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 and 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 *in situ* bioremediation intervention in the coastal areas of Nigeria.

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

1. INTRODUCTION

The Niger Delta aquatic environment is mostly sensitive to variations in water quality, such as salinity or contamination. Lowland rain forests, mangroves, freshwater permanent, sandy coastal ridge barriers and

seasonal swamp forests make up the wetland ecological zones in the Niger Delta. The Niger Delta Mangrove Swamps offer lands for wood making, for large scale fishing and biotechnologically essential microbes. However, contamination caused by crude oil and its by-products has seriously induced negative consequences on the mangrove swamps thereby resulting youthful crises and unrest, prostitution, environmental and food security problems, poverty, decrease in biodiversity of macro and micro species in this region [1].

Large scale contaminations of both the terrestrial and aquatic ecosystems in the regions, consequent on deeds of the oil exploration and production have been documented [2, 3, 4]. It is therefore necessary to evaluate all remediation alternatives on the basis of their capacity to eliminate organic pollutants effectively. This is because most of these products especially the polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, and xylene (BTX) are toxic, mutagenic and carcinogenic [5]. The non-biological methods such as chemical oxidation, volatilization, photooxidation, and bioaccumulation are rarely effective in speedy elimination and cleaning up as well as environmentally friendly and cost effective in comparison to microbial bioremediation [6]. An improved and healthier way is to use enhanced biodegradation. Bioremediation is a economical and maintainable biotechnology for the management of polluted coastal and marine locations [7]. Over twenty genera of bacteria of marine source have been recognized 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 dynamics that hinders the course of bioremediation is bioavailability of hydrophobic pollutants to the hydrocarbon utilizing microbes due to poor solubility, resulting to its increase with associated toxic and carcinogenic effects [6, 8]. The utilization of hydrocarbon will be aided by enhanced interaction between hydrophobic organic compounds and the cells. The ability to adhere on to hydrocarbon is associated with cell surface hydrophobicity (CSH). Statistical studies revealed a significantly high association between the ability of crude oil degradation and CSH [9]. Cells with greater values of hydrophobicity have improved and enhanced adherence to oil droplets than cells with lesser

hydrophobicity. Thus, cells showing higher hydrophobicity may have better prospects to degrade hydrocarbon [8].

It has been studied earlier that this major drawback can be overcome by exploiting chemotactic bacteria. Microbial chemotaxis plays significant part in surface settlement and biofilm formation. Microbes have a normal trend of forming multicellular groups 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 stimulating feature for biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) in that biofilm ensured higher bioavailability of PAHs, and enhanced PAHs degradation rate [10]. A fascinating method that can enhanced the success of bioremediation of hydrocarbon-contaminated environment is the application of biosurfactants. Biosurfactants are surface-active amphipathic metabolites produced by several species of microorganisms. They have wide structural multiplicity, ranging from glycolipids, lipopeptides and lipoproteins to fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants. They reduce surface tension (ST) and critical micelle dilution (CMD) in both aqueous solution and hydrocarbon mixtures, thereby supporting 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 their 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 as well as evaluating the relationship between biofilm formation and biosurfactant production.

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.

2.2 Collection of Sample

Ten samples of sediment and water samples were collected randomly at ten (10) designated points in the three studied areas 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 wetted with the sediment and water samples before collecting them. 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 Isolation of Aromatic Hydrocarbon Degrading Bacteria

The hydrocarbon degraders were isolated from sediment and water samples of the three sampling sites using modified mineral basal agar (MBA) (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 °C and 15 psi for 15 minutes. Thereafter, 0.2 mL acetone solution containing 0.1 % w/v of the selected representative hydrocarbons (xylene, anthracene and pyrene) were aseptically pipetted and uniformly spread on the agar surface of the Petri dish plates. The acetone was allowed to evaporate under aseptic 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 inoculated plates were incubated at 28.00 ± 0.20 °C for 14 days [14, 15].

2.4 Preservation of Cultures

Colonies that developed on MBA plates were sub-cultured onto new MBA plates and incubated for another 14 days. Forty - eight isolates that grew on these plates were selected as xylene, anthracene and pyrene degraders. They were later sub - cultured on Bijou bottles and preserved at 4.00 °C in refrigerator [14].

2.5 Screening Test for the Most Potent Marine Degrading Bacterial Strains

In order to screen and chose the most potent degrading bacterial strains, the growths of the forty-eight isolates was tested by growing 5 mL of each isolates in large test tubes containing 25 mL of the modified mineral basal medium (MBM) augmented with 100 mg /L of xylene, anthracene and pyrene hydrocarbons which were dissolved in acetone (as previously stated) and added to each tube after autoclaving. Thereafter, the test tubes were incubated at 28.00 ± 2.00 °C for five days. After incubation, growths of bacterial cultures as indicated by turbidity were measured at 600 nm using a UV - VIS spectrophotometer (Astell, UV - Vis Grating, 752 W) and the cultures with the highest optical densities on each hydrocarbon in three studied areas were chosen as the most potent xylene, anthracene and pyrene degrading bacteria [14,16, 17].

2.6 Phenotypic Identification of Selected Hydrocarbon Utilizing Bacterial Strains

2.6.1 Morphological characteristics

2.6.1.1 Colonial morphology

The colonial properties such as shape, elevation, margin, optic, texture, colour, size and surface characteristics of the chosen 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 chosen 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 phenotypic characterization, the strains 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

In order to determine the concentration effects of the aromatic compounds on the growth of the isolates, 100 mL of MBM was dispensed into forty - five (45) 250 mL flasks and sterilized by autoclaving. The flasks were then distributed into nine sets of seven flasks. Thereafter, 50, 100, 200 and 300 ppm levels of xylene, anthracene and pyrene hydrocarbons which were individually dissolved in acetone (as previously stated) were exposed to each strains. The fifth, sixth and seventh flasks served as the controls for each the hydrocarbons (without aromatic compounds). Inoculated and control flasks were then incubated at 28.00 ± 2.00 °C for 5 days. After incubation, 5 mL of each sample was aseptically pipetted from each flask and assayed for their level of bacterial 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).

2.8 Measurement of Surfactant Production and Activity

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 measured in triplicates using xylene, anthracene and pyrene as hydrocarbon sources in MBM for 5 days at 28 ± 2 °C. Briefly, after centrifugation, 4 mL of n-hexane were added to 4 mL of the culture supernatants 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:

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

2.8.2 Surface tension measurement

For surface tension measurements in accordance to Viramontes- Ramos [25], the marine bacterial isolates were grown in MBM containing xylene, anthracene and pyrene hydrocarbons and centrifuged. After centrifugation, the supernatants were transferred to a glass tube and a capillary tube was dipped in this liquid. This procedure was done at 28 ± 2 °C. The height reached by the liquid through the 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 tested using the slight modified method of Abu and Chikere [3], and Thavasi *et al.* [22]. The bacterial isolates above were cultured in marine broth for 48 hrs to attain high cell density. Cultivation of cells without aromatic hydrocarbons treatment 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 vigorous shaken manually 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 Nutrient agar plates were inoculated in 3 mL of Brain Heart Infusion (BHI) medium with 1 % glucose and incubated for 24 hrs at 37 °C in stationary conditions and diluted in the ratio 1:10 with fresh medium. Individual wells of sterile 96 microplate wells 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 microtitre plate to serve as a control. After incubation for 24 hrs at 37 °C, the microtitre plates content of each well were removed by beating the bottom plates. In order to remove the planktonic bacteria, the wells were washed four times with 200 µL of phosphate buffer saline (1 × PBS pH 7.2). The plates were then inverted and blotted on paper towels and allowed to air-dry for 15 mins. The biofilm formed cells in the plates were fixed and stained with sodium acetate (2 %) and crystal violet (0.1 % w/v) and later incubated at 28 ± 2 °C for 15 mins. After draining of the crystal violet solution, wells were washed thrice with 1 × PBS in order to remove unbound dye. Finally, all the wells were filled each with 200 µL of 33 % (v/v) glacial acetic acid in order to resolubulized and separate 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 triplicate values were expressed as mean ± standard deviation. Test of significance at 95 % (*P* = .05) was conducted using ordinary one-way analysis of variance (ANOVA) followed by post Tukey's, multiple comparison test. Pearson correlation analysis were performed on the data obtained using Graph-Pad Prism version 7.00. [10, 16, 32].

3. RESULTS

3.1 Screening and Selection Test

The result of the growth profile (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 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 profile (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 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 profile (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 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 chosen as the most potent degraders of xylene, anthracene and pyrene hydrocarbons.

3.2 Taxon of the Degrading Bacterial Strains

The result of the colonial properties 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 microscopic and biochemical characteristics of the aromatic hydrocarbon degrading bacterial strains is presented in Table 5. From the result, most bacterial strains 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 highest 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 highest 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 highest 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 Biosurfactant Production and Activity

The result of the emulsifying activity of marine aromatic hydrocarbon degrading bacteria is shown in Figure 7. 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 6. 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 7. 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 8. 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 Biofilm Formation

The result of the biofilm formation by marine aromatic hydrocarbon degrading bacteria is shown in Figure 5. 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 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). Esedafe *et al*. [33] reported that an occurrence of 3/41 representing 7.32 % isolates

from refinery effluent were capable of utilizing phenanthrene and anthracene as sole carbon and energy sources.

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.* [34] who reported that a diverse microbial population can be isolated from hydrocarbon contaminated samples. 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 Irshaid and Jacob [35], Wanjohi *et al*. [36], Isiodu *et al.* [32] and Fagbemi and Sanusi [37] that two - third of most petroleum hydrocarbon degraders are Gram negatives with one - third being Gram positives but contradict the finding of 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 (Chikere *et al*. [12], Irshaid and Jacob [35], Wanjohi *et al*. [36], Isiodu *et al.* [32] and Fagbemi and Sanusi [37]) and similar result was obtained in this study.

The results in Figures 1, 2 and 3 revealed that the utilization and degradation of these compounds led to increase in optical density (cell mass) of the bacterial strains; however, increase in concentration of these hydrocarbons led to decline in optical density (cell mass) of the bacterial strains with strong significant negative relationship. It is obvious from the findings that most potent strains isolated on xylene (XYL2 and XYL8) and anthracene (ANT1) hydrocarbons were able to adapt and degrade better on the three tested aromatic hydrocarbons than the most potent strains isolated on pyrene (PYR3, PYR5 and PYR9) hydrocarbons. In general, the nine strains utilized and degraded all the aromatic hydrocarbons

demonstrating manifold and diverse catabolic possibilities but with different efficiencies. Previous studies revealed that the utilization and growth of bacterial strains on mono and poly - aromatic hydrocarbons is depended on the concentrations of these compounds and may lead to the concurrent acclimation to certain but not all structurally connected molecules [14, 38, 39]. The adaptive and catabolic (degradative) features of these strains demonstrate that they possess ring fission enzymes [40].

Surface action and emulsification capability of bacterial culture provide a solid indication of biosurfactant production [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*. [41] 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. 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*.. Mulligan [42] 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 [43]. The reports of these authors support the finding 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 hydrophobicity 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 [41]. In an early investigation, cells exhibiting highest hydrophobicity 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. 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 hydrophobicity and also on the properties of the hydrocarbons. 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.

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.* [44] 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. 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 profile 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 profile 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 profile of the aromatic hydrocarbon - degraders isolated from Onne sampled location

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

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

Table 4. Colonial properties of the aromatic hydrocarbon degrading bacterial strains

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

Table 5. Microscopic and biochemical properties of the aromatic hydrocarbon degrading bacterial strains

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 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

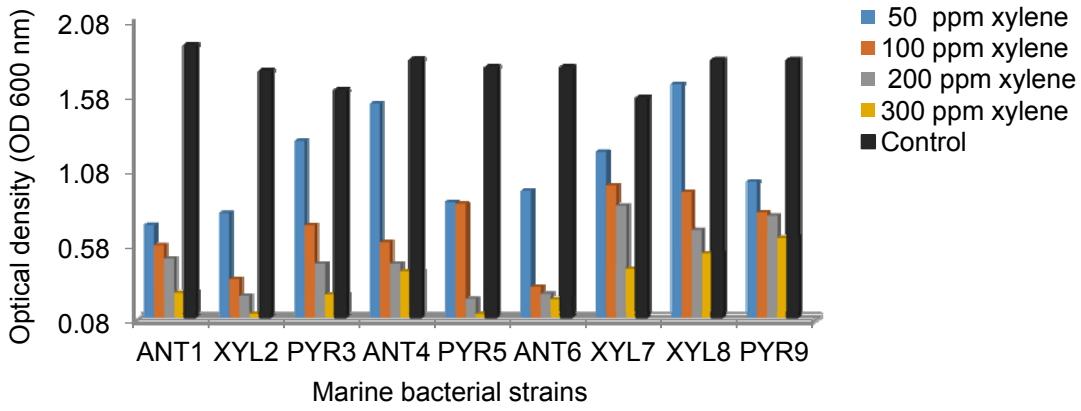

Fig.1.Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of xylene

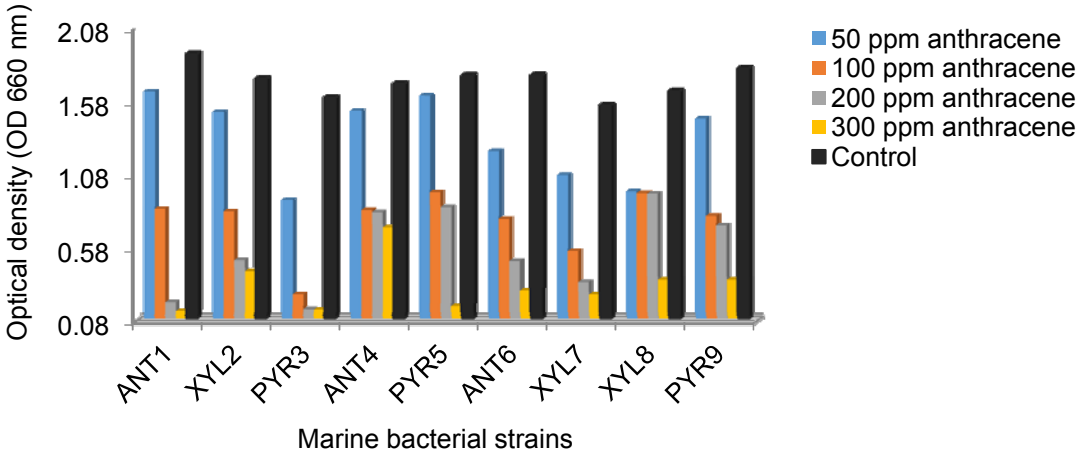

Fig. 2. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of anthracene

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

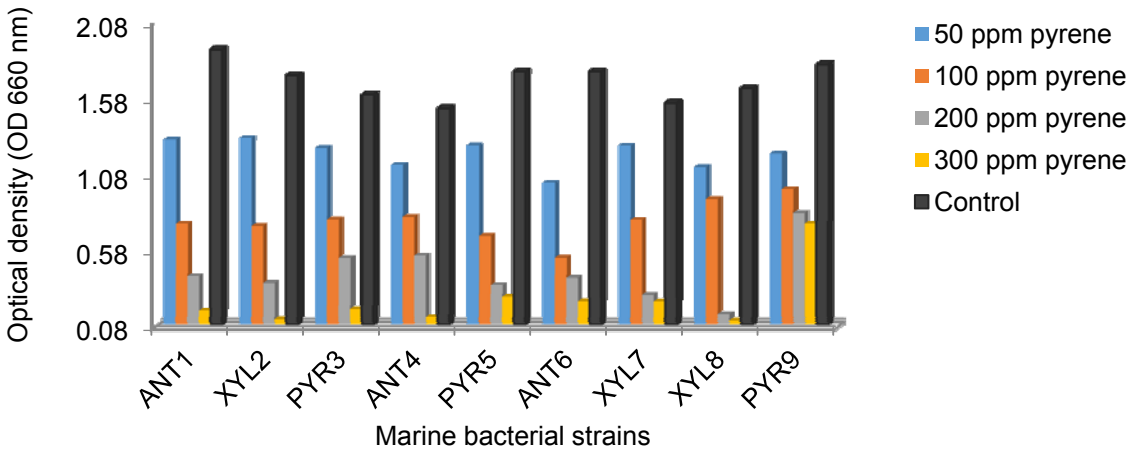

Fig. 3. Growth of the selected aromatic hydrocarbon degrading bacteria on different concentrations of pyrene

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

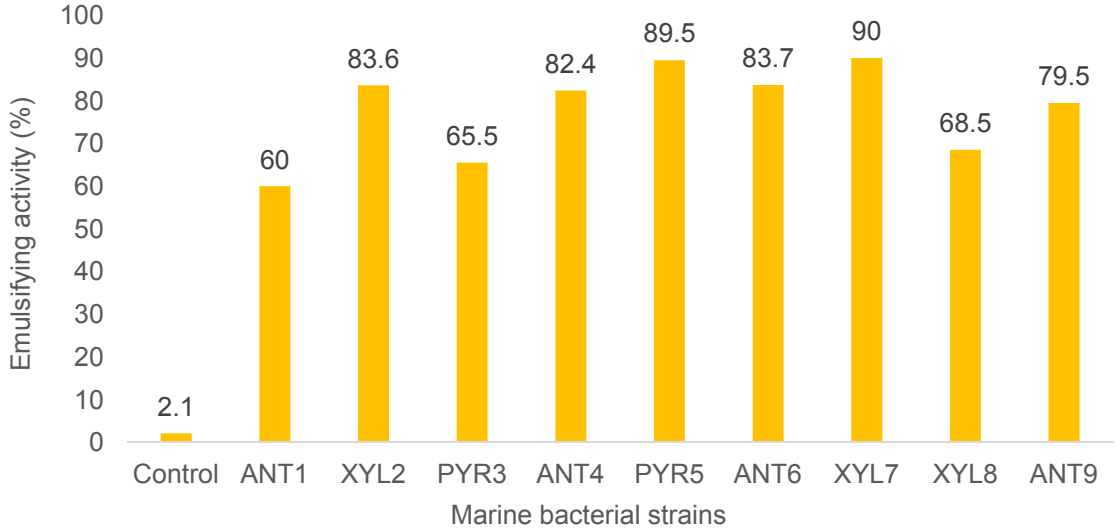

Fig. 4. Emulsifying activity of marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triplicate determination.

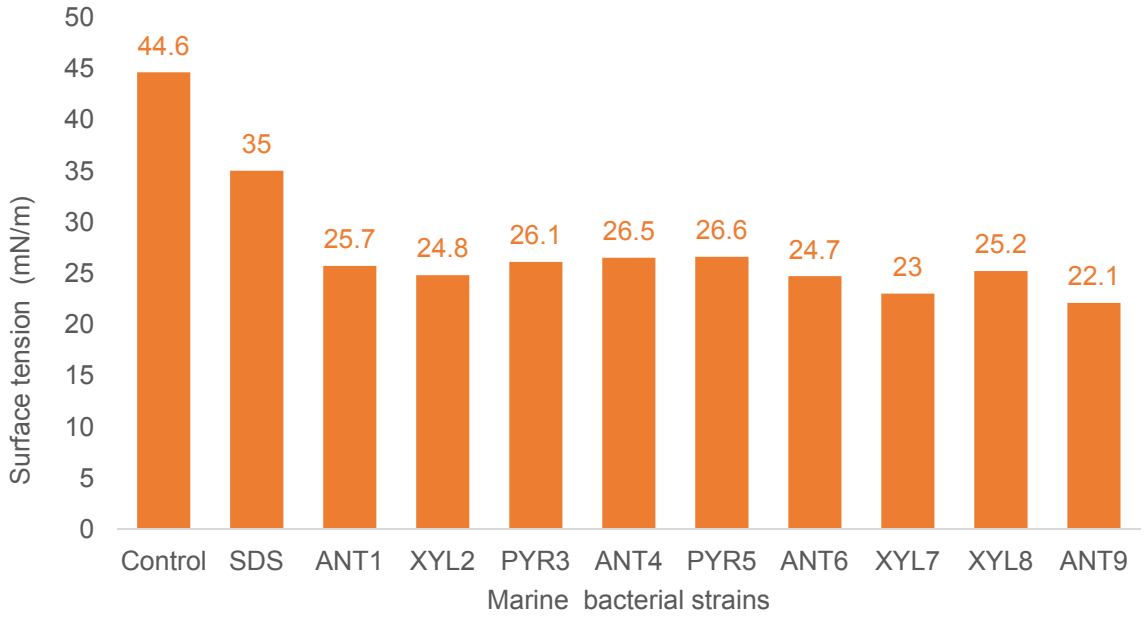

Fig. 5. Reduction in surface tension of the medium by marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triplicate determination.

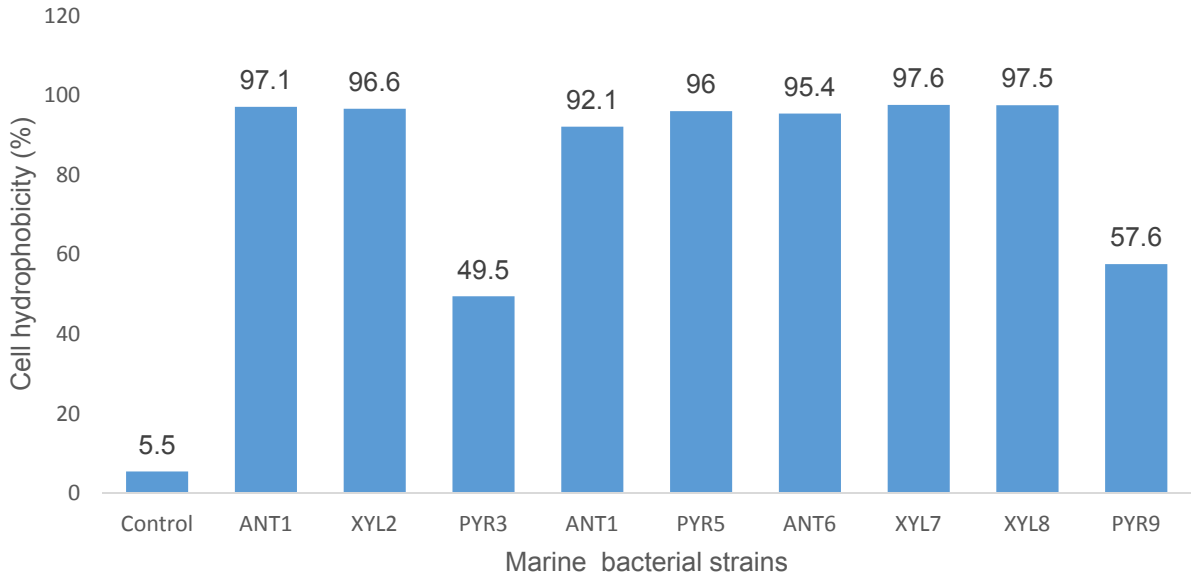

Fig. 6. Marine bacterial adherence to hydrocarbons (BATH) assay

Values are mean ± standard deviation of triplicate determination

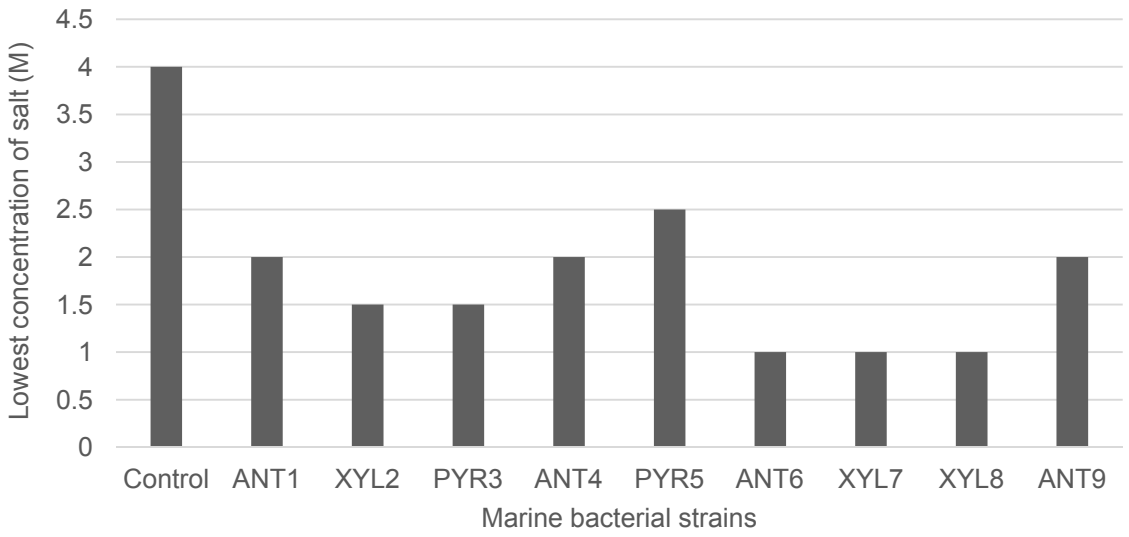

Fig. 7. Salt aggregation test (SAT) of marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triplicate determination.

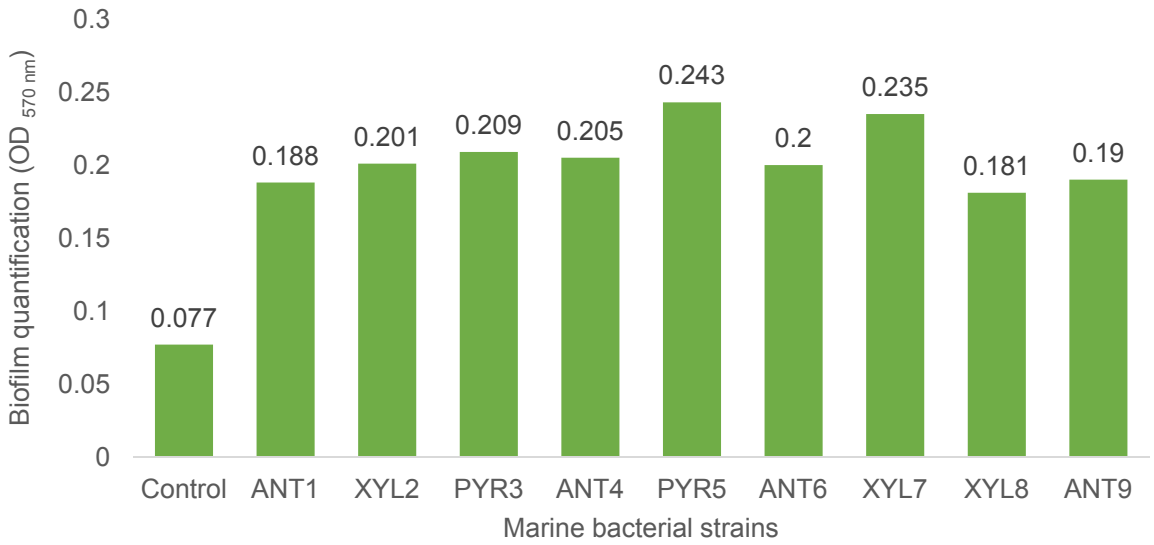

Fig. 8. Biofilm formation by marine aromatic hydrocarbon degrading bacteria

Values are mean ± standard deviation of triplicate determination

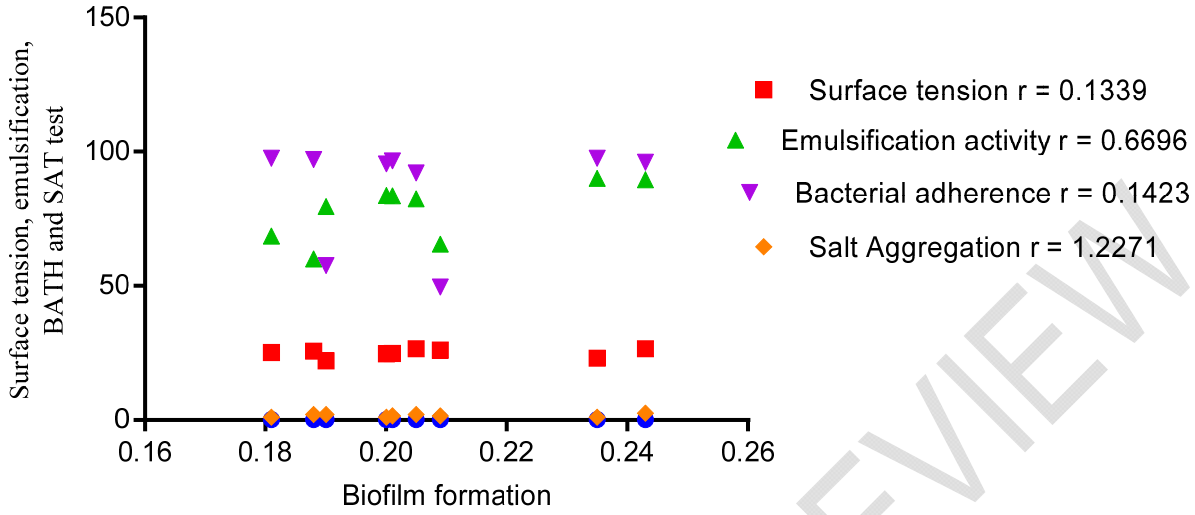

Fig. 9. Biofilm correlation between surface tension, emulsification and cell hydrophobicity

5. CONCLUSION

The entire study revealed that the three studied areas are reservoirs of potential aromatic hydrocarbon degrading bacterial strains belonging to the genera: *Providencia, Alcaligenes, Brevundimonas, Myroides, Serratia, and Bacillus.* It also revealed that the isolated bacteria were able to utilize and degrade mono and poly - aromatic hydrocarbons. These strains were found to be good formers and producers of biofilm and biosurfactant signifying their promising utilization in future biotechnological purposes, either directly as field - released microorganisms or as biofilm formers and biosurfactant producers under regulated settings.

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