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Biotreatment of Crude Oil Contaminated Soil

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Biodegradation of hydrocarbons by microorganisms represents one of the primary mechanisms by which petroleum and other hydrogen pollutants are eliminated from the environment. This work was carried out on the effect of microorganisms on the biotreatment of oil in crude oil contaminated soil. Microorganisms were isolated from two experimental soil samples contaminated with Bonny Crude and normal uncontaminated soil as a control over a period of seven months. The microbial as well as the physico-chemical parameters of the soil samples were all analyzed using standard methods. Changes in total petroleum hydrocarbon level were measured appropriately. Treatments used were the microbial isolates.

Forty-four microorganisms were isolated from the contaminated soils and identified as species of *Pseudomonas* (7), *Flavobacterium* (6), *Bacillus* (8), *Proteus* (4), *Klebsiella* (1), *Pencillium* (5), *Aspergillus* (7), *Fusarium* (3), *Trichypton* (2) and *Neurospora* (1). Ten of the forty-four isolates had ability to degrade crude oil in the laboratory. On contamination a value of $1.0X10^5$ cfu/g in microbial counts were obtained followed by a subsequent increase in population levels after a period of 2months with a value of $1.0X10^6$ cfu/g. Oil application to the soil resulted in an increase in total petroleum hydrocarbon from 0.31 ppm to 5.53 ppm; organic matter from 0.41% to 7.34%; available phosphorus from 1.75 ppm to 2.84 ppm. The treatment measures all showed progressive decrease in oil concentration in the soil. Mixture of bacterial and fungal isolates as a treatment measure proved to be more favourable above all others, it brought the concentration from 5.53 ppm to 0.31

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ppm after a period of 5 weeks of treatment, which is same value with the normal soil (uncontaminated).
Species of *Pseudomonas, Bacillus, Flavobacterium, Proteus, Klebsiella, Penicillium, Aspergillus, Fusarium, Trichyphyton* and *Neurospora* had potential for the degradation of bonny crude oil. They could therefore be employed in environmental cleanup of petroleum spill site.

Keywords: Biodegradation; petroleum spill site; bioaccumulation; treatment.

1. INTRODUCTION

1.1 Crude Oil Chemical Composition and Its Classification

Oil is an exceedingly complex substance composed literally of thousands of different kinds of organic molecules. It varies in composition depending on age as well as conditions of its formation. Despite the complexity and variability of crude oil, some generalizations about its composition can be made. According to [1], crude oil is a naturally occurring, unrefined petroleum product composed of hydrocarbon deposits and other organic materials. [2], say it consists of hydrocarbons of various molecular weights and other organic compounds, it constitute mainly of parrafins (30-50%), cycloparrafins (20-50%) and aromatics (6-14%). It also contains concentration of metals such as nickel, aluminium, magnesium, iron, cobalt, zinc, gold, mercury, chromium, molybdenium and lead.

Petroleum contains hundreds of individual compounds and their components are generally grouped into four classes according to their differential solubility in organic solvents: The saturates (N and branched chain alkanes and cycloparrafins), the aromatic compounds containing alkyl side chains and (or fused cycloparrafin rings), the resins (aggregates with a multitude of building blocks such as pyridines, quinolines, carbazoles, thiophenes, sulfoxides, and amides), and the asphaltenes (aggregates of extended polyaromatics, naphthenic acids, sulfides, polyhydric phenols, fatty acids and mettalophyrins) [3].

1.2 Environmental Pollution Due To Petroleum Hydrocarbon Spillage and Fate in Soil

The impact of petroleum prospecting and production operations has produced ecological problems of great dimensions. The significance of any given spill is dependent on the amount of oil spilled and on the impact on the environment. But the transmission of the oil from the point of contamination to other points depends on many factors, which includes the spill volume, hydrocarbon viscosity, temperature, wind speed, land contouring, rainfall, extent of cultivation, fluctuations in the level of water table, and the nature of the hydrocarbon and soil [4].

Spilled oil has deleterious effects on flora and fauna of the ecosystem. The economic life of the people in the affected areas is usually disrupted, such as farmlands, navigational activities and fishing efforts as well as the disruption of the eco-balance in the affected ecosystem. Also, pollution of the environment due to accidental seepages, rupture of pipelines, blow out of terrestrial oil wells and sabotage has been reported [5,6]. The resulting spillages have brought about economic losses as well as contamination of the aquatic and terrestrial ecosystems.

The soil microflora generally responds to changes induced due to petroleum hydrocarbon spillage, such as a rapid increase in size of hydrocarbon metabolizing portion of the community, possible concomitant increase in the non-hydrocarbon utilizing population, inhibition of ATP production, dehydrogenase activity, nitrogen fixation and microbial respiration [7]. Therefore to reduce the hazardous effect of petroleum hydrocarbon, their control and treatment strategies are required.

1.3 Treatment and Degradation of Petroleum Hydrocarbon Contaminated Soil

The problem of petroleum hydrocarbon contaminated soil could be solved by removal of the contaminated soil or reclean in site.

A variety of techniques are available for the physiochemical treatment of hydrocarbon-contaminated soil, this includes flooding; excavation; thermal disruption and incineration

[7]. Also, the use of seeding or biodegradation, which shows a promising level of success have been reported [8]. Other techniques include use of straw or plant material as an absorbent for oil; biosurfactants to clean oiled surfaces [9] and addition of material to encourage microbiological biodegradation of oil [10]. Others have produced materials even in commercial and patented products.

Bioremediation, which is the productive use of microorganism to remove or detoxify pollutants, usually as contaminants of soils, water or sediments that otherwise threaten public health [11] is one of the other biological methods for treatment of all pollutants.

Biodegradation is the degradation caused by biological activities especially by enzymatic action, leading to a significant change in the chemical structure of the exposed material and resulting in the production of carbon dioxide water, mineral salts,(mineralization) and new microbial cellular constituents (biomass) [12]. [13] define biodegradation in relation to petroleum and chemical changes in parent hydrocarbon to petroleum and chemical changes in parent hydrocarbon of spilled oil usually accompanied by a reduction in pH, but the product are not necessarily harmless.

The breakdown of hydrocarbon mixtures depends on the nature of the petroleum hydrocarbon, nature of the microbial community, and on a variety of environmental factors which influences microbial activities [14]. Microbial degradation involves the interactive effects of mixed populations of microorganisms and relies on the metabolic versatility of bacteria and other microbes.

However, many reports on laboratory testing of the biodegradation of crude oil have not been tested in field conditions because laboratory products tests may not be attained within the natural environment where complex physical, chemical and biological interactions occur.

Hence, this study examines the responses of the indigenous microbial communities in experimentally crude oil contaminated soils, assess the changes in soil physico-chemical properties and evaluate some biological treatments with a view to assessing their usefulness in biotreating soil contaminated with crude oil.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Two experimental plots (1 m by 1 m each) were mapped out at the nursery unit of the department of microbiology, University of Ibadan. One of the plots was deliberately contaminated with 400 mls of Bonny crude oil and the second plot served as the control plot, which is the uncontaminated reference plot. The oil was thoroughly mixed with the upper 15 cm of the soil and tillage was done repeatedly for aeration every other day throughout the experimental period. Soil samples were collected at regular intervals (7 days), the collection was made at random on each of the plot before they were taken for physico-chemical analysis and collection was also taken monthly for microbial analysis.

2.2 Soil Analysis

Physico-chemical analysis: Uncontaminated and contaminated soil samples with crude oil were analyzed for their physico-chemical properties. Parameters such as pH in water, % organic Carbon, Exchangeable Cations (Na, k, Ca, and Mg), total nitrogen and heavy metals were all analysed using standard methods used by [15]. Total petroleum hydrocarbon was according to the method used by [16].

2.3 Microbial Analysis

Isolation and Identification Organisms: Isolations of all the isolates were by the method of [17]. 1 ml inoculum was aseptically transferred into a sterile petri-dish and pour-plated with the appropriate agar medium, nutrient agar for bacterial isolation and potato dextrose agar for fungi isolation. The plates were incubated at room temperature (27°C±2°C) for 24 hours in the case of bacterial isolates, while fungal isolates were incubated for 3 to 5 days. Morphological appearances of the inoculated plates were observed and distinct colonies were subcultured to obtain pure isolates which were then maintained on nutrients agar and potato dextrose agar slants for bacterial and fungal isolates respectively and preserved at 4°C.

Bacterial isolates were identified using morphological procedures and biochemical tests, with reference to Bergey's Manual of Systematic Bacteriology [18].

The isolated fungi were identified according to their micromorphology as well as colour, morphology of sporulating structures, reference was made to compendium of soil fungi.

2.4 Total Oil Degraders Count (TOD)

Total oil degraders counts were carried out on the contaminated experimental plot and the control plot. The agar medium used was the modified oil agar medium of [19]. The oil agar medium consist of basal (mineral salt) medium which consists (g/l) of K_2HPO_4, 1.8; K_2HPO_4, 1.2; NH_4cl, 4.0; $MgSO_4.7H_2O$, 2.0; NaCl, 0.1; $FeCl_2.4H_2O$, 0.05; yeast extract, 0.1; and trace elements H_3BO_3,0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.05; and $MnSO_4.H_20$, 0.04. Two percent of agar was added to the basal medium before autoclaving, after which 2ml of crude oil sterilized through membrane pore filtration as sole carbon source was added to the basal medium. Trace elements solutions was also sterilized separately before a small portion of it was added to the basal medium and mixed together aseptically.

Serial dilution method was carried out and pour plate method was used and the plates were incubated at 35°C, visible bacterial colonies were counted after seven days of incubation. Growths of fungi degrading the oil were also seen after the incubation at room temperature for seven days.

2.5 Testing the Bacterial and Fungal Isolates for Crude Oil Utilization

Oil agar was used for testing according to a modified method of [19]. Fifteen millitre of molten sterile oil agar was aseptically poured into each petri dish and allowed to solidify. Testing of each isolate was done by streaking a portion of the colony (using a sterile inoculating loop) from a previous activated 24 to 48 hours culture of the isolate. Bacterial ability to utilize the oil was indicated by the plates on which bacteria grew. Plates on which fungi grew in the case of fungal isolation were also taken as indicators of the fungal ability to utilize crude oil. Pure cultures of each isolate capable of utilizing crude oil were subjected to various microbiological tests to determine their probable identity.

Bacterial isolates were identified using morphological procedures and various biochemical tests. The result of each test was recorded and the probable identity of the isolate determined using Bergey's Manual of systematic Bacteriology [18].

Fungal isolates were identified according to their micromorphology, as well as colour and morphology of sporulating structures, glass slides preparations were done using lactophenol blue [17]. Microscopic examination of prepared slide was done using low power objective, followed by 40 X magnification objective lens. The probable identity was determined using compendium of soil fungi.

2.6 Biotreatment Experiment

Soil bag preparation: Soil was collected at a site located at the nursery unit of the department of microbiology, university of Ibadan. The soil was collected to a depth of 10 cm different points on the site, the soil samples were then mixed together thoroughly. Soil textural class was determined to be loamy sand.

Seed bags were used as experimental units, they were 16 cm by 12 cm. 500 g each of five soil samples were weighed and then, thoroughly mixed with 10 mls each of the crude oil, one of the samples served as the experimental control and one of the samples was not contaminated at all with crude oil, it serves as the control. The remaining three (3) samples were treated with various treatments.

2.7 Preparation of Inocula, Treatments and Seeding

Test isolates were selected based on their ability to grow well on oil agar medium. Five bacterial and five fungal isolates each a mixture of the cultures were used for the study.

Bacterial and fungal isolates were first cultured in 100 ml erlenmeyer flasks containing nutrient and potato dextrose broths at room temperature and at 100 rpm in a shaker for 48 hours (so as to increase the inoculum size).

The various treatment measures used were as follows:

- Seed bag 1 was treated with 20mls of the bacteria isolated, designated as (B).
- Seed bag 2 was treated with 20mls of the fungi isolated, designated as (F)
- Seed bag 3 was treated with 40mls of the mixture of bacteria plus fungi designated as (B=F).

All the samples were duplicated. Changes in total petroleum hydrocarbon level and physico-chemical analysis were carried out at two weeks interval for two months.

3. RESULTS AND DISCUSSION

Forty-four microbial isolates were obtained from the contaminated soil; these isolates were identified for bacteria as species of *Pseudomonas, Flavobacterium, Bacillus, Proteus* and *Klebsiella* while for fungi as species of *Penicillium, Aspergillus, Fusarium, Trichypton* and *Neurospora* all these are shown on Table 1 in their frequency of occurrence. Several researchers have also reported similar isolates from petroleum hydrocarbon contaminated soil samples [14,20,21,22].

Table 1. Frequency of occurrence of bacterial and fungal isolates from artificially-contaminated soil

Isolate name	Frequency of occurrences
Pseudomonas sp	7 (15.71)
Flavobacterium sp	6 (13.64)
Bacillus sp	8 (18.18)
Proteus sp	4 (9.09)
Klebsiella sp	1 (2.27)
Penicillum sp	5 (11.36)
Aspergillus sp	7 (15.91)
Fusarium sp	3 (6.82)
Trichypton sp	2 (4.55)
Neurospora sp	1 (2.27)
Total	44 (100)

Values in parentheses represent percentage of occurrence

Table 2. Physico-chemical analysis of soil sample (field study)

Parameters	Sample A Uncontaminated soil			Sample B Contaminated soil		
	1wk	4wks	7wks	1wk	4wks	7wks
pH	7.40	6.10	6.50	7.30	6.10	5.80
Calcium (mg/kg)	3.12	1.43	1.20	7.30	2.00	2.00
% Nitrogen	0.30	0.14	0.10	0.41	0.34	0.40
TPH (PPM)	1.80	1.78	1.50	6.50	4.55	4.10
Sulphur (mg/kg)	9.50	9.23	8.70	22.40	24.70	24.10
Magnesium (mg/kg)	0.48	0.46	0.35	0.38	0.45	0.48
Av.P (ppm)	1.89	1.83	1.80	3.18	3.10	3.00

Overall Temperature range 29-31^{o}C; Key: 1, 4, 7, wks. – Numbers of weeks on analysis; TPH (ppm) – Total Petroleum Hydrocarbon (parts per million); Av.p – Available phosphorus

Table 3. Physico-chemical analysis of some heavy metals in soil sample (Field Study)

Parameters	Sample A Uncontaminated soil			Sample B Contaminated soil		
	i	1	7	I	1	7
Copper (ppm)	11.00	13.00	9.00	17.00	14.00	10.00
Lead (ppm)	7.00	7.00	6.00	10.00	6.10	6.00
Iron (ppm)	8.00	9.00	6.00	12.00	10.00	8.00
Chromium (ppm)	6.00	8.00	7.00	10.00	7.00	8.00
Cadmium (ppm)	6.20	7.00	7.00	9.00	6.10	6.00
Nickel (ppm)	9.00	7.00	8.00	9.00	7.00	6.00

Key: Ppm – parts per million; i - Day soil was contaminated; 1, 7- Numbers of week on analysis

Table 4. Total petroleum hydrocarbon in Ppm present in soil sample (Field Study)

Sample	i	Day of sample analysis						
		1	2	3	4	5	6	7
A	1.82	1.80	1.72	1.82	1.78	1.68	1.53	1.50
B	6.78	6.50	5.98	4.73	4.55	4.50	4.30	4.10

Key: A – Uncontaminated soil sample; B – Contaminated soil sample; i – Day of contamination
1 2 3 4 5 6 7 – Numbers of weeks on analysis

Table 5. Physico-chemical parameters of polluted soil sample

Sample	Parameters					
	pH	TPH (ppm)	OM (%)	%OC	%N	Av.p (ppm)
NS	6.20	0.31	0.41	0.24	0.024	1.75
CS	6.00	5.53	7.34	4.26	0.426	2.84

Key: NS - Normal soil (day 0); CS - Contaminated soil (day 0)

Table 6. Total culturable Heterotrophic Bacterial (Thb) and total culturable Fungal Count (Thf) in soil

Sample A, B/THB, THF, TOD	Season /Day/Cfu/g			
	Day 1	1 month	2 months	7 months
A (THB)	$1.1x10^5$	$5.2x10^5$	$2.8x10^5$	$3.4x10^4$
B (THB	$1.0x10^5$	$4.8x10^5$	$1.1x10^6$	$2.4x10^4$
A (THF)	$2.1x10^4$	$5.2x10^4$	$5.6x10^4$	$2.5x10^3$
B (THF)	$2.3x10^5$	$5.4x10^4$	$5.8x10^4$	$1.8x10^3$
A (TOD)	$1.0x10^5$	$1.0x10^5$	$1.0x10^5$	$1.7x10^4$
B (TOD)	$6.1x10^4$	$1.2x10^5$	$1.3x10^5$	$1.8x10^4$

Key: A – Uncontaminated soil sample; B – Contaminated soil sample; 1,2,7 – Days of Culturing

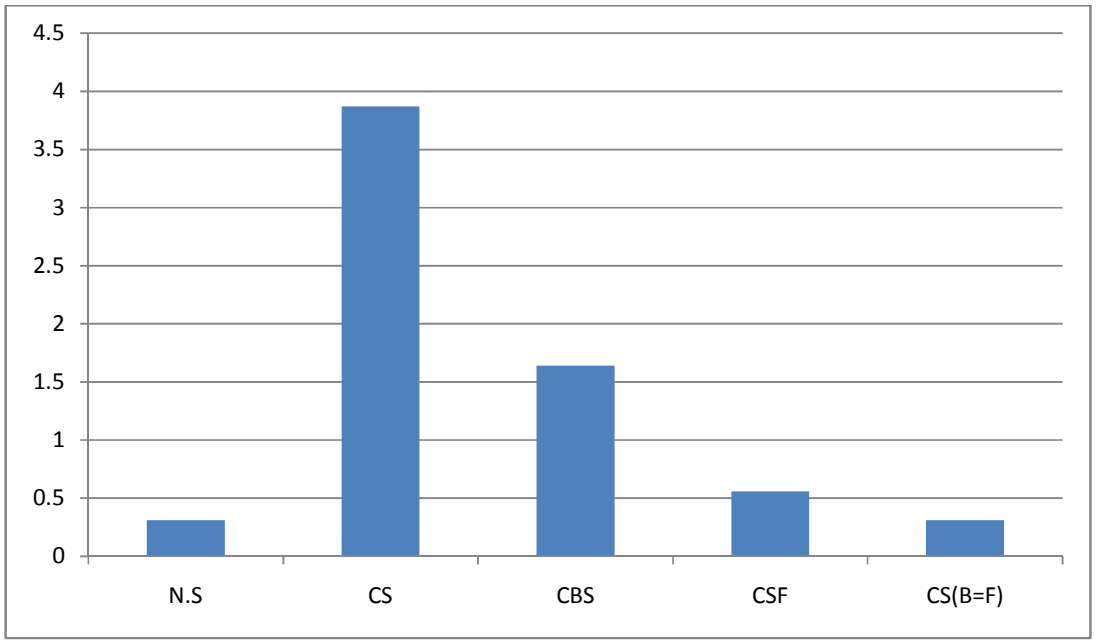

Fig. 1. Effect of various treatments on the total petroleum hydrocarbon in the contaminated soil sample in the soil bags after five weeks

Key: N.S – Normal, uncontaminated soil. CS – Contaminated soil. CBS – Contaminated soil treated with the Bacterial isolates. CSF – Contaminated soil treated with the Fungal isolates. CS(B=F) – Contaminated soil treated with the mixture of Bacterial and Fungal isolates

The physico-chemical analysis of soil samples taken from the field study are shown on Table 2, the pH values for the contaminated soil decreased over the 7 weeks period of contamination from 7.30 on the first week to 5.80 on the seventh week of contamination, this corroborates the work of [23], who found that oil contamination decrease both surface and subsurface soil pH. [24], 2013 however observed that crude oil contamination significantly increases the soil pH up to 8.0 but on the other hand reduced available phosphorus concentration. The oil may have had some direct impact in lowering the pH, it is more likely that

production of organic acids by microbial metabolism is responsible for the difference. Calcium present in the soil also decreases from 7.30 mg/kg to 2.00 mg/kg (first week to seventh week). Table 3 also shows some heavy metals in the soil sample, there were changes in the measures of the different parameters from the day of contamination to the 7th week of contamination. The presence of heavy metals in some environment has attributed to petroleum prospecting and mining as well as oil spills [25].

Changes in physico-chemical and microbiological properties of soil after the addition of hydrocarbons as observed in this investigation seem to be a universal phenomenon. As seen in tables 4 and 5, the deliberately contaminated soil sample contained higher amount of total petroleum hydrocarbon 6.78 PPM compared to the uncontaminated soil sample in the case of the biotreatment experiment. This showed that petroleum hydrocarbon can also be found in an environment that is not contaminated with crude oil. This was in accordance to the report of [26] that hydrocarbon are produced biogenically from the decay of organic material and thus, have origins from sources other than crude oil and petroleum deposits.

Soils contaminated with petroleum product have been observed to show large increase in organic matter, total carbon and nitrogen compared with uncontaminated soils. [27,28], similar observations were made in this study. This was probably due to effect of contamination with petroleum hydrocarbon.

The increase in nitrogen content of oil-contaminated soils could be attributed to the activities of nitrogen-fixing bacteria whose presence have been reported in oil-polluted soils [29]. In situ nitrogen-fixing capabilities of heterotrophic hydrocarbon-degrading bacteria have also been demonstrated [29]. The increase observed in available phosphorus in oil contaminated soils might be due to the existence of reducing conditions at the experimental sites that made iron phosphates more soluble and which brought some phosphorus into solution. The positive correlation between nitrogen content, available phosphorus and the population density of hydrocarbon utilizers in contaminated soils observed in this study corroborated the observation of other workers which promoted the use of N+P fertilizers to increase the rate of oil biodegradation in polluted soils [30,31].

Decrease in microbial numbers in soils immediately after oil application as seen in Table 4 is not an unusual occurrence and has been attributed to the toxic effect or other unfavourable conditions which may occur as a result of the introduction of the oil [19]. The gradual increase in microbial population counts after the initial repression could indicate the adaptation of the organisms to the new environment. In addition, the pollutant oil could have stimulated the growth of the adapted strains. Similar population increases have been demonstrated in soils from temperate and arctic climatic regions using gaseous hydrocarbons and mineral oils [32].

Treatment of the contaminated soil with the mixture of bacteria and fungi favoured the biodegradation of the oil above all others. Similar evidence has been found that microbial degradation involves the interactive effects of mixed populations of microorganisms and relies on the metabolic versatility of bacteria and other microbes [33].

Pinholt et al. [34] observed an increase from 60% to 80% in oil utilizing fungi and an increase from 3% to 50% in oil degrading bacteria during oil decomposition in soil after a fuel oil spill. This can be reflected in this work, which shows that the contaminated soil treated with fungal isolates alone favoured degradation than that treated with bacteria alone.

The trend of degradation observed in this study were as follows, most degraded normal soil {NS (control 1), contaminated soil, treated with bacterial and fungal isolates (CS(B+F)), contaminated soil, treated with fungal isolates (CSF), contaminated soil, treated with bacterial isolates (CSB), and the least degraded being the contaminated soil, (CS (control 2)}.

4. CONCLUSION

Many reports on laboratory testing of biodegradation of crude oil may not have been fully tested under field conditions because laboratory products tests may not be attained within the natural environment where complex, chemical and biological interactions occur. That notwithstanding bioremediation is still an ongoing process in the environment. As observed in this work, the test isolates identified as species of *Pseudeomonas, Flavobacterium, Bacillus, Proteus* and *Klebsiella* for bacteria while for fungi as species of *Penicillium, Aspergillus, Fusarium, Trichypton* and *Neurospora* all these isolates as

well as the mixture of the bacterial and fungal isolates had potential for degrading bonny crude oil as seen in the pilot field study as well as the soil contaminated experimental bags in this study. These isolates could be molecularly characterized for better employment in the treatment of crude oil contaminated sites.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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