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Isolation of Biosurfactant Producing Bacteria from Tannery Effluents in Sokoto Metropolis, Nigeria

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ABSTRACT

Tannery effluents provide an environment for growth of different types of microorganisms. Possibility of occurrence of biosurfactant producers in such effluents collected from local tanneries was investigated in this study. Samples of tannery effluents were collected from three different tanneries. Physicochemical analyses revealed that the effluents contained large amounts of total dissolved solids, total suspended solids, organic and inorganic matter and Chromium beyond the limits stipulated by Federal Environmental Protection Agency (FEPA). Forty seven bacteria species were isolated by enrichment culture technique using Mineral salt media (supplemented with 0.1% v/v Bonny light crude oil) and identified using biochemical characterization. Bacillus (61.70%) and Proteus (12.76%) species were the predominant bacteria, followed by Pseudomonas and Serratia (8.51% each) species. The remaining isolates were Citrobacter diversus, Aeromonas hydrophila, Micrococcus luteus and Enterobacter faecalis which had 2.12% of occurrence each. Hemolysis, oil emulsification, drop collapse and oil spreading methods were used to screening the isolates for biosurfactant production. Of the twenty two isolates tested, only seven (31.8%) and five (22.72%) were hemolytic and able to emulsify the oil respectively. Similarly, only two isolates – Bacillus sphaericus EN3 and Bacillus azotoformans EN16 were positive for drop collapse and oil spreading. Results showed that Bacillus sphaericus EN3 could cause 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 could do same at the rate of 15 and 12 min respectively with 2.9cm diameter. Although Bacillus azotoformans EN16 had higher emulsification index (E_{24} = 58.0 ± 5.5) than Bacillus sphaericus EN3 (E_{24} = 47.8±1.15), the former might have more active surfactants than the latter, considering the period and diameter of oil spreading. Therefore, the two organisms proved to be biosurfactant producers and tannery effluents is a good source for biosurfactant producing bacteria.

Keywords: Biosurfactant, Tannery effluent, Emulsification, Hemolysis

1. INTRODUCTION

Tannery effluents refer to the wastewater resulting from the process of converting skins and hides into leather [1]. The process of tanning requires large volume of water, which is used to either cleanse the hides and skins or to serve as a medium of interaction between the hides and skin [2]. During the tanning process, large amount of effluents are discharged into the surrounding soil as well as water sources. These effluents may contain a variety of chemicals that are used in the tanning process such as sodium sulfate, chromium sulfate, non-ionic wetting agents and may accumulate in the immediate environments of the tanneries [3].

When the effluents are not properly managed, many pathogenic microorganisms and chemicals in the effluents may predispose the inhabitants to serious health hazards [4]. It may alter the physicochemical parameters of soil or water bodies thereby affecting the ecosystems [3, 5]. Another environmental consequence of discharging untreated tannery effluents in the environment is that methanogens may produce excessive methane thus contributing to greenhouse effect and global warming [6].

In order to tackle the menace of tannery effluents to the environment, some treatment methods were employed including bioremediation [7]. In general, potential microorganisms, especially bacterial species can remove heavy metals (the major constituents of the effluents) from solutions by biosorption or bioaccumulation or both. Different bacterial species including *Bacillus*, *Staphylococcus*, *Pseudomonas*, *Schwanella* and *Desulphovibrio* were shown to play an important role in tannery effluent bioremediation through numerous biochemical strategies [8, 9]. The use of biosurfactant for minimizing surface and interfacial tension at the surface and interface respectively has been suggested [7, 10].

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However, there is paucity of information on the production of biosurfactants by bacteria isolated from tannery effluents as well as the use of biosurfactants for the treatment of the effluents. This research was therefore designed to isolate indigenous bacteria from an unpopular source (tannery effluent) that can produce biosurfactants, which could be used for bioremediation of effluents from local tanneries.

2. Materials and Methods

2.1 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).

2.2 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 [11].

Fig. 1. Karaye tannery

Fig. 2. Unguwan Rogo I tannery

Fig. 3. Unguwan Rogo II tannery

2.3 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].

2.4 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.

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3. RESULTS AND DISCUSSION

3.1 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].

3.2 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].

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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 (^{o}C)	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)

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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

3.3 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.

4. Conclusions

Tannery effluents proved to be among the important sources of biosurfactant producing bacteria from contaminated environment. Despite the environmental stress due to heavy metal and low pH, the bacteria were able to thrive and actively involved in degrading organic matter. The low number of biosurfactant producers observed in this study might not be unconnected with series of screening processes that involved many tests as opposed to one or two tests that are normally been reported. More so, the use of crude oil as enrichment substrate in the isolation process might likely contribute to that. Therefore, tannery effluents should not be ignored in search of biosurfactant producers for application in bioremediation. Their application in tannery effluent and heavy metal-contaminated site bioremediation is recommended considering their ability to thrive in high chromium environment.

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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.

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