

# Bioremediation of Crude-Oil Polluted Soil Using Sawdust, Poultry Droppings, and Grass Trimmings through Optimization of Biostimulant Levels Using Box-Behnken Statistical Design

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**Abstract** The escalating issue of crude oil pollution necessitates innovative and sustainable remediation strategies. This study investigated the efficacy of sawdust, poultry droppings, and grass trimmings, both individually and in combination, as biostimulants for bioremediation of crude oil-contaminated soil. Employing the Box-Behnken statistical design, we optimized interactions between these organic amendments to enhance Total Petroleum Hydrocarbon (TPH) removal, identifying optimal levels and synergistic effects. The bacteria identified in the crude oil-contaminated soil included *Bacillus*, *Micrococcus*, *Pseudomonas*, *Staphylococcus*, and *Enterococcus*. The results revealed significant interactions between the biostimulants, with sawdust exhibiting highly significant interactions ( $p < 0.001$ ) and poultry droppings showing significant interactions ( $p < 0.005$ ). The effects of sawdust, grass trimmings, and poultry droppings on TPH removal followed a linear path, underscoring their consistent and predictable impact on the bioremediation process. The model's high coefficient of determination ( $R^2 = 0.9776$ ) confirmed its robustness. Furthermore, the bioremediation process yielded substantial reductions in ecotoxicity levels, corroborating the efficacy of these organic amendments for cost-effective and sustainable remediation of oil-

contaminated sites. This study provides a promising framework for future bioremediation strategies, leveraging locally sourced and eco-friendly materials.

**Keyword** Bioremediation, Crude-Oil, Sawdust, Poultry Droppings, Grass Trimmings, Optimization, Box-Behnken

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## 1. Introduction

Soil pollution from crude oil and petroleum products poses significant global risks to ecosystems and human health, sparking widespread concern [1]. Hydrocarbon contaminants like diesel, kerosene, and polycyclic aromatic hydrocarbons (PAHs) have severely impacted soil fertility, leading to reduced agricultural productivity, soil erosion, and leaching, causing long-term damage [2]. The importance of cleaning up oil-contaminated sites cannot be overstated, given the risk of toxic molecules spreading into the environment, contaminating groundwater, and entering the food chain [3]. The toxicity of spilled petroleum differs with its composition, concentration, and the activity of

microorganisms present in the contamination [4]. Aquatic environments face a persistent and escalating threat from hydrocarbon pollution, disrupting ecological balance and water quality, a significant ecological issue, resulting in substantial loss of biological life and negatively impacting the global economy, especially in regions involved in petroleum production and transportation, a critical global challenge [5]. Traditional physicochemical methods for cleaning up hydrocarbon-contaminated soil, such as evaporation and chemical dispersants, have environmental drawbacks [6,7]. Consequently, researchers are turning to eco-friendly alternatives like bioremediation, harnessing microbial communities to degrade toxic residues, leveraging their low ecological footprint, gentle intervention, and economic viability, a scalable solution for rejuvenating impacted water systems [8]. Research has also shown that repurposing organic waste materials like sawdust can support eco-friendly waste management and environmental cleanliness [9]. Optimization of biostimulant levels refers to determining the most effective combination and concentration of sawdust, poultry droppings, and grass trimmings to maximize Total Petroleum Hydrocarbon (TPH) removal from crude oil-contaminated soil, using the Box-Behnken statistical design, distinct from prior studies that often employed Central Composite Design or single-agent approaches, this work leverages synergistic ternary mixtures, addressing a critical gap in optimizing biostimulant blends for tropical, oil-impacted soils. Oil pollution is a global issue; optimized local biostimulants can inspire strategies in other oil-producing countries, especially tropical regions with similar bioresources. Oil pollution being a global environmental challenge, this study aims to develop a universally applicable, cost-effective bioremediation strategy using locally available biowastes (sawdust, poultry droppings, grass trimmings) in tropical oil-producing regions worldwide, supporting sustainable restoration of oil-impacted soils by finding the appropriate combination that has the most successful bioremediation through its optimization by Box-Behnken statistical design.

## 2. Materials and Methods

### 2.1. Sample Collection

This study utilized various materials sourced locally. Composite soil samples were obtained from the University of Nigeria Nsukka (UNN) agricultural farm. Poultry droppings and sawdust were procured from Ugwoke Poultry Farm in Nsukka, Enugu State. Diesel fuel (AGO) was purchased from a local filling station, while grass trimmings were collected from surrounding fields near UNN.

### 2.2. Sample Preparation

The collected soil samples were oven-dried at 30–40 °C for 24 hours to achieve thorough desiccation, eliminating residual hydration. The oven-dried samples were then sieved through a 1 mm pore size sieve to remove large particles and stones, and the sieved soil was stored in dry, airtight nylon bags until use.

Poultry droppings were passively desiccated under ambient temperature for 24 hours to reduce moisture content. Dried droppings were sieved to remove large particles and stones, and then stored in clean, airtight nylon bags prior to the study. Fresh grass trimmings were cut into smaller sizes and passively desiccated under ambient temperature for 24 hours. Sawdust was mechanically cleaned to remove large particles and debris, and then air-dried under atmospheric conditions for 24 hours.

The physicochemical characteristics of the soil samples were determined before the experiment commenced.

### 2.3. Microcosm Experimental Design

A microcosm study was conducted based on the method of Kramer and Hinckley [10] with modifications. Six kilograms of soil were contaminated with 0.6 L of diesel in aluminum pans. Sawdust (1.5 kg) was added as an organic amendment to the contaminated soil, while unamended contaminated soil served as a control. Moisture content was maintained by adding water, and the experiment was conducted at room temperature in triplicate. The soil was moistened and aerated weekly using a hand trowel. Samples were collected weekly for 8 weeks and analyzed for various parameters, including microbial enumeration, soil enzymatic activities, microbial biomass carbon, nitrogen, and phosphorus, total petroleum hydrocarbon removal, and microbial respiration.

### 2.4. Optimization of Biostimulants Using Box-Behnken Statistical Design

The Box-Behnken response surface methodology (Minitab 17 software) was employed to determine the optimization levels of grass trimmings, sawdust, and poultry droppings to enhance bioremediation of the contaminated soil. This design consisted of 15 experimental runs with 3 factors at varying levels. The organic amendments were applied at varying levels, and treated soil samples were maintained with weekly moistening and aeration. Soil samples were collected weekly for 8 weeks to monitor total petroleum hydrocarbon (TPH) removal. Samples were stored in a refrigerator prior to analysis. The three levels of the factors were set as low (-1), intermediate (0), and high (+1) (See Appendix 1). A total of 15 runs were used to investigate the effects of the three organic amendments/factors (See Appendix 2).

## 2.5. Analytical Procedure

- pH Determination

The soil pH was determined by mixing 7g of soil with 14mL of distilled water (1:2 soil-to-water ratio) and measuring the pH using a glass electrode pH meter (JENWAY).

- Moisture Content Determination

The moisture content was determined by drying a weighed soil sample in a pre-weighed crucible at 105 °C until a constant weight was achieved. The percentage of moisture content was calculated based on the weight difference.

- Soil Particle Size Analysis using Hydrometer Method

The hydrometer method was used to determine the percentage of sand and clay in the soil's inorganic fraction. The soil sample was treated with sodium hexametaphosphate to disperse aggregates, and the density of the suspension was measured using a calibrated hydrometer. Readings were taken after sand and silt settled, following the method of Bouyoucos [11], with corrections made for solution density and temperature.

- Cation Exchange Capacity and Exchangeable Cations by Ammonium Chloride

The cation exchange capacity (CEC) and exchangeable cations of the soil were determined using a method suitable for soils with a pH above 7.5. The soil sample was washed with an ethanol/glycol mixture to remove soluble salts. The cations were then displaced with alcoholic 1M NH<sub>4</sub>Cl at pH 8.5, followed by treatment with 0.05M HCl. Subsequently, NH<sub>4</sub><sup>+</sup> and Cl<sup>-</sup> ions were displaced with a 15% KNO<sub>3</sub> + 6% Ca(NO<sub>3</sub>)<sub>2</sub> solution. The combined nitrate extract was used to determine the cation exchange capacity (CEC), while the combined chloride extract was analyzed to determine the basic exchangeable [12,13].

- Phosphorus Content Determination

Phosphorus content was determined by extracting samples with 25% acetic acid, and the extracts were analyzed using a Unicam UV/visible spectrophotometer at 700 nm. Quality control measures included analyzing a spike sample in every batch and running a standard after each batch to plot a mean control chart.

- Nitrogen Content Determination

Nitrogen content was determined by extracting soil samples with sodium acetate in the presence of sulfuric acid, and the extracts were measured at 470 nm using a Unicam UV/visible spectrophotometer.

- Calcium Content Determination

Calcium content was determined by extracting samples with 25% acetic acid, and the extracts were analyzed using a Unicam UV/visible spectrophotometer at 700 nm.

Quality control measures included analyzing a spike sample in every batch and running a standard after each batch to plot a mean control chart.

## 2.6. Characterization and Identification of Isolates

The bacterial and fungal isolates were identified according to the methods by Cheesbrough [14].

- Gram Staining Procedure

The process involves preparing and heat-fixing a bacterial smear, applying crystal violet stain, treating with Lugol's iodine, decolorizing with acetone, and counterstaining with safranin, before examining the stained sample under a microscope.

- Spore Test

After making a bacterial film on a sterile glass slide and heat-fixing it, 0.5% malachite green was added to boiling water to dye it. The slide was cleaned and counterstained with 0.5% safranin for 30 to 60 seconds after 1 to 10 minutes. After being cleaned and dried, the slide was inspected under a microscope.

- Coagulase Test

The coagulase test detects the enzyme's ability to clot plasma. A bacterial colony was mixed with plasma on a glass slide, and clumping within 10 seconds indicated a positive result.

- Oxidase Test

Bacteria that produce oxidase can be identified using the oxidase test. On filter paper saturated in oxidase reagent (tetramethylphenylenediamine dihydrochloride), a colony was spread. Within ten seconds, a strong purple hue signified a successful outcome.

- Methyl Red Test

Methyl red reagent was applied after the test organism had been cultured for 48 hours in glucose-phosphate peptone water. A favorable result was denoted by a red hue, and a negative result by a yellow tint.

- Voges Proskauer Test

Barritt's reagents (potassium hydroxide and alpha-naphthol) were added after the test organism had been cultured for 48 hours in glucose-phosphate peptone water. A positive outcome was suggested by a pink-burgundy hue.

- Citrate Utilization Test

After being streaked onto Simmon citrate agar, the test organism was cultured for 48 hours. The capacity to use citrate as a single-carbon source was demonstrated by a color shift from green to blue.

- Sugar Fermentation Test

Bromothymol blue was used to assess the isolates'

capacity to ferment carbohydrates in peptone medium. Vapor formation in Durham tubes indicated gas generation, while a shift in color from blue to yellow indicated acid production.

## 2.7. Statistical Analysis

The optimization of organic amendments was conducted using the Box-Behnken design, a statistical experimental approach. This design involved 15 experimental runs with three factors at three levels (-1, 0, and +1), including three central points to assess model robustness. The experimental responses evaluated were total petroleum hydrocarbon (TPH) removal rate and lipase activity. The three-factor levels were categorized as low (-1), intermediate (0), and high (+1), allowing for analysis of the effects of organic amendments on these responses.

## 3. Results and Discussion

Table 1 presents the physicochemical and microbial characteristics of the uncontaminated baseline, revealing a neutral pH of 7.6, cation exchange capacity of 17.2, particle size composition of 96% clay and 1.9% sand, 2.2% moisture content, and nutrient levels of 1.8% nitrogen, 1.0% phosphorus, 1.8% calcium, 0.6% magnesium, and 0.9% organic carbon.

**Table 1.** Physicochemical and microbiological properties of the pristine soil

Parameters	Values
Ph	7.6
Cation exchange capacity (Meg/100g)	17.2
Particle size of clay (%)	96
Particle size of sand (%)	1.9
% moisture	2.2
% nitrogen	1.8
% phosphorous	1.0
% calcium	1.8
% magnesium	0.6
% organic carbon	0.9

Table 2 shows the major bacterial genera identified in this study, with *Bacillus*, *Pseudomonas*, *Staphylococcus*, and *Enterobacter* being predominant in the 5% crude oil environment. All the bacterial genera identified are native

to crude oil environments. Morphological analysis revealed rod-shaped and spherical (cocci) forms. The presence of hydrocarbons influenced the dynamics of microorganisms capable of degrading them, consistent with findings by Ijah et al. [15], who reported similar bacterial genera, including *Bacillus*, *Pseudomonas*, and *Enterobacter*, as effective oil degraders.

Table 3 presents the Total Petroleum Hydrocarbon (TPH) removal results from the bioremediation study. Using response surface methodology, a polynomial equation was derived through multiple regression analysis to model TPH removal as a function of the biostimulants sawdust, grass trimmings, and poultry droppings. In TPH removal, and GT, SW, and PD denote the effects of grass trimmings, sawdust, and poultry droppings, respectively. The model's predictive robustness was evaluated through analysis of variance (ANOVA) coupled with Fisher's F-test.

Table 4 presents the analysis results, where F-values and P-values indicate the significance of each coefficient. The analysis revealed no significant interactive effect between grass trimmings (GT) and poultry droppings (PD) at  $p < 0.05$ . However, significant interactions were observed between GT and sawdust (SW) at  $p < 0.05$  and a highly significant interaction between SW and PD at  $p < 0.001$ , indicating that GT's effectiveness in diesel degradation depends on SW, while SW's impact on diesel removal relies on PD. The regression equation showed a direct relationship between SW and GT, but an inverse relationship between SW and PD, consistent with studies highlighting the importance of C/N ratio in bioremediation. Significantly, using bulking agents like SW in soil restoration can conserve polluted agricultural lands, maintaining soil water balance, biological activity, and fertility replenishment [16]. Response Surface Methodology (RSM) has proven effective in optimizing nutrient levels for enhanced petroleum hydrocarbon removal [17]. Our goal was to identify the optimal nutrient combination for maximum diesel removal while minimizing nutrient levels. The model's high coefficient of determination ( $R^2 = 0.9776$ ) confirmed its goodness of fit, explaining 97.76% of the total variance. This is in agreement with previous studies [18,19] that soil microbial enzymes are recognized as important indicators of soil disturbances and play crucial roles in various ecological processes, including organic matter decomposition. By using a Box-Behnken design to correlate factors with oil removal, Soha et al. [20] demonstrated the bioremediation capacity of *Pseudomonas* sp. sp48 by obtaining a predicted optimum of 89%, which was 2.4 times greater than the baseline medium.

**Table 2.** Morphological and biochemical characteristics of the heterotrophic bacteria isolated

Isolates	Gram stain	Form	Spore stain	Motility	Catalase	Oxidase	Citrate	Indole	Methyl red	Vogesproskauer	Coagulase	Glucose	Sucrose	Lactose	Mannitol	Identity
A	+	Rod	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>Escherichia coli</i>
B	+	Coccus	-	-	+	+	-	-	-	-	-	-	-	-	-	<i>Micrococcus luteus</i>
C	+	Coccus	-	-	-	-	+	-	+	+	-	+	+	+	+	<i>Enterococcus faecalis</i>
D	-	Rod	-	+	+	+	+	-	-	-	-	-	-	-	-	<i>Pseudomonas aeruginosa</i>
E	+	Coccus	-	-	+	-	-	-	+	+	+	+	+	+	+	<i>Staphylococcus aureus</i>
F	+	Rod	+	+	+	-	+	-	-	+	-	-	-	-	+	<i>Bacillus subtilis</i>
G	-	Rod	-	+	+	-	+	+	-	+	-	+	-	-	+	<i>Serratia marcescens</i>

+ = Positive - = Negative

**Table 3.** Observed and predicted TPH responses obtained from the Box-Behnken experimental design

Run no	Observed values (mg/kg)	Predicted values (mg/kg)
1	3993.50	3757.57
2	2509.46	2696.43
3	2423.97	2236.99
4	3528.17	3764.10
5	2308.62	2709.84
6	2080.78	2059.10
7	2217.65	2239.33
8	3757.26	3356.05
9	602.38	437.09
10	2711.20	2496.96
11	2922.40	3136.64
12	458.56	623.85
13	5047.69	5033.36
14	5011.04	5033.36
15	5041.34	5033.36

**Table 4.** Analysis of variance for the RSM quadratic model of TPH

Source	DF	S of squares	Mean square	F-Value	P-Value	Remarks
Model	9	28386943	3154105	24.21	0.001	Significant
Linear	3	552631	184210	1.41	0.342	Not significant
GT	1	108564	108564	0.83	0.403	Not significant
SW	1	102566	102566	0.79	0.416	Not significant
PD	1	341501	341501	2.62	0.166	Not significant
Square	3	20151296	6717099	51.56	0.000	Very significant
GT*GT	1	927025	927025	7.12	0.044	Significant
SW*SW	1	7429589	7429589	57.03	0.001	Very significant
PD*PD	1	13913676	13913676	106.81	0.000	Very significant
2-Way	3	7683017	2561006	19.66	0.003	Significant
<b>Interaction</b>						
GT*SW	1	1674757	1674757	12.86	0.016	Significant
GT*PD	1	780975	780975	6.00	0.058	Not significant
SW*PD	1	5227285	5227285	40.13	0.001	Very significant
Error	5	651343	130269			
Lack-of-Fit	3	650576	216859	565.57	0.002	Significant

Pure Error, 2, 67, 383, DF = Degree of freedom;  $R^2 = 97.76\%$ , adj.  $R^2 = 93.72\%$ , p

## 4. Conclusions

With the help of native microbes and significant ecotoxicity reductions, this study clearly shows that sawdust, poultry droppings, and grass trimmings—all of which were optimized using the Box-Behnken design ( $R^2 = 0.9776$ ,  $p < 0.001$  for sawdust,  $p < 0.005$  for poultry droppings)—are effective biostimulants for improving the removal of Total Petroleum Hydrocarbons (TPH) in Nigerian soils contaminated by crude oil. The conversion of common Nigerian biowastes into an environmentally benign, scalable, and economical bioremediation resource tackles urgent issues with oil pollution in nearby producing areas. Oil-producing areas of Nigeria can use in-situ biostimulation by utilizing these biostimulants and indigenous microorganisms to restore crude oil-polluted soils in a sustainable manner, particularly in areas with a plentiful supply of sawdust, chicken manure, and grass clippings.

## Appendices

**Appendix 1.** Process parameters and their levels

S/N	Factors	Levels		
		-1	0	+1
1	Grass trimmings (g)	130	150	170
2	Poultry droppings (g)	40	50	60
3	Sawdust (g)	570	600	630

**Appendix 2.** Experimental Layout using the Box-Behnken experimental design (3-factors, 3-levels)

S/N	Coded Factor Level			Grass Trimmings (GT)	Sawdust (SD)	Poultry Droppings (PD)
1	-1	-1	0	130	570	50
2	-1	1	0	130	630	50
3	1	-1	0	170	570	50
4	1	1	0	170	630	50
5	-1	0	-1	130	600	40
6	-1	0	1	130	600	60
7	1	0	-1	170	600	40
8	1	0	1	170	600	60
9	0	-1	-1	150	570	40
10	0	-1	1	150	570	60
11	0	1	-1	150	630	40
12	0	1	1	150	630	60
13	0	0	0	150	600	50
14	0	0	0	150	600	50
15	0	0	0	150	600	50

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