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EXPLORING THE EFFECTIVENESS OF *Pseudomonas aeruginosa* ISOLATES FOR BIOREMEDIATION OF CRUDE OIL-CONTAMINATED SOILS USING SOYBEAN HULL AS A BIOSTIMULANT: A FOCUS ON ETPH AND PAHs

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ABSTRACT

The Speciated EPA-16 Polyaromatic Hydrocarbons (PAHs) in crude oil pose significant environmental and health risks due to their harmful properties. This study focused on indigenous bacteria capable of degrading Extractable Total Petroleum Hydrocarbons (ETPHs) and PAHs through a Nutrient-Amended Bioaugmentation Strategy (N-ABS). Crude oil-degrading bacteria were isolated from contaminated soil in Alakiri Community, Rivers State, Nigeria, leading to the identification of four *Pseudomonas aeruginosa* isolates (KUD1-4) via 16S rRNA gene sequencing. Bioremediation treatments, using both bacterial isolates and Soybean hull as a biostimulant, showed that *P. aeruginosa* KUD2 achieved the highest removal efficiencies of 96.59% for ETPHs and 80.00% for PAHs. This study underscores the potential of *P. aeruginosa* KUD2 and Soybean hull in remediating crude oil-contaminated soils.

Keywords: Bioremediation, Indigenous bacteria, Crude oil degradation, Soil pollution, Biosurfactant production

INTRODUCTION

Crude oil, as one of the most coveted natural resources globally, is extensively utilized for energy and raw materials across diverse applications. However, its pervasive usage has resulted in environmental contamination and resource degradation, necessitating urgent remediation efforts (Ossai *et al*., 2018; Adebiyi, 2022). Soil contamination by crude oil, stemming from human activities such as oil spills and improper waste disposal, severely impacts soil fertility, plant growth, and ecosystem health (Odukoya *et al*., 2019; Akhtar *et al*., 2021; Haider *et al*., 2021).This contamination poses a significant threat as it releases polycyclic aromatic hydrocarbons (PAHs) and toxic compounds such as benzene, toluene, ethylbenzene, and pyrene (ETPHs). These pollutants can infiltrate the soil following oil spills, leading to extensive contamination and posing significant risks to both human health and the environment (Gong *et al*., 2015).Crude oil contamination presents critical environmental challenges, persisting in the environment for extended periods and wreaking havoc on terrestrial and aquatic ecosystems (Zabbey *et al*., 2017; Ukhurebor *et al*., 2021). In response, innovative solutions for remediation and ecological restoration are imperative (Ossai *et al*., 2018; Xue *et al*., 2021; Sui *et al*., 2021), with biodegradation techniques gaining traction.

In recent years, bioremediation has emerged as a promising and eco-friendly approach for addressing soil contamination by crude oil. Among bioremediation techniques, the Nutrient-Amended Bioaugmentation Strategy has gained attention for its potential to enhance the biodegradation of crude oil contaminants in soil. This approach harnesses indigenous microbial communities and nutrient supplements to expedite hydrocarbon biodegradation in contaminated soils, aiming to mitigate the adverse impacts of crude oil contamination on ecosystems and human well-being.

Nutrient-amended bioaugmentation aims to mitigate these impacts by introducing hydrocarbon-degrading microbial species and essential nutrients, thereby enhancing biodegradation rates.

Given the increasing frequency of crude oil spills and the consequential environmental damage, there is a pressing need for effective and sustainable remediation strategies. Through this study, we aim to investigate the potential of nutrient-amended bioaugmentation in addressing the growing demand for eco-friendly and cost-effective soil remediation approaches. Furthermore, as environmental regulations become stricter, there is a growing demand for such approaches. Thus, this research aims to provide valuable insights for policymakers, environmental engineers, and stakeholders involved in soil remediation efforts.

MATERIALS AND METHODS

Soil Samples Collection

Soil was collected from four locations in Alakiri community in Okrika Local Government, Rivers State, Nigeria. The soil was sieved with a mesh of 2 mm to get rid of larger particles. The soil was kept at room temperature in a sterile polythene bag to avoid contamination.

Isolation of Crude Oil Degrading Bacterial Strains

Bacterial strains were isolated from the crude oil contaminated soil. The oil degrading bacteria were screened following the method of Diallo *et al.* (2021), Okoye *et al.* (2020) and Ejaz *et al.* (2021). Ten grams of the sample was inoculated into 100 ml Bushnell-Hass medium (BH) (g /L: KH_2PO_4 1; K_2HPO_4 1; $MgSO_4$ 0.2; $CaCl_2$ 0.02; NH_4NO_3 1; $FeCl_3$ 0.05; yeast extract 0.05. These were in done triplicate. The cultures were incubated at 30°C by shaking at 160 rpm for 7 days. Then, 5 ml from culture samples was centrifuged at 4000 rpm for 5 min and the pellets were suspended in 1 ml of sterile normal saline The suspended pellets were inoculated into flasks containing 100 ml BH medium supplemented with 1% crude oil. The inoculated flasks were incubated at the same conditions mentioned above (30°C by shaking at 160 rpm for 7 days.). Then 5 ml aliquots was taken from each culture and centrifuged at 4000 rpm for 5 min to obtain pellet which was suspended in 1 ml sterile normal saline and transferred to BH agar supplemented with 1% crude oil. The agar plates were incubated at 30°C for 5 days. After incubation, colonies were further cultured on nutrient agar (NA) plates and incubated at 30°C for 2 days to obtain pure colonies. Each of the colonies was screened for their crude oil degradation capacities.

Screening and Selection of Hydrocarbon Degrading Isolates

The isolates that demonstrated ability to grow in the presence of crude oil, degrade hydrocarbons and resist the toxicity of crude oil compounds were selected. The isolates were grown in nutrient broth, washed and standardized to give 1.5×10^7 cfu/ml as inoculum. The inoculum was transferred into five 250 ml flasks of BH broth and spotted on five BH agar plates containing different concentration (2 % , 4 % 6 % 8 % 10 % (v/v) of crude oil , The flasks and plates were incubated at160 rpm, 30° C for 5 days. The pellets from each culture were spotted on the respective BH agar plates at 30^0C for five days. The grown cultures in/on each flask and plate respectively were sub cultured on separate Nutrient agar plates to generate discrete pure colonies of the screened and selected degrading isolates. The stock cultures of these isolates were kept at 4ºC for further analysis.

Screening of Isolates for biosurfactant production

The extraction of crude biosurfactant and screening of the isolates for the biosurfactant production was carried out using Patowary *et al.* (2017) and *Zargar et al*. (2022) The selected crude oil degrading isolates were grown in different conical flasks containing 100 ml sterile mineral salt medium each and supplemented with 1 % crude oil as carbon source. The flasks were inoculated with pure loopful of 24 hr bacterial culture and incubated at 30ºC, 160 rpm for 5 days. After five days, the culture broths were centrifuged at 7000 rpm, 4ºC for 20 minutes. The supernatant was filtered through 0.45µm pore size filter paper. The filtrates, cell free culture broths, were used as the crude biosurfactant. The screening of biosurfactant production was carried out through haemolytic, drop collapse, oil displacement and emulsification activity tests.

Identification and Characterization of Bacteria

The selected crude oil-degrading bacteria were identified by 16S rRNA gene sequencing. Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR products were purified using the Qiagen PCR Purification Kit and sequenced. The sequences obtained were compared to known sequences in the NCBI GenBank database using BLAST. Phylogenetic analysis was conducted to determine the relationship of the isolates with known species using MEGA 11. The sequences were submitted to NCBI GenBank, and accession numbers were obtained for each isolate.

Collection and Processing of Soy Hull

Soybeans (SB-2024-001D) were obtained from Ago Market, Ilorin, Kwara State, Nigeria and processed to obtain the soybean hull powder (SH).

Preparation and Proximate Analysis of Soybeans Hulls

The soybeans hull (SH) Soybeans hulls were soaked, air dried, powdered, and stored until use as described by Ijah *et al*. (2013) with slight modification.

Experimental Design and Treatment Strategies:

The experimental design followed Ijah et al. (2013) with slight adaptations, incorporating biostimulation, bioaugmentation with a bacterial consortium (KUD 1, KUD 2, KUD 3, KUD 4), and hybrid strategies. Two controls were used: one with polluted soil and water, and another with polluted soil only. Each treatment used 100 g of contaminated soil, 20 g of soybean hull powder, and 5ml of bacterial culture with $OD_{600} = 1$, maintaining 20 % moisture content (16 % for controls). The setup included nine treatments of 500 g polluted soil each, incubated for 5 weeks at room temperature. This allowed for systematic evaluation of bioremediation strategies to address crude oil pollution in soil.

RESULTS AND DISCUSSION

Four bacterial isolates (KUD 1, KUD 2, KUD 3, KUD 4) were selected for their proficiency in crude oil degradation and biosurfactant production. Results show positive traits for surfactant production, including drop collapse, oil displacement, zone formation, blood haemolysis, and emulsification. Variations in zone formation and emulsification index suggest different efficiencies in surfactant production. This screening offers insights into biosurfactant capabilities, vital for their potential in bioremediation detailed in Table 1.

Table 1: Screening of Bacterial Isolates for Biosurfactant Production (Mean ± SE)

Isolates	Drop Collapse Test	Oil Displacement test	Zone Formation (cm)	Blood Haemolysis Test	Emulsification Index (%)
1	+	+	3.35 ± 0.11	+	60 ± 2.661
2	+	+	4.57 ± 0.07	+	70 ± 1.53
3	+	+	4.00 ± 0.28	+	68 ± 2.33
4	+	+	3.17 ± 0.31	+	65 ± 1.82

Keys: +: Detected; SE: Standard Error

All four isolates underwent biochemical tests, including Voges-Proskauer, Citrate, Methyl Red, Starch Hydrolysis, Motility, Hydrogen Sulphide, Casein Hydrolysis, Gas Production, Gelatin Hydrolysis, Indole Formation, Blue, Greenish, Yellow, Fluorescent, and Endospore Formation. The identical results suggested they belong to the same genus and species, identified as *Pseudomonas aeruginosa.* 16S rRNA gene sequencing confirmed their identity, and the isolates were submitted to NCBI with accession numbers KUD1 (OQ144894), KUD2 (OQ144895), KUD3 (OQ144896), and KUD4 (OQ144897).

Figure 1a illustrates the evolutionary relationships among isolates KUD1, KUD2, KUD3, and KUD4 based on their 16S rRNA gene sequences. These isolates cluster closely with known *Pseudomonas aeruginosa* strains, confirmed by high bootstrap values, forming a distinct clade separate from other bacterial genera like *Acinetobacter*, *Escherichia, Burkholderia*, *Staphylococcus* and *Bacillus*. This indicates significant genetic divergence from these groups. The phylogenetic tree (Fig. 1b) shows KUD2 closely related to *Pseudomonas aeruginosa* strain BRPO3 (KX664101.1) with a high bootstrap value of 100. Despite sharing species identity, KUD2 exhibits different catabolic potentials and distinct metabolic capabilities from other *Pseudomonas aeruginosa* isolates, suggesting varied evolutionary paths.

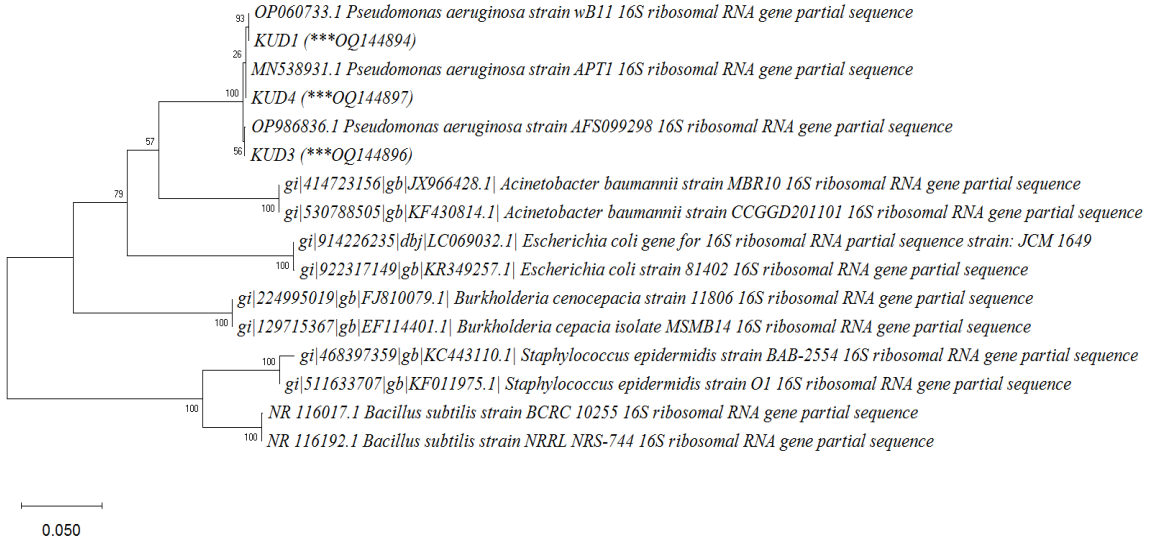

Figure 1a: Phylogenetic tree Oil degrading bacterial isolates and related sequences obtained from NCBI database

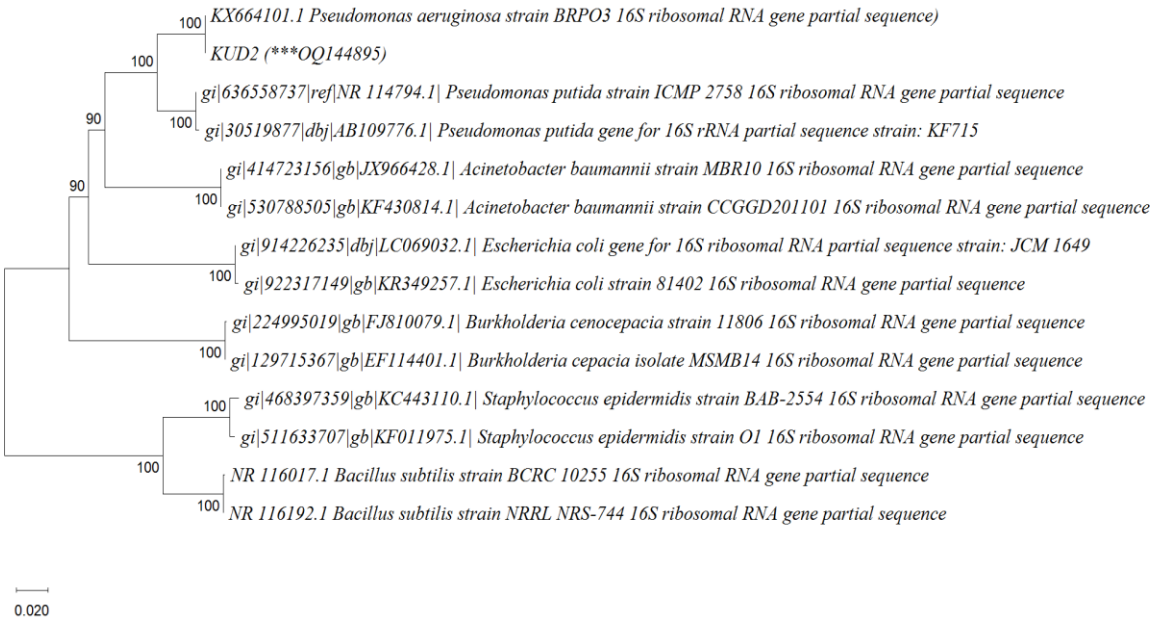

Figure 1b: Phylogenetic tree of Oil degrading bacterial isolates and related sequences obtained from NCBI Database

Figure 2 illustrates how the proximate composition of soybean hulls renders them a potent biostimulant for bioremediation purposes. Their nutrient-rich profile fosters the proliferation and activity of bacteria capable of degrading pollutants, thereby amplifying the efficiency of the bioremediation process and aiding in the restoration of contaminated environments. Specifically, attributes such as carbohydrates, proteins, and fibers play pivotal roles in enhancing soil conditions and facilitating the degradation of crude oil contaminants, underscoring the multifaceted effectiveness of soybean hulls as biostimulants.

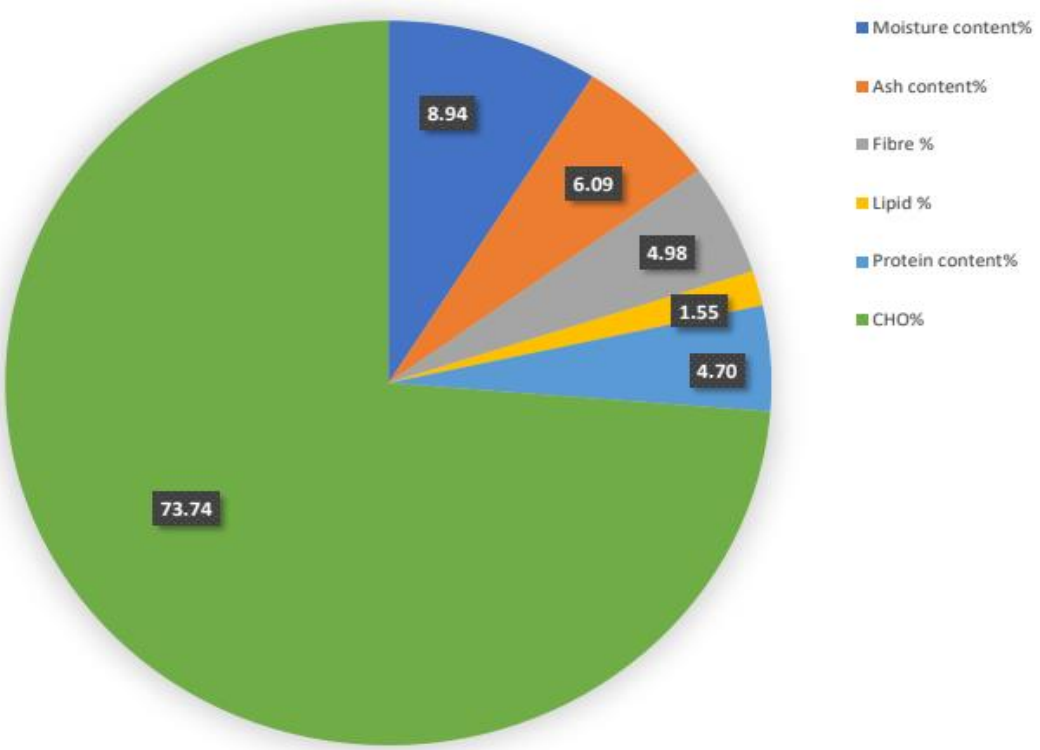

Figure 2: Proximate characteristics of biostimulant (soyabean hull)

The treated soil with pure bacterial cultures showed varied reductions in extractable total petroleum hydrocarbons (ETPHs) and polycyclic aromatic hydrocarbons (PAHs). Specifically, P. aeruginosa KUD1 reduced ETPHs by 62.5% and PAHs by 55.4%, KUD2 by 73.8% and 68.9%, KUD3 by 65.2% and 60.1%, and KUD4 by 67.4% and 61.3%. Combined in a consortium, these isolates achieved an 81.5% reduction in ETPHs and 75.6% in PAHs. Soybean hull alone reduced ETPHs by 45.3% and PAHs by 38.7%. The highest removal efficiency was observed with *P. aeruginosa* KUD2 combined with soybean hull, achieving a 96.59% reduction in ETPHs and 80.00% in PAHs, indicating a synergistic effect.

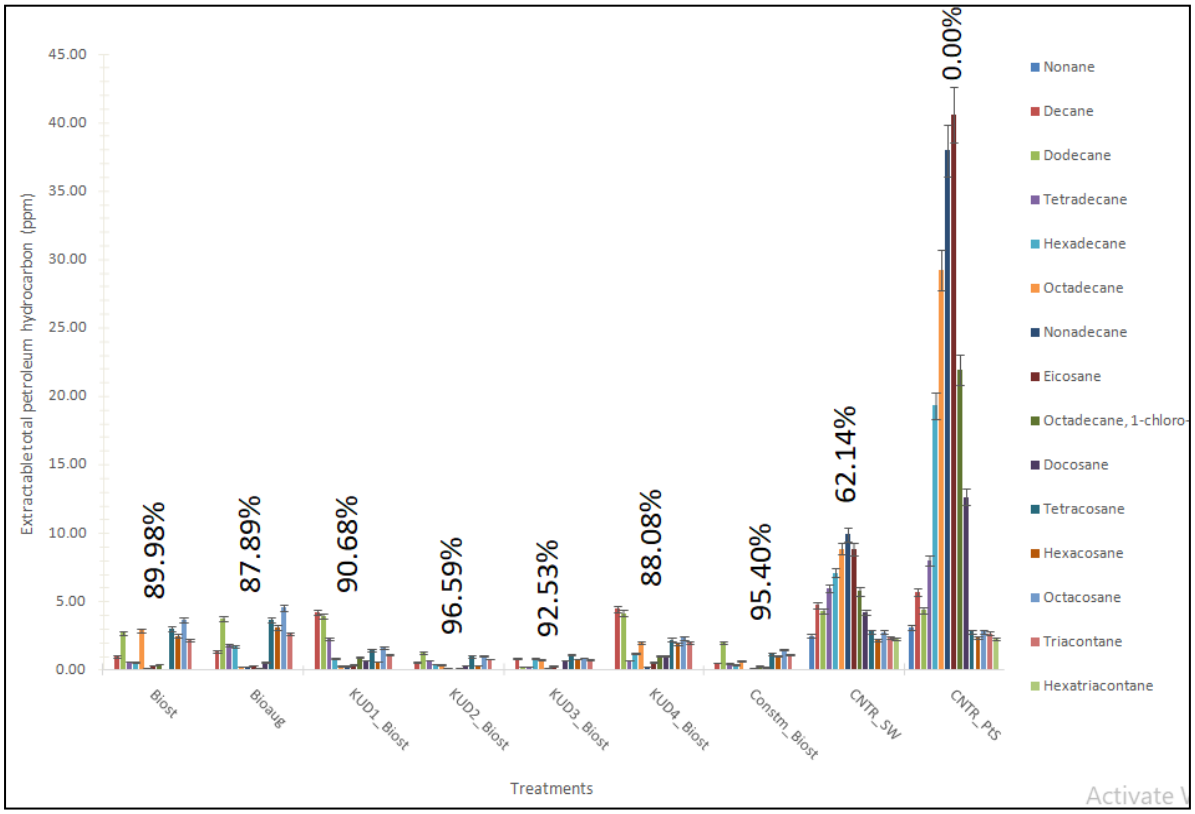

Figure 3: Extractable total petroleum hydrocarbons values obtained in the treated crude oil polluted soil

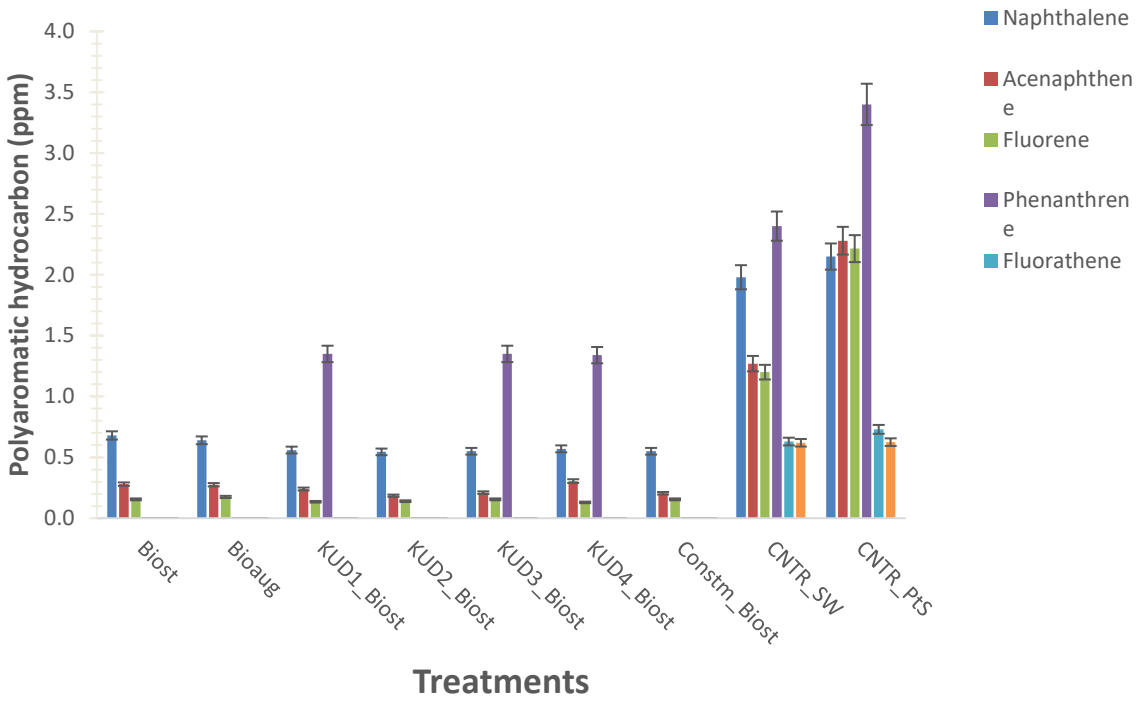

Figure 4: PAHs values obtained in the treated crude oil polluted soil

Discussion

The study investigates the potential of four strains of *Pseudomonas aeruginosa* in the degradation of crude oil and biosurfactant production, highlighting their resilience to crude oil toxicity. The positive surfactant production traits exhibited by these strains suggest their suitability for soil bioremediation. Biosurfactants play a crucial role in the bioremediation of hydrocarbon-contaminated environments. These surface-active molecules reduce the surface tension between oil and water, enhancing the bioavailability of hydrophobic pollutants like crude oil (Karlapudi *et al.*, 2018). This facilitates the emulsification and subsequent microbial degradation of hydrocarbons, making biosurfactant-producing strains particularly valuable in bioremediation efforts (Sah *et al.*, 2022). In our study, the biosurfactants produced by the *P. aeruginosa* strains significantly improved the degradation efficiency, demonstrating their potential in treating crude oil-contaminated soils. The ability of *P. aeruginosa* to produce potent biosurfactants adds to their effectiveness as bioremediation agents, especially in environments where pollutant accessibility limits microbial degradation (Karlapudi *et al.*, 2018).

KUD2 used in this study have shown remarkable resilience to the toxic effects of crude oil, maintaining high levels of activity in contaminated environments. This resilience is partly due to their ability to produce biosurfactants, which not only aid in pollutant degradation but also protect the bacterial cells from the toxic effects of hydrocarbons. The comprehensive profiling and 16S rRNA gene analysis confirm the identity of these strains as *P. aeruginosa*, supporting their role as robust bioremediation agents. The effectiveness of *P. aeruginosa* KUD2 in hydrocarbon degradation, as observed in this study, further solidifies its potential as a key player in environmental restoration efforts (Das & Mukherjee, 2007).

The proximate composition of soybean hulls, which includes high levels of organic matter, proteins, and carbohydrates, makes them an excellent biostimulant for microbial activity in bioremediation. Soybean hulls provide essential nutrients that enhance microbial growth and activity, thereby accelerating the biodegradation process. Addition of soybean hulls significantly boosted the degradation of crude oil, as evidenced by the enhanced microbial performance. The utilization of this agricultural by-product not only offers a cost-effective solution but also aligns with sustainable practices by repurposing waste materials for environmental remediation. The high fiber content in soybean hulls contributes to soil structure improvement, further aiding in the restoration of soil health post-bioremediation (Ijah *et al.*, 2013). This study demonstrates that the proximate composition of soybean hulls is crucial in supporting microbial populations during bioremediation, making it a practical and eco-friendly choice for large-scale applications. The study underscores the potential of indigenous bacteria, particularly *P. aeruginosa* KUD2, in combination with biostimulants, as a sustainable bioremediation approach for crude oil-contaminated soils. The findings offer valuable insights for environmental restoration, promoting practical and eco-friendly solutions for addressing crude oil contamination in soil. The utilization of soybean hulls as biostimulants not only enhances the biodegradation process but also presents a cost-effective and sustainable alternative to synthetic biostimulants. This approach could significantly reduce the environmental impact of crude oil spills and contribute to the recovery of contaminated sites.

CONCLUSION

The study explores the potential of four *P. aeruginosa* strains in crude oil degradation and biosurfactant production, indicating their resilience to crude oil toxicity and suitability for soil bioremediation. Confirmatory 16S rRNA gene analysis identifies KUD2 as a proficient hydrocarbon degrader. Additionally, soybean hulls serve as effective biostimulants, enhancing microbial activity and pollutant degradation. Soil property analysis post-bioremediation shows significant improvements, highlighting the

effectiveness of bioremediation in restoring soil health. This research offers insights into sustainable bioremediation strategies for crude oil-contaminated soils, utilizing indigenous bacteria and cost-effective biostimulants for environmental restoration

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