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Screening and Characterization of Biosurfactant Producing Bacteria from Soil Samples in Ogun-State, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author SOA designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors AKA and SAB reviewed the experimental design and all drafts of the manuscript. Authors AKA and SAB managed the analyses of the study. Authors SOA and AKA performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Biosurfactants are surface active compound that are synthesized by microbes like bacteria, fungi and yeast. Biosurfactant have different applications in the industries. Biosurfactant can be used as emulsifiers, as well as demulsifiers, wetting agents, foaming agents, spreading agents, food ingredients and detergents etc. Ability of the microorganisms to produce biosurfactant was carried out using different screening techniques in a stepwise process.
Study Design: Soil samples were randomly collected from 8 different automobile shops in Ogun State, Nigeria.
Place and Duration of Study: Soil samples were collected from eight different automobile shops located at Oru-Ijebu, Ago-iwoye, Abeokuta and Ijebu-ode in Ogun state, Nigeria during the rainy season in the month of July, 2015.
Methodology: Biosurfactant-producing bacterial isolates were isolated and screened for

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biosurfactant production using haemolytic, oil spreading, drop collapse, bacteria adhesion to hydrocarbon and emulsification index tests (E. I). Bacteria were identified using biochemical tests and best biosurfactant producer bacteria was identified by molecular techniques. Bacteria with highest emulsification potential were selected to be the best biosurfactant producer.

Results: Result of the haemolytic activity test on blood agar showed that 46 isolates (17.4%) out of the 264 isolates showed beta-hemolytic activity, 39 (84.8%) isolates out of the 46 beta haemolytic isolates were positive for oil spreading test, 33 (84.6%) isolates were positive to the drop collapse assay and all the seventeen highly positive isolates for drop collapse test were positive for BATH test. *Pseudomonas taenensis* had the highest E.I of 71.05% at 24 hrs while *Acinetobacter calcoaceticus* had the lowest E.I of 32.6% at 24 hrs. Isolates with highest E. I were chosen to be best biosurfactant producer and are the best choice of isolate to produce biosurfactant.

Conclusion: The present study inferred that bacterial strains of *Pseudomonas taenensis* showed satisfactory results in all the screening tests and can be used as a potential candidates for biosurfactant and bioemulsion production.

Keywords: Biosurfactant; hydrocarbon soil; emulsification; oil spreading; bacteria.

1. INTRODUCTION

Biosurfactants are structurally diverse group of surface-active molecules synthesized by microorganisms which either adhere to cell surfaces or are excreted extracellularly on the growth medium [1]. Biosurfactant molecules can be classified into two major classes such as high molecular weight and low molecular weight biosurfactants. The high molecular weight biosurfactants have molecular weight which include mostly amphiphathic polysaccharide, proteins, lipopolysaccharides and lipoprotein which help to stabilize oil in water emulsion. Low molecular weight biosurfactants have molecular weight which include glycolipid and lippopeptide, which effectively lowers the interfacial and surface tension [2]. Biosurfactants are also surface active compound like chemical surfactants but unlike the chemical surfactant, biosurfactant are synthesized by microbes like bacteria, fungi and yeast.

Chemically synthesized surfactants are toxic, non-degradable and may be accumulated in living tissues leading to the development of cancer diseases. Biosurfactants are preferable to chemical surfactants due to the following characteristics: low or no toxicity, biodegradability, better environmental compatibility, ability to act at wide range of temperature, pH values and salinity levels [3]. Structurally, biosurfactant contain a hydrophilic moiety, comprising an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids [4].

Ability of isolates to produce beta haemolysis on blood agar plate is an indication of its ability to produce biosurfactant. Three types of hemolysis are known to occur: α, β, and γ. Alpha hemolysis (α) is said to occur when a greenish coloration is produced around the colony. Beta hemolysis (β) occurs when a clear zone is produced around the colony and Gamma haemolysis (γ) occurs when no change occurs around the colony [5]. Biosurfactants are further divided into six classes: hydroxylated and cross-linked fatty acids (mycolic acids), glycolipids, lipopolysaccharides, lipoproteins-lipopeptides, phospholipids and the complete cell surface itself [3]. Biosurfactants were used in several industries including organic chemicals, petroleum, petrochemicals, mining, metallurgy (mainly bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals and many others. They can be used as emulsifiers as well as de-emulsifiers, wetting agents, foaming agents, spreading agents, functional food ingredients and detergents. The interfacial surface tension reducing ability of biosurfactants made them to play important role in oil recovery and bioremediation of heavy crude oil [6]. The widespread nature of petroleum products and their use is strongly associated with anthropogenic discharge of hydrocarbons into the environment [7].

Environmental pollution arising from petroleum leakages in storage tanks, spillage during transportation of petroleum products, deliberate discharge of petroleum products and various industrial processes is hazardous to soil and water ecosystems [8]. The aims and objectives of this study are to isolate and characterize bacteria that are capable of producing biosurfactant from hydrocarbon polluted soil and to screen the isolates for biosurfactant production.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Soil samples were collected from 8 different automobile workshops into sterile polythene bags. They were taken at a depth of 0-10 cm, 10-20 cm and 20-30 cm. Collection of soil samples was done aseptically using an auger that was surface sterilized with 75% alcohol prior to use. The soil samples were labelled as (A1-B3) collected from Oru-Ijebu. (C1-D3) collected from Ago-Iwoye, (E1-F3) collected from Abeokuta, (G1-H3) collected from Ijebu-ode. The soil samples used as control were collected at various depths from a soil location not contaminated with hydrocarbon, FUNAAB; X1 (0-10 cm), X2 (10-20 cm) and X3 (20-30 cm) (Table 1).

2.2 Total Heterotrophic Bacterial Count (THBC)

The total heterotrophic bacterial count (THBC) was determined using the method of Rahman et al. [9]. One gram of each of the soil and effluent samples was serially diluted five-fold in sterile distilled water and 1 ml of the diluents was aseptically dispensed into sterile Petri-dishes. Using the pour plate method, Plate Count Agar (Lab M, UK) was poured aseptically on the sterile plates. The plates were incubated at 28℃ for 24 h after which the colonies were counted. This was carried out in replicates. The various colonies were then sub-cultured to obtain pure colonies.

2.3 Total Hydrocarbon Degrading Bacterial Count (THDBC)

The hydrocarbon utilizing bacterial count was carried out on Mineral Salt Medium (MSM) agar on which Dual Purpose Kerosene (DPK) was used as the sole carbon source. Prior to use, the DPK was filtered using a Whatman filter paper No 1, following the method of Jyothi et al. [10]. Two percent (2%) agar was added to solidify the medium. The MSM composition as described by Balogun and Fagade [11] which was made up of Basal Salt Medium (BSM) and Trace element solution.

Table 1. Sampling location for soil samples

Sample	Location	Code	Depth(cm)	Geographic positioning System (GPS) coordinates latitude	Geographic positioning system (GPS) coordinates longitude
1	Oru – Ijebu	A1	0 – 10	06°56'3338"N	03°56'3009"E
2	Oru – Ijebu	A2	10 – 20	06°56'3338"N	03°56'3009"E
3	Oru – Ijebu	A3	20 – 30	06°56'3338"N	03°56'3009"E
4	Oru – Ijebu	B1	0 – 10	06°57'29"N	03°57'2270"E
5	Oru – Ijebu	B2	10 – 20	06°57'29"N	03°57'2270"E
6	Oru – Ijebu	B3	20 – 30	06°57'29"N	03°57'2270"E
7	Ago - Iwoye	C1	0 – 10	06°56'2692"N	03°55'3414"E
8	Ago - Iwoye	C2	10 – 20	06°56'2692"N	03°55'3414"E
9	Ago – Iwoye	C3	20 – 30	06°56'2692"N	03°55'3414"E
10	Ago - Iwoye	D1	0 – 10	06°56'335"N	03°54'629"E
11	Ago – Iwoye	D2	10 – 20	06°56'335"N	03°54'629"E
12	Ago – Iwoye	D3	20 – 30	06°56'335"N	03°54'629"E
13	Abeokuta	E1	0 – 10	07°11'853"N	03°26'16"E
14	Abeokuta	E2	10 – 20	07°11'853"N	03°26'16"E
15	Abeokuta	E3	20 – 30	07°11'853"N	03°26'16"E
16	Abeokuta	F1	0 – 10	07°11'995"N	03°26'3469"E
17	Abeokuta	F2	10 – 20	07°11' 995"N	03°26'3469"E
18	Abeokuta	F3	20 – 30	07°11'995"N	03°26'3469"E
19	Ijebu – Ode	G1	0 – 10	06°51'2243"N	03°55'2963"E
20	Ijebu – Ode	G2	10 – 20	06°51'2243"N	03°55'2963"E
21	Ijebu – Ode	G3	20 – 30	06°51'2243"N	03°55'2963"E
22	Ijebu – Ode	H1	0 – 10	06°51'2493"N	03°55'3026"E
23	Ijebu – Ode	H2	10 – 20	06°51'2493"N	03°55'3026"E
24	Ijebu – Ode	H3	20 – 30	06°51'2493"N	03°55'3026"E
25	Funaab	X1	0 – 10	07°13'2512"N	03°26'623"E
26	Funaab	X2	10 – 20	07°13'2512"N	03°26'623"E
27	Funaab	X3	20 – 30	07°13'2512"N	03°26'623"E

2.4 Surface Active Bacterial Count (SABC)

Screening for surface-active bacteria was done on blood agar. The blood agar was made up of Nutrient Agar containing 5% (v/v) defibrinated rabbit blood. The method was carried out as reported by Tabatabaee et al. [12].

2.5 Screening for Biosurfactant Production

2.5.1 Complimentary test for biosurfactant production

2.5.1.1 Haemolytic activity

The surface active agents producing bacteria and hydrocarbon degraders colonies were used. This was determined according to the method of Tambekar and Gadakh [13]. The plates were incubated at 28℃ for 48 h after which the colonies that showed clear zone of beta-haemolysis were measured and recorded.

2.5.1.2 Oil spreading technique

The surface active agents producing bacteria and hydrocarbon degraders were screened for biosurfactants production using the oil spreading techniques according to Priya and Usharani; Anandaraj and Thivakaran [14,15]. The bacterial isolates were streaked on nutrient agar slant and incubated for 24 hours at 37℃. After 24 hours of growth of the inoculum on nutrient broth, 50 ml of distilled water was added to a large petri dish (25 cm in diameter) followed by the addition of 20 µl of crude oil to the surface of the distilled water and 20 µl of the supernatant of the cultures isolated from the soil. Diameter of the clear zone was taken.

2.5.1.3 Drop collapse test

The surface active agents producing bacteria and hydrocarbon degraders bacterial isolates were screened for drop-collapse test and this was carried out as described by Bodour and Miller [16]. Two microliter of crude oil was added to the microtitre plate and left to equilibrate for 24 h, followed by 5 µL of 48 h cell free supernatant of bacterial strain and the drop size was observed after 1 min. Positive result shows a flat drop and rounded drops were scored as negative which indicate a negative result for biosurfactant production.

2.5.1.4 Bacterial adhesion to hydrocarbon

The surface active agents producing bacteria and hydrocarbon degraders bacterial cells were suspended in phosphate buffer salt solution g/L (K_2HPO_4: 16.9 and KH_2PO_4: 7.3g/L with pH 7 to give an optical density of 0.5 at 600 nm. One hundred microliter of kerosene was added to 2 mls of cell suspension and was vortexed for 2 mins in test tubes. Aqueous phase was allowed to separate for 1 hr. The optical density of the aqueous phase was measured after 10 mins. Hydrophobicity was measured as the percentage of cell adherence to hydrocarbon. The degree of hydrophobicity was calculated as H= 1- $^{A}/_{A_0}$ x 100% Goulart et al. [17] where A is the absorbance of the aqueous phase after hydrocarbon was added and A_0 is the absorbance of the aqueous phase before hydrocarbon was added.

2.5.2 Confirmatory test for biosurfactant production

2.5.2.1 Emulsification index test

Positive bacterial isolates from the above complimentary screening test were grown on MSM, supplemented with 1% DPK for 7 days in an orbital incubator at 180 revolutions per minute (rpm) at 28ºC. Cell free supernatant obtained by centrifuging the broth culture at 15,000 rpm for 15 min was used for the experiment according to Balogun and Fagade [11]. The emulsification index for surface active agents producing bacteria was carried out using the method of Bodour et al. [18]. Two millilitres of the supernatant of each organism was put in reaction tube and 2 ml of DPK added as hydrocarbon substrate tested. The mixture was vortexed at high speed for 2 mins and observed for percentage emulsification at intervals 4 h for 24 h. Emulsification index (EI) was recorded as a percentage of the height of the emulsified DPK to the total height of the mixture after 24 h as described by Tabatabaee et al. [12].

$$E_{24} = \frac{\text{height of emulsion}}{\text{total height}} \times 100\%$$

Where

Height of emulsion = height of emulsified layer
Total height = total height of the liquid column

2.6 Bacterial Characterization

Identification of the isolates were carried out using standard microbiological method. Shape, pigmentation, elevation, size, appearance and motility were used for morphological characteristics. The following biochemical test were carried out: gram stain, catalase test, oxidase test, motility test, indole, coagulase test, nitrate test and urease test and molecular characterization was done on the best isolate that recorded highest biosurfactant producer. Extraction of the genomic DNA of bacteria isolate, amplification by polymerase chain reaction (PCR) using 16SrRNA primer, sequencing of the isolate DNA and DNA sequence was used to reveal the name of the isolate according to the method of Joshi and Deshpande [19].

2.7 Statistical Analysis

Data obtained were subjected to Analysis of Variance and mean were separated with Duncan Multiple Range Test using Statistical Package for Social Sciences (SPSS) version 20.0, ($P<0.05$).

3. RESULTS AND DISCUSSION

The results obtained from the different counts were significantly different at $P<0.05$. Isolation and screening of microorganisms for their efficiency in biosurfactant production before field trials is important and the development of efficient techniques is an important tool in recommending different approaches. The distribution of bacterial isolates obtained from various sampling sites indicates common occurrence of metabolically active strains in the environment suggesting the ability of these microorganisms to utilize hydrocarbons as sole carbon and energy source according to Afuwale [20]. From the result, highest Total Heterotrophic Bacterial Count (THBC) of $17.7x10^6$ CFU/mL was obtained at location H1 and the lowest count of 3.3×10^6 CFU/mL ($P<0.05$) was at location F3. The high range of heterotrophic counts recorded in unimpacted soil might be due to the high level of organic matter usually present in fallow uncultivated soil suggesting that soil is fertile and supports the growth of microorganisms.

Total Hydrocarbon Degrader Count (THDC) exhibited the highest count of 6.8×10^6CFU/mL ($P<0.05$) from sample E2 and the lowest count of 1.2×10^6 CFU/mL ($P<0.05$) from location X1. The range of hydrocarbon degraders recorded was also quite high which could be due to the presence of residual crude oil in the polluted soil which boosts the carbon supply in the soil. The results of total hydrocarbon degrader bacterial count from all contaminated sites were higher than the control soil sample. This agreed with the work of Youssef et al. [21] who reported that the hydrocarbon polluted sites have greater bacterial abundance and a large proportion of bacteria capable of hydrocarbon degradation than unpolluted soils.

Surface Active Bacteria Count (SABC) from sample location C3 showed the highest mean value of $3.2x10^6$ CFU/mL ($P>0.05$) while the lowest value of 2.0×10^5 CFU/mL ($P<0.05$) from G1 and X3 (Table 2).

The high population of surface-active agent producing bacteria recorded in both impacted and non-impacted soil was not in acccordance with the findings of Balogun and Fagade [11] where lower values of 1.2×10^5 was reported. This suggests that surface active agents or the production of biosurfactants does not only occur in impacted soil or polluted soil but also in non-polluted or unimpacted soil. In addition, surface active - agent producing bacteria recorded in the oil impacted soil imply that these bacteria can be of great potential in the remediation of oil impacted soil.

The levels of hydrocarbon/oil present in a contaminated site represent a nutrient rich environment. Also Mandri and Lin [22] reported that the highest number of hydrocarbon utilizing bacteria in the impacted soil is due mainly to the availability of the substrates which these organisms can utilize for their growth and other metabolic activities. The results obtained from the total heterotrophic plate count, total oil degrader count and surface active bacteria count showed high range of values suggesting that soil supported the growth of a wide diversity of bacteria, despite the fact that soil was obtained from the deep subsurface, microorganisms were found surviving there. This affirmed the ubiquity of microorganisms following the method of Willey et al. [23] and that the isolates can metabolize soil.

Ability of the sample to produce beta haemolysis on blood agar signifies the ability of the isolate to lyse red blood cells. Urum and Pekdemir [24]; Rashedi et al. [5] have reported that isolates that haemolyse red blood cells are surface active

Table 2. Bacterial counts of contaminated and uncontaminated soil samples

Location	Depth	Total heterotrophic bacterial count (X 10^5 CFU/mL)	Total hydrocarbon degrader count (X 10^5 CFU/mL)	Surface active bacteria count (X 10^5 CFU/mL)
A1	0 – 10	39.00± 2.31	43.67± 13.20	10.00± 3.46
A2	10 – 20	46.67± 5.70	34.64± 2.43	18.00± 4.04
A3	20 – 30	124.33± 2.91	46.00± 5.20	27.00± 1.73
B1	0 – 10	125.00± 1.73	27.33± 5.81	11.67± 3.84
B2	10 – 20	137.00± 5.20	55.0± 3.46	11.67± 4.91
B3	20 – 30	86.00± 4.04	27.00± 5.20	7.67± 2.60
C1	0 – 10	38.00± 1.15	23.00± 2.89	13.33± 4.40
C2	10 – 20	40.00± 6.93	15.0± 4.62	3.00± 1.53
C3	20 – 30	126.67±32.92	35.63± 9.39	32. 60± 8.41
D1	0 – 10	42.67± 5.81	42.00± 14.43	4.67± 1.86
D2	10 – 20	74.00± 7.51	23.00± 3.46	19.33± 5.24
D3	20 – 30	47.33± 4.91	20.00± 8.08	12.00± 3.46
E1	0 – 10	132.00± 9.81	34.00± 6.90	27.00± 7.51
E2	10 – 20	115.30± 7.51	68.0± 16.74	2.67± 1.20
E3	20 – 30	148.0± 13.28	49.33± 14.15	4.33± 1.76
F1	0 – 10	153.67± 9.24	62.00± 16.44	23.00± 7.0
F2	10- 20	136.33± 3.18	50.67± 10.68	19.0± 5.20
F3	20 – 30	33.65± 6.36	18.00± 5.20	8.00± 3.46
G1	0 – 10	116.00± 8.08	52.00± 4.62	2.0± 0.88
G2	10 – 20	134.00± 6.35	43.00± 4.04	10.00± 4.04
G3	20 – 30	143.00± 67	44.67± 6.57	2.00± 8.88
H1	0 – 10	176.67± 20.92	63.33± 12.99	13.00± 2.89
H2	10 – 20	130.00± 18.72	46.00± 6.93	10.00± 3.46
H3	20 – 30	124.00± 3.79	41.00± 5.20	18.00± 5.20
X1	0 – 10	157.67± 19.38	12.00± 1.73	12.67± 3.46
X2	10 – 20	132.33± 32.27	14.00± 2.31	10.30± 2.3
X3	20 – 30	119.67± 10.20	18.00± 2.33	2.0± 0.73

Values are Mean ± Standard Error of Means

agent producers. Hence, organisms that can lyse red blood cells and displace oil in water surface are biosurfactants producers.

A total of 264 isolates were obtained from the 24 soil samples from the result of total hydrocarbon count (THDC) and surface active bacterial count (SABC).

The result of the haemolytic activity test on blood agar showed that 46 (17.4%) produced beta haemolysis, 58 (22.0%) produced gamma haemolysis and 160 (60.6%) organisms produced alpha haemolysis. Ability of bacterial isolates to produce beta haemolysis is an indication of its ability to produce biosurfactant.

Haemolytic assay or blood agar lysis method had also been recommended by Banat [25] and Yonebayashi et al. [26] as a simple, easy method to test for biosurfactant activity and to screen biosurfactant production by new bacterial isolates while Carrillo et al. [27] recommended the use of haemolytic assay as a primary method to screen for biosurfactant activity. Haemolytic test which is the ability of the cells of the bacteria to lyse red blood cells was not in accordance with the work of Okore et al. [28] where a value between 7 to 19 mm was reported.

Table 3 showed beta haemolytic and oil spreading test. For beta haemolytic test, highest zone of clearance was obtained at location E_2^2 of 26.3±0.58 mm while lowest zone of clearance was obtained at location B_3^1 of 7.0± 0.58 mm.

Screening of the 46 positive isolates using the oil spreading test revealed that 39 (84.8%) isolates were positive while 7(15.2%) were negative.

Oil spreading test was carried out in the study to ascertain if the isolates can displace oil at the surface of water showed that the result gotten in the study was in accordance with the findings of Priya and Usharani [14]; Anandaraj and Thivakaran [15]. The area of oil displacement in an oil spreading assay is directly proportional to the concentration of the biosurfactant in the

solution Morikawa et al. [29]. Also, Youssef et al. [30] recommended the use of oil spreading and drop collapse assay as reliable techniques for testing for biosurfactant production.

Further screening of the 39 positive isolates for biosurfactant using the drop collapse test showed that 33 (84.6%) isolates were positive and 6 were negative (15.4%) (Table 3). Drop collapse result was in accordance with the work of Tambekar and Gadakh [13]. Drop collapse test was suggested to be sensitive and easy method to test production of biosurfactant, however, Said et al. [31] have reported that microorganisms that recorded negative drops-collapse test are not good emulsifiers and

Table 3. Result of haemolytic, oil spreading and drop collapse test by isolates

Isolates	Haemolytic test (mm)	Oil spreading test (mm)	Drop collapse test
A_2^1	12.3± 1.2	3.1± 0.58	++
A_2^2	11.0± 1.15	1.9± 0.89	+
A_2^3	10.0± 0.58	8.4± 6.57	+
A_2^4	16.0± 1.15	3.3± 0.17	++
A_3^2	12.7± 0.67	2.6± 0.15	++
A_3^1	17.0± 1.15	3.3± 0.58	++
A_3^4	19.0± 1.15	2.0± 0.12	+
B_2^1	20.7± 0.33	1.6± 0.32	+
B_2^2	18.3± 0.33	4.2± 0.12	++
B_2^3	12.0± 1.15	1.8± 0.58	+
B_2^4	10± 0.58	4.6± 0.89	++
B_3^1	7.0± 0.58	1.8± 0.12	+
B_3^2	8.3± 0.33	1.4± 0.58	+
B_3^3	18.0± 0.58	2.5± 0.21	++
C_1^1	24.0± 0.58	2.2± 0.15	+
C_1^3	21.67± 0.33	2.6± 0.7	+
C_2^3	20.33± 0.33	3.2± 0.12	++
C_3^4	20.0± 1.15	1.6± 0.88	++
D_2^1	15.0± 0.58	1.9± 0.35	-
D_2^2	15.67± 0.33	3.2± 0.15	++
D_2^3	17.33± 0.33	2.3± 0.89	+
D_2^4	22.0± 1.15	2.1± 0.15	+
E_1^1	14.3± 0.88	-	-
E_1^2	18.0± 1.73	-	-
E_1^3	19.0± 1.73	2.0± 0.18	+
E_1^4	21.0± 1.15	2.3± 0.21	+
E_2^2	26.3± 0.88	3.6± 0.12	++
E_3^4	24.3± 0.88	4.2± 0.12	++
F_1^2	23.7± 0.88	-	-
F_1^3	22.7± 1.45	-	-
F_1^4	21.0± 1.73	2.0± 0.88	+
F_2^1	14.7± 1.45	2.5± 0.12	++
F_2^2	21.0± 2.08	1.6± 0.12	+
F_2^3	14.3± 0.88	2.1± 0.26	+
F_2^4	13.67± 2.03	2.3± 0.33	+
F_3^1	11.3± 1.45	1.7± 0.12	+
F_3^2	14.67± 0.88	2.8± 0.27	++
F_3^3	12.67± 2.33	-	-
H_2^1	18.3± 2.60	2.5± 0.15	++
H_2^2	16.7± 0.67	1.9± 0.15	+
H_2^2	16.7± 0.67	1.9± 0.15	+
H_2^3	21.0± 1.73	1.8± 0.20	+
H_1^2	24.0± 1.73	-	-
H_3^1	26.0± 1.73	-	-
H_3^2	23.0± 2.3	2.2± 0.12	+
H_3^4	23.7± 0.33	3.8± 0.15	++
H_1^3	24.7± 0.88	4.2± 0.15	++

Values are Mean ± Standard Error of Means.

are not good biosurfactant producers; hence some isolates were not selected for further assay. In liquid that contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension.

Seventeen isolates that showed highly positive potential for drop collapse test were finally subjected to bacterial adhesion to hydrocarbon assay (BATH) (Table 4) and result showed that they all had ability for adhesion to hydrocarbon. Ability of the cells that can produce biosurfactant to adhere to hydrocarbon (bath assay) was not in accordance with the work of Padmapriya et al. [32] where maximum activity of 99% bath assay result was recorded.

Isolates were subjected to confirmatory test which is the emulsification index (E.I) test at different time intervals from 0 hr to 24 hrs. Results of emulsification index (E.I) of all the bacteria isolates varies with different time intervals. At 0 hr to 24 hrs, *Pseudomonas aeruginosa* had the highest E.I of 71.05% at 24 hrs while *Acinetobacter calcoaceticus* had the lowest E. I of 32.58% at 24 hrs (Table 5).

Table 4. Result of highly positive drop collapse test isolates for bacterial adhesion to hydrocarbon (BATH) assay

Isolate code	A	H%
A_2^1	0.33±0.02	34
A_2^4	0.26±0.01	48
A_3^2	0.25±0.00	50
A_3^1	0.20±0.07	60
B_2^2	0.32±0.03	36
B_2^4	0.27±0.00	46
B_3^3	0.22±0.00	56
C_2^3	0.29±0.07	42
C_3^4	0.26±0.00	48
D_2^2	0.34±0.01	32
E_2^2	0.22±0.01	56
E_3^4	0.24±0.02	52
F_2^1	0.34±0.06	32
F_3^2	0.33±0.03	34
H_2^1	0.32±0.02	36
H_3^4	0.30±0.01	40
H_1^3	0.31±0.00	38

Key: A = absorbance of the aqueous phase after hydrocarbon was added
A_0= absorbance of the aqueous phase before hydrocarbon was added = 0.5
H% = hydrophobicity

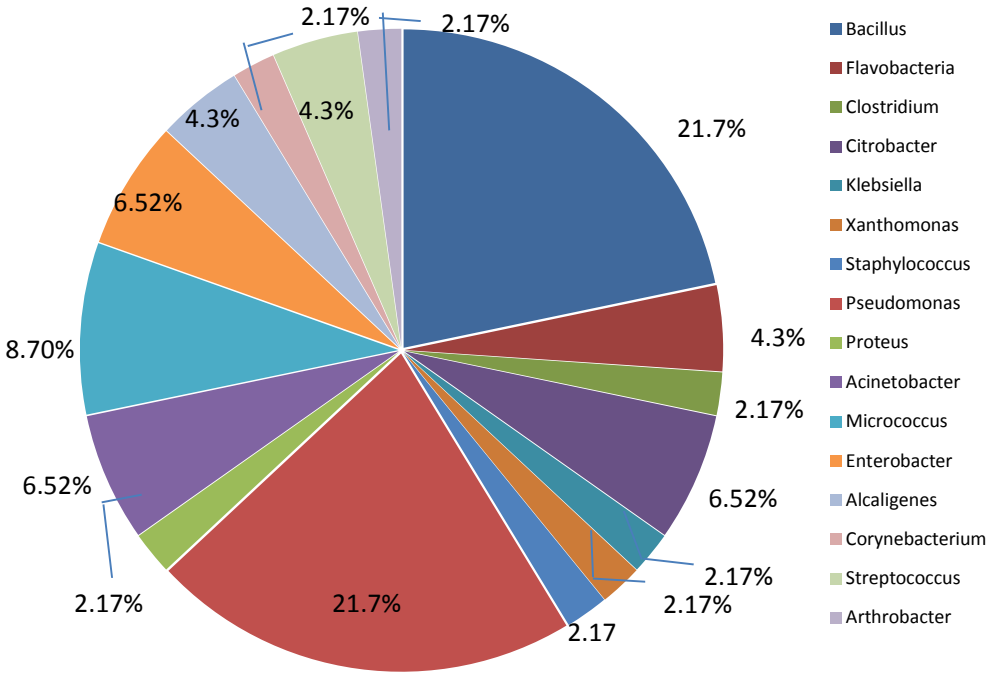

Fig. 1. The occurrence of the various genera identified in the soil samples

Table 5. Emulsification indices test (%) on dual purpose kerosene of isolates

Isolate	Isolate code	0h	4hrs	8hrs	12hrs	16hrs	20hrs	24hrs
Bacillus macerans	A_2^1	73.6±0.5	68.42±1.12	71.43±0.3	62.16±1.0	62.16±1.3	59.46±0.1	57.90±0.8
Bacillus licheniformis	A_2^4	74.36±0.1	69.23±0.3	70.27±0.2	55.0±0.0	56.41±0.9	5 6.41±1.7	58.97±0.2
Bacillus subtilis	A_3^2	65.0±0.02	60.0±0.05	61.54±0.0	46.15±0.04	48.72±0.0 2	48.72±0	51.28±0.2
Citrobacter aerogenes	A_3^1	78.95±0.18	73.0±0.04	66.67±0.0	57.9±0.06	61.54±0.0 4	58.97±0.06	65.79±0.16
Xanthomonas campestris	B_2^2	78.38±0.6	71.05±0.03	63.42±0.08	57.5±1.1	65.79±0.9	68.42±1.7	68.42±1.3
Pseudomonas aeruginosa	B_2^4	81.58±0.0	74.36±0.01	74.36±0.0	65.85±0.1	71.80±0.0 5	67.80±0.02	65.80±0.04
Acinetobacter calcoaceticus	B_3^3	35.90±1.8	31.0±0.04	36.83±0.0	32.77±0.02	34.77±0.0	35.64±0.10	32.60±0.0
Pseudomonas fluorescence	C_2^3	56.41±0.01	51.28±0.01	51.28±0.01	42.5±0.04	40.0±0. 02	42.5±0.01	48.72±0.03
Bacillus licheniformis	C_3^4	56.41±0.06	50.0±0.0	46.15±0.02	46.34±0.01	44.74±0. 06	47.37±0.03	52.63±0.01
Acinetobacter calcoaceticus	D_2^2	37.5±0.0	31.71±0.0	25.0±0.0	35.71±0.04	35.0±1.7	32 .5±0.0	35.90±0.0
Pseudomonas aeruginosa	E_2^2	75.68±0.1	68.42±0.06	58.56±0.03	69.23±0.01	71.05±0 .0	71.06±0.0	71.05±0.0
Citrobacter aerogenes	E_3^4	43.24±0.01	44.74±1.02	37.5±0.01	30.0±0.05	33.33±0.0 2	33.33±0.18	34.21±0.35
Streptococcus mutans	F_2^1	50±0.35	46.15±0.0	40.0±0.3	35.90±0.17	39.47±0.9	36 .84±0.08	42.11±1.0
Bacillus subtilis	F_3^2	48.72±0.0	46.15±0.1	39.47±0	41.03±0.04	43.59±0.02	43.59±0	44.74±0.16
Bacillus macerans	H_2^1	63.16±0.01	58.97±0.01	54.05±0.03	42.11±0.18	51.28± 0.06	46.15±0.03	48.72±0.0
Arthrobacter globiformis	H_3^4	72.5±0.0	65.0±0.0	60.0±0.02	57.14±0.03	57.5±0.0	57 .5±0.06	61.54±0.08
B. laterosporus	H_1^3	70±0.01	66.67±0.02	56.41±0	68.30±0.3	60.98±0.0	58. 54±0.0	66.67±0.04
Control	Ctrl	0	0	0	0	0	0	0

The ability of the biosurfactants produced by the isolates to emulsify points to the fact that they are emulsifiers and can reduce the surface tension of the water/oil mixture. Zero emulsification index recorded for the negative control sample indicated that they are poor emulsifiers and hence do not have the ability to reduce the surface tension of the mixture. Similar result of the emulsification index was recorded by Sriparna et al. [33].

Highest emulsified isolate was identified using molecular methods after the BLAST on the NCBI database to establish identity of the microorganism. The molecular identification result obtained showed that the highest emulsified bacterial isolate was *Pseudomonas taenensis*. The description of the closest identity of the isolate was revealed and assigned the accession numbers: isolate P_T, the isolate was designated as *Pseudomonas taenensis* [accession numbers NZ_AWSQ01000002.1] in lane 1 as shown in Table 6.

Plate 2 shows the polymerase chain reaction of the bacterial isolate.

Ability of some strains to show positive biosurfactant production following one method and negative following other methods makes it very difficult to confirm biosurfactant production using only one method. In view of this, several screening methods have to be considered in order to identify the potential organism that can produce biosurfactant. Compared to other isolates, *Pseudomonas taenensis* showed better potential in the entire screening test for biosurfactant production. Hence, it's chosen as a promising organism for biosurfactant production based on this study.

Table 6. Sequences obtained from NCBI Database Gene Bank

Isolate code	Description of closest identity	Maximum score	Total score	E-value	% Identity	Accession
P_T	*Pseudomonas taenensis* MS-3	37.4	37.4	3e-13573	96%	NZ_AWSQ01000002.1

(NCBI, accessed on September 28, 2015)

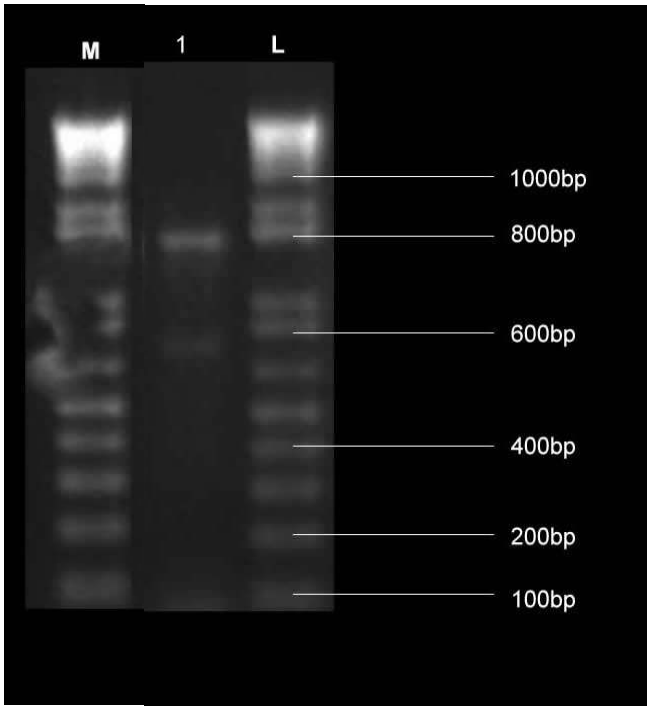

Fig. 2. PCR Amplification of Isolate 1 (B_2^4)

L= ladder, Lane 1: Band showing the presence of Pseudomonas taenensis with highest molecular weight of 800bp, Lane M= +ve control

4. CONCLUSION

Interest in biosurfactants has led to the development of a multitude of methods for the screening of biosurfactant producer strains. Emulsion capacity of *Pseudomonas taenensis* makes it new potential candidates for biosurfactant and bioemulsion production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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