Isolation, Screening and Identification of Biosurfactant-producing Bacteria from Hydrocarbon-polluted and Pristine Soils within Ogoniland, Nigeria

Ijeoma Vivian Nwaguma[1]*, Chioma Blaise Chikere[1] and Gideon Chijioke Okpokwasili[1]

[1]*Department of Microbiology, Faculty of Science, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria.*

Original Research Article

ABSTRACT

Aim: This study investigated the production of biosurfactant from bacteria isolated from hydrocarbon-polluted and pristine soils within Ogoniland in the Niger Delta region of Nigeria.
Methods: Baseline physicochemical parameters of the soil (total petroleum hydrocarbon (TPH), the nature of the soils, pH and temperature) were examined. The biosurfactant-producing bacteria were screened using emulsification assay, emulsification index (E_{24}), lipase activity, haemolytic assay, oil spreading and tilted glass slide. The biosurfactant-producing bacteria were characterized by phenotypic, biochemical and molecular means.
Results: The respective baseline TPH, temperature and pH were 9,419 mg/kg, 28.5±0.4°C and

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*Corresponding author: E-mail: ijeomanwaguma@gmail.com;

5.7±0.1 for hydrocarbon- polluted soil and 1.28 mg/kg, 27.5±0.3°C and 3.7±0.1 for pristine soil. Meanwhile, the respective soil types for the polluted and pristine were humus soil mixed with oil and ordinary humus. Six isolates (IVN-02, IVN-45, IVN-51, IVN-61, IVN-67 and IVN-74) out of forty one (41) distributed within the two different soil samples were found to produce biosurfactant. Phylogenetic analysis based on the 16S rRNA genes classified the six isolates as *Pseudomonas* sp. IVN02, *Alcaligenes faecalis* IVN45, *Klebsiella pneumoniae* IVN51, *A. faecalis* IVN61, *Enterobacter sacchari* IVN67 and *P. aeruginosa* IVN74 respectively. The isolates have been deposited at the GenBank under the accession numbers KT254065, KT254066, KT254060, KT254063, KT254061 and KT254059.
Conclusion: This study demonstrated efficient biosurfactant production from bacterial isolate from hydrocarbon-polluted and pristine soils in Ogoniland within the Niger Delta region of Nigeria. The ability of the bacterial isolates from this region to produce biosurfactant is important considering the level of pollution in Ogoniland and the need to use indigenous and ecologically friendly products in the remediation process.

1. INTRODUCTION

Surfactants or surface-active agents are amphipathic molecules consisting of hydrophobic and hydrophilic moieties. As amphiphilic molecules, surfactants, consists of two parts, a polar (hydrophilic) moiety and a non-polar (hydrophobic) group. The hydrophilic group consists of mono-, oligo-, or poly-saccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols [1]. They can be classified into two main groups; synthetic surfactant and biosurfactant. Synthetic surfactants are produced by chemical reactions, while biosurfactants are produced by biological processes.

The recent interest in biosurfactants is due to the fact that chemically synthesized surfactants are not biodegradable and can be toxic to the environment. Biosurfactants have been used in the oil industry to aid clean-up of oil spills, as well as to enhance oil recovery from oil reservoirs [2]. Biosurfactants have several advantages over synthetic surfactants. These advantages include high biodegradability, low toxicity, good biocompatibility with eukaryotic organisms, effectiveness at wider range of temperatures, pH values and salinities, low irritancy, ability to be produced from renewable and cheaper substrates and synthesis under user-friendly conditions (e.g., low temperature and pressure) [3]. Several types of biosurfactant, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids and lipopolysaccharides have been isolated and characterized.

Hydrocarbons are composed of complex chemical structure such as aliphatic and aromatic hydrocarbons. Individual organisms are able to metabolise a limited range of hydrocarbon substrates [4]. Very often the growth of microorganisms on hydrocarbon is accompanied by the emulsification of the hydrocarbon in the medium and in most cases this has been attributed to the production of surface-active compounds. Biosurfactant-producing microorganisms are naturally present not only in hydrocarbon-polluted soils, but are also present in pristine environment rich in organic matter suitable for the growth of diverse organisms. The majority of known biosurfactants are synthesized by microorganisms grown on water-immiscible hydrocarbons, but some have been produced on such water-soluble substrates as glucose, glycerol and ethanol [5]. Most of the bacteria frequently isolated from hydrocarbon-polluted sites belong to the genera *Pseudomonas, Sphingomonas, Acinetobacter, Alcaligenes, Micrococcus, Bacillus, Flavobacterium, Arthrobacter, Alcanivorax Mycobacterium, Rhodococcus* and *Actinobacter* [6,7].

The microorganisms that produce biosurfactant abound in nature; they inhabit both water (fresh water, groundwater and sea) and land (soil, sediment and sludge). In addition, they can be found in extreme environments (e.g., oil reservoirs) and thrive at wide range of temperatures, pH values and salinity [8]. Furthermore, they can be isolated from undisturbed environments where they have physiological roles, not involving the solubilisation of hydrophobic pollutants, such as antimicrobial activity, biofilm formation or processes of motility and colonization of surfaces [9]. However, hydrocarbon-degrading microbial communities remain the most implicated

environment on widespread capability for biosurfactant production.

Covering around 1,000 km^2 in Rivers State, southern Nigeria, Ogoniland has been the site of oil industry operations since the late 1950s [10]. Ogoniland has a tragic history of pollution from oil spills and oil well fires and this has resulted in tremendous hazardous effect on agricultural practice; giving rise to communal hostility in the region. Cleaning up Ogoniland would require the use of environmentally best practices and products and considering the successful use of biosurfactants in environmental remediation, they would find relevance in the process. Obtaining the biosurfactants from the immediate environment will both reduce the cost of the clean-up process and ensure that ecologically friendly products are used for the remediation process. Hence, the study evaluated the biosurfactant production capacity of bacteria from hydrocarbon-polluted and pristine environments within Ogoniland in Niger Delta Basin of Nigeria.

2. MATERIALS AND METHODS

2.1 Sample Collection

The crude oil-contaminated soils used in this study were obtained from Kporghor community of Tai Local Government Area (Ogoniland), in the lower Niger Delta Basin of Nigeria.

For each soil, samples were randomly collected from different points at depths between 0 - 15 cm using a hand-held soil auger and then bulked to obtain a composite sample. The samples were transported aseptically in sterile polythene bags to the laboratory, for analysis. Thereafter, the samples were stored at ambient temperature for further use [11].

2.2 Physicochemical Analysis

The parameters analysed included pH, temperature and total petroleum hydrocarbon (TPH). Gas Chromatographic analysis was carried out as described by Chikere et al. [12].

2.2.1 Total petroleum hydrocarbons (TPH)

Dried soil samples were powder sieved and cold-extracted in conical flask for a total of two hours in each case using 100% dichloromethane. The solvent from the resultant solution was removed by means of rotary evaporator under vacuum (pressure not greater than 200 mbar) and finally by a flow nitrogen at not more than 30℃ to yield the extracted organic matter (EOM).

The semi volatile compounds were introduced into the GC/MS by injecting the sample extract into a gas chromatograph (GC) equipped with a narrow-bore fused silica capillary column. The GC column was temperature-programmed to separate the analytes, which were then detected with a mass spectrometer (MS) connected to the gas chromatograph.

Analytes eluted from the capillary column, were introduced into the mass spectrometer via a jet separator. Identification of target analytes was accomplished by comparing their mass spectra with the electron impact spectra of authentic standards. Quantitation was accomplished by comparing the response of a major (quantitation) ion relative to an internal standard using an appropriate calibration curve for the intended application.

2.2.2 Chromatographic condition

The GC/MS system comprised Agilent 6890 GC, (Agilent Technologies, Wilmington, USA) with 5975B MSD and MSD chemstation (version D. 03.00). Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min and at a pressure of 75 kpa. The injector temperature was set at 250℃. The program used was; 2 min hold time, ramp to 240℃ at 7℃/min and a final ramp to 285℃ at 12℃ with an 8 min hold time. Column—30-m × 0.25-mm ID × 0.25 μm film thickness silicone-coated fused-silica capillary column. Solvent delay: 4 min, Mode-Scan at 3.54, Solvent delay: 3 min, Quard temp: 150℃, Source temp: 230℃, Transfer line temp: 280℃, Sampling: 2, Low mass: 45.0 amu, High mass: 450 amn, Threshold: 150.

2.3 Serial Dilution

Serial dilution was performed on the samples according to the method described by Nanhini and Josephine [13]. Nine millilitres (9 mL) of normal saline (0.85% NaCl w/v in distilled water) was first dispensed into each clean test tube, sterilized by autoclaving at 121℃ for 15 min at 15 psi and allowed to cool. Ten grams (10 g) of the dry soil sample was dissolved in 90 mL of sterile normal saline to make a stock solution; from this stock solution 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were made.

2.4 Isolation and Enumeration of Bacteria

Hundred microlitres of 10^{-3}, 10^{-5} and 10^{-6} dilutions were spread-plated on the modified mineral salt medium (MSM) described by Techaoei, et al. [14], containing the following ingredients (in 1 L distilled H_2O): glycerol 5 g; Asparagine 1 g; K_2HPO_4 1 g; $MgSO_4.7H_2O$ 5 g; KCl 1.0 g; agar 15 g; and 1 mL of trace solution containing (1L distilled water) $MgSO.7H_2O$ 0.5 g, $CuSO_4.5H_2O$ 0.16 g and $FeSO_4.7H_2O$ 0.015 g, and incubated at 30℃ for 72 h. Morphologically distinct colonies were identified and purified. The total viable cell counts (TVC) of the strains were determined. The selected bacterial isolates were stored in MSM slants and kept under refrigerated condition (4℃) for further experimentation.

2.5 Screening of Biosurfactant-producing Bacteria

Biosurfactant activity was determined for the pure cultures using haemolytic activity, oil spreading technique, lipase activity using tributyrin clearing zone (TCZ), emulsification stability (E_{24}) test, emulsification assay and tilted glass slide test.

2.5.1 Haemolytic activity

This is a qualitative screening test for the detection of biosurfactant producers [15]. Nutrient agar (NA) supplemented with 5% (v/v) fresh human blood was used according to Banat [16] and Carrillo et al. [15]. The plates were incubated at 37℃ for 24 h; after incubation, the plates were then observed for the presence of clear zone around the colonies.

2.5.2 Oil spreading technique

This is one of the best methods to detect the presence of biosurfactant (BS) producers [15]. Twenty microliters (20 µl) of crude oil was added to 40 mL of distilled water (DW) in a petri plate. Culture supernatant of 10 µl was added on the oil-coated water surface. A colony surrounded by an emulsified halo was considered positive for BS production [17]. The diameter of the clearing zone on oil surface was visualized under visible light and measured after 30 sec. This value obtained, correlates to surfactant activity and is also known as displacement activity [18].

2.5.3 Lipase activity by tributyrin clearing zone (TCZ)

Lipolytic activity was observed directly by changes in the appearance of substrates such as tributyrin and triolein, which were emulsified mechanically in various growth media poured into petri dish. The isolates were screened for lipolytic activity on mineral salt agar containing 1% tributyrin (w/v). The pH of the medium was adjusted to 7.3 − 7.4 using 0.1 M of HCl and incubated at 35℃ for 7 days. The plates were examined for zones of clearance around the colonies [19].

2.5.4 Emulsification stability (E_{24}) test (emulsification index)

The emulsification index (E_{24}) provides a rapid and reliable measure of the quantity of biosurfactant. The E_{24} was determined as described by Nitchke and Pastore [20]. Two millilitres (2 mL) of kerosene was added to the same amount of cell-free broth. The mixture was vortexed at high speed for 2 min. After 24 h, the height of the stable emulsion layer was measured. E_{24} index is defined as percentage of the height of emulsified layer divided by the total height of the liquid column [20]. In this study, sodium dodecyl sulphate (SDS) and water were used as positive and negative controls, respectively.

$$E_{24} (\%) = \frac{\text{total height of the emulsified layer}}{\text{height of the liquid layer}} \times 100$$

2.5.5 Emulsification assay

Culture broth was centrifuged at 10,000 rpm/15 min/RT. Supernatant (3 mL) was mixed with hydrocarbon (0.5 mL) and vortexed vigorously for 2 min. This was left undisturbed for 1 h to separate aqueous and hydrocarbon phase [21]. Uninoculated broth was used as blank. Absorbance of the aqueous phase was measured with a spectrophotometer at 400 nm [22].

2.5.6 Tilting glass slide test

This technique is effectively a modification of the drop collapse method [15]. Isolates were grown for 24 h on nutrient agar plates. A sample of colony was mixed with a droplet of 0.85% NaCl at one end of the glass slide. The slide was tilted and droplet observed. Biosurfactant producers were detected by observation of droplet collapsing down [15].

2.6 Biochemical and Phenotypic Characterization

The screened biosurfactant-producing bacteria were identified by morphological and biochemical techniques using Bergey's Manual of Determinative Bacteriology [23].

2.7 Molecular Characterization

Bacterial genomic DNA extraction was performed using ZR Soil Microbes DNA Mini-Prep extraction kit (Zymo Research Corporation, South Africa). The quantity and purity of the extracted genomic DNA bacterial isolates were analysed using an ND-1000 spectrophotometer (Thermoscientific, Inqaba Biotech, South Africa) and agarose gel electrophoresis. The genomic DNA was stored at -20°C. The amplification of the 16S rRNA gene of the isolates was carried out using primer set 27F (5[1]AGAGTTTGATCMTGGCTCAG-3[1]) and 1492R (5[1]GGGTTACCTTGTTACGACTT3[1]). The PCR reaction was carried out in 25 µl volumes containing 12.5 µl of the Master Mix (Zymo Master Mix), 0.4 µl of each primer, mixed with 5µl of the DNA template. Sterile nuclease free water of volume, 6.7 µl was added. The following PCR conditions were used: Initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec., annealing at 52°C for 30 sec., extension at 72°C for 45 sec., final extension step at 72°C for 3 min and cooled to 4°C. Five microliters (5 µl) of the amplified products was run on agarose gel electrophoresis at 120 V for 15 min to determine the quality of the products. The amplified products were also purified using DNA clean and concentrator (DCC) kit (Zymo Research Institute, South Africa) and were ready for sequencing. PCR products of the bacterial DNA were sequenced using Sanger method of sequencing with 3500 ABI Genetic Analyser, performed at Inqaba Biotechnical Industries, South Africa. The sequences generated by the sequencer were visualized using Bioinformatic Algorithms such as Chromaslite for base calling. Bioedit was used for sequence editing, before performing a Basic Local Alignment Search Tool (BLAST) using NCBI (National Center for Biotechnology Information) database. Similar sequences were downloaded and aligned with ClustalW and phylogenetic tree was drawn with MEGA 6 software.

3. RESULTS

3.1 Physiochemical Characteristics of the Soil Sample

The physiochemical characteristics of the soil are presented in Table 1. The hydrocarbon-polluted and pristine (unpolluted) soils had respective pH values of 5.7±0.1 and 3.7±0.1. The temperatures were 28.5±0.4 and 27.5±0.3°C for the hydrocarbon-polluted soil and unpolluted soil, respectively. The soil types ranged from humus soil to humus soil mixed with crude oil.

3.2 Selection and Identification of the Biosurfactant Producers

Out of the 37 bacterial isolates screened (Table 3), 6 isolates were selected as the biosurfactant producers based on their ability to give positive results to all the screening methods employed. Twenty six (26) isolates were isolated from hydrocarbon polluted soils while eleven (11) isolates were isolated from unpolluted soils (Table 2). Table 3 shows the 6 selected isolates and their screening results. The isolates codes were IVN-02, IVN-45, IVN-51, IVN-61, IVN-67 and IVN-74. Four (4) isolates from the hydrocarbon polluted soil were positive to all the screening tests while two (2) isolates from unpolluted soils yielded positive results to all the screening tests (Table 4).

The cultural and colonial characteristics of the six biosurfactant-producing isolates are given in Table 5. Table 6 presents the biochemical characteristics of the biosurfactant-producing bacterial isolates. All the isolates were Gram negative bacilli, belonging to four genera viz., *Pseudomonas*, *Alcaligenes*, *Enterobacter*, and *Klebsiella*.

Table 1. Physiochemical characteristics of the soil samples

Parameter	Hydrocarbon polluted soil (HPS)	Pristine (unpolluted) soil (UPS)
pH	5.7±0.1	3.7±0.1
Temperature (°C)	28.5±0.4	27.5±0.3
Types of soil	Humus soil mixed with crude oil	Humus soil
TPH (mg/kg)	9,419	1.28

TPH – Total Petroleum Hydrocarbon

Table 2. Distribution of the bacterial isolates within different soil samples

Soil sample	Number of isolates	cfu/g
Hydrocarbon polluted soil (HPS)	26	$4.65\pm0.73\times10^{7}$
Pristine (unpolluted) soil (UPS)	11	$5.0\pm1.6\times10^{6}$

Legend: Cfu/g = colony forming unit per gram

Table 3. Screening results of the selected bacterial isolates

Isolate code	Isolate source	Lipase test (mm)	Emulsification assay (A_{400} nm)	Tilted glass Slide test	E_{24}(%) (using kerosene)	Oil spreading mm^2 (using crude oil)	Haemolytic assay
IVN-01	HPS	-	0.5285±0.0025	+	-	-	γ
*IVN-02	HPS	13±2	0.5045±0.025	+	11.1±2.1	28.3±3.1	3±2 mm (β)
IVN-04	HPS	-	0.4805±0.0055	+	10.7±1.2	19.6±0.8	γ
IVN-10	HPS	-	0.4480±0.001	-	-	-	α
IVN-14	HPS	-	0.3980±0.0200	-	-	-	2±1 mm (β)
IVN-15	HPS	7±1	0.4225±0.0015	+	-	-	4±2 mm (β)
IVN-17	UPS	6±2	0.5360±0.000	-	-	-	α
IVN-19	UPS	9±1	0.4055±0.0025	-	-	-	γ
IVN-20	HPS	12±2	0.5315±0.0015	+	18±2	-	γ
IVN-21	HPS	16±1	0.4745±0.0045	+	16.6±1.2	-	3±1 mm (β)
IVN-23	UPS	6±1	0.4455±0.0005	-	7.4±1.5	19.6±3.1	γ
IVN-24	HPS	-	0.4780±0.0020	+	10±2	-	2±1 mm (β)
IVN-25	UPS	-	0.5365±0.0055	-	-	-	α
IVN-27	HPS	-	0.5095±0.0165	+	20±2	-	α
IVN-28	UPS	14±2	0.4400±0.010	+	14.8±1.2	-	α
IVN-31	HPS	-	0.4950±0.000	+	-	95.0±3.2	α
IVN-36	HPS	-	0.4735±0.0025	-	-	28.3±0.8	1±0.5 mm (β)
IVN-39	HPS	21±1	0.4295±0.0005	-	11±1	19.6±0.8	γ
IVN-40	HPS	12±1	0.4460±0.000	-	-	-	α
IVN-44	HPS	-	0.3540±0.008	+	14.2±1.1	3.1±0.2	γ
*IVN-45	HPS	18±2	0.5085±0.0015	+	40±1	19.6±3.0	12±2 mm (β)
IVN-47	UPS	-	0.4195±0.0025	+	66.6±1	78.5±0.8	α
*IVN-51	HPS	11±1	0.4020±0.000	+	47.8±1	78.6±2.4	6±2 mm (β)
IVN-53	HPS	-	0.4260±0.002	-	16.7±1.1	3.1±0.8	α
IVN-54	HPS	8±1	0.4450±0.000	-	7.6±1.2	113.0±0.8	γ
IVN-58	UPS	-	0.4230±0.003	+	12±1	-	γ
IVN-59	HPS	-	0.4100±0.000	+	-	-	α
IVN-60	HPS	-	0.4390±0.001	+	48±2	19.6±0.8	α
*IVN-61	UPS	6±1	0.4075±0.0015	+	15.4±1.1	50.3±4.2	9±1 mm (β)
IVN-63	HPS	-	0.3150±0.003	-	14.2±1	7.1±0.8	γ
IVN-65	HPS	16±1	0.3420±0.001	-	-	-	α
IVN-66	UPS	-	0.4050±0.001	-	-	12.6±3.1	γ
*IVN-67	HPS	16±1	0.3220±0.001	+	24±1	176.6±6.3	2±0.5 mm (β)
IVN-69	HPS	13±2	0.2200±0.010	-	19.2±1.1	153.9±7.1	γ
IVN-70	UPS	-	0.3720±0.001	-	42.3±1	12.6±0.8	γ
IVN-71	HPS	-	0.3110±0.000	-	25±1	19.6±0.8	α
*IVN-74	UPS	5±2	0.4360±0.010	+	15.3±1.1	201.1±3.2	3±1 mm (β)

*Legend: *size and type of haemolysis; Values represent mean and standard deviation for duplicate experiments;*
α = alpha haemolysis; β = beta haemolysis; γ = gamma haemolysis

Table 4. Screening results of the selected bacterial isolates

Isolate codes	Source of sample	Lipase test(mm)	Emulsification assay($A_{400 nm}$)	Emulsification index (E_{24}) %	Tilting glass slide test	Haemolytic assay (mm)	Oil spreading test (mm^2)
IVN-02	HPS	13.0±2.0	0.5045±0.0025	11.1±2.1	+	3.0±2.0	28.3±3.1
IVN-45	HPS	8.0±2.0	0.5085±0.0015	40.0±1.0	+	12.0±2.0	19.6±3.0
IVN-51	HPS	11.0±1.0	0.4020±0.002	47.8±1.0	+	6.0±2.0	78.6±2.4
IVN-61	UPS	6.0±1.0	0.4075±0.0015	15.4±1.1	+	9.0±1.0	50.3±4.2
IVN-67	HPS	16.0±2.0	0.3220±0.001	24.0±2.0	+	2.0±0.5	176.8±6.28
IVN-74	UPS	5.0±1.0	0.4360±0.015	15.3±1.1	+	3.0±1.0	201.1±3.18

Legend: HPS = hydrocarbon polluted soil; UPS = unpolluted soil.
+ = positive test; Values represent mean and standard deviation of duplicate experiments

Table 5. Colony morphology of biosurfactant-producing bacteria

Isolate code	IVN-02	IVN-45	IVN-51	IVN-61	IVN-67	IVN-74
Gram reaction	-	-	-	-	-	-
Morphology	Rod	Rod	Rod	Rod	Rod	Rod
Size	Large	Small	Large	Medium	Small	Large
Shape	Circular	Irregular	Round	Irregular	Round	Circular
Margin	Irregular	Entire	Entire	Entire	Irregular	Irregular
Elevation	Raised	Convex	Unbonate	Convex	Convex	Flat
Pigment	Green	-	-	-	-	Green
Colour	Cream	White	Cream	White	Cream	Cream
Texture	Mucoid	Slimy	Mucoid	Slimy	Dry	Mucoid
Surface	Rough	Smooth & Shiny	Smooth & Shiny	Smooth & Shiny	Shiny	Smooth
Opacity	Opaque	Opaque	Translucent	Opaque	Opaque	Opaque

Legend: - = Negative

3.3 Molecular Characterization of Isolates

The result of the genomic DNA quantification showed that the entire DNAs extracted from the six (6) isolates were pure using the Nanodrop spectrophotometer and agarose gel electrophoresis. All the 6 isolates showed amplification with an amplicon size of 1400 bp. Table 7 shows the molecular and 16S sequence matches for bacterial isolates recovered from pristine and hydrocarbon-polluted.

4. DISCUSSION

This study evaluated the biosurfactant production capacity of bacteria from hydrocarbon-polluted and pristine soils within Ogoniland in the Niger Delta Area of Nigeria. Many researchers have reported the isolation and distribution of biosurfactant-producing bacteria in hydrocarbon-polluted sites [24-26] and uncontaminated (unpolluted) soils [27]. Meanwhile, Bodour and Miller-Maier [28] showed that hydrocarbon-contaminated soils are more yielding than uncontaminated soils.

Six (6) methods (emulsification assay, emulsification index, tilting glass slide, haemolytic assay, oil spreading and lipase activity) were employed in the screening of the bacterial isolates for biosurfactant production. These methods have been previously used by other researchers to identify biosurfactant-producing bacteria; tilted glass slide [28,29], haemolytic assay [15,16], emulsification assay [22], lipase activity [30], oil spreading [29] and emulsification index [31,32]. The isolates screened in this study showed varying results for the different screening methods. Haemolysis, lipase production, emulsification assay and tilted glass slide are qualitative detection techniques while emulsification index and oil spreading (displacement) techniques are quantitative and their values correlates with the quantity of biosurfactant produced.

Table 6. Biochemical characteristics of the biosurfactant-producing bacterial isolates

Isolate code	IVN-02	IVN-45	IVN-51	IVN-61	IVN-67	IVN-74
Gram's stain	- (rods)	- (rods)	- (rods)	- (rods)	- (rods)	- (rods)
Citrate	-	+	+	+	+	+
Motility	+	+	-	+	+	+
Oxidase	+	+	-	+	+	+
Catalase	+	+	+	+	+	+
Indole	-	-	-	-	-	-
Urease	+	-	+	-	-	+
MR	-	-	-	-	-	-
VP	-	+	+	+	+	+
TSI						
Slant	K	K	A	K	K	A
Butt	-	A	A	-	A	-
H$_2$S	-	-	-	-	-	-
Starch hydrolysis	-	-	+	-	-	-
Gelatin hydrolysis	+	-	-	-	-	-
Sugar fermentation						
Maltose	-/A	-/A	+/A	-/-	+/A	+/A
Glucose	-/-	-/A	+/A	-/A	+/A	-/A
Lactose	+/A/	-/A	+/A	+/A	-/A	+/-
Mannitol	-/-	+/A	+/A	+/A	+/A	-/A
Sucrose	-/-	+/A	+/A	+/A	-/A	+/-
Probable genus	*Pseudomonas*	*Alcaligenes*	*Klebsiella*	*Alcaligenes*	*Enterobacter*	*Pseudomonas*

*Legend: + = Positive; - = Negative; K = Alkaline; A = Acid; MR = Methyl red; VP = Vogues Proskauer;
TSI = Triple sugar iron*

Table 7. Molecular and 16S rRNA sequence matches for bacteria isolates recovered from pristine and hydrocarbon-polluted soils

Isolates code	Isolate name	GenBank accession number	GenBank closest cultured organism
IVN02	*Pseudomonas* sp. IVN02	KT254065	*Pseudomonas* sp. G4
IVN45	*Alcaligenes faecalis* IVN45	KT254066	*Alcaligenes faecalis* B17
IVN51	*Klebsiella pneumoniae* IVN51	KT254060	*Klebsiella pneumoniae* QLR-3
IVN61	*A. faecalis* IVN61	KT254063	*Alcaligenes faecalis* B1
IVN67	*Entrobacter sacchari* IVN67	KT254062	*Entrobacter sacchari* SP1
IVN74	*P. aeruginosa* IVN74	KT254059	*Pseudomonas aeruginosa* GIM32

The biosurfactant-producing bacteria were selected based on their ability to give positive results to haemolytic assay, tilting glass slide, oil spreading, lipase and emulsification index. Satpute et al. [29] suggested that single method is not suitable to identify all types of biosurfactants and recommended combination of methods. After selection, the six (6) biosurfactant-producing bacteria were identified using molecular approach. The polluted site had four (4) isolates (*Pseudomonas* sp IVN02,

Alcaligenes faecalis IVN45, *Enterobacter sacchari* IVN67 and *Klebsiella pneumoniae* IVN51) and unpolluted soil had two (2) isolates (*P. aeruginosa* IVN74 and *Alcaligenes faecalis* IVN61). *Pseudomonas aeruginosa* has been widely reported [33-38] for its ability to produce biosurfactant especially rhamnolipid. Bouchez-Naïtali, et al. [39] reported the production of biosurfactant by *Alcaligenes faecalis*. There have been reports [40,41] also, on biosurfactant production by *Enterobacter* sp. Meanwhile, there

is dearth of information on the production of biosurfactant by *Klebsiella pneumonia*e. However, Mishra et al. [42] and Jamal [43] all reported the production of biosurfactant (phospholipid) by *Klebsiella pneumoniae.*

5. CONCLUSION

This study demonstrated efficient biosurfactant production from bacterial isolate from hydrocarbon-polluted and pristine soils in Ogoniland within the Niger Delta region of Nigeria. The ability of the bacterial isolates from this region to produce biosurfactant is important considering the level of pollution in Ogoniland and the need to use indigenous and ecologically friendly products in the remediation process.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Pacwa-Plociniczak M, Plaeza GA, Piotrowska-Seget Z, Cameotra SS. Environmental applications of biosur-factants: Recent advances. Int J Mol Sci. 2011;12:633-654.
2. Tabatabaee A, Assadi MM, Noohi AA, Sajadian VA. Isolation of biosurfactant bacteria from oil reservoirs. Iranian J Env Health Sci Eng. 2005;2(1):6-12.
3. Banat IM, Makkar RS, Cameotra SS. Potential commercial application of microbial surfactants. Appl Microbiol Biotechnol. 2000;53:495-508*.*
4. Marin MA, Pedrogosa A, Laborda F. Emulsifier production and microscopical study of emulsions and biofilms from by the hydrocarbon utilizing bacteria *Acinetobacter calcoaceticus* MM5. Appl Microbiol Biotechnol. 1996;44:660–667.
5. Abu-Ruwaida AS, Banat IM, Haditirto S, Salem A, Kadri M. Isolation of biosurfactant- producing bacteria, product characterization and evaluation. Acta Biotechnol. 1991;11:315–324.
6. Atlas RM. Petroleum microbiology. In: Lederberg J, (ed). Encyclopedia of microbiology. Academic Press, Baltimore. 1991;363–369
7. Okoh AI, Trejo-Hernandez MR. Remediation of petroleum hydrocarbon polluted systems: Exploiting the bio-

8. Chirwa EMN, Bezza FA. Petroleum hydrocarbon spills in the environment and abundance of microbial community capable of biosurfactant production. J Pet Environ Biotechnol. 2015;6:237.
9. Van Hamme JD, Singh A, Ward OP. Physiological aspects. Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnol Advances. 2006;24:604-620.
10. UNEP. Environmental setting in Ogoniland and the Niger Delta. In: Environmental Assessment of Ogoniland. United Nations Environment Programme, Nairobi, Kenya. 2011;30-33.
11. Ezebuiro V, Ogugbue CJ, Oruwari B, Ire FS. Bioethanol production by an ethanol-tolerant *Bacillus cereus* strain GBPS9 using sugarcane bagasse and cassava peels as feedstocks. J Biotechnol Biomater. 2015;5:213.
    DOI: 10.4172/2155-952X.1000213
12. Chikere CB, Obieze CC, Okerentugba P. Molecular assessment of microbial species involved in the biodegradation of crude oil in saline Niger Delta sediments using bioreactors. J Bioremed Biodeg. 2015;6:307.
    DOI: 10.4172/2155-6199.1000307
13. Nanhini B, Josephine RMA. Study on bacterial and fungal diversity in potted soil. Int J Curr Microbiol App Sci. 2013;2(2):1-5.
14. Techaoei S, Lumyong S, Prathumpai W, Santiarwarn D, Leelapornoisid P. Screening, characterization and stability of biosurfactant produced by *Pseudomonas aeruginosa SCMU*106 isolated from soil in Northern Thailand. Asian J Bio Sci. 2011;4(4):340-351.
15. Satpute SK, Arun GB, Prashant KD, Banat IM, Chopade AC. Methods for investigating biosurfactants and bioemulsifiers: A review. Crit Rev Biotechnol. 2010;30(2):127–144.
    DOI: 10.3109/07388550903427280
16. Carrillo PG, Mardaraz C, Pitta-Alvarez SI, Giuliett AM. Isolation and selection of biosurfactant-producing bacteria. World J Microbiol Biotechnol. 1996;12:82-84.
17. Morikawa M, Hiratr Y, Imanaka TA. Study on the structure, functional relationship of lipopeptide biosurfactants. Biochim Biophys Acta. 2000;1488(3):211-218.

18. Huy N, Jin S, Amada K. Characterization of petroleum-degrading bacteria from oil-contaminated sites in Vietnam. J Biosci and Bioeng. 1999;88(1):100-102.

19. Gandhimathi R, Seghal K, Hema TA. Production and characterization of lipopeptide biosurfactant by a sponge-associated marine actinomycetes *Nocardiopsis alba* MSA10. Bioprocess Biosys Eng. 2009;32(6):825-835.

20. Nitschke M, Pastore GM. Biosurfactant production by *Bacillus subtilis* using cassava-processing effluent. Appl Biochem Biotechnol. 2004;112:163–172.

21. Jagtap S, Yavankar S, Pardesi K, Chopade B. Production of bioemulsifier by *Acinetobacter* sp. from healthy human skin of tribal population. Ind J Expt Biol. 2010;48:70–76.

22. Patil JP, Chopade BA. Distribution and *in vitro* antimicrobial susceptibility of *Acinetobacter* sp. on the skin of healthy humans. Nat Med J India. 2001;14:204-208.

23. Buchanan RE, Gibbons NE. Bergey's manual of determinative bacteriology, eighth edition. The Williams and Wilkins C, Baltimore. 1974;1146.

24. Bodour AA, Drees KP, Rain M, Maier RM. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl Environ Microbiol. 2003;69(6):3280-3287.

25. Saravanan V, Vijayakumar S. Isolation and screening of biosurfactant- producing microorganisms from oil contaminated soil. J Acad Indus Res. 2012;1(5):264-268.

26. Zou C, Wang M, Xing Y, Lan G, Ge T, Yan X, Gu T. Characterization and optimization of biosurfactants produced by *Acinetobacter baylyi* ZJ2 isolated from crude oil-contaminated soil sample toward microbial enhanced oil recovery applications. Biochem Eng J. 2014;14:49-58.

27. Jennings EM, Tanner RS. Biosurfactant-producing bacteria found in contaminated and uncontaminated soils. Proceedings of 2000 conference on Hazardous Waste Research; 2000.

28. Bodour A, Miller-Maier R. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. J Microbiol Methods. 1998;32(3):273-280.

29. Satpute SK, Bhawsar BD, Dhakephalkar PK, Chopade BA. Assessment of different screening methods for selecting biosurfactant- producing marine bacteria. Indian J Marine Sci. 2008;37(3):243-250.

30. Deepika L, Kannabiran K. Isolation and Characterization of antagonistic action-mycetes from marine soil. J Microbial Biochem Technol. 2010;2:1-6.
DOI: 10.4172/1948-5948.1000015

31. Haba E, Espuny MJ, Busquets M, Manresa A. Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. J Appl Microbiol. 2000;88:379-387.

32. Ellaiah P, Prabhakar T, Sreekanth M, Taleb AT, Raju PB, Saisha V. Production of glycolipids-containing biosurfactant by *Pseudomonas* species. Indian J Experi Biol. 2002;40:1083-1086.

33. Santa Anna LM, Sebastian GV, Menezes EP, Alves TLM, Santos AS, Pereira JN, Freire DMG. Production of biosurfactants from *Pseudomonas aeruginosa* PA1 isolated in oil environments. Braz. J. Chem Eng. 2002;19:159–166.

34. Davey ME, Caiazza NC, Tootle GAO. Rhamnolipid surfactant production affects biofilms architecture in *Pseudomonas aeruginosa* PA-01. J Bacteriol. 2003;185: 1027-1036.

35. Rashedi H, Assadi MM, Jamshidi E, Bonakdapour B. Production of rhamnolipid by *Pseudomonas aeruginosa* growing on carbon sources. Intern J Environ Sci Tech. 2006;3:297–303.

36. Silva SN, Farias CB, Rufino RD, Luna JM, Sarubbo LA. Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. Colloids Surf B Biointerfaces. 2010;79(1): 174-183.

37. Femi-Ola TO, Oluwole OA, Oluwomofe TO, Yakubu H. Isolation and screening of biosurfactant bacteria from soil conta-minated with domestic waste water. Brit J Environ Sci. 2015;3(1):58-63.

38. Kumar G, Kumar R, Sharma A. Rhizoremediation of petrol engine oil using biosurfactants producing microbial consortium in mustard crop. Int J Appl Sci Biotechnol. 2015;3(2):261-266.

39. Bouchez-Naitali M, Blanchet D, Dardin D, Vandecasteele JP. Evidence for interfacial uptake in hexadecane degradation by *Rhodococcus equi*: The importance of cell flocculation. Microbiol SGM. 2001;147: 2537-2543.

40. Jadhav U, Kadu S, Thokal N, Padul M, Dawkar V, Chougale A, Salve A, Patil.

Degradation of tannic acid by cold-adapted *Klebsiella* sp. NACASA1 and phytotoxicity assessment of tannic acid and its degradation products. Environ Sci Pollut Res. 2011;18:1129–1138.

41. Sarafzadeh P, Hezave AZ, Ravanbakhsh M, Niazi A, Ayatollahi S. *Enterobacter cloacae* as biosurfactant-producing bacterium: Differentiating its effects on interfacial tension and wettability alteration mechanisms for oil recovery during MEOR process. Colloids Surf. B. Biointerfaces. 2013;105:223-229.

42. Mishra S, Jyot J, Kuhad RC, Lal B. Evaluation of inoculum addition to stimulate *in situ* Bioremediation of oily–sludge–contaminated soil. Appl Environ Microbiol. 2001;67(4):1675–1681.

43. Jamal P, Alam MZ, Zainuddin EA, Nawawi WMFW. Production of biosurfactant in 2L bioreactor using sludge palm oil as a sub-strate. IIUM Eng J. 2011;12(4):109-114.

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