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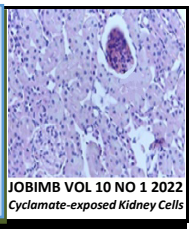

Hydrocarbon Degradation Competence of Bacterial Consortium Isolated from Oil Polluted Soil in Azuabie Town, Port Harcourt, Nigeria

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ABSTRACT

Spills from artisanal hydrocarbon processing in Port Harcourt, Nigeria's Niger Delta, have devastated the local flora and fauna. Microbial consortiums can modify metabolic pathways and reduce hydrocarbon pollution. This study investigated the degradation efficiency of a bacterial consortium composed of *Escherichia coli., Klebsiella* sp., *Lactobacillus* sp., *Enterobacter* sp., *Proteus* sp., and *Serratia* sp., isolated from recurrently polluted soil in Azuabie Town Trans Amadi, Port Harcourt, with total petroleum hydrocarbon (TPH) concentration of 1487.24181 mg/kg and polycyclic aromatic hydrocarbons (PAHs) concentration of 12.852 mg/kg, a value higher than the Nigerian Midstream and Downstream Petroleum Regulatory Authority (NPRA), intervention limit of 5000 mg/kg and 40 mg/kg. Soil samples were aseptically collected with a sterile spatula at 15 cm depth. Microbial isolation was done using enrichment and enumerated via the vapour phase transfer techniques in the Bushnell Haas medium. A colorimetric assay using a 2,6-dichlorophenol indophenol (DCPIP) redox indicator was employed to determine the biodegradation potentials of the isolates. Total diesel degrading bacteria ranged at 2.2×10^6 cfu/g, kerosene degrading bacteria ranged at 3.2×10^6 cfu/g, premium motor spirit oil-degrading bacteria ranged at 4.4×10^6 cfu/g, and engine oil-degrading bacteria ranged at 2.0×10^6 cfu/g. Bacterial strain from the genera *Escherichia coli., Klebsiella* sp., *Lactobacillus* sp., *Enterobacter* sp., *Proteus* sp., and *Serratia* sp., utilized diesel, kerosene, premium moto spirit oil and engine oil as carbon sources. The polluted site's indigenous bacterial community can perform natural attenuation and breakdown hydrocarbons for in situ and ex situ bioremediation.

INTRODUCTION

Pollution caused by petroleum and petrochemical products; a complex mixture of hydrocarbons has been recognized as one of the most serious environmental threats of the 21st century [1]. The consequences of environmental pollution of hydrocarbons involve widespread and long-term destruction of terrestrial and aquatic life and serious human health impairment issues [2]. Oil spillage in Nigeria causes damage to the environment severely due to a lack of logistical support and a trained workforce to put

in place laws, and prompt measures to effectively detect spillage most especially in the Niger Delta region where illegal bunkering has course severe damage to the ecosystem [3,4]. The high incidence of oil leakages and accidental discharges into the environment is enormous. Often oil leakages from pipelines and incidence of oil discharge from illegal bunkering occur in the mangrove swamp forest, the most reproductive ecosystem [5]. Therefore, the exploration and production of petroleum and its derivatives through transportation and distribution in the Niger Delta region has led to serious environmental pollution of terrestrial and aquatic habitats causing a threat to associated flora and fauna [6].

Studies have shown that more than 70 protected areas in the Niger Delta region of Nigeria have lost substantial portions of their areas, including biodiversity depletion due to oil exploration and illegal bunkering [7]. Petroleum oil exploitation activities in the Niger Delta region of Nigeria have in decades increased deforestation and illegal cataloguing activities. This has led to alterations in aquatic and terrestrial habitats, loss of biodiversity, and vegetation fragmentation. These incidences led the United Nations Human Development to make the following declaration about the Niger Delta region of Nigeria, "there is a strong feeling in the region that the degree and rate of environmental degradation are pushing the delta towards ecological disaster [8].

Petroleum products and their fraction emissions and associated pollution occurrence from the activities of multinational corporations in the Niger Delta region have toxic harmful impacts on the region. The oil fractions also known as petroleum hydrocarbons can bioaccumulate and bioconcentrate in lower forms and can be passed on through the food chain to upper trophic level organisms, including humans [9]; [10]; [11]. This study isolated and investigated indigenous microorganisms carrying out the natural attenuation process of bioremediation/biodegradation from oil-contaminated soil in Azuabie Town, Port Harcourt, Rivers State, Nigeria, and their ability to degrade refined petroleum hydrocarbons using 2, 6 Diclorophenol indophenol redox indicator.

MATERIALS AND METHODS

Soil samples were collected from a location contaminated with artisanal refined hydrocarbon oil in Azuabie Town, Trans Amadi Port Harcourt, Nigeria from a depth of 15 cm, put in a sterile bag and taken to the National Agency for Food and Drug Administration and Control Port Harcourt Area laboratory for analysis.

Isolation of Hydrocarbon-degrading bacteria

Hydrocarbon degrading microorganisms were isolated by the enrichment method. A weighed gram of each collected soil sample was added to 100 mL of freshly prepared Bushnell Haas Broth. The composition of (BHB) was done by adding (g/L): KH_2PO_4 1; K_2HPO_4, 1; NH_4NO_3, 1; $MgSO_47H_2O$, 0.2; $FeCL_3$, 0.05; $CaCL_22H_2O$, 0.02. The medium was autoclaved at 121 °C for 15 min. Sterile crude oil was used as a carbon source at 1% (w/v). Enrichment was carried out with a rotary shaking at 180 rpm for several weeks at 37 °C before hydrocarbon-degrading microorganisms were enumerated by pour plating on Standard plate count agar [12].

Characterization of Total petroleum hydrocarbons and polycyclic aromatic hydrocarbons

Sample pre-treatment was conducted according to US-EPA (Method 3050B) [13]. 1 g of hydrocarbon contaminated soil sample was ashed in a muffle furnace at a temperature of 630 °C for 3 hours.

The ashed sample was dissolved in 10 mL concentrated HCl and was heated on an electro-thermal heater hot plate. The solution of the ash was diluted to 50 mL with distilled water and analysed for metal ions by atomic absorption spectrophotometer. TPH and PAH were analysed and determined by GC/FID analysis.

Colorimetric Screening Test for Hydrocarbon Biodegradation with Bacteria Consortium

Bacteria were tested for their potential to utilize different hydrocarbons. The test was based on the ability of the microbes to oxidize hydrocarbons aerobically with the transfer of electron acceptors like O_2 decolorizing the redox indicator 2,6-Dichlorophenol indophenol (DCPIP) from blue to colourless. The redox indicator was prepared by adding 1 g of DCPIP powder to 1 litre of distilled water in a volumetric flask for a final redox potential of +0.217v [14]. Colonies of 48 hours old isolates stored in peptone broth were homogenized and 2 mL homogenate was transferred into a test tube. The final experimental solution contained 2 mL of the isolate homogenate solution, 8 mL of BHB, 0.5 mL kerosene, diesel oil, premium motor spirit oil and engine oil (filtered sterilized) and 1 mL of redox indicator [15]. A similar set-up containing 0.5 mL of each hydrocarbon was used as a control to evaluate degradation. Hydrocarbon degradation was monitored for changes in colour from blue to colourless (**Fig. 1**).

Fig. 1. Biodegradation screening set up of hydrocarbon utilizing bacteria. Tube 1: Has a control tube with BHM, DCPIP and a similar tube with BHM, DCPIP, Engine oil and consortia isolates. Tube 2: Has a control tube with BHM, DCPIP and a similar tube with BHM, DCPIP, PMS and consortia isolates. Tube 3: Has a control tube with BHM, DCPIP and a similar tube with BHM, DCPIP, Diesel and consortia isolates. Tube 4: Has a control tube with BHM, DCPIP and a similar tube with BHM, DCPIP, Kerosene and consortia isolates.

RESULTS

Results of the physiochemical properties of the polluted soil are shown in **Table** 1. The soil is chronically contaminated with petroleum hydrocarbons with a neutral pH of 6.6. Nutrient availability of nitrogen and phosphorus absorption is extremely low as shown in **Table 1**. Total petroleum hydrocarbon (TPH) of the polluted soil was 1487.2 mg/kg with carbon lengths of C_{12}, C_{14} through C_{40} while polycyclic aromatic hydrocarbon (PAH) was 12.8 mg/kg with known carcinogenic compounds. The concentration values gotten are higher than the acceptable limit stipulated by EGASPIN. Hydrocarbon utilization bacteria data is shown in **Table 2**. **Figs. 2**, and **3** shows the chromatogram of total petroleum hydrocarbons and polycyclic aromatic hydrocarbon

concentrations in the polluted soil. Based on biochemical reactions [5] distinct bacterial species isolated were namely *Escherichia coli., Klebsiella* sp.*, Enterobacter* sp.*, Lactobacillus* sp. and *Proteus* sp.

Table 1. Physiochemical properties of soil.

parameters	Concentration in the soil sample	NPRA/NURC intervention limit
TPH (mg/kg)	1487.24181	5,000
PAH (mg/kg)	12.85287	40
pH	6.6	_
Conductivity (µs/cm)	125	_
Temperature (^{O}C)	27.3	_
Moisture (%)	7.72	_
Total Nitrogen (%)	0.056	_
Phosphate (mg/kg)	1.554	_
Potassium (mg/kg)	145.87	_
Lead (mg/kg)	7.02	520
Cadmium (mg/kg)	0.41	12
Nickel (mg/kg)	1.96	210
Copper (mg/kg)	1.14	190

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= No of Nigerian Midstream and Downstream Petroleum; Regulatory Authority and the Nigerian Upstream Regulatory Commission (NPRA and NURC) Intervention limit in Soil.

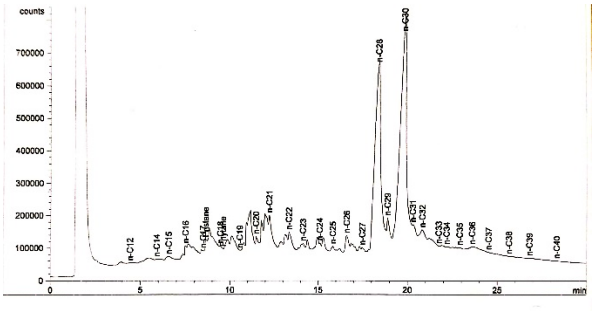

Fig. 2. Chromatogram of total petroleum hydrocarbons (TPH) concentration in the polluted soil.

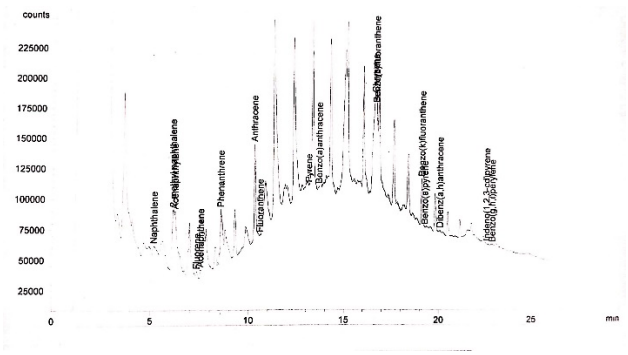

Fig. 3. Representative GC/FID chromatogram of PAH compounds in the polluted soil

Table. 2 Hydrocarbon degrading microbial properties.

Hydrocarbon degrading bacteria	Dilution	Colonies counted	CFU/gm
Diesel degrading bacteria (Automotive gas oil)	10^{-5}	101	2.2×10^{6}
Kerosene degrading bacteria (Paraffin oil)	10^{-5}	160	3.2×10^{6}
Premium motor spirit oil degrading bacteria (PMS)	10^{-5}	220	4.4×10^{6}
Engine oil degrading bacteria (Lubricating oil)	10^{-5}	100	2.0×10^{6}

DISCUSSION

This study investigated the ability of microorganisms used as a consortium to degrade arrays of hydrocarbons which could serve as important agents for the bioremediation of hydrocarbon-polluted soil in the Niger Delta region, Nigeria. The intermittently polluted soil's total petroleum hydrocarbon and polycyclic aromatic hydrocarbon concentrations were 1487.242 mg/kg and 12.852 mg/kg against the regulatory intervention limit of 5000 mg/kg and 40 mg/kg in the soil as regulated in EGASPIN [16]. Total diesel degrading bacteria ranged at 2.2×10^{6}, kerosene degrading bacteria ranged at 3.2×10^{6}, premium motor spirit oil-degrading bacteria ranged at 4.4×10^{6}, while engine oil-degrading bacteria ranged at 2.0×10^{6}. The consortiums show more degradative capability in premium motor spirit oil degradation followed by kerosene before diesel and engine oil degradation.

The discharge of hydrocarbons into terrestrial and water bodies is becoming a real threat. Hydrocarbons disposed of in soil or water cause substantial ecological deteriorations such as damaging the photosynthesis process, whereby changing soil texture and water quality and bringing about adverse aesthetic influences. These issues are further compounded by the gradual accumulation of hydrocarbon in the tissues of terrestrial animals and aquatic creatures, which consequently causes serious health impairment issues throughout the food chain [17].

Petroleum hydrocarbon alters the pH of the ecological environment mostly soil, it changes the oxygen proportion by creating anoxic precincts whereby exerting pressure on the diversity of microbial populations, causing a shift in the microbial community resident in the soil but allowing few microbial species to survive in such ecological niches because of their ability to modify inherent structure and functions which enable them to adapt to the chemical constituents in hydrocarbon contaminated soil [18]. In this study, the microbial consortia were employed to biodegrade diesel, kerosene, premium motor spirit oil and engine oil. The process began with the accurate selection of microbes that possess bio-degradative competencies in the previous study [19].

The selected genus *Escherichia coli, Klebsiella* sp.*, Lactobacillus* sp.*, Enterobacter* sp.*, Serratia* sp.*,* and *Proteus* sp.*,* exhibited auspicious biodegradation traits within a time range of 20-24 hours, either by mutualism, commensalism, and complementary metabolic activities has improved the co-existence stability, achieving complete degradation in 24 hours with the use of Bushnell Haas medium and 2, 6-dichlorophenol indophenol (DCPIP). Interestingly, the presence of compatibility and mutually beneficial corporation in the mixed co-culture system accelerates the biodegradation process, as stipulated by Ibrahim *et al.,* [20].

Compared to a single strain, consortia have broad metabolic potential to degrade various hydrocarbons [21]. This metabolic cross-feeding in a microbial consortium ensures complete mineralization of contaminants which involves multiple catalytic enzymes that generally do not exist in a single strain, thus, the microbial consortium is a feasible alternative for real-world application due to its intrinsic advantages, allocation of the metabolic burden through division of labour, more stability to environmental fluctuations, broader metabolic capabilities and greater robustness to facilitate intermediate compound removal and enhance swift biodegradation process due to cooperated and complementary metabolic activities. [21].

The high absorption of total petroleum hydrocarbon 1481.242 mg/kg against the 5000 mg/kg regulation limit by EGASPIN [16], further depleted nutrient availability in the polluted soil; carbon-nitrogen, carbon-phosphorus percentage as well as the percentage of carbon to other micronutrients availability in the polluted soil, creating disproportion. The presence of lead also shows a high degree of toxicity since lead is a known toxicant. Even though it has a low concentration in the contaminated soil, it can impact negatively diverse microbial species [22]. Lead can also accumulate in soil for a longer period and be converted into lead sulphate to form inert compounds due to organic matter interaction in the soil [22]. The concentration of cadmium and nickel in both oxides and hydroxides forms are less harmful to organisms, but due to chemical reduction, oxidation and alterations, soil pH toxicity can be accelerated [23].

The presence of copper in the soil signifies a strong correlation that exists between copper, organic carbons and oxides in the polluted soil, though the concentration is lower than the value obtained by Okafor *et al.,* [14], which could be attributed to the variations in the soil structure and texture in association to heavy crude oil pollution. Phosphorus and nitrogen are the most essential nutrients required for bacterial metabolism which when depleted in absorptions will negatively affect the effective nature of these microbes to bio-remediate recalcitrant and xenobiotic. [24]; and [25], stipulated the use of NPK fertilizers, nitrogen-rich agricultural wastes and bio-fertilizers to balance the nutrient availability of polluted soil and ensure effective and robust nutrient cycling during the bioremediation of hydrocarbon polluted soil.

The isolates used as bio consortium in this study had been found to degrade crude oil in the previous study [19]. Among the bacteria sp., isolated *Enterobacter* sp., *Serratia* sp., *Klebsiella* sp., and *Proteus* sp., are widely reported as hydrocarbon degraders [26]; [27]. Other isolates *Escherichia coli* was reported by [28]; [29], while *Lactobacillus* sp., was reported by [30] for their polycyclic aromatic hydrocarbon degradative capability. [31], reported in their findings, the ability of probiotic *Lactobacillus* in Benzo[a]pyrene degradation. These hydrocarbon degraders identified in this study can be harnessed, and genetically studied to decipher their catabolic genes, enzymes and hydrocarbon specificities for future application in the clean-up of hydrocarbon oil impacted sites in the Niger Delta region of Nigeria.

CONCLUSION

Bioremediation efforts have mainly focused on exploiting a single strain superbug to achieve the desired goals of biodegradation. However, a single bacterium utilizing the entire hydrocarbon is very unlikely because a complete mineralization process involves multiple catalytic enzymes that generally do not exist in a single bacterial strain. The advancement of biotechnology can help to engineer microbial consortia with desired functions associated with bioremediation. This engineering process can oblige a natural microbial community to accomplish the desired functions by modifying environmental variables, reconfiguring the metabolic pathway and reprogramming social interactions among different species of microorganisms. These approaches could be used for microbial consortia to achieve an effective bioremediation process. In this study, we constructed an effective bacterial consortium composed of *Escherichia coli, Klebsiella* sp., *Enterobacter* sp., *Proteus* sp., and *Lactobacillus* sp., obtained from the site used for artisanal refined oil activities to degrade diesel, kerosene, premium motor spirit oil and engine oil. The consortium exhibited a significant ability to assimilate these hydrocarbons as a carbon source in Bushnell Haas medium with stronger efficacy using 2,6-Dichlorophenol indophenol oxidation reagent. The trend of the degradation of arrays of refined hydrocarbons and the presence of these heterophilic organisms in this study area is proof of in-situ bioremediation of hydrocarbons in the polluted soil. Further studies should be focused on the metabolic interactions between species brought together as microbial consortia to make accurate control over bioremediation processes to obtain desired functions and stable results.

INSTITUTIONAL REVIEW BOARD STATEMENT

This study was conducted according to the Environmental guideline and Standard for the Petroleum Industries in Nigeria (EGASPIN), 1992, Revised 2002. Issued by the Nigerian Midstream and Downstream Petroleum; Regulatory Authority and the Nigerian Upstream Regulatory Commission (NPRA and NURC).

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