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Biosurfactant Production by Bacteria Isolated From Local Tannery Effluent in Sokoto Metropolis

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Abstract: *With increasing demand of Biosurfactants for environmental and industrial application, there is increased interest in prospecting of microorganisms and substrates for production of substantial quantities of Biosurfactants. A study was carried outto investigate the ability of Bacillus sphaericus EN3 and Bacillus azotoformans EN16 to produce large quantities of Biosurfactants and determine a suitable substrate for its production. The organisms were previously screened for biosurfactant production and found to be good candidates. Three carbon sources including glucose, diesel and crude oil were used and incorporated into modified Bushnell – Haas medium at 2% w/v concentration. Large quantities of Biosurfactants were observedwhen glucose was used as substrate with a mean production of 1.01 ± 0.3 g/L and 0.58 ± 0.17 g/L for B. sphaericus EN3 and B. azotoformans EN16 respectively after 10 days. The surfactants were shown to be stable at various pH (2 - 12), temperature (50°C – 100°C) and salinity (2 – 10% NaCl) ranges. The Biosurfactants were however more effective at pH of 8 to 10, 50^{0}Cand salinity of 2 to 4% NaCl. Chromatographic analysis revealed that the surfactants produced by both organisms were principally phospholipids. Therefore, the organisms were capable of producing substantial amounts of surfactants and harnessing their potentials would be essential.*

Keywords: *Biosurfactant, Bacteria, Effluent & Organism*

INTRODUCTION

Biosurfactants are surface active agents produced by microorganisms. They reduce surface and interfacial tension by accumulating at the interface of immiscible fluids and thus increase the solubility, bioavailability and subsequent biodegradation of the hydrophobic or insoluble organic compounds (Van Hamme et al., 2006). They consist of heterogeneous group oforganic compounds including glycolipids, lipopolysaccharides, oligosaccharides and lipopeptides (Thavasi et al., 2011). Biosurfactants vary in chemical properties and molecular size. They contain hydrophobic and hydrophilic moieties that give them the ability to interact between

fluid phases, thus minimizing surface and interfacial tension at the surface and interface respectively (Kranth et al., 1999;Satpute et al., 2009).

Currently most of surfactants being used are chemical surfactants derived from petroleum. However, the interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environment friendly, the possibility of their production through fermentation and their potentialapplications in areas such as environmental protection, crude oil recovery, health care and food processing industries (Makkar et al., 2011).

Production of biosurfactants has increased in recent years due to their biodegradability, reduced toxicity and diverse application compared to synthetic surfactants (Seghal Kiran et al., 2010). The diverse applications of biosurfactants in different sectors such as environmental, pharmaceutical and industrial sectors, etc. lead to high demand of the product. High production cost of biosurfactants has been a great challenge in meeting its global demand (Krepsky et al., 2007).

Many attempts were made to produce huge amountof biosurfactants at lower costs. Much attention focused on obtaining efficient microbial strain and cheap and readily available substrate (Saghel Kiran et al., 2010). Majority of biosurfactant production involved the use of microorganisms grown on water immiscible hydrocarbons, and some water- soluble substrates (Abu- Ruwaida et al., 1991; Desai and Banat, 1997; Tabatabaee et al., 2005; Makkar et al.2011). Bacteria especially *Bacillus* species, are among the best known biosurfactant producers. Previous studies have shown the potentials of *Bacillus subtilis* and *Bacillus licheniformis* to produce large quantities of biosurfactants using different substrates (Joshi et al., 2008; Jaysree et al., 2011). *Bacillus sphaericus* and *Bacillus azotoformans* are among many spore forming bacilli although with no previous report of biosurfactant production prior to current work (Adamu et al., 2015). In addition to determining the best substrate for biosurfactant production, this study also studied the ability of the two bacterial species to produce large quantities of biosurfactants under laboratory settings.

Table 1: The amount of biosurfactant produced by *Bacillus* species

Yield of Biosurfactant Produced g/L (mean ± SD)						
	Bacillus sphaericus EN3			*Bacillus azotoformans* EN16		
Time	Glucose	Crude oil	Diesel	Glucose	Crude oil	Diesel
2	0.22±0.02	0.11± 0.01	0.11±0.01	0.11±0.01	0.05±0.05	0.05±0.05
4	0.51±0.01	0.21±0.01	0.31±0.01	0.22±0.25	0.05±0.05	0.21±0.01
6	0.71±0.01	0.32±0.02	0.31±0.01	0.51±0.01	0.11±0.01	0.22±0.25
8	2.25±0.20	0.49±0.01	0.79±0.01	1.25±0.25	0.21±0.01	0.41±0.01
10	1.50±0.30	0.43±0.03	0.79±0.01	0.82±0.02	0.20±0.00	0.40±0.05
*Mean	1.01 ± 0.3^{a}	0.31 ± 0.07^{a}	0.46 ± 0.01^{a}	0.58 ± 0.17^{a}	0.12 ± 44^{b}	0.26 ± 0.07^{ab}

*Values with different superscript in raw differ significantly ($p \leq 0.05$) for a particular species

Materials and Methods

Collection of Samples

Tannery effluents were collected from three local tanneries in Sokoto, Sokoto State, Nigeria. Sokoto is located to the extreme Northwest of Nigeria between longitudes 4º 8'E and 6º54'E and latitudes 12ºN and 13º 58'N. Samples were collected in sterile sample bottles and transported in ice box to Microbiology laboratory, Usmanu Danfodiyo University, Sokoto. The triplicate effluents samples were collected by simple random sampling from the tanneries. The tanneries were: Karaye, Ungwarogo I and Ungwarogo II (Figs. 1, 2 and 3 respectively).

Determination of Physicochemical Properties of the Tannery Effluents

The pH, biochemical oxygen demand (BOD), chemical oxygen demand (COD), temperature, total solid (TS), total dissolved solid (TDS), total suspended solid (TSS), hardness, electrical conductivity, color, chromium content and other physicochemical properties were determined using standard methods for waste water, as described by American Public Health Association, (APHA, 2005).

Fig. 1. Karaye tannery

Fig. 2. Unguwan Rogo I tannery

Fig. 3. Unguwan Rogo II tannery

Microbiological Analysis of Tannery Effluent

Ten-fold serial dilutions of the effluent suspension were carried out as described by Benson, [12]. By spread plate technique, 1ml aliquots of samples dilutions were inoculated in triplicates on Nutrient agar (NA) for the enumeration of total aerobic heterotrophic bacteria. The NA plates were incubated at 37^{o}C for 24 - 48 hours. Mineral salt media (MSM) of Ijah *et al*. [13] (1.2g KH_2PO_4, 1.8g K_2HPO_4 4.0g NH_4Cl, 0.2g $MgSO_4.7H_2O$, 0.1g NaCl, 0.01g $FeSO_4.7H_2O$ and 20g agar per liter at pH 7.4; supplemented with 0.1% v/v Bonny Light crude oil) was used for the isolation of biosurfactant producing bacteria. Colonies which appeared on the plates were counted and expressed as colony forming units per milliliter (cfu/ml) of sample. Pure isolates were obtained by repeated subculturing on fresh mineral salt media plates. The pure isolates were maintained on agar slants in a refrigerator (8°C). The isolates were identified by biochemical characterization using the schemes of Barrow and Feltham [14] and Bergey's Manual identification flow chart [15].

Screening of Bacterial Isolates for Biosurfactant Production

Four methods were used to screen the bacterial isolates for potentials to produce biosurfactant. The methods were blood hemolysis test, emulsification index, oil spreading, and drop collapse methods as described elsewhere [16]. Isolates were grown in MSM containing the crude oil as mentioned above. The culture was incubated for 10 days at 30°C with regular shaking. After the incubation period, the broth of each isolate was centrifuged at 6000 rpm for 10 minutes and the supernatants separated by filtration in order to obtain cell-free supernatants. The supernatants were used for emulsification, drop collapse and oil spreading tests.

a. Blood Hemolysis test

The bacterial isolates were inoculated on blood agar containing 5% (v/v) human blood. The plates were incubated at 30^{o}C for 48 hours. Hemolytic activity was detected as the presence of a clear zone around a colony. The clear zone (hemolytic activity) suggests the presence of biosurfactant [17].

b. Drop collapse test

Drop collapse test was carried out according to the method described by Youssef *et al.* [17]. A drop of crude oil (Bonny light) was placed on a grease free slide and one drop of the cell free supernatant was placed at the center of the oil drop. Collapse of the drop was due to reduction of interfacial tension between the liquid drop (containing biosurfactant) and the hydrophobic surface of the oil. The time it took the oil drop to collapse was also recorded.

c. Oil spreading method

Oil spreading technique was carried out according to the method described by Youssef *et al.* [17]. Fifty milliliters of distilled water were added to a Petri dish followed by addition of 100 micro liters of crude oil (Bonny light) to the surface of the water. Then one drop of the supernatant was dropped on the crude oil surface. The diameter of clear zone on oil surface was measured using a meter rule and the time taken to achieve the spread was also noted.

d. Emulsification capacity test

Emulsification activity was carried out using the method of Tabatabaee *et al.* [18] and Techaoei *et al*. [19]. Four 4ml of the crude oil was added to equal amount of cell free supernatant and vortexed at 500 r.p.m for 10 minutes. After 24 hours, the height of the stable emulsion layer was measured using meter rule. The emulsification index (E_{24}) was calculated as the ratio of the height of the emulsion layer and the total height of liquid, as given by the expression:

$$E_{24} = \frac{\text{h emulsion}}{\text{h total}} \times 100$$

Where:
E_{24} is emulsion index after 24 hours,
h emulsion is the height of emulsion layer,
h total is the total height of the liquid.

RESULTS AND DISCUSSION

Physicochemical analysis

Table 1 shows the physicochemical qualities of the tannery effluents analyzed. The color of the effluent samples was found to be gray to dark brown while the odor was disagreeable. This can also be observed in figures 1, 2 and 3 above. The color and disagreeable odor of the effluent could be due to the tanning chemicals that are known to have a pungent or choking smell and the decomposition of the skin and hides during the tanning process. Imamulhaqq [2] made similar observation in some tannery effluents in Bangladesh. The temperature of the samples ranged from 22.5 to 32^{o}C, while the pH ranged between 4.2 and 5.9. The temperature

of the tannery effluent was within the permissible limit prescribed by FEPA (Nigeria) while pH of the effluent was acidic and was below the permissible range (6.0 – 9.0) allowed by FEPA [20]. The acidic pH of the effluent could be due to the effect of chemicals used in the tanning process and the accumulation of acidic metabolites [8].

The electrical conductivity (EC) ranged from 13586 to 15500 s/m, while the total hardness (TH) ranged from 2400 to 3500mg/L. The total dissolved solids (TDS) and total suspended solids (TSS) were determined and they ranged from 170 to 943.5mg/L and 170.7 - 1057mg/L respectively. The chromium (Cr) and dissolved oxygen (DO) contents of the effluents were in the range of 0.7 to 86.5 and 0.2 to 1.8 respectively. Karaye tannery had higher Cr contents whereas Unguwan Rogo II had highest DO (Table 1). The values obtained for EC, TSS, BOD, COD and Cr contents of the effluent were higher than the limit allowed by the FEPA [20] as shown in Table 1. Only values for DO were within the recommended limit.

Similar reports on excess physicochemical parameters from tannery effluents were reported by Tudunwada *et al*. [3]. The high level of hardness of the tannery effluent is attributed to the presence of magnesium sulfate and calcium bicarbonate. This agreed with finding of Tudunwada *et al*. [3], who suggest that sodium sulfate, chromium sulfate and non-ionic wetting agents are the major constituents of tannery effluent and may accumulate in the immediate environments of the tanneries.

The high BOD in the tannery effluent indicates the presence of large amount of biodegradable materials while high COD indicates that non-biodegradable materials are much higher than the biodegradable (organic) matter [3]. Chromium content of the tannery effluent was quite high (0.7 – 86.5mg/L). This may be due to the accumulation of residual chromium compound used in the tanning process. The chromium compound could provoke phytotoxic and genetoxic effects [21].

Microbiological analysis

Total heterotrophic bacterial counts revealed that Unguwan Rogo II (8.5×10^6 cfu/ml) tannery had the highest bacterial counts followed by Karaye (5.3×10^6 cfu/ml), while Unguwan Rogo I (4.64×10^5 cfu/ml) had the lowest count as shown in Table 2. These high counts could be due to available nutrients and favorable temperature of the effluent as well as the ability of the organisms to withstand, tolerate or adapt to the unfavorable condition of the effluent. The count also corresponded to BOD values in which the tannery with lowest BOD (Unguwan Rogo II) had the highest count, indicating active biodegradation of organic matter.

A total of 47 bacteria were isolated and identified. *Bacillus licheniformis* had the highest frequency of occurrence (19.1%), followed by *Bacillus azotoformans* (12.8%), P*roteus mirabilis* (12.8%)*, Bacillus leterosporus* (8.5%), *Bacillus sphaericus* (8.5%) and *Bacillus cereus* (6.4) in that order. In addition, *Serratia fonticola*, *Bacillus larvae*, *Serratia liquefaciens* had 4.3% of occurrence each. Other species isolated include *Citrobacter diversus, Aeromonas hydrophila, Enterococcus faecalis, Micrococcus luteus, Bacillus papilliae* and *Pseudomonas aeruginosa* with 2.1% each. The presence of these organisms in tannery effluent might not be surprising especially due to ubiquity and versatility of bacteria, despite the presence of toxic substances such as chromium and other heavy metals. Some of the organisms identified were reported by some researchers as part of tannery biota [3, 7].

Table 1: physicochemical properties of the tanneries

Parameter	Values (mean of triplicates)			Range	Recommended Limit (FEPA)
	Karaye	Unguwan Rogo I	Unguwan Rogo II		
Color	Dark brown	Dark brown	Gray brown	Gray - brown	NIL
Odor	disagreeable	Disagreeable	Disagreeable	Disagreeable	NIL
Temperature (ºC)	26.35	32.0	20.7	22.5-32.0	<40
pH	5.9	4.2	5.3	4.2-5.9	6.0-9.0
Electrical Conductivity (s/m)	13856	15500	13676	13586-15500	200
Total Hardness (mg/L)	2910	3500	2900	2400-3500	125
Total Dissolved Solids (mg/L)	170	943.5	357.5	170-943.5	500
Total Suspended Solids (mg/L)	1057	170.7	181.4	170.7-1057	≤200
Dissolved Oxygen (mg/L)	1.0	0.2	1.8	0.2-1.8	≤2.0
Biochemical Oxygen Demand (mg/L)	940	631	25.58	25.58-940	15
Chemical Oxygen Demand (mg/L)	2362	2521	3439	2362-3439	40
Chromium (mg/L)	86.5	21.5	0.7	0.7-86.5	<1.0

mg/L; milligram per litre, FEPA: Federal Environmental Protection Agency (Nigeria)

Table 2: Aerobic heterotrophic bacteria

Tannery site	Bacterial count (cfu/ml)
Karaye	$5.3\times10^{6} \pm 0.02$
Unguwan Rogo I	$4.64\times10^{5} \pm 0.1$
Unguwan Rogo II	$8.5 \times 10^{6} \pm 0.05$

cfu: colony forming unit

Screening of Bacteria for Biosurfactant Production

A total of 22 bacterial isolates were screened for ability to produce biosurfactants (Table 3). Seven (31.8%) were shown to be hemolytic on blood agar. Six out of the seven isolates were members of the *Bacillus* species including *Bacillus sphaericus* (EN1 and EN3), *Bacillus larvae* EN7, *Bacillus licheniformis* EN11, *Bacillus cereus* EN13 and *Bacillus azotoformans* EN16; while the remaining one was *Citrobacter diversus* EN4. Thavasi *et al*. [16] have previously demonstrated the hemolytic activity of *Bacillus* and *Citrobacter* species on blood agar. Hemolytic activity is been regarded by some authors as indicative of biosurfactant production and used as a rapid method for bacterial screening [18].

Results for emulsification of the crude oil revealed that five bacterial isolates emulsified the oil at varying rates as shown in Table 3. *Bacillus azotoformans* EN16 had the highest (58.0 ± 5.5) emulsification index (E_{24}) after 24 hours period. In contrast, *Bacillus larvae* EN7 was shown to be the least (14.3 ± 3.35) in terms of E_{24} value after the same period of exposure. All the species that were hemolytic were also able to emulsify the oil with the exception of *Bacillus licheniformis* EN11 and *Bacillus sphaericus* EN1. The inability of the two organisms to emulsify the oil indicated their inability to produce biosurfactants and thus, the hemolytic ability could be attributed to extracellular secretions. This agreed with the findings of Thavasi *et al*. [16] and Elemba [22].

It was observed that, the supernatant of only two isolates were positive for drop collapse and oil spreading tests (Table 3). *Bacillus sphaericus* EN3 collapsed the oil drop in 10 minutes while *Bacillus azotoformans* EN16 collapsed the drop in 15 minutes. Similarly, *Bacillus sphaericus* EN3 and *Bacillus*

azotoformans EN16 caused the spreading of the crude oil at the rate of 7 and 12 minutes with a diameter of 3.4cm and 2.9cm respectively. The results also showed that the remaining three isolates that were positive for hemolysis and emulsification could neither spread the oil nor caused the collapse of the oil drop (Table 3).

Negative drop collapse and oil spreading observed in the remaining three isolates might be associated with the fact that some bacterial species do not produce extracellular materials as biosurfactants but the cells exist as biosurfactants themselves. This agreed with the work of Hommel [23] who made similar observations. Therefore, *Bacillus sphaericus* EN3 and *Bacillus azotoformans* EN16 emerged as the best tannery isolates capable of producing biosurfactants. Emergence of these species was not surprising especially that some authors [18, 7, 16] reported the occurrence of *Bacillus* species as important biosurfactant producers.

Table 3: Screening of biosurfactant producing bacteria

Isolate	Hemolysis	Emulsification index E_{24} (%)	Drop collapse		Oil spreading		
			Result	Time (min)	Result	Diameter (cm)	Time (min)
Bacillus sphaericus (4)[a]	+ (2)	47.80± 1.15*	+	10	+	3.4	7
Serratia fonticola EN2	-	21.25± 1.25	-	-	-	-	-
Citrobacter diversus EN4	+	17.10± 2.10	-	-	-	-	-
Aeromonas hydrophila EN5	-	-	-	-	-	-	-
Proteus mirabilis EN6	-	-	-	-	-	-	-
B. larvae EN7	+	14.30±3.35	-	-	-	-	-
Enterococus faecalis EN8	-	-	-	-	-	-	-
Micrococus luteus EN9	-	-	-	-	-	-	-
B. licheni formis (2)[b]	+ (1)	-	-	-	-	-	-
B. papilliae EN12	-	-	-	-	-	-	-
B. ceraus EN13	+	-	-	-	-	-	-
P. aeruginosa EN14	-	-	-	-	-	-	-
B. leterosporus EN15	-	-	-	-	-	-	-
B. azotoformans EN16	+	58.00± 5.50	+	15	+	2.9	12
P. putrefaciens EN17	-	-	-	-	-	-	-
P. syringae EN18	-	-	-	-	-	-	-
Serratia liquefaciens EN19	-	-	-	-	-	-	-
P. putida EN20	-	-	-	-	-	-	-

[a] contain 4 isolates: EN1, EN3, EN20 and EN22. [b] contain 2 isolates: EN10 and EN11. * from this point onward, only EN3 is positive.

Bacterial isolates

The present study was carried at Postgraduate Research laboratory of Usmanu Danfodiyo University, Sokoto, Nigeria (long. 4º 8'E and 6º54'E; lat. 12ºN and 13º 58'N.). Two bacterial species: *Bacillus sphaericus* EN3 and *Bacillus azotoformans* EN16 were used in the study. These species were previously isolated from Tannery effluents from Sokoto metropolis tanneries (Adamu et al., 2015). The bacteria were screened for biosurfactant production and found to be positive for hemolysis, oil emulsification, drop collapse and oil spreading test. *Bacillus sphaericus* EN3 caused the collapse and spread of oil drop at the rate of 10 and 7 min respectively with an average diameter of 3.4cm, while *Bacillus azotoformans* EN16 was able to do same at the rate of 15 and 12 min respectively with 2.9cm diameter.

Biosurfactant Production

In order to test the ability of the two isolates to produce large quantities of biosurfactants, three carbon substrates were used. These include crude oil (Bonny light – a Nigerian type of crude oil), dieseland glucose. Using conical flasks, 500ml modifiedBushnell – Haas medium of Ismail et al. (2014) (composed of 1.2g KH_2PO_4, 1.8g K_2HPO_4 4.0gNH_4Cl, 0.2g $MgSO_4.7H_2O$, 0.1g NaCl, 0.01g

$FeSO_4.7H_2O$ and 20g agar per liter at pH 7.4) were prepared and dispensed into three flasks. To each of the flask, 2% w/v of crude oil, diesel and glucose wereadded respectively (Seghal Kiran et al., 2010). The setup was replicated three times and incubated at room temperature for 10 days. During the period of incubation, the amount of biosurfactant produced was monitored after two days interval.

Extraction and purification of thebiosurfactants

Bacteria cells were removed by centrifugation (12000 rpm for 10 minutes) using a centrifuging machine (HME GLOBAL 800D, ENGLAND), and culture

supernatant was acidified with HCl (0.1M) to obtain the pH of 2.0. The extraction of the biosurfactants was performed with a mixture of chloroform: methanol (2:4 v/v) which was added to the supernatant, after being vigorously shaken, and allowed to stand until phase separation. Extracts (lower phase) were concentrated by rotary evaporation and then anhydrous sodium sulfate was added to remove water (Thampaayak et al., 2008). The biosurfactantappeared as white crystals.

Biosurfactant stability test

Stability studies were carried out as described by Obayori et al. (2009). Cell free broth was obtained by centrifuging the cultures at 5000 rpm for 20 minutes. The stability of the biosurfactants against pH, temperature and salt (NaCl) was determined.

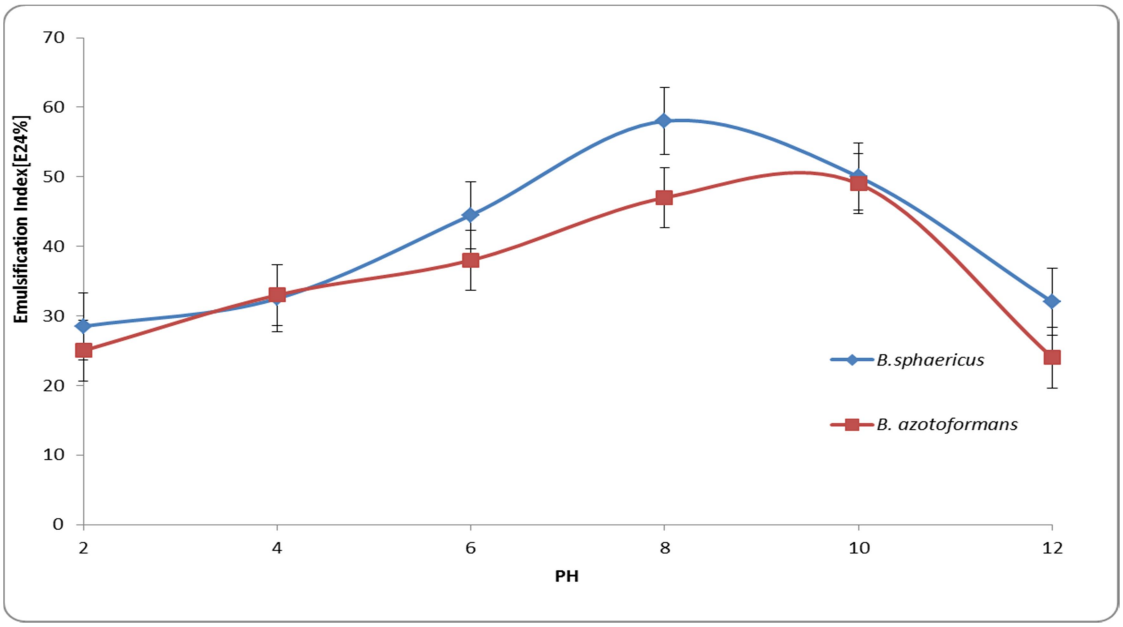
(i) The pH of the biosurfactant was adjusted toacidic (2, 4 and 6 using HCl) and alkaline (8, 10 and 12 using NaOH). Emulsification index (E_{24}) was laterdetermined after 24 hours.

(ii) To test the heat stability of the biosurfactant, the supernatant was heated at 50, 70 and 100^{o}C for 15 minutes using water bath, then cooled to room temperature and E_{24} was determined.

(iii) The effect of sodium chloride (NaCl) on the biosurfactant was also assayed at concentrations of 2, 4, 6, 8 and 10% of NaCl and E_{24} was also determined

Table 2: Characteristics of the produced biosurfactants

Organism	Biosurfactant	Component
Bacillus sphaericus EN3	Phospholipid	Phosphate Leucine Alanine Stearic acid Palmtic acidSerine Arginine
Bacillus azotoformas EN16	Phospholipid	Phosphate Leucine glutamine Stearic acidOleic acid Glycine Valine



2. Fig. 1: Stability of biosurfactants to pH

Determination of Emulsification index

Emulsification activity was carried out using the method of Tabatabaee et al. (2005) and Techaoei et al. (2011). Four 4ml of the crude oil was added to equal amount of cell free supernatant and vortexed at 500

r.p.m for 10 minutes. After 24 hours, the height of the stable emulsion layer was measured using meter rule. The emulsification index (E_{24}) was calculated as the ratio of the height of the emulsion layer and the total height of liquid, as given by the expression:

$E_{24} = \frac{h\ emulsion}{} \times 100h\ total$

Where: E_{24} is emulsion index after 24 hours, h emulsion is the height of emulsion layer, h total is the total height of the liquid.

Characterization and Identification ofBiosurfactants

The biosurfactants produced by the *Bacillus* strains were characterized due to the properties they displayed such as high E_{24} index, oil displacement andtheir stability over wide range of pH, temperature and salinity. The biosurfactants were characterized and identified using high performance liquid chromatography (HPLC). The HPLC analysis was carried out using HPLC machine (model 4340, JANWAY, UK) at 210 nm wavelength. Standard solutions of fatty acids, amino acids, and sugars phosphates were prepared and analyzed. The crude biosurfactants from the bacteria were also analyzed and compared to the standards. The components of the crude biosurfactants were identified upon interpretation of the results.

RESULTS

The result from Table 1 showed that the amount of biosurfactants produced by *Bacillus sphaericus* EN3 using crude oil as carbon source was 0.11±0.01 - 0.49±0.01 g/L, 0.11±0.01 - 0.79±0.01 g/L for diesel
and 0.22±0.20 - 2.25±0.20 g/L for glucose after 10 days period. The time for optimum production of biosurfactant by this organism was 8 days. Similarly, the amount of biosurfactant produced by *Bacillus azotoformans* EN16 using crude oil was 0.05±0.05 - 0.21±0.01 g/L, after 10 days. Using diesel and glucose, the quantity produced was 0.05±0.05 – 0.41±0.01g/L and 0.11±0.01 – 1.25±0.25g/L
respectively (Table 1). Optimum production of biosurfactant by this organism was observed on the 8th day. However, the highest yield was recorded when glucose was used as carbon source by both the organisms, followed by diesel and crude oil although without significant difference ($p \leq 0.05$) in case of *B*. *sphaericus* EN3.

The effect of pH on the stability of the biosurfactants produced was determined at pH range of 2-12 (Fig. 1). Biosurfactant produced by *Bacillus sphaericus* EN3 was stable at all pH level tested with highest activity at pH 8.0 (E_{24}= 58.0%). The same applies to that produced by *Bacillus azotoformans* EN16 with highest activity at pH 10.0 (E_{24}= 49.5%). There was variations in E_{24} values with regards todifferent pH levels ($P<0.05$).

The effect of temperature on the stability of the biosurfactants produced revealed that the biosurfactant activity occurred best at the temperature range of 50 – 100^{o}C with higher activity at 50^{o}C (E_{24} = 61.0% for *Bacillus sphaericus* EN3 and E_{24} = 48.0% for *Bacillus azotoformans* EN16). These results are shown in Fig. 2 and no significant difference was observed at the various temperature levels ($P < 0.05$).

The effect of different concentrations of NaCl on the stability of biosufactants produced by *B. sphaericus* EN3 and *B. azotoformans* EN16 are presented in Fig. 3. The results revealed that biosurfactant activity occurred at NaCl concentrations ranging from 2.0 to 12.0%. However, slightly higher activity occurred at 4.0% NaCl (E_{24}= 39.8%) for EN3 and at 2.0% NaCl (E_{24}= 40.5%) for EN16 (Fig.3). These values (E_{24}= 39.8% and 40.5%) were significantly different ($P>0.05$).

Chromatographic analysis revealed that the biosurfactant produced by *Bacillus sphaericus* EN3 was phospholipid and amino acids made up of palmitic acid, leucine, alanine, serine, and arginine. Similarly, *Bacillus azotoformans* EN16 produced phospholipid with the following components: glutamine, stearic acid, oleic acid glycine, valine and arginine. Phosphate, leucine, stearic acid, and arginine were the common components observed in both of the biosurfactants (Table 2).

DISCUSSIONS

The production of biosurfactants by microorganisms has been a subject of increasing interest in recent years, especially due to their increasing potential application. In the present study, result showed that *Bacillus sphaericus* EN3 and *Bacillus azotoformans* EN16 yielded a higher amount of biosurfactants when glucose was used compared to crude oil and diesel as carbon sources. The reason may be that glucose is easily-metabolized carbon source than crude oil and diesel. Several investigators have identified crude oil, diesel and glucose as good source of carbon for biosurfactant production (Desai and Banat, 1997; Rahman et al., 2002; Makkar et al., 2011). The finding of the present study that glucose gave a higher yield of biosurfactant than crude oil and diesel is however contrary to the report of Mata-Sandoval et al. (2000) who found that hydrophilic substance like glucose gave a poor yield of biosurfactant while hydrophobic substrates like corn oil and long chain alcohols caused a higher yield of biosurfactant. Crude oil and diesel are hydrophobic substances and biosurfactant yield of 0.21 – 0.79g/L was obtained as compared to 1.25 – 2.25g/L for glucose in the present study. The yield of biosurfactants by the *Bacillus* species observed was lower than the one reported by Ijah and Olarinoye (2012) and Ibrahim *et al*. (2013).

Stability studies indicated that the biosurfactants were stable at different pH, temperature and salinity (Figs. 1, 2, and 3). Biosurfactant from *B. sphaericus* EN3 were more active at pH 8.0, (E_{24} = 58%), 50°C (E_{24} = 61%) and 4% NaCl (E_{24} = 39%). For biosurfactant from *B. azotoformans* EN16 however, the activity was more pronounced at pH 10, 50°C and 2% NaCl with E_{24} of 49.5%, 48% and 40.5%respectively. This indicated that the compounds present in the biosurfactants are not degraded within the prevailing physicochemical conditions. Akhavan Sepahy et al. (2005) reported that *Bacillus* species could produce biosurfactant at various ranges of salinity and temperature with optimum at 3 - 5% and 50°C respectively. There are several reports on thestability of biosurfactants at extreme conditions (Obayori et al., 2009; Davishi et al., 2011; Ibrahim et al., 2013). Taking into cognizance the optimum conditions for the biosurfactants' activity, one could suggest the potential applicability of these surfactants in microbial enhanced oil recovery (MEOR) since these conditions (high temperature, pH, and salinity) prevail in oil reservoirs.

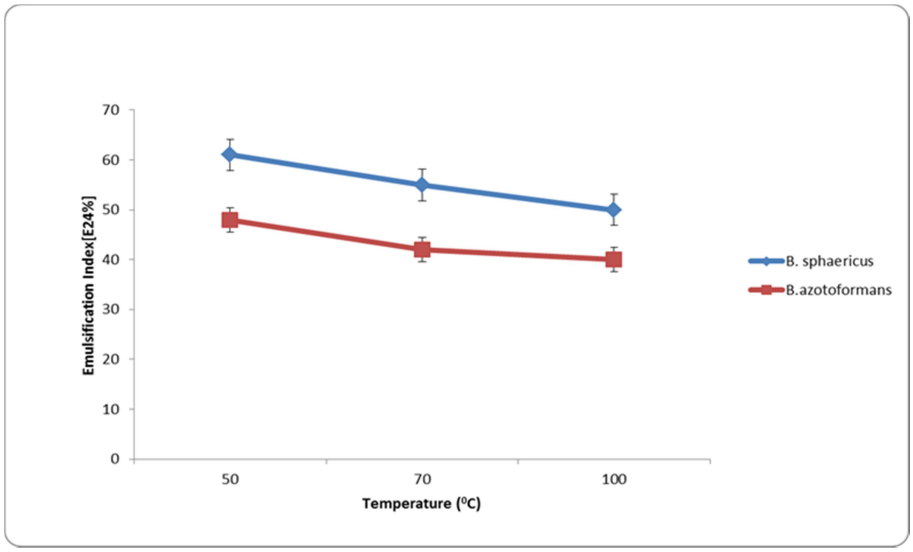


Fig. 2: Stability of biosurfactants to temperature

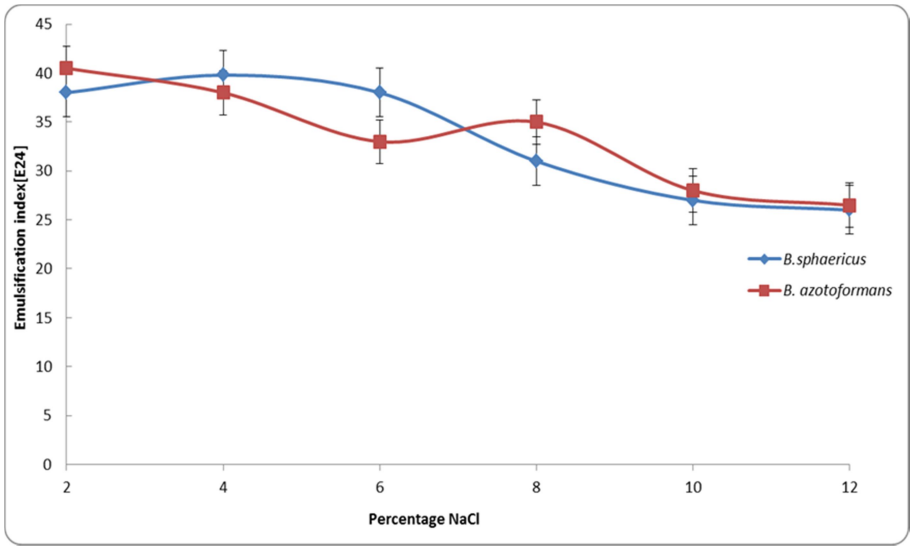


Fig. 3: Stability of biosurfactants to NaCl

The biosurfactants produced by the two organisms were identified as phospholipids with different compositions. However, some components were common to both phospholipids. These include phosphates, leucine, stearic acid and arginne (Table 2). Several researchers reported the production of phospholipids biosurfactants produced by *Bacillus* species of different strains. Davis et al. (2003) reported the production of phospholipid by *Bacillus subtilis* ATCC and composed of inorganic phosphate, stearic acid, arginine and palmitic acid. Abdel-Mawghoud et al. (2008) reported the production of phospholipid biosurfactant by *Bacillus subtilis* B55and was composed of inorganic phosphate, proteinand stearic acid. Similarly Joshi

et al. (2008) reported phospholipid production by *Bacillus subtilis* 20B and composed of inorganic phosphate, alanine and oleic acid. Ijah and Olarinoye (2012) reported phospholipids production by *Bacillus* R07 and *Bacillus* R28. This strengthens the fact that most of microbial surfactants are phospholipids in nature.

However, contrary to present study, most of investigators (for instance McInerney et al., 1990; Maneerat and Phetrong, 2007; Joshi et al., 2008; Jaysree et al., 2011) have listed *Bacillus subtilis* and *Bacillus licheniformis* as most important biosurfactantproducers among the *Bacillus* species. Our efforts to find previous researches that listed either or both of *B.sphaericus* and *B. azotoformans* as biosurfactant producers was fruitless, except current recent findings (Adamu et al., 2015).. This might be attributed to the fact that the number and type of biosurfactant producing organisms is not completely exhausted and many more organisms are underway to be discovered as long as researches are sustained in this direction. Investigations have shown that biosurfactant producers are diverse (Maneerat and Phetrong, 2007), present in different environments (Jaysree et al., 2011) and could make up to 35% of aerobic heterotrophs (Jennings and Tanner, 2000).

CONCLUSION

In this work, *B. sphaericus* EN3 and *B. azotoformans* EN16 were shown to be potent biosurfactant producers. Glucose proved to be the best substrate for large quantities of surfactants followed by diesel and crude oil. Also the biosurfactants were mainly madeup of phospholipids and are stable at various ranges of pH, temperature and salinity although could be used effectively at certain conditions. Therefore, the two *Bacillus* species were important biosurfactant producers and application of their products in bioremediation and MEOR should be exploited.

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