Evaluating the degradation effect of bacterial consortium from the feces of *Bos tarus* and fecal matter on Crude oil polluted soil

ABSTRACT

Aim: This study evaluated the effect of degradation of bacterial isolate from feces of *Bos tarus* and fecal matter on crude oil polluted soil in a laboratory scale.

Study design: This study covered the isolation of bacterial consortium from the fecal matter of *Bos tarus* obtained from Giriki Farms located at Gariki harness Uti, Delta State, Nigeria used to treat soil sample spiked with crude oil obtained from the Warri Refining and Petrochemical Company (WRPC)

Place and Duration of Study: The study was conducted in the Environmental Science laboratory, Federal University of Petroleum Resources, Effurun, Delta State, Nigeria. The research lasted for four months.

Methodology: Hydrocarbon utilizing bacteria consortium was isolated from the dung of *Bos tarus* using a carbon free medium employing pour plate/vapor phase techniques. The identified consortiums were screened to select the fastest degrader of hydrocarbons by DCPIP. The best fit consortium was subjected to purification and identification of its bacterial make up using standard microbiological procedures. The selected consortium was used to treat soil spiked with crude oil alone and with fecal matter. The degradation effect of the residual crude oil was determined by Gas chromatography.

Results: Results showed that isolates were capable and effective in degrading crude oil with performance enhanced in crude oil polluted soil with bacterial consortium and fecal matter (90.29%) and 78.00% in polluted soil with only bacterial isolates. This clearly points to the roles of elemental constituents of the fecal matter acting as a catalyst and bio-stimulant to hasten the rate of degradation of crude oil although not undermining the role of microorganisms.

Conclusion: The study revealed that microorganisms from the waste of *Bos tarus* are capable of degrading short and long chain hydrocarbons present in crude oil and the degradation process is enhanced if the ecological niche from which the isolates are drawn is maintained.

Keywords: *crude oil, Bos tarus, fecal matter, bio-stimulant, degradation.*

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1. INTRODUCTION

Crude oil together with its multiplex derivatives since its discovery in Nigeria has improved the economy of the country as well as enhanced the livelihood of man. However, its explorative, exploitative including transportation and storage usage has created a newer challenge of pollution spreading the menace of health challenges not just to man and other living organisms but also the environment. (1).
Petrogenic-based pollution has caused critical concerns in the environment distorting environmental aesthetics, tourism and human health. A major concern to the environmentalist is the fact that petroleum hydrocarbons pollution is characterized by the presence of recalcitrant compounds that produce intermediates that pose their own danger to humans and the environment (2). Microorganisms metabolize compound break their complex bonds and released more toxins into the environment thereby posing deleterious environmental and health challenges (3). Furthermore, petroleum hydrocarbons contain many substances that are harmful. These substances range in degree of toxicity from acute to chronic. (4).
Despite the economic gain of crude oil, pollution by it affects many living things including their ecological niches (5). The environmental hazards resulting from oil spill include alteration of soil quality, soil fertility, plant physiology and water quality (6). Sadly crude oil and its derivatives leach into soil and contaminate ground water changing the normal flora and fauna of the top soil and subsoil decrease sing the agricultural productivity of the soil (4).
Animal waste has been recycled in various facets of life in the bid to reuse and recover ensuring that very minimal litter is left for disposal. Feces of animals have recorded enormous breakthrough in restoring the integrity of polluted soils. The search for efficacious and cutting edge strategies to recover contaminated sites has intensified in recent years especially in the midst of food scarcity and terrible diseases (7). It has been documented that the addition of bacterial isolates from poultry and pig waste including addition of commercial quantities of nutrients like nitrogen and phosphorous enhances the rate of petroleum hydrocarbon biodegradation (8,3). There is however paucity of data as to the use of isolates from the fecal matter of *Bos tarus* retaining the fecal material as part of substrate for bioremediation purpose. It was therefore economically and ecologically important to ascertain the extent to which microorganisms isolated from feces of *Bos tarus* will degrade petroleum hydrocarbons in in the presence of its fecal matter.

2. methodology

2.1 Sample collection

Crude oil (Sweet Crude) so named because of its low sulphur content was collected from Warri Refining and Petrochemical Company (WRPC) in a sterile container and safely transported the laboratory. Soil sample was collected from a farmland in Federal University of Petroleum Resources (FUPRE) Ugbomro, Delta State using a hand soil auger after removing debris at various depths of 5cm, 10cm and15cm. The soil samples were bulked together and homogenized. It was subsequently sun-dried and sieved using a 2mm pore size wire mesh to remove debris and stones. The fecal matter was obtained from Giriki Farms located at Gariki harness Uti, Delta State, Nigeria. The animal waste was sun dried like the soil sample.

2.2 Determination of physical and chemical characteristics of soil and animal wastes

Physicochemical parameters of soil sample and fecal matter were analyzed prior to the contamination with crude oil following standard chemical and physical testing protocols: pH was determined with a digital pH meter (Winlab model 290A ASTM D1293B) standardized with pH 4, 7 and 9 buffer solution and recalibrated before repeat use. Moisture content was determined by oven dry weight method (gravimetry) (9), Temperature was determined with a mercury thermometer (MRC 201, Israel) calibrated 0-100^{o}C, Electrical conductivity (EC) measured in (µs/cm) was determined using Data Log Conductivity Meter (SPER SCIENTIFIC 850039). Total organic carbon and total nitrogen (kjeldahl method) were determined following standard procedures. Total phosphorus was determined by (hypochorate method) using available phosphorus, Bray's No. 1 method (10).

2.3 Isolation and identification of bacteria

The vapour phase transfer technique was used for the enumeration of hydrocarbon utilizing bacteria (HUB) in mineral salts medium of the following composition (1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.05 g $FeCl_2$, 0.02 g $CaCl_2.2H_2O$ in 1000 ml of distilled water at pH 7.0 (11). The molten medium was poured into sterile petri dishes and allowed to solidify. The mineral salt agar was inoculated with 0.1ml supernatant from serially diluted fecal matter samples in petri dishes and inverted over the inside cover containing crude soaked sterile Whatman No.1 filter paper. Consortiums were picked around clear zones and purified by continuous subculture for pure cultures. Microscopic, macroscopic and biochemical examination of the isolates were undertaken for identification purposes. The isolates were identified by comparing their characteristics with those of known species, as recommended in Berge's Manual of Determinative Bacteriology.

2.4 Screening of Consortium

To test the potential of isolates to degrade hydrocarbons, isolates were subjected to 2,6-Dichlorophenol indophenol testing, adopting the method of (12, 13). The assay mixture containing 2.5ml of carbon free Bushnell Haas medium, 150 µl of $FeCl_3.6H_2O$ suspension and 150 µl of 2, 6-DCPIP was mixed with 300 µl of bacteria consortium standardized at optical density (600 nm to 0.1) and 25 µl of crude (1000 mg/L in acetone). The reaction mixture was incubated at 32℃ under shaking conditions (120 rpm) for 4 days. Degradation ability of the bacterial consortium was observed by recording the discoloration of the medium from blue to colorless. A negative control was also set up containing only the mineral salt medium and the dye without microorganisms and crude. The positive control contained mineral salt medium, the dye and crude. Population of the bacterial consortium was measured using a spectrophotometer at OD_{600nm} (14).

2.5 Degradation of polluted soil by bacterial consortium and fecal materials

The experimental setup contained a microcosm with sterile soil 500g, spiked with crude oil 50ml and inoculated with 0.5ml of standardized inoculum appropriately labelled as 'A'. The second microcosm 'B' contained the 500g soil, 50ml crude oil and 50g of fecal material no organism. The third microcosm 'C' contained same contents as in A but with 50g of the fecal material homogenized into the soil sample. A positive control contained the soil and crude oil while the negative control was the soil, no crude and no treatment.

- ve ctrl Soil 500g
+ve ctrl Soil (500g) + crude oil (50ml)
A Soil (500g) + crude oil (50ml) + isolates (50g)
B Soil (500g) + crude oil (50ml) + fecal material(50g)
C Soil (500g) + crude oil (50ml) + isolates () + fecal material (50g)

The mixtures were manually turned over for aeration with 5ml of sterile distilled water added at 4days intervals to prevent desiccation. On fortnight basis, 5g each of samples were withdrawn from the containers and analysed for microbial growth, pH, and total organic carbon (TOC). Residual crude oil (TPH) was determined by GC-FID analysis

2.6 Estimation of Residual Petroleum Hydrocarbon

The residual petroleum hydrocarbons were determined using a Gas chromatography (GC) system 6890 series. Samples were extracted in a sterile beaker with equal proportion of acetone and methylene chloride in the ratio 50:50. The sample was placed in a sonicator and sonicated for about 10-15 minutes at 70^{o}C. Thereafter, 10g of anhydrous sodium sulphate was added to the mixture and left until a clear extract developed. The extract was poured in a round bottom flask and procedure repeated. The solvent was concentrated using hexane in 1 to 3ml ratio and the mixture was ready for analysis. Before samples were analyzed, the GC was calibrated using a series of TPH standards commercially obtained products of (AccuStandard, USA). An aliquot of the sample was injected into the cuvette of the GC and a five-point calibration curve was prepared having a range of 12.5-200.0 µg/mL. The response factor (RF) was calculated using the area response and the amount of standard material. A blank was used for initial analysis before the ready to use mixture was injected into the Gas chromatograph at a temperature of 250^{o}C and fractionated into

the aliphatic and aromatic fractions. The n-alkanes were quantitated with ranges C_8-C_{36} and the loss of each C-chain was calculated by subtracting the final concentration from the initial concentration and expressing it as percentage (%) loss. Depicted thus (A-B/A) x100

3.0 Results and Discussion

The physicochemical parameters of both the soil and the fecal materials are presented in Table1 and reveal a clear distinction in the nutrient supplementation between the arable soil and fecal matter. In comparison to the soil, pH of the feces of *Bos tarus* was near neutral unlike the soil which was acidic in line with reports of the acidic nature of the Niger Delta Soil area in Nigeria (15). The pH of the fecal matter helped to regulate the homeostasis of the soil and aided the proliferation of bacteria culminating into the loss of the hydrocarbon as seen in study. Without an iota of doubts the richness of the fecal matter plays a role in the enhancement of biodegradation.

Table 1: Physical and chemical characteristics of soil and animal wastes used for hydrocarbon biodegradation tests

Physiochemical parameters	Soil	feces of *Bos tarus*
Ph	6.25 ± 0.11	7.75 ± 0.03
Moisture content (%)	23.1 ± 0.14	69.32 ± 0.31
Total organic carbon (%)	27.1 ± 0.01	44.3 ±0.01
Total nitrogen (%)	0.51 ±0.56	0.87 ± 0.12
Phosphorus (%)	0.01 ± 0.01	0.14 ± 1.27
Calcium (%)	0.11 ± 0.00	0.52 ± 0.01
Magnesium (%)	0.00 ± 0.00	0.05 ± 0.01
Sodium (%)	0.00 ± 0.00	1.04 ± 0.00

*Values in the table represent Mean±SD

Successfully isolating bacteria and evaluating them is essential for microbiological experimental work therefore in choosing isolation methods, care has to be taken to obtain viable organisms needed for specific roles. Bacteria are ubiquitous and can proliferate within unique ecological niches where they are exposed to multiplex compounds. These compounds provide intrinsic nutrient supplementation that aid their survival in various niches they carve for themselves (20). Thus in this study vapour phase technique was applied to spread plate to specifically isolate hydrocarbon degraders. Growth of the organisms was seen as clear zones on plate and on purification/identification the identities of each organism in a group was revealed. The opaque spaces when subculture revealed a consortium of organisms This finding tallies with works by (21)

A consortium of hydrocarbon utilizing bacteria (HUB) was isolated from the fecal matter from *Bos tarus* also known as cow dung identified by the clear zones that appeared on the plates of the applied vapour technique. This was subsequently purified/screened/identified and tested for degradative ability (Table 3). The three isolates that made the strongest consortium by DCPIP were *Bacillus subtilis*, *Citrobacter fendii* and *Enterobacter aerogenes*.

Table 2 presents the screening result for the best hydrocarbon degrader by 2, 6-Dichlorophenol indophenol (DCPIP) method. The general observation of the indicator is that oxidized forms are coloured and reduced forms are colourless (12). These organisms subjected to the DCPIP were all from the Enterobacteriaceae family probably due to the fecal source from where the isolates were drawn. This finding tallies with studies of many researchers who have found these organisms and many more are resident in feces of ruminants (16). Furthermore, it is conceivable that the feces of animals are largely products of what was consumed by them. For nomadic animals that feed from the polluted environment, hydrocarbon utilizing bacteria (HUB) would be habited in their egested materials. This feat has been reported that hydrocarbon utilizing organisms, is found in grasses and fecal matter in areas chronically exposed to hydrocarbons from anthropogenic sources as the case of the Niger Delta region of Nigeria (17).

It has been documented that *Bacillus subtilis* is a suitable candidate for biodegradation because it possesses a rigid cell structure which is composed of peptidoglycan, a polymer of sugars and amino acids and also resistant endospores, lipoprotein and glycoprotein which protects the organism while it naturally utilizes hydrocarbons (18). This may be the reason for the better performance in the DCPIP screening test in which consortium with *Bacillus subtilis* performed better than consortium without it (Table 2) Similarly, *Citrobacter fendii* and *Entrobacter aerogenes* have been reported to possess affinity to degrade hydrocarbons (19) aligning with findings in this study.

Table 2: Quantitative screening of consortium by 2, 6-Dichlorophenol indophenol(DCPIP) redox indicator using UV-VIS @600nm

Label	Microorganisms	Coloritry change after(Days): 1	2	3	4
Control	Not applicable	1.94±0.02[a]	1.88±1.46[a]	1.87±2.02[a]	1.87±2.36[a]
A	*Azomonas agillis* *Cellulomonas flavigenes* *Bacillus subtillis*	1.94±0.02[a]	1.16±0.03[b]	0.56±0.04[b]	0.21±0.07[c]
B	*Citrobacter fendii* *Bacillus Subtilis* *Enterobacter aerogenes*	1.94±0.02[a]	0.99±0.03[b]	0.23±0.02[c]	0.10±0.01[c]
C	*Arthrobacter globiformis* *Cellulomonas flavigenes* *Salmonella chloleraesuis*	1.94 ± 2.3 [a]	1.73 ± 0.6[a]	1.64 ± 1.49 [a]	1.11 ± 0.04[b]

Table 3: **Morphological, Biochemical and sugar fermentation of isolates from the best performing consortium.**

Morphology	Microscopy	Biochemical							Sugar Fermentation							
Shape	Grams reaction	Urease	Oxidase	Catalase	Coagulase	Indole	VP	MR	Citrate	Glucose	Lactose	Sucrose	H_2S	Acid	Gas	Probable Organism
Rod	-	-/+	-	+	-	-	-	+	+	+	+	+	+	+	+	*Citrobacter fendii*
Rod	-	-	+	+		-	+	-	+	-	-	-	-	-	-	*Bacillus Subtilis*
Cocci	-	-	-	-	-	-	+	-	+	+	+	+	-	+	+	*Enterobacter aerogenes*

Table 4 shows the results of the degradation of total petroleum hydrocarbon which is the main constituent of crude oil. Biodegradation by microorganisms was observed by the reduction in the concentration of TPH in the crude oil. The bacterial consortium in cow dung reduced the crude oil concentration to a level that will have less or no impact on the soil.

It was observed that there was no significant effect in the concentration of TPH for the medium containing only soil otherwise known as the negative control and the medium containing soil and crude known as positive control. This is because there was no addition of crude and/or microbes in the soil medium to give any effect throughout the time period for observation. This is an indication that the untreated sterile soil could not degrade the crude oil

There were indications of changes in the TPH concentrations in mediums A, B and C. The degradation effect of the bacteria consortium alone on the crude oil was high (78%) after 42days. However, the degradation protocol of the bacterial consortium and fecal matter, C was significantly different in comparison to only the bacterial consortium showing a 90.28% loss of crude. This shows that the reduction in the concentration of the total petroleum hydrocarbon was more effective in the mixture of the soil, crude, consortium and fecal material. The infinitesimal loss in the sample containing only the fecal matter B was not surprising because the soil, crude and even the fecal matter was sterilized (see materials and methods). This fact gives credence to the effect of bacteria in degradation of crude oil as in its absence there was basically next to nothing loss in the concentration of crude. This result collaborates with other research findings that reports the biodegradation of petroleum hydrocarbon is enhanced in soil treated with animal wastes (22)

These observations show the effectiveness of introducing microbial organisms needed to catalyze the degradation process. It is known that alkanes of moderate size are easily degraded (23) consequently microorganisms are prone to begin degradation with these intermediate chain lengths before the longer chains. This assertion may not be far from the fact that degradation of crude oil complemented by soil treated with animal waste which contain the needed strains of microbes for this action (3).

Table 4: Degradation of crude oil by bacterial isolates in sterile soil amended sterile cow dung

Bioremediation Protocol		Mean TPH (ppm ± SD) after 42days	Overall reduction of TPH (%)
Soil (negative control)	-ve ctrl	3.59 ± 0.02	Not applicable
Soil + Crude (Positive control)	+ve ctrl	124.59 ± 2.53	3.08
Soil + Crude + Consortium	A	28.08 ± 1.59	78.16
Soil + Crude + fecal matter	B	117.24 ± 2.11	8.78
Soil + Crude + consortium t fecal matter	C	12.48 ± 3.22	90.29

Initial TPH 128.56ppm. [abc]Values across the table with similar superscripts are not significantly different at 5% based on ANOVA

4.0 Conclusion

Crude oil hydrocarbon pollution has altered the normal constituent of the fauna and flora of the ecological niches in which crude oil explorative and exploitative activities take place. This study has revealed the effectiveness of microbial consortium together with fecal materials (bio-stimulant) as a useful contributory tool that can help restore the integrity of damages caused by crude oil spills. The feces of *Bos tarus* is rich in essential nutrients, needed to correct nutrient limitations in the growth environment of the hydrocarbonistic bacteria (24) and revitalizes the organisms aiding in their proliferation and utilization of hydrocarbons. Interestingly, the decrease in TPH was high and the peak attenuations obtained were just in 42 days. This suggests that the use of fecal material of *Bos tarus* otherwise known as cow dung could be a cost cutting measure and streamlined process option for hydrocarbon polluted soils. Thus combinations of cow dung and the mixed cultures of *Bacillus. Subtilis, Micrococcus specie* and *Entrobacter aerogenes* effectively degraded long chain hydrocarbons as indicated by the analysis of n-alkanes.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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