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Multiple Degradation and Resistance Capabilities of Marine Bacteria Isolated from Niger Delta, Nigeria on Petroleum Pollutants and Heavy Metals

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine multiple degradation and resistance capabilities of marine bacteria isolated from Rivers State, Nigeria on petroleum pollutants and heavy metals.
Study Design: Nine treatments and the controls designs were set up in triplicates containing 100 mL of sterile modified mineral basal medium in 250 mL conical flasks supplemented with 50, 100, 200 and 300 ppm of xylene, anthracene and pyrene each; 1 % of other petroleum pollutants and 300 ppm of heavy metals, nine marine hydrocarbon degraders and incubated at 24ºC for 5 - 7

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days. The nine treatments and controls set ups designated as ANT1, XYL2, PYR3, ANT4, PYR5, ANT6, XYL7, XYL8, PYR9 and CTRL (Without hydrocarbons) were used to determine the multiple degradability of the marine bacteria.

Place and Duration of Study: Department of Microbiology, Faculty of Natural Sciences, Chukwuemeka Odumegwu Ojukwu University, Uli Nigeria between September, 2014 and March, 2017.

Methodology: A laboratory scale study was carried on six composite samples of the sediment and water samples from the three studied areas using enrichment, screening, selection, molecular, growth effect and substrate specificity techniques.

Results: The findings revealed that screening and selection for the indigenous bacterial isolates from the three studied areas resulted in the isolation of nine out of forty eight (9/48) of the potent strains representing 18.75 % of the total isolates with significant (*P* = .05) multiple degradation and resistance potentials but with different efficiencies on xylene, anthracene and pyrene, other petroleum products and heavy metals at 50 – 300 pm and 1 %. All the nine potent strains were fully characterized molecularly and phylogenetically and belong to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus.*

Conclusion: Thus, these selected potent bacterial strains could significantly contribute in the development of a cost - effective bioremediation process on aromatic hydrocarbons and heavy metals contaminated environments in Nigeria.

Keywords: Petroleum products; heavy metals; marine bacteria; multiple degradation and resistance; Niger Delta.

1. INTRODUCTION

The world marine ecosystem has been studied extensively since the second half of the last century. The marine environment is subject to contamination by organic pollutants from a variety of sources. Organic contamination results from uncontrolled releases from manufacturing and refining installations, spillages during transportation, direct discharge from effluent treatment plants and run-off from terrestrial sources [1]. Environmental pollutants such as petroleum hydrocarbons, heavy metals and pesticides have been known to have direct toxic effects when released into the aquatic environment. There is a direct link between surface water and sediment contamination. Accumulated heavy metals or organic pollutants in sediment could be released back into the water with deleterious effects on human health [2].

The impact of these wastes in the Niger Delta ecosystems of Nigeria is an obvious environmental concern particularly with regards to persistence and ecotoxicity. Soil and ground water contamination by crude oil are becoming increasingly sensitive issues in Nigeria, since most of her portable water supply is derived from shallow and unconified aquifers. It is therefore, important to assess all remediation options on the basis of their ability to remove organic contaminants successfully. This is because most of these products especially the polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene and xylene (BTEX) and heavy metals are toxic, mutagenic and carcinogenic [3]. The physical and chemical methods like volatilization, photooxidation, chemical oxidation, and bioaccumulation are rarely successful in rapid removal and cleaning up and also these methods are not safe and cost effective when compared to microbial bioremediation [4]. A better way is to use enhanced biodegradation.

Extensive studies have been done on the biodegradation of isolated bacteria from the natural environment leading to isolation of some bacteria which have the ability of using PAHs compounds as the sole carbon and energy source. Isolating the bacteria with necessary performance for degradation of organic pollutants such as xylene, anthracene and pyrene in soil and water ecosystems can be the perfect solution for improving the microbial population in areas contaminated by hydrocarbons [5]. Several species of bacterial genera *Pseudomonas, Serratia, Marinobacter, Providencia, Alcaligenes, Salmonella, Nocardia, Mycobacterium, Cunninghamella, Rhodococcus, Beijerinckia, Lysinibacillus, Corynebacterium, Diaphorobacter, Pseudoxanthomonas, Bacillus* and *Sphingomonas;* have been found highly capable of degrading petroleum hydrocarbons as well as heavy metals are well documented [6-14].

There are many reports by several investigators on multiple degradation and resistance potentials of bacteria from soil and fresh water ecosystems on petroleum pollutants and heavy metals but few reports regarding multiple degradation and resistance capabilities of bacteria from marine origin of crude oil - impacted Niger Delta ecosystem are published and hence necessitated and justifies this study. This study was undertaken to determine multiple degradation and resistance potentials of marine bacteria isolated from Rivers State, Nigeria on petroleum pollutants and heavy metals.

2. MATERIALS AND METHODS

2.1 Description of the Sampling Sites

The studied areas were Abonema Wharf Water Front (Plate 1) in Akuku - Toru Local Government Area, Nembe Water - Side (Plate 2) in Port Harcourt Local Government Area and Onne Light Flow Terminal Seaport (Plate 3) located in Eleme Local Government Area of Rivers State. Abonema town is 53 Km and Abonema Wharf Water Front is 3 - 5 Km from Port Harcourt capital city; Nembe water side is located within Port Harcourt capital city of Rivers State, while Onne Light Flow Terminal is about 35 Km east from Port Harcourt capital city of Rivers State and 7 Km from Onne town. These sites were geo - referenced using Handheld Global Positioning System (GPS) GPSMAP 76 sc with the coordinates obtained from the sampling points or positions. Abonema Wharf Water Front, Nembe Water - Side and Onne Light Flow Terminal Seaport were located between latitude 4°46'15.82"N to latitude 4°46'38.01"N and longitude 7°0'0.54"E to longitude 7°0'34.82"E with average elevation of 4.1 m , latitude 4°45'8.72"N to latitude 4°45'26.42"N and longitude 7°1'11.37"E to longitude 7° 2'14.54"E with average elevation of 2.7 m and latitude 4°41'32.58"N and 4°41'58.18"N and longitude 7°9'26.34"E and 7°10'48.82"E with average elevation of 2.3 m, respectively. These water - ways are subjected to human - induced pressures resulting from urbanization, industrialization and intensive navigation. Abonema Wharf Water Front community is a popular and busy commercial but dangerous jetty area close to Port Harcourt city inhabiting tens of thousands of different families living close to petroleum tank farms and tankers queue up daily to load refined petroleum products. Nembe Water - Side is situated very close to Creek road market, Port Harcourt, Nigeria. It shares boundary with Bayelsa and links Port Harcourt city with Bonny Island where most of the oil installations in Rivers State are. It also links the Island directly with the Atlantic ocean through which crude oil is exported by massive oil tankers [15]. Onne Light Flow Terminal Seaport is a port of Nigeria and the largest oil and gas free zone in the world supporting exploration and production for Nigerian activities. It is situated on the Bonny River Estuary along Ogu Creek and accounts for over 65% of the export cargo through the Nigerian Sea Port.

Plate 1. Geoeye satellite image (2016) showing the Abonema sample points

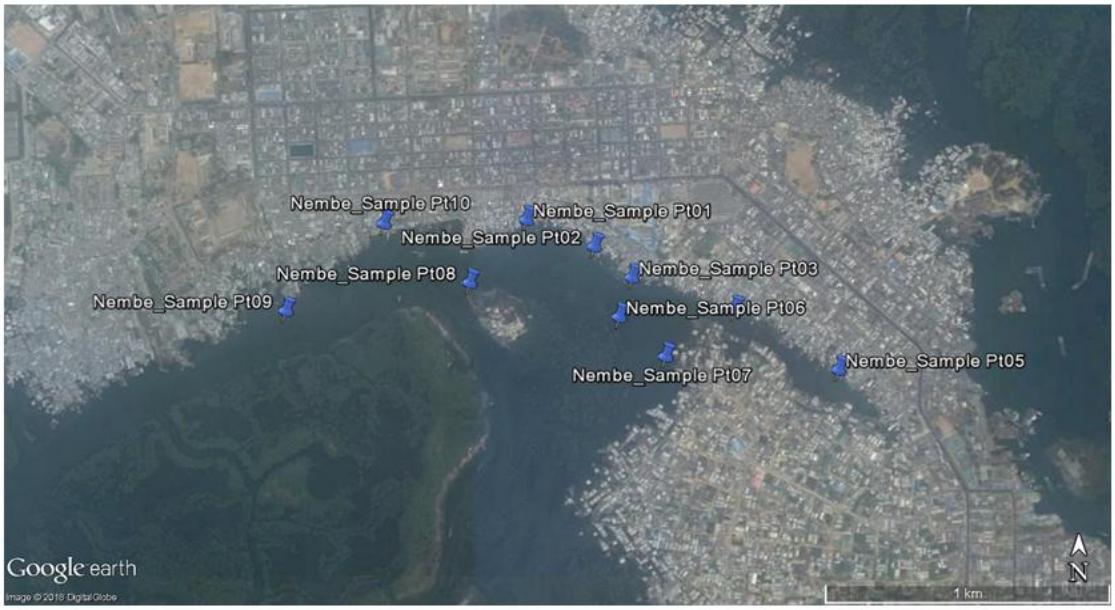

Plate 2. Geoeye satellite image (2016) showing the Nembe sample points

Plate 3. Geoeye satellite image (2016) showing the Onne sample points

2.2 Sample Collection and Processing

Selection of sampling sites is depended on activities occurring in the region as described above (Plates 1, 2 and 3). Ten samples each of the marine sediment and water were collected randomly per point of the designated ten (10) points of the three sampling sites. The samplings were done once in each of the three sampling sites in September, 2014. The samples were mixed together after which a total of six composite /representative sediment and water samples were taken for the analysis. The surface aerobic sediment samples were collected with a 95 % ethanol - sanitized plastic spatula at 5 cm depth inside 95 % ethanol - sanitized clean, dry,

leak - proof, wide mouthed plastic containers that is lined with aluminum foil. The water samples were collected at the air-water interface by hand dipping the 95 % ethanol - sanitized clean, dry, leak - proof, cylindrical shaped 2 L plastic containers. The containers with lids slightly opened were rinsed with the samples thrice before aseptically collecting the samples. All the composite or representative sediment and water sample containers were labelled with sample type, date, time and place of collection. They were placed into a sterile polythene bags in ice packed coolers to keep them under a temperature not more than 4°C. Then transported to the laboratory for microbiological analyses and stored at 4°C in refrigerator for 12 – 24 hrs [16-18].

2.3 Enrichment, Culturing and Isolation of Aromatic Hydrocarbon Degrading Bacterial Strains

The hydrocarbon degraders were isolated from sediment and water samples of the three sampling sites using modified mineral basal agar (4 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 1.8 g KH_2PO_4, 0.1 g $FeSO_4$, 0.1 g NaCl, 0.2 g $CaCl_2$, 15 g Agar agar and distilled water 1,000 mL at pH 7.00 ± 0.20) enriched with xylene, anthracene and pyrene as sole carbon and energy source. The medium was sterilized by autoclaving at 121 $^{\circ}$C and 15 psi for 15 minutes. Thereafter, 0.2 mL acetone solution containing 0.1 % w/v of the selected hydrocarbons (xylene, anthracene and pyrene) were aseptically pipetted and uniformly spread on the agar surface of the pre - dried Petri dish plates. The acetone was allowed to evaporate under sterile condition and 0.1 mL aliquots of the 10^{-3} dilutions were plated on the solidified media with a glass spreader. The spreader was sterilized after each successive spreading by dipping it in 70 % ethanol and then passing it through flame of a Bunsen burner. The inoculated plates were sealed using adhesive tape and foil to prevent contamination and photolysis and later placed in black polythene bags, and then incubated in the dark at 28.00 ± 0.20°C for 14 days [18, 19].

2.4 Purification and Maintenance of Cultures

Colonies that developed on hydrocarbon - coated plates were replicated onto fresh hydrocarbon - coated agar plates and incubated for 14 days. Isolates that grew on these plates were selected as xylene, anthracene and pyrene degraders and sub - cultured on Bijou bottles where they are preserved at 4°C in refrigerator [18].

2.5 Screening and Selection Test

In order to screen and select the best and strongest degrading strains, growth of the different organisms were tested by growing 5 mL of each desired isolates in large test tubes containing 25 mL of the modified mineral basal medium with 100 mg /L of xylene, anthracene and pyrene hydrocarbons which were dissolved in acetone and added to each tube after autoclaving. Thereafter, the test tubes were incubated at room temperature (28.00 ± 2.00 °C) for five days. Bacteria that started growing fast with high turbidity in the vicinity of the medium containing aromatic compounds measured at 600 nm using a UV - VIS spectrophotometer (Astell, UV - Vis Grating, 752 W) were selected as the candidate of xylene, anthracene and pyrene degrading bacteria [5,18,20].

2.6 Characterization and Identification of Selected Hydrocarbon Utilizing Bacterial Isolates

2.6.1 Molecular characteristics

2.6.1.1 Identification of bacteria

Further identification was carried out using the Gram - reaction test and molecular techniques. The Gram - reaction test was first used to ascertain the morphological characterization of the colonies before proceeding to do molecular identification which includes DNA extraction, polymerase chain reaction (PCR), gel electrophoresis, sequencing and blasting [21].

2.6.1.2 Genomic DNA extraction of the bacterial isolates

DNA extraction was conducted using conventional method of Cetyltrimethyl Ammonium Bromide (CTAB) protocol in sterile Eppendorf tubes. About 10 mL of the pure cultures from nutrient broth was vortexed and 1.5 mL of it was transferred into 2 mL Eppendorf tubes and centrifuged with a microcentrifuge (Eppendorf Minispin plus, 12 x 1.5/2.0 mL) at 14,000 rpm for 5 minutes. The supernatant was discarded to recover the pellets, which was then resuspended in a solution containing 567 µL of tris ethylene diamine tetraacetic acid buffer (tris EDTA or TE buffer), 30 µL of 10 % sodium dodecyl sulphate (SDS) and 3 µL of proteinase K

(20 mg /mL) and was incubated in Accu block digital dry bath incubator (Labnet International, USA) at 65 °C for 1 hr. Then 180 µL of 5 M NaCl and 80 µL of 10 % CTAB solutions were added to the mixture and incubated for 10 minutes at 65 °C. After which an equal volumes (400 mL) of phenol and chloroform was added to each tube and centrifuged at 14, 000 rpm for 15 minutes and then 300 µL of the supernatant was transferred into new sterile Eppendorf tubes and the DNA was precipitated by adding 0.6 mL cold isopropanol to each tube. The precipitate was collected by spinning the tube in a centrifuge at 14, 000 rpm for 15 minutes and the supernatant was discarded. Then 200 µL of freshly prepared 70 % ethanol was added to the tube to wash DNA pellets by spinning at 14, 000 rpm for 10 minutes. The supernatant was carefully removed to air - dry the DNA pellets and 100 µL of TE buffer was added to the dried DNA pellets and incubated at 37 °C for 60 minutes to dissolve the DNA pellets. Then 1 µL of RNAase was added to the tube and incubated at 37°C for 60 minutes. The DNA was separated electrophoretically with 1 % agarose gel stained with 0.1 µg /mL ethidium bromide running at 80 V for 60 minutes using tris acetate EDTA (TAE) electrophoresis buffer. The DNA was visualised by UV fluorescence to determine the success of the extraction process [21].

2.6.1.3 Polymerase chain reaction (PCR) and sequencing of the extracted DNA

The master mix aliquot for the PCR was dispensed into individual PCR tube and the different DNA samples were added to each tubes. The negative control was used to check for contamination in the master mix. The PCR reagents in each tube amounted to 50 µL containing: buffer (5 µL), $MgCl_2$ (1.5 µL), primer 1 (forward 16S - P1 PCR 5′AGAGTTTGATCCTGGCTCAG3′) (2 µL), primer 2 (reverse 16S - P2 PCR 5′AAGGAGGTGATCCAGCCGCA3′) (2 µL), dNTP mix (1 µL), Dream Taq (0.25 µL), sterile sabax water (35.25 µL) and DNA samples (3 µL). The PCR reactions was performed using MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). The cycling conditions were set at (a) initial denaturation 10 mins at 95°C for 1 cycle. (b) Denaturation at 95°C for 30 seconds, (c) Annealing cycling at 94°C for 30 seconds, (d) Elongation at 54°C for 2 mins. All steps in denaturation, annealing and elongation was for 35 cycles and (e) final elongation 10 mins at 72°C for 1 cycle. The reaction was held at 4°C for 1 hr in the thermal cycler. The PCR products was separated electrophoretically with 1% agarose gel stained with 0.1 µg /mL ethidium bromide running at 80 V for 60 minutes in 100 mL of 1x TAE electrophoresis buffer. The PCR products were visualised by UV fluorescence to determine the size of the amplified bands. Then the PCR products (20 µL each) were cleaned up later using 160 µL of 13% polyethylene glycol (PEG) 8000, 20 µL of 5 M NaCl solution and 200 µL of 70 % ethanol. The cleaned PCR products were sent for sequencing and was conducted using the automated DNA sequencer (Perkin-Elmer) which was carried out according to the manufacturers' instruction. This was done at the Forestry and Agricultural Biotechnology Institute (FABI) Sequencing Facility, University of Pretoria, South Africa [21].

2.6.1.4 Blasting and phylogenetic correctional analyses

The basic local alignment search tool (BLAST) of DNA sequences was performed by editing the sequences of the 16S rRNA region obtained using BioEdit software. The edited sequences were copied in a FASTA format form. Then, blasting was done on National Centre for Biotechnology Information (NCBI) website. Homologies of the 16S rRNA sequences were checked and compared with the sequences of those on the database (Ubani et al., 2016). From the list of many identical sequences, four of the sequences were selected for each bacterium from the GenBank for phylogenetic analyses. The bacterial sequences from the treatments were aligned using online version of MAFFT software. The phylogenetic correctional analyses were done using Mega 7 software and evolutionary distance of the isolates were computed using neighbour - joining (NJ) methods. The bootstrap consensus tree was inferred from 100 replicates and all positions containing gaps as well as missing nucleotide data were eliminated from the data - set [10,21-23].

2.6.1.5 GenBank Accession Number

NCBI accession numbers were assigned to the nine selected aromatic hydrocarbon degrading bacterial strains and the nucleotide sequence details of 16S rRNA genes of the isolates reported in this study have been deposited in the GenBank databases since 01/12/16 under the accession numbers from KY171979 - KY171987 [22,24,25].

2.7 Determination of Concentration Effect of Aromatics on the Growth of the Isolates

To determine the effects of aromatic hydrocarbon concentrations on the growth of the isolates, precisely 100 mL of modified mineral basal medium was dispensed into forty - five (45) 250 mL flasks and sterilized by autoclaving. The flasks were then divided into nine sets of seven flasks. Thereafter, 50, 100, 200 and 300 ppm levels of xylene, anthracene and pyrene which were separately dissolved in acetone (as before) were exposed to each isolates. The fifth, sixth and seventh flasks served as the controls for each hydrocarbons and contained no xylene, anthracene and pyrene. The inoculated and control flasks were then incubated as previously described at 28.00 ± 2.00°C for 5 days. After 5 days of incubation, 5 mL sample was aseptically collected from each flask and monitored for the level of microbial growth which was indicated by increase in turbidity (optical density OD) of the medium measured in triplicate determinations at 600 nm using a UV - VIS spectrophotometer (Astell UV - Vis Grating, 752 W) [19].

2.8 Substrate Specificity Test

In addition to growth on xylene, anthracene and pyrene, the purified strains were also tested for growth on other petroleum products and heavy metals. The petroleum products include: crude oil, diesel oil, kerosene, engine oil, benzene, toluene, hexane, parabenzene, ethyl benzene, and phenol while the heavy metals include: copper (II) chloride ($CuCl_2$), arsenic trioxide (AsO_3), lead (II) sulphate ($PbSO_4$), mercury (II) chloride $HgCl_2$, potassium dichromate ($K_2Cr_2O_7$), potassium iron cyanide (KFeCN), cadmium oxide (CdO), manganese (II) chloride ($MnCl_2$) and zinc sulphate ($ZnSO_4$). Modified mineral basal medium (4 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 1.8 g KH_2PO_4, 0.1 g $FeSO_4$, 0.1 g NaCl, 0.2 g $CaCl_2$, 15 g Agar agar and distilled water 1,000 mL at pH 7.00 ± 0.20) containing 1 % v/v of the above petroleum products were inoculated and incubated for 7 days at 28.00 ± 2.00°C while Seawater Nutrient medium containing 300 ppm of the heavy metals were inoculated and incubated for 5 days at 28.00 ± 2.00°C. After 5 days of incubation, 5 mL samples were aseptically collected from each flask and monitored for the level of microbial growth which was indicated by increase in turbidity (optical density OD) of the medium measured in triplicate determinations at 600 nm using a UV - VIS spectrophotometer (Astell UV - Vis Grating, 752 W). Cultures without increase in turbidity over initial optical density and non-inoculated control were scored as no growth (-) while cultures with increased turbidity significantly greater than the control (i.e. growth attenuation, optical density OD reading above 0.2) were scored as growth (+) [12,19].

2.9 Data Analysis

The data were analyzed using Graph - Pad Prism statistical software version 7.00 (GraphPad software Inc. San Diego, California). All values were expressed as mean ± standard deviation. Ordinary one - way analysis of variance (ANOVA) followed by post Tukey's, multiple comparison test was performed on the data obtained. The results were considered statistically significant at 95 % confidence intervals (P = .05) [5,23].

3. RESULTS

3.1 Isolation and Selection Test

The result of the growth performance (OD_{600} nm) of the aromatic hydrocarbon - degraders isolated from Abonema sampled location is presented in Table 1. From the result, 13 isolates were obtained with strains XYL2, ANT4 and PYR3 having the highest significant *(P* = .05) absorbance values of 0.952 ± 0.004, 0.775 ± 0.007 and 1.041 ± 0.008 on xylene, anthracene and pyrene hydrocarbons. The result of the growth performance (OD_{600} nm) of the aromatic hydrocarbon - degraders isolated from Nembe sampled location is presented in Table 2. From the result, 17 isolates were obtained with strains XYL7, ANT1 and PYR5 having the highest significant (P = .05) absorbance values of 1.055 ± 0.002, 0.816 ± 0.007 and 0.933 ± 0.007 on xylene, anthracene and pyrene hydrocarbons. The result of the growth performance (OD_{600} nm) of the aromatic hydrocarbon - degraders isolated from Onne sampled location is presented in Table 3. From the result, 18 isolates were obtained with strains XYL8, ANT6 and PYR9 having the highest significant (P = .05) absorbance values of 0.741 ± 0.007, 1.433 ± 0.013 and 0.871 ± 0.001 on xylene, anthracene and pyrene hydrocarbons. On the basis of these results, strains ANT1, XYL2, PYR3, ANT4, PYR5, ANT6, XYL7, XYL8 and PYR9 were selected as the best and strongest degraders of xylene, anthracene and pyrene hydrocarbons.

3.2 Molecular Characteristics of Bacterial Isolates

The result of the PCR - amplification of 16S rRNA genes of the aromatic degrading bacteria genomic DNA is shown in Plate 4. From the result, it was confirmed that the PCR products obtained using universal primer 16S-P1 PCR (27F 5′–3′: AGA GTT TGA TCC TGG CTC AG) and 16S-P2 PCR (1492R 5′–3′: ACG GCT ACC TTG TTA CGA CTT) have the molecular weight of 1 kbp visualized by UV fluorescence under agarose gel electrophoresis. The result of the percentage similarity and GenBank accession numbers of 16S rRNA sequences of the closest relative for the aromatic degrading bacterial isolates is presented in Table 4. From the result, *Alcaligenes faecalis* was the most blasted bacteria with high similarity (98 - 99 %) followed by *Providencia* spp. (95 - 97 %), *Brevundimonas diminuta* (100 %), *Myroides odoratus* (90 %), *Serratia marcescens* (97 %) and *Bacillus cereus* (98 %) using NCBI BLAST software. The result of neighbour - joining phylogenetic relationship among the 16S rRNA sequence of the aromatic degrading bacterial isolates constructed by MEGA 7.0 is shown in Fig. 1. From the result, it revealed that the nine bacterial isolates (KY171979, KY171984, KY171987, KY171980, KY171982, KY171981, KY171985, KY171986 and KY171983) show the same ancestry as they arise from the same node and hence they are evolutionary related to each other.

3.3 Effect of Aromatic Hydrocarbon Concentrations

The results of the growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of xylene, anthracene and pyrene are shown in Figs. 2, 3 and 4. From the xylene result, the isolate *Alcaligenes faecalis* PYR5 had the least growth of 0.122 ± 0.003 (OD_{600nm}) observed at xylene of 300 ppm while *Providencia* sp. XYL8 had the best growth of 1.661 ± 0.297 recorded when exposed to 50 ppm of xylene for 5 days. From the anthracene result, the isolate *Providencia vermicola* ANT1 had the least and best growth of 0.048 ± 0.003 and 1.660 ± 0.020 (OD_{600nm}) when exposed to anthracene at 300 ppm and 50 ppm for 5 days, respectively. From the pyrene result, the isolate *Providencia* sp. XYL8 had the least growth of 0.123 ± 0.001 (OD_{600nm}) observed at pyrene of 300 ppm while *Alcaligenes faecalis* XYL2 had the best growth of 1.330 ± 0.002 recorded when exposed to 50 ppm of pyrene for 5 days. There were extreme significant differences among group of cell growth suspensions and the concentration of hydrocarbons (P = .05) with very strongly significant negative correlation (P = .05; r = - 0.783 to - 0.980).

3.4 Substrate Specificity Test

The result of the growth specific test of the marine bacterial isolates on crude oil and different hydrocarbons is presented on Table 5. From the result, the abilities of the isolates to degrade different hydrocarbons were found to vary. Isolate ANT1 had moderate/heavy growth on all the substrates while the other isolates had heavy, moderate, poor and no growth on the hydrocarbons substrates. The result of the growth specific test of the marine bacterial isolates on different heavy metals is presented on Table 6. From the result, the abilities of the isolates to resist heavy metals were also found to vary. All the isolates had growth and resisted all metals except PYR3, ANT4 and ANT6 that were sensitive (–) to AsO_3 and CdO compounds. Isolates ANT1, XYL2, PYR3, ANT4, PYR5, ANT6, XYL7, XYL8 and PYR 9 resisted and had heavy growths (+++) on $HgCl_2$, KFeCN, CdO, $PbSO_4$, $CuCl_2$, $K_2Cr_2O_7$, AsO_3, $MnCl_2$ and $ZnSO_4$ metallic compounds at 300 mg/ L.

4. DISCUSSION

In this study, a total of nine (9) isolates ANT1, XYL2, PYR3, ANT4, PYR5, ANT6, XYL7, XYL8 and PYR9 out of the 48 isolates (9/48) representing 18.75 % of the isolates were screened and selected as best and strongest degraders of xylene, anthracene and pyrene hydrocarbons which they significantly (P = .05) utilize as source of carbon and energy and is indicated by absorbance values below of each isolates (Tables 1, 2 and 3). This study agrees with the explanation of Mao et al. [26] that the most important aspect of microbial degradation of PAH is enrichment and isolation of indigenous PAH degraders because the indigenous PAH degrading bacteria are already adapted to utilizing PAH. Pathak and Bhatnagar [27], argued that enrichment culturing is important for the success of hydrocarbon bioremediation because the process leads to selection of microorganism accustomed to hydrocarbon degradation. Esedafe et al. [28] reported that an occurrence of 3/41 representing 7.32 % isolates from refinery effluent were capable of utilizing phenanthrene and anthracene as sole carbon and energy sources. It also indicates that only these isolates

had the physiological capabilities to metabolize the aromatic hydrocarbons.

Akinbankole et al. [11], obtained 1,500 bp PCR product from anthracene and pyrene isolates isolated from oil contaminated water and soil in Malaysia. Yuliani *et al.* [20], obtained in their research 1,489 bp PCR product from phenanthrene and pyrene isolates isolated from marine area of Indonesia. Isiodu *et al.* [23], reported that all the seven (7) polyaromatic hydrocarbon utilizing bacterial isolates isolated from Bodo Creek brackish water in Nigeria showed amplification with an amplicon size of 500 bp. Our findings were within range of their findings. The result in Table 6 showed that *Alcaligenes faecalis* was the most occurring organism after blasting with high sequence similarity (98 - 99 %) followed by *Providencia* species (95 - 97 %), *Brevundimonas diminuta* (100 %), *Myroides odoratus* (90 %), *Serratia marcescens* (97 %) and *Bacillus cereus* (98 %) using NCBI BLAST software. Akinbankole et al. [11], found out that *B. cereus* was the most blasted organism with sequence homology (99 %). The phylogenetic tree depicts the evolutionary relationship among hydrocarbon metabolizing bacteria isolated in this study. The result shown in Fig. 1 revealed that the nine bacterial isolates (KY171979, KY171984, KY171987, KY171980, KY171982, KY171981, KY171985, KY171986 and KY171983) show the same ancestry as they arise from the same node and hence they are evolutionary related to their relatives and their nucleotide sequences of their 16S rRNA genes have been deposited in GenBank database since first of December two thousand and sixteen (01/12/16). They belong to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia,* and *Bacillus*; and families of: Enterobacteriaceae, Alcaliginaceae, Caulobacteriaceae, Flavobacteriaceae, and Bacillaceae; and phyla of: Proteobacteria, Bacteroidetes and Firmicutes which members have been implicated in petroleum and aromatic hydrocarbon biodegradation by several authors [7,8,10,11,13, 15,18,20,23,29-32].

The results in Figs. 2, 3 and 4 showed that the utilization and degradation of these compounds resulted in increase in optical density (cell mass) of the organisms; however, increase in concentration of these compounds led to decrease in optical density (cell mass) of the organisms with very strongly significant negative correlation (P = .05; r = - 0.783 to - 0.980). It is apparent from the results that strains isolated on xylene (*Alcaligenes faecalis* XYL2 and *Providencia* sp. XYL8) and anthracene (*Providencia vermicola* ANT1) hydrocarbons were able to grow better on the three tested aromatic hydrocarbons than all the strains (*Brevundimonas diminuta* PYR3, *Alcaligenes faecalis* PYR5 and *Bacillus cereus* PYR9) that were isolated on pyrene hydrocarbons. Also, all the nine strains degraded all the three aromatic hydrocarbons and grew well indicating multiple biodegradation potentials but with different efficiencies hence termed multiple degraders. Also, these strains especially *Alcaligenes faecalis* XYL2 and *Providencia* sp. XYL8 degraded xylene and anthracene hydrocarbons equally but less than pyrene hydrocarbon in the order of degradation: xylene = anthracene > pyrene, Comparatively, *Providencia* sp. XYL8, *Providencia vermicola* ANT1 and *Alcaligenes faecalis* XYL2 had better degradation efficiencies than the rest of the other six (6) strains (*Brevundimonas diminuta* PYR3, *Alcaligenes faecalis* ANT4, *Alcaligenes faecalis* PYR5, *Myroides odoratus* ANT6, *Serratia marcescens* XYL7 and *Bacillus cereus* PYR9. The result is in consistent with the research carried out by Poornachander et al. [33], who reported that growth of *Bacillus cereus* CPOU13 decreased with increasing PAHs concentrations (phenanthrene, anthracene and pyrene) from 10 ppm to 250 ppm in MSM. Also, similar was the work carried out by John *et al.* [18], in which they found out that the growths of all the test isolates (*Alcaligenes faecalis* AFS-5, *P. putida* AFS-3 and *M. varians* AFS-2) were PAH - dependent and provide strong evidence for selective PAH degradation by bacteria. The acclimation of microbial community to one substrate, may lead to the simultaneous acclimation to some but not all structurally related molecules. Akinbankole et al. [11], isolated and identified *B.thuringiensis, B.megaterium* and *B.cereus* in both pyrene and anthracene enriched medium and the three bacteria have the metabolic adaptability of utilizing low and high molecular weight PAH. Bahobail et al. [14] reported that three isolates *Pantoea agglomerans* (BDCC-TUSA-8), *Acinetobacter lwoffii* (BDCC-TUSA-12) and *Bacillus thuringiensis* (BDCC-TUSA-18) showed multiple degradation potentials with remarkably fast reaction rates on n-Hexadecane, phenol and phenanthrene, representing the major types of hydrocarbon pollutants. Their abilities to utilize both low and high molecular weight PAHs is an indication of the possession of ring fission enzymes [34].

The result in Table 5 revealed that the abilities of the bacterial strains to degrade different hydrocarbons were found to vary. These differences might be attributed to the membrane toxicity and non - possession of the necessary enzymes [35]. The result agrees with the report of Fagbemi and Kehinde, [8] that the abilities of the bacterial hydrocarbon degraders to degrade hydrocarbons varied and *C. koseri, S. ficaria* and *B. coagulans* had moderate/ strong growth on crude oil which is similar in this study but differ in species (*Serratia marcescens* XYL7 and *Bacillus cereus* PYR9). John and Okpokwasili [19], report that nitrifying bacteria are excellent degraders of crude oil. Also, the result in Table 6 revealed that all the isolates had growth and resisted all metals except PYR3, ANT4 and ANT6 that were sensitive (–) to AsO_3 and CdO compounds. The result agrees with the report of Jaysanker et al. [12], that single bacterial strains can be resistant

Table 1. Growth performance of the aromatic hydrocarbon - degraders isolated from Abonema sampled location

Isolate	Optical density (OD_{600} nm)		
	Xylene	Anthracene	Pyrene
AB1	0.657 ± 0.008 [a]	0.657 ± 0.001 [a]	0.580 ± 0.003 [a]
ANT4*	0.676 ± 0.004 [a]	0.775 ± 0.007 [a]	0.822 ± 0.002 [a]
AB3	0.701 ± 0.021 [a]	0.467 ± 0.029 [a]	0.666 ± 0.003 [a]
AB4	0.715 ± 0.004 [a]	0.598 ± 0.024 [a]	0.841 ± 0.001 [a]
PYR3*	0.598 ± 0.005 [a]	0.511 ± 0.003 [a]	1.041 ± 0.008 [a]
AB6	0.641 ± 0.001 [a]	0.494 ± 0.002 [a]	0.653 ± 0.001 [a]
AB7	0.687 ± 0.004 [a]	0.690 ± 0.002 [a]	0.803 ± 0.016 [a]
AB8	0.618 ± 0.001 [a]	0.638 ± 0.007 [a]	0.782 ± 0.003 [a]
AB9	0.457 ± 0.002 [a]	0.475 ± 0.001 [a]	0.573 ± 0.004 [a]
XYL2*	0.952 ± 0.004 [a]	0.312 ± 0.002 [a]	0.838 ± 0.021 [a]
AB11	0.793 ± 0.014 [a]	0.495 ± 0.002 [a]	0.970 ± 0.003 [a]
AB12	0.647 ± 0.002 [a]	0.446 ± 0.001 [a]	0.621 ± 0.005 [a]
AB13	0.328 ± 0.001 [a]	0.415 ± 0.001 [a]	0.451 ± 0.001 [a]

** = Isolates with highest degradability; values are mean ± standard deviation of triplicate determination; superscript a = Non-significant difference;*
F (12, 24) = 2.09; P = .06

Table 2. Growth performance of the aromatic hydrocarbon - degraders isolated from Nembe sampled location

Isolate	Optical density (OD_{600} nm)		
	Xylene	Anthracene	Pyrene
NW1	0.885 ± 0.003 [a]	0.236 ± 0.005 [a]	0.708 ± 0.008 [a]
PYR5*	0.710 ± 0.003 [a]	0.216 ± 0.005 [a]	0.933 ± 0.007 [a]
NW3	0.466 ± 0.007 [a]	0.201 ± 0.000 [a]	0.806 ± 0.004 [a]
NW4	0.893 ± 0.002 [a]	0.356 ± 0.008 [a]	0.827 ± 0.008 [a]
NW5	0.750 ± 0.004 [a]	0.132 ± 0.005 [a]	0.767 ± 0.008 [a]
NW6	0.644 ± 0.004 [a]	0.246 ± 0.004 [a]	0.724 ± 0.008 [a]
NW7	0.561 ± 0.003 [a]	0.193 ± 0.005 [a]	0.808 ± 0.001 [a]
NW8	0.628 ± 0.008 [a]	0.472 ± 0.001 [a]	0.826 ± 0.008 [a]
XYL7*	1.055 ± 0.002 [a]	0.588 ± 0.005 [a]	0.927 ± 0.001 [a]
NW10	0.809 ± 0.002 [a]	0.785 ± 0.002 [a]	0.881 ± 0.004 [a]
NW11	0.826 ± 0.001 [a]	0.444 ± 0.002 [a]	0.891 ± 0.001 [a]
NW12	0.625 ± 0.005 [a]	0.563 ± 0.001 [a]	0.728 ± 0.006 [a]
NW13	0.374 ± 0.008 [a]	0.775 ± 0.001 [a]	0.760 ± 0.001 [a]
NW14	0.701 ± 0.001 [a]	0.622 ± 0.003 [a]	0.788 ± 0.007 [a]
NW15	0.705 ± 0.008 [a]	0.529 ± 0.004 [a]	0.830 ± 0.002 [a]
NW16	0.769 ± 0.002 [a]	0.380 ± 0.001 [a]	0.822 ± 0.001 [a]
ANT1*	0.804 ± 0.003 [a]	0.816 ± 0.007 [a]	0.583 ± 0.001 [a]

** = Isolates with highest degradability; values are mean ± Standard deviation of triplicate determination; superscript a* = Non-significant difference;
F (16, 32) = 1.08; P = .410

Table 3. Growth performance of the aromatic hydrocarbon - degraders isolated from Onne sampled location

Isolate	Optical density (OD_{600} nm)		
	Xylene	Anthracene	Pyrene
ON1	0.721 ± 0.001 [a]	0.884 ± 0.007 [a]	0.500 ± 0.001 [a]
ON2	0.204 ± 0.001 [a]	0.660 ± 0.011 [a]	0.454 ± 0.001 [a]
ON3	0.473 ± 0.003 [a]	0.476 ± 0.036 [a]	0.561 ± 0.013 [a]
ON4	0.207 ± 0.001 [a]	0.766 ± 0.001 [a]	0.565 ± 0.033 [a]
ON5	0.477 ± 0.002 [a]	0.457 ± 0.001 [a]	0.378 ± 0.005 [a]
ON6	0.409 ± 0.005 [a]	0.489 ± 0.100 [a]	0.562 ± 0.021 [a]
ON7	0.251 ± 0.003 [a]	0.428 ± 0.014 [a]	0.728 ± 0.001 [a]
ON8	0.111 ± 0.005 [a]	0.429 ± 0.014 [a]	0.425 ± 0.021 [a]
ON9	0.463 ± 0.008 [a]	0.357 ± 0.011 [a]	0.281 ± 0.006 [a]
PYR9*	0.106 ± 0.001 [a]	0.335 ± 0.001 [a]	0.871 ± 0.001 [a]
ON11	0.700 ± 0.001 [a]	0.901 ± 0.005 [a]	0.417 ± 0.002 [a]
ANT6*	0.511 ± 0.006 [a]	1.433 ± 0.013 [a]	0.568 ± 0.009 [a]
ON13	0.273 ± 0.002 [a]	0.386 ± 0.002 [a]	0.527 ± 0.001 [a]
ON14	0.278 ± 0.005 [a]	0.553 ± 0.022 [a]	0.684 ± 0.003 [a]
ON15	0.291 ± 0.003 [a]	0.748 ± 0.009 [a]	0.522 ± 0.010 [a]
ON16	0.662 ± 0.001 [a]	0.919 ± 0.002 [a]	0.494 ± 0.002 [a]
XYL8*	0.741 ± 0.007 [a]	0.510 ± 0.013 [a]	0.602 ± 0.004 [a]
ON18	0.354 ± 0.002 [a]	1.004 ± 0.001 [a]	0.478 ± 0.001 [a]

** = Isolates with highest degradability; values are mean ± standard deviation of triplicate determination; superscript a = Non-significant mean; F (17, 34) = 1.19; P = .320*

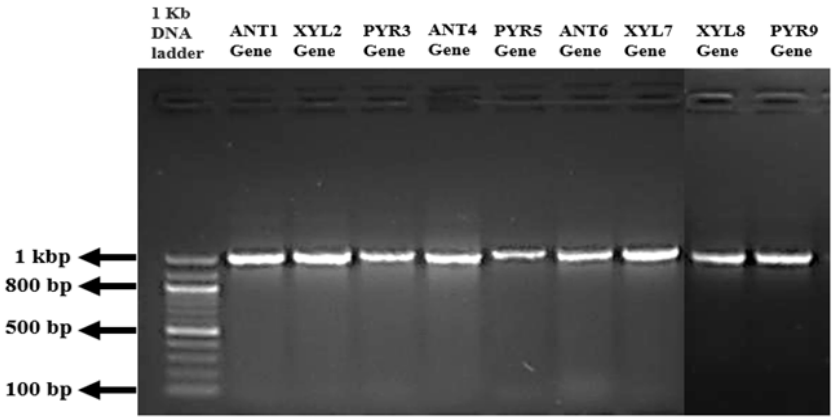

Plate 4. PCR - amplification of 16S rRNA genes of the aromatic degrading bacteria genomic DNA

ANT1 - *Providencia vermicola*, XYL2 - *Alcaligenes faecalis*, PYR3 - *Brevundimonas diminuta*, ANT4 - *Alcaligenes faecalis*, PYR5 - *Alcaligenes faecalis*, ANT6 - *Myroides odoratus*, XYL7 - *Serratia marcescens*, XYL8 - *Providencia* sp. and PYR9 - *Bacillus cereus*

to many metals and that the multi - metal resistant bacteria highly resistant to mercury possess the genetic components for dealing with many toxic metal ions. These isolates are of interest for molecular characterization of mechanisms for resistance to multiple metals and hold promise for bioremediation of toxic heavy metals, including in environments that are contaminated by several metals. Also, Athar et al. [13], published that the aromatic hydrocarbon degrading bacterial isolates were capable of degrading a variety of different hydrocarbons with its ability to grow in different metals stress environment. It was observed that all the bacteria sensitive to the metal compounds ($PbNO_3$, $ZnCl_2$, $COCl_2$, $CdCl_2$, $K_2Cr_2O_7$, Hg and $NiCl_2$) have shown sensitivity even at lowest used concentration (50 mg/ mL), whereas the resistant bacteria showed resistance to the highest concentration (150 mg/ mL).

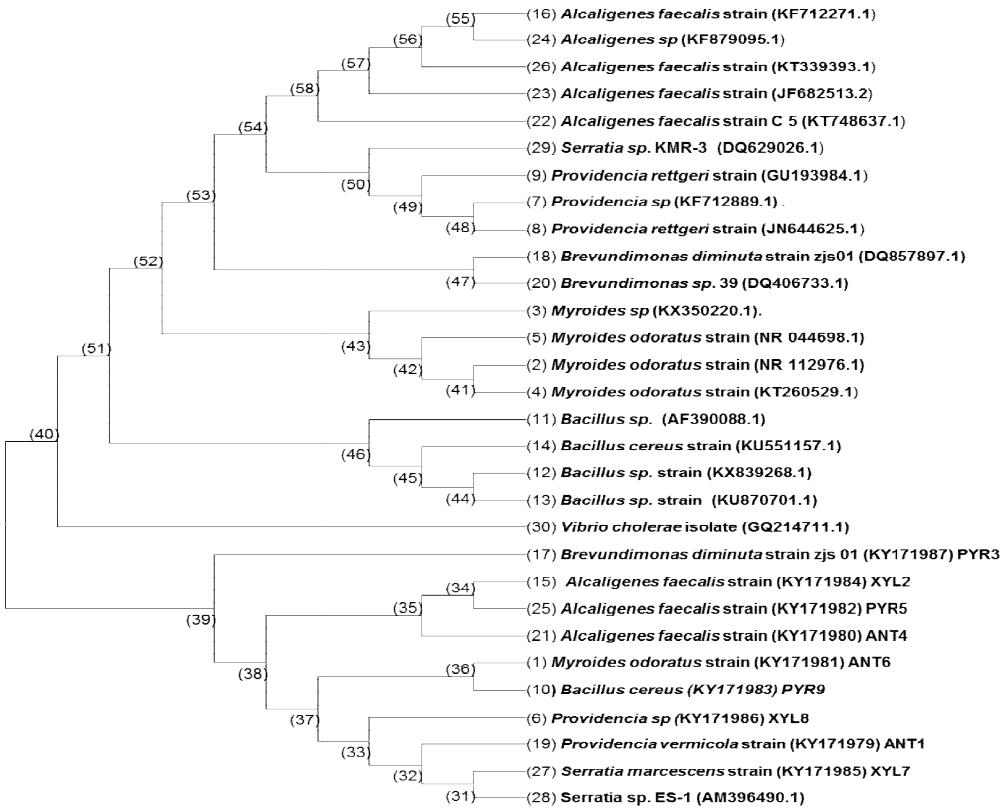

Fig. 1. Neighbour - joining phylogenetic relationship among the 16S rRNA sequence of the aromatic degrading bacterial isolates constructed by MEGA 7.0

Numbers at the nodes indicate bootstrap support (%) based on 1000 replicates. The sum of branch length = 0.93646865 using p - distance method involving 9 nucleotide sequences with total of 303 positions. GenBank accession numbers are given in parenthese

Table 4. Percentage similarity and GenBank accession numbers of 16S rRNA sequences of the closest relative for the aromatic degrading bacterial isolates

Isolate code	Closest relative in GenBank	Max score	Total score	Query coverage	E. value	Max identity	Accession number
ANT1 Nembe	*Providencia vermicola* strain MTCC 5578	544	544	93%	6e-151	95%	KY171979
XYL2 Abonema	*Alcaligenes faecalis* strain MOR02	1559	4679	99%	0.0	99%	KY171984
PYR3 Abonema	*Brevundimonas diminuta* strain zjs 01	1489	1489	97%	0.0	100%	KY171987
ANT4 Abonema	*Alcaligenes faecalis* strain MOR02	1594	1594	99%	0.0	99%	KY171980
PYR5 Nembe	*Alcaligenes faecalis* strain MOR02	1537	4612	98%	0.0	98%	KY171982
ANT6 Onne	*Myroides odoratus* strain D25T	1194	1194	95%	0.0	90%	KY171981
XYL7 Nembe	*Serratia marcescens* strain SM6	1476	1476	98%	0.0	97%	KY171985
XYL8 Onne	*Providencia* sp. strain X1	1491	1491	98%	0.0	97%	KY171986
PYR9 Onne	*Bacillus cereus* strain B4	1543	16940	98%	0.0	98%	KY171983

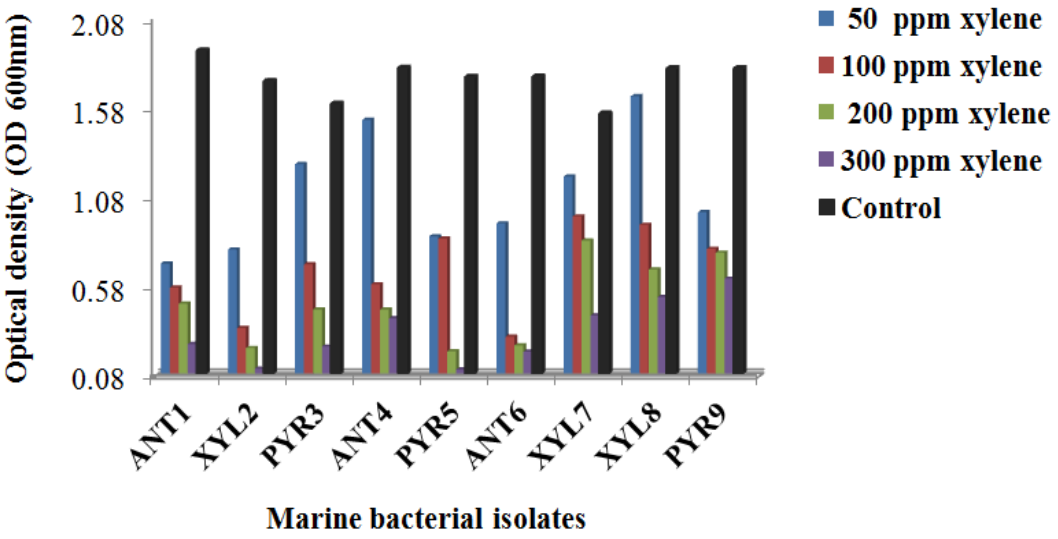

Fig. 2. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of xylene

PPM = Part Per Million; XYL = Xylene; ANT = Anthracene; PYR = Pyrene; ANT1 - *Providencia vermicola*, XYL2 - *Alcaligenes faecalis*, PYR3 - *Brevundimonas diminuta*, ANT4 - *Alcaligenes faecalis*, PYR5 - *Alcaligenes faecalis*, ANT6 - *Myroides odoratus*, XYL7 - *Serratia marcescens*, XYL8 - *Providencia* sp. and PYR9 - *Bacillus cereus; F (9, 40) = 5.75; P < 0.0001; R2 =0.614 - 0.944*

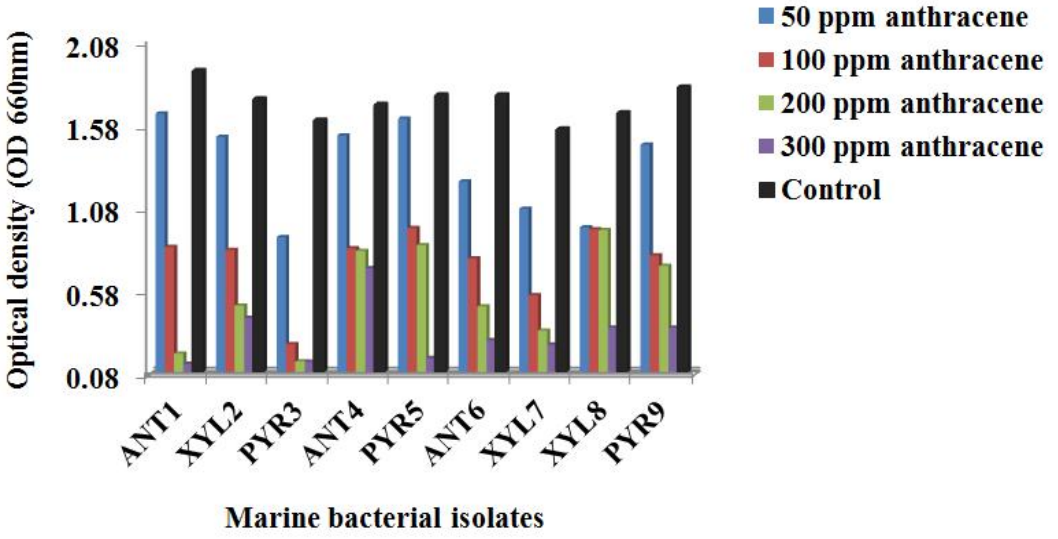

Fig. 3. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of anthracene

PPM = Part Per Million; XYL = Xylene; ANT = Anthracene; PYR = Pyrene; ANT1 - *Providencia vermicola*, XYL2 - *Alcaligenes faecalis*, PYR3 - *Brevundimonas diminuta*, ANT4 - *Alcaligenes faecalis*, PYR5 - *Alcaligenes faecalis*, ANT6 - *Myroides odoratus*, XYL7 - *Serratia marcescens*, XYL8 - *Providencia* sp. and PYR9 - *Bacillus cereus; F(9, 40) = 5.74; P < 0.0001; R2 =0.676 - 0.932*

Table 5. Growth specific test of the marine bacterial isolates on crude oil and different hydrocarbons

Substrate	Isolate								
	***Providencia vermicola* strain ANT1**	***Alcaligenes faecalis* strain XYL2**	***Brevundimonas diminuta* strain PYR3**	***Alcaligenes faecalis* strain ANT4**	***Alcaligenes faecalis* strain PYR5**	***Myroides odoratus* strain ANT6**	***Serratia marcescens* strain XYL7**	***Providencia* sp. strain XYL8**	***Bacillus cereus* strain PYR9**
Crude oil	++	+++	+++	+++	++	+++	++	+	+++
Toluene	+++	+++	++	+++	–	–	–	–	–
Petrol	+++	–	++	++	+	–	–	+++	++
Petroleum ether	++	++	+	+++	–	++	+++	–	+
Engine oil	+++	++	+++	+++	++	+++	+	++	+++
Diesel	+	+	+++	–	+	+++	++	+++	+++
Hexane	+++	–	–	+++	+	+++	+++	+	+++
Kerosene	++	+	+++	++	+	+	+++	++	++
Catechol	+++	+	+	+	+	+++	+	++	+
Parabenzene	++	+	++	+++	++	++	+++	+++	+++

Growth was followed by measuring the increase of OD at 600 nm of the culture for 7 days; + + + Heavy growth: OD 600 nm > 0.2; + + Moderate growth: OD 600 nm 0.1 ≤ 0.2 + Poor growth: OD 600 nm 0.02 ≤ 0; - No growth: OD 600 nm < 0.02

Table 6. Growth specific test of the marine bacterial isolates on different heavy metals

Isolate	Substrate (300 mg/ L)								
	$CuCl_2$	**AsO_3**	**$PbSO_4$**	**$HgCl_2$**	**$K_2Cr_2O_7$**	**KFeCN**	**CdO**	**$MnCl_2$**	**$ZnSO_4$**
Providencia vermicola strain ANT1	++	+	+	+++	++	++	+	+	+
Alcaligenes faecalis strain XYL2	++	+	+	++	++	+++	+	+	+
Brevundimonas diminuta strain PYR3	+	–	+	+	+	+	+++	+	+
Alcaligenes faecalis strain ANT4	+	-	+++	+	+	+	+	+	++
Alcaligenes faecalis strain PYR5	+++	+	+	++	++	++	+	+	+
Myroides odoratus strain ANT6	++	–	+	++	+++	++	–	+	+
Serratia marcescens strain XYL7	+	+++	+	+	+	+	–	+	+
Providencia sp. strain XYL8	+	+	+	+	+	+	+	+++	+
Bacillus cereus strain PYR9	+	+	++	+	+	+	+	+	+++

Growth was followed by measuring the increase of OD at 600 nm of the culture for 5 days; + + + Heavy growth: OD 600 nm > 0.2 + + Moderate growth: OD 600 nm 0.1 ≤ 0.2; + Poor growth: OD 600 nm 0.02 ≤ 0.1; - No growth: OD 600 nm < 0.02

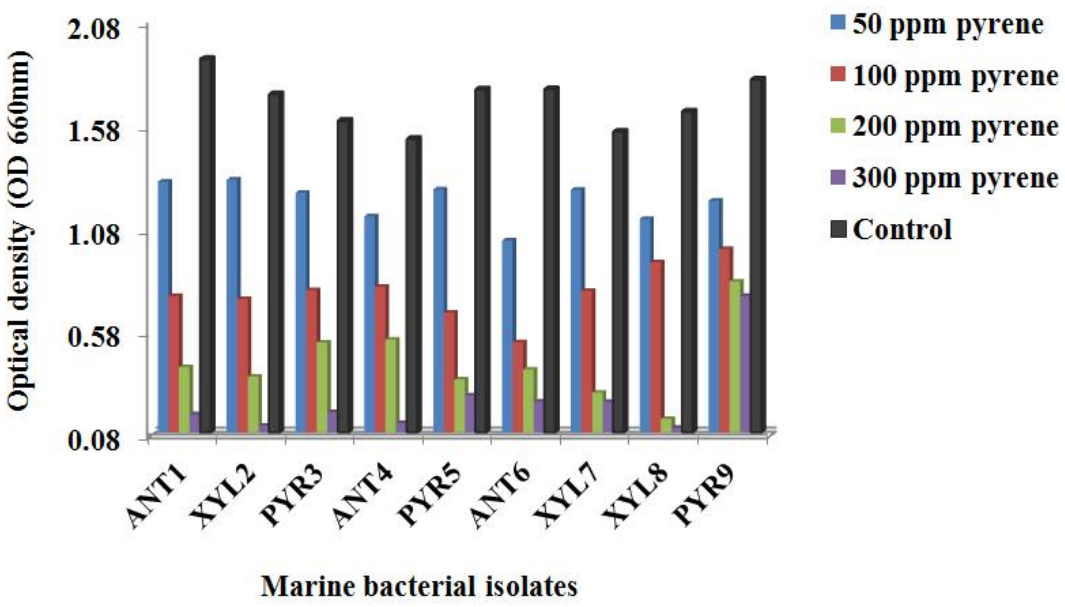

Fig. 4. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of pyrene

PPM = Part Per Million; XYL = Xylene; ANT = Anthracene; PYR = Pyrene; ANT1 - *Providencia vermicola*, XYL2 - *Alcaligenes faecalis*, PYR3 - *Brevundimonas diminuta*, ANT4 - *Alcaligenes faecalis*, PYR5 - *Alcaligenes faecalis*, ANT6 - *Myroides odoratus*, XYL7 - *Serratia marcescens*, XYL8 - *Providencia* sp. and PYR9 - *Bacillus cereus; F(9, 40) = 5.75; P < 0.0001; R2 = 0.739 - 0.961*

5. CONCLUSION

It can be concluded that the screening for indigenous bacterial isolates from the three studied areas resulted in the isolation of nine out of fourty eight (9/48) potent isolates with multiple degradation and resistance potentials on xylene, anthracene, pyrene, other petroleum products and heavy metals. All the nine potent strains were fully characterized, identified and belong to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus.* Together with their demonstrated multiple degradation and resistance potentials, the nine selected potent bacterial strains could significantly contribute in the development of a cost - effective bioremediation process on petroleum hydrocarbons and heavy metals contaminated environments in Nigeria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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