*Original Research*

# Bioremediation of Crude Oil-Polluted Soil Using Bacteria and Poultry Manure Monitored through Soybean Productivity

**Alfreda O. Nwadinigwe*, Ekene G. Onyeidu**

Department of Botany, University of Nigeria, Nsukka, Enugu State, Nigeria

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**Abstract**

Bioremediation of crude oil-contaminated soil was carried out using endogenous oil-degrading bacteria isolated from deliberately polluted soil and poultry manure. Isolates were subjected to morphological, physiological and biochemical tests and identified as *Pseudomonas* sp. and *Bacillus* sp. The results showed that the control (without crude oil treatment) significantly ($P<0.01$) produced the most pronounced result in germination, growth and productivity of the soybean plants used for monitoring bioremediation efficacy. This was followed by the consortium, *Pseudomonas*, *Bacillus*, nutrient broth, poultry manure, and crude oil ($P<0.01$ significance). This indicates that bioaugmentation with bacteria and biostimulation with poultry manure were remediative to crude oil-polluted soil, with bioaugmentation performing better than biostimulation.

**Keywords:** bacteria, bioremediation, crude oil, manure

## Introduction

Environmental pollution by petroleum is an increasing global problem. Crude oil-polluted soil may remain unsuitable for plant growth for months or years depending on the degree of contamination. Natural rehabilitation of crude oil-contaminated soils may take time to accomplish and because of high demand on land it may be difficult or impossible to allow polluted soils to go fallow or rehabilitate naturally [1]. Restoration of fertility to agricultural land previously polluted by oil is of great importance.

Biological systems have evolved natural ways of recycling and self-remediation. Among the current techniques available for decontaminating crude oil-polluted soils, bioremediation appears to be receiving greater emphasis. This may be due to the fact that bioremediation techniques do not leave any negative effects on the soil [2, 3]. Bioremediation may be regarded as a clean-up technology that uses naturally occurring micro-organisms to degrade hazardous substances into less toxic or non-toxic compounds [4]. It is the optimization of natural biodegradation in which micro-organisms chemically alter and break down organic molecules into other substances such as carbon dioxide, fatty acids, and water, in order to obtain energy and nutrients [5]. Bioaugmentation and biostimulation are two approaches to bioremediation geared toward enhancing and speeding up the process [6, 7].

Bioaugmentation involves the addition of external microbial population (endogenous or exogenous) to the polluted site. Bacteria are the most common bioaugmentation organisms [8]. Biostimulation involves the addition of appropriate microbial nutrients to a polluted site to increase nutrient and microbial activities of indigenous microbial flora [1, 7, 9].

Soybean is a global proteinous staple crop that has contributed to the nutritional and economic requirements of

*e-mail: fredanwad@yahoo.com

many nations, hence crude oil should not be allowed to spill on arable soils. However, in the case when a plant is growing in soil polluted with crude oil, efforts should be made to bioremediate the soil. There is the need to carry out more work on the bioaugmentation and biostimulation as part of bioremediation of crude oil-polluted plants. Bioremediation is important to oil-producing nations where oil spillage is rampant and often imparts devastating global environmental pollution. Bioremediation helps to mitigate climate change caused by pollution. So, knowledge gained from any bioremediative work can go a long way to relieving affected nations, distressed land owners, environmentalists, other stakeholders and indeed the whole earth, if applied. Not much comparative work has been carried out on biostimulation and bioaugmentation, and no bioremediative work has been monitored using soybean productivity. Moreover, no work has exhausted the isolation of microorganisms that might be involved in crude oil degradation. There is the need to have a pool of microbial strains with this degradative ability from which scientists can chose when studying this environmental pollution in greater detail.

The objective of this study, therefore, was to investigate the efficacy of bioaugmentation (using bacteria) and biostimulation (using poultry manure) in bioremediating crude oil-polluted soil. This was monitored through their influence on the vegetative and reproductive parameters of soybeans.

## Experimental Procedures

### Bacteria Isolation, Biochemical Tests, Characterization, and Identification

The bacteria used in this work were isolated from the top 1 cm sandy-loamy soil of the Botanical Garden, University of Nigeria, Nsukka that had been previously and deliberately contaminated with crude oil. Four grams of the contaminated soil were introduced into 20 ml of sterile nutrient broth [10]. Sterilization was carried out by autoclaving at 121ºC for 25 minutes (the sterile liquid media were stored in bijou bottles). They were incubated for 24 hours at 37ºC. Streaking was carried out on the solid nutrient agar in Petri dishes and incubated for 18 hours at 37ºC [11]. Organisms with distinct colony characteristics were isolated and sub-cultured on sterile nutrient agar plates, repeatedly, until pure colonies were obtained. The plates were placed in an incubator for 18 hours at 37ºC. To screen the isolates for hydrocarbon utilization, 1 ml of crude oil was added (as the only carbon source) to 4 ml of synthetic medium [12].

The synthetic medium was made up of 1% of NaCl, 0.042% $MgSO_4 \cdot 7H_2O$, 0.042% $NaNO_3$, 0.029% KCl, 0.083% $KH_2PO_4$, and 0.125% $NaHPO_4$, all in one liter of deionized water. The pH was adjusted to 7.4 with 0.1M NaOH. The cultures were incubated at 27ºC for five days and observed daily for biosurfactant production [13]. Two, out of eight distinct isolates showed the ability to utilize hydrocarbons and these two were used subsequently for characterization and identification. For characterization and identification of the isolates, the morphology, staining reaction, microscopic examination, motility test, growth at 41ºC and 4ºC, and biochemical tests were carried out according to Bergey's Manual of Determinative Bacteriology [14]. The results are in Table 1. The two organisms were identified as *Pseudomonas* sp. and *Bacillus* sp. and deposited in the culture collection facility of the Department of Microbiology, University of Nigeria[1].

Table 1. Results of biochemical tests and colony characteristics for identification of the bacterial isolates.

| Test | Result | |
|---|---|---|
| Gram stain | Gram positive | Gram negative |
| Endospore | + | - |
| Motility | + | + |
| Glucose | + | + |
| Trehalose | + | - |
| Sucrose | + | - |
| L-Rhamnose | + | - |
| D-ribose | - | - |
| D-Xylose | - | + |
| Oxidase | + | + |
| Catalase | + | + |
| Growth at 41ºC and 4ºC | - | + |
| Colony characteristics | White, raised, round and mucoid colony | Yellow-orange color, irregularly shaped with smooth edges |
| Possible identification | *Bacillus* | *Pseudomonas* |

**Inoculum build-up**: *Pseudomonas* was inoculated into bijou bottles, each containing 5 ml of nutrient broth and incubated at 37ºC. The bacterial count was carried out by measuring absorbance using a spectronic 20 spectrophotometer at an absorbance of 560 nm wavelength, until a cell concentration of $1.5 \cdot 10^8$ colony forming units (CFU)/ml (1 McFarland Standard) was achieved. The 5 ml culture was transferred into 1 liter culture nutrient broth. This was repeated using *Bacillus* instead of *Pseudomonas*. The bacterial consortium was prepared by mixing equal volumes (500 ml) of the culture of the above cell concentration of each isolate. These were used for field work.

**Field Work**: Topsoil (0-15 cm depth) from the botanical garden of the university, was used to fill 56 perforated black polythene bags (39,745 $cm^3$ average volume each). Each soil bag weighed 8 kg. Each soil surface was sprayed with 200 ml of crude oil, except the control. One of the soil

[1]In a subsequent work, DNA-based identification will be carried out on isolates *Pseudomonas* and *Bacillus* and published as an addendum.

Table 2. Vegetative parameters of soybeans planted in crude oil-polluted soil treated with bacteria, poultry, and nutrient broth.

| Treatment | Mean No. germ. | Mortality No. | Mean Height | Mean leaf area |
|---|---|---|---|---|
| Control | 2.9±0.1[a] | 1 | 57.7±0.8[c] | 82.3±0.9[e] |
| Crude oil (only) | 1.3±0.2[b] | 14 | 33.2±1.2[d] | 39.6±0.4[g] |
| Consortium | 2.8±0.2[a] | 2 | 56.3±0.6[c] | 79.8±0.5[e] |
| *Pseudomonas* | 2.4±0.3[a] | 5 | 54.1±0.6[c] | 74.0±1.4[e] |
| *Bacillus* | 2.3±0.4[a] | 6 | 53.5±0.5[c] | 73.1±1.5[e] |
| Nutrient broth | 1.5±0.3[b] | 12 | 42.4±6.1[d] | 55.8±8.1[f] |
| Poultry manure | 1.4±0.3[b] | 10 | 40.2±5.8[d] | 50.0±7.2[f,g] |

Values represent means ±standard error, except mortality number.
Mean values with the same letters in each column are not significantly different.
($P<0.05$)
No. germ. – number that germinated.

Table 3. Reproductive parameters of soybeans planted in crude oil-polluted soil treated with bacteria, poultry manure, and nutrient broth.

| Treatment | Mean no. of flowers | Mean no. of pods |
|---|---|---|
| Control | 18.1±0.6[a] | 15.5±0.8[e] |
| Crude oil (only) | 4.8±0.5[b] | 3.3±0.4[f] |
| Consortium | 15.6±0.5[a] | 13.0±0.8[g] |
| *Pseudomonas* | 14.0±0.8[c] | 10.5±0.7[h] |
| *Bacillus* | 14.3±0.6[c] | 10.1±0.7[h] |
| Nutrient broth | 8.9±1.4[d] | 7.3±0.6[j] |
| Poultry manure | 9.1±1.5[d] | 6.1±1.0[j] |

Values represent means ±standard error.
Mean values with the same letters in each column are not significantly different.
($P<0.05$)

bags sprayed with crude oil was contaminated with 1 litre culture ($1.5 \cdot 10^8$ cell concentration) of *Pseudomonas*. This was repeated using *Bacillus*, consortium, 1 L of nutrient broth, and 2 g of poultry droppings, respectively (instead of *Pseudomonas*). Nutrient broth was used to check its effect as a culture medium. One crude oil-contaminated soil bag was left without any treatment and one bag was left without crude oil contamination or any other treatment (control mentioned above). Each bag was mixed manually and the experiment was carried out in 8 replicates. The bags were kept under the sun and were watered by rainfall since the experiment was carried out during the rainy season. After two weeks, three soybean (*Glycine max* (L.) Merr.) (IT90K-277-2) seeds obtained from the university's Crop Science Department were sown in each bag after testing the seeds for viability.

Germination count, shoot height, leaf area, and the number of flowers and pods in each plant were determined. The emergence of plumule was taken as germination, which started 4 days after sowing. The maximum height and leaf area were recorded at the age of 60 days. The leaf area was determined using the formula: length × maximum width × 0.75 [15]. Pod count started at the age of 80 days. Color changes in the plants and death were observed. The soil used was analyzed for clay, silt, fine sand, coarse sand, organic carbon, organic matter, nitrogen, sodium, calcium, potassium, magnesium, phosphorus, pH, and cation exchange capacity, before the experiment, in the Department of Soil Science. After the experiment the crude oil- and the crude oil-polluted soil treated with consortium and poultry manure were also analyzed for these soil properties. The results were subjected to analysis of variance (ANOVA) and multivariate analysis was used to test the differences between means [16].

## Results

The results of the characterization and identification of the isolates showed that *Bacillus* and *Pseudomonas* were the micro-organisms that could utilize the hydrocarbons (Table 1). The results of the vegetative parameters (Table 2) showed that the control, the consortium (a combination of *Pseudomonas* and *Bacillus*), *Pseudomonas,* and *Bacillus* treatments gave the highest mean number of germination, mean plant height, and mean leaf area, while the nutrient broth, poultry manure, and crude oil (only) treatments produced the lowest of these vegetative parameters (significant at $P<0.01$). The control, consortium, *Pseudomonas* and *Bacillus* treatments were not significantly different from each other. Also, the nutrient broth, poultry manure, and crude oil (only) treatments were not significantly different from each other.

Soybean plants grown in soil treated with only crude oil showed yellowing of leaves two to three weeks after germination and produced the highest plant mortality of 14, while the other plants showed leaf yellowing 3 months after germination, that is after pod maturity. These other plants had lower mortality numbers (Table 2).

The results of the reproductive parameters (Table 3) showed that the control produced the highest mean number of flowers and pods per plant (significant at $P<0.01$).

Table 4. Analyses of some soil properties in crude oil, poultry manure, and consortium treatments.

| Soil Properties | Before the experiment | After the experiment | | |
|---|---|---|---|---|
| | | Crude oil treatment | Crude oil + Poultry manure treatment | Crude oil + consortium treatment |
| % clay | 16.72 | 16.64 | 16.20 | 16.67 |
| % silt | 2.0 | 2.7 | 3.1 | 2.9 |
| % Fine sand | 51.3 | 50.6 | 50.3 | 50.9 |
| % coarse sand | 29.98 | 29.86 | 29.89 | 29.92 |
| % organic carbon | 1.8 | 2.3 | 1.98 | 1.9 |
| % organic matter | 2.3 | 2.1 | 2.0 | 2.2 |
| % total Nitrogen | 0.15 | 0.07 | 0.53 | 0.7 |
| Sodium (ppm) | 0.36 | 0.38 | 0.38 | 0.41 |
| Calcium (ppm) | 3.4 | 3.2 | 3.3 | 3.2 |
| Phosphorus (ppm) | 0.29 | 0.18 | 0.21 | 0.23 |
| Potassium (ppm) | 0.05 | 0.09 | 0.06 | 0.07 |
| Magnesium (ppm) | 1.4 | 1.3 | 1.2 | 1.4 |
| pH | 6.4 | 4.4 | 5.3 | 6.1 |
| Cation Exchange capacity | 9.2 | 9.1 | 9.1 | 9.0 |

This was followed by the consortium treatment. Crude oil (only) produced the lowest mean number of flowers and pods per plant ($P<0.01$ significant). The mean number of flowers and pods produced by *Pseudomonas* and *Bacillus* treatments were not significantly ($P<0.01$) different from each other. Also, the mean number of flowers and pods produced by nutrient broth and poultry manure treatments were not different from each other. The plants grown in soil treated with only crude oil started flowering from 9 to 10 weeks old, while the rest started flowering earlier, from 7 to 8 weeks.

## Soil Properties

The results of soil properties (Table 4) indicated an increase in percentage nitrogen in soils treated with the consortium (0.7%) and poultry manure (0.53%) when compared with 0.15% nitrogen before the experiment. There was a decrease in % nitrogen (0.07%) in the soil treated with only crude oil. pH decreased from 6.4 before the experiment, to 6.1 for consortium, 5.3 for poultry manure and 4.4 for crude oil (only). Phosphorus also decreased in the soil treated with only crude oil but comparatively increased in the soils treated with poultry manure and consortium.

## Discussion

In the present work, the consortium, *Pseudomonas,* and *Bacillus* treatments demonstrated bioremediating capabilities since their effects on germination, height, leaf area, and the production of flowers and pods in soybean were not significantly different or close to those of the control (without crude oil pollution). Thus, this bioaugmentation with bacteria produced bioremediating effects. The toxic components of the crude oil might have been metabolized by these bacteria, which might have produced enzymes that could use petroleum as a substrate, thus degrading the crude oil. This agrees with the suggestion of Onwurah [17], who reported that crude oil-degrading bacteria such as *Pseudomonas*, *Micrococcus*, and *Bacillus* could metabolize the toxic components of crude oil, leading to its degradation. Odokuma and Inor [18] reported that bioaugmentation with *Bacillus* and *Azotobacter* increased the germination and height of *Phaseolus vulgaris* in oil-polluted soil.

The comparatively high enhancement of the vegetative and reproductive parameters of soybean by the consortium, *Pseudomonas,* and *Bacillus* in the present investigation, might also have been as a result of increases in the population and microbial activities of indigenous hydrocarbon-degrading organisms. Coates and Anderson [19] stated that the appropriate specific pollutant-degrading micro-organisms are always present at the polluted site, but at a low concentration. The stimulation of the biodegrading potential of these indigenous microbial communities through bioaugmentation with mixed bacterial cultures (consortium) had been reported [6, 17, 18]. Studies on bacteria sequencing indicated that some groups of bacteria that commonly occur in crude oil-contaminated environments are in syntrophic association [8]. Banat [20] also reported that the organic compounds in crude oil could be metabolized by oil-degrading bacteria to release secondary substrates that could support the growth and activities of other bacteria. This bacterial association may be the reason for the higher productivity recorded in plants treated with the consortium when compared with plants treated with single cultures of *Pseudomonas* and *Bacillus* in the present work.

Both poultry manure and nutrient broth combated the adverse effects of crude oil pollution, but not as much as the consortium, *Pseudomonas* and *Bacillus*, as regards the vegetative and reproductive parameters in the present investigation. This is because the productivity of soybeans treated with poultry manure and nutrient broth (biostimulation) was higher than that of crude oil alone, but lower than that of the bacteria. The relative effectiveness of this biostimulation may be caused by increased nutrient supply, which is usually insufficient or unavailable to plants in crude oil-contaminated soil. This agrees with the work of Nwadinigwe and Ezeamama [9], who reported that biostimulation with poultry manure increased nutrient supply to *Senna obtusifolia* and combated the adverse effects of crude oil pollution on its germination and productivity. Poultry manure improves the soil structure by cementing the soil particles together [7]. Danffonchio et al. [21] reported that biostimulation leads to metabolic activation of some functional oil degraders that may respond to treatment at a slow rate.

Treatment with only crude oil adversely affected the vegetative and reproductive parameters in the present investigation. Hence, crude oil pollution should not be tolerated in any environment. This is similar to the work of Nwadinigwe and Onwumere [22], who reported that crude oil, kerosene, and petrol significantly inhibited the germination, growth, and productivity of soybeans. Ogboghodo et al. [1] also reported that crude oil affected the growth and productivity of *Zea mays*. Ayotamuno and Kogbara [23] suggested that the reduction in yield as a result of petroleum product pollution was due to interference with water and nutrient supply of the crop, rather than any direct toxic effect. Agunwamba et al. [3] reported that in crude oil-polluted soils, oil fills up the air spaces in the soil, thereby excluding oxygen and water from being available to the plants. This interference with oxygen, water, and nutrient supply was combated by the addition of bacteria, poultry manure, and nutrient broth in the present studies. The micro-organisms in the present work might have degraded the toxic components of the crude oil, added nutrients, and increased oxygen and water availability to the soil. The non-availability of nutrients in soil polluted with crude oil (without any treatment) might have contributed to the yellowing of the leaves earlier than those that received treatment in the present work. This agrees with the report of Ogboghodo et al. [1], who observed that the yellowing of leaves of *Zea mays* was due to nutrient deficiency in crude oil-polluted soils. In the present investigation, the highest mortality was recorded for plants treated with only crude oil. This agrees with the work of Nwadinigwe and Uzodinma [24], who reported the wilting and death of some groundnut seedlings polluted with crude oil.

In the present investigation, decreases in nitrogen, phosphorus, and pH recorded for the soil treated with only crude oil might have contributed to the retardation of growth and productivity in the soybeans planted therein. Nitrogen and phosphorus are among the most important mineral elements needed for growth and productivity in plants. High acidity may not favour the germination, growth and productivity of the soybean. Similar observations were reported by Ogboghodo et al. [1], who stated that there were decreases in nitrogen and pH in crude oil-polluted soil. In the present study, poultry manure might have supplied nitrogen and phosphorus to the soil, hence the increase of the two elements in poultry manure-treated soil. Since phosphorus is needed for plant growth and productivity, the increased phosphorus supplied by poultry manure might have been absorbed by soybeans for growth and productivity. Moreover, the presence of crude oil added to the poultry manure might have stimulated the plant to carry out even greater absorption of phosphorus to compensate for the adverse effects of the crude oil. In the case of increased nitrogen in the consortium, the bacteria might have degraded the crude oil and encouraged atmospheric nitrogen fixation by micro-organisms.

## Conclusion

The present investigation showed that the use of bacteria (bioaugmentation) and poultry manure (biostimulation) were remediative to crude oil-polluted soil. Also, bioaugmentation performed a better bioremediation than biostimulation. Bioremediation with a mixed culture of *Pseudomonas* and *Bacillus* (consortium) was more efficient than a single culture of *Pseudomonas* or *Bacillus*. Testing with a combination of bioaugment and biostimulant will be carried out in future works.

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