**A STUDY OF OCEAN-SOURCE BIOSURFACTANT-PRODUCING AND PETROLEUM HYDROCARBON DEGRADING BACTERIAL RESOURCE OF THE ATLANTIC OCEAN, ARAROMI SEA-SIDE, ONDO STATE, NIGERIA**

**Abstract**

Bioavailability of hydrocarbons to microorganisms is a serious threat to the efficiency of biodegradation process for the decontamination of polluted environments. The objective of this research is to characterize ocean-source biosurfactant-producing bacterial resource with potential to degrade hydrocarbons. The oceanwater sample was collected from the Atlantic Ocean on latitude 6.3250338 and Longitude 4.4834361 in Ilaje Local Government Area, Ondo State, Nigeria. The physicochemical properties of the sample were estimated by standard physical and chemical analytical techniques while GC-MS analysis was used for the determination of TPHs. Quantification and identification of bacterial isolates were carried out by standard morphological, biochemical and molecular methods based on the 16S rRNA gene sequencing. Four autochthonous marine-source bacteria were evaluated for their petroleum hydrocarbon utilization potentials by spectrophotometry method at 600 nm wavelength of culture in Bushnell Haas medium fortified with 2% petroleum hydrocarbon and then assayed for biosurfactant activity by oil collapse, foaming activity, emulsification and oil drop tests. The population of hydrocarbon degrading bacteria was 9.80×104 - 3.00×105 (CFU/mL) constituting 2.09 – 6.38% of the total heterotrophic bacterial population. *Lysinibacillus fusiformis* (OM179766.1) *Citrobacter werkmanii* (CP019986.1), *Lysinibacillus sphaericus* (CP015224.1) and *Pseudomonas putida* (MT604992.1) with 94.07-99.40% percentage homology were identified from the sample and exhibited varying responses to petroleum derivatives. The isolates exhibited positive responses to biosurfactant activity except *Citrobacter werkmanii* which showed negative response to drop collapse test. The results of this study conclude that there exist in the marine ecosystem diversity of biosurfactant-producing bacteria that can be exploited to enhance pollutant bioavailability for the remediation of petrochemical wastewater.

**Keywords:** Bacterial resource, Biosurfactant, Bioavailability, Biodegradation, Ocean-source, Petroleum hydrocarbons

**1. Introduction**

The environment is frequently contaminated by different types of pollutants which vary in type, nature, structure, toxicity and persistence. Petroleum and its products are major sources of energy to drive the economy and our daily activities (Ikuesan *et al*., 2022). Pollution of land and sea by petroleum hydrocarbons is an inevitable consequence of diverse anthropogenic activities of exploration, exploitation, transportation, distribution activities, oil bunkering and accidental spills which compromises environmental quality with significant threat to all forms of biomass dependent on the environment through toxicity, bioaccumulation, and habitat destruction.

The restoration of hydrocarbon contaminated environment (soil and water) requires the elimination and removal of the pollutants through physicochemical and biological techniques. The physicochemical methods of environmental restoration such as *in-situ* burning, thermal desorption, coker, chemical oxidation, solvent extraction and excavation have process dependent limitations including ineffectiveness, high cost of application, destruction of soil structure and secondary pollution effects (Jain *et al*., 2011) and are usually labour intensive. The biological strategies of environmental remediation also called bioremediation are ecofriendly, relatively cost effective, simple and leverages on the diverse catabolic activities of certain microorganisms to metabolize different hydrocarbons pollutants to fulfil their nutritional requirements (Dzionek *et al*., 2016; Farber *et al*., 2019). The removal or detoxification of contaminants by biological techniques can be achieved by the stimulation of autochthonous microorganisms through supplementation with nutrient or nutrient-rich materials (Boboye *et al*., 2023) and bioaugmentation with known pollutant degraders to synergize with the indigenous microbial population. Al-Marri *et al*. (2023) reported that microbial communities exposed to polluted environment possess efficient biodegradation potential due to their genetic and metabolic adjustments over time in extreme environments.

Biodegradation process is influenced not only by factors such as the nature and the population of indigenous microorganisms and physicochemical conditions but also by the nature and bioavailability of xenobiotics. Sajna *et al*. (2015) and Chebbi *et al*. (2017) reported that low bioavailability of hydrocarbons to the microorganisms is a serious threat to the efficiency of biodegradation process. However, enhancement of degradation can be achieved byincreasing bioavailabilityof organic contaminants to the microflora through the addition of biosurfactants to the polluted medium. Biosurfactants are surface-active molecules with both hydrophilic and hydrophobic moieties that are either present on the surface of the microorganisms or secreted extracellularly as secondary metabolites under certain conditions (Vijayakumar and Saravanan, 2015; Santos *et al*., 2016; Sarubbo *et al*, 2022). The unique property of biosurfactants is their ability to minimize surface and interfacial tension between two phases such as oil and gas thereby causing them to emulsify and spread easily as emulsions in water or other liquids (Sarubbo *et al* 2022; Hassanshahian and Ravan; 2018). The distinctive qualities of microbial surfactants over chemical surfactants are linked to their surface activity, pH tolerance, temperature and ionic strength, susceptibility to microbial action, low-toxicity, emulsifying and demulsifying ability and [antimicrobial activity](https://ascidatabase.com/result.php?searchin=Keywords&cat=&ascicat=ALL&Submit=Search&keyword=antimicrobial+activity) (Fakruddin, [2012](https://scialert.net/fulltext/?doi=jm.2015.181.192#19862_tr)). Chebbi *et al*. (2017) reported that some bacteria growing on hydrocarbons demonstrated the capability to produce biosurfactants that can efficiently dissolve hydrocarbons in the water phase making them accessible and predisposed to biodegradation.

Several groups of microorganisms including by bacteria, fungi and yeasts with ability to degrade complex pollutants due to a broad array of catabolic activities have been identified from different ecological habitats. Marine microbial resources have unmatched physiological properties and novel metabolites such as biosurfactant to inhabit unusual environment such as petroleum tainted ecosystem (Hassanshahian and Ravan, 2018). Ocean-source biosurfactant-producing bacteria, in particular, have shown significant potential in enhancing petroleum hydrocarbon degradation in marine environments (Silva *et al*., 2014). Marine environments, with their high salinity, variable pressure, and nutrient scarcity, are home to a unique set of microbial communities that have adapted to these challenging conditions. These challenges favor leveraging on readily biodegradable and environmentally innocuous biosurfactants to reduce the severity of petroleum hydrocarbon (Hassanshahian and Ravan, 2018). Ocean-source bacteria that produce biosurfactants and evolved to utilize hydrocarbons as their primary carbon source, especially in oil-contaminated waters include species of *Alcanivorax*, *Pseudomonas*, *Rhodococcus*, *Bacillus*, and *Marinobacter* (Martinotti *et* *al*., 2010). *Alcanivorax borkumensis* is one of the well-studied marine hydrocarbon degraders, recognized for its ability to produce glycolipid biosurfactants. Marine *Pseudomonas* species produce rhamnolipids, a type of glycolipid biosurfactant known for its efficiency in reducing surface tension and emulsifying hydrophobic compounds. These bacteria can degrade several hydrocarbons, including those with aromatic rings, making them versatile in oil spill bioremediation (Das and Chandran, 2011). *Rhodococcus erythropolis* is known for theproduction of trehalolipids, *Rhodococcus* species can degrade aliphatic and aromatic hydrocarbons, especially in cold and saline conditions. Biosurfactants produced by *Rhodococcus erythropolis* enhance the solubilization of hydrophobic pollutants, promoting biodegradation in marine ecosystems (Perfumo *et al*., 2006). These marine bacteria not only survive but thrive in the presence of hydrocarbons due to their unique metabolic pathways and biosurfactant production, which allow them to outcompete other microorganisms in oil-contaminated waters (Silva *et al*., 2014). The effectiveness of microbial surfactant-producing bacteria in degrading petroleum hydrocarbons lies in their ability to increase the bioavailability of these hydrophobic compounds based on the mechanisms of enhanced solubilization and emulsification, increased microbial adhesion, micellization and hydrocarbon uptake and enzymatic degradation (Banat *et al*., 2010). Hence, there is a growing interest in exploring eco-friendly and sustainable approaches, such as bioremediation using biosurfactant-producing bacteria sourced from oceanic environments. The ultimate aim of this research is to isolate and characterize ocean-source biosurfactant-producing bacterial resource with potential for hydrocarbon utilization.

**2. Materials and methods**

**2.1 Collection of Oceanwater Sample**

Oceanwater samples from the Atlantic Ocean at Araromi Sea-Side (6.3250338 Latitude, and 4.4834361 Longitude) in Ilaje Local Government, Ondo State. The oceanwater sample was collected in sterile glass bottle and transported in ice chest maintained at 4oC for analyses.

**2.2 Physicochemical and Total Petroleum Hydrocarbons Analyses of the Assayed Oceanwater Sample**

The standard physical and chemical analytical techniques by (Ekpo and Ebeagwu, 2009; Obayori *et al*., 2008; Osuji and Nwoye, 2007; APHA, 2005) were used to estimate the physical and chemical properties of the oceanwater sample. Determination of the levels of TPHs in the sample was carried out using GC-MS by operating MSD in selective ion monitoring (SIM) and Scan mode to ensure low level detection of the target constituents. Agilent 7820A gas chromatograph coupled to 5975C inert mass spectrometer (with triple axis detector) with electron-impact source (Agilent Technologies) was used. After calibration, the samples were analyzed and corresponding TPH concentration obtained.

**2.3 Microbiological Analysis of Sample**

**2.3.1 *Media Preparation***

Dehydrated (28 g) of nutrient agar medium was prepared in accordance with manufacturer’s instructions and Bushnell Hass mineral salt medium (BHA) was composed in line with the specification of Borah and Yadav (2017) by dissolving K2HPO4 (1.0 g/L), FeCl3. (0.05 g/L), NH4NO3 (1 g/L), MgSO4 (0.2 g/L), KH2PO4 (1.0 g/L), CaCl2 (0.02 g/L), agar- agar (20 g/L), in distilled water (1000 mL). Both media were then stirred to properly dissolve and poured (20 mL) into MacCartney bottles and autoclaved at 121oC for 15 minutes, fortified with 10 mg/l fungisol and then maintained at about 44oC. The MSM was supplemented with 1% (v/v) membrane filter (0.22 μm) sterilized petroleum derivative (diesel, kerosene and petrol) to serve as the only carbon and energy source (Ikuesan *et al*., 2022).

**2.3.2 *Enumeration of Bacteria from the Oceanwater Sample***

Exactly one milliliter (1 mL) aliquot of serially diluted (10-1-10-5) oceanwater sample into sterile Petri dishes and then separately overlaid them with pre-sterilized NA and BHA. The plates were allowed to gel and then incubated at 37°C for 48 hours and seven days respectively for total heterotrophic and hydrocarbon degrading bacteria (Veerapagu *et al*., 2019). After incubation, the number of colonies within the range of 30-300 that developed on the NA and BHA plates were counted as the total heterotrophic (THC) and hydrocarbon degrading bacterial (THD) counts, expressed as colony forming unit per milliliter (CFU/mL) of sample respectively. Distinct colonies on BHA plates were sub-cultured by repeated streaking on fresh NA medium obtain pure cultures and then maintained on agar slants at 4oC.

**2.3.3 Screening of test isolates for biosurfactant production**

Each bacterial isolate obtained from the oceanwater sample was grown in 100-ml Erlenmeyer flasks containing 50 ml Bushnell Haas medium (BHM) supplemented with 1% diesel and then, incubated at 35°C for 72 h. The cultures in BHM were centrifuged at 7000×g for 10 min (Gomaa and El-Meihy, 2019) and cell-free supernatant were used to assay for surface-active compounds by the Drop collapse, Oil spread, Emulsification activity (E24) and foaming activity assay methods.

**2.3.4 Drop collapse test**

A thin film of diesel oil was created on oil-free slide and one drop of the supernatant was then introduced onto the diesel oil-coated surface and then observed after 1 minute for oil collapse and spread or was rejected by the oily surface. If the drop spreads or collapses, it indicates the presence of biosurfactant, as biosurfactants reduce surface tension, causing the drop to collapse. A positive result indicates biosurfactant production, while a stable drop suggests the absence of biosurfactant. (Lei *et al*., 2023).

**2.3.5 Oil spread assay**

The oil spread assay was performed following the procedure of Youssef *et al*. (2004) and Sharma *et al*. (2014). I added 100 µL diesel oil unto 50 mL distilled water in a clean glass Petri dish, followed by adding 10µL of the cell-free culture supernatant to the oil surface. The appearance of a clear zone (or halo) around the supernatant drop implies biosurfactant activity and diameter of the clear zone was measured. The larger zone is indicative of higher biosurfactant activity.

**2.3.6 Emulsification activity (E24)**

Emulsification activity was determined by the procedure described by Ibrahim (2016). Two milliliters (2 mL) of hydrocarbon (diesel oil) to 2 ml of culture supernatant in a culture tube and then mixed thoroughly for 2 minutes with a vortex, and then allowed to stand for 24 hours. The height of emulsified layer and entire height of the liquid column (in millimeters) were measured and the emulsification index (E24) was calculated as the percentage of the height of the emulsion layer relative to the total height of the liquid column.

E24 (%) = (Height of emulsion layer / Total height of liquid) × 100

# **2.3.7 Foaming Activity in Biosurfactant Assay**

This assay determines whether the biosurfactant produced by the isolates in the culture medium has foaming properties. Foaming activity of the isolates was assessed by the procedure described by Borah and Yadav (2017). Cell-free soup of overnight nutrient broth culture was used to assess the ability of a biosurfactant to generate and stabilize foam when agitated. Two milliliters (2 ml) of the supernatant were introduced in a test-tube and vigorously agitated for 2 minutes to generate foam and allowed to stand for 1 minute and observed for steady foam. Broth without cells was employed for control. The foaming activity based on percentage foam formation was quantified by measurement of the initial foam height and the foam stability over time using the formula:

Foaming (%) = (height of the foam layer/total height) x 100

Higher and more stable foam formation suggests stronger biosurfactant production and effectiveness.

**2.3.8 *Evaluation of Hydrocarbon Degradation Potential of Biosurfactant – Producing Bacterial Isolates from the Assayed Oceanwater***

This was performed by measuring the significant growth of the isolates in BHM separately enriched with 1% (v/v) diesel oil, Kerosene and petrol (Ikuesan and Olugbode, 2023; Olukunle *et al*., 2020) and then incubated on a rotary shaker at 35oC and 110 rpm in 200 mL Erlenmeyer flasks for 15 days. I measured the optical density of triplicate samples of bacteria culture in BHM after 6 hours of incubation as day 1 using the spectrophotometer at 600 nm wavelength and subsequently at 2 days interval to determine microbial growth and petroleum hydrocarbon utilization potential of the isolates.A set of uninoculated BHM supplemented with petroleum hydrocarbon was served as control.

**2.3.9 *Characterization and Identification of Hydrocarbon Degrading Bacterial Isolates from the Assayed Oceanwater***

Four bacterial isolates that exhibited best growth pattern on BHA were selected and purified by repeated unto fresh nutrient agar. Purified microbial colonies were then identified using cultural, morphological and biochemical characteristics. Biochemical tests carried out include; catalase test, citrate test, hydrogen sulphide (H2S) production, indole, urease, methyl red, VP test, motility, starch hydrolysis and sugar fermentation using Cheesbrough (2006) and Holt *et al*., 1994 as standard references.

**2.3.10 *Molecular Identification of the Bacterial Isolates***

The molecular identification of bacterial isolates was performed using 16S rRNA sequencing, following DNA extraction with commercial DNA extraction kits (Zymo Research). The concentration of the extracted bacterial DNA was measured using a Nanodrop at 260 nm and 280 nm, while the quality and size of the DNA were assessed using 1% agarose gel electrophoresis and visualized under a UV light source (Ikuesan *et al*., 2020). Amplification of the extracted DNA with appropriate primers, sequencing, sequence analysis, and phylogeny construction were carried out following the methods described by (Ikuesan *et al*, 2020; Kumar *et al*., 2016; Tamura *et al*., 2004)

**3. RESULTS**

**3.1 Physicochemical and Heavy Metal Composition of Oceanwater of Araromi Sea-side**

The results of the different physicochemical parameters of the assayed oceanwater sample presented in Table 1 indicated that the pH of the ocean water was 8.23 and the electrical conductivity of 175.34 (µS/cm) suggesting slight alkalinity and relatively high electrical conductivity respectively. The DO, BOD and COD were in relatively high amount of 6.68 (mg/L), 10.93(mg/L) and 12.46 (mg/L). The mineral elements and heavy metals shown in Fig. 1 also vary and occurred in the range 10.65 -45.66 (mg/L) and 0.017-1.323 (mg/L) respectively with the concentration of iron being highest among the metals.

**3.2 Quantification of Total Petroleum Hydrocarbon in the Oceanwater of Araromi Sea-side**

The gas chromatographic-mass spectrometry analysis of the sample for TPH are shown in Table 2 and the Total Ion Chromatography (TIC) in Fig. 2. The results revealed varying concentration among the 30 target compounds at the retention time of 3.894-29.288 mins. The target TPH in the oceanwater sample vary in their concentrations in the range of 0.01-1.33 (mgL) with Hentriacontane having the highest concentration of 1.33 (mg/L) and Q value of 26 as shown in Table 2. The Q value also called quality value was highest (87) for Octacosane while Nonane, Dodane and Tetradecane had Q values of 76, 69 and 69 respectively as indicated in Table 2

Table 1: Physicochemical Composition of Oceanwater of Araromi Sea-side

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Parameter | | | | | | | |
| pH | EC (US/cm) | Color (Hazen) | TDS  (mg/l) | TSS  (mg/l) | Turbidity  (NTU) | Salinity  (mg/l) | T. Hard.  (mg/l) |
| 8.233±  0.002 | 175.335±  0.031 | 5.000±  0.000 | 97.285±  0.005 | 1.315±  0.002 | 6.936±  0.006 | 9.249±  0.003 | 110.517±  0.004 |
| Acidity  (mg/l) | T. Alkalinity  (mg/l) | Chloride  (mg/l) | DO  (mg/l) | BOD  (mg/l) | COD  (mg/l) | Nitrate  (mg/l) | Phosphate  (mg/l) |
| 33.751±  0.001 | 142.612±  0.003 | 6.879±  0.004 | 6.679±  0.004 | 10.928±  0.005 | 12.462±  0.003 | 1.393±  0.005 | 0.884±  0.003 |

Figure 1: Mineral and Heavy Metal Composition of Oceanwater of Araromi Sea-Side

Table 2: Quantification of Total Petroleum Hydrocarbon in the Oceanwater of Araromi Sea-side

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| S/N | Target Compound | R. T | Concentration (mg/l) | Q value |
| 1 | Nonane | 3.894 | BC | 76 |
| 2 | Decane | 5.370 | BC | 55 |
| 3 | Undecane | 6.840 | BC | 1 |
| 4 | Dodecane | 8.317 | 0.02 | 69 |
| 5 | Tridecane | 9.719 | BC | 1 |
| 6 | Tetradecane | 11.063 | BC | 69 |
| 7 | Pentadecane | 12.305 | BC | 1 |
| 8 | Hexadecane | 13.455 | 0.02 | 1 |
| 9 | Heptadecane | 14.840 | 0.07 | 12 |
| 10 | Pentadecane, 2,6,10,14... | 14.840 | 0.05 | 3 |
| 11 | Octadecane | 15.601 | 0.02 | 12 |
| 12 | Hexadecane, 2,6,10,14-... | 15.601 | 0.06 | 24 |
| 13 | Nonadocane | 16.722 | BC | 8 |
| 14 | Eicosane | 17.667 | 0.01 | 27 |
| 15 | Heneicosane | 18.273 | 0.04 | 5 |
| 16 | Docosane | 19.480 | 0.09 | 63 |
| 17 | Tricosane | 20.264 | 0.43 | 48 |
| 18 | Etracosane | 0.000 | ND | - |
| 19 | Pentacosane | 21.958 | 0.12 | 1 |
| 20 | Hexacosane | 22.805 | 0.06 | 46 |
| 21 | Heptacosane | 23.503 | 0.13 | 30 |
| 22 | Octacosane | 24.253 | 0.05 | 87 |
| 23 | Nonacosane | 24.934 | 0.09 | 33 |
| 24 | Triacontane | 25.328 | 0.22 | 22 |
| 25 | Hentriacontane | 26.278 | 1.33 | 26 |
| 26 | Dotriacontane | 26.696 | 0.68 | 22 |
| 27 | Tritriacontane | 27.463 | BC | 33 |
| 28 | Tetratriacontane | 28.041 | 0.11 | 58 |
| 29 | Pentatriacontane | 28.682 | 0.01 | 32 |
| 30 | Hexatriacontane | 29.288 | 0.85 | 63 |

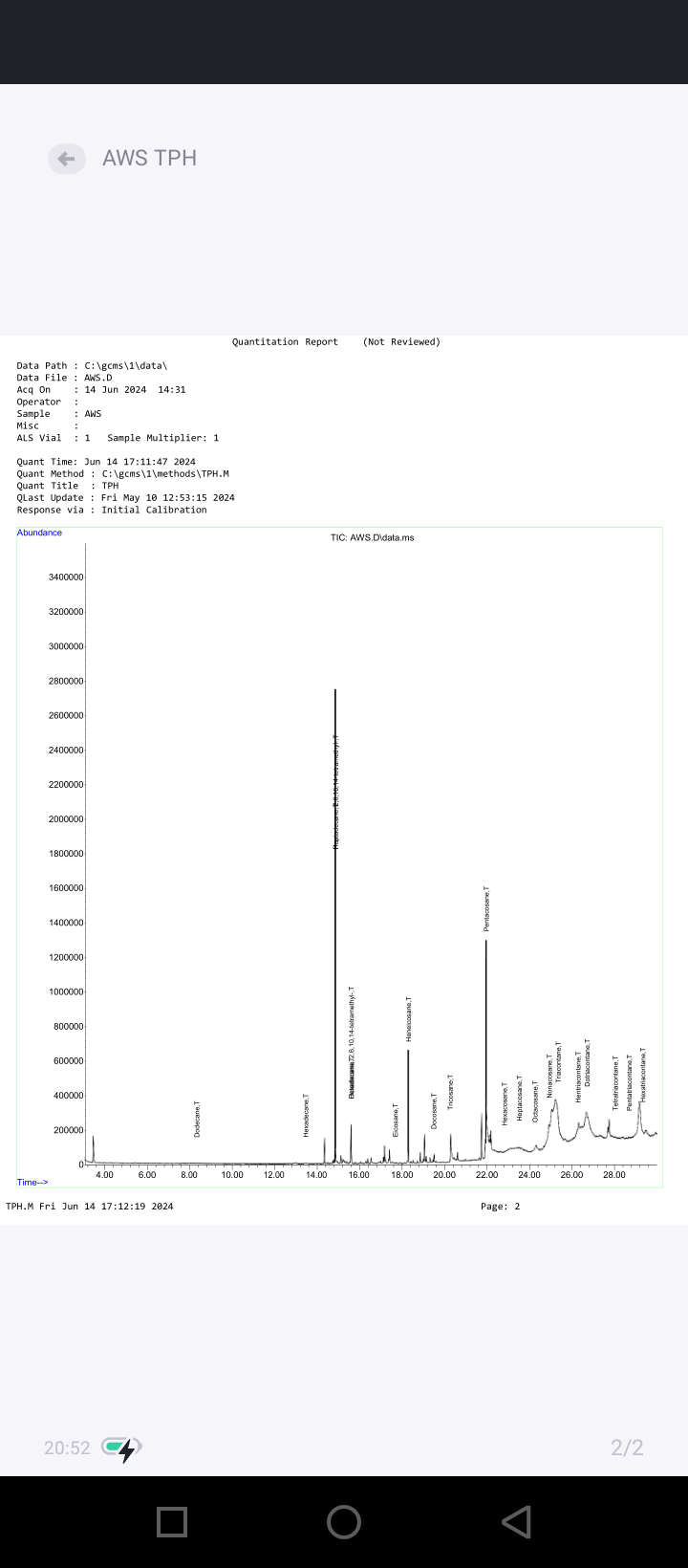


Figure 2: Total Ion Chromatography (TIC) of the GC-MS analysis of the oceanwater sample

**3.3 *Load and Identity of Bacteria in the Assayed Oceanwater Sample***

The load of heterotrophic (THB) and hydrocarbon utilizing (TUB) bacteria are presented in table 3. Results revealed that using diesel oil, kerosene and petrol as choice petroleum hydrocarbon, the HUB population in the oceanwater sample were 3.00×105 (CFU/mL), 2.67×105 (CFU/mL), and 9.80×104 (CFU/mL) representing 6.38%, 5.68% and 2.09% for diesel, kerosene and petrol respectively relative to the total heterotrophic load of 4.7×106 (CFU/mL). Two species of *Lysinibacillus*, *Citrobacter* sp. and *Pseudomonas* sp. bacteria were tentatively identified based on their morphological and biochemical characteristics. Among these bacteria, *Citrobacter* sp. and *Pseudomonas* sp. were non-sporing motile rods, Gram negative, negative to indole, urease tests, but positive to catalase, nitrate reaction and citrate reactions. The bacterial species identified as *Lysinibacillus* were spore forming Gram positive motile rods, negative to indole and nitrate reduction tests but showed positive reactions to urease, catalase, citrate tests.

**Table 3:** Bacterial Population of the Assayed Oceanwater Sample

|  |  |  |
| --- | --- | --- |
| **Bacterial Type** | **Load (CFU/mL)** | **% Hydrocarbon Utilizer** |
| THB | 4.7×106 | NA |
| DUB | 3.00×105 | 6.38 |
| KUB | 2.67×105 | 5.68 |
| PUB | 9.80×104 | 2.09 |

**Legend:** THB; Total Heterotrophic Bacteria, DUB; Diesel Utilizing Bacteria, KUB; Kerosene Utilizing

Bacteria, PUB; Petrol Utilizing Bacteria

**3.4 *Assay for Biosurfactant Production by Oceanwater Isolated bacteria***

The four oceanwater bacterial isolates screened for biosurfactant production demonstrated positive responses of varying degree to oil spread, emulsification activity, drop collapse test and foaming activity as shown in Table 4. The result shows that among the isolates, *Lysinnibacillus* (AQE 1) exhibited the least foaming activity of 26% and *Pseudomonas* sp. (AQE 4)had the foaming activity of 66% while *Lysinibacillus* sp. (AQE 3) followed by *Pseudomonas* sp*.* (AQE 4) demonstrated the highest emulsification (E24) of 83% and 76% respectively. The oil spreading assay was highest (8.1) with AQE 2 and all the isolates except AQE 2 showed positive responses to drop collapse test.

**Table 4: Biosurfactant activity of ocean-source bacteria**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Isolate Code** | Oil spread (mm) | E24 (%) | Drop collapse | Foaming activity (%) |
| AQE 1 | 6.7 | 74 | + | 26 |
| AQE 2 | 8.1 | 44 | - | 56 |
| AQE 3 | 6.0 | 83 | + | 60 |
| AQE 4 | 6.5 | 76 | + | 66 |
| Control | 0.00 | 0.00 | 0.00 | 0.00 |

**Legend:** AQE 1**;** Lysinibacillus sp. AQE 2; Citrobacter sp., AQE 3; Lysinibacillus sp.,

AQE 4; *Pseudomonas* sp*.*

**3.5 Hydrocarbon Utilization Potential of Biosurfactant- Producing Bacterial Resource from the Assayed Atlantic Oceanwater**

The petroleum hydrocarbon utilization potentials of the four bacterial isolates of Atlantic Ocean- origin determined by measurement of optical density at 600 nm is shown in Fig. 3-5. The individual biosurfactant-producing isolates exhibited variation in their potentials to metabolize diesel, kerosene and petrol as sole source of carbon for growth during the study period with *Pseudomonas* sp. (AQE 4) exhibiting highest ability from day 9-15

Figure 3: Diesel Oil Utilization Potential of Biosurfactant- Producing Bacteria from the Assayed Oceanwater.

Figure 4: Kerosene Oil Utilization Potential of Biosurfactant- Producing Bacteria from the Assayed Oceanwater.

Figure 5: Petrol Oil Utilization Potential of Biosurfactant- Producing Bacteria from the Assayed Oceanwater.

**3.6 Molecular characterization and identity of the bacterial isolates from the assayed oceanwater**

Molecular identification of the four oceanwater bacterial resource were performed by amplification and sequencing the 16S rRNA gene sequencing and comparison with the NCBI database of known 16S rRNA sequences. Plate 1 shows the photographic image of an agarose gel indicting the amplification of the 16S target region. Fig. 1 shows the nanodrop graph to determine the purity of the bacterial strains at A 260/A280. The concentration and purity of the extracted DNA of bacterial isolates shown in table 2 were 274.1-443.4 (ng/µL) and 1.91-1.93 (A260/A280) respectively. The amplicons were about 300 base pairs relative to the molecular ladder. The information on the molecular characteristics and identity revealed that these biosurfactant- producing and hydrocarbon degrading bacterial strains had sequence length (base pair) of 1 512bp, 1 510bp, 1 940bp and 693bp (Table 3). The phylogenetic tree revealed clustering patterns that correspond to the evolutionary and taxonomic relationships of the studied organisms. The inclusion of closely related sequences enabled the identification of meaningful evolutionary linkages and highlighted divergence points among the isolates and their homologues. From the phylogenetic tree constructed, Fig. 2 shows the evolutionary relatedness of the isolates when clustered and compared with 24 other nucleotide sequences in the NCBI. The bacteria strains were identified as *Lysinibacillus fusiformis* (OM179766.1), *Citrobacter werkmanii* (CP019986.1), *Lysinibacillus sphaericus* (CP015224.1) and *Pseudomonas putida* (MT604992.1)respectively and and percentage identity in the range 94.07 – 99.40%.

0

5

10

10

mm Absorbance

220

240

260

280

300

320

340

Wavelength (nm)

AOE 1

AOE 2

AOE 3

AOE 4

Figure 1: Nanodrop graph of extracted genomic DNA purity

Table 5: Concentration and purity of extracted genomic DNA of the ocean-source bacterial isolates.

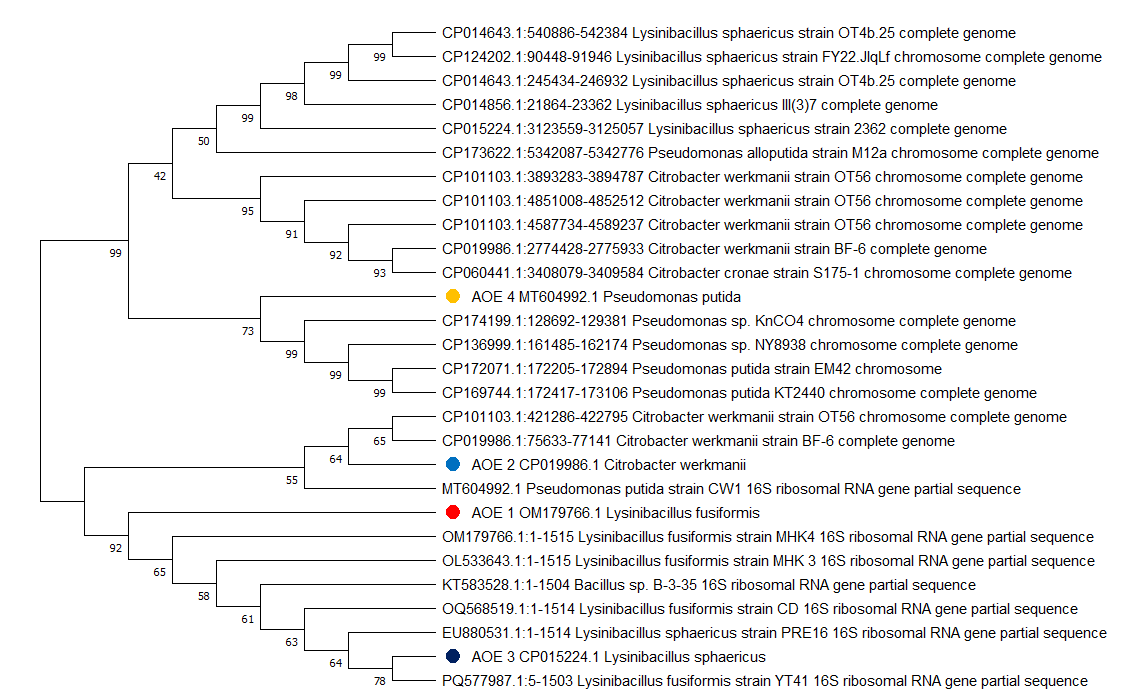
|  |  |  |  |
| --- | --- | --- | --- |
| Sample ID | Isolates | Concentration ng/µL | Absorbance (A260/A280) |
| AOE1 | *Lysinibacillus fusiformis* | 443.4 | 1.91 |
| AOE2 | *Citrobacter werkmanii* | 414.6 | 1.93 |
| AOE3 | *Lysinibacillus sphaericus* | 360.7 | 1.91 |
| AOE4 | *Pseudomonas putida* | 274.1 | 1.92 |



Figure 1: Photographic image of an agarose gel indicting the amplification of the 16S target region.

Table 6: The identities of isolates based on 16Sr RNA sequencing

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Isolate code | Predicted organism | Sequences length (bp) | % identity | Ascension no of Blast hit (NCBI) |
| AOE 1 | *Lysinibacillus fusiformis* | 1 512 | 94.07% | OM179766.1 |
| AOE 2 | *Citrobacter werkmanii* | 1 510 | 99.40% | CP019986.1 |
| AOE 3 | *Lysinibacillus sphaericus* | 1 940 | 96.73% | CP015224.1 |
| AOE 4 | *Pseudomonas putida* | 693 | 98.41% | MT604992.2 |



**Figure 1:** Phylogenetic tree of 16S rRNA sequences of the four bacterial resource isolated obtained from the Atlantic Oceanwater sample. The tree was constructed using sequences of comparable region of the 16S rRNA gene sequences available in NCBI databases. Neighbor-joining analysis using 1,000 bootstrap replicates was used to infer tree topology.

**4. Discussion**

The marine ecosystem is complex and mainly affected by various physical, chemical and biological factor. Temperature, pH, dissolved oxygen, total suspended and dissolved solids, total alkalinity and acidity and heavy metal contaminants are among important physicochemical parameters that influence the survival of aquatic organisms. The physiological conditions can vary and be impaired by different types of environmental pollutants. The release of petroleum and its derivatives into the marine environment by human activities is frequent with overburden effects on the marine ecosystem and significant negative consequences on marine lives. The pH (hydrogen ion concentration) is a measure of acidity or alkalinity of a medium on a scale of 0-14. The pH of 8.23 obtained in the study is considered alkaline and is within the pH range of 6.5-8.5 supportive of aquatic lives and optimal for biodegradation in most aquatic and terrestrial ecosystem (Joutey *et al*., 2013). Also, the total hardness of the assayed oceanwater is within the 61-120 (ppm) considered as moderate hardness

The relatively high EC of 175.34 (µS/cm) is indicative of fairly high concentration of dissolved ions or salt. The low salinity of 9. 25 (mg/L) observed in this study can be ascribed to the influence of freshwater from rivers and runoff since the sample was collected from a coastal community. The concentration of DO is within the normal range (7-8.0 mg/L) in ocean water and indicative of good water quality and ecosystem balance necessary for the survival of aquatic life including microorganisms. The nutrient characteristics of seawater provide an insight into the existing health status of the Deepsea environment of the Atlantic Ocean. The result of this study shows the abundance of nutrient which are essential for the growth and survival of ocean microbial life. Microorganisms can use inorganic N and P in the form of nitrate and phosphate respectively and the low concentration of these essential macronutrients can negatively affect ocean ecosystem balance and biological productivity of the ocean.

Microorganisms are widespread in every compartment of the environment and their activities are very significant for the renewal, maintenance and overall sustenance of the environment.  The marine environment provides habitat for diverse groups of aquatic lives and physiologically supports the health of diverse groups of marine lives including microorganisms. The oceanwater sample contain considerable load of heterotrophic and oil degrading bacteria with the hydrocarbon degraders constituting 2.08-6.38% of the total heterotrophic bacterial population. The counts of total heterotrophic bacteria were higher relative to the pollutant degrading bacterial population. Ikuesan *et al*. (2015) reported that the higher counts of total heterotrophs are particularly expected since the hydrocarbon degraders are also member of the heterotrophic community. The detection of hydrocarbon degrading bacteria from the assayed sample agrees with the report of Dasgupta *et al*. (2013) that bacteria have long been considered as one of the predominant hydrocarbon degrading agents found in the environment. The load of heterotrophic and hydrocarbon degrading bacteria in this study indicated that the oceanwater provides habitat for diverse groups of microorganisms that can tolerate the salinity of marine or salt water ecosystem with a proportion of the indigenous bacteria having the potential to metabolize petroleum hydrocarbon for growth and survival. The load and proportion of hydrocarbon utilizing bacteria in this environment is suggestive of previous exposure. This assertion is derived from the report of Atlas (1981) reported that the load of hydrocarbon degrading microorganisms in any environment is reflection of the extent to which the ecosystem is exposed to hydrocarbon contamination. Similarly, Rahman *et al*. (2002) reported that the extent of petroleum pollution of the ecosystem can be determined by the population of hydrocarbon utilizers within the microbial community. This assertion agrees with the report of Gkorezis *et al*. (2016) that bacteria in the petroleum hydrocarbon polluted environment have ability to metabolize hydrocarbons and utilize them as sole carbon source. Four petroleum utilizing bacteria types belonging to three genera were isolated from the assayed sample are considered to be adapted for continued existence, growth and multiplication in polluted ocean environment. The result further agrees with the report of Nduka, (2022) that microorganisms have enzyme systems that degrade and utilize different hydrocarbons as source of carbon and energy. Das and Chandran (2011) also reported that the efficiency of microorganisms in biodegradation varies and ranged from 0.003% to 100% for marine bacteria. The occurrence of petroleum utilizing bacterial isolates in the assayed oceanwater sample implies that these native microbes can metabolize and decompose the pollutants, utilizing them as source of carbon and energy. Joutey *et al*. (2013) ascribed the ability of these microorganisms to degrade organic pollutants to their metabolic machinery and capacity to adapt to harsh environments, thus suggesting their potentials as bioremediation agents.

Biodegradation of pollutants in natural environment is lesser as a result of variables which include competition among microorganisms, limited availability of essential substrates, adverse external conditions (aeration, moisture, pH, temperature), and low bioavailability of the pollutant. Biosurfactants are known for their ability to increase bioavailability of xenobiotics to microorganisms. The biosurfactant assay methods adopted in this study were among those previously reported by (Chandran and Das, 2011; Satpute *et al*. 2008). The biosurfactant assay results suggests that the isolates vary in the their biosurfactant activity. The ability to isolate these biosurfactant-producing bacteria from the oceanwater with indication of hydrocarbon contamination agrees with the assertion of Nwaguma *et al*. (2016) that biosurfactant producing bacteria are ubiquitous and are commonly found in hydrocarbon contaminated environments. The findings in this study also agree with the assertion of Chebbi *et al*. (2017) that some bacteria growing on hydrocarbons possess the capability to produce biosurfactants that can efficiently dissolve hydrocarbons and predisposed them to biodegradation.

Although *Citrobacter werkmanii*, is a known biosurfactant-producing bacterium, however, it showed a negative response to oil collapse test in this study. Drop collapse test relies on the ability of the biosurfactant to reduce surface tension between the droplet and the sealing film reduces thus producing droplets that are flat in shape against spherical in shape if negative. Importantly, low biosurfactant production can result in negative response to oil collapse test. This negative response of *Citrobacter werkmannii* to drop collapse test is in line with the report of Satpute *et al*. (2008) that a single method is unsuitable for the identification all the types of biosurfactant and suggested that a combination of assay methods will be appropriate. Among the four isolates, the emulsification activity (E24) which relates to the ability of the biosurfactant to form and stabilize emulsion was highest (83%) for *Lysinibacillus sphaericus* followed by 76%, 74% and 44% respectively for *Pseudomonas putida*, *Lysinibacillus fusiformis* and *Citrobacter werkmanii* suggesting that biosurfactant of *Lysinibacillus sphaericus* will form more stable emulsion that the others.

The A260/A280 ratio is quantitatively used to determine the purity of DNA. The purity at A260/A280 ratio and concentration of the extracted genomic DNA used in this study demonstrated that it was free of impurities and proteins that could interfere and mislead the molecular identification process of the bacterial isolates.

**5. Conclusion**

The Atlantic Ocean possesses favourable physiological properties that support the survival of diversity of bacterial species which are both biosurfactant-producers and petroleum hydrocarbon degraders. The ocean experiences exposure to petroleum hydrocarbon contamination evidenced by the occurrence of total petroleum hydrocarbon in the assayed oceanwater sample. Pollution of the ocean is suggestive of oil spill in the sea which could result from crude oil exploitation and exploration activities of oil multinationals, ocean-going boat operations and related human activities. The results of this study conclude that there exist in the marine ecosystem diversity of biosurfactant-producing bacteria with mechanisms for oil degradation and could be harnessed to enhance pollutant bioavailability for the remediation of petrochemical wastewater and oil polluted environment as a cheap, ecofriendly and non-invasive approach.

**COMPETING INTERESTS DISCLAIMER:**

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

**References**

1. Al-Marri, S., Eldos, H. I., Ashfaq, M. Y., Saeed, S., Skariah, S., Varghese, L., Mohamoud, Y. A.
   1. Sultan, A. A. and Raja, M. M. (2023) *Biotechnology Reports*, 39: 1-14
2. APHA (2005). Standard Methods for the Examination of Water and Waste Water, American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF), Washington, DC, USA,
3. Atlas, R. M (1981). Microbial degradation of petroleum hydrocarbons: An environmental
   1. perspective. *Microbiology Review* **45:** 180 – 209
4. Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M. G., Fracchia, Marchant, R. (2010).
   1. Microbial biosurfactants production, applications and future potential. *Applied Microbiology and Biotechnology*, 87(2), 427-444.
5. Boboye, B. E., Ikuesan, F. A. and Olukunle O. F (2023) Comparative efficiency of different biological
   1. techniques in the remediation of petroleum oil polluted soil, *International Research Journal of Environmental Sciences* 12(2), 13-20
6. Borah, D. and Yadav, R. N. S. (2017) Bioremediation of petroleum-based contaminants with biosurfactant
   1. produced by a newly isolated petroleum oil degrading bacterial strain. *Egyptian Journal of Petroleum*, 26(1), 181–188.
7. Chebbi, A., Elshikh, M. Haque, F., Ahmed, S., Dobbin, S., Marchant, R., Sayadi,S Chamkha, M., Banat,
   1. I.M. (2017) Rhamnolipids from *Pseudomonas aeruginosa* strain W10; as antibiofilm/antibiofouling products for metal protection, J. Basic. Microbiol. 57 (5) 364–375, <https://doi.org/10.1002/jobm.201600658>.
8. Cheesbrough, M. (2006). District Laboratory Practice in Tropical Countries. 2nd Edition., Cambridge
   1. University Press, Cambridge, UK., ISBN- 13:9781139449298. 50: 165-176.
9. Das, N., and Chandran, P. (2011). Microbial degradation of petroleum hydrocarbon contaminants:
   1. An Overview. *Biotechnology Research International*, 1-13.
10. Dasgupta, D., R. Ghosh and T. K. Sengupta (2013) Biofilm-mediated enhanced crude oil degradation by newly isolated *Pseudomonas* species ISRN. *Biotechnology*, 2013, Article ID 250749
11. Dzionek, A., Wojcieszynska, D. and Guzik, U. (2016). Natural carriers in bioremediation: A Review.
    1. *Electronic Journal of Biotechnology,* 23, 28-36.
12. Fakruddin Md (2012) Biosurfactant: Production and Application *Petroleum & Environmental* 
    1. *Biotechnology* 3:4
13. Farber, R., Rosenberg, A., Rozenfeld, S., Banet, G. and Cahan, R. (2019). Bioremediation of artificial
    1. diesel- contaminated soil using bacterial consortium immobilized to plasma-pretreated wood waste. *Microorganisms*; 7(1-17).
14. Gkorezis, P, Daghio, M, Franzetti A, Van Hamme, J. D, Sillen, W. 2016The Interaction between Plants and
    1. Bacteria in the Remediation of Petroleum Hydrocarbons: An Environmental Perspective. Frontiers in Microbiology; 7:1836.
15. Gomaa, E. Z. and Rasha M. El-Meihy, R. M. (2019)Bacterial biosurfactant from *Citrobacter freundii* 
    1. *MG812314.1* as a bioremoval tool of heavy metals from wastewater *Bulletin of the National Research Centre* 1-14
16. Hassanshahian, M. and Ravan, H. (2018) Screening and identification of biosurfactant producing marine
    1. bacteria from the Caspian Sea. *Caspian J. Environ. Sci.*16(2):179~189.
17. Holt, J. G., Kreig, N. R., Sneath, P. H., Stanley, J. J. and William S. T (1994). Bergey’s Manual of
    1. determinative Bacteriology. *Baltimore.*
18. Ibrahim, H. M. M. (2016). Biodegradation of used engine oil by novel strains of Ochrobactrum
    1. anthropiHM-1 and Citrobacter freundii HM-2 isolated from oil-contaminated soil. 3 Biotech, (2016); 6:226.
19. Ikuesan, F. A., Boboye, B. E. and Adetuyi, F. C. (2015) Cow dung as inoculum carrier for the
    1. degradation of crude oil *Sky Journal of Microbiology Research*, 3(4), pp. 047 - 054
20. Ikuesan, F. A., Olukunle, O. F., Oyelakin, O. O. and Boboye, B. E. (2020).Molecular characterization of
    1. crude oil degrading bacterial isolates from polluted soils and cow dung.*International Journal of Environment, Agriculture and Biotechnology,* 5(3): 533-541 *https://dx.doi.org/10.22161/ijeab.53.6 533*
21. Ikuesan, F. A., Ehuwa, I. and Adeleke, B. S (2022) ‘Petroleum Derivatives’ Toxicity: Influence on The
    1. Growth of Soil Nitrifying Bacteria, *Dutse Journal of Pure and Applied Sciences* (DUJOPAS*),*8 (3b): 45-54
22. Ikuesan, F. A. and Olugbode, J. E. (2023) Assessment of the Potentials of Autochthonous Lipase-Producing
    1. Bacteria in the Treatment of Palm Oil Mill Effluent, *Biological and Environmental Sciences Journal for the Tropics* 20(3):106-120
23. Jain, D., Collins-Thompson, D. and Lee, H. (1991). A drop-collapsing test for screening surfactant-
    1. producing microorganisms. *Journal of Microbiology Methods*, 13(4):271-279.
24. Jain, P. K, Gupta, V. K., Gaur, R. K., Lowry, M, Jaroli, D. P. and, Chauhan, U. K. (2011)
    1. Bioremediation of petroleum oil contaminated soil and Water. Res. J. Environ. Toxicol.;5(1): 1-26.
25. Joutey, N.T., Bahafid, W., Sayel, H. and Ghachtouli, N. E. (2013) Biodegradation: Involved
    1. Microorganisms and Genetically Engineered Microorganisms. Biodegradation-Life Sciences, DOI: 10.5772/56194
26. Kumar, S., Stecher G. and Tamura K. (2016). MEGA7: Molecular evolutionary genetic analysis
    1. version 7.0 for bigger datasets. *Mol. Biol. Evol*.,; *33*, 1870-1874
27. Lei, L., Zhang, Y., Wang, Z., Chen, Q., and Li, X. (2023). Insights into the roles of Enterobacteriaceae in
    1. petroleum hydrocarbon degradation: Mechanisms, challenges, and future directions. Environmental Science and Pollution Research, 30(4), 12345-12360.
28. Nduka, J. K, Umeh, L. N., Okerulu, I. O., Umedum, L. N, Okoye, H. N. (2012). Utilization of different
    1. microbes in bioremediation of hydrocarbon contaminated soils stimulated with inorganic and organic fertilizers. Journal of Petroleum and Environmental Biotechnology, 3(2):1–9.
29. Nwaguma, I. V., Chioma B. C. and Gideon C. O. (2016). Isolation, characterization and application of
    1. biosurfactant by *Klebsiella pneumoniae* strain IVN51 isolated from hydrocarbon-polluted soil in Ogoniland, Nigeria. *Journal of Bioresource & Bioprocess*, 3:40. Available from: doi: 10.1186/s40643-016-0118-4.
30. Okoh, A. I, Trejo- Hernandez, M. R. (2006) Remediation of petroleum hydrocarbon polluted systems:
    1. Exploiting the bioremediation strategies. Afr. J. Biotechnol., 5(25):2520.
31. Olukunle, O. F., Oyelere, B. R. and Sanni, I. O. (2020) Screening for Biodegradative Activity of Diesel
    1. Oil by Microorganisms Isolated from Petroleum Polluted Soil of a Mechanic Workshop at AKAD Community, Akure, Nigeria *African Scientist (21)2:*
32. Perfumo, A., Banat, I. M., Canganella, F., and Marchant, R. (2006). Rhamnolipid production by a bacterial
    1. consortium isolated from an oil-contaminated lagoon. *Bioresource Technology*, 97(11), 1632-1640.
33. Sajna, K.V Sukumaran, R. K. Gottumukkala, L. D. and Pandey, A. (2015) Crude oil biodegradation aided
    1. by biosurfactants from Pseudozyma Sp. NII 08165 or its culture broth, Bioresource. Technol 191 (2015) 133–139, https://doi.org/ 10.1016/j.biortech.2015.04.126.
34. Santos, D. Rufino, R. Luna, J. Santos, V. Sarubbo, L. (2016) Biosurfactants: multifunctional biomolecules
    1. of the 21st century, Intern. J. Mol. Sci. 17 (3):401, https:// doi.org/10.3390/ijms17030401.
35. Sarubbo L. A , Silva, M. G. C , Durval , Karen, I. J. B., Bezerra, G. O., Ribeiro, B. G , Ivison A. Silva, I. A
    1. Matthew S. Twigg, M. S. and Ibrahim M. Banat, I. M (2022) Biosurfactants: Production, properties, applications, trends, and general perspectives, Biochemical Engineering Journal 181 (2022) 108377
36. Sharma, A., Soni, J., Kaur, J. and Kaur, G. (2014). A Study on biosurfactant production in Lactobacillus
    1. and *Bacillus* sp. *Int. J. Curr. Microbiol.* *Appl. Sci.* 3 (11) 723-733.
37. Satpute S. K., Bhawsar B. D., Dhakephalkar P. K. and Chopade B. A. (2008). Assessment of different
    1. screening methods for selecting biosurfactant producing marine bacteria. Indian *Journal of Marine Science*, 37(3):243–250.
38. Silva, R. C., F. S., Almeida, D. G., Rufino, R. D., Luna, J. M., Santos, V. A., and Sarubbo, L. A. (2014).
    1. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. International Journal of Molecular Sciences, 15(7), 12523-12542.
39. Sneath, P. H. A. and Sokal, R. R. (1973). Numerical Taxonomy; The principles and practice of
    1. numerical classification. San. Francisco, Free, 573 pp
40. Tamura, K., Nei M. and Kumar S. (2004). Prospect for inferring very large phylogenies by using
    1. the neighbor-joining method. Proc Natl Acad Sci USA, *101*, 1030-11035
41. Veerapagu, M., Jeya, K. R., Kalaivani, R., Jeyanthi, K.A. and Geethanjali, S. (2019) Screening of
    1. hydrocarbon degrading bacteria isolated from oil contaminated soil, *The Pharma Innovation Journal* 2019, 8(6): 69-72
42. Vijayakumar, S and Saravanan, V (2015). Biosurfactants-Types, Sources and Applications. *Research Journal of Microbiology, 10: 181-192.*
43. Youssef, N. H, Duncan, K. E, Nagle, D. P, Savager, K. N, Knapp, R. M. and McInerney, M. J. (2004),
44. Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal Microbiology* *Meth*ods. 56: 339– 346.