Original Research Article

Biofilm and Biosurfactant Mediated Enhanced Aromatic Hydrocarbons by Marine Bacteria Isolated from Contaminated Marine Environments of Niger Delta

ABSTRACT

Aims: To examine the effects of biofilm and biosurfactant associated cells of marine bacteria isolated from contaminated marine environments of Niger Delta on aromatic hydrocarbon degradation.

Study Design: Nine treatments and the controls designs were set up in triplicates containing 100 mL of sterile modified mineral basal medium in 250 mL conical flasks supplemented with 50, 100, 200 and 300 ppm of xylene, anthracene and pyrene each and nine marine hydrocarbon degraders; incubated at 24 °C for 5 - 7 days. The nine treatments and control set ups designated as ANT1, XYL2, PYR3, ANT4, PYR5, ANT6, XYL7, XYL8, PYR9 and CTRL (without hydrocarbons) were used to examine the effects of biofilm and biosurfactant produced by the marine bacteria on aromatic hydrocarbon degradability.

Place and Duration of Study: Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, Uli Nigeria between September, 2014 to August, 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, selection, morphological, biochemical, growth effect, emulsification, surface tension, bacterial adherence to Hydrocarbon (BATH), salt aggregation (SAT) and microtitre plate biofilm formation tests.

Results: The findings revealed that the three sampling sites harbour a lot of efficient aromatic degrading bacterial strains belonging to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus* able to significantly (P = .05) degrade the aromatic hydrocarbons. Significant positive correlation between biofilm formation and emulsification activity (r = 0.670; P = .05), was observed while surface tension (r = 0.134; P > .05), BATH (r = 0.142; P > .05) and SAT (r = 0.227; P > .05) had no significant positive correlation with biofilm formation.

Conclusion: Thus, the metabolic traits potentials of these strains could be exploited for

Keywords: Aromatic hydrocarbons, aquatic pollution, marine bacteria, bioremediation, biosurfactants, biofilm formation, Niger Delta.

1. INTRODUCTION

The Niger Delta ecosystem is particularly sensitive to changes in water quality, such as salinity or pollution. The Niger Delta is a wetland containing a number of ecological zones: sandy coastal ridge barriers, mangroves, freshwater permanent and seasonal swamp forests, and lowland rain forests. The

Niger Delta Mangrove Swamps provide grounds for commercial fishing, timber production, biotechnologically important microorganisms. However, pollution caused by petroleum and its by-products has greatly impacted negatively on the mangrove swamps leading to reduction in seafood output, increased food security challenges, reduction in biodiversity of mangrove biota, youth restiveness and violence in this region [1].

Large scale pollutions of both the terrestrial and aquatic environment in the area, consequent on activities of the oil industry have been documented [2, 3, 4]. It is therefore important to assess all remediation options on the basis of their ability to remove organic contaminants successfully. This is because most of these product especially the polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, and xylene (BTX) are toxic, mutagenic and carcinogenic [5]. The physical and chemical methods like volatilization, photooxidation, chemical oxidation, and bioaccumulation are rarely successful in rapid removal and cleaning up and also these methods are not safe and cost effective when compared to microbial bioremediation [6]. A better way is to use biodegradation. Bioremediation is a cost-effective and sustainable biotechnology for the treatment of contaminated coastal and marine sites [7]. 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 [6].

One of the major factors that impedes the process bioremediation is bioavailability of hydrophobic contaminants to the hydrocarbon utilising microorganisms due to poor solubility, leading to its accumulation with accompanying toxic and carcinogenic effects [6, 8]. The utilization of hydrocarbon will be aided by improved contact between hydrophobic organic compounds and the cells. The ability to adhere on to hydrocarbon is correlated with cell surface hydrophobicity (CSH). Statistical analyses showed a significantly high correlation between the ability of crude oil degradation and CSH [9]. Cells with higher hydrophobicity have better chance to adhere to oil droplets than cells with lower hydrophobicity. Thus, cells showing higher hydrophobicity may have better potential to degrade hydrocarbon [8].

It has been investigated earlier that this major limitation can be improved by exploiting chemotactic bacteria. Microbial chemotaxis plays important role in surface colonization and biofilm formation. Microbes have a natural tendency to form multi-cellular aggregates being glued to form biofilm. Biofilm can be formed by single bacterial species or even by a group of bacteria, fungi, algae, and protozoa [6]. Also, several studies reported that biofilm formation, a surface life style for many bacteria, was a promoting factor for biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) in that biofilm ensured higher bioavailability of PAHs, and improved PAHs degradation rate [10]. A promising method that can improve the effectiveness of bioremediation of hydrocarbon-contaminated environment is the use of biosurfactants. Biosurfactants are surface-active amphipathic metabolites produced by a plethora of microorganisms. They have wide structural diversity, ranging from glycolipids, lipopeptides and lipoproteins to fatty acids, neutral lipids, phospholipids and polymeric and particulate biosurfactants. Biosurfactant reduces surface tension (ST) and critical micelle dilution (CMD) in both aqueous solution and hydrocarbon mixtures, thereby facilitating the creation of micro-emulsions with the formation of micelle, in which hydrocarbons can solubilize in water or water in hydrocarbons [11].

Several investigators have reported the enhancement of biodegradation through biofilm formation and biosurfactant production and much of the publications centred on the terrestrial ecosystems with little information on biofilm and biosurfactant mediated biodegradation by marine microorganisms especially in the Niger Delta since the metabolic features are less toxic and environmentally friendly compare to their synthetic counterparts. In this study, we report the effects of biofilm and biosurfactant associated cells of marine bacteria isolated from contaminated marine environments of Niger Delta on aromatic hydrocarbon degradation.

2. MATERIALS AND METHODS

2.1 Description of the Sampling Sites

The studied areas were Abonema Wharf Water Front in Akuku-Toru Local Government Area, Nembe Water-side in Port Harcourt Local Government Area and Onne Light Flow Terminal Seaport 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 [12]. Onne Light Flow Terminal Seaport is a port of Nigeria and the largest oil and gas free zone in the world supporting exploration and production for Nigerian activities. It is situated on the Bonny River Estuary along Ogu creek and account for over 65 % of the export cargo through the Nigerian Sea Port.

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 [3, 13, 14].

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 [14,15].

2.4 Purification and Maintenance of Cultures

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

2.5 Screening and Selection Test

In order to screen and select the best and strongest degrading strains, growth of the different organisms were tested by growing 5 mL of each desired isolates in large test tubes containing 25 mL of the modified mineral basal medium with 100 mg /L of xylene, anthracene and pyrene hydrocarbons which were

dissolved in acetone and added to each tube after autoclaving. Thereafter, the test tubes were incubated at room temperature (28.00 ± 2.00 °C) for five days. Bacteria that started growing fast with high turbidity in the vicinity of the medium containing aromatic compounds measured at 600 nm using a UV - VIS spectrophotometer (Astell, UV - Vis Grating, 752 W) were selected as the candidate of xylene, anthracene and pyrene degrading bacteria [14,16, 17].

2.6 Characterization and Identification of Selected Hydrocarbon Utilizing Bacterial Isolates

2.6.1 Morphological characteristics

2.6.1.1 Colonial morphology

After sub - culturing and incubation, culturing morphological properties such as shape, elevation, margin, optic, texture, colour, size and surface characteristics of the selected bacterial strains were observed and noted [18].

2.6.1.2 Microscopic morphology

The standard methods of Gram staining and endospore staining were carried out on the selected bacterial strains as described in Cheesbrough [19] and Health Protection Agency [20].

2.6.2 Biochemical characteristics

The standard methods of testing for catalase, indole, motility, methyl red – Voges Proskauer, citrate, urease, starch hydrolysis, gelatin, nitrate reduction, coagulase, Hydrogen sulphide production, Sugar fermentation, Oxidase and Casein hydrolysis tests were carried out as described in Willey *et al*. [18] and Cheesbrough [19].

2.6.3 Identification of the marine bacterial isolates

Following morphological and biochemical characterization, the isolates were identified using Bergey's manual for determinative bacteriology, as described in Holt *et al.* [21].

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

To determine the effects of aromatic hydrocarbon concentrations on the growth of the isolates, precisely 100 mL of modified mineral basal medium was dispensed into fourty-five (45) 250 mL flasks and sterilized by autoclaving. The flasks were then divided into nine sets of seven flasks. Thereafter, 50, 100, 200 and 300 ppm levels of xylene, anthracene and pyrene which were seperately dissolved in acetone (as before) were exposed to each isolates. The fifth, sixth and seventh flasks served as the controls for each hydrocarbons and contained no xylene, anthracene and pyrene. Inoculated and control flasks were then incubated as previously described at 28.00 ± 2.00 °C for 5 days. Five millilitre sample was aseptically collected from each flask and assayed for the level of microbial growth which was indicated by increase in turbidity of the medium measured at 600 nm using a UV - VIS spectrophotometer (Astell UV - Vis Grating, 752 W) [15].

2.8 Measurement of Surfactant Production

2.8.1 Measurement of emulsifying activity

According to the slight modification of the method described by Thavasi *et al*. [22, 23] and Dhail [24], bioemulsification activity of the surfactants were determined in triplicates using xylene, anthracene and pyrene as hydrocarbon sources in mineral basal medium for 7 days at 28 ± 2 °C. Briefly, after centrifugation, 4 mL of n-hexane were added to 4 mL of the culture supernatants or biosurfactant crude extracts and centrifuged at 4000 rpm for 2 mins. The mixtures were allowed to stand for 10 mins prior to measurement. The negative controls were maintained with xylene, anthracene and pyrene hydrocarbons (X + A + P in 1:1:1 ratio) without the organisms. The emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as percentage below:

$$\text{Emulsification activity} = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times \frac{100}{1}$$

2.8.2 Surface tension measurement

For surface tension measurements in accordance to Viramontes- Ramos [25], the marine bacterial isolates were grown in mineral basal medium containing xylene, anthracene and pyrene and after

centrifugation, the supernatants were transferred to a glass tube and a capillary tube was dipped in this liquid. This procedure was done at a room temperature. The height reached by the liquid trough a capillary tube was measured in triplicates and surface tension calculated according to the following formula:

$$\gamma = ½ rh\delta g$$

Where: γ = surface tension (mN /m); δ = Density (0.99 g /mL); g = gravity (980 cm /s^2); r = capillary radius (0.09 cm); h = height of the liquid column (cm)

2.8.3 Bacterial adherence to hydrocarbon (BATH) assay

Bacterial cell surface hydrophobicity was examined by the modified BATH test of Abu and Chikere [3], and Thavasi *et al.* [22]. The bacterial isolates above were grown in marine broth for 48 hrs to achieve high cell density. Cultivation of cells without aromatic hydrocarbons exposure were also carried out as a control. The cultures were centrifuged to harvest the cells. The cells were washed twice with PUM buffer which comprised 16.87 g K_2HPO_4, 7.26 g KH_2PO_4, and 0.2 g $MgSO_4.7H_2O$ and filter sterilized with 1.8 % urea in 1000 mL of distilled water. The PUM buffer was used to suspend the cells after washing. To the different cell suspensions (2 mL) in test tubes (Pyrex), 100 µL of xylene, anthracene and pyrene were added and vortex-shaken for 3 minutes. After shaking, the hydrocarbons and aqueous phases were allowed to separate for 1 hr. OD of the aqueous phase was then measured at 610 nm in a spectrophotometer (Astell, UV- Vis Grating, 752W). For a given sample, three independent determinations were made and the mean value was calculated. Cells adhering to hydrocarbon droplets were verified and visualized. Briefly, a few drop of 2- (4-iodophenyl) 3-(4-nitrophenyl)-5- phenyltetrazolium chloride (INT) solution was added to the BATH assay culture broths and observed under the microscope. The INT turned red if it was reduced inside the cells, indicating the viability and adherence of cells with xylene, anthracene and pyrene droplets. From the OD values, percentage of cells attached to the hydrocarbons which is an indication of the hydrophobicity of the isolates were calculated using the following formula:

% of bacterial cell adherence = (1- (OD shaken with hydrocarbon/OD original)) x 100

Where: OD shaken with hydrocarbon = OD of the mixture containing cells and hydrocarbons

OD original = OD of the cell suspension in the buffer solution (before mixing with hydrocarbon)

When hydrophobicity is between 0 % and 30 %, it is assumed that the cell surface of the microorganism has hydrophilic properties; from 30 % to 40 %, the surface has mixed hydrophobic and hydrophilic properties; above 40 %, the cell surface of the microorganism has hydrophobic properties [26, 27].

2.8.4 Salt aggregation test (SAT)

The salt aggregation test was carried out by adopting the modified method of Abu and Chikere [3] and Abu and Tepikor [28]. Bacterial cell suspensions were prepared by cultivating the isolates in marine broth for 48 hrs to achieve high cell density. The cells were harvested by centrifuging and washed twice with 0.002 M Na_2HPO_4 buffer solutions. The buffer also served as the suspending medium. Agar-agar plates were prepared in triplicates and eight holes bored on each plates using sterile cork borer to take the different molar concentrations of ammonium sulphate solution. Then 50 µL of 0.5 M, 1.0 M, 1.5 M, 2.0 M, 2.5 M, 3.0 M, 3.5 M and 4.0 M solutions of ammonium sulphate solution were separately dispensed into the eight wells. This was followed by the addition of 50 µL of the bacterial cell suspensions in 0.002 M Na_2HPO_4 to each wells. This was rocked sideways for 2 mins and the degree of aggregation was recorded as the salt aggregation test value for that organism against the control containing solutions of ammonium sulphate solution without organism. The reaction mixture causing maximum agglutination was considered positive whereas absence of agglutination was considered as negative. Lowest concentration of $(NH_4)_2SO_4$ in the reaction mixture causing clumping of cells is expressed as hydrophobicity. Classification was expressed as:

< 2.0 M = strongly hydrophobic, 2.0 – 3.5 M = moderate Hydrophobic, > 3.5 M = Hydrophilic

2.9 Determination of Biofilm Formation

2.9.1 Microtiter plate biofilm formation assay

According to the slight modification method described by Lotfi *et al.* [29], strains from fresh agar plates were inoculated in 3 mL of Brain Heart Infusion (BHI) with 1 % glucose and incubated for 24 hrs at 37°C

in stationary conditions and diluted 1 in 20 with fresh medium. Individual wells of sterile 96 well microplates were filled with 200 µL of the diluted cultures and 200 µL aliquots of only BHI + 1 % glucose were dispensed into each of eight wells of the column 10 of microtiter plate to serve as a control (to check non-specific binding and sterility of media). After incubation (24 hrs at 37 °C), the microtiter plates content of each well were removed by tapping the bottom plates. The wells were washed four times with 200 µL of phosphate buffer saline (1 × PBS pH 7.2) to remove planktonic bacteria. The plates were then inverted and blotted on paper towels and allowed to air-dry for 15 mins. Adherent organisms' forming-biofilms in plate were fixed with sodium acetate (2 %) and stained with crystal violet (0.1 % w/v) and allowed to incubate at room temperature for 15 mins. After removing the crystal violet solution, wells were washed three times with 1 × PBS to remove unbound dye. Finally, all wells were filled by 200 µL of 33 % (v/v) glacial acetic acid per well to resolubulized and release the dye from the cells. The optical density (O.D.) of each well was measured at 570 nm using an automated micro plate reader (MR – 96A MINDRAY, Germany) [30] in triplicates. The OD values of non-inoculated sterile medium were taken as control. The data obtained were used to classify the strains as high producers (OD > 0.500), producers (OD between 0.500 and 0.100) or poor producers (OD < 0.100) [31].

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 as well as Pearson correlation analysis were performed on the data obtained. The results were considered statistically significant if the probability is less than .05 (P = .05) [10,16, 32].

3. RESULTS

3.1 Isolation and Selection Test

The result of the growth performance (OD_{600} nm) of the aromatic hydrocarbon-degraders isolated from Abonema sampled location is presented in Table 1. From the result, 13 isolates were obtained with strains XYL2, ANT4 and PYR3 having the highest significant *(*P = .05) absorbance values of 0.952 ±

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

3.2 Morphological and Biochemical Characteristics of Bacterial Isolates

The result of the colonial morphology of aromatic hydrocarbon degrading bacteria is presented in Table 4. From the result, most colonies were circular and irregular in shape, flat in elevation, undulate in margin, translucent in optic, smooth in texture, creamy in colour, 4 mm in size and glistering in surface description.

The result of the morphological and biochemical properties of the aromatic hydrocarbon degrading bacterial isolates is presented in Table 5. From the result, most bacterial isolates were Gram negative in Gram reaction, rod shaped arranged in single or pair, negative to spore, indole, methyl red, Voges Proskauer, urease, gelatin, nitrate reduction, coagulase, hydrogen sulphide production, xylose, lactose, arabinose, maltose and casein hydrolysis tests while positive to catalase, motility, citrate, starch hydrolysis, mannitol, glucose, sucrose, saccharose and oxidase tests.

3.3 Effect of Aromatic Hydrocarbon Concentrations

The results of the growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of xylene, anthracene and pyrene are shown in Figures 1, 2 and 3. From the xylene result, the isolate *Alcaligenes faecalis* PYR5 had the least growth of 0.122 ± 0.003 (OD_{600nm}) observed at

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

3.4 Measurement of Biosurfactant Production

The result of the emulsifying activity of marine aromatic hydrocarbon degrading bacteria is shown in Figure 4. From the result, *Serratia marcescens* XYL7 had the highest emulsifying activity of 90.00 ± 0.01 % followed by *Alcaligenes faecalis* PYR5 89.50 ± 0.03 % while the least is *Providencia vermicola* ANT1 with 60.00 ± 0.01 %. The control set-up had 2.10 ± 0.00 %. The result of the reduction in surface tension of the medium by marine aromatic hydrocarbon degrading bacteria is shown in Figure 5. From the result, *Bacillus cereus* PYR9 had the highest reduction (22.10 ± 8.9 mN/m) in surface tension followed by *Serratia marcescens* XYL7 with 23.00 ± 8.50 and *Alcaligenes faecalis* PYR5 had the least reduction of 26.60 ± 8.90. All the strains reduced the surface tension below that of the positive control (SDS) of 35.00 ± 0.10 % synthetic surfactant and the negative control of 44.60 ± 0.03 %. The result of the marine bacterial adherence to hydrocarbons (BATH) assay is shown in Figure 6. From the result, isolate *Serratia marcescens* XYL7 had the highest cell adhesion of 97.60 ± 0.10 % while *Brevundimonas diminuta* PYR3 had the least cell adhesion of 49.50 ± 0.02 %. The control had 5.50 ± 0.20 %. The result of the salt aggregation test (SAT) of marine aromatic hydrocarbon degrading bacteria is shown in Figure 7. From the result, all the isolates had their lowest concentration of salt giving aggregations below 3.50 in the concentration range of 1.00 ± 0.50 – 2.50 ± 0.50 M of ammonium sulphate solutions which is indicative of hydrophobic cell surfaces. The control had 4.00 ± 0.00 M

3.5 Determination of Biofilm Formation

The result of the biofilm formation by marine aromatic hydrocarbon degrading bacteria is shown in Figure 8. From the result, *Alcaligenes faecalis* PYR5 had the highest biofilm quantification with $OD_{570\ nm}$ of 0.243 ± 0.002 followed by *Serratia marcescens* XYL7 with $OD_{570\ nm}$ of 0.235 ± 0.001 and the least is *Providencia* sp. XYL8 with $OD_{570\ nm}$ of 0.181 while the control had $OD_{570\ nm}$ of 0.077 ± 0.001. Statistically, there was very strong to perfect significant positive correlation (*P* = .05) between biofilm formation and emulsification activity (r = 0.670), while surface tension (r = 0.134), BATH (r = 0.142) and SAT (r = 0.227) had no significant (*P* >.05) weak positive correlation with biofilm formation (Figure 9).

4. DISCUSSION

In the last few decades, the research focus has been on surfactant-mediated bioremediation. One of the approaches to enhance this technology is to use biosurfactants [33] which could increase solubility of oils in water to enhance the bioavailability of the hydrophobic substrates. Also, biofilm-mediated bioremediation presents a proficient and safer alternative to bioremediation with planktonic microorganisms because cells in a biofilm have a better chance of adaptation and survival (especially during periods of stress) as they are protected within the matrix [34].

In this study, a total of nine (9) isolates ANT1, XYL2, PYR3, ANT4, PYR5, ANT6, XYL7, XYL8 and PYR9 out of the 48 isolates (9/48) representing 18.75 % of the isolates were screened and selected as best and strongest degraders of xylene, anthracene and pyrene hydrocarbons which they significantly (*P* = .05) utilize as source of carbon and energy and is indicated by absorbance values below of each isolates (Tables 1, 2 and 3). This study agrees with the explanation of Mao *et al*. [35] 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 [36], 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*. [37] 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.

The result in Table 4 showed that most colonies were circular and irregular in shape, flat in elevation, undulate in margin, translucent in optic, smooth in texture, creamy in colour, 4 mm in size and glistering in surface description. The results of this research agree with the research carried out by Al -Thani *et al.* [38] who reported that a diverse microbial population can be isolated from hydrocarbon contaminated samples. In agreement with research carried out by Arulazhagan *et al*. [39] and Akinbankole *et al.* [40] who reported that bacteria isolated from pyrene and anthracene enriched medium are known to utilize pyrene and anthracene as their sole carbon source for growth and energy. The result in Table 5 showed that most of the marine bacterial isolates were Gram negative in Gram reaction, rod shaped arranged in single or pair, with variable reactions to different biochemical tests. The marine bacteria were identified as *Providencia vermicola* strain ANT1, *Alcaligenes faecalis* strain XYL2, *Brevundimonas diminuta* strain PYR3, *Alcaligenes faecalis* strain ANT4, *Alcaligenes faecalis* strain PYR5, *Myroides odoratus* strain ANT6, *Serratia marcescens* strain XYL7, *Providencia* sp. strain XYL8 and *Bacillus cereus* strain PYR9 using Bergey's manual for determinative bacteriology by Holt *et al.* [21]. These findings agree with the reports of Mrozik *et al*. [41], Okerentugba and Ezeronye [42], Chikere *et al*. [12] John *et al.* [14), Irshaid and Jacob [43], Wanjohi *et al*. [44], Isiodu *et al.* [32] and Fagbemi and Sanusi [45] that two - third of most petroleum hydrocarbon degraders are Gram negatives with one - third being Gram positives but contradict the findings of Akinbankole *et al*. [40], Kafilzadeh *et al*. [46] and Kafilzadeh and Pour [16], who reported that more of Gram positive bacteria were isolated than Gram negative bacteria. A lot of rod - shaped bacteria have also been implicated in hydrocarbon degradation studies (Okoh [47], Perfumo *et al*. [48], Chikere *et al*. [12], Alfreda and Ekene [49]; John *et al*., [14], Akinbankole *et al*. [40], Irshaid and Jacob [43], Wanjohi *et al*. [44], Isiodu *et al.* [32] and Fagbemi and Sanusi [45] and similar result was obtained in this study.

The results in Figures 1, 2 and 3 showed that the utilization and degradation of these compounds resulted in increase in optical density (cell mass) of the organisms; however, increase in concentration of these

compounds led to decrease in optical density (cell mass) of the organisms with very strongly significant negative correlation (*P* = .05; r = - 0.783 to - 0.980). It is apparent from the results that strains isolated on xylene (XYL2 and XYL8) and anthracene (ANT1) hydrocarbons were able to grow better on the three tested PAHs than the strains isolated on pyrene hydrocarbons. Generally, the nine isolates degraded all the aromatic hydrocarbons and grew well indicating multiple biodegradation potentials but with different efficiencies. The result is in consistent with the research carried out by Poornachander *et al*. [50], who reported that growth of *Bacillus cereus* CPOU13 decreased with increasing PAHs concentrations (phenanthrene, anthracene and pyrene) from 10 ppm to 250 ppm in MSM. Also, similar was the work carried out by John *et al.* [14], in which they found out that the growths of all the test isolates (*Alcaligenes faecalis* AFS-5, *P. putida* AFS-3 and *M. varians* AFS-2) were PAH - dependent and provide strong evidence for selective PAH degradation by bacteria. The acclimation of microbial community to one substrate, may lead to the simultaneous acclimation to some but not all structurally related molecules. Akinbankole *et al.* [40], isolated and identified *B.thuringiensis, B.megaterium* and *B.cereus* in both pyrene and anthracene enriched medium and the three bacteria have the metabolic adaptability of utilizing low and high molecular weight PAH. Bahobail *et al.* [51] reported that three isolates *Pantoea agglomerans* (BDCC-TUSA-8), *Acinetobacter lwoffii* (BDCC-TUSA-12) and *Bacillus thuringiensis* (BDCC-TUSA-18) showed multiple degradation potentials with remarkably fast reaction rates on n-Hexadecane, phenol and phenanthrene, representing the major types of hydrocarbon pollutants. Their abilities to utilize both low and high molecular weight PAHs is an indication of the possession of ring fission enzymes [52].

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 [53]. Surface activity and emulsification capability of bacterial culture give a strong indication of biosurfactant production. Surface tension reduction, emulsification property and stabilising capacity are the most important properties of microbial surfactants with potential industrial applications [25]. The result in Figure

4 revealed that *Serratia marcescens* XYL7 had the highest emulsifying activity of 90.00 ± 0.01 % followed by *Alcaligenes faecalis* PYR5 89.50 ± 0.03 % while the least is *Providencia vermicola* ANT1 with 60.00 ± 0.01 %. The control set-up had 2.10 ± 0.00 %. Similar observation was found by Pandey *et al*. [54] who reported that *Serratia marcescens* L-11 was able to emulsify crude oil (Castrol 2T oil) in synthetic medium, and produced 0.88 g L−1 of crude biosurfactant. The culture-liquid emulsion indices (EI48) obtained in the presence of PAH compounds were 2 – 3 folds higher than those of the PAH free controls indicating the significant role of surfactants, produced by the bacterium, in PAH solubilisation and degradation. Kurniati *et al*. [55] reported that a pyrene degrading strain *G. cholesterolivorans* AMP 10 formed a stable emulsion in used lubricant oil with an emulsification index (E24) of 74%. Thavasi *et al.* [22] observed emulsification of different hydrocarbons by crude biosurfactant isolated from *B. megaterium, C. kutscheri* and *P. aeruginosa* were in the order of wastemotor lubricant oil > crude oil > peanut oil > kerosene > diesel > naphthalene > anthracene > xylene. Also, the result in Figure 5 revealed that all the strains reduced the surface tension below that of the positive control (SDS) of 35.00 ± 0.10 % synthetic surfactant and the negative control of 44.60 ± 0.03 %. It can be concluded from the study that biosurfactants produced by these isolates have low molecular weights and reduction of the surface tension of the media indicates the production of surface – active compounds which will enhance the aromatic hydrocarbons biodegradation by mobilization, solubilization and emulsification. The result agrees with research work of Meliani and Bensoltane [10] who reported that all their isolates were able to lower the surface tension, presumably via biosurfactant production of the medium containing xylene or benzene hydrocarbons. The reduction of surface tension values ranges from 75 mN /m to 55 mN /m, 45 mN /m and 36 mN /m, respectively for *P. aeruginosa, P. fluorescens* and *P. putida*. Similar result was observed by Dhail [24] who reported that reduction of surface tension measurements by all the isolated bacteria from oil spilled marine water and marine sediments of Arabian Sea (Mumbai) indicates the production of surface-active compounds. Amer *et al.* [53] in their publication reported that all the isolates in their collections from the Mediterrenean Sea, Egypt were able to reduce surface tension when compared to the non-inoculated medium surface tension (65.66 ± 4.00 mN /m); in particular those isolated on the ONR7a medium (average value within the collection: 27.4 ± 10.2), namely *M. hydrocarbonoclasticus* demonstrated higher ST reduction compared to those belonging to the ASW

collection (average value within the collection: 47.25 ± 10.36). Mulligan [56] reported that the low molecular weight biosurfactant are able to reduce the surface tension below 40 mN /m while the high molecular weight bioemulsifiers can form and stabilize emulsions without remarkable surface tension reduction [57]. Kurniati *et al*. [55] reported that a pyrene degrading strain *G. cholesterolivorans* AMP 10 lower the surface tension of medium from 71.3 mN /m to 24.7 mN /m. Safary *et al.* [58] observed that CpA1 a biosurfactant producing bacterium from Caspian Sea was able to reduce the surface tension of the medium to 39.4 mN /m below that of the positive control 1 % SDS, a synthetic surfactant. Thavasi *et al.* [23] reported that highest surface tension reduction was observed with 5 strains namely *Bacillus megaterium* (30.80 ± 1.13 mN /m)*, B. subtilis* (38.75 ± 0.30 mN /m)*, Corynebacterium kutscheri* (36.90 ± 0.77 mN /m)*, C.xerosis* (37.80 ± 0.42 mN /m)*, Lactobacillus delbrueckii* (32.50 ± 0.70 mN /m)*, Pseudomonas aeruginosa* (28.70 ± 0.98 mN /m) and *P. fluorescens* (34.70 ± 0.35 mN /m). The reports of these authors support the finding in this study.

Cell adherence with hydrophobic compounds like crude oil is considered as an indirect method to screen bacteria for biosurfactant production, because cells attach themselves with oil droplets by producing surface active compounds called biosurfactants [23] and in this study, the result in Figure 6 showed that all the isolates had relatively strong hydrophobic cell surfaces [26, 27]. These differences in BATH hydrophobicities may be attributed to high production of hydrophobic cell surface proteins. Such high aromatic hydrocarbon affinity observed with *Serratia marcescens* XYL7 correlated with the maximum biodegradation potential observed for this strain. Several species of *Rhodococcus* and *Mycobacterium* have reportedly changed their cell surface hydrophobicity to facilitate the uptake of hydrophobic substrates [54]. Amer *et al*. [53] observed that the highest hydrophobicity (77.3 %) was recorded for the strain *M. hydrocarbonoclasticus* SCS6 isolated from highly contaminated marine sediment of Mediterreanean Sea, Egypt. Nwanyanwu and Abu [59] reported that all the phenol- utilizing bacterial organisms isolated from petroleum refinery effluent expressed moderately hydrophobicity in BATH test when grown in TSB, NB, PW and BH media containing *p*-xylene hydrocarbon respectively. Cell surface properties are important factors that determine the rate of degradation of hydrophobic substrates. In an early investigation, cells exhibiting highest hydrophobicities were among the fastest hydrocarbon

degraders [22]. Therefore, isolates with high hydrophobicity are likely to be more efficient degraders, as reported in this study for *Serratia marcescens* XYL7. Positive cell hydrophobicity was reported as an indication of biosurfactant production [22]. Visualization of bacterial cells adherence to aromatic compounds as presented in Table 6 confirmed the affinity of cells towards hydrocarbon droplets as all the isolates were positive to the visualization test under the microscope. To ensure validity of result, it is always a good practice to employ more than one method in assessing cell surface hydrophobicity i.e. relying on SAT or BATH as the only method for assessing hydrophobicity may produce misleading results with some bacteria strains because a particular bacteria may be hydrophobic for BATH assay and also give a hydrophilic result in SAT assay leading to misleading results [28]. The SAT is a "a salting out" or in this case "salting in" phenomenon where ammonium sulphate is causing aggregation (salting out) of the cells as it (the ammonium sulphate) goes into solution and in this study the result in Figure 7 revealed that the strains had different hydrophobic cell surfaces. These differences may be due to the differences on cell surface charges and alterations on the surface of the organisms by toxic effects of these aromatic compounds especially pyrene. The study also showed that there was a good agreement in establishing the hydrophobicity of the isolates between BATH and SAT assays for all the nine isolates. Abu and Tepikor [28] observed that *E. coli* cell surface had the most hydrophobic with a SAT value below 3.5 M of the five isolates from oil contaminated soil sample. For bacteria to utilize aromatic compounds that have formed phase boundary with water, it will depend on their hydrophobicities and also on the properties of the hydrocarbons. Nwanyanwu and Abu [59] reported that all the phenol- utilizing bacterial organisms isolated from petroleum refinery effluent expressed moderate SAT hydrophobicity but *Pseudomonas sp*. RWW showed strong SAT hydrophobicity in all the growth media than other test organisms. Therefore, the highly hydrophobic bacteria such as *Serratia marcescens* XYL7 would be attracted more to the organic phase of the aromatic hydrocarbons than the water phase because of their non-affinity for water.

Bacterial adhesion is one of the first steps of a biofilm formation. Cell Surface Hydrophobicity (CSH) has been considered as an important factor in the stability of microbial aggregates [10]. Biofilm quantification was used to further verify the the BATH and SAT assay and the result in Figure 8 revealed that all the isolates were categorized as being biofilm producer and the control has no biofilm production according to

Stepanovic *et al.* [30] and Chowdhury and Ray [31] classification. Similar observations were obtained by Amer *et al.* [53] who published that five (5) strains representing 10 % of the collection of isolates from Mediterrenean Sea Egypt were found to be biofilm producers and the strains belong to the *M. hydrocarbonoclasticus, A. faecalis, B.cereus,* and *P. vermicola* species. The result agrees with the observation of Meliani and Bensoltane [10] that *P. fluorescens* and *P. aeruginosa* isolates exhibited an important biofilm mass with increased biofilm formation. The *Pseudomonas* isolates had OD_{590} nm readings ranging from 0.29 to 0.35 in the presence of xylene or benzene hydrocarbons. Microbial EPS is crucial to the formation of biofilm and cell aggregates which contribute to protect cells from hostile environments and can bind significant amounts of heavy metals. Forming biofilms by these strains is considered a natural strategy to maintain a favorable niche in stressful environments with increased hydrocarbon concentrations [10]. Results obtained in the present study indicate that increase in cells hydrophobicity (BATH, Visualization, SAT) does not necessarily enhanced the attachment of viable cells (biofilm) on the surface or lead to stronger biofilms (Figure 9).

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

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

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

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

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

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

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

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

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

Table 5. Morphological and biochemical properties of the aromatic hydrocarbon degrading bacterial isolates

Property	Isolate								
	ANT1	XYL2	PYR3	ANT4	PYR5	ANT6	XYL7	XYL8	PYR9
Gram reaction	–	–	–	–	–	–	–	–	+
Cellular morphology	Paired short rods	Singled long rods	Singled long rods	Singled short rods	Singled long rods	Single longer rods and long chains	Paired short rods	Single paired long Rods	Singled short rods
Spore test	–	–	–	–	–	–	–	–	+
Catalase	+	+	+	+	+	+	+	+	+
Indole	+	–	–	–	–	–	–	+	+
Motility	+	+	+	+	+	–	+	+	+
Methyl red	–	–	–	–	–	–	–	–	+
Voges-Proskauer	–	–	–	–	–	–	+	–	–
Citrate	–	+	–	+	+	+	+	–	+
Urease	+	–	–	–	–	–	–	+	+
Starch hydrolysis	+	+	–	+	–	+	–	+	+
Gelatin hydrolysis	–	–	–	–	–	–	+	–	–
NO_3 reduction	+	–	–	–	–	–	+	+	+
Coagulase test	+	–	–	–	–	–	+	+	–
H_2S production	+	–	–	–	–	+	–	+	+
Mannitol	+	–	–	–	–	+	+	+	+
Glucose	+	+	–	+	+	+	+	+	+
Xylose	–	–	–	–	–	–	+	–	+
Lactose	–	–	–	–	–	–	–	–	+
Sucrose	–	+	–	+	+	–	+	–	+
Arabinose	+	–	–	–	–	+	+	–	–
Maltose	–	–	+/–	–	–	–	–	–	+
Saccharose	+	+	–	+	+	–	+	+	+
Oxidase	–	+	+	+	+	+	+	–	+
Casein hydrolysis	–	–	–	–	–	–	+	–	–

NO_3 = Nitrate; H_2S = Hydrogen sulphide; – = Negative result; + = Positive result

Table 4. Colonial morphology of the aromatic hydrocarbon degrading bacterial isolates

Isolate	Colonial description						
	Shape	Elevation	Margin	Optics	Texture	Colour	Size	Surface
ANT1	Circular	Flat	Undulate	Translucent	Smooth	Creamy	4.0 mm	Dull
XYL2	Irregular	Raised	Erose	Translucent	Smooth	Creamy	3.0 mm	Dull
PYR3	Circular	Flat	Undulate	Translucent	Smooth	Creamy	2.0 mm	Glistering
ANT4	Circular	Flat	Erose	Translucent	Smooth	Creamy	5.2 mm	Glistering
PYR5	Circular	Flat	Undulate	Translucent	Smooth	Creamy	4.0 mm	Dull
ANT6	Rhizoid	Flat	Lobate	Translucent	Rough	Yellow	4.2 mm	Glistering
XYL7	Irregular	Flat	Undulate	Translucent	Rough	Red	4.0 mm	Glistering
XYL8	Irregular	Flat	Undulate	Translucent	Smooth	Creamy	4.2 mm	Glistering
PYR9	Irregular	Raised	Undulate	Translucent	Smooth	Creamy	4.0 mm	Glistering

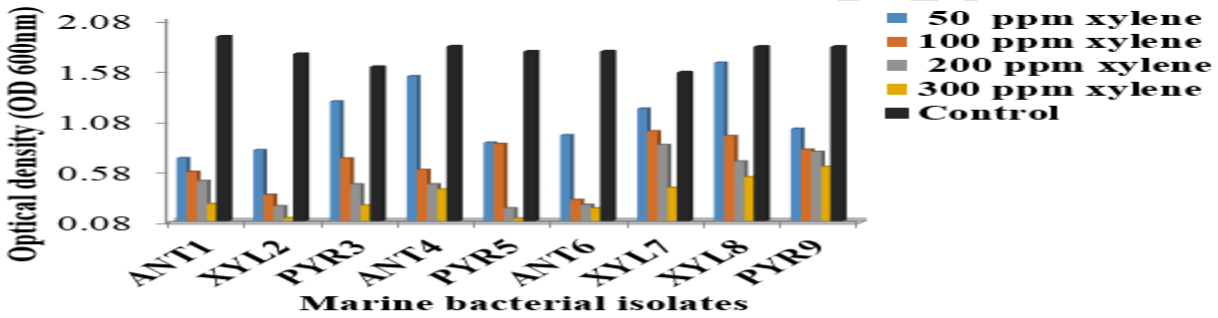

Fig. 1. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of xylene

PPM = Part Per Million; XYL = Xylene; ANT = Anthracene; PYR = Pyrene

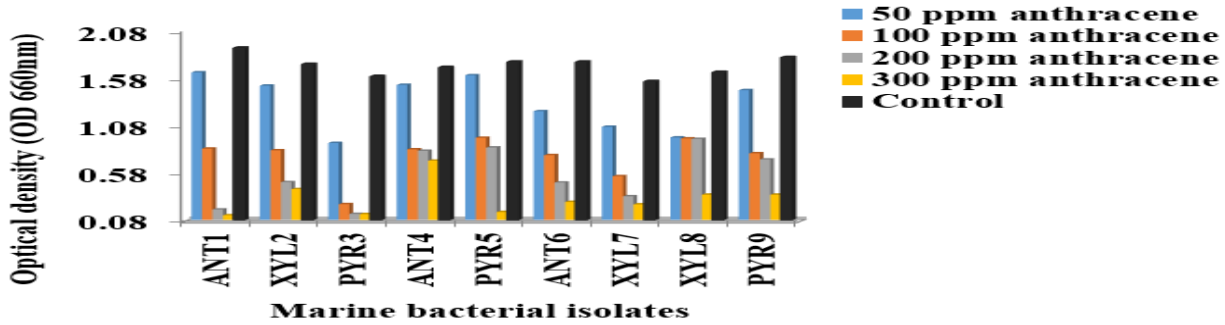

Fig.2. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of anthracene

PPM = Part Per Million; XYL = Xylene; ANT = Anthracene; PYR = Pyrene

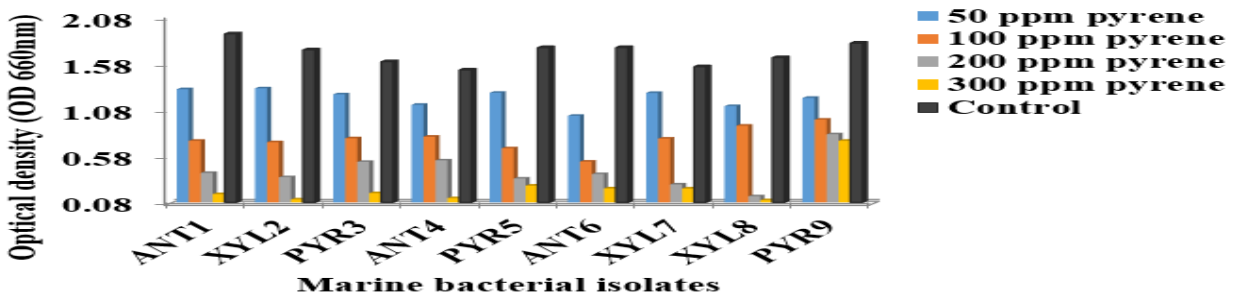

Fig. 4. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of pyrene

PPM = Part Per Million; XYL = Xylene; ANT = Anthracene; PYR = Pyrene

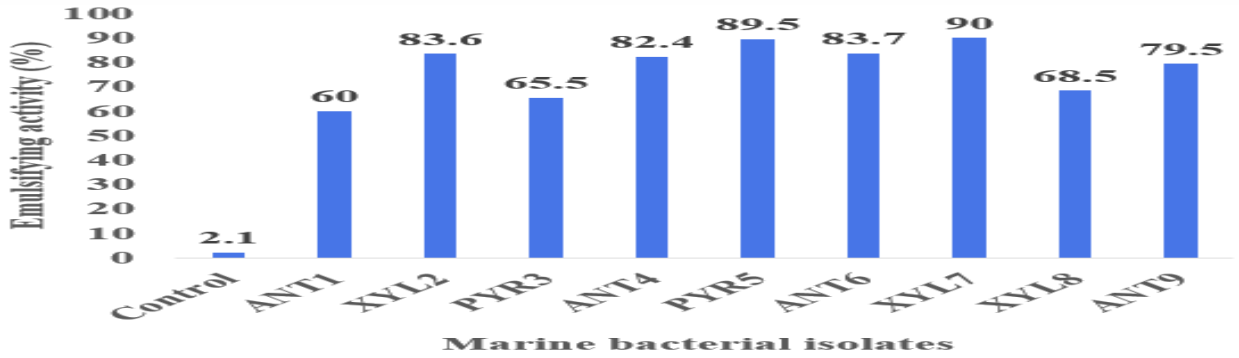

Fig. 4. Emulsifying activity of marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triplicate determination.

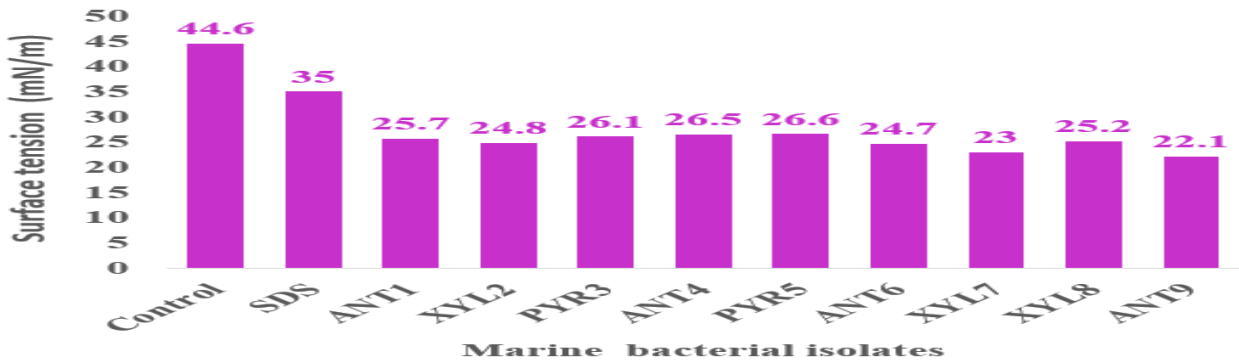

Fig.5. Reduction in surface tension of the medium by marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triolicate determination.

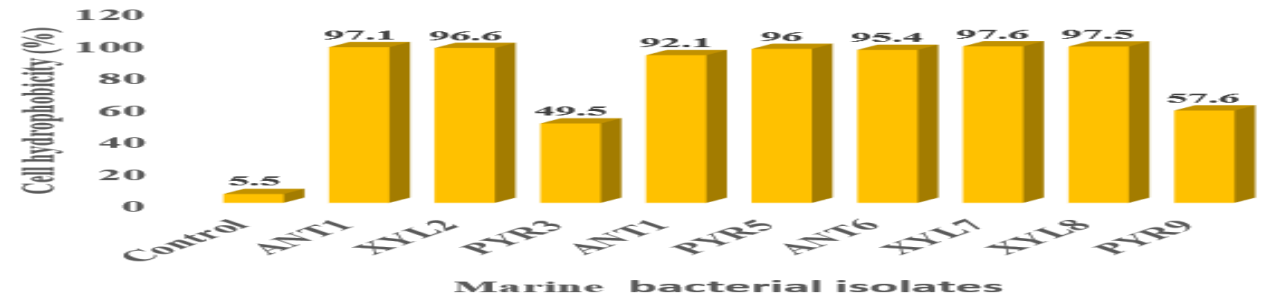

Fig. 6. Marine bacterial adherence to hydrocarbons (BATH) assay

Values are mean ± standard deviation of triplicate determination

Table 6. Visualization of bacterial cells adherence to aromatic compounds

Isolate	Reaction
ANT1	+ ve
XYL2	+ ve
PYR3	+ ve
ANT4	+ ve
PYR5	+ ve
ANT6	+ ve
XYL7	+ ve
XYL8	+ ve
PYR9	+ ve

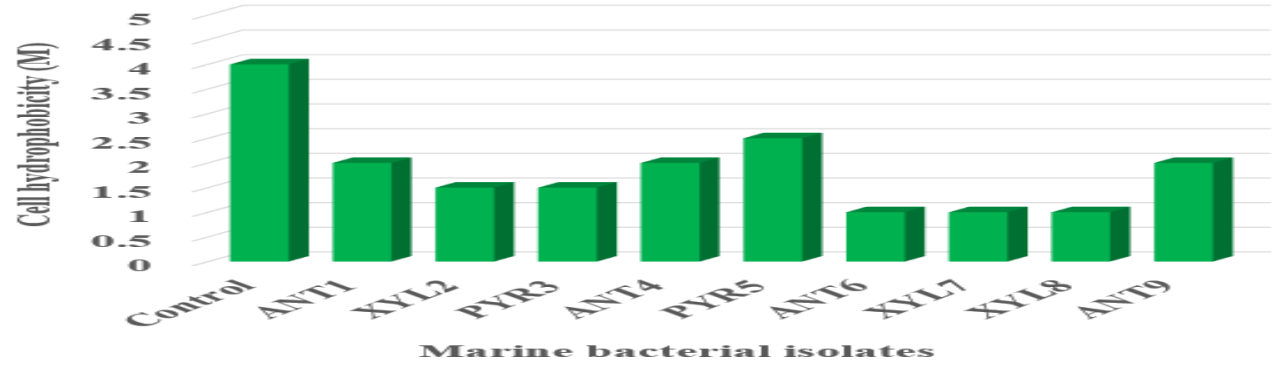

Fig.7. Salt aggregation test (SAT) of marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triplicate determination

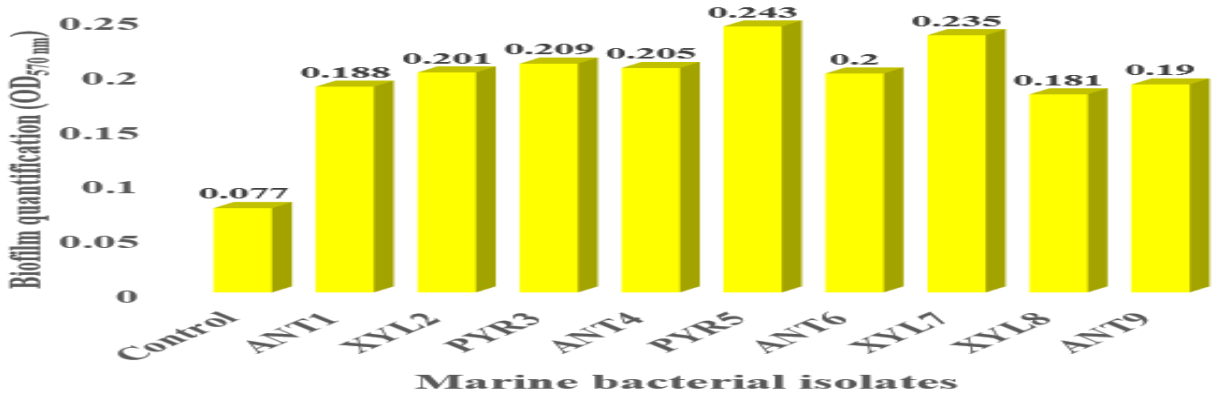

Fig.8. Biofilm formation by marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triplicate determination

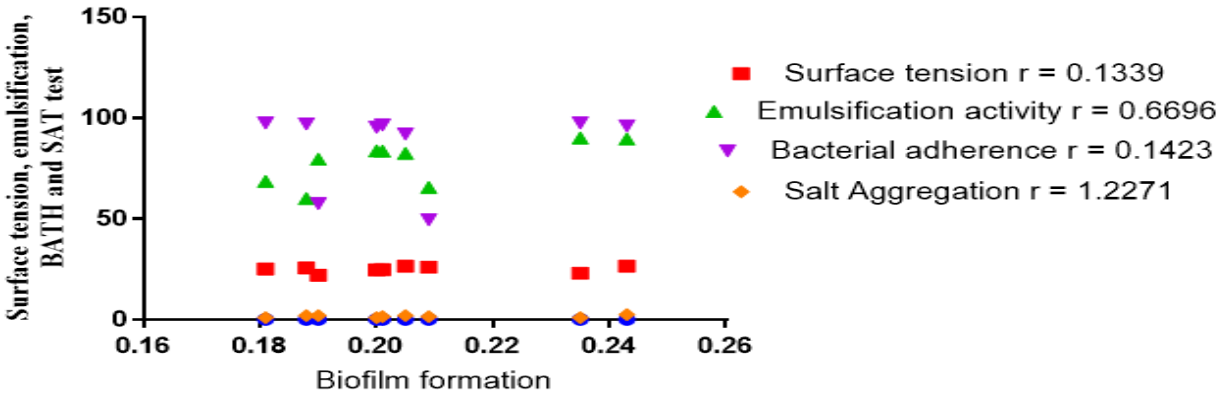

Fig.9. Biofilm correlation between surface tension, emulsification and cells hydrophobicities

5. CONCLUSION

The whole study revealed that the three sampling sites harbour a lot of efficient aromatic hydrocarbon degrading bacterial strains belonging to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus.* It also revealed that the isolated bacteria especially *Serratia marcescens* XYL7 were able to significantly (*P* =.05) degrade simple, low and high molecular weights aromatic hydrocarbons.

These strains were found to be good formers and producers of biofilm and biosurfactant suggesting their possible exploitation in future biotechnological processes, either directly as field-released microorganisms or as biofilm formers and biosurfactant producers under controlled conditions.

REFERENCES

1. Orji FA, Abiye AI, Dike EN. Laboratory scale bioremediation of petroleum hydrocarbon - polluted mangrove swamps in the Niger Delta using cow dung. Mal J Microbiol. 2012;8(4):219-28.
2. Odokuma LO, Ibor MN. Nitrogen fixing bacteria enhanced bioremediation of a crude oil polluted soil. Glob. J. Pure Appl. Sci. 2002;86:455-68.
3. 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.
4. Okpokwasili GSC. Microbes and the environmental challenge. Inaugural lecture series No. 53. Port Harcourt: University of Port Harcourt Press; 2006.
5. Akpe AR, Ekundayo AO and Esumeh FI. Degradation of crude oil by bacteria: A role for plasmid-borne genes. Glob J Sci Front Res 2013;13(6):20-6.
6. 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.
7.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.

8. Vyas TK, Dave BP. Production of biosurfactant by *Nocardia otitidiscaviarum* and its role in biodegradation of crude oil. Internat J Environ Sci Tech. 2011;8(2):425-32.
9. Obuekwe CO, Al-Jadi ZK, Al-Saleh ES. Hydrocarbon 1 degradation in relation to cell surface hydrophobicity among bacterial hydrocarbon degraders from petroleum contaminated Kuwait desert environment. Internat Biodet Biodeg. 2009;63:273-9.
10. Meliani A, Bensoltane A. Enhancement of hydrocarbons degradation by use of *Pseudomonas* biosurfactants and biofilms. J Pet Environ Biotech. 2014;5:168.
11. Patowary K, Saikia RR, Kalita MC, Deka S. Degradation of polyaromatic hydrocarbons employing biosurfactant-producing *Bacillus pumilus* KSs2. Ann Microbiol. 2015;65:225-34.
12. Chikere CB, Okpokwasili GC, Ichiakor O. Characterization of hydrocarbon utilizing bacteria in tropical marine sediments. Afr J Biotech. 2009;8(11):2541-4.
13. 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.
14. 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.
15. 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. 2012:88:1020-26.
16. 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.
17. 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.
18. Willey JM, Sherwood IM, Woolverton CJ. Prescott, Harley and Kleins Microbiology. 7th ed. New York: McGraw-Hill companies; 2008.
19. Cheesbrough M. District laboratory practice in tropical countries. 2nd edn. New York (USA): Cambridge University Press; 2006.
20. Health Protection Agency. Staining procedures BSOP TP 39: National standard method, Issue 1. United Kingdom:Standards Unit, Evaluations and Standards Laboratory Centre for Infections; 2007.
21. Holt JG, Kreig, NR, Sneath PHA, Staley JT, Williams ST. Bergey's manual of determinative bacteriology. 9th edn. Maryland (USA): A Waverly Company; 1994.
22. Thavasi R, Jayalakshmi S, Balasubramanian T, Banat IM. Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolates of *Bacillus megaterium, Corynebacterium kutscheri* and *Pseudomonas aeruginosa.* Biores Tech. 2011a;102:772-8.
23.Thavasi R, Sharma S, Jayalakshmi S. Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. J Pet Environ Biotech. 2011b;S1:001. doi:10.4172/2157-7463.S1-001.
24. Dhail S. Isolation of potent biosurfactant producing bacteria from oil spilled marine water and marine sediments. Afr J Biotech. 2012;11(103):16751-7.
25. Viramontes-Ramos S, Portillo-Ruiz MC, Ballinas- Casarrubias MDL, Torres-Muñoz JV, Rivera-Chavira BE, Nevárez-Moorillón GV. Selection of biosurfactant/ bioemulsifier-producing bacteria from hydrocarbon-contaminated soil. Bra J Microbiol. 2010;41(3):668-75.
26. Basson A, Flemming LA, Chenia HY. Evaluation of adherence, hydrophobicity, aggregation, and biofilm development of *Flavobacterium johnsoniae*-like isolates. Microb Eco.2008;55:1-14.
27. Kaczorek E, Olszanowski A. Uptake of hydrocarbon by *Pseudomonas fluorescens* (P1) and *Pseudomonas putida* (K1) strains in the presence of surfactants: A cell surface modification. Wat Air Soil Poll. 2011;214:451-9.
28. Abu GO, and Tepikor, G.O. (2006). Physicochemical properties of some Nigerian crude oil samples and some bacterial isolates in relation to cell engineering for bioremediation application. *Nigerian Journal of Microbiology,* 20 (3): 1435-1442.
29. Lofti G, Hafida H, Nihel K, Abdelmonaim K, Nadia A, Fatima NAS, Walter Z. Detection of biofilm formation of a collection of fifty strains of *Staphylococcus aureus* isolated in Algeria at the University Hospital of Tlemcen. J Bact Res. 2014;6(1):1-6.
30. Stepanovic´ S, irkovic´ IC´, Ranin L, _Svabic´-Vlahovic M. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol. 2004;38:428-32.
31. Chowdhury S, Ray RR. A study of biofilm production by a multi-metal tolerant strain of *Klebsiella* sp. Internat J Adv Res. 2015;3(6):744-50.
32. 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.
33. Rahman KS, Rahman TJ, Kourkoutas Y, Petsas I, Marchant R, et al. Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Biores Tech. 2003;90:159-68.

34. Decho AW. Microbial biofilms in intertidal systems: an overview. Cont. Shelf Res. 2000;20:1257-73.
35. 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.
36. Pathak H, Bhatnagar K. *Alcaligenes*-the 4T engine oil degrader. J Biorem and Biodeg. 2011;2(4):2-5.
37. 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.
38. 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.
39. Arulazhagan P, Yeom IT, Vasudevan N. Biodegradation of polycyclic aromatic hydrocarbon by a halotolerant bacterial consortium isolated from marine environment. Internat J Environ Sci Tech. 2011;7(4):639-52.
40. 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.
41. Mrozik A, Piotrowska-Seget Z, Łabuzek S. Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. Pol J Environ Studies. 2003;12:15-25.
42. Okerentugba PO, Ezeronye OU. Petroleum degradating potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigerias. Afr J Biotech. 2003;2(9):288-92.
43. 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.
44. Wanjohi L, Mwamburi L, Too E, Aloo B, Kosgei J. Isolation and identification of bacteria with bioremediation potential of oil spills in lake Nakuru, Kenya. Asi J Microbiol Biotech Environ Sci. 2015;17(4):831-8.
45. 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.
46. 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.
47. Okoh AI. Biodegradation of Bonny light crude oil in soil microcosm by some bacterial strains isolated from crude oil flow stations saver pits in Nigeria. Afr J Biotech. 2003;2(5):104-8.
48. Perfumo A, Banat IM, Marchant R, Vezzulli L. Thermally enchanced approaches for bioremediation of hydrocarbon-contaminated soil. Chem.2007;66(1):179-84.
49. Alfreda ON, Ekene GO. Bioremediation of crude oil polluted soil using bacteria and poultry manure monitored through soybean productivity. Poll J Environ Stud. 2012;21(1):171-6.
50. Poornachander RM, Anitha Y, Satyaprasad K. Abilities of Bacillus Cereus CPOU13 in biodegradation of polycyclic aromatic hydrocarbons (PAHs). J Pharm Chem Biol Sci. 2016;4(1):54-64.
51. Bahobail A, Gad El-Rab SMF, Amin GA. Locally isolated bacterial strains with multiple degradation potential capabilities on petroleum hydrocarbon pollutants. Adv Microbiol. 2016;6:852-66.
52. Amund OO, Ilori MO, Adebusoye SA, Musa KJ. Utilization of alicyclic compounds by soil bacteria. Nat Sci. 2006;4(3):65-8.
53. Amer RA, Mapelli F, El-Gendi HM, Barbato M, Goda DA, Corsini A et al. 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.
54. Pandey AK, Chaudhary P, Singh SB, Arora A, Kumar K, Chaudhry S et al. Deciphering the traits associated with PAH degradation by a novel *Serratia marcescens* L-11 strain, J Environ Sci Heal. 2012;47(5):755-65.
55. Kurniati TH, Rusmana I, Suryani A, Mubarik NR. Degradation of polycyclic aromatic hydrocarbon pyrene by biosurfactant-producing bacteria *Gordonia cholesterolivorans* AMP 10. Biosaintifika: J Biol Biol Edu. 2016;8(3):336-43.
56. Mulligan CN. Environmental applications for biosurfactants. Environ Poll. 2005;133:183-98.
57. Batista SB, Mounteer AH, Amorim FR, Totola MR. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. Biores Tech. 2006;97: 868 – 875.
58. Safary A, Ardakani MR, Suraki AA, Khiavi MA, Motamedi H. Isolation and characterization of biosurfactant producing bacteria from Caspian Sea. Biotech. 2010;9(3):378-82.
59. Nwanyanwu CE, Abu GO. Influence of growth media on hydrophobicity of phenol-utilizing bacteria found in petroleum refinery effluent. Internat Res J Biol Sci. 2013;2(10):6-11.