Original Research Article

Growth Profile and Metabolic Pathways Involved in Degradation of Aromatic Hydrocarbons by Marine Bacteria Isolated from Niger Delta, Nigeria

ABSTRACT

Aims: To determine the growth profile and metabolic pathways involved in degradation of aromatic hydrocarbons by marine bacteria isolated from Niger Delta, Nigeria

Study Design: Nine treatments designs were set up in triplicate in conical 500 mL containing 100 mL of sterile modified mineral basal medium supplemented with three aromatic hydrocarbons (100 mg /L each) and nine marine aromatic hydrocarbon degraders. The treatment were kept in the laboratory and incubated at 24 °C for 24 days degradation period. The nine treatment and control set ups designated as ANT1, XYL2, PYR3, ANT4, PYR5, ANT6, XYL7, XYL8, PYR9 and CTRL were used to determine the growth profile and metabolic pathways involved in degradation of aromatic hydrocarbons by marine bacteria.

Place and Duration of Study: Biotechnology Laboratory, Institute for Science and Technology Education, University of South Africa, Pretoria, South Africa between September, 2015 to June, 2018.

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 characterization, degradation and GC-MS assays.

Results: The findings revealed that the sampling sites harbour a lot of efficient aromatic degrading bacterial strains belonging to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus*. The bacterial strains were able to significantly (*P* =.05) degrade simple, low and high molecular weights aromatic hydrocarbons as source of carbon and energy. These strains utilized 100 mg /L of xylene (one ring), anthracene (three rings), and pyrene (four rings) via two main pathways and catechol is the major constant product that appeared during the degradation period (24 days).

Conclusion: Thus, the biodegradation capacities and metabolic pathways obtained from these results suggest that the bacteria especially *Serratia marcescens* XYL7 could be used efficiently to bioremediate aromatic hydrocarbon contaminated aquatic ecosystems in Nigeria.

Keywords: Aromatic hydrocarbons, marine bacteria, growth profile, biodegradation, biochemical pathways, Niger Delta.

1. INTRODUCTION

The Niger Delta region is the centre of petroleum production and development activities in Nigeria. Oil spill incidents have occurred in various parts and at different times in the Niger Delta's aquatic and terrestrial environments. These spills have been associated with sabotage, corrosion of pipes, carelessness during oil production and oil tanker accidents [1]. The release of crude oil and refined petroleum products in the

terrestrial and aquatic environments result in a long term threat to all forms of life [2]. Most of these compounds are considered as carcinogenic, mutagenic and potent immunotoxicants and classified as priority environmental pollutant by the US Environmental Protection Agency [3].

Aromatic hydrocarbons (AHs) are a class of hydrophobic organic compounds made of casted aromatic rings with linear and angular molecular arrangements, and they are found in the environment due to the processing of wood, char, creosote, petroleum mud, asphalt and pesticides. These substances can be bioactive, but persist in ecosystems for years, due to the low solubility in water and to the adsorption on solid particles [4]. Aromatic hydrocarbons are common environmental pollutants with toxic, genotoxic, mutagenic and carcinogenic properties. They mainly occur in petroleum industry activities. Oil spills because of pipeline breakages, tanks leakages or storage and transportation accidents can be considered as the most frequent causes of hydro-carbon release, included PAHs into soils. BTEX compounds are components of gasoline and aviation fuels that are carcinogenic and neurotoxic to most organisms [5].

Usually, contaminated sites are polluted by a mixture of PAHs [6]. Besides, the physical processes are often limited to aquatic environments only. The physical and chemical methods like volatilization, photooxidation, chemical oxidation, and bioaccumulation are rarely successful in rapid removal and in cleaning up PAHs, and also these methods are not safe and cost effective when compared to microbial bioremediation [7] metabolic. Bacteria play a major role in hydrocarbon degradation. The reason for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs [5]. Thus, for an efficient remediation process, it is important that the bacteria involved have a complete degradation pathway so that no potentially toxic degradation products accumulate [6]. Over twenty genera of bacteria of marine origin have been documented to be hydrocarbon degrading. Bacteria belonging to subphyla α -, β -, and δ - proteobacteria are well established to be of such nature [7]. The bacterial genera include *Pseudomonas, Serratia, Marinobacter, Providencia, Alcaligenes, Pseudomonas, Salmonella, Nocardia, Mycobacterium, Cunninghamella, Rhodococcus, Beijerinckia, Lysinibacillus, Corynebacterium, Diaphorobacter, Pseudoxanthomonas, Bacillus* and *Sphingomonas* have been found highly capable of degrading xylene, anthracene and pyrene hydrocarbons and well documented [8,9,10,11,12,13,14].

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Strategies to improve bioremediation technologies for BTEX and PAH-contaminated soils require a broader understanding of the biochemical pathways involved in degradation and in the eventual formation of partially oxidized products [15]. Particularly, degradation pathway of xylene by *Pseudomonas* sp. proceeds by oxidation at the methyl group akin to that of toluene to the corresponding methylbenzyl-alcohols, tolualdehydes, toluic acids and methylcatechols [16] and key reactions connected to benzoate and β – ketoadipate catabolism [17]. Biotransformation pathways for anthracene have been elucidated by various microoorganisms suggesting dioxygenation and dehydration by which 1, 2-dihydroxylanthracene is formed [18]. Brinda and Velan [18] reported that anthracene metabolites were identified using high performance liquid chromatography and gas chromatophy mass spectrum analyses to elucidate the biodegradation pathways. Three degradation pathways were proposed, one of which has been demonstrated as is a new branch in anthracene degradation pathways because of 9 hydroxyl fluorene a novel metabolite. Pathway of pyrene degradation has been proposed for Mycobacterium PYR-1 growing on pyrene which involves ring oxidation and ring cleavage metabolites. This pathway was later confirmed and studies by many investigators and also in other microorganisms that use pyrene as a sole source of carbon and energy [15].

However, considering the complexity and ubiquity of the aromatic compounds in the Niger Delta environment, it necessitates the further understanding of different environmental pathways for their degradation and identification of metabolites by new strains especially *Serratia marcescens* XYL7 in this study since several investigators reported BTEX and PAH degradation but the catabolic pathways are not described. This will assist in designing bioremediation strategies for remediation and reclamation of sites contaminated with such hazardous compounds in the Niger Delta.

In this study we report the growth profile of marine bacteria when grown in xylene, anthracene and pyrene as source of carbons and energies. Identification of intermediate metabolic products obtained during their degradation when used as carbon source by marine bacteria was also reported. Based on the intermediates identified in this study, an attempt was also made to propose metabolic pathways for xylene, anthracene and pyrene biodegradations.

2. MATERIALS AND METHODS

2.1 Description of the Sampling Sites

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The studied areas were Abonema Wharf Water Front (Figure 1) in Akuku-Toru Local Government Area, Nembe Water-side (Figure 2) in Port Harcourt Local Government Area and Onne Light Flow Terminal Seaport (Figure 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 Portharcourt 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 Waterside 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. Onne Light Flow Terminal Seaport (Plate 16) 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 account for over 65 % of the export cargo through the Nigerian Sea Port. Anthropological survey revealed the presence of human activities such as transportation of petrochemical products through tankers, canoes, boats and ships to neighboring villages, towns, cities, states and nations due to the presence of multinational petrochemical and oil servicing industries such as Chevron Nigeria Limited, Cameron Offshore services, Exon Mobil Nigeria Limited, Socotherm Pipecoaters, Beker Hughes Oil Servicing Company, Aiteo Energy Resource, Sorelink Oil and Dozzy Oil and gas et cetra that generate the wastes that contaminate the sites above.

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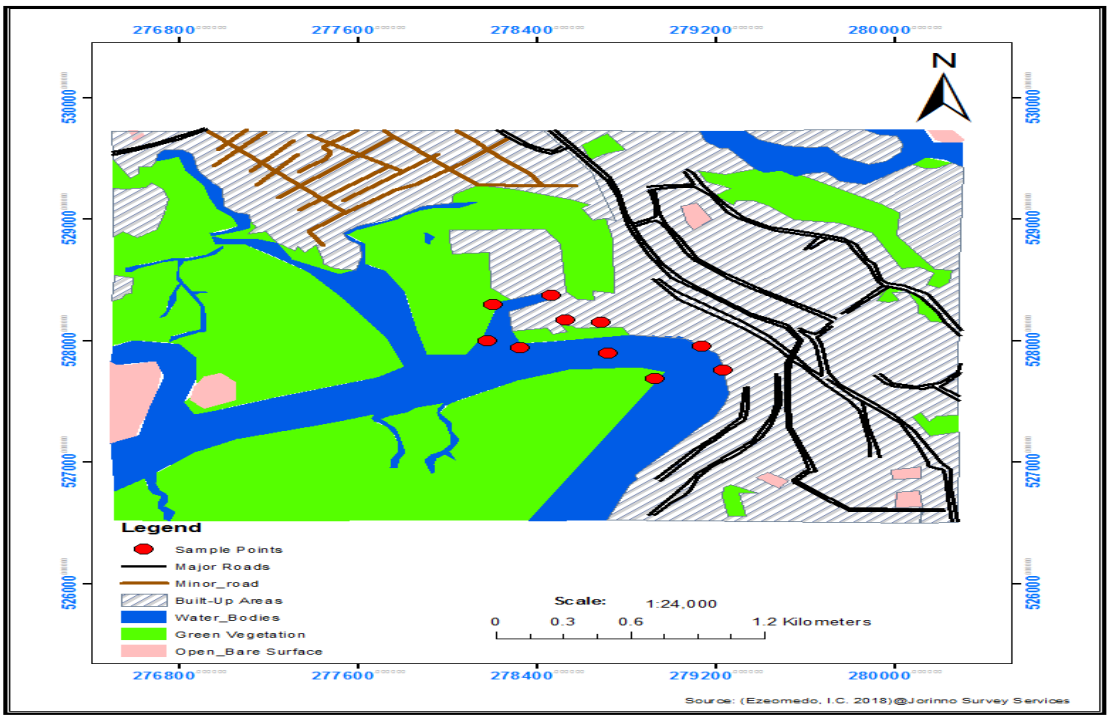

Fig. 1. Thematic map indicating the locations of sample points and its land cover in Abonema study area

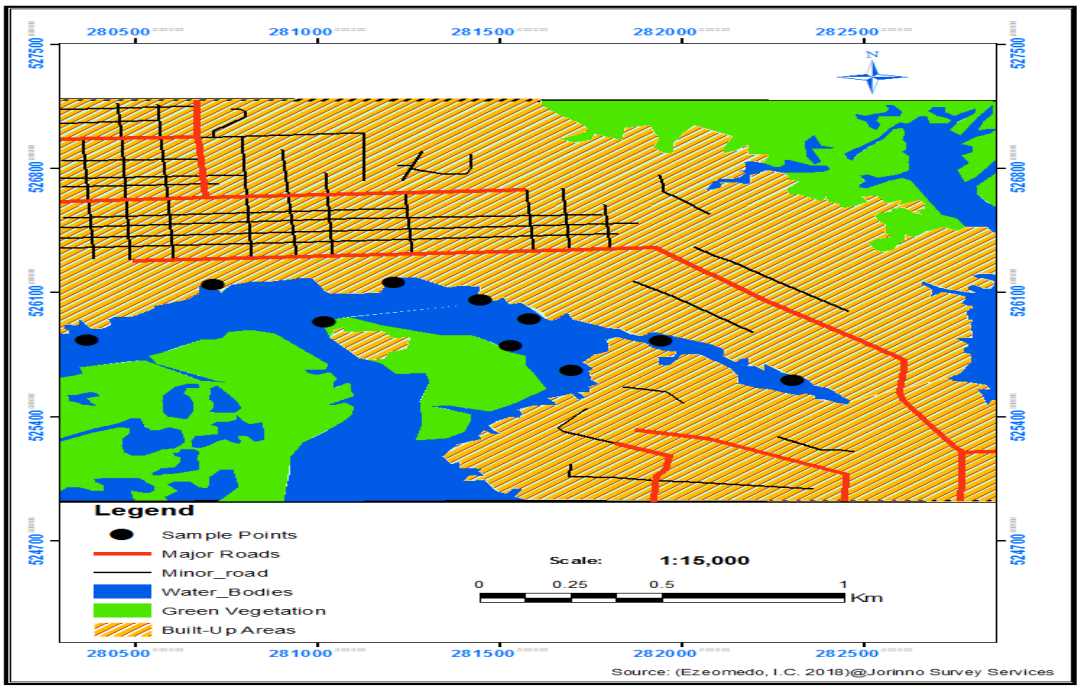

Fig.2. Thematic map indicating the locations of sample points and its land cover in Nembe study area

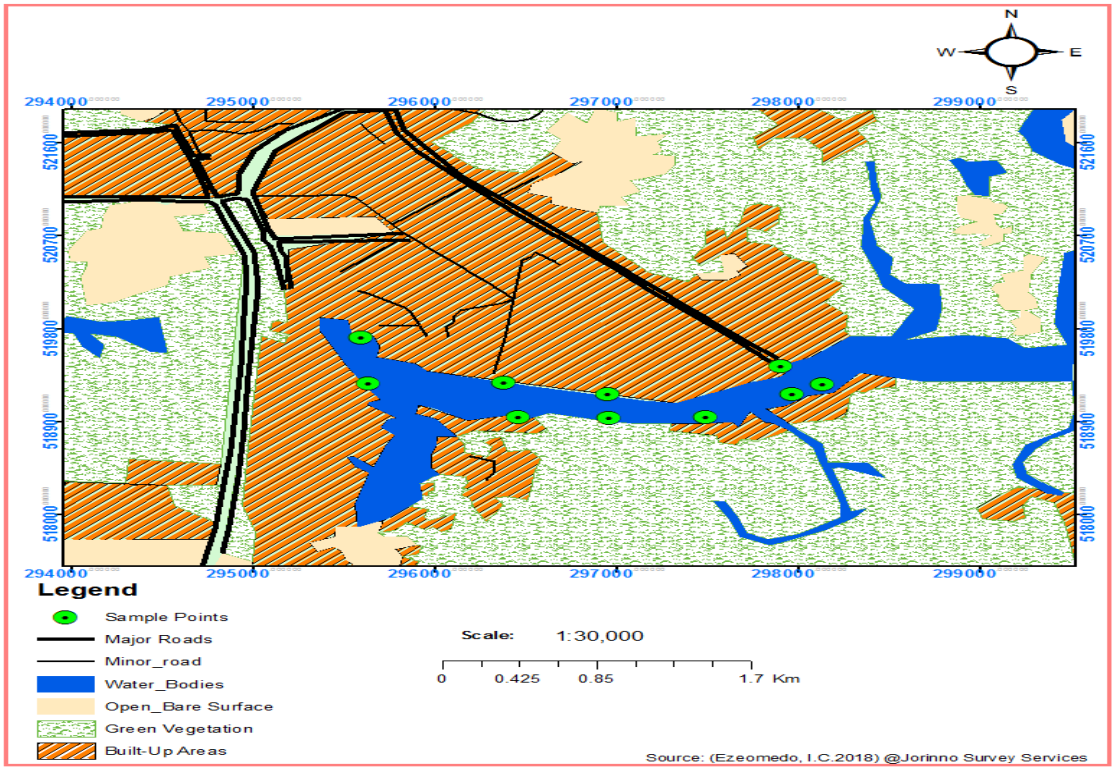

Fig. 3.Thematic map indicating the locations of sample points and its land cover in Onne study area

2.2 Sample Collection and Processing

Ten samples were collected randomly at each designated points in the three particular sampling sites (Figures 1, 2 and 3) and mixed together after which a total of six 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 wide mouthed plastic containers. The water samples were collected at the air-water interface by hand dipping the 95 % ethanol - sanitized cylindrical shaped 2 L plastic containers. The containers were rinsed with the sediment and water samples before collecting the samples. All the composite or representative sediment and water samples containers were placed into a sterile polythene bag and then transported to the laboratory for microbiological analyses [19, 20, 21].

2.3 Enrichment, Culturing and Isolation of Aromatic Hydrocarbon 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 $FeSO_4$,

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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^2 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 spreaded 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 spread plated on the surfaces of the solidified media with the aid of 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 [21, 22].

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 Bjou bottles where they are preserved at 4 °C in refrigerator [21].

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 [21, 22, 23].

2.6 Characterization and Identification of Selected Hydrocarbon Utilizing Bacterial Isolates

2.6.1 Molecular characteristics

2.6.1.1 Identification of bacteria

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Further identification was carried out using the Gram - reaction test and molecular techniques. The Gram - reaction test was first used to ascertain the morphological characterisation and purity of the colonies before proceeding to do molecular identification which includes DNA extraction, Polymerase chain reaction (PCR), gel electrophoresis, sequencing and blasting [24].

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 [24].

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 tubes 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),

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primer 1 (forward 16S - P1 PCR 5/AGAGTTTGATCCTGGCTCAG3/) (2 µl), primer 2 (reverse 16S - P2 PCR 5/AAGGAGGTGATCCAGCCGCA3/) (2 µl), dNTP mix (1µl), 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 was set at (a) initial denaturation 10 minutes 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, using 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 [24].

2.6.1.4 Sequencing and blasting analyses

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 [28]. The basic local alignment search tool (BLAST) of DNA sequences was performed by editing the sequences of the 16S rDNA 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 [12, 24, 25, 26].

2.6.1.5 Gene Bank 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 [13, 25, 27].

2.7 Degradation assay

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Following the methods of Swaathy *et al*. [12] and John and Okpokwasili [22] as modified in this study, the degradation rates of bacterial isolates were determined using hydrocarbon supplemented 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). Precisely, 1 ml of 48 hrs old cultures of each bacteria was introduced into 28 sterile 200 ml capacity conical flasks (4 sets of 7 flasks) in triplicates containing 100 ml of sterile modified mineral basal medium supplemented with 100 mg /L of xylene, anthracene and pyrene hydrocarbons respectively as source of carbon at 24 $^{\circ}$C for 24 days.

2.7.1 Identification of metabolites formed/degraded products using instrumental analysis

2.7.1.1 Extraction of the aromatic hydrocarbon fractions

The samples were extracted after 24 days of incubation by adopting the standard method described by AOAC [28]. The sample mixtures were separated using separating funnel and the dichloromethane layer was concentrated in rotary evaporator. One millilitre of acetonitrile was added into the concentrate and transferred into a vial ready for analysis. The florisil was heated in an oven at 130 $^{\circ}$C overnight (ca.15 h) and transferred to a 250 ml size beaker and placed in a desiccator. A 0.5 g anhydrous Na_2SO_4 was added to 1.0 g of activated flosiril (magnesium silicate) (60 – 100 nm mesh) on an 8 ml column plugged with glass wool. The packed column was filled with 5 ml n – hexane for conditioning. The stopcock was open to allow N – hexane run out until it just reaches top of sodium sulphate into a receiving vessel whilst tapping gently the top of the column till the florisil settled well in the column. The extract was transfered onto the column with disposable Pasteur pipette from an evaporating flask. Each evaporating flask was rinsed twice with 1 ml portions of n – hexane and added to the column. The eluate was collected into an evaporating flask and rotary evaporator to dryness. The dried eluate was dissolved in 1 ml n – hexane and made ready for AH GC-MS analysis.

2.7.1.2 Gas chromatography – mass spectrometry (GC – MS) analysis

The aromatic hydrocarbons present in the extracts were quantified using GC/MS as described by Bobak [29]. The stock standard was restek cat. No 8270-1 which contains semivolatile mix. It was purchased from Sigma Aldrich, South Africa. The concentration of the stock standard was 1000 ppm and it was used to prepare the calibration standards of 10 ppm, 30 ppm and 50 ppm. Working standard solution was prepared from the surrogate standard using dichloromethane. Calculation of the required concentrations was based

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on the chemical formula: $C_1V_1 = C_2V_2$; Where C_1 = Concentration of stock solution, C_2 = Concentration to be made, V_1 = Volume to be determined, V_2 = Volume required. These standards were first analysed using the GC/MS to register a known retention time to match with each compound. The qualitative and quantitative analyses of the semivolatile compounds present in the sample extracts were carried out with the GC/MS Agilent 7860 GC system and 5975C MSD, equipped with a 7683B autosampler (Agilent Technologies, USA). The sampler syringe was 5.0 µl and splitless injection was 1.0 µl. The carrier gas used was helium 30 cm/second and at a constant flow rate of 1 ml/minute. The inlet, splitless, 260 ^{o}C, purge flow was 50 ml/minute at 0.5 minute and gas saver was at 80 ml/minute at 3 minutes. Inlet liner was the deactivated dual taper direct connect. The column was Agilent HP-5 ms ultra inert 30 m x 0.25 mm x 0.25 µm film thickness. The oven program was started at 40 ^{o}C for 1 minute to 100 ^{o}C (15 ^{o}C/min), 10 ^{o}C/minute to 210 ^{o}C (1 minute), 5 ^{o}C/minute to 310 ^{o}C and it was held for 8 minutes. The detection was MSD source at 300 ^{o}C, quadrupole at 180 ^{o}C, transfer line at 290 ^{o}C, scan range 45 to 450 amu. The vials were amber screwed top glass vials and the vial cap was blue screwed cap. The vial inserts were 100 µl glass/polymer feet. The septum was advanced green. The ferrules were 0.4 mm id short; 85/15 vespel/graphite. The magnifier was 20X magnifier loupe. This instrument works on principle that a small amount of liquid extract injected into the instrument id volatilized at the hot injection chamber. The volatilized molecules are swept by a stream of inert carrier gas through a heated column that holds a high boiling liquid as the stationary phase. As the mixture flows along the column, the components bombard each other at different rates between the gaseous phase, dissolved in the high boiling liquid and it is then separated into pure components. The compounds are passed through a detector which sends an electronic signal to the recorder which responds by peak formations. The peaks formed are quantified by mass selective detector using the retention time of the relative compounds registered from a known standard. Aromatic hydrocarbons are identified by retention times matching to standards concentration. The value of the chromatogram was quantified using peak area integration. The extraction procedure and extract analysis on all samples as well as the quality control and assurance of gas chromatographic – mass spectroscopic analyses were described by Bobak [29] and the mass spectra obtained after the analysis were compared to the mass spectra in the the PubChem and NIST libraries /databases [30] .

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2.8 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 if the probability is less than .05 (P = .05) [23, 26].

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 percentage similarity and Gen bank 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.

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3.3 Identification of Metabolites Formed/Degraded Products

The results of GC analysis of metabolites obtained during the incubation of marine isolates in the presence of xylene, anthracene and pyrene hydrocarbons; GC Retention data and electron impact mass spectral properties of metabolites formed from xylene, anthracene and pyrene utilization as well as proposed metabolic pathway for the degradation of xylene, anthracene and pyrene based on the degraded products identified using GC-MS by *Alcaligenes faecalis* strain XYL2, *Serratia marcescens* strain XYL7, *Providencia* sp. strain XYL8, *Providencia vermicola* strain ANT1, *Alcaligenes faecalis* strain ANT4, *Myroides odoratus* strain ANT6, *Brevundimonas diminuta* strain PYR3, *Alcaligenes faecalis* strain PYR5 and *Bacillus cereus* strain PYR9 are presented and shown in Tables 5, 6 and 7 and Figures 4, 5, 6, 7, 8 and 9 . From the results, m – toluic acid, 4 - methoxylbenzenethiol, α – toluic acid, α – methylbenzylmethanol, catechol, propionic acid and acetaldehyde were the degraded metabolites of xylene; paranaphthalene, 2 – Ethyl – 9, 10 – anthraquinone, ethyl 6, 8 – difluoro – 4 - hydroxyquinoline – 3 – carboxylate, 1,2 - bis(trimethylsilyl) benzene, 1, 2, 3-trihydroxylbenzene, benzenecarboxylic acid and catechol were the degraded metabolites of anthracene; while 9, 10 - dihydroanthracene, 4 – nitrophthalic acid, benzenecarboxylic acid, hydroxylmethylbenzene and catechol were the degraded metabolites of pyrene.

4. DISCUSSION

PAHs are serious pollutants and health hazards, and they occur as complex mixtures, including low and high molecular weight; therefore, degradation of PAHs in the environment is becoming more necessary and interesting. Low molecular weight is more easily degraded, while high molecular weight is more recalcitrant and requires specific microorganisms to perform the degradation [25].

In this study, sediment and water samples obtained from Abonema Wharf water front, Nembe water side and Onne light terminal flow studied areas were analyzed microbiologically for the presence of potential aromatic hydrocarbon degraders. 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*. [31] that the most important aspect of microbial degradation of PAH is enrichment and isolation of indigenous PAH degraders

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because the indigenous PAH degrading bacteria are already adapted to utilizing PAH. Pathak and Bhatnagar [32], 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*. [33] reported that an occurence 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.

Molecular characterization of bacteria helps in the quantification and detection of their phylogenetic diversity. Molecular identification of bacteria is highly sensitive and specific as compared to a biochemical approach of identification. Molecular characterization of the 16S rRNA gene using polymerase chain reaction (PCR) is a well - known method of identifying a species and genera of bacteria [34]. 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* spp. (95 - 97 %), *Brevundimonas diminuta* (100 %), *Myroides odoratus* (90 %), *Serratia marcescens* (97 %) and *Bacillus cereus* (98 %) using NCBI BLAST software. Akinbankole *et al*. [14], found out that *B. cereus* was the most blasted organism with sequence homology (99 %). The result also revealed that the nine bacterial isolates were assigned accession numbers: KY171979, KY171984, KY171987, KY171980, KY171982, KY171981, KY171985, KY171986 and KY171983) and the nucleotide sequences of their 16S rRNA genes have been deposited in Genbank database since first of December thousand and sixteen (01/12/16). They belong to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus*; and families of: Enterobacteriaceae, Alcaliginaceae, Caulobacteraceae, Flavobacteriaceae, and Bacillaceae; and phyla of: Proteobacteria, Bacteroidetes and Firmicutes which members have been implicated in petroleum and aromatic hydrocarbon biodegradation by several authors [9, 10, 12, 14, 21, 35, 26, 36, 37, 38, 39, 40, 41].

The results presented and shown in Tables 5, 6 and 7 and Figures 4, 5, 6, 7, 8 and 9 showed that m – toluic acid, 4 - methoxylbenzenethiol, α – toluic acid, α – methylbenzylmethanol, catechol, propionic acid and acetaldehyde were the degraded metabolites of xylene; paranaphthalene, 2 – Ethyl – 9, 10 – anthraquinone, ethyl 6, 8 – difluoro – 4 - hydroxyquinoline – 3 – carboxylate, 1,2 - bis(trimethylsilyl) benzene, 1, 2, 3 -trihydroxylbenzene, benzenecarboxylic acid and catechol were the degraded metabolites of anthracene; while 9, 10 - dihydroanthracene, 4 – nitrophthalic acid, benzenecarboxylic acid,

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hydroxylmethylbenzene and catechol were the degraded metabolites of pyrene. Metabolites I, II and I (m – toluic acid, paranaphthalene and 9, 10 - dihydroanthracene) eluted at 6.30, 9.82 and 11.61 minutes had the highest base molecular ion peak of 136 (100), 236 (100) and 180 (100) m/zs, and other major fragment ions at m/z values of 91 (97) and 119 (57); 221 (30) and 193 (28) and 179 (94) and 178 (51.5) while metabolites VI and IV (acetaldehyde, benzenecarboxylic acid and hydroxylmethylbenzene) had the lowest base molecular ion at 29 (100), 105 (100) and 79 (100) m/zs with 6.04, 5.83 and 4.58 minutes retention time and the other major fragment ions at m/z values of 44 (82) and 43 (48); 122 (84) and 77 (67.5); and 108 (89) and 107 (71) respectively during xylene, anthracene and pyrene degradations. These mass fragments were in agreement with published spectra of Kim *et al.* [30]. Kim *et al*. [17], reported that the degradation of *m* - xylene to 3 - methylbenzoate and 3 - methylcatechol with further cleavage to propionaldehyde and pyruvate. Morasch *et al*. [42], reported that 3 - methylphenylitaconic acid, *m* - toluic acid and methylbenzylsuccinate were extracted from supernatants of *m* – xylene grown cultures. Otenio *et al.* [43], reported the degradation of *m* - xylene to 3 - methylbenzenealcohol and Brinda and Velan [18], who reported four metabolites having base ion at m/z 73 and the other specific ion at m/z 147 from anthracene extracts. Ahmed *et al*. [44], observed the formation of anthrone by alkaliphilic bacteria at C9 and C10 position and further leads to the formation of quinone product of PAHs and two quinone products were observed in this study. Swaathy *et al*. [12], found out that their mass spectral analyses and the library details suggested that (i) naphthalene (m/z - 128), (ii) naphthalene- 2-methyl (m/z - 142), (iii) benzaldehyde-4-propyl (m/z - 148), (iv) 1, 2, benzene di-carboxylic acid (m/z - 167) and (v) benzene acetic acid (m/z - 137) were the major degraded products detected. The pathways of the aromatic hydrocarbon degradation were proposed based on the metabolites mass spectrum analyses. The aromatic hydrocarbon (AH) metabolism pathways varies depending on the species used in this degradation study. Furthermore, it seems likely that the degradation of individual AH compounds by the selected bacteria proceeds via independent pathways [45]. From the metabolic pathways of these bacteria, it could be concluded that these strains utilized 100 mg /L of xylene (one ring), anthracene (three rings), and pyrene (four rings) via two main pathways and catechol is the major constant product that appeared during the degradation period (24 days). Thus, xylene, anthracene and pyrene degradation by strains XYL7 and PYR5 produced several metabolites which are similar to the ones produced by other xylene, anthracene and pyrene degraders such as catechol by *Leclercia adecarboxylata* PS4040 [15], Phthalic acid by *Sphingomonas koreensis* Strain ASU-06; *Alcaligenes faecalis* MVMB1, *P. vulgaris* 4Bi and *P. fluorescens* 29 L [25, 18, 46, 47, 48]; toluic acid by

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Desulfotomaculum [42] and benzylalcohol by *P. putida* CCMI 852 and *Pseudomonas putida* mt-2 [43, 17] and 9, 10 – dihydroanthracene (9,10 – anthraquinone) by marine *Bacillus licheniformis* MTCC 5514 [12, 48].

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	0.657 ± 0.001	0.580 ± 0.003
ANT4*	0.676 ± 0.004	0.775 ± 0.007	0.822 ± 0.002
AB3	0.701 ± 0.021	0.467 ± 0.029	0.666 ± 0.003
AB4	0.715 ± 0.004	0.598 ± 0.024	0.841 ± 0.001
PYR3*	0.598 ± 0.005	0.511 ± 0.003	1.041 ± 0.008
AB6	0.641 ± 0.001	0.494 ± 0.002	0.653 ± 0.001
AB7	0.687 ± 0.004	0.690 ± 0.002	0.803 ± 0.016
AB8	0.618 ± 0.001	0.638 ± 0.007	0.782 ± 0.003
AB9	0.457 ± 0.002	0.475 ± 0.001	0.573 ± 0.004
XYL2*	0.952 ± 0.004	0.312 ± 0.002	0.838 ± 0.021
AB11	0.793 ± 0.014	0.495 ± 0.002	0.970 ± 0.003
AB12	0.647 ± 0.002	0.446 ± 0.001	0.621 ± 0.005
AB13	0.328 ± 0.001	0.415 ± 0.001	0.451 ± 0.001

** = Isolates with highest degradability; values are mean ± standard deviation of triplicate determination.*

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

Isolate	Optical density (OD_{600} nm)		
	Xylene	Anthracene	Pyrene

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

** = Isolates with highest degradability; values are mean ± Standard deviation of triplicate determination.*

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

** = Isolates with highest degradability; values are mean ± standard deviation of triplicate determination.*

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

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

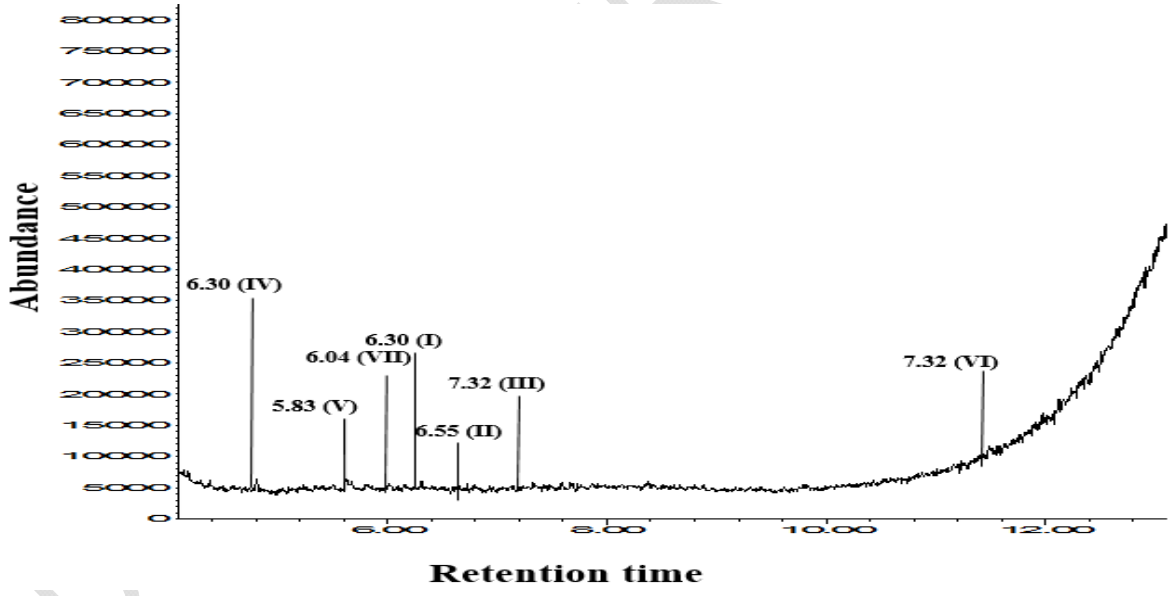

Fig. 4. GC analysis of metabolites obtained during the incubation of marine isolates in the presence of xylene hydrocarbon. I. m – Toluic acid II. 4 – Methoxylbenzenethiol III. α – Toluic acid IV. α – Methylbenzylmethanol V. catechol VI. Propionic acid and VII. Acetaldehyde

Table 5. GC Retention data and electron impact mass spectral properties of metabolites formed from xylene utilization by *Alcaligenes faecalis* strain XYL2, *Serratia marcescens* strain XYL7 and *Providencia* sp. strain XYL8

Metabolite	tR (min)	m/z ions (relative intensity/abundance)	Structural suggestion	Isolate code
i	6.30	136(100), 91(97), 119(57)	M – toluic acid	XYL7 and XYL8
ii	6.55	40(100), 125(78), 97(35)	4 - Methoxylbenzenethiol	XYL7 and XYL8
iii	7.32	91(100), 136(38), 92(19)	α – Toluic acid	XYL2, XYL7 and XYL8
iv	4.81	107(100), 122(86), 79(58)	α - Methylbenzylmethanol	XYL7 and XYL8
v	5.83	110 (100), 108 (15.55), 82 (18.37), 64(28.13),	Catechol	XYL2, XYL7 and XYL8
vi	11.56	74(100), 28(93), 45(90)	Propionic acid	XYL2 and XYL7
vii	6.04	29(100), 44(82), 43(48)	Acetaldehyde	XYL2, XYL7 and XYL8

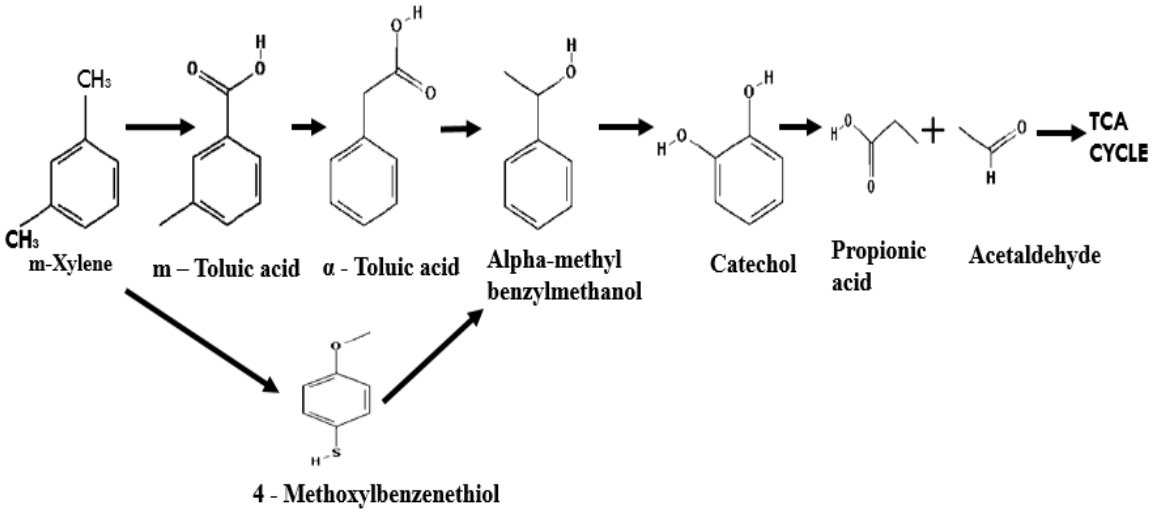

Fig.5. Proposed metabolic pathway for the degradation of the xylene by *Alcaligenes faecalis* strain XYL2, *Serratia marcescens* strain XYL7 and *Providencia* sp. strain XYL8 based on the degraded products identified using GC - MS

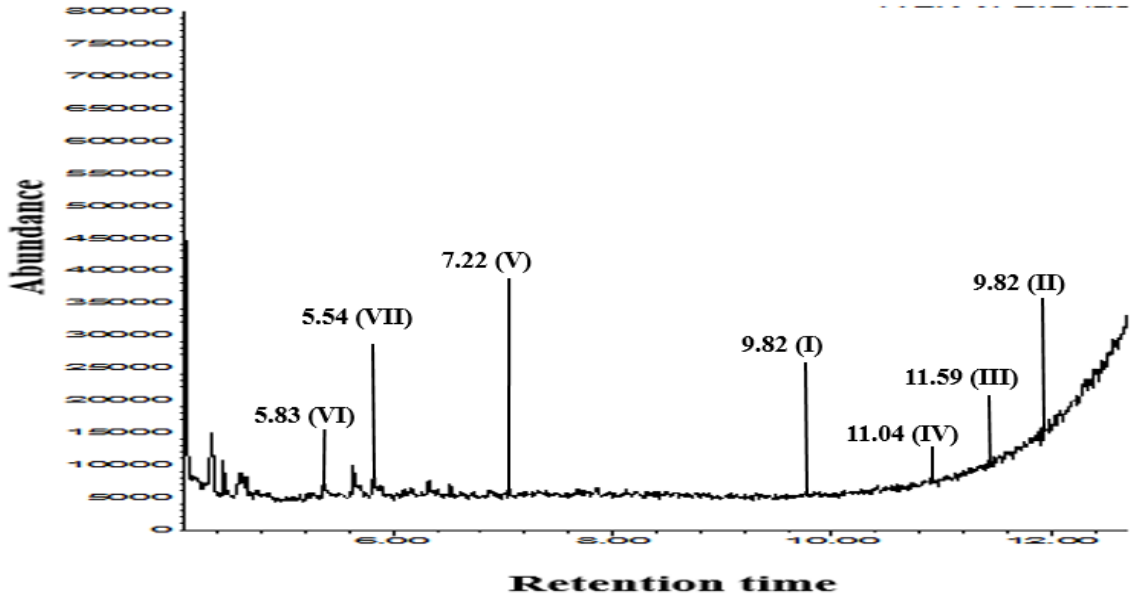

Fig.6. GC analysis of metabolites obtained during the incubation of marine isolates in the presence of anthracene hydrocarbon. I. Paranaphthalene, II. 2 – Ethyl – 9, 10 – anthraquinone III. Ethyl 6, 8 – difluoro – 4 - hydroxyquinoline – 3 – carboxylate IV. 1, 2 – Bis (trimethylsilyl) benzene V. 1, 2, 3 - Trihydroxylbenzene VI. Benzenecarboxylic acid and VII. Catechol

Table 6. GC Retention data and electron impact mass spectral properties of metabolites formed from anthracene utilization by *Providencia vermicola* strain ANT1, *Alcaligenes faecalis* strain ANT4 and *Myroides odoratus* strain ANT6

Metabolites	tR (min)	m/z ions (relative intensity/abundance)	Structural suggestion	Isolate code
i	9.82	178(100), 179(15), 176(14)	Paranaphthalene	ANT4 and ANT6
ii	11.98	236(100), 221(30), 193(28)	2 – Ethyl – 9, 10 – anthraquinone	ANT1
iii	11.59	207(100), 253(25), 151(23)	Ethyl 6, 8 – difluoro – 4 - Hydroxyquinoline – 3– carboxylate	ANT1
iv	11.04	207(100), 73(27.5), 208 (22.5)	1,2 - Bis(trimethylsilyl) benzene	ANT4 and ANT6
v	7.22	126 (100), 52(47.5), 80(35)	1, 2, 3-trihydroxylbenzene	ANT1
vi	5.54	236(100), 221(30), 193(28)105(100), 122(84), 77(67.5)	Benzenecarboxylic acid	ANT1, ANT4 and ANT6
vii	5.83	110 (100), 108 (15.55), 82 (18.37), 64 (28.13),	Catechol	ANT1, ANT4 and ANT6

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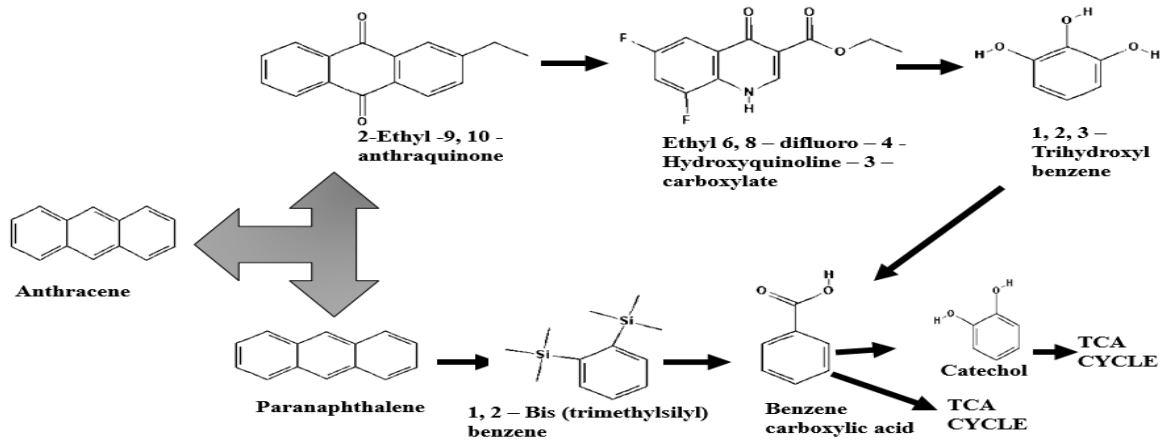

Fig.7. Proposed metabolic pathway for the degradation of the anthracene by *Providencia vermicola* strain ANT1, *Alcaligenes faecalis* strain ANT4 and *Myroides odoratus* strain ANT6 based on the degraded products identified using GC-MS

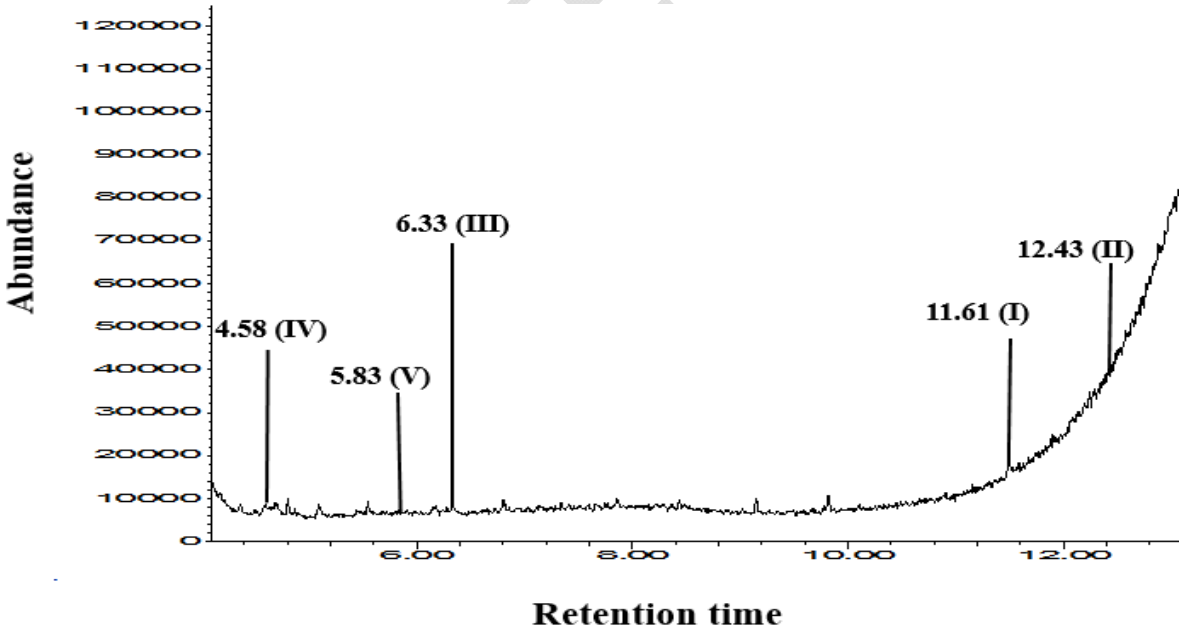

Fig. 8. GC analysis of metabolites obtained during the incubation of marine isolates in the presence of pyrene hydrocarbon. I. 9, 10 - Dihydroanthracene II. 4 – Nitrophthalic acid III. Benzenecarboxylic acid IV. Hydroxylmethylbenzene V. Catechol

Table 7. GC Retention data and electron impact mass spectral properties of metabolites formed from pyrene utilization by *Brevundimonas diminuta* strain PYR3, *Alcaligenes faecalis* strain PYR5 and *Bacillus cereus* strain PYR9

Metabolite	tR (min)	m/z ions (relative intensity/abundance)	Structural suggestion	Isolate code
i	11.61	180(100), 179(94), 178(51.5)	9, 10 - Dihydroanthracene	PYR9
ii	12.43	149(100), 75(67), 103(65)	4 – Nitrophthalic acid	PYR3 and PYR5
iii	6.33	105(100), 122(84), 77(67.5)	Benzenecarboxylic acid	PYR3, PYR5 and PYR9
iv	4.58	79(100), 108(89), 107(71)	Hydroxylmethylbenzene	PYR3 and PYR5
v	5.83	110 (100), 108 (15.55), 82 (18.37), 64 (28.13),	Catechol	PYR3, PYR5 and PYR9

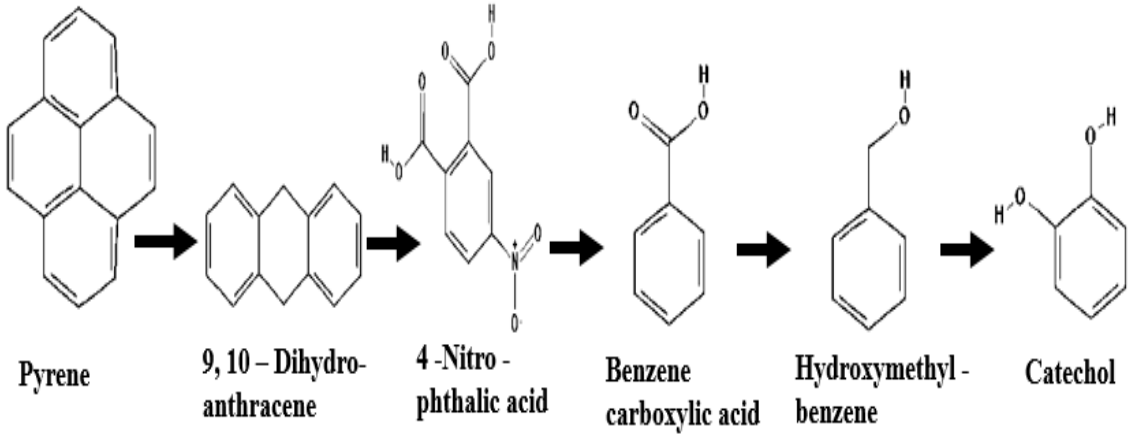

Fig.9. Proposed metabolic pathway for the degradation of the pyrene by *Brevundimonas diminuta* strain PYR3, *Alcaligenes faecalis* strain PYR5 and *Bacillus cereus* strain PYR9 based on the degraded products identified using GC - MS

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5. CONCLUSION

The whole study revealed that the three sampling sites harbour a lot of efficient aromatic hydrocarbon degrading bacterial strains belonging to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus.* It also revealed that the isolated bacteria especially *Serratia marcescens* XYL7 were able to significantly (*P* =.05) degrade simple, low and high molecular weights aromatic hydrocarbons as source of carbon and energy. It could also be concluded that these strains utilized 100 mg /L of xylene (one ring), anthracene (three rings), and pyrene (four rings) via two main pathways and catechol is the major constant product that appeared during the degradation period (24 days). Thus, the growth and biodegradation capacities as well as metabolic pathways obtained from these results suggest that the bacteria especially *Serratia marcescens* XYL7 could be used efficiently to bioremediate aromatic hydrocarbon contaminated aquatic ecosystems in Nigeria.

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