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: Department of Environmental Sciences, 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, biodegradation, biodiversity, catabolic genes, marine bacteria, Niger Delta, surfactant genes.

1. INTRODUCTION

The Niger Delta region is the centre of petroleum exploration, production and development accomplishments in Nigeria. Several cases of oil spills have been reported thereby promoting and increasing the pollution of the Niger Delta's aquatic and terrestrial environments. These spills have been associated with disruption, deterioration of pipes, negligence, mishaps during oil exploitation, production,

rupture of storage tanks and accidents brought about by transportation [1]. Most of these compounds especially the aromatic hydrocarbons are considered as toxicants and are classified as priority environmental pollutants by the US Environmental Protection Agency [2].

Biodiversity is the result of evolution that occur billions of years ago leading to multiplicity of species of living organisms on the earth. Molecular phylogenetic studies have shown that the chief diversity of life is in the microbial world and it is circulated amongst the three main domains: eubacteria, eukarya, and archaea. The methods of single cell sequencing, metatranscriptomics, metaproteomics and metagenomics are used not only to reveal the community structure (species taxanomy, abundance and distribution) but also of the useful and biological niche of a community [3]. Most of biotechnological products such as biosurfactants, antibiotics, compatible solutes, exoplolysacccharides, enzymes etcetera with enormous medical, environmental and industrial uses are obtained from microbes of marine ecosystems or sources. This has attracted significant attention and interest on the marine bacteria as they produces novel metabolites of diverse applications than the terrestrial and other sources. Several reports have implicated marine bacteria in the production of metabolites such as surfactant that have purposeful and useful roles in bioremediation especially in the aspect of enhanced oil recovery [4].

As polluted sites are contaminated by a mixture of PAHs and other environmental contaminants [4], considerable investigation has concentrated on the bioremediation of aromatics hydrocarbons (Ahs) through metabolism and co-metabolism. The mechanism of degradation process chiefly involves enzyme machinery: dehalogenases, dehydrogenases, oxygenases and hydrolases system [5]. Thus, for an effectual remediation process to exist, it is imperative that the bacteria involved should have a comprehensive degradation pathway so that no possible noxious metabolites accrue [6]. Researches have shown that a cluster of genes for whole degradation of aromatic compounds are found in several PAH - degrading bacteria through genetic analyses of PAH catabolic pathways. The diversity of aromatic-dioxygenase genes in several PAH degrading bacterial isolates have been studied through PCR amplification using genes specific primers or degenerate primers to revealed them [4]. Even though numerous bacteria capable of degrading PAHs have already been isolated and documented, it is still important to search, monitor and select more efficient and potent strains especially of the marine origin that can absolutely and rapidly mineralize PAHs under the deplorable and unfriendly environmental conditions of Niger Delta. Thus,

exploring the phylogenetic markers such as 16S rRNA genes to disclose the microbial diversity and further investigation of gene expression to unveil the metabolic power of the marine microbes in the Niger Delta 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 anthropological - induced stresses brought about by expansion, industrial development and rigorous transportation. Abonema Wharf Water Front community is a popular and busy commercial but dangerous jetty area close to Port Harcourt city inhabiting tens of thousands of different families living close to petroleum tank farms and tankers queue up daily to load refined petroleum products. Nembe Waterside is located adjacent to Creek Road Market, Port Harcourt, Nigeria. It shares boundary with Bayelsa and connects Port Harcourt city with Bonny Island where greater percentage of petroleum connections in Rivers State are located. Also, it connects the Island straight to the Atlantic Ocean where petroleum is shipped by gigantic oil vessels [7]. Onne Light Flow Terminal Seaport is a port of Nigeria and the leading petroleum and gas unrestricted zone in the world supportive of exploration and production for Nigerian accomplishments. It is located on the Bonny River

Estuary beside Ogu Creek and accounts for more than 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, Exxon Mobil Nigeria Limited, Socotherm Pipecoaters, Beker Hughes Oil Servicing Company, Aiteo Energy Resource, Sorelink Oil and Dozzy Oil and gas etcetera that generate the wastes that contaminate the sites above.

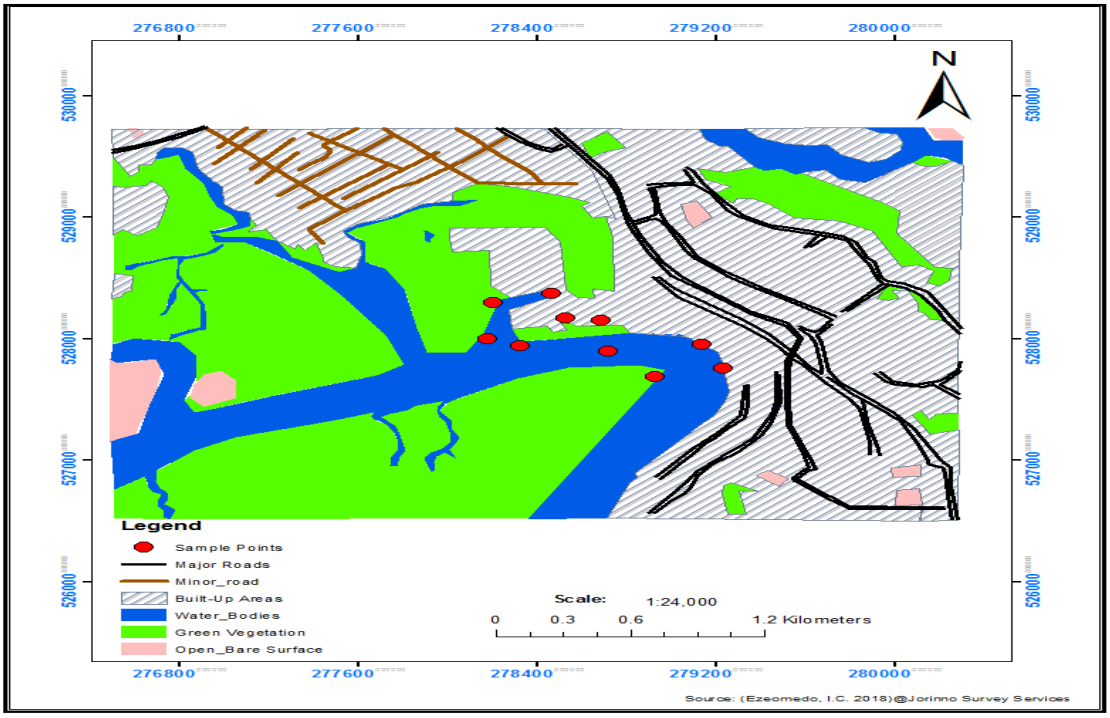

Fig. 1. Thematic map indicating the locations of sample points and its land cover in Abonema study area

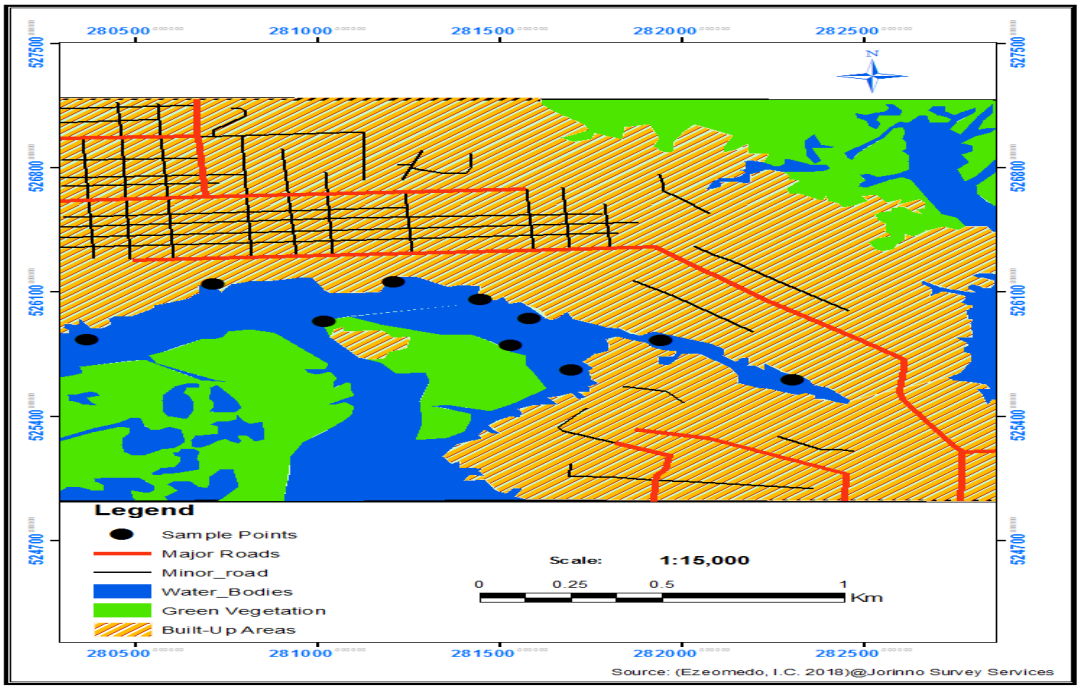

Fig.2. Thematic map indicating the locations of sample points and its land cover in Nembe study area

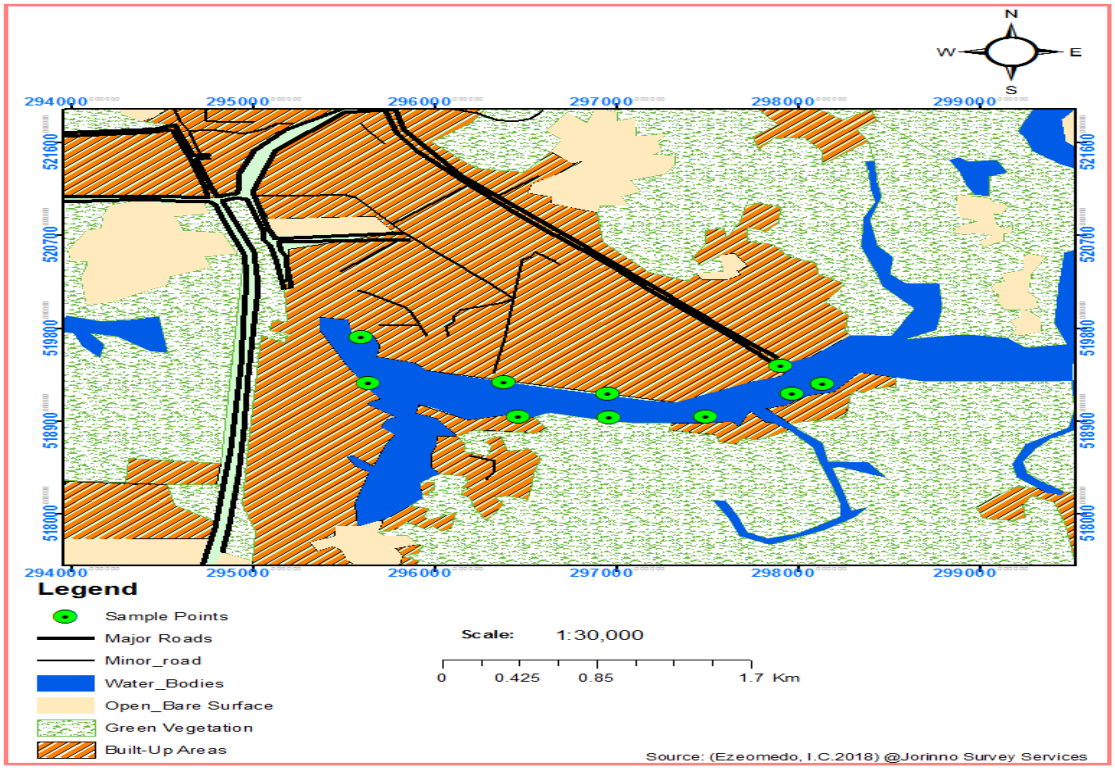

Fig. 3.Thematic map indicating the locations of sample points and its land cover in Onne study area

2.2 Collection and Processing of Sample

Ten samples each of the marine sediment and water were collected randomly per point of the designated ten (10) points of the three sampling sites (Figures 1, 2 and 3). The samplings were done once in each of the three sampling sites in September, 2015. The samples were mixed together after which a total of six composite /representative sediment and water samples were obtained and 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 Microbiology Laboratory, Chukwuemeka Odumegwu University, Uli Campus, Nigeria for microbiological analyses [8 - 10].

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

The hydrocarbon degraders were isolated from sediment and water samples of the three sampling sites using modified mineral basal agar (4 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$, 1.8 g KH_2PO_4, 0.1 g $FeSO_4$, 0.1 g NaCl, 0.2 g $CaCl_2$, 15 g Agar agar and distilled water 1,000 mL at pH 7.00 ± 0.20) enriched with xylene, anthracene and pyrene aromatic hydrocarbons. Sterilization of the medium was done by autoclaving at 121 $^{\circ}$C and 15 psi for 15 minutes. Thereafter, 0.2 mL acetone solution containing 0.1 % of the selected hydrocarbons (xylene, anthracene and pyrene) were aseptically pipetted and spread on the surface of the agar contained in Petri dish plates. The acetone was allowed to evaporate aseptically and 0.1 mL aliquots of the 10^{-3} dilutions were plated on the solidified media with a glass spreader. The spreader was sterilized after each successive spreading by dipping it in 70 % ethanol and then passing it through flame of a Bunsen burner. The inoculated plates were sealed using adhesive tape and foil to prevent contamination and photolysis and later placed in black polythene bags, and then incubated in the dark at 28.00 ± 0.20 °C for 14 days. Also, Nutrient agar plates without hydrocarbons were inoculated and incubated at 28.00 ± 0.20 °C for 24 – 48 hrs [7, 10 - 12].

2.4 Total Viable Count Technique

2.4.1 Determination of total non-hydrocarbon - utilizing bacteria (TNHUB)

The spread plate technique was used to determine the total non-hydrocarbon - utilizing bacteria on nutrient agar medium. All the plates yielding 30 - 300 colonies were counted and the average number of colonies per plates were determined. The number of total heterotrophic bacteria was expressed at CFU /g and CFU /mL [7, 12 - 15].

2.4.2 Determination of total viable hydrocarbon - utilizing bacteria (TVHUB)

Following the technique described above, total viable hydrocarbon - utilizing bacteria were determined on the modified mineral basal medium. All the plates yielding 30 - 300 colonies were counted, the average number of colonies per plates were determined and expressed at CFU /g and CFU /mL [7, 12, 14].

2.5 Purification and Maintenance of Cultures

Colonies that developed on hydrocarbon enriched plates was duplicated onto new hydrocarbon enriched plates and incubated at 28.00 ± 0.20 °C for 14 days. The newly developed colonies were carefully chosen as xylene, anthracene and pyrene degraders and finally sub - cultured on Bijou bottles where they are preserved at 4 °C in refrigerator [10].

2.6 Screening and Selection Test

In order to screen and select the best and strongest degrading strains, the bacterial isolates were grown using 5 mL of all the isolates in test tubes comprising 25 mL of the modified mineral basal medium supplemented with 100 ppm of xylene, anthracene and pyrene hydrocarbons as described sub-section 2.3 above. Thereafter, the set ups were incubated at 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, quantified at 600 nm using a UV - VIS spectrophotometer (Astell, UV - Vis Grating, 752 W) were selected as xylene, anthracene and pyrene degraders [10, 16, 17].

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

2.7.1 Molecular characteristics

2.7.1.1 Identification of bacteria

The Gram - reaction test was first used to verify the morphological characterization of the bacterial colonies prior to molecular identification [18].

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. The DNA was separated electrophoretically with 1 % agarose gel containing 0.1 µg /mL ethidium bromide stain. The 20 cm long gels were ran at 80 V /cm for 60 minutes in 100 mL of 1x tris acetate EDTA (TAE) electrophoresis buffer. The DNA was visualised by UV fluorescence to determine the success of the extraction process [18].

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 tube and the different DNA samples were added to each tubes. The negative control was used to check for contamination in the master mix. The PCR reagents in each tube amounted to 50 µL containing: buffer (5 µL), $MgCl_2$ (1.5 µL), primer 1 (forward 16S - P1 PCR 5′AGAGTTTGATCCTGGCTCAG3′) (2 µL), primer 2 (reverse 16S - P2 PCR 5′AAGGAGGTGATCCAGCCGCA3′) (2 µL), dNTP mix (1 µL), Dream Taq (0.25 µL), sterile sabax water (35.25 µL) and DNA samples (3 µL). The PCR reactions was performed using MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). The cycling conditions were set at (a) initial denaturation 10 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 The PCR products (genes) were separated at 1 kbp size and visualized as stated above (Figure 1) [18]. Then the PCR products (20 µL each) were cleaned up later using 160 µL of 13 % polyethylene glycol (PEG) 8000, 20 µL of 5 M NaCl solution and 200 µL of 70 % ethanol. The cleaned PCR products were sent for sequencing and was conducted using the automated DNA sequencer (Perkin-Elmer) in line with the sequencing kit protocol at the Forestry and Agricultural Biotechnology Institute (FABI) Sequencing Facility, University of Pretoria, South Africa [18].

3.7.1.4 Blasting and phylogenetic correctional analyses

The blasting of DNA sequences was performed by revising the sequences of the 16S rRNA genes obtained using BioEdit software, corrected sequences duplicated in a FASTA format form and completed in the National Centre for Biotechnology Information (NCBI) website. Homologies of the gene sequences were checked and compared with the sequences of the NCBI database and finally aligned using MAFFT software [19]. The taxonomic correctional studies were done using Mega 7 software and evolutionary distance of the isolates were calculated using neighbour - joining (NJ) methods [4, 15, 18, 19].

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 these strains have been submitted to the NCBI/Genbank database since 01/12/16 under the accession numbers from KY171979 - KY171987 [3, 4, 15, 19, 20, 21].

2.8 Detection of Catabolic and Surfactant Genes by PCR Analyses

2.8.1 PCR primer design

Following the method of Swaathy *et al.* [19] 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′ (Figure 3). Likewise, according to the method of Qazi *et al*. [21] and Swaathy *et al*. [3] 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') (Figure 3). They were subjected to the same PCR and gel electrophoretic conditions and protocols stated above [18].

2.9 Data Analysis

All values were expressed as mean ± S.D. and ordinary one-way analysis of variance (ANOVA) followed by post Tukey's, multiple comparison test was performed on the data obtained using Graph-Pad Prism version 7.00. The results were considered statistically significant at 95 % confidence intervals (P = .05) [15, 16].

3. RESULTS

3.1 Bacterial Enumeration

The result of the mean total non-hydrocarbon utilizing bacterial (TNHUB) 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 (*P* =.05) mean TNHUB 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 (*P* =.05) mean TNHUB 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 viable hydrocarbon utilizing bacterial (TVHUB) 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 (*P* =.05) mean TVHUB count of log 30.20 ± 0.12 CFU / g with highest percentage of occurrence (17.30 ± 0.12 %) and the lowest significant (*P* =.05) mean TVHUB count of log 04.35 ± 0.04 CFU /mL with lowest percentage of occurrence (02.50 ± 0.04 %) respectively.

3.2 Degradability 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 Characterization of Bacterial Isolates

The result of the PCR - amplification of 16S rRNA genes of the aromatic degrading bacteria genomic DNA is shown in Figure 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 2. From the result, it revealed that the nine bacterial isolates (KY171979, KY171984, KY171987, KY171980, KY171982, KY171981, KY171985, KY171986 and KY171983) arise from similar clusters and node establishing that they are correlationally connected to one another with common origin.

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 Figure 3. 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

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 surface water harbours more non –hydrocarbon utilizing 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 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 NHUB 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.* [7] who reported that the waterways are unceasingly open to petroleum hydrocarbons due to navigational actions and this possibly will have augmented the sediment with hydrocarbon utilizing bacteria. However, the insufficiency of the HUB counts could be ascribed to the lack of nutrients at that depth especially nitrogen and phosphorus which reduce with input of hydrocarbons. Additional factor that decreases existing metabolic nutrients in marine ecosystem according to Xu *et al.* [22], is substantial percolation initiated by tidal flood and wave action. The existence of hydrocarbon utilizers in the midst of the heterotrophic population in the samples is a sign of earlier contamination owing to hydrocarbon pollution [12, 23].

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 are indicated by absorbance values of each isolates (Tables 3, 4 and 5). The result is justified by the finding of Mao *et al*. [24], who reported that the enrichment and isolation of native PAH degraders is the most essential feature of microbial degradation of PAH as the native PAH degraders possess the adaptive traits to these hydrocarbons. Pathak and Bhatnagar [25], reported that enrichment culturing is very imperative towards the attainment of hydrocarbon bioremediation as it leads to selection of microbial degraders that are better adapted to hydrocarbons. Esedafe *et al*. [12] reported that an occurrence of 3/41 representing 7.32 % isolates from refinery effluent were efficient in degrading the polyaromatic hydrocarbons (PAHs).

The result in Figure 1 confirmed that the PCR products of the genomic DNA of the isolated bacteria had the molecular weight of 1 kbp. Akinbankole *et al.* [26], obtained 1,500 bp PCR product from anthracene and pyrene degrading bacteria isolated from petroleum polluted water and soil in Malaysia in their research finding. Yuliani *et al.* [17], obtained in their research 1,489 bp PCR product from phenanthrene and pyrene degrading bacteria isolated from marine region of Indonesia. Isiodu *et al.* [15], 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*. [26], found out that *B. cereus* was the most blasted organism with 99 % sequence similarity. The phylogenetic tree showed the evolutionary correlation among hydrocarbon metabolizing bacteria isolated in this study. The result shown in Figure 2 revealed that the nine bacterial isolates (KY171979, KY171984, KY171987, KY171980, KY171982, KY171981, KY171985, KY171986 and KY171983) had similar lineage as they arise from analogous node and hence they are evolutionary connected to their relatives in the Genebank. The sequences of these genes have been submitted to NCBI/Genbank database since first of December two thousand and sixteen (01/12/16). They belong to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus*; and families of: Enterobacteriaceae, Alcaligenaceae, Caulobacteriaceae, Flavobacteriaceae, and Bacillaceae; and phyla of: Proteobacteria, Bacteroidetes and Firmicutes which members have been implicated in petroleum and aromatic hydrocarbon biodegradation by several authors [7, 10, 15, 17, 19, 26 - 33].

In order to broaden the description 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 Figure 3. 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. Even though biosurfactants aid in

solubilizing or mediating the interface between the bacterial degraders and the aromatic compounds, the catabolic responses detected in our study were effected by the dioxygenase genes as revealed 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 showed that these strains (ANT1, XYL2, XYL7 and XYL8) synthesize dioxygenases for either complete or partial breakdown of aromatic hydrocarbons (xylene, anthracene and pyrene) and a probable reason could be that the detected genes were highly preserved among diverse Gram - negative bacteria; hence support the research work of Hesham *et al*. [4], who confirmed the existence of both monooxygenase and dioxygenase in *S. koreensis* strain ASU - 06. *C12O* and *C23O* dioxygenases has been known to contribute a significant part in the catabolism of aromatic rings by the bacteria as they are responsible for splitting of aromatic C – C bond at ortho or meta positions. The implication of the gene *C23O* as an essential gene in the catabolism of low and high molecular weights PAHs has been reported by Swaathy *et al*. [19]. Moreso, the detection of rhamnolipid (*rhlB*) and surfactin/lichenysin (*SrfA3/LicA3*) genes in these strains support the continuous argument among the scientists about the occurrence of *rhlB* and *SrfA3/LicA3* genes in bacteria other than *Pseudomonas* and *Bacillus* species and several new strains of bacteria having exceptional ability to synthesize rhamnolipids have in recent times been described. There are dearth of reports documented on rhamnolipid production among pathogens especially *Burkholderia mallei* and *B. pseudomallei* and the non - pathogenic especially *B. thailandensis*. This observation contradicts the findings of Swaathy *et al.* [3, 19], 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 the aromatic hydrocarbons but had unsuccessful amplification, could be as a result of incompatibilities among the tested primers and gene sequences [34] and may contain other catabolic and surfactant aside the tested genes.

Table 1. Mean total non – hydrocarbon utilizing bacterial (TNHUB) 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 viable hydrocarbon utilizing bacterial (TVHUB) 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.*

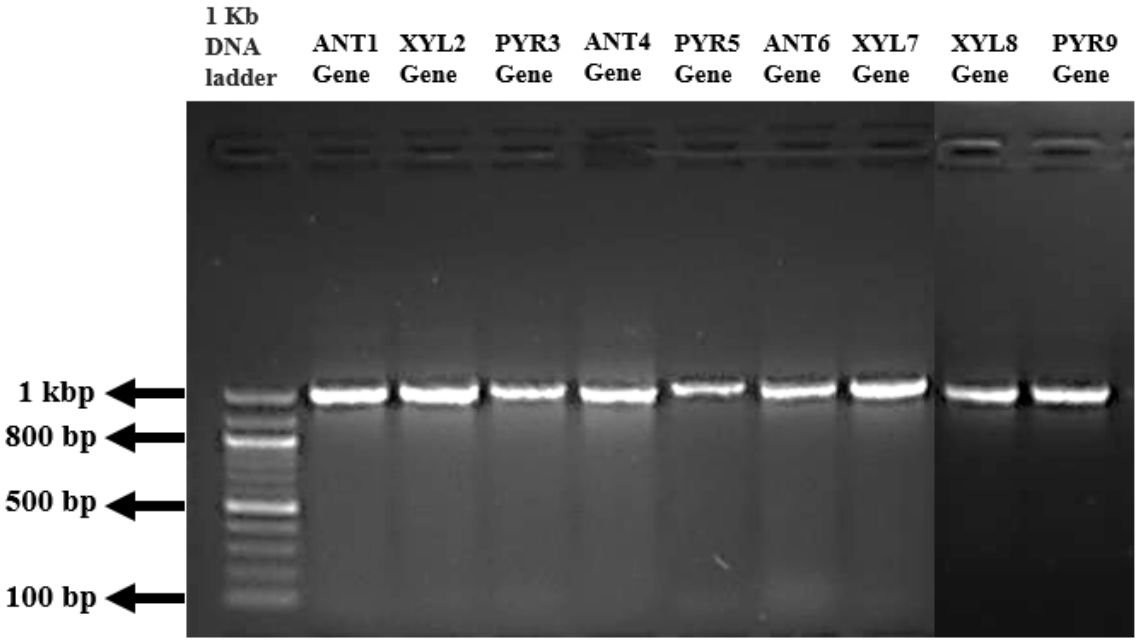

Fig. 1. PCR - amplification of 16S rRNA genes of the aromatic degrading bacteria genomic DNA

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

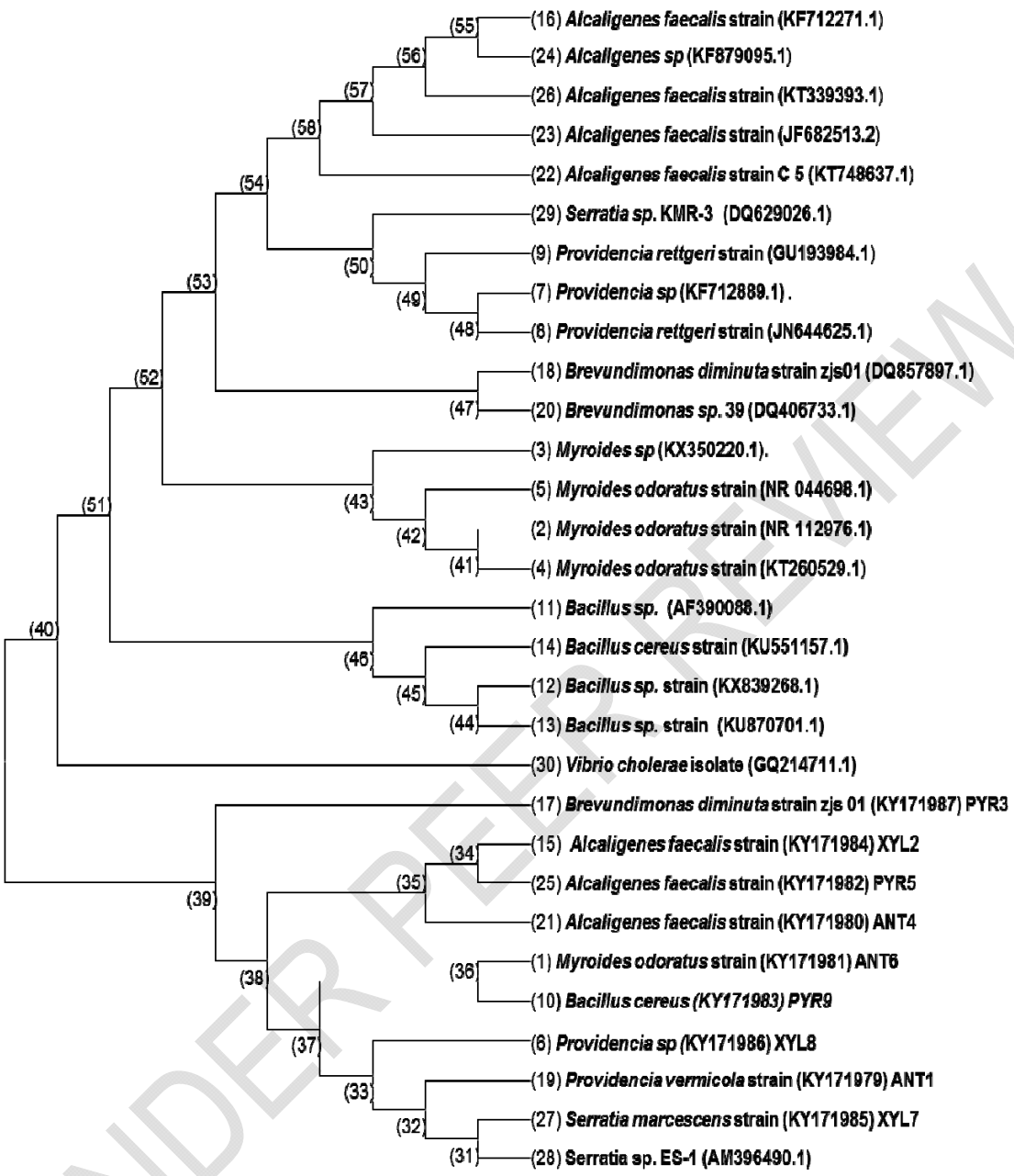

Fig. 2. Neighbor-joining phylogenetic relationship among the 16S rRNA sequence of the aromatic degrading bacterial isolates constructed by MEGA 7.0.

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

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

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*	-	-	-	-

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

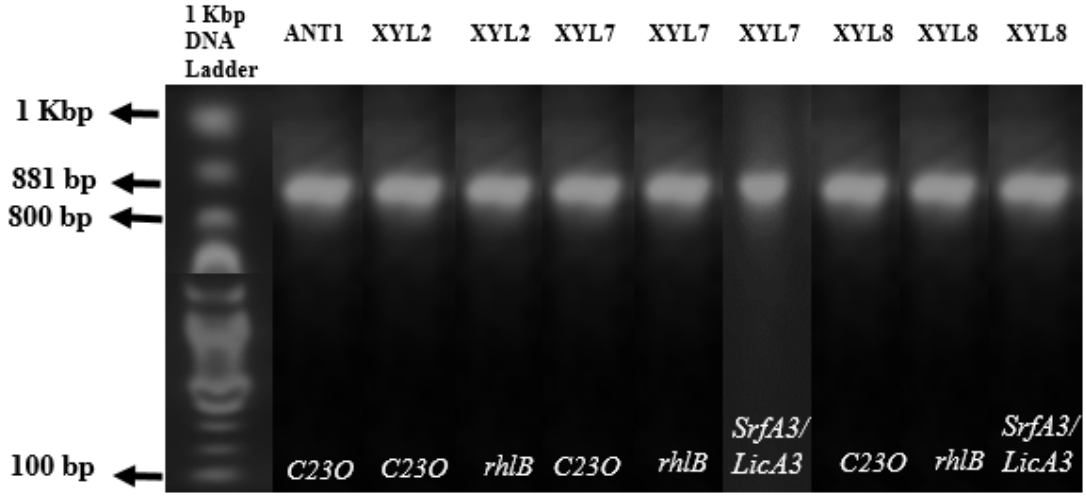

Fig. 3. PCR – amplification of primers specific for catabolic gene (*C23O*) and surfactant genes (*rhlB, SrfA3/LicA3*) of *Providencia vermicola* strain ANT1, *Alcaligenes faecalis* strain XYL2, *Serratia marcescens* strain XYL7 and *Providencia* sp. strain XYL8

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 may possibly play a significant part 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.

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