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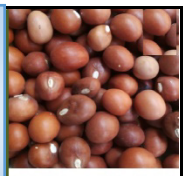

Isolation and Characterization of Biosurfactant-producing Bacteria from Ternary Effluent within Maiduguri Metropolitan Council

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ABSTRACT

Almost all surfactants currently in use are chemically derived from petroleum. However, the diversity of biosurfactants, environment friendly characteristics, the possibility of their production through fermentation and their potential applications in areas such as environmental protection, surface crude oil recovery, health care and food processing industries, has made its interest steadily increasing in recent years. This research is targeted towards the isolation of indigenous biosurfactant producing bacteria from the local tanneries in Maiduguri. Physico – chemical parameters such as temperature and pH of all samples were tested at the site of collection, while others such as Biochemical Oxygen Demand (BOD), Desolved Oxygen (DO) and Electrical Conductivity (EC) were tested ex – situ. Simple and selective microbiological media were used for the isolation. A total of ten (10) different bacterial isolates were obtained from the eighteen (18) samples tested. Identification of the isolates was achieved through morphological and Biochemical analysis. The isolates obtained included *Providencia rettgeri*, *Salmonella enterica*, *Enterobacter cancerogenus*, *Leminorella richardii*, *Bacillus licheniformis*, *B. niacini*, *Paenibacillus residui*, *Proteus mirabilis*, *Lysinibacillus massiliensis and L. fusiformis.* Of the ten (10) isolates obtained, only seven (7) were found positive for biosurfactant production. They incuded *P. rettgeri*, *E. cancerogenus*, *L. richardii*, *B. niacini*, *P. residui*, *L. massiliensis and L. fusiformis*, of which *L. fusiformis* showed the highest emulsification activity of 25% at 4cm emulsion height. The results suggest that the Majema tannery effluents harbour biosurfactant - producing bacteria. Thus, tannery effluents can be possibly remediated using the biosurfactants and bacteria producing the biosurfactants.

INTRODUCTION

When microorganisms are fed a carbon source like diesel, crude oil, glucose, sucrose, glycerol, etc., they make biosurfactants, which are amphiphilic surface-active agents. They are able to orient across fluid phases thanks to the presence of hydrophobic and hydrophilic moieties, reducing surface and interfacial tension [1]. Because of their many advantageous properties—including being non-toxic, biodegradable, and resistant to environmental factors like temperature, pH, and ionic strength—microbial surfactants have been widely adopted as a viable alternative to synthetic surfactants in a wide variety of industries and applications [2]. Bodour *et al*. [3] classified biosurfactants based on their chemical structure and microbial origin as glycolipids, lipopeptides, sophorolipids, surface active antibiotics, neutral lipids, fatty acids, polymeric biosurfactants and particulate biosurfactants [4,5]. Modern surfactants are almost entirely synthesized from petroleum. The interest in biosurfactants has been growing significantly in recent years due to their various properties and possible uses in fields like environmental protection, surface crude oil recovery, healthcare, and the food processing industry [6].

Features of biosurfactant include environmental friendliness, biodegradability, and low toxicity [7], higher foaming [8], Biosurfactants are preferable to chemical surfactants due to their enhanced selectivity and specific activity

throughout a wide range of temperature, pH, and salinity conditions [9].

Tannery manufacturing as a whole involves a number of processes that are harmful to both the natural environment and workers. Treatment of tannery effluent is crucial because of the high environmental pollution rate caused by the volume, nature, and concentration of pollutants such tanning chemicals (chromium and tannin), color, organic matter, and others [10]. Imamulhaqq [11], chemicals used in the soaking, tanning, and post tanning processes of hides and skins, such as sodium sulphite and basic chromium sulphate, as well as non-ionic wetting agents, soda ash, bactericides, CaO, ammonium chloride, ammonium sulphide, and enzymes. Leather businesses use chromium compounds extensively in the tanning process, and as a result, they often release chromium-laden effluents into neighboring water bodies around the world [12].

Finishing techniques and solvents/auxiliary materials can be rather diverse [13]. Imamulhaq [11] It has been reported that only around 20% of the several chemicals employed in the tanning process actually make it into the leather. American Public Health Association [14] The majority of the chemicals used in the tanning process are not absorbed by the leather and are instead discarded [15]. Furthermore, in some regions of Bangladesh, the chromium-based solid wastes from tanneries are transformed into chicken feed, which can have negative effects on both livestock and humans [13]. Tannery workers have a higher than average risk of developing gastrointestinal, dermatological, and other ailments; more than 90% of them will die before reaching age 50 [16]. In this regard, biosurfactants may prove advantageous. However, there is a lack of data on the utilization of biosurfactants in the treatment of tannery effluent and the synthesis of biosurfactants by bacteria isolated from this wastewater.

MATERIALS AND METHODS

Study area

Majema, the only surviving tannery in the whole of Maiduguri, Borno State after the folding up of the State owned Neital Shoe Factory in 2015 as a result of rising case of Boko Haram insurgency is situated along Shehu Umar Garbai Road Mafoni, Lawan Bukar Ward on the coordinate 11°51'08.8"N 13°09'10.8"E. The tannery consists of six (6) major sections from which the various tanning processes are carried out. Soaking, dehairing, bating, pickling, finishing, and discharging are all parts of the process. The raw hides that have been maintained return to their original moisture levels throughout the soaking process.

They clean it so that it doesn't have any dirt, manure, blood, or preservatives (sodium chloride, bactericides), etc. During a Dehairing section, any excess tissue is cut away. Hair and epidermis are dissolved chemically in an alkaline media containing sulfide and lime to accomplish dehairing. If the hide still seems too meaty after it's been skinned at the butcher, it'll need to be fleshed before it's ready for dehairing and liming. In the Bating department, the alkalinity of the dehaired, fleshed, and alkaline hides is neutralized by the deliming process using acid ammonium salts, and the remaining hair and protein are removed and digested using enzymes similar to those in the digestive system. This procedure eliminates both hair roots and pigmentation. This enzyme treatment softens the hides slightly.

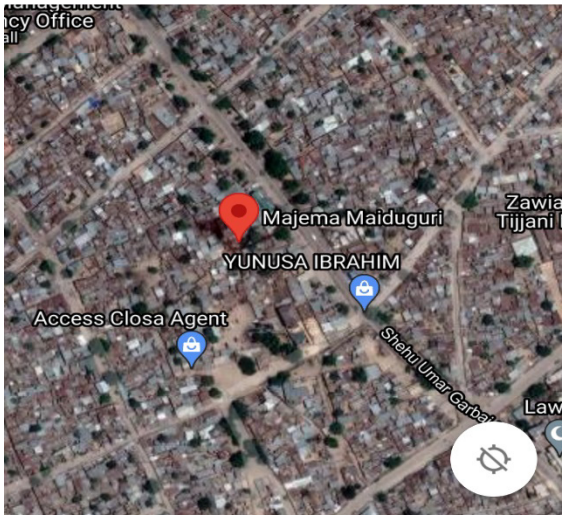

Fig. 1. Arial view of Majema Tannery, Mafoni Ward, Maiduguri, Borno State.

Collection of tannery effluent Samples

Using a Fifty milliliters (50ml) sample bottle, each of the Tannery effluent was collected from six (6) different sections of the Majema tannery at different times (morning, afternoon and evening) namely; the Soaking section (designated as SK), Dehairing section (designated as DH), Bating section (designated as BT), Pickling section (designated as PK), Finishing section (designated as FN) and the discharge (designated as DC). All samples were collected aseptically using a scoop. The temperature and pH of each of the samples was taken before transporting them to the laboratory in an ice box for further analysis.

Physico-chemical analysis of the tannery effluent samples

The temperature, pH and other parameters such as BOD, DO, and EC were determined using standard procedures.

Microbiological analysis of the tannery effluent samples

Exactly 1 mL from each of the tannery effluent sample (SK, DH, BT, PK, FN and DC) was introduced into a test tube containing 9ml of sterile distilled water. Ten-fold serial dilution for each of the effluent sample suspension was prepared [17]. By spread plate technique, 1ml aliquots of sample dilutions were seeded in triplicates on both Nutrient agar (NA) and Bushnell Hass medium (Mineral Salt Media) for the enumeration of total aerobic heterotrophic bacteria. The Nutrient Agar and Mineral Salt media (MSM) plates were incubated at 37 ºC for 24 h and 72 h respectively. Colonies which appeared on the plates were counted and expressed as colony forming units per milliliter (cfu/mL) of the test effluent sample. Pure isolates were obtained by further subculturing onto fresh Nutrient agar (NA) plates and subsequently maintained on NA slants in a refrigerator at a temperature of 8^0C for preservation and subsequent analysis.

Morphological and biochemical characteristics of the bacterial isolates

Biochemical test

The tests conducted included Gram staining, spore staining, citrate utilization, indole, Methyl red, Voges Proskauer (MR-VP) tests. Production of urease, oxidase and catalase, and utilization of various carbohydrates by the isolates were also tested. The results were recorded and the isolates identified by comparing their characteristics with those of known taxa using the schemes of Barrow and Feltham [17].

Screening of bacterial isolates for biosurfactant production
Drop collapse test
Drop collapse test was carried out according to the method described by Youssef *et al*.[18]. In this method, a drop of crude oil is placed on a grease free slide and one drop of the cell free supernatant (obtained by centrifugation) placed at the center of the oil drop. The oil drop is seen to collapse due to the presence of biosurfactant in the supernatant (which reduces the force of interfacial tension between the liquid drop and the surface due to the hydrophobic property of the biosurfactant.).

The collapse was recorded as positive and also the time taken for the oil drop to collapse was also recorded. The crude oil drops that remained rounded were recorded as negative indicating absence of biosurfactant.

Oil spreading method
Oil spreading technique was carried out according to the method described by Youssef *et al*. [18]. The method involves adding 50ml of distilled water to a Petri dish followed by the addition of 100 micro liters of crude oil to the surface of the water. One drop of supernatant of the nutrient broth culture is then dropped on the crude oil surface. The diameter of clear zone on oil surface is measured using a meter rule.

Emulsification capacity test
In the emulsification capacity test, 4 mL of kerosene it was added to equal amount of cell-free supernatant and vortexed at 5000 ×g for 10 min. After 24 h, the height of the stable emulsion layer was measured using meter rule [8,19]. The emulsification index (E_{24}) was calculated as the ratio of the height of the emulsion layer and the total height of liquid given by the expression:

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

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

RESULTS

Results obtained from the physico – chemical analysis as summarized in **Table 1** show that sample taken from SK had temperature range of between (31.2 – 33.85) ºC with a mean temperature of (32.35±1.36) ºC. The pH of the site was between (6.55 – 6.84) and the average pH (6.61±0.21). The BOD of the sample ranges from (0.77 – 0.82) ppm with a mean BOD of (0.76±0.07) ppm. DO varied between (6.92 –7.02) ppm and mean DO was (6.83±0.25) ppm. The EC of the sample ranged between (204.9 – 205.6) µs/cm^{3}with an average EC of (204.9±0.70)µS/cm3.

Sample from site DH had a temperature range of between (32 – 34.04)ºC and with a mean temperature of (32.78±1.10)ºC. The pH of the site ranged from (6.23 – 6.36) and the mean pH was (6.38±0.34). BOD of the site varied between (1.07 – 1.34) ppm with an average BOD of (1.13±0.19) ppm. The DO of ranged between (6.37 – 6.95) ppm and mean DO of (6.66±0.29) ppm. The EC ranged between (217.9 – 218.2) µS/cm3 with a mean of (217.9±0.30) µS/cm3.

Sample from site BT had a temperature range of (31.45 – 33.35)ºC with a mean temperature of (32.29±0.97)ºC. pH also varied between (6.98 – 7.45) with a mean pH of (7.17±0.25). The BOD also ranges between (0.834 – 0.872) ppm with an average BOD of (0.83±0.05) ppm. DO from the effluent sample ranged between (7.03 – 7.45) ppm and the mean DO was (7.49±0.48) ppm. EC of the site ranged between (198.64 – 201.87) µS/cm3 with a mean of (200.18±1.62) µS/cm3.

The temperature of the sample from site PK ranged between (30.4 – 34.75)ºC with a mean temperature (32.16±2.29)ºC. The pH of the site was between the ranges of(3.85 – 4.45) with a mean pH of (3.99±0.41). BOD of the site also varied between (0.786 – 1.024) ppm with a mean BOD of (0.93±0.13) ppm. DO varied between (6.68 – 7.32) ppm and with mean DO of (7.01±0.32) ppm. The sample had an EC range of between (200.47 – 202.20) µS/cm3 with a mean EC of (202.62±2.39) µS/cm3. The temperature of the sample from site FN varied between (31.2 – 33.3)ºC with mean temperature of (32.0±1.14)ºC. pH of the site also varied between (6.55 – 6.75) and the mean pH was (6.54±0.22). Also, BOD ranged between (1.12 – 1.27) ppm with a mean BOD of (1.19±0.07). The DO of the site ranged from (7.05 – 7.35) ppm, with a mean DO of (7.18±0.15) ppm. The EC of the sample ranged between (208.67 – 210.5) µS/cm3. Mean EC of the site was (209.59±0.92) µS/cm3.

The temperature of site DC varied between (30.35–34.00)ºC and with a mean temperature of (31.92±1.88)ºC. The pH of the site ranged from (6.02 – 7.09) and mean pH was (6.52±0.54). BOD of the site varied between (1.06 – 1.46) ppm with an average BOD of (1.21±0.22) ppm. The DO of the site ranged from (6.89 – 7.02) ppm, with a mean DO of (6.94±0.07) ppm. The EC ranged from (214.9 – 215.8)µS/cm3 with a corresponding mean EC of (215.37±0.45) µS/cm3.

Results obtained from the bacterial colony count of the 3rd, 4th and 5th serial dilution on Nutrient Agar culture plate of each of the samples showed that samples from the Discharge section of the tannery (coded as DC) had the highest number of bacteria colony of 72±4.58 cfu/mL with the Soaking section (coded as SK) having the lowest of the six (6) sections with a mean count of 34.67±2.08 cfu/mL. The result from colonies obtained from the MSM culture plates showed that DC had the highest number of bacteria colony with a mean count of 30.67±3.51 cfu/mL, DH section having the lowest of 4.33±3.21 cfu/mL (**Fig. 2**)

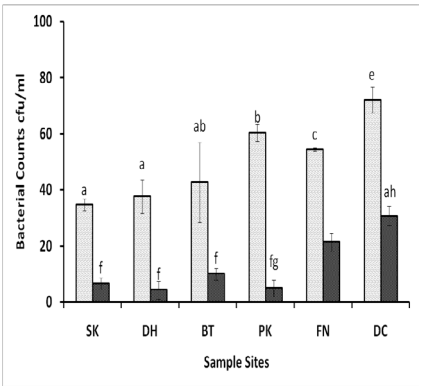

Fig. 2. Enumeration of total aerobic heterotrophic bacteria and biosurfactant producing bacteria bars (means + SD, n=3) with different letters within treatments are significantly different based on LSD ($p < 0.05$).

Table 1. Physico-chemical parameters of Majema tannery effluent samples.

Parameters (Mean±S.D)	Soaking (SK)	Dehairing (DH)	Bating (BT)	Pickling (PK)	Finishing (FN)	Discharge (DC)	Recommended Limit (FEPA)
Temp (°C)	32.35±1.36	32.78±1.1	32.29±0.97	32.16±2.29	32.0±1.14	31.92±1.88	<40
pH	6.61±0.21	6.38±0.34	7.17±0.25	3.99±0.41	6.54±0.22	6.52±0.54	6.0-9.0
BOD (ppm)	0.76±0.07	1.13±0.19	0.83±0.05	0.93±0.13	1.19±0.07	1.21±0.22	15
DO (ppm)	6.83±0.25	6.66±0.29	7.49±0.48	7.01±0.32	7.18±0.15	6.94±0.07	≤2.0
EC(µS/cm3)	204.9±0.7	217.9±0.3	200.18±1.62	202.62±2.39	209.59±0.92	215.37±0.45	200

Note: DO=Dissolved Oxygen BOD=Biological Oxygen Demand EC=Electrical Conductivity.

Table 2. Biochemical characteristics of the bacterial isolates from the tannery effluent samples.

Isolate code	Indole	M.R	V.P	citrate	Urease	Catalase	Oxidase	Gas	H2S	Maltose	Sucrose	Glucose	Suspected isolate
SK01	+	+	-	+	+	+	-	-	+	-	-	-	*Providencia rettgeri*
SK02	-	-	-	+	+	+	-	-	+	-	-	-	*Salmonella enterica*
SK03	+	+	-	+	+	+	-	-	+	-	+	-	*Lysinibacillus fusiformis*
DH01	+	+	-	+	+	+	-	-	+	-	-	-	*L. fusiformis*
DH02	-	-	-	+	+	+	-	+	-	+	-	+	*Enterobacter cancerogenus*
DH03	-	-	-	+	+	+	-	-	+	-	-	+	*Leminorella richardii*
BT01	-	-	-	+	-	+	+	-	-	-	-	-	*L. fusiformis*
BT02	-	-	-	+	+	+	+	-	+	-	-	-	*L. fusiformis*
BT03	-	-	+	+	-	+	+	+	-	-	-	-	*L. massilliensis*
PK01	-	+	-	+	+	+	-	-	+	-	-	+	*L. fusiformis*
PK02	+	-	-	+	-	+	-	-	-	-	+	+	*Bacillus niacini*
PK03	-	+	-	+	-	+	-	-	-	+	-	-	*L. fusiformis*
FN01	+	-	-	+	-	+	-	+	-	-	+	-	*L. fusiformis*
FN02	-	-	+	+	-	+	+	+	-	+	-	-	*L. massiliensis*
FN03	-	-	-	+	-	+	-	-	-	-	-	-	*L. fusiformis*
DC01	-	-	-	+	-	+	+	+	-	+	-	-	*Paenibacillus residui*
DC02	-	-	+	+	-	+	+	+	-	-	-	-	*L. massilliensis*
DC03	-	-	-	+	+	+	+	-	+	-	-	-	*L. fusiformis*

+ : positive - : negative

Results obtained from the Biochemical and Morphological analysis of all the positive isolates as represented in **Tables 2** and **3** respectively indicates *Lysinibacillus fusiformis* as the predominant bacteria present in the positive samples obtained from the enumeration using MSM with a percentage occurrence (as summarized in **Table 4**) of 33.3% s. Other bacteria isolates present included *Providencia rettgeri* (8.3%), *Salmonella enteric* (8.3%), *Enterobacter cancerogenus* (4.17%), *Leminorella richardii* (4.17%), *L. masilliensis*(12.5%), *Bacillus niacin*(8.3%),*Paenibacillus residui* (4.17%), *Bacillus licheniformis* (8.3%), and *Proteus mirabilis* (4.17%) .

Of the twenty-four (24) positive samples, it was observed that only about seven (7) samples showed positive result as summarized in **Table 5**. Supernatants of effluent obtained from the DC section of the Tannery showed the highest positivity with a collapse time of about 1.35 mins while supernatants of sample from the SK section had the lowest positive result of about 4.28 mins. Previous results of the Biochemical analysis (**Table 5**) indicate *L. fusiformis* as the organism occupying the DC01 position and, hence having the highest result.

The seven (7) samples which were tested positive were further subjected to the oil spreading test. The test showed that *L. fusiformis* (DC01) has the highest zone of oil spreading and the lowest oil spreading time of about 7 cm in diameter and 0.56min respectively. *P. rettgeri* (SK01) has the least of all the positive samples with a diameter 2.6 and a time of 2.45 min. Summary of the result can be seen in **Table 5**.

Table 3. Morphological analysis of effluent samples of Majema tannery.

Isolate Code	Size (µm)	Colour	Elevation	Margin	Consistency
SK01	2	Creamy	Raised	Smooth	Swarming
SK02	2	Creamy	Raised	Smooth	Swarming
SK03	2	Creamy	Raised	Smooth	Swarming
DH01	2	Creamy	Raised	Smooth	Swarming
DH02	3	Creamy	Raised	Serrated	Dry
DH03	2	Creamy	Raised	Smooth	Swarming
BT01	3	Creamy	Raised	Smooth	Dry
BT02	2	Creamy	Raised	Smooth	Swarming
BT03	1	Creamy	Raised	Smooth	Mucoid
PK01	2	Creamy	Raised	Smooth	Swarming
PK02	3	Creamy	Raised	Smooth	Dry
PK03	4	Creamy	Raised	Smooth	Mucoid
FN01	2	Creamy	Raised	Smooth	Dry
FN02	3	Creamy	Flat	Smooth	Mucoid
FN03	3	Creamy	Raised	Smooth	Mucoid
DC01	4	Creamy	Flat	Smooth	Mucoid
DC02	3	Creamy	Flat	Smooth	Dry
DC03	2	Creamy	Flat	Smooth	Mucoid

Table 4. Percentage occurrence of bacteria isolates from tannery effluent.

Isolated organism	No of occurrence	% of occurrence
Providencia rettgeri	2	8.3
Salmonella enteric	2	8.3
Lysinibacillus fusiformis	8	33.3
Enterobacter cancerogenus	1	4.17
Leminorella richardii	1	4.17
L. massilliensis	3	12.5
Bacillus niacin	2	8.3
L. massiliensis	1	4.17
Paenibacillus residui	1	4.17
Bacillus licheniformis	2	8.3
Proteus mirabilis	1	4.17
Total	24	100

Table 5. Drop collapse test results of effluents from different sites of the tannery.

Specimen No	Isolate	Time taken (min)
DH01	*Enterobacter cancerogenus*	2.63
DH02	*Leminorella richardii*	3.68
DC02	*Lysinibacillus. Massilliensis*	2.43
SK01	*Providencia rettgeri*	4.28
FN01	*Bacillus niacin*	3.58
FN02	*Paenibacillus residui*	4.02
DC01	*L. fusiformis*	1.35

Table 5. Oil spreading test results of effluents from different sites of the tannery.

Specimen No	Isolate	Diameter of collapse zone(cm)	Time taken (min)
DH01	*Enterobacter cancerogenus*	5	1.1
DH02	*Leminorella richardii*	4.1	3.7
DC02	*Lysinibacillus. Massilliensis*	4.8	1.21
SK01	*Providencia rettgeri*	2.6	2.45
FN01	*Bacillus niacin*	3.3	3.5
FN02	*Paenibacillus residui*	2.9	2.85
DC01	*L. fusiformis*	7	0.56

The emulsification activity as recorded in **Table 7** is seen to have *L. fusiformis* (DC01) with the highest emulsification index (E_{24}) of 50%at 4cm emulsion height. In contrast, *P. rettgeri* (SK01) was seen to have the lowest $E_{24}$of 25% at 2cm emulsion height.

Table 7. Emulsification test results of effluents from different sites of the Tannery.

Specimen No	Isolates	Initial Emulsion level (cm)	Final Emulsion level (cm)	h-emulsion level (cm)	E_{24} (%)
DH01	*Enterobacter cancerogenus*	1	4.5	3.5	43.75
DH02	*Leminorella richardii*	1	4	3	37.5
DC02	*Lysinibacillus. massilliensis*	1	4.5	3.5	43.75
SK01	*Providencia rettgeri*	1	3	2	25
FN01	*Bacillus niacin*	1	4	3	37.5
FN02	*Paenibacillus residui*	1	3.5	2.5	31.25
DC01	*L. fusiformis*	0.5	4.5	4	50

Threats to the environment and human health are inherent to the tannery industry as a whole. Treatment of tannery effluent is crucial because of the high environmental pollution rate caused by the volume, nature, and concentration of pollutants such tanning chemicals (chromium and tannin), color, organic matter, and others [10]. Only about 20% of the large number of chemicals used in the tanning process is absorbed by leather, the rest is released as waste [11]. Chromium is a known human carcinogen [15].

The temperature and pH of the tannery effluents were within the permissible limit prescribed by FEPA (1991). However, the pickling section had an acidic pH of 3.99 which can be attributed to the effect of chemicals used for tanning (sodium chloride and hydrogen sulphate.) and the accumulation of acidic metabolites in the effluent [13]. Dissolved oxygen (DO), and Electrical conductivity (EC), of the effluents were higher than the limits allowed by FEPA [20] while the Biochemical oxygen demand (BOD) was lower than the FEPA standard (**Table 1**).

Several investigators have made similar observations [8,21,22]. The high level of conductivity of the tannery effluents is attributed to the presence of magnesium sulphate and calcium bicarbonate in the Tannery while low BOD indicates a low amount of biodegradable materials in the effluent [21]. There were 4.33-30.6 cfu/mL of total aerobic heterotrophic bacteria in the tannery effluents. The high numbers could be because the effluents are nutrient-rich and warm, or because the organisms have learned to thrive in spite of the effluent's harsh conditions [21].

The bacteria identified were predominantly members of the genus *Lysinibacillus*. Predominance of this genus of bacteria is linked to its ability to utilize chromium as substrate, which helped it to thrive well in the environment [23]. The occurrence of other isolates of the genera *Providencia, Salmonella, Enterobacter, Leminorella, Bacillus* and *Paenibacillus* (**Table 3**) in tannery effluents has been previously reported [8,21,22,24,25]. Drop collapse test, oil spreading technique and Emulsification assay are indirect method used to screen biosurfactant production [22,26].

The crude oil showed positive results to these tests due to the presence of the biosurfactants. These findings agree with the report of Youssef *et al*. [18], who recommended that drop collapse, oil spreading and emulsification capacity assay methods were reliable for testing biosurfactant production. *L. fusiformis* showed a higher emulsification index than the other positive isolates. This could be because the isolate metabolized the diesel used to mask the effluent's odor at the discharge point. *Lysinibacillus fusiformis* is a naturally occurring bacterium with many isolated strains from a wide variety of habitats, including agricultural soils and industrial effluent [18].

CONCLUSION

The results of this study indicated that *L. fusiformis* could produce biosurfactants. However, the organism was not as effective as many other *Baccillus* species reported in the literature. The isolate produced phospholipid biosurfactant, which was stable at high pH and temperature, pointing to the fact that the biosurfactant could be used in extreme environments. The results obtained in this study constitute a stimulus for future research in this area. Thus, more interest should be given to other bacteria in the tannery effluents.

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