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Enhanced Bioremediation of Brass Crude-Oil (Hydrocarbon), Using Cow Dung and Implication on Microbial Population

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ABSTRACT: The present study has used soil samples from Nigeria, contaminated with Brass crude-oil, to determine its biodegradation through enhanced biostimulation with cow dung and periodic aeration. Over a period of twenty-eight days, the hydrocarbon-utilizing bacteria (HUB) and hydrocarbon-utilizing fungi (HUF) have been counted and identified. Results from biodegradation of the brass crude-oil over the aforementioned period show that amended crude-oil-spiked soil has had 54.82% degradation while for amendment and periodic turning this has been 55.90%, not significantly higher than the former at $p \leq 0.05$. Also degradation of spiked soil without cow dung amendment has been 16.13%. The identified HUB are *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis,* and *Streptococcus thermophillus,* with individual occurrence of 18.52% as well as *Proteus vulgaris* and *Micrococcus luteus* with 11.11% and 14.81% occurrence, respectively. Also, the occurrence rate of HUF like *Aspergillus flavus, A. niger, Penicillium chrysogenum, Trichothecium roseum,* and *Penicillium citrinum* have been 15.63% each; while for *Alternaria alternata* and *Neurospora crazza* it has been 6.25% and for *Saccharomyces cerevisae* and *A. fumigatus,* 9.38% and 3.13%, respectively. The study concludes that amendment with cow dung and periodic turning of the soil enhance degradation of Brass crude-oil significantly. What is more, aeration by periodic turning slightly improves degradation only with cow dung treatment on Days 21 and 28.

Keywords: Bacteria, Biodegradation, Organic amendment, Crude-oil, Fungi.

INTRODUCTION

Release of crude-oil into the soil always distorts its both physical and chemical characteristics (Udeh *et al*., 2013). Also reports show that the presence of crude-oil in soil hinders proper aeration, since the oil film serves as physical obstacle between air and soil, thus causing a distortion in soil texture (Atuanya, 1987). Due to the fact that crude-oil is a complex combination of hydrocarbon and non-hydrocarbon compounds, it has varied effects on soil micro-organisms (Udeh *et al*., 2013). These detrimental effects are due to the altered soil redox potential ratio, which increases soil pH, leading to suffocation and toxicity of soil biota (Udeh *et al*., 2013).

Several bioremediation techniques have been developed to enhance degradability of crude-oil (hydrocarbon) in the soil. One of these techniques is biostimulation, which involves supplying nutrients for the soil in order to promote the capability of indigenous microorganisms to degrade petroleum (Chang *et al*., 2013; Wu *et al*., 2016). It has

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been reported that the population of micro-organism drops, when subjected to hydrocarbons from petroleum, whereas Obire (1990) stated that the overall effects of hydrocarbons on total microbial diversity remain unclear in most cases. Various microbes such as *Pseudomonas spp.*, *Vibrio spp.*, *Corynebacterium spp.*, *Arthrobacter spp*, *Brevibacterium spp*, *Staphylococcus spp*, *Bacillus spp*, and *Thiobacillus spp* are reportedly capable of degrading hydrocarbons in media (Snape *et al*., 2001). Snape *et al*. (2001) also reported that among the fungi, *Penicillium* and *Aspergillus* spp are capable of bioremediation of hydrocarbon, while Fatuyi *et al.* (2012) isolated *Penicillium italicum* and *Aspergillus niger* from the oil-polluted site. The role of amendment in biostimulation, reportedly an effective approach in bioremediation process, is to augment native fertility status of such soil samples, promoting the rate of degradation, thereby reducing soil contamination (Ijah *et al*., 2013).

This study aims at investigating the effect of cow dung and periodic aeration by turning on degradation and microbial population of Brass crude-oil-polluted soil.

MATERIALS AND METHODS

Cow dung was selected as the organic component to be added into Brass crude-oil contaminated soil. The crude-oil (Brass blend) was collected from Mid-Western Oil and Gas Limited, Victoria Island, Lagos, Nigeria, while cow dung was gathered from nomadic herdsmen in Ilorin, north-central Nigeria. Table 1 gives physico-chemical parameters of cow dung.

Approximately 2 kg of soil (sieved with 2-mm mesh) was placed in plastic containers, labelled Pots A, B, C, and D according to the method of Olabisi *et al.* (2009). The soil in Pots A, B, and C received 200 ml of crude-oil, while the content of Pot D did not receive any to serve as the Control. Each treatment was replicated three times. The soils were left for 2 days to age, after which the content of both A and B was supplemented with 200 g dried cow dung and thoroughly homogenized. In addition to organic waste supplement, the soil in B was periodically turned over to increase aeration. The content of individual containers was watered with 300 ml of distilled water each, thence to be thoroughly mixed and incubated at room temperature for 28 days. Periodic sampling from each pot was carried out at seven-day intervals throughout the entire incubation period. The samples were analyzed for changes in pH, moisture, crude-oil loss, and microbial counts.

The pH of the spiked soil was determined in 1:2.5 (w/v) soil-water suspension while the soil moisture content was determined based on the calculation below:

$$\%\ \text{soil moisture} = \frac{\textit{Initial weight of soil} - \textit{oven} - \textit{dried weight of soil}}{\textit{Initial weight of soil}} \times 100$$

Biodegradation of crude-oil (crude-oil loss) was determined based on weight loss method of Bossert & Bartha (1984). To put it briefly, 3 g of soil was suspended in 10 ml diethyl ether in a universal bottle and shaken vigorously to extract the oil. The mixture (solvent-oil) was subjected to natural evaporation of solvent at room temperature overnight, and the weight of the residual oil and the beaker was taken. The percentage of degraded oil was calculated according to Ijah & Ukpe (1992).

$$\text{Biodegradation}(\%) = \frac{\textit{weight of oil}(\textit{Control}) - \textit{weight of oil degraded}}{\textit{weight of oil}(\textit{Control})} \times 100$$

Samples of soil from each treatment was taken every 7 days for enumeration and identification of Total Heterotrophic Bacterial (THB) count, using the Spread Plate Method (Abu & Ogiji, 1996; Agbor *et al.*, 2012). Briefly, soil suspensions were prepared by 5-fold serial dilution with 1 g of soil and 1 ml of 10^{-5} dilution, spread on the plates in triplicate. The Colony Forming Units (CFU) of the bacteria were counted after incubation at 37 ^{o}C for 24 hours.

Crude-oil utilizing bacteria (HUB) in

the soil samples were counted, using Surface Spreading Technique (Hwang *et al*., 2001; Hamamura *et al*., 2006). Soil suspensions were prepared with 5-fold serial dilution of 1g of soil, while 1 ml of 10^{-5} dilution was used for inoculation of agar plates in triplicate. After inoculation, a sterile filter paper (Whatman No.1), saturated with crude-oil, was aseptically introduced into the inner part of the Petri dish covers. The oil-saturated filter paper served as a sole carbon/energy source for organisms' growth via transferring the vapor phase. The plates were all incubated in an inverted position at room temperature for 7 days before the average counts were measured. Isolated bacterial colonies were then characterized in the microbiology laboratory, University of Ilorin, Nigeria. The bacterial isolates were characterized and identified once their pure culture was obtained through repeated sub-culturing by means of colonial morphology, cellular morphology, and biochemical reactions.

The Surface spreading techniques (Jidere & Akamigbo, 2009) were employed to count the total number of heterotrophic fungi (THF). Soil samples were serially diluted from 10^{-1} to 10^{-3}, with 1 ml of 10^{-3} dilution being plated in triplicate into Sabouraud Dextrose Agar (SDA) plates that has been supplemented with streptomycin (to inhibit bacterial growth). The plates were incubated at 28 ^{o}C for 2 days before the established colonies got counted and expressed as colony forming units per gram of the soil sample (CFU/g) (Agbor *et al.*, 2012). Crude-oil utilizing fungi (HUF) got measured, using the earlier discussed Surface Spreading technique, though with minor modification. In addition, the isolates were sub-cultured and isolated colonies were further purified by sub-culturing, having been identified in the microbiology laboratory, University of Ilorin, Ilorin. Fungal isolates were further identified, based on surface texture, pigmentation, and under-surface characteristics.

The data were subject to analysis of variance (ANOVA), conducted in Statistical Package for the Social Sciences (SPSS v. 20.0). The treatments were compared statistically with ANOVA and got separated, using Duncan's Multiple Range Test (DMRT). Figures were presented, using Origin 7 software and the means were separated at $p<0.05$.

RESULTS AND DISCUSSION

Table 1 presents physico-chemical and microbial properties of the cow dung, used in the bioremediation trial. The pH of the cow dung was neutral (7.2) which is quite important as most microbial species can survive only within a certain pH range. Organic matter content was high (6.36%), with K, N, and P contents being 7.00%, 9.66%, and 12.87%, respectively.

Average counts of HUB and HUF were 49.3×10^{6} CFU/g and 23.0×10^{4} CFU/g, respectively. The HUB species identified in the dung were *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Micrococcus luteus*, whereas the identified HUF species included *Aspergillus niger*, *Saccharomyces cerevisae*, *Aspergillus flavus*, *Penicillum chrysogenum*, and *Neurospora crazza*. The number of HUB exceeded that of HUF species (Table 1).

Table 1. Physicochemical and microbial properties of cow dung

	pH	K (%)	N (%)	P (%)	Moisture (%)	Org. C (%)	Org. M (%)
Cow dung	**7.20**	**7.00**	**9.66**	**12.87**	**1.10**	**3.70**	**6.36**
	Microbial Count				**Organisms**		
HUB (10^{6} CFU/g)	49.3±3.76				*Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Micrococcus luteus*		
HUF (10^{4} CFU/g)	23.0±2.89				*Saccharomyces cerevisae, Aspergillus flavus, Aspergillus niger, Penicillium chrysogenum, Neurospora crazza*		

Note: K=Potassium, N=Nitrogen, P=Phosphorous, Moisture=Moisture content, Org. C= Organic Carbon, Org. M=Organic Matter, %=Percentage.

Fig. 1 shows the pH and moisture content of amended soils during the sampling period. The highest mean pH (pH=7.27) was observed in the soil with cow dung amendment (A) on day 14, while the lowest mean pH (pH=6.19) belonged to spiked soil without any amendment (C) on day 28. Soil pH is very important as it determines nutrient bioavailability and micro-organism activities (Wang *et al*., 2013). Amendment with cow dung increased soil pH to near neutral, which usually encourages the growth of hydrocarbon-utilizing micro-organisms, thus enhancing degradability of crude-oil. Moisture content was significantly the highest ($P<0.05$) in spiked soils amended with only cow dung (A) and cow dung plus frequent turning (B) on days 0, 7, and 14, while significantly higher moisture content was observed in A, compared to B, on days 21 and 28. This shows that amendment reduced the rate of moisture loss in crude-oil biodegradation, possibly because of increased water-holding capacity of the soil from addition of organic amendment.

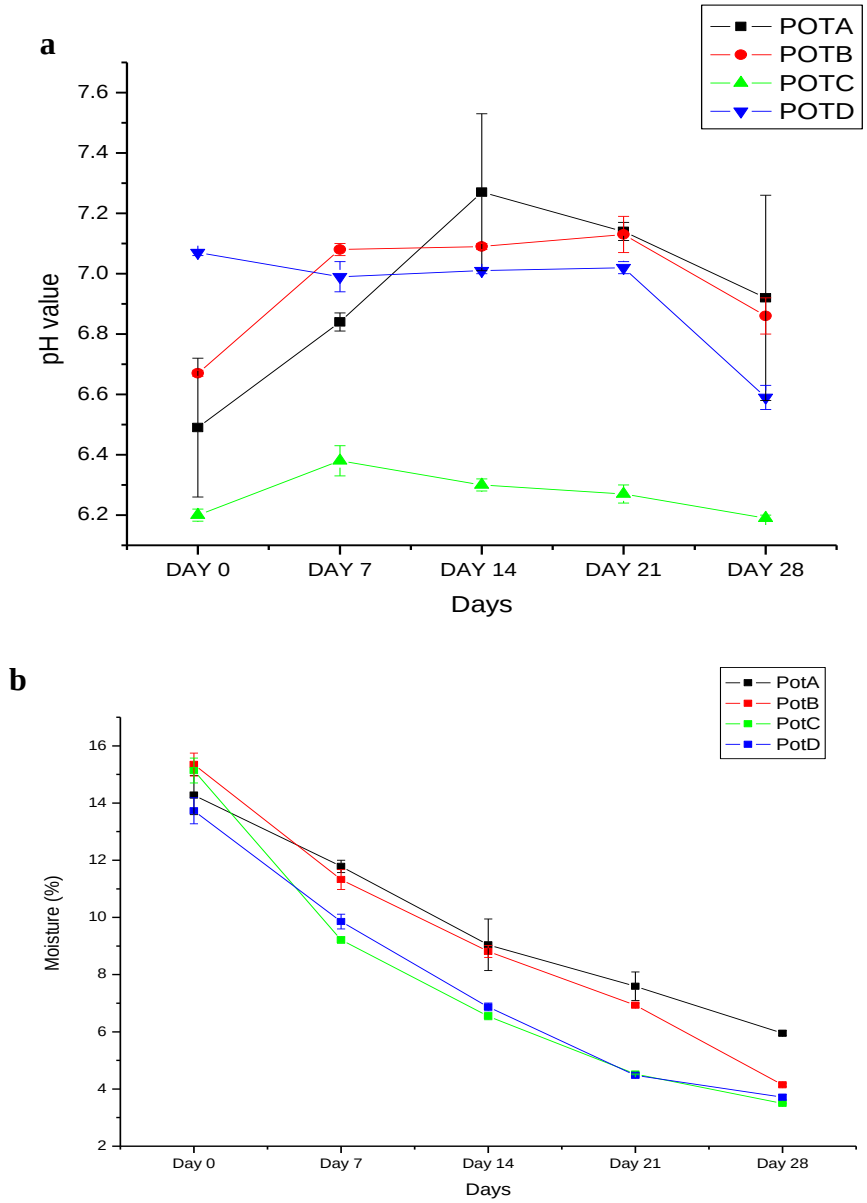

Fig. 1. pH (a) and moisture (b) of crude-oil spiked soil with or without amendment over a period of 28 days (in percent)

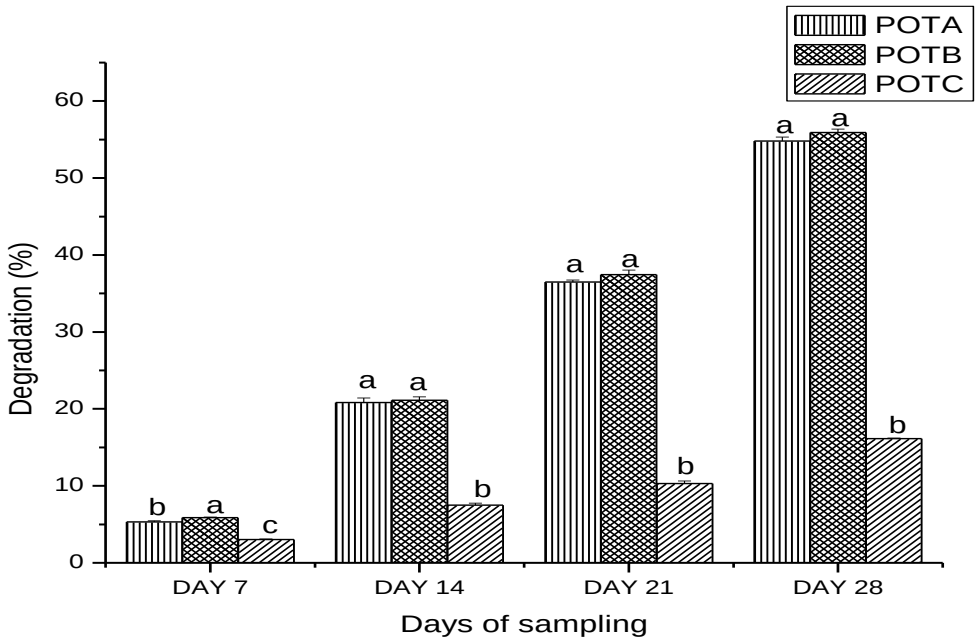

Fig. 2. Percentage degradation of crude oil after organic amendment of spiked soil for 28-day duration (in percent)

Fig. 2 illustrates biodegradation of crude-oil in the soil, throughout the study period, i.e., 28 days. Degradation in A (amendment only) and B (amendment + periodic turning) ascended significantly ($P<0.05$) throughout the entire period of study. At the end of the 28 days, crude-oil amended with cow dung with periodic turning (B) had the highest (55.9%) oil degradation, followed by crude-oil soil, amended only with cow dung (A) (54.8%). The least oil degradation was recorded in non-amended soil, spiked with oil (C) (16.1%). Addition of cow dung to the spiked soil raised the degradability of crude-oil in the spiked soil, which was consistent with several other reports, saying that addition of organic nutrients stimulates degradation potential of micro-organisms to break down organic pollutants at a faster rate (Ausma *et al*., 2002).

Table 2 gives colony characteristics for hydrocarbon-utilizing bacteria (HUB) isolates in crude-oil spiked soils. Six bacterial isolates were characterized, based on the colony shape, edge, pigmentation, optical characteristics, elevation, shape of the cell, consistency, and surface texture. Table 3 presents biochemical tests to identify the bacteria isolates. Based on positive or negative responses of these isolates to the tests, probable organisms were identified, revealing that more gram-positive bacteria were isolated, compared to gram-negative ones.

Table 4 shows colony characteristic for hydrocarbon-utilizing fungi (HUF) isolates and the corresponding probable organisms. Fungal isolates were labelled k_A-k_L and were identified, based on surface texture, pigmentation, and sub-surface characteristics. Table 5 presents the occurrence (%) of HUB and HUF in the samples of spiked soil over a period of 28 days. An 18.55% occurrence was consistent for *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Streptococcus thermophillis*, followed by *Proteus vulgaris* and *Micrococcus luteus* with 11.54% occurrences. Also Table 5 gives occurrence of HUF species in the soil samples over the period of 28 days, showing that *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Trichothecium roseum*, and *Penicilium citrinum* had the highest occurrence (i.e., 15.63%, each) while the lowest rate belonged to *Aspergillus*

fumigatus (3.13%). High number of HUB and HUF in amended spiked soil might be due to the presence of appreciable quantities of nitrogen (9.6%) and phosphorus (12.9%) in the cow dung, allegedly necessary nutrients for bacterial degradative activities (Lee *et al*., 2003; Adesodun *et al*., 2008).

Table 2. Morphological characteristics of HUB isolates from cow-dung-amended spiked soil

Isolate	Colony shape	Edge	Pigmentation	Optical characteristic	Elevation	Cell shape	Consistency	Surface texture
K_1	Circular	Entire	Golden Yellow	Opaque	Raised	Cocci in cluster	Butyrous	Smooth
K_2	Irregular	Lobate	Bluish-green	Translucent	Flat	Rod	Viscid	Smooth
K_3	Swarming	Lobate	Creamy	Opaque	Flat	Rod	Butyrous	Smooth
K_4	Circular	Entire	Creamy white	Opaque	Raised	Cocci in chains	Butyrous	Smooth
K_5	Irregular	Entire	Creamy	Translucent	Flat	Rod	Butyrous	Smooth
K_6	Circular	Entire	Orange	Opaque	Raised	Cocci in pair	Butyrous	Smooth

Keys: k_1-k_6 = Isolates 1-6

Table 3. Identification of HUB isolates from cow-dung-amended spiked soil

Isolates	Gram reaction	Catalases	Motility	Oxygen relationship	Spore test	Methyl red	Voges-Proskauer test	Indole test	Urease test	Citrate utilization	Coagulase test	Starch hydrolysis	Oxidase test	Sugar fermentation						Probable organism
														H_2S production	Glucose	Sucrose	Lactose	Maltose	Mannitol	
K_1	+	+	+	FA	_	+	+	+	_	_	_	_	_	_	A	AG	AG	AG	AG	*Staphylococcus aureus*
K_2	_	+	+	FA	_	_	_	_	+	+	_	_	_	_	_	_	_	_	_	*Pseudomonas aeruginosa*
K_3	+	+	+	FA	+	+	+	_	_	+	_	+	_	_	A	A	A	A	A	*Bacillus subtilis*
K_4	+	+	+	FA	_	+	_	+	_	_	_	+	_	_	AG	A	A	A	A	*Streptococcus thermophillus*
K_5	_	+	+	AE	_	+	_	+	+	_	_	_	_	+	AG	AG	_	_	_	*Proteus vulgaris*
K_6	+	+	+	FA	_	_	_	_	+	_	_	_	_	_	A	A	A	A	A	*Micrococcus luteus*

Keys: (+) = Positive, (AE) = Aerobic, (A) = Acid Production, (-) = Negative, (FA) = Facultative Anaerobic, (AG) = Acid & Gas Production. k_1-k_6 = Isolates 1-6.

Table 4. Morphological characteristics of HUF isolates from cow-dung-amended spiked soil

Fungal Isolate	Surface texture	Pigmentation	Under-surface	Probable organism
K_A	Powdery	Greenish-yellow	Creamy	*Aspergillus flavus*
K_B	Powdery	Blackish	Creamy	*Aspergillus niger*
K_C	Powdery	Greyish-black	Black	*Alternaria alternate*
K_D	Powdery	Pinkish	Creamy	*Neurospora crazza*
K_E	Powdery	Creamy	Creamy	*Saccharomyces cerevisae*
K_F	Powdery	Bluish-green with broad white margin	Creamy	*Penicillium chrysogenum*
K_G	Powdery	Greenish	Creamy	*Trichothecium roseum*
K_H	Powdery	Brownish	Creamy	*Aspergillus fumigates*
K_I	Powdery	Bluish-green with narrow white margin	Creamy	*Penicillium citrinum*

K_A-k_I = Isolates A-I

Table 5. Occurrences of HUB and HUF species from cow-dung-amended spiked soil within a period of 28 days

Bacterial species	Sampling period (Day) 0	7	14	21	28	Occurrence (%)
			HUB			
Staphylococcus aureus	+	+	+	+	+	18.52
Pseudomonas aeruginosa	+	+	+	+	+	18.52
Bacillus subtilis	+	+	+	+	+	18.52
Streptococcus thermophillus	+	+	+	+	+	18.52
Proteus vulgaris	+	+	_	+	_	11.11
Micrococcus luteus	+	+	+	+	_	14.81
			HUF			
Aspergillus flavus	+	+	+	+	+	15.63
Aspergillus niger	+	+	+	+	+	15.63
Alternaria alternate	+	_	_	_	+	6.25
Neurospora crazza	+	_	_	+	_	6.25
Saccharomyces cerevisae	+	_	+	_	+	9.38
Penicillium chrysogenum	+	+	+	+	+	15.63
Trichothecium roseum	+	+	+	+	+	15.63
Aspergillus fumigatus	_	_	_	_	+	3.13
Penicillium citrinum	+	+	+	+	+	15.63

Key: (+) = Present, (-) = Absent

CONCLUSION

The present study demonstrated that cow dung is a good organic substrate, possessing nitrogen, phosphorus and potassium, which have great potentials to enhance bioremediation of crude-oil-polluted soil, as it improves bioremediation by increasing microbial activities, if applied in appropriate quantity. The high level of degradation, observed just in 28 days of the study, suggests that the use of cow dung and enhanced remediation (periodic turning) could be efficient in bioremediation of crude-oil-polluted soils. Cow dung is available in large quantities in Nigeria, especially in the northern parts of the country; therefore, it can serve as a potential option for cleaning up sites, polluted by crude oil.

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