fluctuations during the incubation period suggest metabolic adjustments in response to environmental conditions. The mass chromatograms highlighted the strain's effectiveness in degrading a broad spectrum of aliphatic hydrocarbons and PAHs, confirming its potential as a viable bioremediation agent. These findings emphasize the importance of tailored bioremediation strategies and contribute valuable insights into the enzymatic mechanisms employed by *P. aeruginosa* KUD2 for environmental cleanup applications.

## CONFLICT OF INTEREST

Authors declare no conflict of interest

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