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Assessment of enhanced biodegradation potentials of animal wastes on diesel-contaminated soil

Tosan Akiri-Obaroakpo[1]✉, Emmanuel Imarhiagbe[1] and Frederick Ekhaise[2]

[1]University of Benin, Faculty of Life Sciences, Department of Environmental Management and Toxicology, Benin City, Nigeria; [2]University of Benin, Faculty of Life Sciences, Department of Microbiology, Benin City, Nigeria;
✉***Corresponding author, E-mail:*** *mercy.akiri-obaroakpo@uniben.edu.*

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Abstract. Oil spillage is a menace, crippling most oil-producing regions around the globe**.** The aim of this study was to access the role of poultry litter and cow dung in enhancing biodegradation of diesel-contaminated soil. The treatment sets were split into three levels of diesel pollution (50 mL, 100 mL and 150 mL) amended with poultry litters, cow dung and a mixture of both amendments. The microbiological properties**-**and the total petroleum hydrocarbon content was analyzed for a period of six months using the pour plate techniques and Gas Chromatography (GC-FID), respectively. Agarose gel electrophoresis was used for plasmid detection. Mean total heterotrophic bacterial counts ranged between 40.5±0.5 x10^4 cfu^{-1} and 102.0 ±4.0 x10^4 cfu^{-1}, for C_1 (soil with poultry litter and cow dung with 50mL diesel) and Control 2. The mean total hydrocarbonoclastic bacterial counts ranged from 42.0±2.0 x10^4 cfu^{-1} to 66.5±2.5 x10^4 cfu^{-1} for B_1 (soil with cow dung with 50mL diesel) and C_3 (soil with poultry litter and cow dung with 150mL diesel). *Bacillus subtilis* (25.7%) and *Staphylococcus aureus* (4.73%) were reported as the isolates with the highest and least percentage frequency of occurrence. The percentage of diesel oil degradation was highest in C_1 (98.5%) and lowest in Control 1 (31.6%). Plasmid extraction studies carried revealed that two out of the five hydrocarbonoclastic bacteria had both plasmids and chromosomal genes. The result has indicated the enhanced capacity of mixed amendments relative to individual waste treatment used in this study and should be recommended for bioremediation application.

Key words: Diesel oil pollution, animal wastes, total petroleum hydrocarbon, plasmid profile.

Introduction

Oil spill is a term which defines the discharge of liquid petroleum hydrocarbons into the environment and it is a form of pollution. The term oil spill applies mostly to the marine environment where oil is released into the water body (ocean or coastal waters), but it could also occur on land; Adelana *et al.* (2011). This could be through pipeline and tankers corrosion, sabotage, oil production operations and the spill of any oily refuse or waste oil; Adelana *et al.* (2011). The effect of oil spillage on terrestrial environment has damaging impact on crop grown on it (Pepper *et al.,* 1996). The physical properties of floating oil may hinder respiration, photosynthesis or feeding of plants and animals. Bioremediation is the use microorganisms to breakdown contaminant into less harmful compounds (Omotayo *et al*., 2012). In bioremediation, the microorganisms used may be autochthonous to the polluted site or they may be brought from elsewhere to the contaminated site to complement the action of the resident microbial population. Many plasmids carry genes that confer a selective advantage to their hosts in certain environment (De Magalhaes *et al.,* 2008; Mohania *et al.,* 2008). In recent times, organic wastes are being used to stimulate microbial consortium in order to enhance the biodegradation process. Organic wastes contain large amounts of nitrogen because of the presence of high levels of proteins and amino acids. In this study, the aim is to determine the role of poultry litter and cow dung in enhancing biodegradation of diesel-contaminated soil.

Materials and methods

Soil and diesel collection

Soil samples were collected from the Animal and Environmental Biology (AEB) Experimental Garden, Faculty of Life Sciences from a depth of 0-15 cm. The animal wastes, cow dung and poultry litters, were collected from University of Benin Agricultural Farm and the cattle market at Benin Technical College Road, Benin City, Edo state, Nigeria, at coordinates (6° 23'49"N, 5°36'55"E), (6°24'11"N, 5°36'37"E). The petroleum (diesel) used for the experiment was from a filling station in Benin, Edo State, Nigeria.

Treatment set up

Triplicates of eleven samples was set up for the experiment:
Control 1: 2.0 kg of uncontaminated soil only
Control 2: 2.0 kg soil contaminated with 50 mL of diesel

Treatment A_1: 2.0 kg of soil contaminated with 50mL diesel with 100 g of poultry litter; A_2: 2.0 kg of soil contaminated with100 mL diesel with 100 g of poultry litter; A_3: 2.0 kg of soil contaminated with 150mL diesel with 100 g of poultry litter.

Treatment B_1: 2.0 kg of soil contaminated with 50mL diesel with of 100 g of cow dung; B_2: 2.0 kg of soil contaminated with 100 mL diesel with 100 g of cow dung; B_3: 2.0 kg of soil contaminated with 150 mL diesel with 100 g of cow dung.

Treatment C_1: 2.0 kg of soil contaminated with 50 mL diesel with ~~of~~ 100 g of poultry litter and cow dung; C_2: 2.0 kg of soil contaminated with 100 mL diesel with 100 g of poultry and cow dung; C_3: 2.0 kg of soil contaminated with 150mL diesel with 100 g of poultry litter and cow dung.

The experiment was carried out for a period of six months (three months of rainy season and three months of drought). The perforated buckets containing the soil samples were kept in the open but protected from the direct effect of rain. During this period, the soil samples were stirred and the temperature of the soil taken at regular interval. Soil samples were bimonthly collected for analysis.

Enumeration of total heterotrophic and hydrocarbon utilizing bacterial counts

Enumeration of bacterial isolates was carried out on treatments by measuring 10 g of soil sample into 90 mL of distilled water. This mixture was agitated and 1mL was taken from each mixture representing each treatment and was serially diluted to make a ten-fold diluent. Aliquot of 1 mL of the 10^{-4} and 10^{-6} dilutions was seeded unto Nutrient Agar, Mannitol Salt Agar and Mackonkey Agar for the isolation of heterotrophic bacteria. In screening for total hydrocarbon utilizing bacterial counts, vapour phase transfer technique was used (Chikere and Azubuike, 2014). An aliquot of 1mL (10^{-4} and 10^{-6} dilution) of the crude oil soil suspension was seeded onto Bushnell Haas agar, sterile Whatman filter papers soaked in diesel were aseptically placed into the lids of each inoculated Bushnell-Haas Agar plates and incubated at room temperature for 6 days. The colonies, after incubation, were visualized on the agar plates and counted; they are expressed in $cfug^{-1}$; the colonies isolated were further purified by sub-culturing unto agar slant; Sharma, (2009).

Cultural test and Gram stain reaction was carried out on bacterial isolates. Their features were compared with related species from Bergey's Manual of Determinative Bacteriology for bacterial cells, and their identities were confirmed (Holt *et al*., 1994). The biochemical tests of the samples were carried out on: production of catalase, coagulase, citrate utilization, indole and oxidase enzymes. Fermentation of sugars was also examined.

Determination of total petroleum hydrocarbons (TPH)

TPH was analyzed using organic solvent extraction procedures (Onyeonwu, 2000). The TPH was performed using Gas Chromatograph Agilent 6890 Series, with an Agilent FID detector. The percentage of crude oil degraded after six months was determined from the equation:

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

Weight of crude oil degraded = Original weight of crude oil – Weight of residual crude oil.

Plasmid DNA isolation

Isolation of hydrocarbonoclastic bacterial plasmid DNA was carried out to ascertain the molecular weight of plasmids based on their movement through agarose gel in comparison with a molecular marker. Plasmid DNA was excised from a bacterial cell by alkali treatment method as described by (Crosa *et al.,* 1994).

Plasmid curing

All isolates that were observed to harbour plasmid were subjected to plasmid curing suggested by Sheikh (2003). The bacterial isolates were cultured in broth medium containing the preferred hydrocarbon in which the bacteria had shown the highest growth. 0.1 mL of the culture was added to 100 mL of nutrient broth containing 1% SDS (Sodium Dodecyl Sulphate). This was incubated at 37^0C for 24 h. Thereafter, the broth was shaken vigorously to homogenize the content and loopfuls of the broth medium were sub-cultured on Nutrient Agar plate and also on Bushnell Haas Agar containing 1% (v/v) diesel. The plates were incubated at 37^0C for 24 h and the colonies counted. Colonies that failed to grow on Bushnell Hass Agar containing 1% (v/v) diesel plates were considered cured.

Statistical analysis*:* Results obtained were computed using Two Way Analysis of Variance (ANOVA) without replication to test the level of significance between the groups of means for the different treatment samples.

Results

The mean total heterotrophic bacterial counts (Table 1) ranged between 40.5±0.5 x10^4 cfu^{-1}and 102.0 ±4.0 x10^4 cfu^{-1}, the minimum being recorded for C_1 in August, 2016 and the maximum Control 2 in December, 2016. The mean total hydrocarbon utilizing bacterial counts ranged between 42.0 ± 2.0 x10^4 cfu^{-1} to 66.5±2.5 x10^4 cfu^{-1}, the minimum being recorded for B_1 in July, 2016 and the maximum C_3 in September, 2016.

Table 1. Mean total heterotrophic bacterial counts (x10^4 cfu^{-1})
(July 2016 – December 2016)

Treatments/ Months	July	August	September	October	November	December	P-value
Control1	65.0±7.0	73±13.0	69.5±2.5	52.5±2.5	54.0±2.0	45.0±3.5	P<0.05[a]
Control2	55.5±0.5	55.5±3.5	51.0±1.0	44.5±1.5	46.5±3.5	40.5±0.5	P<0.05[a]
A_1	72.5±7.5	79.5±9.5	81.0±9.0	77.0±15.0	68.0±1.0	64.0±8.0	P<0.05[a]
A_2	66.5±2.5	73.5±5.5	77.0±5.0	70.0±2.0	64.5±4.5	64.0±8.0	P<0.05[a]
A_3	62.5±2.5	67.0±2.0	72.0±3.0	68.5±3.5	65.5±2.5	62.5±2.5	P<0.05[a]
B_1	64.0±2.0	64.5±2.5	68.0±4.0	65.0±3.0	67.5±0.5	64.5±2.5	P<0.05[a]
B_2	58.0±2.0	61.5±2.5	57.5±5.5	57.5±2.5	56.0±2.0	53.5±1.5	P<0.05[a]
B_3	59.0±1.0	60.5±1.5	58.5±0.5	52.5±2.5	51.0±1.0	51.0±1.0	P<0.05[a]
C_1	98.5±2.5	102.0±4.0	99.5±10.6	94.5±2.5	92.5±2.5	77.0±10.0	P<0.05[a]
C_2	93.0±1.0	93.0±3.0	95.5±0.5	91.5±1.5	88.5±1.5	80.0±2.0	P<0.05[a]
C_3	92.0±2.0	95.0±2.0	93.5±1.5	85.5±1.5	83.0±3.0	79.5±6.5	P<0.05[a]

P < 0.05 – significant difference; 'a' connotes values that are significant
Keys: control 1: soil only, control 2: soil + 50mL diesel, A_1: soil + 100g poultry litter + 50mL diesel, A_2: soil + 100g poultry litter + 100mL diesel, A_3: soil + 100g poultry litter + 150mL diesel, B_1: soil + 100g cow dung + 50mL diesel, B_2: soil + 100g cow dung + 100mL diesel, B_3: soil + 100g cow dung + 150mL diesel, C_1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C_2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C_3: soil + 50g cow dung + 50g poultry litter + 150mL diesel

Table 2. Mean total hydrocarbon utilizing bacterial counts (x10^4 cfu^{-1})
(July 2016 – December 2016)

Treatments/ Months	July	August	September	October	November	December	P-value
Control1	59.0±1.0	56.0±4.0	56.5±3.5	51.5±1.5	51.5±0.5	46.5±1.5	P>0.05[b]
Control2	50.5±4.5	55.5±3.5	61.5±2.5	59.0±2.0	57.0±1.0	51.5±1.5	P>0.05[b]
A_1	50.5±1.5	56.0±3.0	62.0±2.0	61.0±1.0	59.5±2.5	57.5±2.5	P>0.05[b]
A_2	52.0±3.0	57.5±0.5	63.5±1.5	63.0±0.0	62.0±1.0	57.2±0.5	P>0.05[b]
A_3	53.0±5.0	59.0±1.0	64.0±3.0	63.5±0.5	62.0±3.0	61.0±3.0	P>0.05[b]
B_1	42.0±2.0	51.5±4.5	56.5±0.5	54.5±1.5	50.5±0.5	51.0±2.0	P>0.05[b]

Treatments/ Months	July	August	September	October	November	December	P-value
B_2	43.0±2.0	51.5±3.5	54.0±0.0	60.5±4.5	52.5±0.5	52.5±2.5	P>0.05[b]
B_3	43.0±5.0	53.0±7.0	60.5±2.5	62.0±2.0	57.0±1.0	55.0±1.0	P>0.05[b]
C_1	58.5±0.5	61.5±1.5	62.5±0.5	63.5±2.5	58.5±1.5	53.5±0.5	P>0.05[b]
C_2	60.0±5.0	65.0±3.0	64.5±0.5	63.5±3.5	53.5±1.5	52.0±2.0	P>0.05[b]
C_3	61.5±2.5	65.5±2.5	66.5±2.5	65.0±1.0	64.5±0.5	62.5±2.5	P>0.05[b]

P > 0.05 – No significant difference; 'b' connotes values that are not significant
Keys: control 1: soil only, control 2: soil + 50mL diesel, A1: soil + 100g poultry litter + 50mL diesel, A2: soil + 100g poultry litter + 100mL diesel, A3: soil + 100g poultry litter + 150mL diesel, B1: soil + 100g cow dung + 50mL diesel, B2: soil + 100g cow dung + 100mL diesel, B3: soil + 100g cow dung + 150mL diesel, C1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C3: soil + 50g cow dung + 50g poultry litter + 150mL diesel

Seven bacterial species were isolated. Of these heterotrophic bacteria isolated, five isolates were hydrocarbon degrading bacteria. *Bacillus subtilis* (25.7%) was reported to record the highest percentage frequency of occurrence and the least frequency was recorded for *Staphylococcus aureus* (4.73%) (Table 3).

Table 3. Percentage frequency of occurrence of the bacterial isolates

Isolates	Control 1	Control 2	A (A_1,A_2,A_3)	B (B_1,B_2,B_3)	C (C_1,C_2,C_3)	Total
*Bacillus subtilis**	5	7	9	5	12	38 (25.7%)
Bacillus sp*	3	5	6	6	9	29 (19.6%)
Staphylococcus aureus	1	-	1	4	1	7 (4.73%)
Klebsiella sp*	3	3	4	3	6	19 (12.84%)
Escherichia coli	-	-	2	3	5	10 (6.76%)
*Enterobacter aerogenes**	1	1	3	4	6	15 (11.03%)
*Pseudomonas aeruginosa**	2	1	5	2	8	18 (12.16%)
Total	17 (12.50%)	15 (11.03%)	30 (22.06%)	27 (19.85%)	47 (34.82%)	136 (100%)

Keys: control 1: soil only, control 2: soil + 50mL diesel, A_1: soil + 100g poultry litter + 50mL diesel, A_2: soil + 100g poultry litter + 100mL diesel, A_3: soil + 100g poultry litter + 150mL diesel, B_1: soil + 100g cow dung + 50mL diesel, B_2: soil + 100g cow dung + 100mL diesel, B_3: soil + 100g cow dung + 150mL diesel, C_1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C_2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C_3: soil + 50g cow dung + 50g poultry litter + 150mL diesel.

* Hydrocarbon degrading bacteria

Effect of soil amendments on Total Petroleum Hydrocarbon

Table 4 shows the initial and the final TPH utilization with treated soil. Diesel oil degradation was highest in C_1 (98.5%) and lowest in control1 (31.6%).

Table 4. Percentage (%) Total Petroleum Hydrocarbon (TPH) degradation (July 2016 – December 2016)

Treatments	**Cntrl 1**	**Cntrl 2**	**A_1**	**A_2**	**A_3**
Initial TPH (mg/kg)	3.8917	13292.4	11362.3	12140.5	13595.4
Final TPH (mg/kg)	2.6618	8056.41	592.25	848.766	1392.57
% Degradation	31.6	39.3	94.8	93.0	89.8

Treatments	**B_1**	**B_2**	**B_3**	**C_1**	**C_2**	**C_3**	**P-value**
Initial TPH (mg/kg)	11656.7	12193.7	12576.5	11447.1	11558.2	11622.3	P<0.05
Final TPH (mg/kg)	796.067	957.015	1774.65	169.69	270.164	446.83	P< 0.05
% Degradation	93.2	92.2	85.9	98.5	97.7	96.2	P< 0.05

Keys: control 1: soil only, control 2: soil + 50mL diesel, A_1: soil + 100g poultry litter + 50mL diesel, A_2: soil + 100g poultry litter + 100mL diesel, A_3: soil + 100g poultry litter + 150mL diesel, B_1: soil + 100g cow dung + 50mL diesel, B_2: soil + 100g cow dung + 100mL diesel, B_3: soil + 100g cow dung + 150mL diesel, C_1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C_2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C_3: soil + 50g cow dung + 50g poultry litter + 150mL diesel

Plasmid profile of bacterial isolates

Figure 1 shows the electrophoretic profile of all five hydrocarbon degrading bacterial isolates having plasmids. The hydrocarbonoclastic bacterial isolates designated B, C, D, E, and F showed positive growth on Bushnell Hass medium before curing, but upon curing, only isolates D and E (*Bacillus subtilis and Enterobacter aerogenes*) had scanty growth. No growth was seen in isolates B, C and F (*Klebsiella* sp., *Pseudomonas aeruginosa* and *Bacillus* sp). Positive growth on media after curing indicates the ability of the isolate to utilize the petroleum present on the media as a sole source of carbon. This enzymatic ability could only be enhanced by the gene that codes for degradation present in the chromosome. No growth indicates that the gene that codes for degradation is not present in the chromosome but in the plasmid which has been cured.

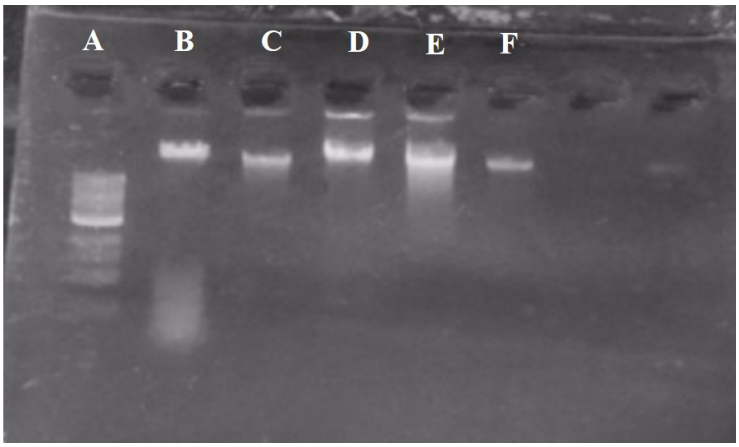

Figure 1. Electrophoretic separation profile plasmid DNAs of isolates before curing (A = 10kb Ladder; B= *Klebsiella* sp.; C=*Pseudomonas aeuginosa* ; D= *Bacillus subtilis*; E=*Enterobacter aerogenes;* F=*Bacillus* sp.) All isolates had plasmids greater than 10kb.

Discussion

Bioremediation is often used in pollution control because of its ecofriendly nature and there are also readily available microbes that effectively carry out the remediation process. The total heterotrophic bacterial counts were in this order; C_1> C_2> C_3> A_1> A_2> A_3> B_1> B_2> B_3> Control 1 > Control 2. High bacterial count observed in the combined amendment could be due to the increased nutrient as a result of the combination of organic wastes.

There was observable increase in the first three months (July-September) and decrease from October to December. This agrees with the work of Stephen *et al*. (2015), who observed that bacterial count reduces with time. This could be attributed to the exhaustion of nutrients with time and also to the fact that bacteria grow well when moisture content is adequate. It was observed that bacterial counts were higher in poultry amended soil than in soil amended with cow dung; this agrees with the report of Obasi *et al.* (2013), which showed a higher bacterial count in poultry litter amended soil than soil amended with cow dung, sawdust and horse manure.

The bacterial counts enumerated from the uncontaminated soil (Control 1) were above those from soil incorporated with diesel (Control 2). This difference in bacterial counts could be attributed to the incorporation of diesel to control 2 which reduced the capacity of the bacterial population that could not utilize hydrocarbon as carbon source for growth. Similar were the findings of Stephen *et al,* (2015), which observed higher bacterial counts in oil-free soil than in oil-

polluted soil. There was significant difference ($P < 0.05$) in heterotrophic bacterial and fungal counts between July to December, 2016 indicating that moisture and nutrients influenced microbial growth. The reverse was the case in total petroleum hydrocarbon utilizing bacterial counts (TPHUB), as the bacteria were able to utilize the hydrocarbon as sole source of carbon and energy and thus increased in number as the volume of hydrocarbon increased. There was significant difference in bacterial counts from July to December, 2016. This finding corroborates with the results of Adebusoye *et al.* (2007), who reported an increase in TPHUC; although there was no significant difference ($P> 0.05$) between THBC and TPHBC.

It was observed that soil amended with poultry litters and cow dung had greater percentage degradation than the individual waste treatments. Although poultry litter amended soil had greater percentage degradation than that of soil amended with cow dung. This is in agreement with earlier reports by Tanee and Kinako (2008) who reported a marked decrease in the total hydrocarbon content of amended crude oil polluted soils relative to the control soils. Higher loss of TPH was evident in the combined compost amendment followed by poultry litter amended soil and then cow dung amended soil. This corroborates the findings of Umar *et al*. (2012), who reported that bioremediated soil using cow dung and chicken droppings have high removal rate of TPH compared to control soil.

The result of the electrophoretic profile of plasmid DNAs for the five hydrocarbonoclastic bacteria (*Klebsiella* sp., *Bacillus subtilis*, *Bacillus* sp.*, Enterobacter aerogenes* and *Pseudomonas aeruginosa*) showed plasmids greater than 10kb. *Enterobacter aerogenes* and *Bacillus subtilis* revealed scanty growth after curing. The ability of the cured isolates to grow in crude oil medium thereby retaining their ability to degrade crude oil have been earlier reported by John and Okpokwasili (2012) and Akpes *et al.* (2013), These findings correlate with work done by Akpes *et al.* (2013) whose plasmid-cured isolates retained the ability to degrade crude oil. This observation suggests, however, that some bacteria isolates possess both plasmid and chromosomal DNAs that code for the degradation of crude oil. Since these isolates were cured of their plasmids, it means that the genes that possess the enzyme for degradation were encoded in both the plasmid and the chromosome.

Conclusions

This study has shown that organic wastes play key roles in stimulating microorganisms in order to achieve enhanced biodegradation. The combined compost amendment had better percentage degradation and should be recommended for bioremediation processes. Poultry litters offered better degradation potentials than cow dung and would be preferred when one is faced to choose between either of them.

More studies should be carried out on hydrocarbon degrading bacteria whose gene that codes for the enzymes for degradation are located in both chromosomes and plasmids. It is believed that these groups of bacteria will be most effective in carrying out the remediation process. The Federal Government by way of encouragement should give financial support to scientists so as to fast track the cleanup process of oil-contaminated rivers and soil in some part of Nigeria, especially in Niger-Delta region.

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