Original Research Article

Phylogenetic Framework and Metabolic Genes Expression Analysis of Bacteria Isolated from Contaminated Marine Environments of Niger Delta

ABSTRACT

Aims: To explore the phylogenetic framework of bacteria isolated from contaminated marine environments of Niger Delta and the expression of the metabolic genes coding for aromatic hydrocarbon degradation and surfactant production.
Study Design: Nine treatments designs were set up in triplicates containing 25 mL of sterile modified mineral basal medium supplemented with nine marine hydrocarbon degraders incubated at 24 °C for 5 days. Three of the set ups were supplemented each with 1 mg /L of xylene, anthracene and pyrene.
Place and Duration of Study: Biotechnology Laboratory, Institute for Science and Technology Education, University of South Africa, Pretoria, South Africa between September, 2015 to December, 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, characterization, and PCR assays to explore the phylogenetic framework and metabolic genes expression of the marine bacteria for aromatic hydrocarbon degradation and surfactant production.
Results: The findings revealed that there was significant abundance of THB (P = .05) more than TCHUB and more xylene degraders than anthracene and pyrene degraders in the sediment and water samples respectively. The phylogenetic correlational analysis revealed that all the nine selected best degraders out of 48 isolates from the studied area were evolutionary related belonging to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus;* able to significantly (P = .05) utilize the all the aromatic hydrocarbons. The existence of catabolic and surfactant genes namely catechol dioxygenase (*C23O*), rhamnolipid enzyme (*rhlB*) and surfactin/lichenysin enzyme (*SrfA3/LicA3*) genes were detected in only four (4) out of the nine (9) marine aromatic degrading bacteria with 881 base pairs sizes.
Conclusion: Thus, the study revealed that these bacterial strains especially *Serratia marcescens* XYL7 might possess metabolic genes for *in situ* aromatic hydrocarbon degradation and surfactant production.

Keywords: Aromatic hydrocarbons, marine bacteria, biodiversity, biodegradation, catabolic genes, surfactant genes, 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 especially the aromatic hydrocarbons are considered as carcinogenic, mutagenic and potent immunotoxicants and classified as priority environmental pollutant by the US Environmental Protection Agency [3].

Biodiversity is given by a variety of species living on earth resulting from billions of years of evolution. Molecular phylogenetic studies have revealed that the main diversity of life is microbial and it is distributed among three domains: prokaryotes, eukaryotes, and archaea. Metagenomics, metatranscriptomics, metaproteomics, and single cell sequencing are the approaches providing a view not only of the community structure (species phylogeny, richness, and distribution) but also of the functional (metabolic) potential of a community [4]. Among the various sources, microbes of marine waters/sludges received good attention because of the wide potential biological products with immense applications. Most of the products are used to recover oil. In addition, the interest in marine bacteria arises from their biotechnological potential in bioremediation and in oceanic biogeochemical cycles and as a source of novel metabolites different from those isolated from terrestrial bacteria, such as antibiotics and products for industrial use like exopolysaccharides, enzymes, biosurfactants, and compatible solutes [5].

Usually, contaminated sites are polluted by a mixture of PAHs [5]. Polycyclic Aromatic Hydrocarbons (PAHs) are found in considerable amounts in crude oil and oily effluents of petroleum refineries. Low molecular weight (LMW) PAHs are relatively volatile, high soluble in water and more degradable than high molecular weight (HMW) PAHs [6]. Aromatic with one, two or three aromatic rings (xylene and anthracene) are also efficiently biodegraded; however, those with four or more aromatic ring (pyrene) are quite resistant biodegradation [7]. 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 [8]. A better way is to use biodegradation. Thus, much research has focused on the biological degradation of aromatics hydrocarbons (Ahs) through metabolism and co-metabolism. The degradation process involves enzyme machinery: dehalogenases, dehydrogenases, oxygenases and hydrolases system [9]. Bioremediation is a cost-effective and sustainable biotechnology for the treatment of contaminated coastal and marine sites [10]. 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 [8]. 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 [6,11,12,13,14,15].

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 [16]. Genetic analyses of PAH catabolic pathways in several PAH - degrading bacteria revealed the presence of a group of genes for complete degradation of aromatic compounds. PCR amplification using genes specific primers or degenerate primers has been used to detect and study the diversity of aromatic-dioxygenase genes in PAH degrading bacterial isolates [5]. Although many bacteria capable of degrading PAHs have already been isolated, it is still important to screen for more efficient and potent strains that can completely and rapidly decompose PAHs under the deplorable and unfriendly environmental conditions of Niger Delta. Thus, exploring the phylogenetic markers such as 16S rRNA genes to reveal microbial diversity and further exploration of gene expression to reveal the functional power of the microbes is essential. Therefore, the aim of the present study was to explore the phylogenetic framework of bacteria isolated from contaminated marine environments of Niger Delta and the expression of the metabolic genes coding for aromatic hydrocarbon degradation and surfactant production.

2. MATERIALS AND METHODS

2.1 Description of the Sampling Sites

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.

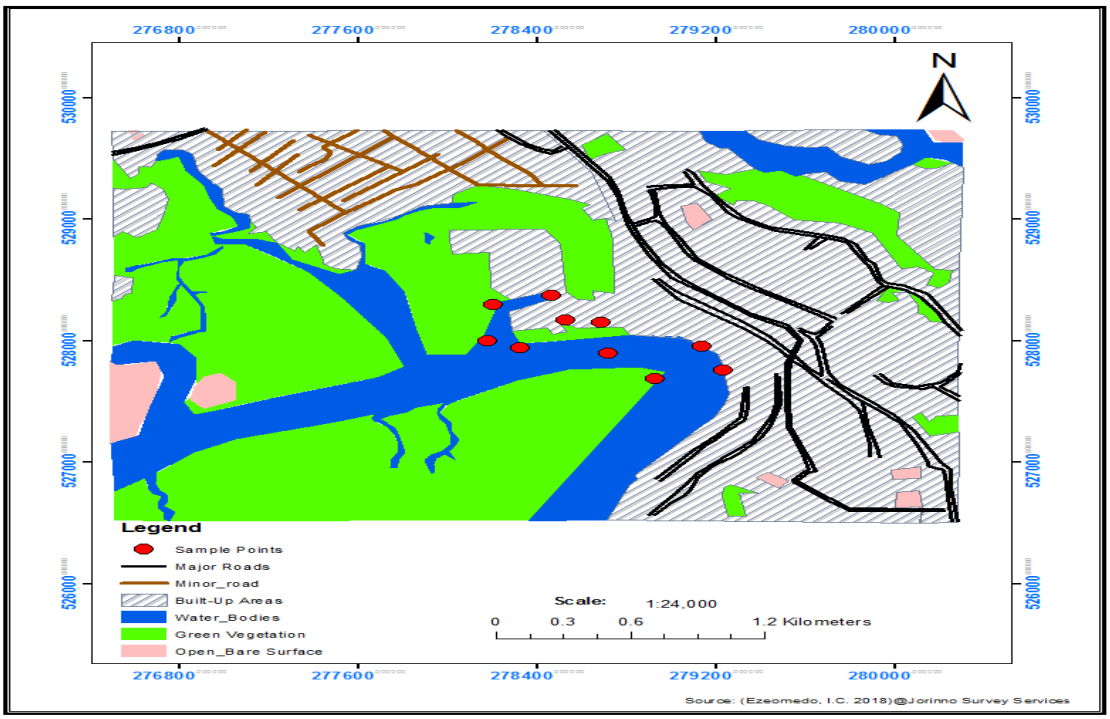

Fig. 1. Thematic map indicating the locations of sample points and its land cover in Abonema study area

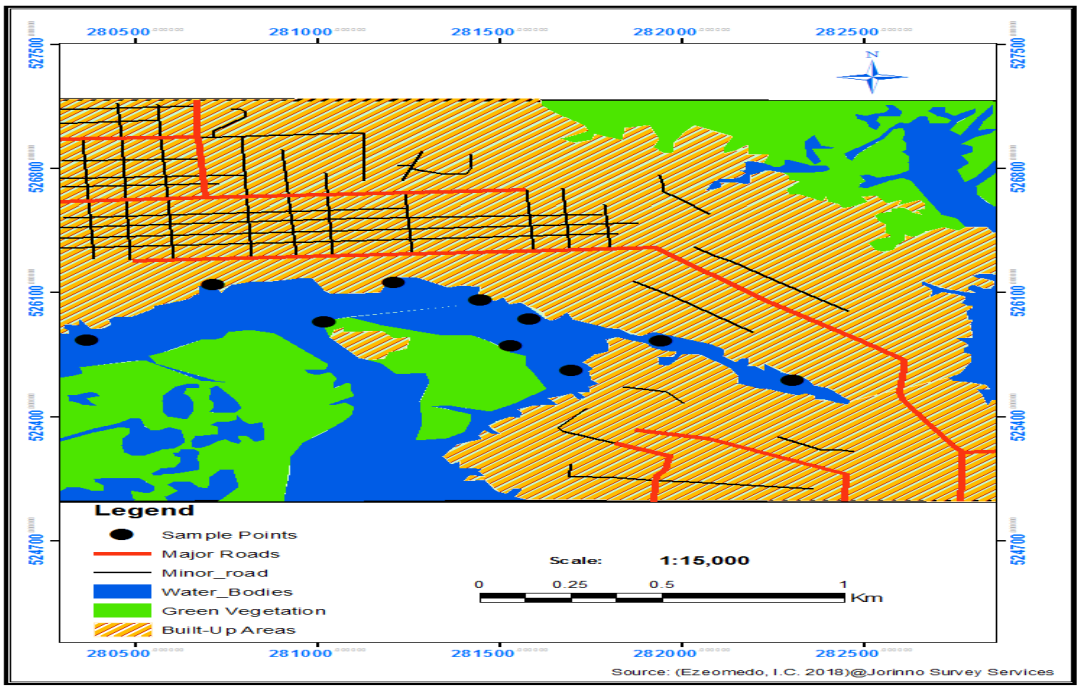

Fig.2. Thematic map indicating the locations of sample points and its land cover in Nembe study area

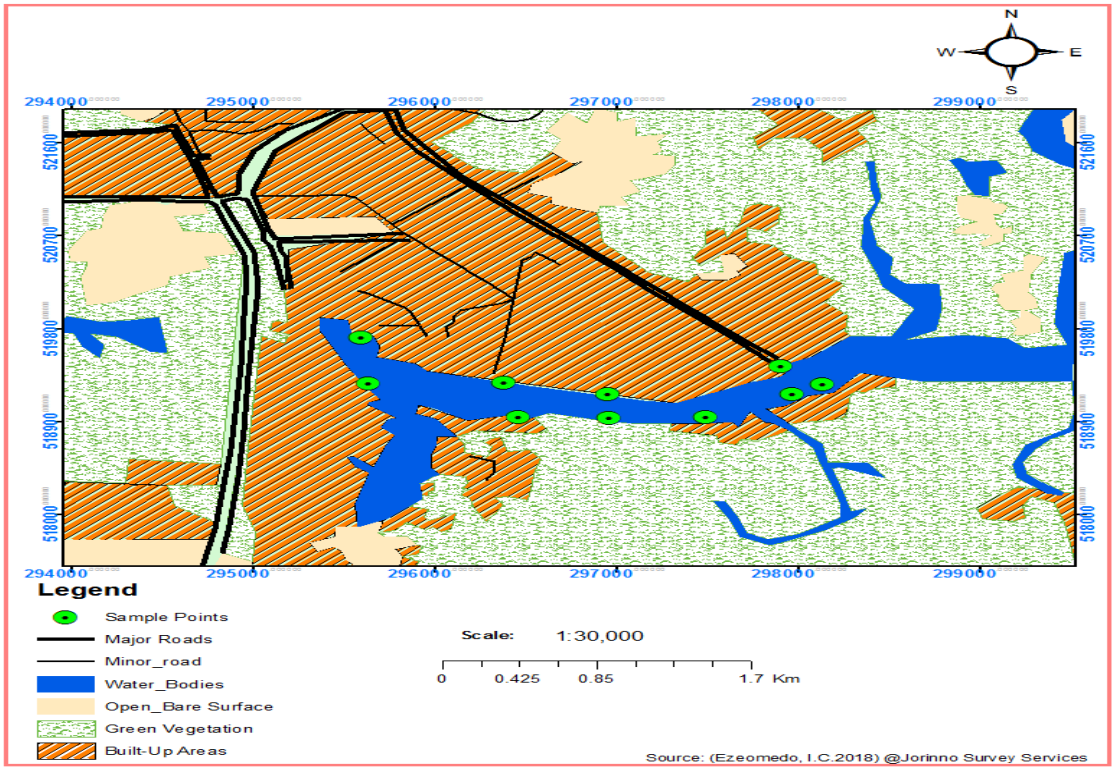

Fig. 3.Thematic map indicating the locations of sample points and its land cover in Onne study area

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 [17,18, 19].

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 $FeSO_4$,

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 Also, Nutrient agar plates without hydrocarbons were inoculated and incubated at 28.00 ± 0.20 °C for 24 – 48 hrs [19, 20, 21, 22].

2.4 Total Viable Count Technique

2.4.1 Enumeration of total heterotrophic bacteria (THB)

The total heterotrophic bacterial count was determined on nutrient agar using spread plate method. All the plates yielding 30 - 300 colonies were counted and the average number of colonies per plates were determined. The numbers of total heterotrophic bacteria were expressed at CFU/g and CFU/ml [21, 22, 23, 24, 25].

2.4.2 Enumeration of total culturable hydrocarbon - utilizing bacteria (TCHUB)

The total culturable hydrocarbon-utilizing bacteria were enumerated on the modified mineral basal agar using the spread plate method. All the plates yielding 30 - 300 colonies were counted and the average number of colonies per plates were determined. The numbers of total hydrocarbon utilizing bacteria were expressed at CFU/g and CFU/ml [21, 22, 24].

2.5 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 [19].

2.6 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 [19, 26, 27].

2. 7 Characterization and identification of selected hydrocarbon utilizing bacterial isolates

2.7.1 Molecular characteristics

2.7.1.1 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 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.

2.7.1.2 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 [28].

2.7.1.3 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), 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. 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].

3.7.1.4 Blasting and phylogenetic correctional analyses

The basic local alignment search tool (BLAST) of DNA sequences was performed by editing the sequences of the 16S 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 (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 [5, 14, 25, 28].

3.7.1.5 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.

2.8 Detection of Catabolic and Surfactant Genes by PCR Analyses

2.8.1 PCR primer design

Following the method of Swaathy *et al.* [14] as modified in this study, a portion of catechol gene 881 bp (C23O) was pulled out from the genomic DNA using F: 5′- ATG AGC AAC AAA TAC GAA TT- 3′ and R: 5′- TCA AAC GGT CAA TCT GAT AT- 3′ primers with 1.5 U of Taq DNA polymerase in a 25 ml reaction mixture, consisting of 100 ng of genomic DNA, 20 pmol of each primer, 200 µM dNTPs and 1X Taq buffer with 1.5 mM $MgCl_2$. PCR was conducted using the following temperature profile: initial denaturation at 93 °C for 2 minutes, then 30 cycles of 1 minutes at 93 °C, 35 seconds at 45 °C and 1.5 minutes at 72 °C;

and finally an extension reaction of 5 minutes at 72 °C. Likewise, according to the method of Qazi *et al*. [29] and Swaathy *et al*. [4] as modified in this study, the primer pair of microsurf gene *srfA3/licA3F*: CAAAAKCGCAKCATATGAG and *srfA3/licA3R*: AGCGGCAYATATTGATGCGGYTC was designed to amplify a 881 bp portion of the *srfA3* or the homologous *licA3* gene present in surfactin/lichenysin and gene-specific primers of rhamnolipid gene (kpd - F 5'-GCCCACGACCAGTTCGAC-3' and kpd - R 5' CATCCCCCTCCCTATGAC-3') with 2.5 µl of Taq DNA polymerase in a 25 ml reaction mixture, consisting of 100 ng of genomic DNA, 20 pmol of each primer, 200 µM dNTPs and 1X Taq buffer with 2 mM $MgCl_2$. PCR was conducted using the following temperature profile: initial denaturation at 93 °C for 2 min., then 38 cycles of 35 seconds at 93 °C, 35 seconds at 48 °C, and 45 seconds at 72 °C; and finally an extension reaction of 5 minutes at 72 °C.

2.8.2 Gel electrophoresis

The PCR products were 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 [28]

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 if the probability is less than 0.05 (P = .05) [25, 26].

3. RESULTS

3.1 Total Viable Count

The result of the mean total heterotrophic bacterial (THB) count of sediment and water samples from the three sampled locations is presented on Table 1. From the result, Nembe water had the highest significant mean THB count of log 18.95 ± 0.04 CFU / mL with highest percentage of occurrence (32.40 ± 0.16 %) while Onne sediment had the lowest significant mean THB count of log 05.34 ± 0.02 CFU / g with lowest percentage of occurrence (02.90 ± 0.06 %) respectively. Similarly, the result of the mean total hydrocarbon utilizing bacterial (THUB) count sediment and water samples from the three sampled locations is presented

on Table 2. From the result, Abonema sediment with xylene and Abonema water with xylene had the highest significant mean THUB count of log 30.20 ± 0.12 CFU / g with highest percentage of occurrence (17.30 ± 0.12 %) and the lowest significant mean THUB count of log 04.35 ± 0.04 cfu/ml with lowest percentage of occurrence (02.50 ± 0.04 %) respectively.

3.2 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 3. 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 4. 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 5. 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.

3.3 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 1. 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 Gen bank accession numbers of 16S rRNA sequences of the closest relative for the aromatic degrading bacterial isolates is presented in Table 6. 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 neighbor-joining phylogenetic relationship among the 16S rRNA sequence of the aromatic degrading bacterial isolates constructed by MEGA 7.0 is shown in Figure 4. 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.

3.4 Detection of Catabolic and Surfactant Genes

The result of the catabolic and surfactant genes detected in some of the marine aromatic degrading bacterial isolates using specific primers for each gene is presented in Table 7 while the result of the PCR – amplification of primers specific for catabolic gene (*C23O*) and surfactant genes (*rhlB, SrfA3/LicA3*) of *Providencia vermicola* strain ANT1, *Alcaligenes faecalis* strain XYL2, *Serratia marcescens* strain XYL7 and *Providencia* sp. strain XYL8 is shown in Plate 2. From the results, catabolic gene (*C23O*) was detected in only four (4) out of the nine marine aromatic degrading bacteria with name stated above while surfactant genes (*rhlB, SrfA3/LicA3*) were detected only in three (3) out of the nine marine aromatic degrading bacteria all of which had 881 base pairs sizes of PCR products of the catabolic and surfactant genes visualized by UV fluorescence under agarose gel electrophoresis respectively

4. DISCUSSION

It has been established by many studies that bioremediation i.e. the exploitation of microorganisms for detoxifications of heavy metal ions, aromatic hydrocarbons, petroleum products, pesticides and other toxic organic molecules is the method of choice owing to fewer secondary hazards and generally low cost. Studies showed that hydrocarbon degrading bacteria are ubiquitously distributed in soil and aquatic environments. However, their populations constitute less than 1 % of total microbial communities. Many of the microorganisms proposed for biodegradation and bioremediation have been isolated from contaminated soils and waters [30]. In this study, an attempt was made to explore the diversity, catabolic and surfactant genes of marine hydrocarbon utilizing bacteria from our three study areas and the result in Tables 1 and 2 showed that Nembe water had the highest significant (P =.05) mean THB count of log 18.95 ± 0.04 CFU / mL with highest percentage of occurrence (32.40 ± 0.16 %) while Onne sediment had the lowest significant mean THB count of log 05.34 ± 0.02 CFU / g with lowest percentage of occurrence (02.90 ± 0.06 %)

respectively revealing that surface water harbours more heterotrophic bacteria which could possibly be due to the more nutrient and oxygen levels in surface water than the sediment samples. The result in Table 2 showed that Abonema sediment with xylene and Abonema water with xylene had the highest significant (*P* =.05) mean THUB count of log 30.20 ± 0.12 CFU / g with highest percentage of occurrence (17.30 ± 0.12 %) and the lowest significant mean THUB count of log 04.35 ± 0.04 CFU / ml with lowest percentage of occurrence (02.50 ± 0.04 %) respectively revealing that sediment surface harbours more hydrocarbon utilizing bacteria than surface water possibly due to the high accumulative nature of sediment to aromatic compounds than water. Aromatic compounds tend to have low solubility to water hence making them adsorbed more to sediment than surface water. Comparatively, there was significant abundance of THB more than (HUB) and more xylene degraders than anthracene and pyrene degraders in the both samples respectively. The result uphold the findings of Chikere *et al.* [21] who reported that the waterways are continuously exposed to petroleum hydrocarbons owing to navigational activities and this may have enriched the sediment with hydrocarbon utilizing bacteria. However, the paucity of the HUB counts may be attributed to the inadequacy of nutrients at that depth especially nitrogen and phosphorus which deplete with input of hydrocarbons. Another factor that reduces available metabolic nutrients in marine environment according to Xu *et al.* [31], is heavy leaching caused by tidal inundation and wave action. The occurrence of hydrocarbon utilizers among the heterotrophic population in the samples is an indication of previous contamination due to hydrocarbon pollution [22, 32].

Following isolation, 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 3, 4 and 5). This study agrees with the explanation of Mao *et al*. [33] 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 [34], 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*. [22] 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 [35]. The result in Plate 1 confirmed that the PCR products obtained have the molecular weight of 1 kbp visualized by UV fluorescence under agarose gel electrophoresis. Akinbankole *et al.* [15], obtained in their research 1,500 bp PCR product from anthracene and pyrene isolates isolated from oil contaminated water and soil in Malaysia. Yuliani *et al.* [27], obtained in their research 1,489 bp PCR product from phenanthrene and pyrene isolates isolated from marine area of Indonesia. Isiodu *et al.* [25], 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. 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*. [15], 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 Figure 4 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 thousand and sixteen (01/12/16). They belong to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus*; and families of: Enterobacteriaceae, Alcaligenaceae, 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 [11, 12, 14, 15, 19, 21, 25, 27, 30, 36, 37, 38, 39].

The biotechnological potential of the strains inhabiting oil - polluted ecosystems does not rely exclusively on their ability to degrade a certain hydrocarbon mixture but it includes additional features. Different microorganisms were reported to possess multiple adaptations to facilitate oil degradation procedures, such as the synthesis of biosurfactants or emulsifiers and biofilm formation, processes that enhance the bacterial adhesion to hydrocarbons, increasing their solubility and thus promoting their degradation [11]. Degradation of aliphatic and PAHs was mostly carried out by the mono - and dioxygenases produced by bacteria [5]). Inorder to widen the characterization of the aromatic hydrocarbon - degrading bacteria isolated from the Rivers marine environment, PCR assays were performed to explore for functional genes coding for the catechol 2, 3 - dioxygenase enzyme (*C23O*) and genes related to surfactant production namely rhamnolipid enzyme (*rhlB*) and surfactin/lichenysin enzyme (*SrfA3/LicA3*) and the results are presented and shown in Table 7 and Plate 2. From the results, catabolic gene (*C23O*) was detected in only four (4) out of the nine marine aromatic degrading bacteria with name stated above while surfactant genes (*rhlB, SrfA3/LicA3*) were detected only in three (3) out of the nine marine aromatic degrading bacteria all of which had 881 base pairs sizes of PCR products of the catabolic and surfactant genes visualized by UV fluorescence under agarose gel electrophoresis respectively. Although biosurfactants help to solubilize or mediate the interaction between the organisms and the aromatic compounds, the catabolic reactions observed in the present study have been executed by the dioxygenase genes as observed from the amplified product of 881 bps. The detection of *C23O* gene in some of the Gram negative bacteria and not in the Gram positive bacteria indicates these isolates produce dioxygenase for the oxidation of aromatic hydrocarbons (xylene, anthracene and pyrene) and a possible reason may be because these detected genes are highly conserved among different Gram - negative bacteria and support the research work of Hesham *et al*. [5], who confirmed the presence of both monooxygenase and dioxygenase in *S. koreensis* strain ASU - 06. *C12O* and *C23O* dioxygenases play a key role in the metabolism of aromatic rings by the bacteria because they are responsible for cleavage aromatic C – C bond at ortho or meta positions. This gene *C23O* was identified by Swaathy *et al*. [14], as an important gene responsible for catabolizing low molecular weight as well as high molecular weight PAHs. Moreso, the detection of rhamnolipid (*rhlB*) and surfactin/lichenysin (*SrfA3/LicA3*) genes in these isolates support the constant debate among the scientists about the prevalence of rhamnolipid and surfactin/lichenysin genes in bacteria other than *Pseudomonas* and *Bacillus* species and some novel strains of bacteria having unique ability to produce rhamnolipids have recently been reported. Rhamnolipid production in few pathogens including *Burkholderia mallei* and *B. pseudomallei*

and the non - pathogenic *B. thailandensis,* has also been reported. This observation contradicts the findings of Swaathy *et al.* [4, 14], who found out that the expression of *srf* and *licA3* genes were only realized in all of the five *Bacillus* species and marine *Bacillus licheniformis* MTCC 5514. On the other hand, those isolates that could grow on the aromatic hydrocarbons but failed to give positive amplification, may probably be due to mismatches between the tested primers and gene sequences [40] and may contain other catabolic and surfactant aside the tested genes.

Table 1. Mean total heterotrophic bacterial (THB) count of sediment and water samples from the three sampled locations

Samples	Log CFU/g/ml	Percentage (%)
Abonema water	13.60 ± 0.02	20.80 ± 0.16
Abonema sediment	12.70 ± 0.02	18.90± 0.20
Nembe water	18.95 ± 0.04	32.40 ± 0.16
Nembe sediment	11.50 ± 0.04	16.30 ± 0.16
Onne water	08.00 ± 0.16	08.70 ± 0.20
Onne sediment	05.34 ± 0.02	02.90 ± 0.06

The values are mean ± standard deviation of triplicate determination.

Table 2. Mean total hydrocarbon utilizing bacterial (THUB) count of sediment and water samples from the three sampled locations

Sample	Log CFU/g/ml	Percentage (%)

Abonema water + xylene	04.35 ± 0.04	02.50 ± 0.04
Abonema sediment + xylene	30.20 ± 0.12	17.30 ± 0.12
Abonema water + anthracene	09.20 ± 0.08	05.30 ± 0.08
Abonema sediment + anthracene	08.05 ± 0.01	04.60 ± 0.02
Abonema water + pyrene	04.42 ± 0.04	02.50 ± 0.04
Abonema sediment + pyrene	09.45 ± 0.01	05.40 ± 0.02
Nembe water + xylene	12.35 ± 0.02	07.10 ± 0.06
Nembe sediment + xylene	09.50 ± 0.01	05.50 ± 0.01
Nembe water + anthracene	10.50 ± 0.01	06.00 ± 0.12
Nembe sediment + anthracene	06.09 ± 0.03	03.50 ± 0.02
Nembe water + pyrene	15.35 ± 0.15	08.80 ± 0.02
Nembe sediment + pyrene	08.25 ± 0.02	04.70 ± 0.02
Onne water + xylene	07.60 ± 0.01	04.40 ± 0.02
Onne sediment + xylene	07.70 ± 0.08	04.40 ± 0.08
Onne water + anthracene	05.20 ± 0.02	03.00 ± 0.06
Onne sediments + anthracene	08.50 ± 0.02	04.90 ± 0.01
Onne water + pyrene	10.50 ± 0.02	06.00 ± 0.02
Onne sediment + pyrene	06.95 ± 0.12	04.00 ± 0.12

The values are mean ± standard deviation of triplicate determination.

Table 3. 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 4. 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	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 5. 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.*

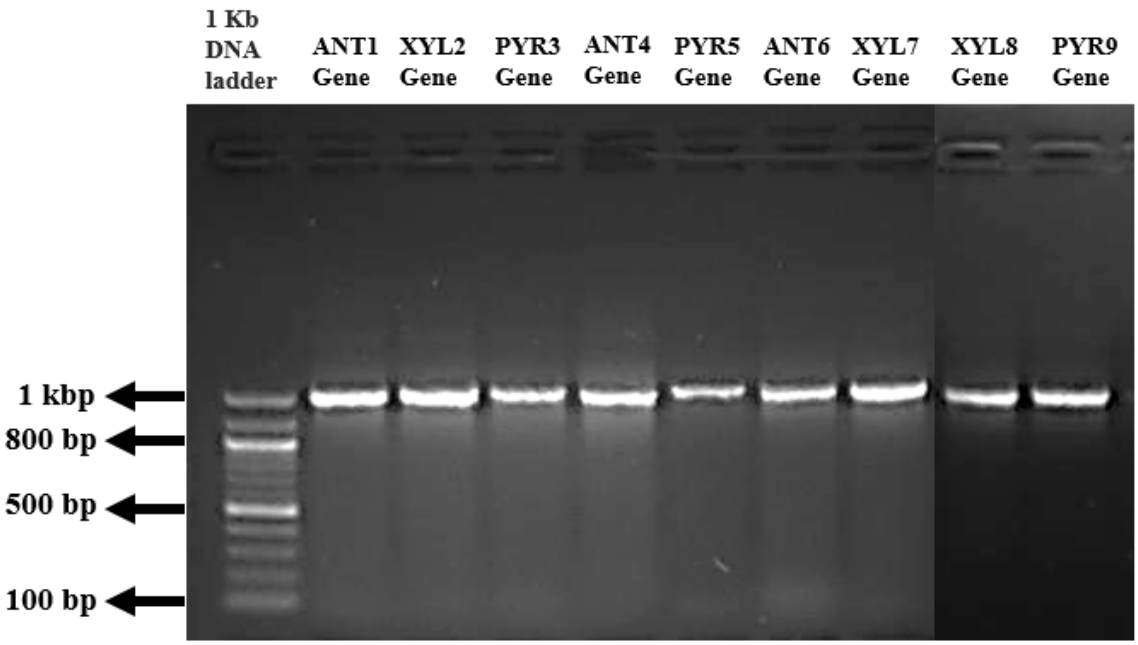

Plate 1. PCR - amplification of 16S rRNA genes of the aromatic degrading bacteria genomic DNA

Table 6. Percentage similarity and Gen Bank accession numbers of 16S rRNA sequences of the closest relative for the aromatic degrading bacterial isolates

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

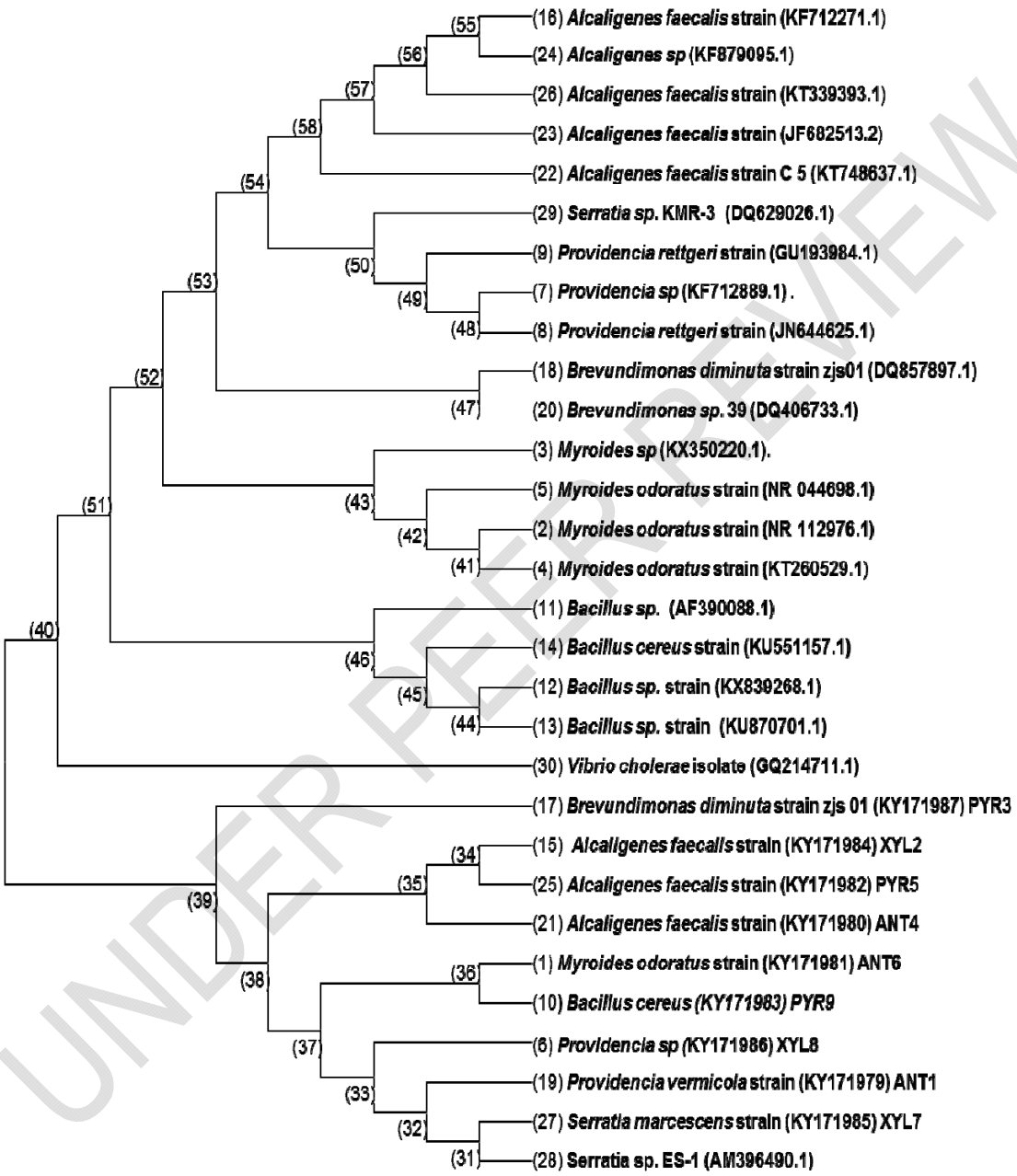

Fig. 4. Neighbor-joining phylogenetic relationship among the 16S rRNA sequence of the aromatic degrading bacterial isolates constructed by MEGA 7.0.

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 parentheses

Isolate code	Bacterial name	Expected band (bp)	*C23O*	*rhlB*	*SrfA3/LicA3*
ANT1	*Providencia vermicola*	881 bp	+	-	-
XYL2	*Alcaligenes faecalis*	881 bp	+	+	-
PYR3	*Brevundimonas diminuta*	-	-	-	-
ANT4	*Alcaligenes faecalis*	-	-	-	-
PYR5	*Alcaligenes faecalis*	-	-	-	-
ANT6	*Myroides odoratus*	-	-	-	-
XYL7	*Serratia marcescens*	881 bp	+	+	+
XYL8	*Providencia* sp.	881 bp	+	+	+
PYR9	*Bacillus cereus*	-	-	-	-

Table 7. Catabolic and surfactant genes detected in some of the marine aromatic degrading bacterial isolates using specific primers for each gene

+ sign indicates the PCR product was detected and – sign means PCR product was not detected.

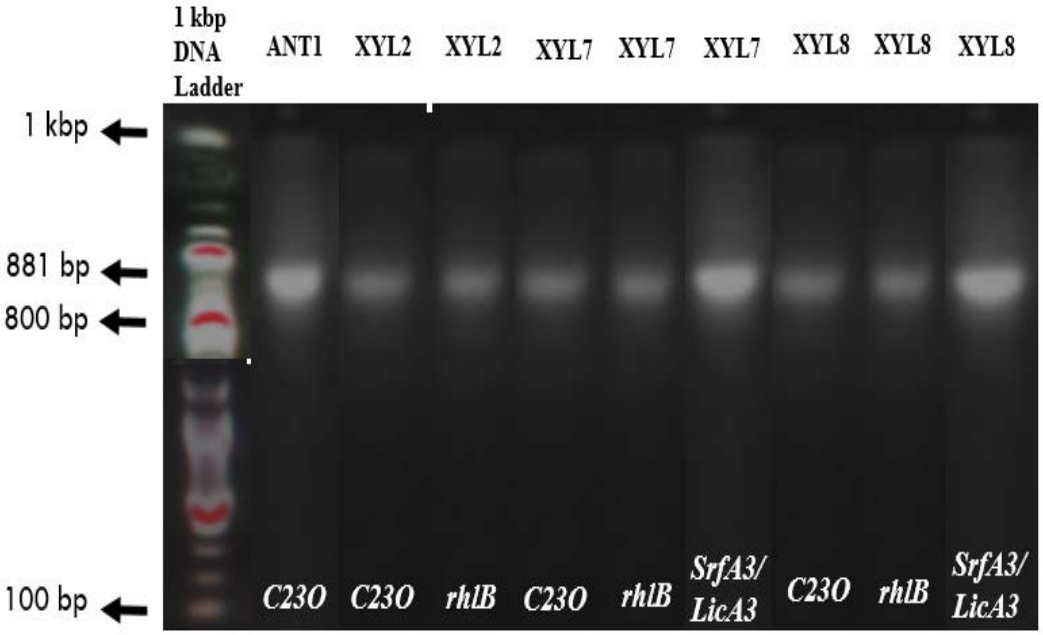

Plate 2. PCR – amplification of primers specific for catabolic gene (*C23O*) and surfactant genes (*rhlB, SrfA3/LicA3*) of *Providencia vermicola* strain ANT1, *Alcaligenes faecalis* strain XYL2, *Serratia marcescens* strain XYL7 and *Providencia* sp. strain XYL8

5. CONCLUSION

The whole study revealed that the three sampling sites harbour a lot of phylogenetically related aromatic hydrocarbon degrading bacterial strains belonging to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus* which are able to utilize xylene, anthracene and pyrene as source of carbon and energy. It also revealed that some of the isolated bacteria especially *Serratia marcescens* XYL7 possess functional genes coding for aromatic degradation and surfactant production. Our results suggest that these bacteria could played an important role in degradation of simple, low and high molecular weight aromatic hydrocarbons and could be recommended for *in situ* aromatic hydrocarbon bioremediation in the Niger Delta environment.

REFERENCES

1. Nwilo PC, Badejo OT. Oil spill problems and the management in the Niger Delta. International Oil Spill conference, Miami, Florida, USA. 2005;20-2.
2. Balba MT, Al-Awadi R, Al-Daher R. Bioremediation of oil contaminated soil: Microbiological methods for feasibility and field evaluation. J. Microbiol. Meth.1998;32:155-64.

3. Rajaei S, Seyedi SM, Raiesi F, Shiran B, Raheb J. Characterization and potentials of oil-degrading bacteria inhabiting the rhizosphere of Wild Oat (*Avena Fatua* L.) in South West of Iran. Iran J Biotech. 2013;11(1):32- 40.
4. Swaathy S, Kavitha V, Pravin AS, Sekaran G, Mandal AB, Gnanamani A. (2014a). Phylogenetic framework and biosurfactant gene expression analysis of marine *Bacillus* spp. of Eastern Coastal Plain of Tamil Nadu. Internat J Bact. 2014a;2014: 860491.
5. Hesham AE, Mawad AMM, Mostafa YM, Shoreit A. Biodegradation ability and catabolic genes of petroleum-degrading *Sphingomonas koreensis* strain ASU-06 isolated from Egyptian oily soil. BioMed Res Internat. 2014;2014:127674.
6. Babita K, Singh SN, Deeba F, Sharma M, Pandey V, Singh DP. Elucidation of pyrene degradation pathway in bacteria. Adv Biores. 2013;4(2):151-60.
7. Sutiknowati LI. Hydrocarbon degrading bacteria: Isolation and identification. Mak Sains. 2007;11(2):98-03.
8. Dasgupta D, Ghosh R, and Sengupta TK. Biofilm-mediated enhanced crude oil degradation by newly isolated *Pseudomonas* species. ISRN Biotech. 2013;2013(250749):1-13.
9. Nakamura FM, Germano MG, Tsai SM. Capacity of aromatic compound degradation by bacteria from Amazon Dark Earth. Diver. 2014;6:339-53.
10. Paniagua-Michel J, Rosales A. Marine bioremediation: A sustainable biotechnology of petroleum hydrocarbons biodegradation in coastal and marine environments. J Biorem Biodeg. 2015;6:273.
11. Amer RA, Mapelli F, El-Gendi HM, Barbato M, Goda DA, Corsini A, Cavalca L, Fusi M, Borin S, Daffonchio D, Abdel-Fattah YR. Bacterial diversity and bioremediation potential of the highly contaminated marine sediments at El-Max District (Egypt, Mediterranean Sea). Biomed Res Internat 2015;2015:981-9.
12. Fagbemi OK, Sanusi A. I. Chromosomal and plasmid mediated degradation of crude oil by *Bacillus coagulans, Citrobacter koseri* and *Serratia ficaria* isolated from the soil. Afr J Biotech. 2017;16(21):1242-53.
13. Lily MK, Bahuguna A, Bhatt KK, Dangwal K. Degradation of anthracene by a novel strain *Brachybacterium paraconglomeratum* BMIT637C (MTCC 9445). Internat J Environ Sci. 2013;3(4):1242-52.
14. Swaathy S, Kavitha V, Pravin AS, Mandal, AB, Gnanamani A. Microbial surfactant mediated degradation of anthracene in aqueous phase by marine *Bacillus licheniformis* MTCC 5514. Biotech Rep. 2014b;4:161-70.
15. Akinbankole AS, Tunung R, Tennant AM. Biochemical and molecular characterization of pyrene and anthracene metabolizing bacteria isolated from oil contaminated water and soil in Malaysia. J Appl Environ Microbiol. 2015;3(1):25-30.
16. Ma J, Xu L, Jia L. Degradation of polycyclic aromatic hydrocarbons by Pseudomonas sp. JM2 isolated from active sewage sludge of chemical plant. J Environ Sci. 2012; 24(12): 2141-8.
17. Abu GO, Chikere BO. Cell surface properties of hydrocarbon utilizing bacterial isolates from the Port Harcourt marine environment. Nig J Microbiol. 2006;20(1):809-16.
18. Gorleku MA, Carboo D, Palm LMN, Quasie WJ, Armah AK. Polycyclic aromatic hydrocarbons (PAHs) pollution in marine waters and sediments at the Tema Harbour, Ghana. Acad J Environ Sci. 2014;2(7):108-15.
19. John RC, Essien JP, Akpan SB, Okpokwasili GC. Polycyclic aromatic hydrocarbon degrading bacteria from aviation fuel spill site at Ibeno, Nigeria. Bull Environ Contam Toxicol. 2012;88:1014-9.
20. John RC, Okpokwasili GC. Crude oil-degradation and plasmid profile of nitrifying bacteria isolated from oil impacted mangrove sediment in the Niger Delta of Nigeria. Bull Environ Contam Toxicol. 2012;88:1020-6.
21. Chikere CB, Okpokwasili GC, Ichiakor O. Characterization of hydrocarbon utilizing bacteria in tropical marine sediments. Afr J Biotech. 2009;8(11):2541-44.
22. Esedafe WK, Fagade OE, Umaru FF, Akinwotu O. Bacterial degradation of the polycyclic aromatic hydrocarbon (PAH) -fraction of refinery effluent. Internat J Environ Biorem Biodeg. 2015;3(1):23-7.
23. Bahig AE, Aly EA, Khaled AA, and Amel KA. Isolation, characterization and application of bacterial population from agricultural soil at Sohag Province, Egypt. Mal J Microbiol. 2008;4(2):42-50.
24. Kafilzadeh F, Pour FH, Tahery Y, Azad HN. Bioremediation of pyrene by isolated bacterial strains from the soil of the landfills in Shiraz (Iran). Ann Biol Res. 2012;3(1):486-94.
25. Isiodu GG, Stanley HO, Ezebuiro V, Okerentugba PO. Role of plasmid-borne genes in the biodegradation of polycyclic aromatic hydrocarbons (PAHs) by consortium of aerobic heterotrophic bacteria. J Petr Environ Biotech. 2016;7(1):264.
26. Kafilzadeh F, Pour FH.Degradation of naphthalene, phenanthrene and pyrene by *Pseudomonas* sp. and *Corynebacterium* sp. in the landfills. Internat J Biosci. 2012;2(9):77-84.
27. Yuliani H, Sahlan M, Hermansyah H, Wijanarko A. Selection and identification of polyaromatic hydrocarbon degrading bacteria. World Appl Sci J. 2012;20(8):1133-8.

28. Ubani O, Atagana HI, Thantsha MS, Rasheed A. Identification and characterisation of oil sludge degrading bacteria isolated from compost. Arch Environ Prot. 2016;42(2):67-77.
29. Qazi MA, Malik ZA, Qureshi GD, Hameed A, Ahmed S. Yeast extract as the most preferable substrate for optimized biosurfactant production by *rhl*B gene positive *Pseudomonas putida* SOL-10 isolate. J Biorem Biodeg. 2013;4(7):204.
30. Athar MA, Akbar A, Khan YH, Ali I, Mehmood U, Sabri AN, Hasnain S. Characterization of hydrocarbon degrading bacteria isolated from hydrocarbon contaminated soil. J Pure Appl Microbiol. 2014;8(6):1-9.
31. Xu R, Lau NLA, Ng KL, Obbard JP Application of a slow-release fertilizer for oil bioremediation in beach sediments. J Environ Qual. 2004;33:1210-6.
32. Al-Thani RF, Abd-El-Haleem DAM, Al-Shammri M. Isolation and characterization of polyaromatic hydrocarbons-degrading bacteria from different Qatari soils. Afr. J. Microbiol. Res. 2009;3(11):761-6.
33. Mao J, Luo Y, Teng Y, Li Z. Bioremediation of polycyclic aromatic hydrocarbon-contaminated soil by a bacterial consortium and associated microbial community changes. Internat Biodet Biodeg. 2012;70:141-7.
34. Pathak H, Bhatnagar K. *Alcaligenes*-the 4T engine oil degrader. J Biorem and Biodeg. 2011;2(4):2-5.
35. Srujana K, Khan BA. Isolation and characterization of polycyclic aromatic hydrocarbon degrading soil microbes from automobile workshop sediments. J Environ SciTech. 2012;5(1):74-83.
36. Ichor T, Okerentugba PO, Okpokwasili GC. Biodegradation of total petroleum hydrocarbon by aerobic heterotrophic bacteria isolated from crude oil contaminated brackish waters of Bodo Creek. J Biorem Biodeg 2014;5(5):236-41.
37. Irshaid FI, Jacob JH. Screening and characterization of aerobic xylene-degrading bacteria from gasoline contaminated soil sites around gas stations in Northern Jordan. J Biol Sci. 2015;15(4):167-76.
38. Louvado A, Gomes NCM, Simões MMQ, Almeida A, Cleary DFR, Cunha A. Polycyclic aromatic hydrocarbons in deep sea sediments: microbe–pollutant interactions in a remote environment. Sci Tot Environ. 2015;526:312-28.
39. Patowary K, Saikia RR, Kalita MC, Deka S. Degradation of polyaromatic hydrocarbons employing biosurfactant-producing *Bacillus pumilus* KSs2. Ann Microbiol. 2015;65:225-34.
40. Ding GC, Heuer H, Zühlke S, Spiteller M, Pronk GJ, Heister K et al. Soil type-dependent responses to phenanthrene as revealed by determining the diversity and abundance of polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenase genes by using a novel PCR detection system. Appl Environ Microbiol. 2010;76 (14):4765-71.