Hydrocarbon degrading potentials of *Chromobacterium violaceum, Bacillus subtilis* and *Micrococcus luteus* isolated from lemna waste dumpsite, Cross River State, Nigeria

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Abstract- The quest to harness the vast species of microorganisms inherent in waste dumpsites in Calabar metropolis for bioremediation of hydrocarbon polluted sites formed the focus of this study. Three bacterial species isolated fromLemna dumpsite, Calabar were identified and characterized as *Chromobacterium violaceum, Bacillus subtilis* and *Micrococcus luteus* using standard microbiological procedures. The bacterial isolates were then investigated for their hydrocarbon degrading potentials by subjecting them to 5 days fermentation programme in a continuous agitation using three hydrocarbon substrates (Kerosene, diesel and petrol). The results obtained surprisingly revealed *Chromobacterium violaceum*as the best hydrocarbon degrader as it uses mostly petrol and kerosene as its most favorable carbon sources in the order petrol > kerosene > diesel.*Bacillus* subtilis were more prolific in degrading diesel and kerosene than petrol in the order Diesel > kerosene > petrol, while *Micrococcus luteus* was observed as the least hydrocarbon degrading bacteria species amongst the isolates as they tend to adapt slowly to the three different hydrocarbon substrates used.The high presence of these bacterial species in decomposing solid waste, dumpsite leachates and soil depicts their ability to biodegrade mixed volumes of heterogeneous wastes comprising both organic, inorganic and most times hydrocarbon fractions inherent in the wastes. These however are attributes for the hydrocarbon degrading potentials of organisms isolated from such dumpsites when employed for bioremediation studies. Hence the need explore these bacterial species for large scale oil clean-up exercise in the field.

Index Terms- *Chromobacterium violaceum, Bacillus, Micrococcus luteus,*Lemna dumpsite

I. INTRODUCTION

Municipal solid waste dumpsites harbors a verse diversity of microorganisms most of which are hydrocarbonoclastic with great potentials to biodegrade different fractions of hydrocarbons. Hydrocarbons in the Environment are biodegraded primarily by bacteria and fungi (Leahy *et al.*, 1990).

In Calabar as well as other parts of Nigeria and to a larger extent most developing countries, the metropolis are littered with wastes of various kinds; garbage, plastics, bottles, disposable cups, discarded tires and even human and live-stock faces (Bassey *et al.*, 2015). These wastes are aesthetically unpleasant, constitute eyesores, produce unpleasant odor especially decomposed by bacteria. These dumpsites thus constitute a habitat for vector and other nuisance organisms capable of transmitting or causing diseases such as typhoid, infantile diarrhea and cholera in humans and animals. Waste dumpsites refer to areas or land sites where material wastes from several sources and processes (domestic, industrial, etc) are deposited. Waste dumps include both municipal solid wastes and industrial wastes including liquid effluents containing heavy metals (Olanrewaju, 2002).

Waste dumpsites provide a rich source of microorganisms most of which are pathogenic and also hydrocarbonoclastic (Odeyemi *et al*., 2011). Activities involving the disposal of solid wastes even if properly controlled with proper precautionary measures adopted may have adverse impact on the environment especially air since most of the dumps are open.

Microorganisms present in the waste example; *Chromobacterium violaceum, Bacillus sp and Micrococcus sp* use the waste as a food source. Under anaerobic conditions typical in most dumps, these microorganisms convert the organic material in the waste to methane and carbon dioxide. These gases rise through the dump and escapes into the atmosphere. The presence of large amounts of these gases in uncontrolled environment may subsequently lead to global warming and other environmental challenges.

The major objective of this study was the isolation of bacteria present in Lemna Dumpsite in Calabar, Nigeria and determining their level of hydrocarbon degrading potentials for environmental management processes such as bioremediation.

II. MATERIALS AND METHODS

The study area and sampling site

The study area is Calabar municipality which is located between latitude $4^0$13’ E and $5^0$15’ E and longitude $8^0$15 S and $8^0$21’S in Nigeria. The area is characterized by wet and dry

seasons with high annual rainfall in the range of 350 – 400mm, and run-off estimated to reach 90%. It has mangrove vegetation.

The sampling site was an open dump, popularly called Lemna dumpsite and located in IkotEffangaMkpa ravine, in Calabar municipality. The ravine is located North-East of Calabar municipality. It is bounded on the south by Eburutu Barracks which is a residential area. Its topography is plane except a few slopy terrain which ends in the ravine. The waste dumpsite is managed by Patson Environmental Services Limited (PESL) and receives wastes on daily basis. Most of these disposed wastes are mainly domestic and market wastes. The waste dump has an area of about 3,265m^2 and close to a small stream and river used as water board intake and many households within the area for domestic services.

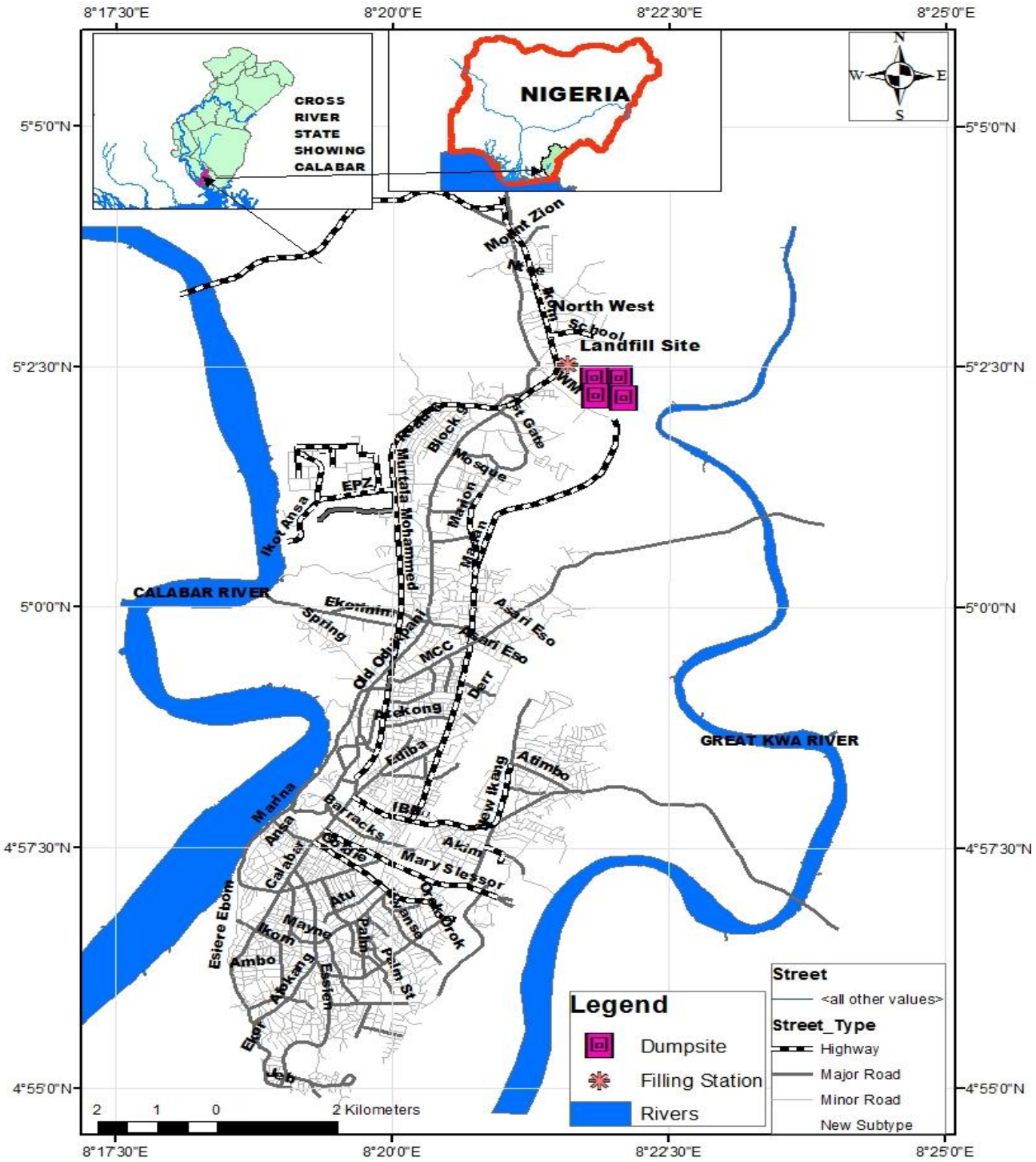

PLATE 1: map of study area showing sampling site in Calabar Municipality
Source: GIS Unit, University of Calabar, 2012

III. SAMPLE COLLECTION

Solid waste sample collection

A large wooden spatula that had been properly rinsed with sterile distilled water was used to collect the decomposing wastes into sterile glass Petri dishes and sealed with masking tape and properly labeled. The samples were labeled with the abbreviations SW1, SW2, SW3, and SW4. These samples were collected from the Ikot–EffangaMkpa dumpsite of Calabar metropolis. The entire samples were then taken to the laboratory for analysis or stored in a refrigerator at 4^{O}C until they were needed for analysis.

Soil sample collection

With the aid of a garden rake, the wastes were removed to expose the soil under the waste dump from where waste and leachate samples would be collected. The soil sample were taken at about 15cm depth with the use of a hand-driven auger and then taken to the laboratory in labeled polythene bags in ice-cold boxes at approximately 4^{0}C for both microbiological and physicochemical analysis. A control sample was taken from a location bout 500 meters away from the dumpsite.

Leachate sample collection

The method described by Basseyet *al.,* 2015 was employed. Here the PVC pipes were bought and cut into four parts, each of 1m and 0.5m in length. The base end of each pipe was permanently sealed with a pipe cover and an adhesive while the top ends were just fitted with pipe covers. The pipes (both 1m and 0.5m length) were perforated evenly at considerable distances from their base ends to allow for water percolation and collection. The whole pipe lengths were then buried into an already dug ground in each sampling point with small allowances at the top for access to top ends (which were only temporarily sealed). The dug out soil and wastes were replaced back to the ground and made to properly cover the pipes. This was left for a period of three to four (3-4) weeks before sampling for the percolated leachates. Sterile enema pumps were used for the leachate collection into sterile bottles and labeled properly as L1, L2, L3 and L4 respectively. The samples were then taken to the laboratory for microbiological and physicochemical analysis or stored in the refrigerator maintained at 4^{0}C until required for analysis.

PLATE 2: Perforated PVC pipes used for sampling of leachates from the dumpsite

Isolation, identification and confirmation of organisms

For decomposing solid waste (DSW) and dumpsite soil sample (DS) One gram (1g) was dissolved in 9ml sterile distilled water. From the solution, ten-fold serial dilutions in the range of 10^{-1} – 10^{-9} were prepared (Atlas and Bartha, 1992; Ejaet *al.,* 2006, Bassey et al., 2015). One milliliter (1ml) aliquot of the sample dilution from 10^{-4} – 10^{-6} was then seeded into sterile Petri dishes and total heterotrophic bacterial count was determined by pour plate technique using tryptone soy agar (APHA, 1998). For the recovery of aerobes, tryptone soy agar was used while tryptone soya supplemented with 1%(w/v) cysteine hydrochloride (BDH Chemicals, U.K) was used for the recovery of anaerobes. Aerobic cultures were incubated at 35°C for 48 hours while anaerobic cultures were incubated in Baird and Tat-lock anaerobic jar at 30°C for (48 to 72 hours. Visible colonies of between 30–300 were multiplied by the reciprocal of the dilution factors and recorded as colony forming units per gram (cfu/g) of waste.Characteristic colonies on the tryptone soy agar were picked by a sterile wire loop and streaked on nutrient agar and incubated at 37°C for 48 hours. This was repeated until pure cultures were obtained and preserved at 4°C in agar slants for biochemical identification. The bacteria isolated are then identified using Bergey's Manual of Determinative Bacteriology (Buchanam and Gibons, 1974).

Hydrocarbon Utilizationand Growth Experiment

Distinct colony of the confirmed test organisms were subcultured unto nutrient broth in universal sterile bottles and incubated at bench for 16-24hrs to obtain viable colonies. Turbidity of the broth was thereafter determined. Hydrocarbon utilization by the test bacterial species with respect to the petroleum fractions used (kerosene, diesel and petrol) were determined using colony forming unit and optical density for a

fermentation period of 5 days in flasks subjected to vigorous agitations.

Minimal salt brothThe enrichment procedure as described by Nwachukwu (2000) was used in the estimation of hydrocarbon utilizers. A minimal salt broth containing 2.0g of NA2HPO4, 0.17g of K2SO4, 4.0g of NH4NO3, 0.53g of KH2PO4, 0.10g of MgSO4, 7H2O and 5.0g of agar – agar dissolved in 1000ml of distilled water was prepared. The solution was sterilized by autoclaving. Twenty-eight test tubes were sterilized and placed in test tube racks,

IV. RESULTS AND DISCUSSION

The frequency of occurrence of the three bacterial species isolated from Lemna waste dumpsite soil (DS), decomposing solid waste (DSW) and leachate sample (LS) are presented in table 1. Identification of the isolates as *Bacillus subtilis*, *Chromobacteriumviolaceum and Micrococcus luteus*was done using Bergey's Manual of determinative Bacteriology as revised by Buchanam and Gibbon (1974).

The optical densities for *Chromobacteriumviolaceum*when subjected to fermentation in three different hydrocarbon fractions shows that the bacterium utilizes petrol and kerosene better than diesel fraction in the order petrol>kerosene>diesel with optical density of Fig. 1.

Represented in Fig. 2 are the optical densities in absorbance for *Bacillus subtilis*

Table 1: Frequency of occurrence of bacterial species isolated from Lemna waste dumpsite

Bacterial isolates	**DS**	**DSW**	**LS**
Bacillus subtilis	52(41.27%)	30(28.85%)	36(47.37%)
Chromobacteriumviolaceum	53(42.06%)	44(42.31%)	30(39.47%)
Micrococcus luteus	21(16.67%)	30(28.85%)	10(5.68%)
Total	126	104	76

DS= dumpsite soil, DSW= Decomposing solid waste, LS= Leachate sample

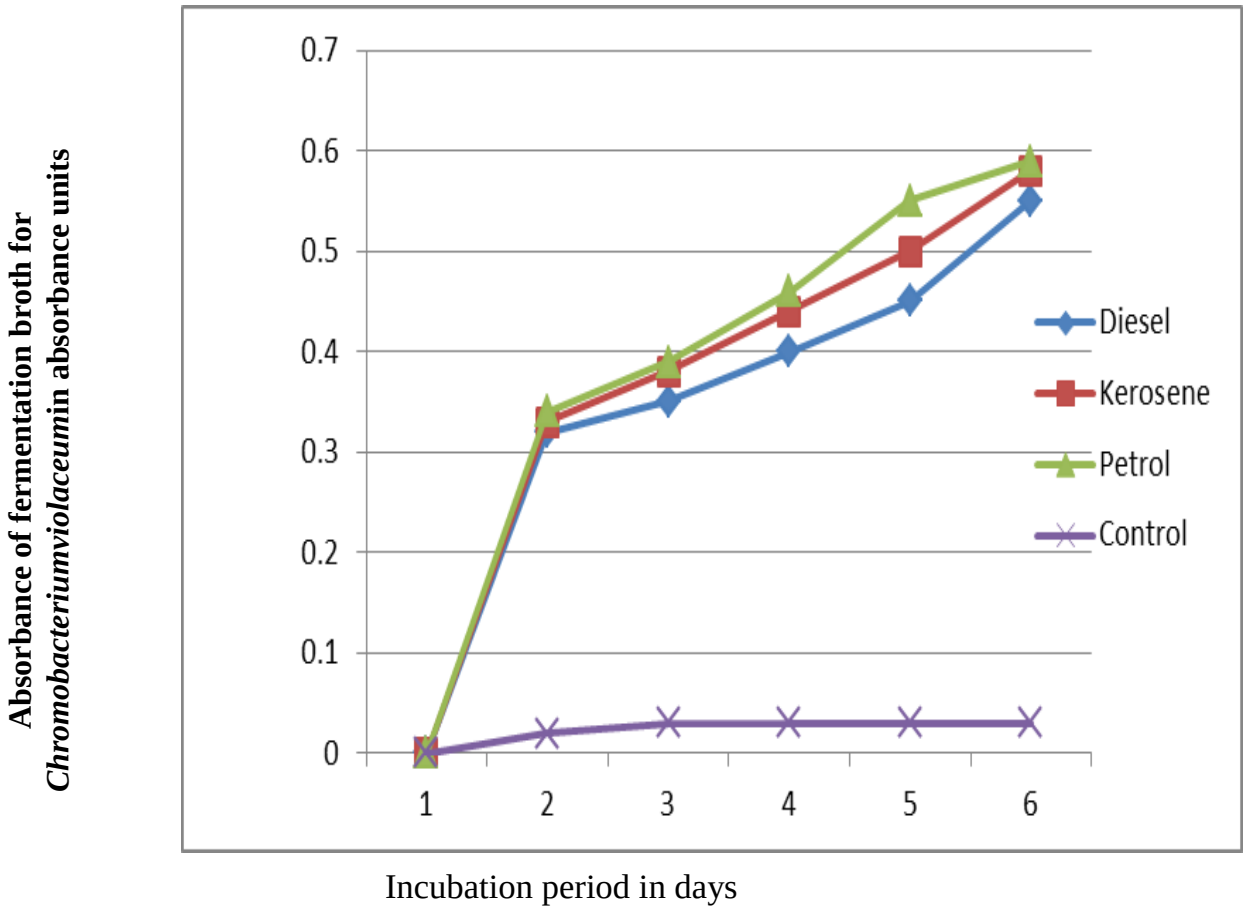

Figure 1: Graphical representation of optical density of *Chromobacteriumviolacium* grown in different hydrocarbon substrates.

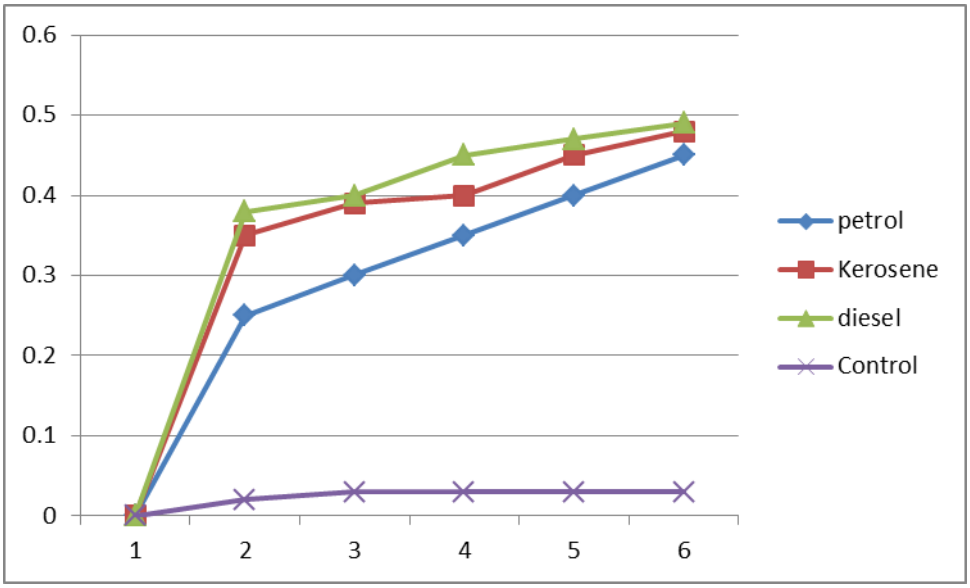

Figure 2: Graphical representation of optical density of *Bacillus subtilis* grown in different hydrocarbon substrates.

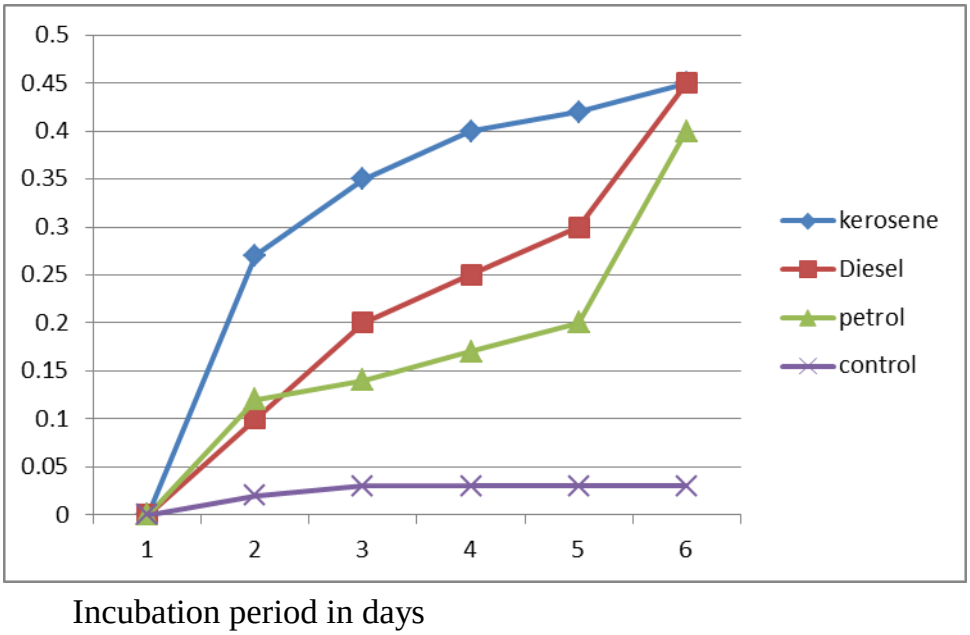

Figure 3: Graphical representation of optical density of *Micrococcus* spgrown in different hydrocarbon substrates.

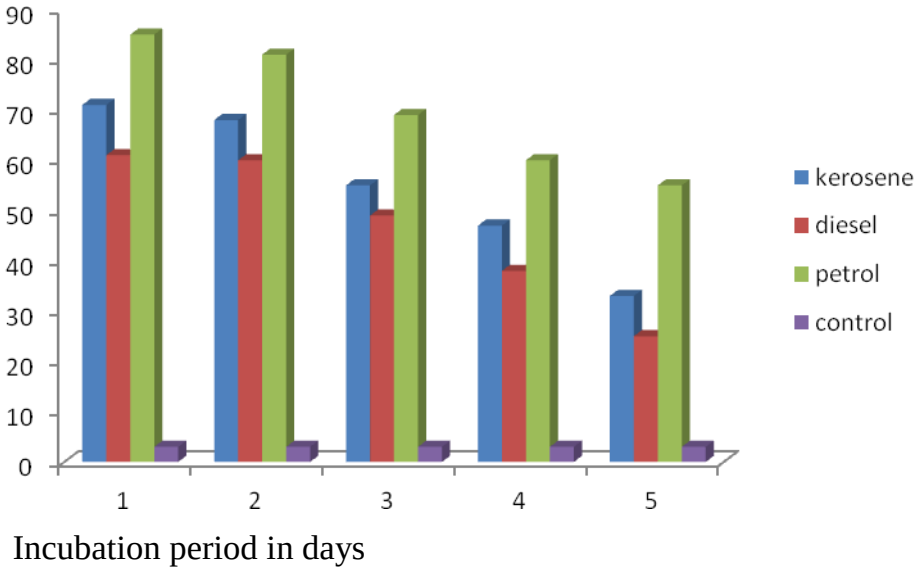

Figure 4: Bar chart showing Colony Forming Units of *Chromobacteriumviolaceum*grown in different hydrocarbon substrates.

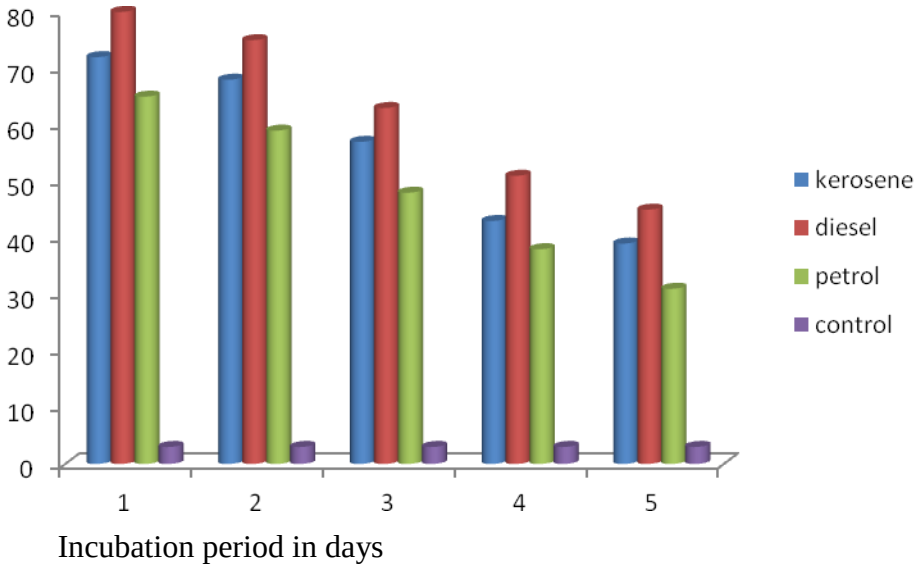

Figure 5: Bar chart showing Colony Forming Units of *Bacillus subtilis* grown in different hydrocarbon substrates.

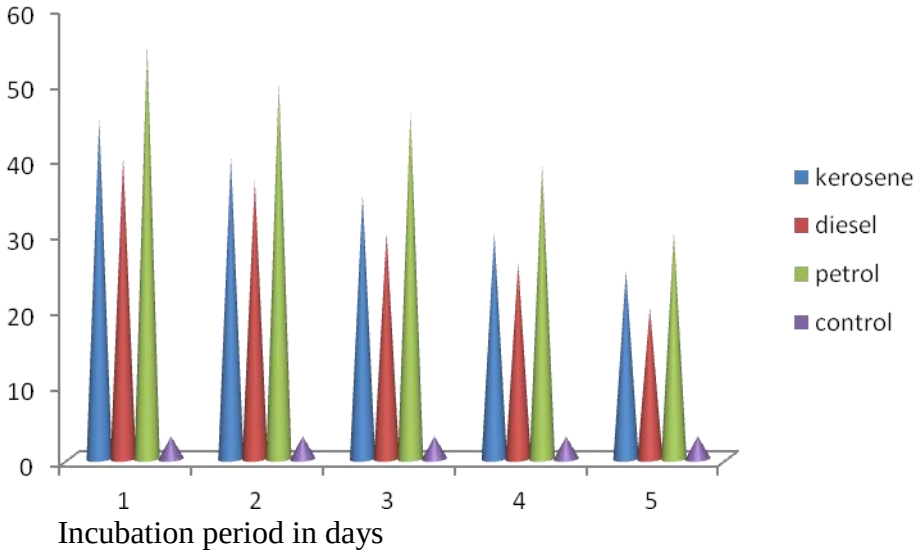

Figure 6: Bar chart showing Colony Forming Units of *Micrococcus luteus* grown in different hydrocarbon substrates.

V. Discussion

Oil exploration in most parts of the world has impacted both positively and negatively on microbial flora and fauna and the environment at large. The need to reduce the negative impacts of hydrocarbon pollutants and contaminants in the environment has long provoked researchers on studying hydrocarbon degradation using a consortium of microorganisms that possess hydrocarbon degrading abilities. Microorganisms inherent in waste dumpsites are major components of microbial food web, biochemical cycles and energy flow. Most of these microorganisms are highly involved in wastes biodegradation and emissions of core greenhouse gases (Carbondioxide, methane and nitrous oxide) into the environment with high potentials of global warming (Bassey et al., 2016). Their biodiversity is determined largely by the anthropogenic and natural variability of physicochemical indices (Unimke *et al.,* 2014; Bassey *et al.,* 2015).

In this study, the results obtained showed that most of the microbial species isolated from Lemna waste dumpsites were capable of degrading hydrocarbons, though with varying levels of efficiency. *Chromobacterium violaceum* displayed characteristic activity as the best hydrocarbon degrader in the range of petrol>kerosene>diesel as shown in Figure 1. The result also indicates that *Bacillus subtilis* were excellent in diesel and kerosene degradation than petrol in the range of diesel>kerosene>petrol as shown in Figure 2. In another study by Bassey et al., 2015, C. violaceum was rather seen as a degrader of broad spectrum beta lactam antibiotics including cefotaxime, ceftazidime and cefuroxime as against the present study were the organism displayed a characteristic ability in degrading all the fractions of hydrocarbons used. The presence of these hydrocarbonoclastic bacteria in mostly decomposing solid waste (DSW) of Lemna dumpsite heavily laden with disposed oil cans and used engine oils is an indication of their persistence and utilization of these fractions of oil as their carbon source over time. However, *C. violaceum* isolated from an environment without a history of oil spill may not be an active hydrocarbon degrader unlike *Bacillus* sp and *Micrococcus* sp with much published literatures as efficient hydrocarbon degraders (Uaboi-Egbenni and Olanipekun, 2006). The isolation of *C. violaceum* in decomposing solid waste (DSW) and dumpsite leachates (DSL) was first reported in 2015 by Bassey et al.,. Their re-isolation in same dumpsite confirms their abundance and possibly their affinity to both organic and inorganic substrates that constitute the heavy pile of waste in the dumpsite.

Furthermore *Micrococcus luteus* had the least potential of degrading hydrocarbon displaying a characteristic slow rate of adaptation to the 3 substrate applied as shown in Figure 3. The varying levels degradative capability shown by the isolates could be attributed to molecular differences as well prior exposure.

In this study the microbial load were significantly high and varied with samples (dumpsite soil, decomposing solid waste and leachate sample) as shown in Table 1. The occurrence of the 3 genera of bacteria indicates their active role in environmental self-sustainability. Biogeochemical cycling and carbon flux, especially in the study site (Lemna waste dumpsite, Calabar). The decomposition of organic and inorganic wastes provides high level of nutrients making dumpsites among the most productive microbial habitats in the world. *Chromobacterium violaceum* displayed characteristic reduction in colony forming unit (cfu) in relation to duration of incubation in descending order from day 1-5 as shown in Figure 4. This observation could be attributed to factors such as nutrient depletion and other environmental factors. Careoeto et al., (2004) and Bassey et al., (2016) reported that three sets of genes related to arsenic resistance, cyanate degradation, and acid dehalogenation are found in the *C. violaceum* genome and could be explored biotechnologically for both environmental pollution control and bioremediation. In another study, Tiku et al., (2016), Bassey et al., (2015) and Okereke et al., (2007) reported that heavy metal tolerability among bacteria isolates from auto-mechanic workshop pristine soil investigated showed high microbial bioload for the hydrocarbon polluted soils obtained within the

vicinity of the auto-mechanic workshops and could be a suggestive ability of this micro flora to proliferate in these environments despite the deliberate exposure of these soils to varying doses of petroleum or its refined products. This is also line with our study that confirmed a high load of *C. violaceum*, *Bacillus subtilis* and *Micrococcus luteus* with varying potentials of degrading different fractions of hydrocarbons

Similar trends were observed for *Bacillus species* and *Micrococcus species* Figure (5 & 6) grown in the various substrates, while the factors responsible for this observation could be same as stated above.

VI. Conclusion

The detection of microbial diversity and there inert capabilities in biodegradation of hydrocarbon is of great practical relevance especially in bioremediation study. The result of this study will go a long way in ascertaining the self-degradative and sustaining capabilities of the waste dumpsites as well the application of the microbial consortium in oil clean-up activities. The excellent degradative potentials displayed by *Chromobacterium violaceum* and to a lesser extent *Bacillus species* and *Micrococcus specie* is of great importance in environmental management and sustainability.

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