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Bio-stimulation of soil enzymes using diammonium phosphate and urea fertilizer on crude oil contaminated sandy-loam soil

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ABSTRACT

Pollution caused by crude oil is one of the most prevalent environmental problems in oil-rich countries. Bioremediation processes usually exploit the ability of microorganisms to degrade and/or detoxify organic contaminants. A widely used bioremediation strategy is bio-stimulation of the soil's indigenous microbes by the addition of nutrients, as crude oil contamination tends to result in the rapid depletion of the available pools of major inorganic nutrients such as nitrogen and phosphorus. This study examined the bio-stimulation effect of diammonium phosphate and urea fertilizers on crude oil-contaminated soil enzymes. Soils were artificially contaminated with 3000, 5000, or 8000 ppm of crude oil and treated with Diammonium phosphate (DAP) and urea fertilizers. The activities of soil enzymes such as laccase, lipase, catalase, and peroxidase were analyzed every 6^{th} day for 30 days. The results indicated that the activity of laccase for all the treated soils was significantly higher than the untreated group on days 18, 24, and 30, while those of peroxidase and catalase peaked at day 12, with a sharp decline on days 18 to 30 when compared to the untreated soil. However, the activity of lipase continued to increase until the 30^{th} day in all the treated soils, and the increase was higher in contaminated soils treated with DAP and urea. The decreased activities of peroxidase and catalase in the treated soils may be related to a decrease in the microbial load of the soil. Furthermore, the increase in the activities of soil enzymes, especially in the treated soils, suggested that the treatments contributed to enhancing the activities of the enzymes, and hence may help in the bioremediation process through bio-stimulation of the soil enzymes that function in the breakdown of environmental contaminants.

1. Introduction

Petroleum-based products are a major source of energy for daily life and industry. Pollution arising from crude oil is a recurrent anomaly [1]. Leaks and spills during drilling, refining, transport, and storage of petroleum and its products, as well as pipeline vandalism, result in soil and water pollution. Soil and water pollution by crude oil remain an issue of concern because of its negative impact on the environment; thus, the need for

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speedy, dependable, and efficient clean-up techniques for the restoration of oil polluted environments becomes imperative. Bioremediation is a rapidly developing method for the removal and breakdown of petroleum pollutants. It is a microorganism-aided (bacteria, yeast, and fungi) transformation or breakdown of petroleum (hydrocarbon-based) contaminants into less-hazardous or non-hazardous compounds [2]. This method of soil restoration helps to control pollution in an environmentally friendly manner. Aerobic (oxygen requiring) and anaerobic (non-oxygen requiring) microorganisms may be used in these pollution control methods [3]. Several bioremediation strategies have been explored: land farming, pump and treat, composting, bioreactors, bio-stimulation, bioaugmentation, etc. [4]. Bio-stimulation involves the addition of limiting nutrients such as phosphorus and nitrogen, as well as oxygen, to soil microbes to improve environmental conditions and enhance bioremediation. This is achieved by optimizing ecological conditions for the purpose of stimulating the growth of resident pollutants-degrading microorganisms. Crude oil pollution increases the concentration of carbon in the soil and consequently makes nitrogen and phosphorus major limiting nutrients. During bio-stimulation, fertilizers supply the appropriate concentration of nitrogen, phosphorus, and other nutrients needed by hydrocarbon degrading microorganisms for their metabolic processes and degradation of hydrocarbons present in the soil. These nutrients aid in microbial metabolism and the biosynthesis of amino acids, proteins, and nucleic acids. Additional nutrients in the form of fertilizers may, therefore, be added to polluted soils to optimize the major limiting nutrients, and the availability of varying sources of such nutrients will be highly beneficial to the bioremediation process [5]. Urea fertilizer is known to be a very good source of nitrogen that is required for proper plant growth; DAP [$(NH_4)2HPO_4$] has been widely reported to be an excellent source of two essential macronutrients, phosphate and nitrogen, needed for plant growth. Its popularity is due to its relatively high content of nitrogen and phosphorus in addition to its excellent physical properties. It has a high solubility (588 g/L at 20 °C) and dissolves quickly in soil to release phosphate and ammonium useable by plants. A very important characteristic of DAP is its alkaline pH (7.5–8) that develops around the dissolving granule. Effective bioremediation has been documented to require a carbon, nitrogen, and phosphorus (C: N: P) ratio of 100:10:1 - 100:1:0.5, while the recommended values for OSRRMS [6] are 100:2:0.2 - 100:1:0.1. The ratios of 100:10:1 - 100:1:0.5 are said to be excessive because all the carbons present in the soil are not used for biomass formation and the constant recycling of nitrogen and phosphorus by microorganisms. It should be noted however that when nutrient concentration in the soil exceeds what is required, it can inhibit the process of biodegradation. A large number of microorganisms in the soil play significant roles in biodegradation by secreting extracellular enzymes that breakdown crude oil (substrate) into forms that are utilizable by the microbes, thereby aiding the subsequent removal from the environment and replacement with beneficial or less toxic products [7,8]. Induction of synthesis and the secretion of crude oil degrading enzymes are triggered by the increased level of irregular polymers and/or toxins in the soil. The presence and concentration of these enzymes in the soil are indicators of the level of bioremediation efficiency and biomarkers for the presence of microbial life in the soil [8]. The enzymes include microbial oxidoreductases, microbial peroxidases, microbial laccases, microbial hydrolytic enzymes, microbial lipases, microbial cellulases, and microbial proteases [9]. In this study, we investigated the bio-stimulation possibilities of DAP and urea fertilizer in enhancing the bioremediation of crude oil contaminated soil.

2. Materials and methods

2.1. Collection and preparation of soil sample

The soil was collected from a site at the University of Benin, Nigeria that was known to have remained unexposed to crude oil pollution over the years. The soil samples were collected into sterile polythene bags from a depth of 0 to 50 cm and transported to a greenhouse. The soil was air dried and sieved using a 2 mm sieve to remove plant material, stones, and other large particles. The Hydrometer method [10] was used to determine the particle size distribution of the soil, the percentage of sand, silt,

and clay, and to assign a textural class using the USDA textural triangle.

2.2. Determination of Soil Total Organic Carbon

The Walkley-Black method, also known as the wet oxidation method [11], was used to determine the total organic carbon in the soil. 10 mL of 1 N $K_2Cr_2O_7$ was added by means of a pipette to a 500 mL Erlenmeyer flask containing 1 g of the soil sample, followed by the addition of 20 mL of concentrated H_2SO_4 (96%). The mixture was swirled gently to mix and placed on an asbestos sheet to avoid rapid heat loss; it was left to stand for 30 min. Then, 10 mL of 85% H_3PO_4 (85%) and 0.2 g of ammonium fluoride were added, followed by 10 drops of the ferroin indicator to give a yellow-orange to dark green color. The indicator was added just prior to titration to avoid deactivation or adsorption onto the clay surfaces. Titration was done with 0.5 N Fe^{++} to a burgundy endpoint. A reagent blank following the above procedure without soil was also run. The formula below was used to calculate the total organic carbon of the soil.

$$\%\ \text{Easily oxidizable organic carbon (EOOC)} = \frac{(B-S)\times N\ of\ Fe++}{g\ of\ Soil}\times\frac{12}{4000}\times 100 \quad (1)$$

here: B = ml of Fe^{++} solution used to titrate blank, S = ml of Fe^{++} solution used to titrate sample, and 12/ 4000 = milliequivalent weight of C in g.

$$\text{Total Organic Carbon (TOC)} = \frac{\text{EOOC}}{0.77}$$

2.3. Soil Contamination and Treatment

The soil sample was weighed into eight plastic bowls with a diameter of (30 cm). The soil (5 kg) was contaminated with or without crude oil and labeled as follows: group A was not contaminated with crude oil, group B was prepared by adding 3 g of crude oil to 1 kg of soil (3000 ppm), group C was prepared by adding 5 g of crude oil to 1 kg of soil (5000 ppm), group D was prepared by adding 8 g of crude oil to 1 kg of soil (8000 ppm), group E was comprised DAP + Urea-treated soil, crude oil-contaminated soil (3000 ppm) and DAP+Urea treatment (group F), crude oil-contaminated soil (5000 ppm) and DAP+Urea treatment (group G), and crude oil-contaminated soil (8000 ppm) and DAP+Urea treatment (group H). Groups A to H were kept in a greenhouse and treated as shown in Table 1. The C: N: P ratio of 100:2:0.2 was constituted using the total organic carbon available in the soil. DAP served as the sole source of phosphorus and a minor contributor of nitrogen, while urea served as the major source of nitrogen. Soil contamination was done by adding the appropriate amount of crude oil and mixing properly with the soil until a homogenous mixture was obtained. Table 1 shows the various groups and treatments.

Table 1. Soil Contamination with crude oil and treatments.

Groups	Crude oil added per kg of soil	Fertilizer treatment (nutrient supplement at C: N: P ratio of 100:2:0.2)	
		DAP (g)	UREA (g)
A	-	-	-
B	3 g	-	-
C	5 g	-	-
D	8 g	-	-
E	-	2.25	8.88
F	3 g	0.50	1.97
G	5 g	0.56	2.21
H	8 g	0.78	3.08

Group A, which served as the control, received neither crude oil nor nutrient supplements; it is an indicator of possible natural (changes not induced by pollution or nutrient supplementation) microbial, enzymatic, and physicochemical changes in soil separated from its natural habitat and kept under laboratory conditions. Groups B-D were contaminated with crude oil at concentrations of 3000, 5000, and 8000 ppm, respectively, but received no nutrient supplementation. They showed the course of enzyme activities that occurred in the polluted soil and will reflect the possibility of natural recovery of soil from pollution without external assistance in the form of bio-stimulation. Group E received only nutrient supplementation without crude oil contamination. It showed possible changes in the enzymatic properties of soils upon fertilizer application. Groups F-H received nutrient supplementation as well as crude oil contamination at concentrations of 3000, 5000, and 8000ppm, respectively, to reflect the effect of nutrient bio-stimulation on crude oil polluted soils. The

experiment lasted for 30 days during which each of the soil samples was assayed for activities of laccase, lipase, catalase, and peroxidase every 6th day.

2.4. Soil enzymes assays

2.4.1. Soil laccase activity

Laccase activity was measured using pyrogallol (25 mM) and an acetate buffer (50 mM, pH 5.0) [12-14]. The soil sample (1 g) was weighed into a 250 mL conical flask, and 50 mL of acetate buffer was added. The flask was incubated at room temperature for 1 h with vigorous shaking every 20 min. The volume of the buffer was increased to 125 mL, and the flask was shaken vigorously. An aliquot of 10 mL of the soil suspension was transferred to a centrifuge tube and centrifuged at 4000 rpm for 10 min. The supernatant was used for the enzyme assay. The tube containing 2mL of the supernatant and I mL substrate was incubated in the dark at room temperature for 1 h. The control sample containing 2 mL of supernatant and 1 mL of buffer, as well as the substrate control containing 1 mL substrate and 2 mL buffer, were incubated in the dark at room temperature for 1 h. The absorbance of the control and test samples were measured at 460 nm using a UV-Visible spectrophotometer.

$$\text{Laccase activity (µmol/h/g)} = \frac{A \times V1}{E \times V2 \times T \times W}$$

Where:

A = Net absorbance = Test – (sample control – substrate control)

V_1 = Volume of buffer used

E = Molar extinction coefficient for pyrogallol = 4.2 per µmol

V_2 = Volume of soil suspension

T = Substrate incubation time

W = Mass of soil sample

2.5. Data analysis

All data are expressed as mean ± SEM. The data were analyzed using Windows' SPSS statistics 23 software. ANOVA was used to compare the means, followed by Duncan's Multiple Range (DMRT). Statistical significance was taken as $p < 0.05$.

3. Results and discussion

Laccase, lipase, catalase, and peroxidase activities were assayed because they were synthesized by oleophilic microbes for the mineralization and use of hydrocarbon pollutants present in their habitat (soil). They serve as biomarkers of bioremediation of contaminated soils [7,8,17,18]. Table 2 shows the baseline enzyme activities of the soil (enzyme activity on day 1 of the experiment).

Table 2. Baseline enzyme activity of soil samples.

Parameter	Uncontaminated soil	Crude oil-contaminated soil			Treated soil (100:2:0.2)	Contaminated soil + Treatment (100:2:0.2)		
		3000 ppm	5000 ppm	8000 ppm		3000 ppm	5000 ppm	8000 ppm
Laccase (µmol/h/g)	0.11 ± 0.01	0.12 ± 0.01	0.16 ± 0.01	0.13 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.12[a] ± 0.01
Lipase (µmol FFA released/g)	0.16 ± 0.44	2.67 ± 0.33	3.33 ± 0.67	3.00 ± 0.58	0.31 ± 0.57	0.53 ± 0.07	0.93 ± 0.13	0.73 ± 0.07
Catalase (U/g)	0.62 ± 0.01	0.33 ± 0.00	0.28 ± 0.02	0.16 ± 0.00	0.73 ± 0.01	0.47 ± 0.02	0.44 ± 0.01	0.45 ± 0.02
Peroxidase(µmol/h/g)	0.94 ± 0.05	1.13 ± 0.05	1.19 ± 0.06	1.18 ± 0.09	1.39 ± 0.09	1.26 ± 0.01	1.54 ± 0.02	1.47 ± 0.01

Values are expressed as mean ± SEM (n = 3).

Table 3. Soil laccase activity.

Laccase Activity (µmol/h/g)						
GROUPS	**DAY 0**	**DAY 6**	**DAY 12**	**DAY 18**	**DAY 24**	**DAY 30**
A	0.11 ± 0.01	0.28 * ± 0.02	1.02 * ± 0.03	1.65 * ± 0.08	0.76 * ± 0.03	0.30 * ± 0.04
B	0.12 ± 0.01	0.19 ± 0.02	[a] 0.83 * ± 0.02	[a] 2.79 * ± 0.10	[a] 1.64 * ± 0.11	[a] 0.61 * ± 0.03
C	[a] 0.16 ± 0.01	0.37 * ± 0.04	[a] 1.24 * ± 0.10	1.70 * ± 0.04	1.00 * ± 0.08	0.42 * ± 0.05
D	0.13 ± 0.01	0.25 ± 0.02	0.91 * ± 0.02	[a] 2.66 * ± 0.06	[a] 1.52 * ± 0.08	0.28 ± 0.07
E	[a] 0.19 ± 0.01	0.17 ± 0.05	1.02 * ± 0.04	[a] 2.69 * ± 0.07	[a] 1.27 * ± 0.06	[a] 0.49 * ± 0.05
F	[a] 0.17 ± 0.01	[ab] 0.58 * ± 0.10	[ab] 2.13 * ± 0.02	[a] 2.65 * ± 0.07	[ab] 1.59 * ± 0.07	0.39 * ± 0.08
G	[ab] 0.15 ± 0.01	0.16 ± 0.02	[ab] 2.78 * ± 0.01	[a] 2.75 * ± 0.05	[a] 1.06* ± 0.10	[b] 0.31 * ± 0.03
H	[b] 0.12 ± 0.00	0.18 ± 0.01	[ab] 1.63 * ± 0.02	[a] 2.53 * ± 0.06	[a] 1.19 * ± 0.06	[b] 0.24 * ± 0.02

Effect of adding DAP and urea fertilizers at CNP concentration of 100:2:0.2 on Soil Laccase Activity. Superscript a indicates significant difference at $p < 0.05$ between the groups (A-H). Superscript b indicates significant difference at $p < 0.05$ between the groups (E-H), while superscript * indicate significant difference at $p < 0.05$ between the days. Values are expressed as mean ± SEM, (n = 3). The activity of an extracellularly synthesized oxidoreductase called laccase was observed to be on a steady rise in all soil samples from day 0 to day 18, after which a decline was observed, indicating an increase in oxidative enzyme activity that corresponds with decreasing contaminant concentration in the soil. Its highest and lowest activities were in group B (day 18) at 2.79 ± 0.10 and group A (day 0) at 0.11 ± 0.01, respectively. Within the contaminated groups B, C, D, F, G, and H, the laccase activity increased as contaminant concentrations rose from 3000 to 5000 ppm but declined at 8000 ppm, which may be an indication of one or more of several factors. Aromatic compounds of low molecular weight are able to significantly influence the growth and laccase activity of microorganisms by increasing laccase transcription. This result agrees with the report of Gupta *et al.* [19]. Another reason for laccase activity increase could be that laccase is extracellularly secreted by microbes in response to the presence of oil contaminants in its environment, thereby suggesting that as concentrations of the contaminants are reduced over time due to their degradation and utilization, there is a corresponding reduction in the synthesis, secretion, and activity of the enzyme in soil samples [7,14]. On the other hand, the reduction in laccase activity as the contaminant concentration increases from 5000 to 8000 ppm could be a result of the inhibition of the enzyme activity at high contaminant concentrations caused by increased toxicity of the soil and lower levels of aeration [20]. The similar values recorded across untreated and bio-stimulated groups could be an indication that the addition of DAP and urea to polluted soil did not improve the synthesis of laccase for any of the reasons discussed below. Firstly, laccase synthesis is strongly affected by the nature of nutrients and the quantity of nutrients present, especially nitrogen and trace elements in the growth medium; these reports are supported by the study of Viswanath *et al.* [21]. They stated that laccases were synthesized at not just high nitrogen concentrations but at a high ratio of carbon to nitrogen. Heinzkill *et al.* [22] earlier reported that a greater synthesis of laccase was recorded using nitrogen-rich media compared to the laccase yield resulting from the use of nitrogen-limited media. All these suggested that a very high concentration of nitrogen was needed to improve laccase synthesis. In this study, the limiting source of nitrogen used possibly resulted in the reduced laccase activity observed. Secondly, soil pH also critically contributes and plays a significant role in the synthesis and secretion of laccase by fungal microbes. The pHs of soil samples in this study were between 6.5 and 6.7, which is outside the laccase synthesis and secretion optimum pH range. This could also be a contributor to the less than optimum levels of laccase activity observed in this study. These results corroborate the results of past

research where the optimum pH needed for laccase synthesis in many fungi fell in the range of pH 5.0 and 6.0 [23,24]. Fungi cultures in a medium of pH 5 showed high levels of laccase activity [25]. Thirdly, temperature, a vital contributor to the growth of microbes, is stated to be most suitable for the synthesis of laccase at an optimal of 25 °C to 30 °C when cultures are placed in light or the dark, respectively [25]. The soil samples in this study exceeded this optimum temperature range, especially in the presence of light, possibly leading to a reduction in laccase activity during the course of this study. In group B, it was observed that laccase activity on days 0 and 6 was not significantly different. For group C, laccase activities were not significantly different on days 0 and 30, while in group D, laccase activities were not significantly different on days 0, 6, and 30. Similarly, bio-stimulated groups F, G, and H had activities on days 6 and 30 that were not significantly different. These results are clear indications that laccase may either be constitutively expressed or induced. The constitutive group does not readily react with dissolved compounds with properties similar to their substrates (xenobiotics and heavy metals recognizable by and specific for the promoter regions of genes encoding laccase that can bind to the recognition sites of the gene when present in the medium and induce laccase production), single inducers may produce the desired response for laccase synthesis, and a complex mixture of inducers may be required. Several compounds known as inducers may elicit a positive response on laccase production: metal ions; copper or cadmium; cycloheximide; low molecular weight aromatic or organic acids, such as veratric acid and ferulic acid; and other phenolic or aromatic compounds such as 2, 5-xylidine and veratryl alcohol [26-28]. Another important enzyme that plays very crucial role in bioremediation of soils is lipase. The activity of lipase was investigated in this study. The table below, Table 4, shows the activity of lipase in the untreated and treated soils during the study period.

Effect of adding DAP and urea fertilizers at CNP concentration of 100:2:0.2 on Soil Lipase Activity. Superscript a indicates significant difference at $p < 0.05$ between the groups (A-H). Superscript b indicates significant difference at $p < 0.05$ between the groups (E-H), while superscript *s indicates significant difference at $p < 0.05$ between the days. Values are expressed as mean ± SEM (n = 3). Lipase activity, expressed as µmol FFA released/g soil, was assayed throughout the period of this study as a marker for the reduction in toxicity level in bioremediation and an indicator of soil fertility [29,30]. Lipase is an enzyme responsible for the hydrolysis of glycerides and absorption of hydrolysis products into the microbe for metabolic processes. The activity of lipase increased in all groups during the experimental period (Table 4). Similarly, an increased rate of lipase activity during bioremediation over time was reported in a study carried out by Ogbolosingha *et al.* [31], where they showed that the lipase activity increased as the volume of crude oil (contaminant) increased relative to time, although the increment was more prominent in the soil samples remediated with *Arthrobacter species* than *Bacillus species*. The increase in the activity of lipase may be due to the abundance of the enzyme's substrate and the relative affinity of each substrate [32]. The significantly higher levels of lipase activity in group E compared to group A (control) were recorded in this study, possibly because of the higher microbial population that the added fertilizer induced. Reduction in the lipase activity as the contaminant concentration increased could be a result of the relative affinity of each substrate for the microbial enzymes. The non-growth substrate with high enzyme affinity will significantly decrease the rate of the growth substrate degradation, while a non-growth substrate with low enzyme affinity will have lesser effects, which corroborates the findings of Subhas and Robert [33]. In addition to competitive inhibition, unproductive binding of a non-growth substrate could cause uncompetitive inhibition or noncompetitive inhibition of growth substrate degradation. These reasons have also been corroborated by Ugochukwu *et al.* [32]. The activity of catalase was investigated in this study. The table below, Table 5, shows catalase activity in all soils studied.

Table 4. Soil lipase activity.

	Lipase Activity (µmol FFA released/g soil/min)					
GROUPS	**DAY 0**	**DAY 6**	**DAY 12**	**DAY 18**	**DAY 24**	**DAY 30**
A	^{a} 160.44 ^{u} ± 7.92	^{a} 260.33 ^{v} ± 3.59	^{a} 308.39 ^{w} ± 2.8	^{a} 430.66 ^{x} ± 2.31	^{a} 535.29 ^{y} ± 4.55	^{a} 627.30 ^{z} ± 7.63
B	^{b} 124.67 ^{u} ± 2.52	^{b} 136.42 ^{uv} ± 2.21	^{b} 177.28 ^{w} ± 2.06	^{b} 194.76 ^{x} ± 2.49	^{b} 210.79 ^{y} ± 3.02	^{b} 337.57 ^{z} ± 9.96
C	^{c} 102.97 ^{u} ± 2.79	^{c} 116.56 ^{v} ± 3.94	^{c} 139.24 ^{w} ± 2.04	^{c} 169.00 ^{x} ± 2.1	^{c} 190.41 ^{y} ± 3.57	^{c} 263.08 ^{z} ± 4.29
D	^{cd} 91.22 ^{u} ± 1.79	^{cd} 116.17 ^{v} ± 1.45	^{cd} 128.21 ^{w} ± 2.50	^{d} 145.53 ^{x} ± 2.68	^{cd} 177.28 ^{y} ± 1.90	^{d} 215.53 ^{z} ± 5.28
E	^{e} 313.78 ^{u} ± 5.79	^{e} 433.77 ^{v} ± 8.10	^{e} 561.48 ^{w} ± 5.23	^{e} 778.67 ^{x} ± 7.63	^{e} 837.00 ^{y} ± 5.13	^{e} 975.29 ^{z} ± 5.63
F	^{f} 283.78 ^{u} ± 5.39	^{f} 293.13 ^{uv} ± 2.12	^{f} 412.22 ^{w} ± 2.94	^{f} 605.11 ^{x} ± 6.48	^{f} 689.84 ^{y} ± 8.42	^{f} 764.67 ^{z} ± 32.68
G	^{ag} 174.72 ^{u} ± 5.62	^{ag} 250.76 ^{v} ± 5.4	^{g} 341.37 ^{w} ± 9.73	^{g} 411.13 ^{x} ± 3.46	^{ag} 527.50 ^{y} ± 9.3	^{ag} 630.30 ^{z} ± 5.1
H	^{bh} 138.22 ^{u} ± 8.3	^{h} 223.29 ^{v} ± 1.93	^{h} 260.69 ^{w} ± 7.8	^{h} 302.72 ^{x} ± 5.2	^{h} 318.70 ^{xy} ± 2.52	^{h} 427.44 ^{z} ± 9.00

Table 5. Soil catalase activity.

	Catalase Activity (U/g)					
GROUPS	**DAY 0**	**DAY 6**	**DAY 12**	**DAY 18**	**DAY 24**	**DAY 30**
A	0.62 ± 0.01	0.67 ± 0.03	0.74 * ± 0.01	0.66 ± 0.02	0.59 ± 0.01	0.52 * ± 0.00
B	^{a} 0.33 ± 0.00	^{a} 0.43 * ± 0.04	^{a} 0.45 * ± 0.02	^{a} 0.29 ± 0.02	^{a} 0.21 * ± 0.01	^{a} 0.15 * ± 0.02
C	^{a} 0.28 ± 0.02	^{a} 0.37 * ± 0.02	^{a} 0.45 * ± 0.01	^{a} 0.29 ± 0.00	^{a} 0.22 * ± 0.01	^{a} 0.15 * ± 0.02
D	^{a} 0.16 ± 0.00	^{a} 0.21 * ± 0.02	^{a} 0.23 * ± 0.01	^{a} 0.16 ± 0.01	^{a} 0.13 ± 0.00	^{a} 0.09 * ± 0.00
E	^{a} 0.73 ± 0.01	^{a} 0.77 ± 0.01	^{a} 0.91 * ± 0.04	^{a} 0.75 ± 0.02	^{a} 0.67 * ± 0.01	0.55 * ± 0.01
F	^{ab} 0.47 ± 0.02	^{ab} 0.55 * ± 0.02	^{ab} 0.59 * ± 0.01	^{ab} 0.44 ± 0.02	^{ab} 0.34 * ± 0.01	^{ab} 0.27 * ± 0.02
G	^{ab} 0.44 ± 0.01	^{ab} 0.54 * ± 0.02	^{ab} 0.68 * ± 0.01	^{ab} 0.45 ± 0.00	^{ab} 0.33 * ± 0.01	^{ab} 0.24 * ± 0.02
H	^{ab} 0.45 ± 0.02	^{ab} 0.51 * ± 0.03	^{ab} 0.65 * ± 0.03	^{ab} 0.58 * ± 0.02	^{ab} 0.43 ± 0.01	^{ab} 039 * ± 0.00

Effect of adding DAP and urea fertilizers at a CNP concentration of 100:2:0.2 on Soil Catalase Activity. Superscript a indicates significant difference at $p < 0.05$ between the groups (A-H). Superscript b indicates significant difference at $p < 0.05$ between the groups (E-H). Superscript * indicates the significant difference at $p < 0.05$ between the days. Values are expressed as mean ± SEM (n = 3). Catalase is responsible for the breakdown of hydrogen peroxide, a byproduct of aerobic respiration, into water and oxygen. It was observed in this study that catalase activity was significantly reduced after day 12 in all groups (Table 5). The reduction could be due to unfavorable conditions such as hypoxia, changes in pH, oxygen concentration, unavailability of nutrients, and a decrease in the microbial population [34-36]. The significantly lower catalase activity observed in un-remediated soils than in remediated ones was an indication of the effect of nutrient unavailability and oxygen concentration on catalase activity. Polluted soils, whether remediated or not, had low catalase activity when compared to the control and group E, indicating a higher microbial population in these groups (Table 5). These trends corroborate the studies of Achuba and Okoh [36] and Ogbolosingha et al. [31]. Soil catalase activity could also be influenced by other factors such as temperature and moisture content; it has been suggested to experience a resurgence in its activity as microbes adjust to their new environment [37].

Table 6. Soil peroxidase activity.

Peroxidase Activity (µmol/h/g)						
GROUPS	**DAY 0**	**DAY 6**	**DAY 12**	**DAY 18**	**DAY 24**	**DAY 30**
A	0.94 ± 0.05	1.52 * ± 0.02	2.13 *± 0.05	2.15 * ± 0.12	2.29 * ± 0.09	2.74 * ± 0.03
B	[a] 1.13 ± 0.05	[a] 1.51 * ± 0.03	[a] 5.75 *± 0.02	[a] 3.04* ± 0.03	[a] 3.17 *± 0.03	[a] 3.40 * ± 0.12
C	[a] 1.19 ± 0.06	1.60 * ± 0.03	[a] 5.68 * ± 0.00	2.51 * ± 0.06	2.42 * ± 0.03	2.56 * ± 0.06
D	[a] 1.18 ± 0.09	1.64 ± 0.02	[a] 5.60 * ± 0.02	[a] 3.81 * ± 0.41	[a] 3.18 * ± 0.16	2.85 * ± 0.18
E	[a] 1.39 ± 0.09	[a] 2.23 * ± 0.15	[a] 5.64 * ± 0.01	[a] 4.04 * ± 0.49	[a] 4.80 * ± 0.08	[a] 5.24 *± 0.05
F	[a] 1.26 ± 0.01	[b] 1.63 * ± 0.01	[ab] 6.19 * ± 0.02	[ab] 3.21 * ± 0.09	[ab] 3.29 * ± 0.03	[ab] 3.46 * ± 0.09
G	[a] 1.54 ± 0.02	[ab] 1.82 ± 0.01	[ab] 6.11*± 0.01	[a] 3.32 * ± 0.31	[ab] 3.86 * ± 0.06	[ab] 4.21 *± 0.03
H	[a] 1.47 ± 0.01	[ab] 1.79 ± 0.02	[ab] 5.51 * ± 0.00	[a] 3.24 * ± 0.22	[a] 4.61 * ± 0.16	[a] 5.26 *± 0.15

Effect of adding DAP and urea fertilizers at CNP concentration of 100:2:0.2 on Soil Peroxidase Activity. Superscript a indicates significant difference at $p < 0.05$ between the groups (A-H). Superscript b indicates significant difference at $p < 0.05$ between the groups (E-H), while superscript * indicates significant difference at $p < 0.05$ between the days. Values are expressed as mean ± SEM (n = 3). Peroxidase is a ubiquitous enzyme that catalyzes the oxidation of lignin and other phenolic compounds using hydrogen peroxide as an electron acceptor in the presence of a mediator. In this study, the activity of peroxidase decreased in all groups (B, C, D, E, F, G and H) after day 12, indicating a reduction in the TPH content of the soil (Table 6). Previous studies suggest that peroxidase synthesis and secretion are induced in response to oxidative stress caused by pollutants such as crude oil, where they play roles in lignin transformation and phenolic compound detoxification [8]. Therefore, the rapid rise in peroxidase activity from days 0 to 12 was an indication of oxidative stress caused by the TPH presence in the soil, while the subsequent decrease in activity indicated a reduction in the concentration of TPH, followed by a reduction in peroxide synthesis and secretion. Remediated soils (groups F, G, and H) (Table 6) showed higher peroxidase activity when compared to un-remediated soils (groups B, C, and D). And this was probably due to the higher microbial load in remediated soils compared to un-remediated ones. This hypothesis is based on the fact that peroxide is a ubiquitous enzyme in cells, performing several other functions such as immune system and hormonal regulation, auxin metabolism, suberin formation, cell elongation, and defense [38,39]. It suggested that an increased microbial population corresponded to an increased peroxidase presence, as seen in the peroxidase activity recorded in the control group during the experimental period.

4. Conclusions

This study demonstrated that soil polluted with crude oil could be remediated by the application of nutrients such as diammonium phosphate and urea fertilizers. Our findings suggested that the application of diammonium phosphate and urea enhanced the activities of enzymes responsible for biodegrading toxicants.

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