

Bioremediation of Crude Oil-Polluted Soil with a Consortium of *Pseudomonas aeruginosa* and Soybean Hull

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Abstract This study investigates the bioremediation of crude oil-contaminated soil using *Pseudomonas aeruginosa* isolates with soybean hull as a biostimulant. The experimental setup included biostimulation, bioaugmentation, and hybrid approaches with bacterial strains KUD-1, KUD-2, KUD-3, and KUD-4. Molecular identification confirmed these strains as *Pseudomonas aeruginosa*. Over a five-week incubation period, key physicochemical properties, such as pH, electrical conductivity, moisture content, and levels of essential nutrients, were monitored. The results demonstrated significant reductions in toxic metals, with chromium (Cr^{+6}) decreasing from 52.1 mg/kg to 13 mg/kg, iron (Fe) from 16,350 mg/kg to 560 mg/kg, and residual crude oil content from 0.51 g/10g to 0.11 g/10g. Improvements in soil fertility markers were also observed, with total nitrogen increasing from 126.6 mg/kg to 300 mg/kg, and total organic carbon rising from 0.761% to 6.55%. The pH of the soil increased from 6.28 to 7.78. This study underscores the effectiveness of biostimulation and bioaugmentation in enhancing the bioremediation process and restoring soil health, offering a sustainable solution for mitigating crude oil pollution.

Keywords: Bioremediation, Crude Oil, *Pseudomonas aeruginosa*, Soybean Hull, Biostimulation, Bioaugmentation, Soil Health, Environmental Restoration, Toxic Metals

I. Introduction

Crude oil spills pose a significant threat to terrestrial ecosystems, leading to severe environmental degradation and health risks [1]. The presence of polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs) in contaminated soils can persist for decades, adversely affecting soil quality, water resources, and biological diversity [2]. Traditional remediation methods often involve physical and chemical treatments, which can be costly and may result in secondary pollution [3]. In contrast, bioremediation leverages the natural abilities

of microorganisms and presents a sustainable and effective alternative [4].

Despite advancements in bioremediation technologies, the effectiveness of microbial degradation in crude oil-polluted soils remains limited by environmental factors and nutrient availability [5]. Many native microbial communities lack the capacity to efficiently degrade high concentrations of hydrocarbons, necessitating the introduction of specialized bacterial strains [6]. Furthermore, the synergistic effects of combining microbial consortia with organic amendments, such as soybean hull, in optimizing bioremediation processes are not fully understood [7]. This gap in knowledge hinders the development of effective, cost-efficient remediation strategies for oil-contaminated environments.

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Existing literature highlights the potential of *Pseudomonas aeruginosa* for bioremediation; however, there is limited research on the synergistic effects of using a consortium of these strains alongside organic biostimulants like soybean hull [8]. Most studies have focused on single-strain applications, neglecting the advantages that a diverse microbial community could offer [9]. Additionally, the impact of nutrient amendments on the degradation rates of hydrocarbons in varying environmental conditions requires further exploration to optimize bioremediation strategies [10].

This study is crucial for several reasons. First, it addresses the urgent need for effective remediation techniques in the face of increasing oil contamination due to industrial activities and spills. By investigating the use of a consortium of *Pseudomonas aeruginosa* strains combined with soybean hull, this research aims to enhance hydrocarbon degradation rates, potentially leading to more efficient bioremediation practices. Ultimately, this study seeks to provide a sustainable solution to crude oil pollution, promoting ecological restoration and safeguarding public health.

II. Materials and Methods

A. 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.* [11], Okoye *et al.* [12] and Ejaz *et al.* [13]. 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

B. 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'-AGAGTTTGTATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-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.

C. Experimental Design and Treatment Strategies:

The experimental design, based on [14] with slight adaptations, consisted of three main approaches: biostimulation, bioaugmentation with a bacterial consortium, and hybrid strategies combining both methods using specific bacterial strains (KUD 1, KUD 2, KUD 3, KUD 4). Two control groups were included, one with polluted soil and water and another with polluted soil only. Each treatment utilized 100 grams of contaminated soil and underwent a 5-week incubation period at room temperature to simulate real-world conditions. An inoculum size equivalent to 5 % of the optical density at 600 nm (OD600) was used, containing bacterial cell suspensions with an OD600 value of 1. Additionally, 20 grams of soybean hull powder were mixed with 100 grams of soil, and 5 ml of bacterial culture with an OD of 600 nanometers equal to 1 were introduced. The moisture content was adjusted to 20 % in the treated soil, while control samples maintained 16% moisture content. Regular monitoring of soil conditions and microbial growth ensured accurate observation and assessment of the bioremediation process. The setup involved nine treatments, each comprising 500 grams of polluted soil subjected to various combinations of biostimulants, bacterial isolates, or controls. These treatments were incubated for 5 weeks at room temperature, allowing the systematic evaluation of bioremediation strategies, including biostimulation, bioaugmentation, and their combinations, in addressing crude oil pollution in soil environments.

D. Bioremediation Performance Analyses

Bacterial heterotrophic counts, residual crude oil, pH, Electric conductivity (EC), nitrogen (N), phosphorus (P), potassium (K) and total

carbon content (TOC) were evaluated at 7 days interval during all the treatment of the soil. The cations, anions and Residual crude oil from the treated soil and uncontaminated soil were determined using ED-XRF, UV-VIS Spectrophotometric and Gravimetric respectively then confirmed using GC-MS focusing on the ETPH and PAHs.

E. Determination of the Degraded Crude Oil in Soil using Gravimetric Techniques

The amount of crude oil degraded in the soil was determined using the weight loss method of [15] by suspending 10 g of soil in 25 ml of diethyl ether in an Erlenmeyer flask. It was shaken vigorously to extract the oil. The solvent oil mixture was transferred into a preweighed beaker. This was done until all oil was extracted from the soil. The solvent oil mixture was exposed at room temperature overnight to allow the solvent to evaporate completely. The weight of the beaker containing the residual oil was recorded and the percentage of oil degraded was obtained as ratio of the weights of the oil samples. The biodegradation was calculated using equation (1)

$$Biodeg = \frac{crude\ oil(control) - crude\ oil\ (degraded)}{crude\ oil(contr)} \times 100\% \quad (1)$$

F. Soil Sample Preparation for Hydrocarbon Analysis via the Solvent Extraction Method

A 50:50 solvent mix of acetone and methylene chloride was added to a 5-gram dry soil sample in a pear-shaped tube, sonicated for 15 minutes at 70°C, and then anhydrous sodium sulfate was added until a clear extract formed. This process was repeated twice, and the extracts were concentrated to 1.5 ml, exchanged with 20 ml of hexane, and concentrated again. The concentrated extracts were passed through

anhydrous sodium sulfate and collected in sample vials. They were fractionated into aliphatic and aromatic fractions using silica gel cartridges packed with hexane slurry. Gas Chromatography-Mass Spectrometry (GCMS) analysis was conducted using an Agilent J&W HP-5ms UI column, with helium as the carrier gas and specific temperature settings.

G. Physicochemical Characterization of the Polluted soil Magnesium (Mg), Potassium (K), Chromium (Cr⁺⁶), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Zn), Lead (Pb), Sodium (Na)

Soil samples underwent analysis to determine the concentrations of various elements using established methods such as atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectrometry (ICP-MS), as detailed in [16]. Additionally, phosphate, nitrates, and sulphates were assessed using colorimetric or titrimetric methods following procedures outlined by [17]. Total nitrogen content was determined via the Kjeldahl method [18], while total organic carbon content was measured using the Walkley-Black method [19]. Soil pH

was determined using a pH meter [20], and electrical conductivity was measured with a conductivity meter as described by [21]. Moisture content was assessed according to [22], and residual crude oil content was determined through solvent extraction followed by gravimetric analysis [23].

III. Results and Discussion

A. Results

The four isolates (KUD1-4) were characterized using various biochemical tests, yielding identical results that tentatively identified them as *Pseudomonas aeruginosa*. 16S rRNA gene sequencing confirmed this identification, with high homology to *Pseudomonas aeruginosa* entries in GenBank. The 16S sequences of the isolates were deposited in the NCBI Database (accession numbers OQ144894-897) and clustered closely with known *Pseudomonas aeruginosa* strains in the phylogenetic tree, confirming their classification within the *Pseudomonas aeruginosa* species as Illustrated in Fig. 1a

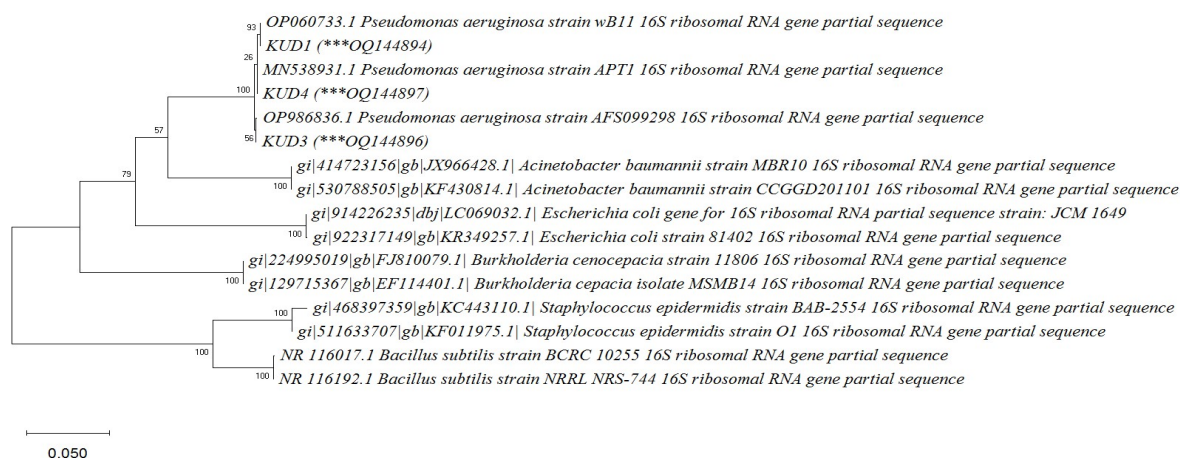


Figure 1a: Phylogenetic Tree Oil of Degrading Bacterial Isolates and Related Sequences Obtained from NCBI Database

The phylogenetic tree (Fig 1b) shows that isolate KUD2 (OQ144895) is closely related to *Pseudomonas aeruginosa* strain BRPO3 (KX664101.1), with a high bootstrap value of 100, confirming its classification as *Pseudomonas aeruginosa*. Other bacterial strains, including *Pseudomonas putida*, *Acinetobacter baumannii*, and *Bacillus subtilis*, form distinct clades, indicating significant genetic divergence. Notably, KUD2 exhibits different catabolic potentials and evolutionary paths despite belonging to the same species as other *Pseudomonas aeruginosa* isolates, highlighting its distinct metabolic capabilities.

The bioremediation process significantly reduced heavy metal concentrations in the crude oil contaminated soil. Chromium (Cr+6) decreased from 52.1 mg/kg to 13 mg/kg (68.6% reduction), iron (Fe) from 16350 mg/kg to 560 mg/kg (95.2% reduction), copper (Cu) from 494 mg/kg to 121 mg/kg (69.4% reduction), and zinc (Zn) from 363 mg/kg to 65 mg/kg (73.1% reduction). In contrast, manganese (Mn) concentrations remained relatively stable, with no significant change between the contaminated soil (178 mg/kg) and bioremediated soil (172 mg/kg). As illustrated in Table 1

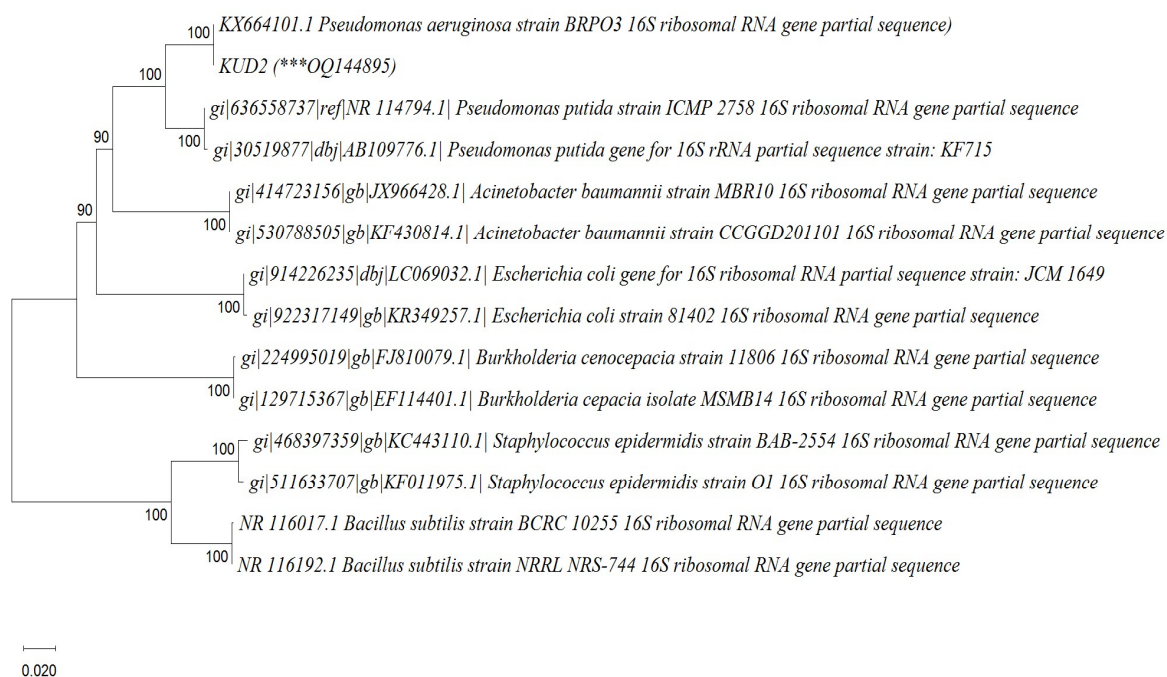


Figure 1b: Phylogenetic Tree of Oil Degrading Bacterial Isolates and Related Sequences Obtained from NCBI Database

Table 1: Heavy Metal Concentrations in Different Soil Treatments

Parameter	Crude Oil Contaminated Soil	Uncontaminated Soil	Bioremediated Crude Oil Contaminated Soil
Chromium (Cr⁺⁶) (mg/kg)	52.1 ± 2.24 ^a	-	13 ± 1.22 ^b
Manganese (Mn) (mg/kg)	178 ± 5.76 ^{ab}	200 ± 0.00 ^a	172 ± 5.1 ^{ab}
Iron (Fe) (mg/kg)	16350 ± 11.44 ^a	300 ± 7.51 ^c	560 ± 4.53 ^b
Copper (Cu) (mg/kg)	494 ± 4.44 ^a	-	121 ± 1.78 ^b
Zinc (Zn) (mg/kg)	363 ± 2.25 ^a	-	65 ± 2.19 ^b

Values are presented as mean ± standard deviation; Values in each column which have different letters are significantly different (p<0.05)

The bioremediation process significantly altered the physicochemical properties of the crude oil contaminated soil. Magnesium decreased from 9800 mg/kg to 7456 mg/kg, and potassium from 1750 mg/kg to 980 mg/kg. In contrast, nitrates increased from 27.8 mg/kg to 42.11 mg/kg, and total nitrogen from 126.6 mg/kg to 30 mg/kg. The pH increased from 6.28 to 7.78, and moisture content increased from 3.75% to 5.92%. Residual crude oil decreased from 0.51 g/10g to 0.11 g/10g, and aerobic heterotrophic bacteria increased from 2.5×10^4 cfu/g to 2.5×10^7 cfu/g, as illustrated in Table 2

B. Discussions

The analysis of the soil samples reveals significant alterations in the physicochemical properties and elemental composition due to crude oil contamination. The contamination markedly increased the levels of several toxic metals, including chromium (Cr⁺⁶), iron (Fe), copper (Cu), zinc (Zn), and lead (Pb). For instance, Cr⁺⁶ levels rose to 52.1 mg/kg in

contaminated soil compared to undetectable levels in uncontaminated soil. Similarly, Fe concentration increased to 16350 mg/kg from just 300 mg/kg in uncontaminated soil. These elevated levels of toxic metals pose a severe threat to soil health and can have detrimental effects on plant growth and microbial communities (13). Additionally, crude oil contamination resulted in a decrease in essential nutrients like magnesium (Mg) and potassium (K). The concentration of Mg dropped to 9,800 mg/kg in contaminated soil from 10,452 mg/kg in uncontaminated soil, and K levels decreased to 1750 mg/kg from 2,160 mg/kg. This reduction in essential nutrients further exacerbates the negative impact on soil fertility and productivity.

Bioremediation significantly improved the soil quality by reducing the concentrations of toxic metals and increasing the levels of essential nutrients. Cr⁺⁶ concentrations decreased to 13 mg/kg after bioremediation, and residual crude oil content was reduced from 0.51 g/10g to 0.11

Table 2: Physicochemical Properties of Different Soil Treatments

Parameter	Crude Oil Contaminated Soil	Uncontaminated Soil	Bioremediated Crude Oil Contaminated Soil
Magnesium (Mg) (mg/kg)	9800 ± 12.23 ^{ab}	10452 ± 21 ^a	7456 ± 14 ^b
Potassium (K) (mg/kg)	1750 ± 7.45 ^b	2160 ± 59 ^a	980 ± 17 ^c
Sodium (Na) (mg/kg)	21100 ± 13.77 ^a	17860 ± 18.51 ^b	14534 ± 11.67 ^c
Phosphate (mg/kg)	4.775 ± 0.54 ^b	11.20 ± 1.09 ^a	0.545 ± 0.00 ^c
Nitrates (mg/kg)	27.8 ± 3.32 ^c	35.45 ± 5.89 ^b	42.11 ± 3.33 ^a
Sulphates (mg/kg)	83.3 ± 5.00 ^c	111.32 ± 8.44 ^b	143 ± 6.43 ^a
Total Nitrogen (mg/kg)	126.6 ± 1.50 ^c	218.30 ± 1.88 ^b	300 ± 6.12 ^a
Total Organic Carbon (%)	0.761 ± 0.12 ^b	5.280 ± 0.92 ^a	6.55 ± 0.00 ^a
pH	6.28 ± 0.45 ^{ab}	6.80 ± 0.23 ^{ab}	7.78 ± 0.56 ^a
Electrical Conductivity (µS/cm)	82 ± 0.28 ^b	101 ± 3.78 ^a	55.40 ± 4.57 ^c
Moisture Content (%)	3.75 ± 0.06 ^b	8.77 ± 0.39 ^a	5.92 ± 1.11 ^{ab}
Residual Crude Oil (g/10g)	0.51 ± 0.01 ^a	-	0.11 ± 0.00 ^b
Aerobic Heterotrophic Bacteria (After 48 hrs) (cfu/g)	2.5x10 ⁴	5.7x10 ¹²	2.5x10 ⁷

Values are presented as mean ± standard deviation; Values in each column which have different letters are significantly different (p<0.05)

g/10g. These results highlight the effectiveness of bioremediation in removing contaminants and restoring soil health [24]. The improvement in nutrient levels post-bioremediation is noteworthy. Total nitrogen content increased from 126.6 mg/kg in contaminated soil to 300 mg/kg, and total organic carbon content rose from 0.761% to 6.55%. These increases are indicative of enhanced soil fertility, which is

crucial for supporting plant growth and maintaining healthy microbial communities [25].

The pH of the soil increased from 6.28 in contaminated soil to 7.78 after bioremediation, indicating a shift towards more neutral conditions. This change is beneficial for many soil microorganisms and plants that thrive in neutral pH environments [26]. Additionally, the decrease in electrical conductivity from 82 µ/ms

in contaminated soil to 55.40 $\mu\text{m/s}$ post-bioremediation reflects reduced salinity, further contributing to improved soil conditions.

The moisture content of the soil showed a moderate improvement after bioremediation, increasing from 3.75% in contaminated soil to 5.92%. This increase in moisture content is crucial for microbial activity and plant growth. The analysis also revealed a significant increase in the population of aerobic heterotrophic bacteria, with counts rising from 2.5×10^4 cfu/g in contaminated soil to 2.5×10^7 cfu/g after bioremediation. This increase in microbial activity is a positive indicator of soil health and the effectiveness of the bioremediation process [27].

IV. Conclusion

The results of this study underscore the potential of bioremediation as an effective strategy for restoring crude oil-contaminated soils. By significantly reducing the levels of toxic metals and residual crude oil, and improving nutrient content, soil pH, and microbial activity, bioremediation demonstrates its capability to rehabilitate degraded soils and enhance their fertility and productivity. The findings of this study confirm that crude oil contamination significantly disrupts the physicochemical and elemental balance of soil, leading to increased levels of toxic metals and decreased levels of essential nutrients. However, the bioremediation process proved effective in mitigating these adverse effects, restoring soil health, and improving its fertility. The success of bioremediation in this study highlights its potential as a sustainable and environmentally friendly approach for managing and rehabilitating contaminated soils, ultimately contributing to ecosystem restoration and agricultural productivity.

Future research should focus on optimizing bioremediation techniques, exploring the use of different microbial consortia and biostimulants, and assessing the long-term impacts of bioremediation on soil health and crop yield.

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