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Enumeration and Identification of Microbial Load in Oil Spilled Soil Remediated using *Eudrilus eugeniae*

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ABSTRACT

The scope of this study was to enumerate and identify microorganisms present in crude oil polluted soil before and after remediation with *Eudrilus eugeniae* using conventional method for enumeration and molecular tools (ITS 1 and ITS 4 primers) for identification. A crude oil polluted soil from Khana Local Government of Rivers State Nigeria and also soil from botanical garden of the University of Lagos were used. These soil samples were measured in four different concentrations (25%, 50%, 75% and 100%). Each of these concentrations was experimental setup of addition of substrate alone, *E. eugeniae* and substrate and *E. eugeniae* alone. These setups were made in triplicate amounting to a total of thirty six experimental setups. Enumeration of microbial load was done before and after addition of each of the content of the experimental setups using conventional method and then identification of microbes was done using molecular tools. The result shows total heterotrophic fungi was 2.9 x 10^4 cfu/m/g, while the total heterotrophic bacteria was 4.4 x 10^5 cfu/m/g before remediation. After remediation, total heterotrophic fungi was 1.6 x 10^5 cfu/m/g while total heterotrophic bacteria was 5.0 x 10^4 cfu/m/g. This shows that microorganisms were present in the soil before and after remediation. The increase in number of microbes suggests a favorable condition for continuity of microbial life. Fungi such as *Trichoderma asperellum, Aspergillus flavus, Penicillum chrysogenum* and another strain of *Aspergillus flavus* were identified. The bacteria sequencing failed suspected to be due to primers used or error with sequencing plates. In conclusion, the results of this study confirmed the presence of microorganisms in crude oil polluted soils. The enumeration and identification of the bacteria and fungi in this experiment will help to enhance *in situ* clean-up of crude oil polluted soil.

1. Introduction

Increase in petroleum derived substances has led to contamination of soil environment by indiscriminate release and accidental spillage of petroleum base substances into farmlands. The pollution of the soil by petroleum derived substances such as diesel oil has therefore becomes a prevalent environmental threat. In order to solve this, Environmental remediation such as bioremediation becomes paramount. Bioremediation is a useful method for soil remediation and vermiremediation is a major tool in bioremediation, if pollutant concentrations are moderate and non-biological techniques are not economical.

Oil spill is the release of a liquid petroleum hydrocarbon or its products into the environment, due to human activity, and it's a form of pollution. Pollution is the introduction of contaminants into the natural environment that causes adverse change [1]. Pollution has always accompanied civilizations. It started from prehistoric times when man created the first fires. According to a 1983 article in the journal Science, "soot" found on ceilings of prehistoric caves provides sample evidence of the high levels of pollution that was associated with inadequate ventilation of open fires [2].

Environmental contamination as a result of oil spill has become a global problem in industrialized and developing countries such as Nigeria. It is one of the most dangerous pollution factors known today. It can cause a threat to the environment. This suggests that the effects of crude oil spill will vary from source to source. However details of the potential biological damage will depend on the ecosystem where the spill occurred [3]. Oil spills in the marine environment may affect organisms found therein by direct toxicity or by physical smothering [4]. Oil spills generally, can cause various damages to the marsh vegetation. It was found to reduce growth, photosynthetic rate, stem height, density, and above ground biomass of *Spartina alterniflora* and *Spartina patens* and may cause their death [5]. Crude oil spill on sea forms a surface slick whose components can follow many pathways. Some may pass into the mass of seawater and evidence suggests they may persist for a long time before their degradation by microorganisms in the water [4]. The slick usually becomes more viscous and forms water-in-oil emulsion. Oil in water causes depletion of dissolved oxygen due to transformation of the organic component into inorganic compounds, loss of biodiversity through a decrease in amphipod population that is important in food chain, and eutrophication. Short-term toxicity in fishes includes lymphocytosis, epidermal hyperplasia, and haemorrhagic septicaemia [6]. In mammals it possesses an anticoagulant potency [7]. It was estimated that some tens of thousands of seabirds were killed as a result of spilled oil in sea [8]. Dying mangrove trees, tarred beaches and declining fish catches, all seem to be threats to long term viability of some ecosystem such as the Niger Delta areas of Nigeria [9]. The contamination of these habitats poses major public health and socio-economic hazards which most often has developed into impetuous protestations between some of the oil companies and the surrounding communities.

On land, crude oil spills have caused great negative impact on food productivity. For example, a good percentage of oil spills that occurred on the dry land between 1978 and 1979 in Nigeria, affected farm-lands in which crops such as rice, maize, yams, cassava and plantain were cultivated [9]. Crude oil affects germination and growth of some plants, it also affects soil fertility but the scale of impact depends on the quantity and type of oil spilled. Severe crude oil spill in Cross-River state, Nigeria, forced some farmers to migrate out of their traditional home, especially those that depend solely on agriculture [10]. This is because petroleum hydrocarbons 'sterilize 'the soil and prevent crop growth and yield for a long period of time. The yield of steroidal sapogenin from tuber tissues of *Dioscorea deltoidea* is adversely affected by some hydrocarbons [11]. The negative impact of oil spillages remains the major cause of depletion of the Niger Delta of Nigeria vegetative cover and the mangrove ecosystem [12]. Therefore, there is a serious need for remediation.

Bioremediation is a treatment process that uses organisms to break down, degrade, hazardous substances into less toxic or nontoxic substances. The ability to utilize hydrocarbons is widely distributed among diverse microbial populations. In general, population levels of

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hydrocarbon utilizers and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons [13]. It has also become a universally acceptable technology for the removal of a wide range of contaminants, especially generated from the petrochemical industries from the environment as it is a relatively cheap and effective method of remediation compared to other methods [14].

The techniques of bioremediation include microbial remediation/ microremediation (microoragnsims), phytoremediation (the use of plants), phycoremediation (the use of algae), mycoremediation (fungi), zooremediation (animals) and vermiremediation (earthworm) [15]. Vermiremediation is the use of earthworms to clean up contaminants from the soil. Research on the potential utilization of earthworms has shown an ability to manage different forms of pollution. It has been discovered that earthworms are tolerant to, and can remove, or aid the removal of a wide range of organic and inorganic contaminants such as pesticides, polycyclic aromatic hydrocarbons (PAH), crude oil and heavy metals from the soil. Few researchers recommended the possible approaches to vermiremediation which include direct application of earthworms to contaminated soils, co-application of earthworms to contaminated soils with another organic media such as compost, application of contaminated media to earthworms as part of a feeding regime and indirect use of earthworms through the application of vermidigested material (vermicompost) [16, 17]. Significantly, earthworms lead to improvement in the quality of soil and land where they inhabit [16].

According to Njoku et al. [18], vermiremediation is very cost effective and environmentally sustainable to alleviate polluted soils and sites contaminated with hydrocarbons in just few weeks to months. Earthworms are considered ecosystem engineers because they affect the physicochemical and biological properties of the soils they inhabit through their activities such as casting and burrowing [18]. Several researches have established the potentials of earthworms to bioremediate crude oil and other petrochemicals from laboratory and field trials polluted soil. Earthworms accelerate the removal of hydrocarbons as they burrow through soil by rendering contaminants available for microbial degradation, by feeding on the organic matter that harbour contaminants, and by improving soil structure and aeration.

Ameh et al.[14] investigated the use of earthworms (*Eudrilus eugeniae*) for vermi-assisted bioremediation of petroleum hydrocarbon-contaminated mechanic workshop soils. After 35 days of treatment, earthworm inoculation affected a higher drop in total petroleum hydrocarbon contents as compared to the samples without worms, indicating that earthworms may be used as biocatalysts in the bioremediation process.

Eudrilus eugeniae is a specie of earthworm originated from West Africa and are popularly called as "African night crawler". They are also found in Srilanka and in the Western Ghats of India, particularly, in Travancore and Poona [19]. *Eudrilus eugeniae* lives on the surface layer (epigeic) of moist soil and are also found wherever organic matter is accumulated [19]. It is nocturnal and lies in the surface layer during the day. The worm is reddish brown with convex dorsal surface and pale white, flattened ventral side. The adult worms are about 25-30 cm in length, 5-7 mm in diameter; consist of about 250-300 segments and weigh 5600 mg of maximum individual biomass.

This study would be useful in determination of microbes present in crude oil polluted soil and how the actions of these microbes can improve and restore the soil. The enumeration and identification of the bacteria and fungi in this experiment will help to enhance *in situ* clean-up of crude oil polluted soil.

2. Experimental Methods

The polluted soil was obtained from a depth of 30 cm in Khana Local Government Area of Rivers state in the Niger Delta region of Nigeria. The earthworm (*Eudrilius eugeniae*) and the unpolluted soil were collected from the botanical garden in the University of Lagos, Nigeria and provided with rice bran and dry leaves as food source (substrate).

The experiment was carried out using 4 concentrations (25, 50, 75 and 100%) of crude oil. Each set up contained 1 kg of mixture of the polluted and non-polluted soils; 25% concentration contained 250 g of crude oil polluted soil and 750 g of unpolluted soil, 50% concentration contained 500 g of crude oil polluted soil and 500 g of unpolluted soil, 75% concentration also contained 750 g of crude oil polluted soil and 250 g non polluted soil, while 100% concentration contained 1000 g of crude oil polluted soil. Each of this was then divided into three form: containing the soil into which only *E. euginiae* was introduced, containing soil into which the *E. euginiae* and their substrate were introduced, containing the soil into which only substrate were introduced. Each of these was then replicated in three forms.

For the microbial analysis, sterile cork borer was used to pick distinct fungal growth from the mixed culture at room temperature for 3-5 days [14]. Distinct colonies were picked each from mixed culture and a smear was made using sterile wire loop**.** The same was done for all different colonies growth to get pure cultures**.** The inoculated were incubated for 24 - 48 hours at 37 °C. Cultural characteristics were recorded [14]. The fungal DNA was extracted and a quantity and quality check was done following [20].

Polymerase chain reaction (PCR) was done using the Internal transcribed spacer (ITS) gene for characterization of fungi, Internal transcribed spacer (ITS) universal primer set which flank the ITS1, 5.8S and ITS2 region was used;

ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'

ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3'

PCR conditions include a cycle of initial denaturation at 94 °C for 5 min, followed by 35 cycles of each cycle comprised of 30 seconds denaturation at 94 °C 30 seconds annealing of primer at 55 °C, 1.5 minutes extension at 72 °C and a final extension for 7 minutes at 72 °C [20]. Then the amplified PCR products were purified at 4 °C and sent for sequencing following Frank et al. [21].

2.1 Procedure for Bacterial Genomic DNA Extraction

For the bacterial genomic DNA extraction, single colonies grown on medium were transferred to 1.5 mL of liquid medium and cultures were grown on a shaker for 48 h at 28 °C, and then cultures were centrifuged at 4600x g for 5 minutes following [20]. The PCR for bacterial DNA was done [21]. The amplified fragments of bacterial DNA were purified [21]. Quality and quantity checks were also recorded and the amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis [20].

Phylogenetic tree using maximum likelihood method was used to compare the relatedness of the four fungi isolated from the samples. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model [22]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 533 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [23]. The result from laborartory analysis were subjected to descriptive analysis using SPSS 22 and Graphpad Prism 7.0.

3. Results and Discussion

After forty-two days of experiment, total heterotrophic fungi count, showed a significant decrease in fungal load at 100% with earthworm and substrate. This may be because the microorganisms could not survive at this concentration for a long period of time. The 50% concentration with earthworm alone had increase in fungal load, this may likely be because the concentration (level of pollution) was suitable for the fungi and they could strive better to continue multiplying at this concentration.

Table 1 Total heterotrophic fungi count at initial and final

Sample code	Average Fungal load	
	Initial THFC (cfu/m/g)	Final THFC (cfu/m/g)
25% A	$1.28\times10^{3}\pm158.99$	$4.03\times10^{3}\pm1125.96$
25% B	$1.28\times10^{3}\pm709.66$	$5.77\times10^{4}\pm41216.23$
25% C	$1.75\times10^{3}\pm525.20$	$4.10\times10^{4}\pm29737.74$
50% A	$2.37\times10^{3}\pm771.54$	$1.63\times10^{5}\pm143271.70$
50% B	$8.5\times10^{2}\pm104.08$	$9.33\times10^{3}\pm1855.92$
50% C	$1.02\times10^{3}\pm109.29$	$2.57\times10^{4}\pm17227.24$
75% A	$1.78\times10^{4}\pm7595.69$	$1.67\times10^{4}\pm4371.63$
75% B	$1.08\times10^{3}\pm684.55$	$5.40\times10^{4}\pm43061.97$
75% C	$1.0\times10^{4}\pm3905.13$	$2.47\times10^{4}\pm10728.98$
100% A	$1.88\times10^{4}\pm6495.73$	$4.33\times10^{3}\pm666.67$
100% B	$2.93\times10^{4}\pm15624.06$	$3.67\times10^{3}\pm6173.42$
100% C	$1.13\times10^{4}\pm5650.07$	$7.67\times10^{3}\pm6173.42$

A= Earthworm alone; B= Earthworm+substrate; C= Substrate alone. Cfu/m/g= colony forming unit per millimetre per gram

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Meanwhile, for total heterotrophic bacterial count, 75% concentration with earthworm alone significantly increased in bacteria load, this may suggest that the concentration was favourable for the bacteria to survive. The 25 % concentration with earthworm and substrate significantly decreased in bacteria load. This may suggest that some of the bacteria that were present initially had been lost due to the earthworms eating up some of the bacteria.

Table 2 Total heterotrophic bacterial count at initial and final

Sample Code	Average Bacterial Load	
	Initial THBC (cfu/m/g)	Final THBC (cfu/m/g)
25% A	$1.95\times10^5\pm11302.66$	$3.58\times10^6\pm3216722$
25% B	$5.0\times10^4\pm0$	$4.38\times10^7\pm19133333$
25% C	$5.55\times10^4\pm4821.83$	$1.77\times10^6\pm1173049$
50% A	$4.88\times10^4\pm26258.86$	$3.50\times10^7\pm13527749$
50% B	$5.78\times10^4\pm28181.46$	$7.00\times10^7\pm6995000$
50% C	$7.0766\times10^4\pm12875.47$	$5.57\times10^6\pm1550871$
75% A	$4.43\times10^5\pm60575.94$	$1.90\times10^6\pm1550871$
75% B	$6.90\times10^4\pm4000$	$2.55\times10^6\pm13755199$
75% C	$2.95\times10^5\pm142243.92$	$2.67\times10^6\pm6765928$
100% A	$4.82\times10^5\pm55251.95$	$1.66\times10^6\pm1179834$
100% B	$3.27\times10^6\pm155025.89$	$1.55\times10^7\pm15231765$
100% C	$2.68\times10^5\pm92300.84$	$3.10\times10^7\pm2952413$

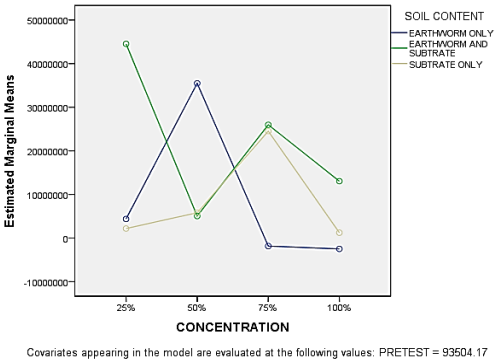

Fig. 1 Estimated Marginal Means of Fungi and Bacteria

After the Gel electrophoresis, pictures of purified PCR products amplified from bacteria and fungi showed samples [9]. These likely tallies with the work of Santhini et al. [24] whereby; seven samples were discovered. This may be as a result of the substrate used. The phylogenetic analysis of the samples was 100 % relatedness between Sample 1 identified as *Trichoderma asperellum* and sample 2 which is *Aspergillus flavus*. Sample 2 and sample 4 which are both *Aspergilli* are different strains. Also from morphological characteristics of the two isolates, there are more sclerotia with fewer spores in sample 4 identified as *Aspergillus terreus* while yellowish green spores with large but few sclerotia were observed with sample 2 identified as *Aspergillus flavus.* Sample 3 identified from BLAST result as *Penicillium chrysogenium* was shown as an out-group. With reference from the work of Santhini et al. [24] and other researchers this evidently confirmed that microorganisms are regular in soils contaminated with crude oil. This entails a great capability for these species to endure in such an environment and therefore indicates the strains could hold a capability of degrading petroleum hydrocarbons from the soils they inhabit. However, the few isolates of this work that included *Trichoderma asperellum, Aspergillus flavus, Penicillum chrysogenum* and another entirely different strain of *Aspergillus flavus* have given an indication to the identity and phylogeny of microorganisms in crude oil polluted soil.

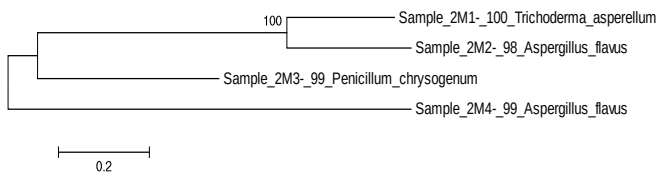

Fig. 1 Molecular phylogenetic analysis by maximum likelihood method

The tree with the highest log likelihood (-2685.2001) is shown in Fig. 1. The percentage of trees in which the associated taxa clustered together is shown next to the branches in Fig. 1 below. In the figure below, the fungi with the longest branch is known as recently evolved while the fungus with the shortest branch is known as the oldest on the tree.

4. Conclusion

The findings of this study confirmed the presence of microorganisms such as bacteria and fungi in crude oil polluted soils. The enumeration and identification of the bacteria and fungi in this experiment suggests trying these microorganisms for remediation of crude oil polluted soil. Further studies could also be done for more novel bacterial and fungal species to be recovered in future.

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