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# Comparative efficiency of different biological techniques in the remediation of petroleum oil polluted soil

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## Abstract

*Spillage of petroleum hydrocarbons affect soil quality with negative consequences on profitable agriculture. Among remediation techniques, bioremediation is relatively non-invasive, efficient and less expensive. Bioremediation efficiency is influenced by the applied techniques, materials, type and the amount of inoculum. This study aimed to evaluate the efficiency of different biological techniques in the remediation of petroleum oil- contaminated soil. The identity of indigenous petroleum utilizing fungi in soil and cow fecal matter samples were determined using the 16S rRNA gene sequencing. Soil was spiked with crude oil (10%w/w) and treated with 5% and 10% NPK for bio-stimulation, formulated microbial consortia for bioaugmentation, combined treatments and monitored for crude oil loss for sixteen weeks using weight loss method. Autochthonous crude oil degrading fungal isolates were Meyerozyma sp. S8CF-18(KP072797.1), Penicillium citrium F14 (MG711907.1), Aspergillus tubingensis Hoba5-41(KC020122.1), A. terreus (KR704571.1) and A. tubingensis MSEF76 (KT310980.1) from cow dung and A. brunneoviolceus F9P2SF19 (MK035983.1) and A. aculeatinus NG202 (MT0237141.1) from soil sample. Results revealed crude oil removal of 27.90% in the control sample, 46.61% and 49.88% respectively for bacterial and fungal consortia and 64.76% and 72.15% for samples amended with 5% and 10% NPK respectively. Combined treatment of polluted sample with microbial consortium, NPK and cow dung showed remarkable increase of 70.74 - 89.83% and 78.38 - 94.39% crude oil removal efficiency over bioaugmentation and bio-stimulation as standalone strategy. This study showed that bioaugmentation coupled with bio-stimulation is the most promising bioremediation technique.*

**Keywords:** Bio-stimulation, Bioaugmentation, Cow dung, Crude oil, Microbial consortia, NPK.

## Introduction

Spillage of petroleum hydrocarbons of varying magnitude is common in the oil producing areas of Nigeria, causing enormous environmental challenges and concerns. Agricultural lands are degraded and become wastelands and unfit for productive agriculture, thereby affecting the economy and health of the people and residents of communities as a result of increased unemployment and escalated poverty resulting in unimaginable protests against government and oil multinationals.

The consequences of crude oil pollution of the environment demand that contaminated sites are returned to their pristine status through a process called remediation with the primary intention of removing or destroying pollutants or modifying them to less harmful forms. Bioremediation strategies remove or transform environmental pollutants by the use of desired living organisms with capabilities of utilizing the contaminant as carbon and energy source, thereby detoxifying or removing the pollutant from the environment. This biological approach proves to be eco-friendly, non-invasive and cheaper relative to the physicochemical methods which apart from being expensive, may also create other environmental problems[1,2].

Among the factors affecting the efficiency of bioremediation process include the quality, type as well as the population of indigenous microorganisms, the nature of xenobiotics, level of nutrients, aerobic conditions, pH, moisture content and other soil properties[3]. Contaminant removal without human intervention (natural attenuation) usually takes a long time due to the population of degrading microorganisms (about 10%) in soil relative to the total heterotrophic microbial population[2]or the quantity and nature of pollutant spilled.

Two foremost approaches to achieve bioremediation of oil spill sites are bio-stimulation and bioaugmentation. In bio-stimulation, indigenous hydrocarbon degraders are stimulated by supplementation with nutrients rich materials such as inorganic fertilizers or fertilizers of organic origin such as animal droppings or biowastes[4-6] whereas in bioaugmentation, known degraders are applied to synergize with the existing soil autochthonous population.

Bioaugmentation is normally achieved by using microbial species that can be cultured and isolated from soils or other carrier materials such as sludges, composts, cow dung, animal wastes or genetically engineered microorganisms (GEMs).

However, the diversity of microbial species present in the natural ecosystem suggests that certain microbes may not be culturable including a large population of microorganisms that have potentials for pollutant degradation. These carrier materials containing the degraders may be added directly to the contaminated soil and then transfer their indigenous microbial population and yet maintain their capacity to degrade pollutants[7].

Therefore, enhancement of degradation either by stimulation of indigenous microbes or addition of exogenous microorganisms is a goal that deserves attention. This research is undertaken to determine the relative efficiency of different *unitalicize* biological options and their combination as strategies of bioremediation in order to restore the soil original and useful values as a step to ameliorate the negative consequences of pollution on farmlands, farmers and residents of oil producing area where crude oil spill is a frequent menace to the economy, health and life of the people.

## Materials and Methods

**Soil and Cow dung:** The experimental soil collected from Ido-Ani ($7^o17'0"$ N, $5^o$ 52'0" E) and cow dung from Shasha, Akure, both in Ondo State, Nigeria were used. Hand auger was used to collect the samples at 15-20 cm depth into separate sterile black cellophane bags. The cow dung was used as stimulant and an inoculum carrier.

**Crude oil sample and NPK:** Standard-grade crude oil with specific gravity of 0.835 was used while NPK (20:10:10) was obtained from Agro Investment Store, Akure, Nigeria.

**Isolation of crude oil - degrading fungi from samples:** Bushnell- Hass mineral salt medium (MSM) supplemented with 1.2% agar was used. The pH of the medium was adjusted to 5.6, autoclaved at 121°C for 15 mins and then fortified with 50mg/L of streptomycin and supplemented with 2% (v/v) of 0.45µm filter -sterilized crude oil to serve as the only source of carbon and energy. One gram (1g) of each sample was rehydrated with 10 ml of previously sterilized distilled water and then serially diluted to the $6^{th}$ dilution and 1 mL from each dilution was separately pour- plated onto sterile Petri dish and overlaid with MSM–Agar and then incubated at $28^oC\pm2^oC$ for 21days. Colonies of crude oil degrading fungi that developed on the MSM Agar were purified on Potato Dextrose Agar (PDA) containing 1 % streptomycin and incubated for 7 days at $28^oC\pm2^oC$. The culture plates were observed for growth and pure cultures were maintained.

**Identification of crude oil utilizers from samples:** Eight purified fungal cultures with crude oil utilization potentials were selected from preliminary screening of polluted agricultural soils and cow dung. The identity of purified fungal isolates from samples was confirmed using the 16S rRNA gene sequencing.

**Extraction of fungal genomic DNA:** The Cetyl Trimetyl Ammonium Bromide (CTAB) method was used for the extraction of genomic DNA from 18 hr old broth culture previously transferred onto eppendorf tubes and then centrifuged at 14,000 revolutions per minute (rpm) for 2 minutes[8].

The supernatant was thereafter carefully disposed and the DNA extraction was performed accordingly[9]. This was then followed by the resuspension of the DNA pellets in 100 µl of distilled water sterilized by autoclaving. The spectrophotometer regulated at 260 nm and 280 nm wavelength was used to measure the concentration of DNA and then determine the purity of the genome. The DNA quality and integrity by size fractionation on 1.0% agarose gel was determined using the Agarose gel electrophoresis and then visualized with UV light source.

**PCR analysis:** MJ Research PTC-200 (model) Thermal Cycler was used to perform the PCR using ITS 1 and ITS 4 primers. The PCR mix was composed and PCR profile are as described[8,]

**16S rDNA products Gel Electrophoresis:** Analysis of PCR products were conducted on 1% buffer solution of Trisbase, acetic acid and EDTA (TAE) agarose gel and then stained for 45-60 min at 80 V using ethidium bromide and the bands were observed using the UV light and thereafter photographed with Kodak UV imaging system.

**PCR products Purification:** Sodium acetate (2M) washing method was used to further purify the amplicon for sequencing following the procedures described [8]. The purified and air-dried amplicon was thereafter transferred into 5µl of previously sterilized distilled water and maintained at $4^oC$ for sequencing.

**Sequencing of DNA:** Sequencing ofpurified gene products (16S rRNA)were thereafter conducted and then followed by cycle sequencing in the forward and backward directions using primers. The PCR mix and profile (rapid profile) and cocktail mix used for the sequencing were as detailed[8]. Sequencing running followed after the resuspension of the clean PCR in previously sterilized distilled water. The DNA sequence (ABI 3100 model) was then loaded with samples and the nucleotide data released in form A, C, T and G were noted.

**Evolutionary Tree Construction:** The sequences of nucleotides obtained were compared with other sequences based on the NCBI BLAST tools. Phylogeny was inferred using the UPGMA method[10] and all the sequences obtained were aligned using the MEGA7 software with the elimination of all positions containing gaps and missing data and evolutionary distances were computed using the Maximum Composite Likelihood method[11]. Thereafter, the evolutionary analyses were conducted MEGA7[12]. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

**Selection of microorganisms for bioaugmentation:** Four bacterial and fungal isolates were selected from those that exhibited best degradative ability based on their optical density at 540nm[13].

**Preparation of microbial consortia:** Bacterial consortium: *Escherichia coli*, *Klebsiella edwardsii*, *Pseudomonas aeruginosa* and *Pseudomonas pseudomallei.* Fungal consortium: *Paecilomycesvariotii*, *Kodamaea ohmeri*, *Cephalosporium* and *Aspergillus glaucus*. Formulation of consortia: Bacterial and fungal consortia were prepared according to the methods given in literatures[14,15].

**Bioremediation experiment:** Two hundred and forty grams (240 g) portion of partially air-dried, sieved and mechanically homogenized soil in plastic bucket was spiked in triplicate with 10% (w/w) crude oil and then thoroughly mixed using sanitized spatula and moisture content adjusted to 25% (w/w) with sterilized water and left for 7 days for acclimatization[4].

The experimental outlay is as shown below. Inoculation with bacterial and fungal consortia was according to the method of Otokunefor *et al.*[16]. The cow dung and NPK were dissolved in the sterile water for moisture adjustment. The experimental buckets were left uncovered, incubated at $28^oC \pm 2^oC$ and stirred twice a week for thorough mixing of nutrients with the contaminated soil and provide the aeration necessary for microbial activities and then moistened weekly with 10ml sterile distilled water[17]. The amount of crude oil remaining in each bucket determined 7 days after contamination as day 0 [18] and then fortnightly for 16 weeks.

**Experimental outlay:** Experimental outlay are as fellows
Soil (240g) + crude oil as control (unamended)
Soil (240g) + crude oil + Bacterial consortium (40ml)
Soil (240g) + crude oil + Fungal consortium (40ml)
Soil (240g) + crude oil + NPK (5 % w/w)
Soil (240g) + crude oil + NPK (10% w/w)
Soil (240g) + crude oil + Bacterial consortium (40ml) + cow dung (10% w/w)
Soil (240g) + crude oil + Bacterial consortium (40ml) + NPK (10% w/w)
Soil (240g) + crude oil + Fungal consortium (40ml) + Cow dung (10% w/w)
Soil (240g) + crude oil + Fungal consortium (40ml) + NPK (10% w/w).

**Determination of crude oil loss:** The quantity of crude oil in sample was estimated by the weight loss method with n- hexane as extraction liquid[19]. The crude oil loss is calculated as follows:

$$\% \text{ Crude oil degraded} = \frac{\text{weight of crude oil degraded}}{\text{Original weight of crude oil added}} \times 100$$

Where the weight of crude oil degraded was measured as original weight of crude oil minus weight of residual crude oil after evaporating the extractant.

**Statistical analyses:** The One - way Analysis of Variance (ANOVA) with SPSS version 18.0 was used to analyze data obtained while means were compared by Duncan's Multiple Range Test (DMRT) at 95% confidence level values.

## Results and discussion

Figure-1 shows the phylogenetic tree of indigenous fungi isolated from soils and cow dung while Table-1 is the complete nucleotide blasts of ITS genes of indigenous crude oil utilizing fungi from soil and cow fecal matter. ITS analysis revealed the identities of the autochthonous crude oil degrading fungal isolates as *Meyerozyma* sp *S8CF-18(KP072797.1), Penicillium citrium F14 (MG711907.1), Aspergillus tubingensis Hoba5-41(KC020122.1), A. terreus (KR704571.1)* and *A. tubingensis MSEF76(KT310980.1)* from cow dung and *A. brunneoviolaceus F9P2SF19(MK035983.1)* and *A. aculeatinus NG202 (MT0237141.1)* from experimental soil sample.

The fungal genera were among the crude oil degrading fungi reported by researchers Odeyemi[3] and Das, N. and Chandran, P.[5] with *Aspergillus* being dominant in the samples. Figure-1 shows the tree with the highest log likelihood (-10558.83) and the percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Jukes-Cantor model, and then selecting the topology with superior log likelihood value.

A total of 17 sequences were involved in this analysis and the final dataset revealed a total of 1315 positions. On phylogeny, all selected accessions segregated into two strongly supported clusters composed of six stable clades in all, as appraised based on bootstrap supports[20].

All except the clades of CF1 and AF1 were members of *Trichomaceae*. The observation of clusters formed four stable phylogenetic clades, three of which included members of the genus *Aspergillus* and *Penicillium* and gather six of the newly identified strains (Figure-1). CF4 is located in a strongly supported clade with *Aspergillus tubingensis* strain MSEF76 and it was 88.69% similar to *Aspergillus terreus*. CF5 is also clustered in the same clade with 97.93% similarity to *A. tubingensis* strain MSEF76 (Table-1). CF1 was closely related to *Penicillium citrium* strain F14 in a well supported a clade (100% bootstrap support). CF3 was 95.83% similar to *Aspergillus tubingensis* strain Hoba 5-41 and was sub-clustered in the same group as CF4 and CF5 which is strongly supported by a bootstrap value of 99%. The phylogeny also confirmed the placement of strain SF2 in *Aspergillus* clade (100% bootstrap support) as part of a well-supported group (99% bootstrap support) with strain CF2-CF5 (Figure-1). Although strain SF2 belongs to this same clade, however, SF1 is paraphyletic to SF2 and is observed to be 97.32% similar to *Aspergillus aculeatinus* strain NG202.

CF1 was clustered in the same clade with *Meyerozyma sp* strain S8CF-18 with a strong bootstrap support of 100%. Consistent with this observation Yurkov, A. M. et al.[21] have also confirmed that newly identified strains join the *Meyerozyma* clade on a bootstrap support of 100% when analyzed using the same method.

The strain AF1 was clustered in the same clade with *Kodamaea ohmeri* strain AMC_K0002 on a strong bootstrap support similar to the *Meyerozyma* clade. The genus *Kodamaea and Meyerozyma* are member of the *Saccharomycetaceae*[22,21]. This may explain the proximity of both clade on the phylogeny compared to the broad clade of *Aspergillus* and *Penicillium* genus. Overall, CF2-CF5, and SF1-SF2 share more similarity. On the other hand, AF1 and CF1 are more associated than the other newly identified strains. The percentage of crude oil removed during the 16 weeks of study for bioaugmentation and bio-stimulation is shown in Table-2. Results revealed that the fungal consortium caused higher crude oil removal of 49.88% over the 27.90% and 46.61% in control and sample bioaugmented with bacterial consortium respectively.

At 10% (w/w) amendment with NPK, higher crude oil removal efficiency (72.15%) was observed against 64.76% for 5% (w/w) of NPK.

Table-3 shows a combination of bio-stimulation and bioaugmentation as treatment options. Treatment of the polluted soil with bacterial consortium and 10% (w/w) of supporting materials; cow dung (PSCDB) and inorganic fertilizer (PSNPKB) revealed enhancement of degradation at 89.83% and 70.74% for samples with NPK and cow dung respectively. Similarly, treatment of the polluted soil with fungal consortium and 10% (w/w) of supporting materials; cow dung (PSCDF) and inorganic fertilizer (PSNPKF) increased degradation from 72.15% with NPK alone to 94.39% when fungal consortium was combined with NPK (Table-2, 3). In all cases, samples treated with fungal consortium yielded higher degradation (49.88%, 78.38% and 94.39%) than those with bacteria (46.61%, 70.74% and 89.83%). Samples treated with NPK also showed higher efficiency (89.83% and 94.39%) than with cow dung (70.74% and 78.38%).

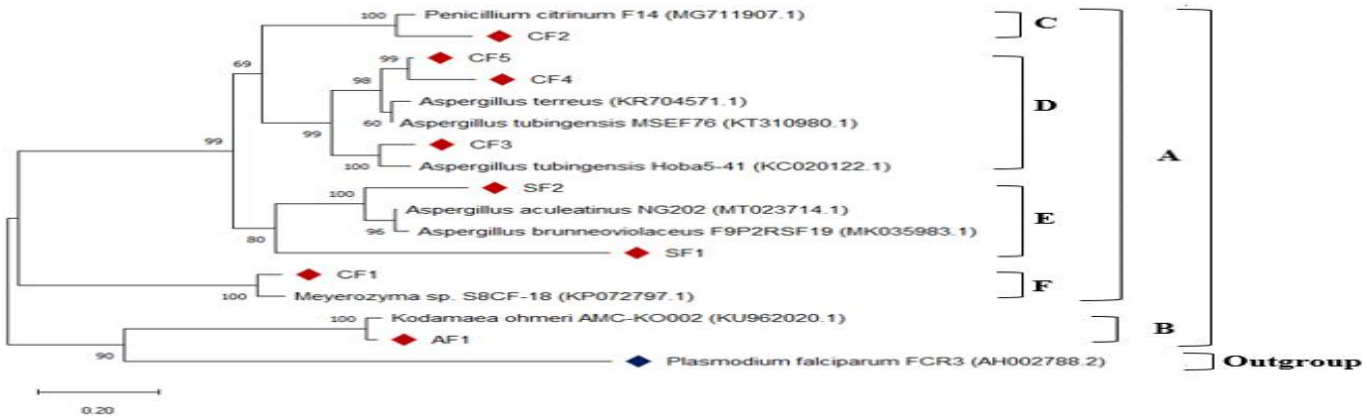


**Figure-1:** Phylogenetic analysis of ITS gene of crude oil degraders by maximum likelihood-method. The numbers at the node indicate bootstrap support (%) on the basis 1000 replication. The scale bar indicates 0.20 nucleotide substitution for each nucleotide position. Accession numbers from the GenBank are shown in parenthesis.

**Table-1:** BLAST results of identified indigenous crude oil degrading fungi from samples.

| Organism Name | Strain | Barcode | Max Score | Total score | Percentage identity (%) | Accession |
|---|---|---|---|---|---|---|
| *Kodamaeaohmeri* | AMC_K0002 | AF1 | 922 | 1974 | 96.45 | KU962020.1 |
| *Aspergillus brunneoviolaceus* | F9P2RSF19 | SF1 | 161 | 161 | 73.48 | MK035983.1 |
| *A. aculeatinus* | NG202 | SF2 | 754 | 754 | 97.32 | MT023714.1 |
| *Meyerozyma*sp. | S8CF-18 | CF1 | 1022 | 1233 | 91.22 | KP072797.1 |
| *Penicillium citrium* | F14 | CF2 | 959 | 959 | 96.27 | MG711907.1 |
| *Aspergillus tubingensis* | Hoba5-41 | CF3 | 1144 | 1567 | 95.83 | KC020122.1 |
| *A. terreus* | - | CF4 | 893 | 893 | 88.69 | KR704571.1 |
| *A. tubingensis* | MSEF76 | CF5 | 1002 | 1002 | 97.93 | KT310980.1 |

Legend: AF1, SF1-SF2= Crude oil degrading fungi from soil; CF1-CF5 = Crude oil degrading fungi from cow dung.

**Table-2:** Percentage crude oil removal by bioaugmentation and biostimulation.

| Period | Control | Bioaugmentation | | Biostimulation | |
|---|---|---|---|---|---|
| WKS | UPS | PSB | PSF | NPK (5%w/w) | NPK (10% w/w) |
| 2 | 1.79 ± 0.01[a] | 5.37 ± 0.02[a] | 6.37± 0.00[a] | 9.04±0.00[a] | 10.99±0.01[a] |
| 4 | 3.91 ± 0.01[b] | 10.12±0.04[b] | 10.77 ±0.02[b] | 13.55±0.01[b] | 18.09±0.01[b] |
| 6 | 8.31 ± 0.01[c] | 16.09± 0.03[c] | 16.80 ±0.01[c] | 22.17±0.01[c] | 29.26±0.01[c] |
| 8 | 13.39 ± 0.01[d] | 24.16 ±0.00[d] | 25.65±0.02[d] | 32.40±0.01[d] | 41.13±0.10[d] |
| 10 | 20.17 ±0.01[e] | 32.64±0.01[e] | 34.56±0.01[e] | 44.26±0.01[e] | 54.74±0.00[e] |
| 12 | 23.36 ±0.01[f] | 38.97± 0.01[f] | 41.55±0.02[f] | 53.46±0.04[f] | 62.85±0.01[f] |
| 14 | 26.03 ± 0.01[g] | 43.28± 0.01[g] | 46.06±0.03[g] | 59.87±0.87[g] | 68.38±0.02[g] |
| 16 | 27.90 ±0.01[h] | 46.61 ±0.17[h] | 49.88±2.45[h] | 64.76±0.07[h] | 72.15±0. 01[h] |

Legend: UPS; untreated polluted soil (control), PSB; Polluted soil + Bacterial consortium, PSF; Polluted soil+ Fungal consortium, NPK (inorganic fertilizer).

**Table-3:** Percentage crude oil removal by combination of bioaugmentation and biostimulation.

| Period | 10% (w/w) Treatment | | + | Microbial Consortium | |
|---|---|---|---|---|---|
| (Wks) | PSCDB | PSNPKB | | PSCDF | PSNPKF |
| 2 | 10.98±0.00[a] | 11.15±0.01[a] | | 11.29±0.43[a] | 12.35±0.43[a] |
| 4 | 18.10±0.01[b] | 23.44±0.02[b] | | 18.63±0.02[b] | 25.32±0.02[b] |
| 6 | 27.05±0.03[c] | 36.95±0.01[c] | | 27.84±9.01[c] | 38.97±0.01[c] |
| 8 | 37.24±0.01[d] | 51.70±0.03[d] | | 39.28±0.01[d] | 53.37±0.09[d] |
| 10 | 51.25±0.01[e] | 67.39±0.05[e] | | 54.48±0.11[e] | 68.44±0.11[e] |
| 12 | 59.02±0.01[f] | 77.51±0.01[f] | | 63.79±0.08[f] | 78.85±0.01[f] |
| 14 | 65.97±0.03[g] | 84.54±0.02[g] | | 72.07±0.02[g] | 87.76±0.01[g] |
| 16 | 70.74±0.01[h] | 89.83±0.02[h] | | 78.38±0.02[h] | 94.39±0.02[h] |

Legend: PSCDB; Polluted Soil + Cow dung + Bacterial consortium, PSNPKB; Polluted Soil + NPK fertilizer + Bacterial consortium, PSCDF; PSCDB; Polluted Soil + Cow dung + Fungal consortium PSNPKF; Polluted Soil + NPK fertilizer + Fungal consortium.

This study revealed that although petroleum contaminant can be removed from the soil environment without human intervention, but it will take relatively long time. The results corroborate the submission of Dzionek *et al*.[2] that contaminant removal through natural attenuation usually takes a long time because of the relatively low number (about 10%) of degrading microorganisms in soil. This result implies that the presence of crude oil degraders in then environment alone may not guaranty effective pollutant removal as their number and types may not be sufficient to warrant speedy contaminants removal. The results revealed the treatments resulted in enhanced attenuation of crude oil with time but vary with treatments options when compared with the untreated polluted sample. Crude oil degradation in terms of amount of crude oil loss in soil sample was higher in samples bioaugmented with fungal consortium than bacterial consortium.

The findings are similar to that of Machida and Gomi[23] that in the ecosystem, consortia of organisms from different kingdoms attack different substrates at different rates. The use of microbial consortia and cow dung also produced higher efficiency of crude oil removal over the control. The cow dung takes advantage of indigenous degraders which are otherwise non-culturable, but possess the appreciable metabolic ability to take part in petroleum hydrocarbon utilization. The result corroborates the reports of Ikuesan *et al.* [24] which reported cow fecal matter as carrier of crude oil degraders. This implies that carrier materials transfer hydrocarbon degraders directly without reducing their population or capacity to metabolize pollutants. Thus, using cow dung in which indigenous crude oil degraders have been enriched has advantage of time and cost needed for isolation which may not be successful.

This report agrees with the report of Kim *et al*.[25] that the use of crude oil degraders in a proportion greater than that of the indigenous population ultimately enhanced biodegradation. This study also infers that mixed microbial consortium from exogenous sources synergized with the indigenous microorganisms to speed up degradation. This assertion agrees with the report of Diaz-Ramirez *et al.*[26] and Venosa and Zhu[27] that successful bioremediation of hydrocarbon contaminated environments depend largely on the native microbial population or applied exogenous microorganisms with capabilities to biodegrade the pollutants. The findings in this report suggest that the isolates constituted into consortium (bacterial and fungal) synergized with the autochthonous degraders to achieve extensive biodegradation. The better performance by fungal consortium may therefore be attributed to increase in acidity with time which favored the proliferation of fungi than bacteria.

It also corroborates the preferential use of fungi over bacteria suggested by Adenipekun *et al*.[28] stating that the use of fungi in remediation of polluted soil was preferred because fungi have the lignin degrading enzyme system which acts extracellularly. Fungi have also been reported as providing food for many soil organisms[23]. The amount of crude oil biodegraded agrees with the report of Das and Chandran[5] which suggested that the efficacy of biodegradation vary between soil fungi and bacteria with soil fungi having higher efficiency (6%-82%) than soil bacteria (0.13% - 50%).

The effects of varying concentration of supporting materials; cow dung (CD) and inorganic fertilizer (NPK) were substantial. Crude oil removal at 10% (w/w) treatment with NPK was higher than in 5% (w/w). This increase could be due to increased provision of nutrients for microbial growth and activity. This result agrees with that of Machida and Gomi[23] that the availability of sufficient nitrogen, phosphorous and other inorganic nutrients result in maximum decomposition of substrates. Similarly, soil samples amendment with NPK fertilizer caused higher crude oil degradation than those with cow dung. This implies that NPK stimulated the indigenous microbial population through the provision of additional nutrients for enhanced bio stimulatory activity. The enhancement of degradation by cow dung relative to control samples may imply that cow dung provides supplemental nutrients, especially nitrogen and phosphorous for the growth and survival of autochthonous crude oil degrading soil microbes as well as exogenous microorganisms that evolve into acclimated consortium to bioaugment the activities of indigenous microorganisms in degradation.

Onuoha *et al*.[18] earlier reported similar results which indicated that the enhancement properties may be due to additional nutrients in the inoculum materials which the degraders readily utilize. These results also support the report of Odokuma and Dickson[29], that availability of nutrients such as nitrogen and phosphorous appears to be the most important limiting factor in biodegradation. Although, the application of NPK inorganic fertilizer to bioremediate crude oil polluted soil has the advantage of relative efficiency, however, it is considered to be a more expensive procedure as it may increase soil acidity thereby requiring other treatments such as liming to ameliorate its effects. In contrast, the cow fecal matter is widely and readily available at almost cost - free with the potential to provide supplemental carbon as energy source for cellular activity and also acts as inoculum carrier and often used as agricultural manure.

## Conclusion

Petroleum hydrocarbon contaminant removal from soil is possible without human intervention. This study revealed that removal of petroleum pollutant is not determined by the presence of native microorganisms alone but also by enhancement of degradation through addition of exogenous microbial population inoculated directly or transferred through supporting materials containing them as well as amendment with nutrient- rich materials.

Although, this research is a laboratory study, it can be applied on field scale with cow dung to remediate crude oil contaminated agricultural soils since the inoculated cow dung already carry consortium of crude oil degraders, thereby having reduced chances of competition from soil indigenous microbial consortia. Any preferential use of NPK fertilizer should be accompanied with the determination of its optimal level that would be required as uncontrolled use of NPK would increase soil acidity and become deleterious to soil microbes when applied in high concentration.

Therefore, a stringent control of the concentration of the stimulating material within the effective range would be necessary if NPK or other form of inorganic fertilizer should be successful as strategy to remediate petroleum contaminated soil. However, results suggest that bioaugmentation coupled with bio stimulation is the most promising bioremediation technique for the restoration of petroleum contaminated soil.

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