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Potential Biosurfactant-producing Bacteria from Pharmaceutical Wastewater using Simple Screening Methods in South-West, Nigeria

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Abstract

Emerging multiple opportunities for industrial production and environmental applications have focused increasing research attention on biosurfactants in recent years. Hydrocarbon-polluted soils have proved a major source of biosurfactant-producing bacteria. In this study an alternative method of producing biosurfactants was established, based on pharmaceutical effluents. Pharmaceutical effluents are a rich source of complex organic compounds with potential as a substrate for microbial biosurfactant production. A successful biosurfactant screening assay is achieved when a combination of different methods are employed. Biosurfactant producing bacteria were isolated from pharmaceutical effluent and identified by biochemical methods. The best biosurfactant producer was identified by a molecular method. The biosurfactant screening techniques employed were drop collapse assay, haemolytic assay, oil spreading assay, bacteria adhesion to hydrocarbon assay and emulsification assay. Analysis of haemolytic activity indicated that 35 isolates (44.30%) produced beta-haemolysis, 12 isolates (15.19%) produced gamma-haemolysis, while 32 isolates (40.51%) produced alpha-haemolysis. The highest zone of clearance was 42.0± 1.73 by isolate DF7 and lowest zone of clearance was obtained from isolate GC^5 of 12.0± 0.73. Screening of the 35 isolates using the oil spreading test showed that 28 isolates (80.0%) were positive, while 7 isolates (20.0%) were negative. The highest zone of clearance for the oil in water was 19.0±0.1 by DF^1 while the lowest zone was 4.3±0.33 by MB^3. The drop collapse test revealed that 22 isolates (78.57%) were positive and 6 isolates (21.43%) were negative. At 0h, the highest emulsification percentage was 74.4% by *Bacillus licheniformis* and the lowest was 42.5% by *Arthrobacter globiformis*. After 24h, the highest emulsification value (69.23%) was obtained for *Bacillus clausis*, while the lowest value (30.84%) was obtained for *Arthrobacter globiformis*. The findings demonstrated the ability of the isolates to produce biosurfactant, and confirmed the capacity of *Bacillus clausis* isolated from pharmaceutical effluents as a potential bacteria for biosurfactant production.

Keywords: Screening method; Characterization; Bacteria; Pharmaceutical wastewater; Emulsification capability; Hydrocarbon

Introduction

The increasing demand for pharmaceuticals in Nigeria in recent years has led to establishment of more pharmaceutical manufacturing companies in Nigeria [1]. Manufacturing processes are diverse, but frequently entail conversion of natural substances into pharmaceutical intermediaries through fermentation and extraction processes as well as chemical synthesis. The resulting active ingredients are then formulated into the finished product and packaged [2].

Surfactants are organic molecules produced either chemically or biologically [3]. Chemically synthesized surfactants are normally derived from petroleum, frequently toxic and resistant to microbial degradation. They therefore pose risks as sources of environmental pollution. Hazards associated with synthetic surfactants have therefore drawn much attention to alternatives such as bio-surfactants [4]. Organic chemicals, petroleum, petroleum chemicals, mining, fertilizers, foods, beverages, cosmetics etc. are some of the several industries that make use of surfactant [5-6].

A number of microorganisms such as filamentous fungi, yeasts, and bacteria produce biosurfactants after feeding on immiscible substances in water [7-8]. The main characteristic of these microbial cultures is their ability to excrete relatively large amounts of surface-active substances that emulsify or wet the hydrocarbon phase, thus making them available for absorption [9]. Chemical structure and microbial origin are used to classify biosurfactants [10]. Biosurfactants produced on the microbial cell surface or excreted extracellularly contain hydrophobic and hydrophilic moieties [11-13]. Bio-surfactants of different chemical nature and molecular sizes are surface-active compounds that can be produced by hydrocarbon degrading microorganisms [14].

Microorganisms that produce biosurfactants can inhabit both water (fresh water, groundwater, and sea) and land (soil, sediment and sludge). Biosurfactants can be found in extreme environments and can thrive across wide ranges of temperatures, pH values, and salinity, even in oil reservoirs [15]. *Pseudomonas, Bacillus, Sphingomonas, Klebsiella* and *Actinobacteria* are frequently found in soils and sediments, while *Pseudoalteromonas, Halomonas, Alcanivorax*, and *Acinetobactern* genera are generally dominated by hydrocarbon-degrading bacterial populations in marine ecosystems [16]. In recent times, attention has been given to biosurfactant production due to their potential application in areas such as food processing, pharmacology, cosmetics, oil exploration and exploitation, environmental management, and agriculture [17-18].

Oil recovery and bioremediation of heavy crude oil are important roles played by biosurfactants due to their ability to reduce interfacial surface tension [19]. Their ability to operate at high pH, temperature and salinity levels also make biosurfactants very effective [20]. An emerging medical application of biosurfactants has been reported by [21] in the area of drug transport to sites of infection, as emulsifying agents and as adjuvants for vaccines.

The six classes of biosurfactants are (1) hydroxylated and cross-linked fatty acids (mycolic acids), (2) glycolipids, (3) lipopolysaccharides, (4) lipoproteins-lipopeptides, (5) phospholipids and (6) the complete cell surface [22]. Easily biodegradable, their unique structures offer new properties not found in chemical surfactants, including low toxicity, high surface activity, ionic strength, biodegradability, emulsifying and demulsifying ability, antimicrobial activity, stability at extreme temperatures and pH. Moreover, biosurfactants can be synthesized at low cost from renewable sources and from by-products or pharmaceutical wastes [23].

A variety of screening methods for new biosurfactant-producing microbes are available [24]. The 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, while Gamma haemolysis (γ) occurs when no change occurs around the colony [25].

For efficient detection of potential biosurfactant producers, a combination of various screening methods are required; five such methods were evaluated during this study: haemolytic, oil spreading assay, drop collapse assay, bath assay and emulsification assay.

Biosurfactants can easily be produced from hydrocarbon-based raw materials such are contained in organic waste streams such as pharmaceutical wastewater, which offers an attractive alternative to use of hydrocarbons as a source.

Due to paucity of prior literature on isolation of biosurfactant-producing bacteria from pharmaceutical wastes water, the main goal of this study was to isolate, characterize and screen potential biosurfactant-producing bacteria from pharmaceutical wastewater.

Materials and methods

1) Collection of samples

Effluent samples were collected in sterile 5 L containers from seven different pharmaceutical industries in Lagos and Ogun States, Nigeria and coded individually. Table 1 shows the geographical positioning system of pharmaceutical effluent samples assigned with their different codes.

2) Total heterotrophic bacterial count (THBC)

The total heterotrophic bacterial count (THBC) was determined using the method of Rahman et al., (2002), [26]. One gram of each of the 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 ºC for 24 h after which the colonies was counted. This was carried out in replicate. The resulting colonies were then sub-cultured to obtain pure colonies.

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 Whatman filter paper No.1 [27]. Two percent agar was added to solidify the medium. The MSM composition as described by Balogun and Fagade [28] was made up of Basal Salt Medium (BSM) and trace element solution.

Table 1 Sampling location for pharmaceutical effluent samples

Sample	Code	Geographic positioning system(GPS) coordinates	
		Latitude	Longitude
1	FM	6°43'2381"N	3°13'764"E
2	FH	6º42'2471"N	3°13'3293"E
3	DF	6°42'990"N	3°13'2606"E
4	WG	6°50"958"N	3°53'222"E
5	GC	6º43'1074"N	3º13'1733"E
6	MS	6°32'2436"N	3°20'3"E
7	MB	6°41'483"N	3°10' 622" E

4) Surface active bacterial count (SABC)

Screening for surface-active bacteria was conducted on blood agar. The blood agar was made up of nutrient agar containing 5% (v/v) defibrinated rabbit blood. The plates were incubated at 28ºC for 48 h after which the colonies that showed a clear zone of beta-heamolysis were counted as surface-active agent producer according to Tabatabaee et al. [29].

5) Screening for biosurfactant production

Preliminary and confirmatory tests for biosurfactant production were carried out as follows.

5.1) Preliminary test for biosurfactant production

- Haemolytic Activity

The surface-active agents producing bacteria and hydrocarbon degrader colonies were used. Haemolysis was carried out using blood agar plate. The purified cultures were inoculated and the blood agar plates incubated at 37°C for 24 hours. Beta-haemolytic activity was distinguished based on the presence of a clear zone around the colonies [30].

- Oil spreading technique

The surface active agents producing bacteria and hydrocarbon degraders were screened for biosurfactant production using the oil spreading technique according to Priya and Usharani [31] and Anandaraj and Thivakaran [32]. The bacterial isolates were streaked on nutrient agar slant and incubated for 24 hours at 37°C. After 24 hours of growth of the inoculum on nutrient broth, 50 mL of distilled water was added to a large petri dish (25cm 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 pharmaceutical effluents. The diameter of the clear zone was recorded in each case.

- Drop collapse test

The surface-active agents producing bacteria and hydrocarbon degrader bacterial isolates were screened for the drop-collapse test, which was carried out as described by Bodour and Miller [33]. Two microliters of crude oil was added to the microtiter plate and left to equilibrate for 24 h, followed by 5 µL of 48 h cell-free supernatant of the bacterial strain; the drop size was observed after 1 minute. A positive result shows a flat drop, while rounded drops were scored as negative (indicating no biosurfactant production).

- Bacterial adhesion to hydrocarbon

The surface active agents producing bacteria and hydrocarbon-degrading bacterial cells were suspended in phosphate buffer salt solution g/L (K_2HPO_4: 16.9 and KH_2PO_4: 7.3 g/L with pH 7 to give an optical density of 0.5 at 600 nm. One hundred microliter of kerosene was added to 2 ml of cell suspension, and vortexed for 2 mins in test tubes. The aqueous phase was allowed to separate for 1 h., and 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 $H= [1-A/A_0]*100\%$ [34] 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.

5.2) Confirmatory test for biosurfactant production

- Emulsification index test

Positive bacterial isolates from the above preliminary screening test were grown on MSM, supplemented with 1% DPK for 7 days in an orbital incubator at 180 revolutions per minute (rpm) at 28°C. 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 [28]. 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 (Eq.1) as described by Tabatabaee et al. [29].

$$E_{24} = \frac{\text{height of emulsion}}{\text{total height}} \times 100\% \qquad \text{(Eq.1)}$$

6) Bacterial characterization

Identification of the isolates was carried out using standard microbiological method. Shape, pigmentation, elevation, size, appearance and motility were used for morphological characteristics. The following biochemical tests 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 16Sr-RNA 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 [35].

7) Statistical analysis

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

Results and discussion

1) Bacteria counts

Effluent samples counts showed that the highest THBC of $1.5x10^7$ CFU/mL was obtained from GC and the lowest count of $4.4x\ 10^6$ CFU/mL ($p<0.05$) was at FD. THDC exhibited the highest count of 2.0×10^6 CFU/mL ($p<0.05$) from FD and the lowest count of $4.0x10^5$ CFU/mL ($p<0.05$) from WT. SABC from FD showed the highest mean value of $1.5x10^6$ CFU/mL ($p<0.05$) while the lowest value of $1.3\ x10^5$ CFU/mL ($p<0.05$) from WT (Table 2). 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 an energy source [36]. The results obtained from the total heterotrophic plate count, total oil degrader count and surface-active bacteria count showed a wide range in values, suggesting the ability of pharmaceutical effluent to support the growth of a wide diversity of bacteria, this affirmed the ubiquity of microorganisms [37] and that the isolates can indeed metabolize the effluent.

Table 2 Bacterial counts (X 10^5 CFU/mL) in pharmaceutical effluent

Location	Total heterotrophic bacteria	Total oil degrader	Surface active bacteria
FM	106.00 ± 3.79^{a}	5.00 ± 1.15^{a}	3.33 ± 1.20^{a}
FD	44.33 ± 0.88^{a}	20.33 ± 1.76^{a}	15.00 ± 1.86^{a}
DG	98.30 ± 6.57^{ab}	19.00 ± 2.31^{a}	2.33 ± 0.33^{ab}
WT	$32.\ 67 \pm 2.91^{b}$	4.00 ± 1.15^{b}	1.30 ± 0.33^{ab}
GC	150.30 ± 1.45^{b}	10.33 ± 0.88^{c}	9.33 ± 0.67^{b}
MP	63.67 ± 6.12^{c}	5.33 ± 0.67^{c}	4.67 ± 0.88^{c}
MB	129.70 ± 35.43^{d}	18.33 ± 1.45^{c}	10.30 ± 0.33^{d}

Note: - Values are mean ± standard error of means
- Mean values with same letter within a column are not significantly different at $p<0.05$
- FM, FD, DG, WT, GC, MP, MB are different codes assigned for the different pharmaceutical companies effluent samples

2) Screening of isolates for biosurfactant production

A total of 79 bacteria were isolated from seven pharmaceutical effluents. The result of haemolytic activity showed that 35 (44.30%) isolates produced beta haemolysis, 12 (15.19%) isolates produced gamma haemolysis and 32 (40.51%) isolates produced alpha haemolysis. Highest zone of clearance was 42.0±1.73 by isolate DG7 and lowest zone of clearance was obtained from isolate GC^5 of 12.0± 0.73 (Table 3).

The blood agar method of screening detects the ability to produce biosurfactant on hydrophilic media. Blood agar is an enriched and selective medium that allows only haemolytic organisms to grow by utilizing blood and hence the production of biosurfactant cause cell lysis, indicating biosurfactant production by these organisms. The zone of inhibition is directly proportional to the concentration of surfactant [38]. Cellular uptake of pharmaceutical wastewater may result in bacterial hyper-respiration, leading to elaboration of haemolytic molecules such as hydrogen peroxide and haemolysis. Biosurfactant can cause lysis of erythrocytes. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies [39].

The hemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad [40] reported that the biosurfactant produced by *B. subtilis*, surfactin, lysed red blood cells.] The use of blood agar lysis as a primary method to screen biosurfactant production has been recommended by Carrillo et al. [41]. However, none of the studies reported in the literatures [41-46] mention the possibility of bio-surfactant production without hemolytic activity.

Screening of the 35 isolates using the oil spreading test showed that 28 of the isolates (80.0%) were positive, while 7 (20.0%) were negative. The highest zone of clearance for the oil in water was 19.0±0.1 by DG^1 while the lowest zone was 4.3±0.33 by MB^3. However, this disagrees with the work of Rabah and Bello [47] where the highest value of 20mm was recorded (Table 3). The oil spreading method showed the activity of the biosurfactant, in which the area of oil displacement is directly proportional to the concentration of biosurfactant in the solution. Diameter of the clearing zone on the oil surface correlates to surfactant activity, also known as oil displacement [48].

The drop collapse test revealed that 22 isolates (78.57%) were positive, while 6 (21.43%) were negative. The drop collapse test relies on destabilization of liquid droplets by the surfactant activity. Presence of the surfactant causes the drops to spread or collapse due to reduced interfacial tension between the liquid drop and the hydrophobic surface [49]. This work corroborates the findings of Saminathan and Rajendran [50] in which strongly positive isolates for the drop collapse test were recorded, indicating good biosurfactant production potential.

The ability of the isolate to produce beta haemolysis on blood agar and to displace oil in water signifies the ability of the isolate to produce biosurfactants [51]. The drop collapse test was suggested as an sensitive and simple method to test production of biosurfactant; however Said et al. [52] have reported that microorganisms recording a negative drops-collapse test were unable to produce biosurfactant.

Sixteen isolates showing strongly positive potential using the drop collapse test were subjected to bath assay. The highest adhesion to hydrocarbon was 58.0% by MB^1, while the lowest was 22.0% by DG^4 (Table 4). These results are in accordance with the work of Chakrabarti et al. [53] who also reported a highest value of 58% of the isolate adhesion to hydrocarbon. This test is based on the degree of adherence of cells to various liquid hydrocarbons. Hydrophobic cells become bound to hydrocarbon droplets and rise with the hydrocarbon [54]. Positive cell hydrophobicity was reported as an indication of biosurfactant production [55].

Table 3 Haemolytic and oil spreading test for bacterial isolates from pharmaceutical effluent

Isolates code	Haemolytic test (mm)	Oil spreading test (mm)	Drop collapse test
FD2	18.0 ± 2.89^{a}	9.67 ± 0.88^{a}	++
FD6	23.0 ± 2.31^{ab}	11.0 ± 0.58^{bcdef}	++
GC1	16.3 ± 3.21^{abc}	-	-
GC2	23.0 ± 1.15^{abc}	6.33 ± 0.33^{bcdef}	-
GC3	20.0 ± 2.89^{abc}	-	-
GC5	12.0 ± 0.73^{abc}	5.0 ± 0.58^{defgh}	+
MP4	19.0 ± 4.04^{abc}	-	-
MP7	22.67 ± 1.45^{abc}	10.67 ± 0.88^{ab}	++
MP2	31.3 ± 0.88^{bcd}	7.0 ± 0.58^{abc}	-
MP1	26.0 ± 2.31^{bcd}	7.0 ± 1.15^{abcd}	++
MP3	26.0 ± 2.0^{bcde}	10.0 ± 0.08^{abcd}	++
MP5	18.0 ± 1.73^{bcde}	5.33 ± 0.33^{ghij}	-
FM1	25.0 ± 2.31^{bcde}	-	-
FM2	18.3 ± 1.45^{bcde}	6.33 ± 0.33^{defgh}	-
WT1	24.3 ± 0.88^{bcde}	-	-
WT2	29.0 ± 4.04^{bcde}	8.33 ± 0.33^{abc}	+
WT3	24.0 ± 1.73^{bcde}	4.67 ± 1.20^{fghi}	++
WT5	21.0 ± 2.89^{bcdef}	7.68 ± 0.88^{ij}	++
MB1	28.0 ± 3.46^{bcdef}	6.0 ± 0.0^{ab}	++
MB2	32.0 ± 1.0^{bcdefg}	5.67 ± 2.88^{ab}	++
MB3	35 ± 4.04^{cdefgh}	4.3 ± 0.33^{abcde}	-
MB4	28.0 ± 1.73^{defghi}	6.0 ± 0.58^{abcde}	+
MB5	29.0 ± 1.15^{efghij}	4.33 ± 0.67^{defg}	++
MB6	33.0 ± 2.65^{defghi}	5.0 ± 1.15^{j}	+
MB7	22.0 ± 1.53^{cdefg}	-	-
MB8	26.0 ± 1.73^{cdefgh}	9.0 ± 1.15^{a}	-
MB9	23.0 ± 1.15^{cdefgh}	-	-
MB10	26.0 ± 1.73^{cdefgh}	11.0 ± 0.58^{efghi}	++
MB11	24.0 ± 2.31^{k}	8.67 ± 0.88^{k}	-
DG1	20.0 ± 4.04^{efghij}	19.0 ± 1.0^{abcd}	++
DG2	18.0 ± 2.08^{jk}	8.67 ± 0.88^{hij}	++
DG3	21.0 ± 1.15^{fghij}	8.33 ± 0.33^{cdefg}	++
DG4	18.0 ± 2.89^{ij}	12.0 ± 1.15^{ij}	++
DG6	36.0 ± 2.31^{hij}	8.33 ± 0.88^{ghij}	++
DG7	42.0 ± 1.73^{ghij}	6.0 ± 0.58^{efghi}	+

Note: - Values are mean ± standard error of means
- Mean values with same letter within a column are not significantly different at $p<0.05$
- ++ means strongly positive
- + means positive
- - means negative

Table 4 Bacterial adhesion to hydrocarbon (bath) test for bacterial isolates from pharmaceutical effluent

Isolate code	A	A_0	H%
FD2	0.26 ± 0.10	0.5	48
FD6	0.21 ± 0.03	0.5	58
GC1	0.30 ± 0.02	0.5	40
GC2	0.34 ± 0.02	0.5	32
GC3	0.27 ± 0.04	0.5	46
GC5	0.24 ± 0.01	0.5	52
MP4	0.24 ± 0.01	0.5	52
MP7	0.33 ± 0.02	0.5	34
MP2	0.32 ± 0.00	0.5	36
MP1	0.26 ± 0.05	0.5	48
MP3	0.27 ± 0.01	0.5	46
MP5	0.28 ± 0.00	0.5	44
FM1	0.36 ± 0.03	0.5	28
FM2	0.22 ± 0.01	0.5	56
WT1	0.29 ± 0.07	0.5	42
WT2	0.39 ± 0.00	0.5	22

Note: Values are mean ± standard error of means

Table 5 shows the occurrence of the various genera of bacterial isolates identified in the effluent samples.

Table 5 Occurrence of Bacteria genera in the effluent samples

Bacteria genera identified in the effluent samples	Percentage occurrence
Bacillus	54.29%
Acinetobacter	14.29%
Alcaligenes	5.71%
Micrococcus	2.86%
Pseudomonas	20.0%
Arthrobacter	2.86%

3) Confirmatory test for screening of biosurfactant production from effluent samples

The emulsification ability of isolates obtained from effluent samples are presented in Table 6 and Figure 1. Isolates from the effluent were subjected to a confirmatory test- the emulsification index (EI) test, at different time intervals. At 0 h, the highest emulsification percentage (74.4%) was obtained by *Bacillus licheniformis*, with the lowest (42.5%) by *Arthrobacter globiformis*. At 24 h, the highest EI value (69.23%) was achieved with *Bacillus clausis* from MB5, while the lowest E.I (30.84%) was obtained by *Arthrobacter globiformis*. The emulsification capacity correlates with surface concentration [56]**.** However, this result disagrees with the work of Sidkey et al. [55] who reported a highest value of 66.8%, and with Saminathan and Rajendran [50] who reported a higher emulsification value of 81%. *Bacillus clausis* was therefore selected as the most promising isolate for biosurfactant production based on the result of all the screening tests.

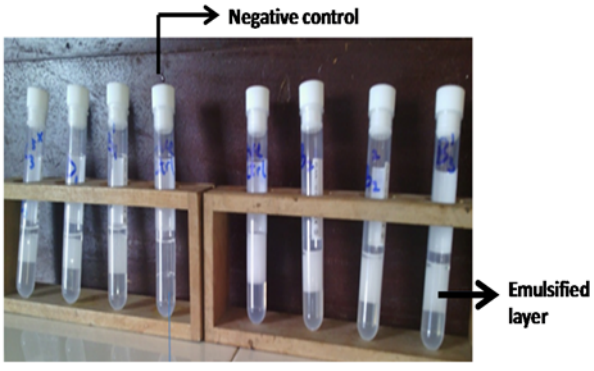

Figure 1 Emulsification ability by isolate

Table 6 Emulsification indices test (%) of effluent isolates

Isolates	Isolate Code	0 h	4 hrs	8 hrs	12 hrs	16 hrs	20 hrs	24 hrs
Bacillus subtilis	FD2	57.90 ± 0.0	48.72 ± 0.1	42.11 ± 0.14	45.95 ± 0.04	42.11 ± 0.03	48.65 ± 0.0	47.38 ± 0.08
Bacillus coagulans	MB1	55.26 ± 0.13	52.63 ± 0.2	46.15 ± 0.11	41.03 ± 0.02	43.59 ± 0.04	47.37 ± 0.2	47.38 ± 0.0
Pseudomonas chlororaphis	MP7	51.35 ± 0.0	38.46 ± 0.0	35.90 ± 0.07	40.54 ± 0.02	38.46 ± 0.0	43.59 ± 0.0	44.73 ± 0.1
Bacillus cereus	MB2	57.5 ± 0.0	52.5 ± 0.32	52.63 ± 0.06	51.28 ± 0.03	51.28 ± 0.02	57.90 ± 0.30	53.85 ± 0.13
Pseudomonas putida	MP3	61.54 ± 0.05	51.28 ± 0.04	51.35 ± 0.0	44.44 ± 0.01	44.44 ± 0.0	51.35 ± 0.01	52.63 ± 0.0
Arthrobacter globiformis	DG1	42.5 ± 0.0	34.15 ± 0.09	30.77 ± 0.06	42.86 ± 0.00	28.57 ± 0.02	29.73 ± 0.12	30.84 ± 0.07
Bacillus coagulans	MP1	65.79 ± 0.01	56.41 ± 0.06	52.63 ± 0.02	47.37 ± 0.03	56.41 ± 0.01	58.98 ± 0.08	55.26 ± 0.05
Bacillus subtilis	FD6	73.68 ± 0.0	64.10 ± 0.01	62.16 ± 0.0	62.16 ± 0.04	63.16 ± 0.11	70.27 ± 0.3	64.10 ± 0.05
Bacillus licheniformis	DG3	51.35 ± 0.01	41.03 ± 0.0	36.84 ± 0.03	44.29 ± 0.05	47.22 ± 0.04	48.65 ± 0.02	42.11 ± 0.00
Bacillus clausis	MB5	68.29 ± 0.0	62.50 ± 0.01	60.53 ± 0.0	66.67 ± 0.03	70.27 ± 0.03	76.32 ± 0.04	69.23 ± 0.0
Bacillus subtilis	MB10	57.5 ± 0.0	51.22 ± 0.03	43.60 ± 0.02	62.86 ± 0.02	57.14 ± 0.03	62.16 ± 0.03	63.16 ± 0.07
Acinetobacter calcoaceticus	WT3	74.36 ± 0.02	72.50 ± 0.06	67.57 ± 0.03	65.71 ± 0.04	69.23 ± 0.03	65.79 ± 0.2	65.79 ± 0.0
Bacillus firmus	DG6	57.5 ± 0.0	50.0 ± 0.05	52.63 ± 0.02	52.78 ± 0.03	52.78 ± 0.0	51.35 ± 0.01	55.26 ± 0.01
Bacillus licheniformis	DG2	51.22 ± 0.06	48.7 ± 0.08	48.65 ± 0.05	54.05 ± 0.2	56.41 ± 0.1	52.63 ± 0.0	56.41 ± 0.0
Bacillus amyloliquefasciens	WT5	65.0 ± 0.09	60.0 ± 0.07	57.90 ± 0.04	62.16 ± 0.03	67.57 ± 0.05	60.53 ± 0.04	61.54 ± 0.11
Bacillus licheniformis	DG4	74.40 ± 0.02	72.47 ± 0.05	65.79 ± 0.03	60.53 ± 0.0	72.22 ± 0.01	64.87 ± 0.0	64.10 ± 0.03
MSM+ Kerosene	Ctrl	0	0	0	0	0	0	0

Note: Values are mean ± standard error of means

4) Molecular characterization and gene sequencing of isolates

Using biochemical characterization, *Bacillus megaterium,* which had the highest emulsified potential, was identified as *Bacillus clausis* when molecular characterization was used**.** The nucleotide sequence of the isolate was submitted to the GenBank database and assigned the accession number KY235235.

Conclusion

In the present study, *Bacillus clausis* isolated from pharmaceutical effluent showed significant biosurfactant activity. Industrial wastes with high content of carbohydrate or lipids satisfy the requirement for use as a substrate for biosurfactant production [57]. This study revealed that the pharmaceutical wastewater samples used harbour a diverse range of microorganisms, predominantly *Pseudomonas* spp*, Bacillus* spp*, Acinetobacter* spp*,* and *Micrococcus, Alcaligenes, Arthrobacter* spp, all with the capability to produce biosurfactants. *Bacillus clausis*, *Acinetobacter calcoaceticus and Bacillus subtilis* showed high emulsification capacity while the least emulsification capacity was recorded for *Arthrobacter globiformis.*

The present investigation also revealed that isolates with the highest emulsification potential have a direct correlation between drop collapse, oil spreading and emulsification stability and this is in full accordance with Mounira and Abdelhadi [58] who found that strains highly active in any one of these methods were also active according to the other three methods. Screening of the biosurfactant producers and selection of the most promising isolate can thus be determined using the aforementioned screening test [58-61]. *B. clausis* was able to produce a clear zone around its colonies of 29.0 mm, equivalent to 2.9 cm for its beta haemolytic activity, a higher zone of clearance for oil in water (4.33 mm) and produce a strongly positive result for drop collapse test with its higher emulsion ability of 69.23%. Interestingly, *B. clausis* was not the highest in blood haemolysis, oil spreading, drop collapse and BATH test but was chosen because of its ability to produce the highest emulsion. Based on the high emulsification ability of *B. clausis*, together with its other excellent screening results, and taking note of the report by Kalyani et al. [9] this study revealed the high potential of *B. clausis* to produce surface-active biosurfactants, with multiple potential applications for various environmental and industrial processes.

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