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Biosurfactant production potential of bacillus obtained from dye effluent

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This study investigated the biosurfactant productions potentials of Bacillus isolated from dye effluent. Samples were collected under aseptic condition from three areas of Sokoto (Marina, Unguwar rogo and Minannata) in Nigeria and transported in an ice bag to microbiology laboratory of Usmanu Danfodiyo University, Sokoto. Enumeration, identification and characterization of the isolates were carried out using standard microbiological methods. The potential and ability to produce biosurfactants was determined using blood haemolytic tests, drop collapse and emulsification techniques. A total of nine organisms were isolated from these three locations sampled, and three were *Bacillus* species which are the predominant bacteria obtained from the three locations. Enumeration results revealed highest bacterial count at Unguwar rogo (17.33×10^5 cfu/ml). Haemolysis results revealed that all the isolated bacterial strains exhibited haemolytic activity. The result of drop collapse test showed that all the isolated organisms had good collapsing ability, and all the isolated organism had positive oil spreading and emulsification ability. This study showed *Bacillus* species are potential biosurfactants producers and should be studied in greater details as strains improvement may enhance the activity of biosurfactants.

Key words: Biosurfactants, drop collapse, emulsification, potential, dye effluent.

INTRODUCTION

Dye effluents are the liquid waste of dye. When the effluents are not properly managed, many pathogenic microorganism and chemicals in the effluents may predispose the inhabitants to serious health hazard (Ogbonna et al*.,* 2004). It may alter the physicochemical parameters of soil or water bodies thereby affecting the ecosystems (Tudunwada et al*.,* 2007). Another environmental consequence of discharging untreated dye

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effluents in the environment is that methanogens may produce excessive methane thus contributing to greenhouse effect and global warming (Faruk et al*.,* 2005). Surfactants are surface active compounds that can be chemically synthesized or biologically formed (biosurfactants). Chemically synthesized surfactants are toxic, non-degradable and may accumulate in living tissues leading to the development of cancer diseases (Seghal et al*.,* 2009; Lakshmipathy et al*.,* 2010). Biosurfactants are preferable to chemical surfactants due to the following characteristics: Low or no toxicity, biodegradability, better environmental compatibility, ability to act at wider range of temperature, pH values and salinity levels.

Furthermore, they may be produced from industrial waste and agriculture products which represent cheap substrates (Deleu and Paquot, 2004; Cho et al., 2005; Dehgan-Noudeh et al*.,* 2009).

Biosurfactants are amphiphilic biological compounds produced extra cellularly or as part of the cell membrane by a variety of bacteria, yeast and filamentous fungi from various substances including sugars, oil and wastes (Mata-sandoval et al*.,* 2000; Chen et al*.*, 2007). Biosurfactants are categorized mainly by their chemical composition and their microbial origin (Banat et al*.,* 2000; Anna et al*.,* 2001). In general, their structure includes hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acid (Costa et al*.,* 2006).

Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants such as increasing the surface area and bio-availability of hydrophobic water insoluble substrates, metal binding bacteria pathogenesis, quorum sensing and biofilm formation (Priya and Usharani, 2009). Unlike synthetic surfactants, microbial – produced compounds (i.e. biosurfactants) are easily degraded and particularly suited for environmental applications such as bioremediation and dispersion of oil spills (Mohan et al*.,* 2006).

Concerning biosurfactants, in order to reduce the production cost of biosurfactants, the yield and product accumulation must be increased through the development of economic engineering process and the use of cost effective substrate for the growth of microorganisms as biosurfactant-producers. The cost of the substrates will greatly influence the economical use of the biosurfactants. Interest in microbial surfactants has been steadily increasing in recent years (Woo and Park, 2004).

The search for biosurfactants producing microorganisms is still an important area of research because of the diversity of their molecules and wide variety of their application. The aim of this study was to isolate and identify biosurfactant producing bacteria from dye effluent, that is, from dye liquid waste.

MATERIALS AND METHODS

Sampling area

Dye effluents were collected from three (3) areas in Sokoto, Sokoto State, Nigeria. Sokoto is located to the extreme North West Nigeria between longitudes 4° 8'E and 6° 54'E and lattitude 12° N and 13° 58'N (Adamu et al*.,* 2015a).

Sample collection

Dye effluents were collected from three areas of Sokoto Township which are Marina, Unguwar rogo and Minannata areas of Sokoto. Samples were collected in sterile sample bottles and transported in ice box to microbiology laboratory, Usman Danfodiyo University, Sokoto. The triplicate dye effluent samples were collected by simple random sampling.

Media preparation

Mineral salt (Bushnell – Haas medium) (MSM)

Mineral salt medium of Isma'il et al. (2014) (composed of 1.2 g KH_2PO_4, 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.1 g Nacl, 0.01 g $FeSO_4.7H_2O$ and 20 g agar per liter at pH 7.4) were prepared and dispensed in three (3) flasks. To each of the flask, 2% $^{w}/_{v}$ of dye and glucose were added respectively (Seghal et al*.,* 2009). Nutrient agar, nutrient broth and blood agar medium were prepared and sterilized according to the manufacturer's instruction.

Microbiological analysis of dye contaminated soil effluent

Fivefold serial dilutions of the effluent suspension were carried out. Using spread plate technique, 1 mL aliquots of dilutions were inoculated in triplicates on nutrient agar plates for the enumeration of total aerobic heterotrophic bacteria. The nutrient plates were incubated at 37°C for 24 h; colonies which appear on nutrient agar plates were sub cultured into mineral salt media (MSM agar). Mineral salt media (MSM agar) with dye as carbon source were used for 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 (Benson, 2001). Pure isolates were obtained by repeated sub culturing of 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 (1993) and Bergey's Manual identifications Plan.

Physicochemical analysis of dye contaminated soil effluent

The pH (hydrogen ion concentration), BOD (Biochemical Oxygen Demand), COD (Chemical Oxygen Demand), DO (Dissolved Oxygen), Temperature TS (Total Solid), TDS (Total Dissolved Solid), TSS (Total Suspended Solid), hardness, color chromium content were determined according to the methods described by Adamu et al. (2015b).

Identification and characterization of the bacterial Isolates

Pure cultures of the heterotrophic bacterial isolates were Identified by cultural, morphological (Gram staining) and biochemical

characteristics (urease activity, indole test, Citrate test, methyl red and Voge's proskauer test, triple sugar iron agar test) according to standard method of Cheesbrough (2000).

Screening of bacteria isolates for biosurfactant production

Four methods were used to screen the bacterial isolates for potential to produce biosurfactant. The methods were the blood hemolysis test, emulsification index, oil spreading, and drop collapse method as described by Thavasi et al. (2011) and Youssef et al. (2004). Isolates were grown in mineral salt medium (MSM) containing the dye as carbon source. The culture was incubated for 10 days at 30°C with regular shaking. After incubation period, the broth of each isolate was centrifuge at 6000 rpm for 10 min and the supernatants separated by filtration in order to obtain cell free supernatants. The supernatants were used for blood hemolysis, emulsification, drop collapse and oil spreading tests.

Blood hemolysis test

The bacterial isolates were inoculated on blood agar containing 5% ($^{v}/_{v}$) human blood. The plates were incubated at 30°C for 48 h (2 days Hemolytic activity was detected as the presence of a clear zone around a colony). The clear zone (Hemolytic activity) suggested the presence of biosurfactant (Youssef et al., 2004).

Drop collapse test

Drop collapse test was carried out according to the method described by Youssef et al. (2004). A drop of crude oil (Bonny light) was placed on a grease free slide and one drop of the 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.

Oil spreading method

Oil spreading technique was carried out according to the method described by Youssef et al. (2004). 50 mL of distilled water was added to Petri – dished followed by addition of 100 uL 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 the clear zone on oil surface was measured using a meter rule and the time taken to achieve the spread was noted.

Emulsification ability/index test

Emulsification activity was carried out using the method of Tabatabaee et al. (2005), and Techaoei et al. (2011). Four (4) mL of the crude oil was added to equal amount of cell free supernatant and vortexed at 500 revolutions per minute for 10 min. After 24 h, the height of the stable emulsion was measured using a meter rule. The emulsification index (E_{24}) was calculated as the rate 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} = emulsion index after 24 h; h emulsion = The height of emulsion layer;h total = The total height of the liquid

Statistical analysis

Data obtained from this research were analyzed using One-way analysis of variance (ANOVA) followed by Duncan Multiple Range Test.

RESULTS

Table 1 shows the physiochemical parameters of dye effluent analyzed. The color was dark-blue, odor was found to be partially agreeable. Temperature of Marina was 3°C, Unguwar rogo is 27°C, and Minannata is 30°C with Unguwar rogo having `significant difference from Marina and Minannata. pH of Marina is 10.50, Unguwar rogo is 10.40 and Minannata is 10.30 There are no significant difference p (<0.05) in the pH from the three sampled areas. COD of Marina is 5.5, Unguwar rogo is 5.3 and Minanata is 5.8. OD, TSS and chromium content all exceeded the limit for the discharge of effluent by the FEPA. BOD of Unguwar rogo and Minanata also exceeded the limit of FEPA for the discharge of effluent. There is significant difference p (<0.05) in the BOD of Marina with that of Unguwar rogo and Minannata.TH and TDS are also within the recommended limit of FEPA. Although the TH of Marina and Unguwar rogo differs significantly p (<0.05) from that of Minannata, the TDS of Marina is also significantly difference from that of Unguwar rogo and Minannata.

Table 2 shows the counts of bacteria from the dye effluent of the three sampled areas. The highest bacterial counts were obtained at Unguwar rogo (17.33×10^5 cfu/ml).

Result of blood hemolysis shown in Table 3 revealed that all the isolated bacterial strains exhibited hemolytic activity; it is always the first test to identify the potential of microorganism's ability to produce biosurfactant. The isolated bacterial strains from Marina show + (2) hemolysis, Unguwar rogo + (3) and Minannata + (2). The result of drop collapse in Table 3 shows that all the isolated organisms have good collapsing ability. Isolated organisms from Marina produced collapsing of drop within 5 s, those from Unguwar rogo produced collapsing of drop within 3 s and those from Minannata within 5 s. The result of oil spreading (oil displacement area) in Table 3 revealed that, isolates from Marina have spreading diameter of 5.7 cm, those from Unguwar rogo have 31.2 cm, and those from Minannata have 10.2 cm The result of Emulsification ability/index in Table 3 revealed that, isolated organisms from Marina have 4.6% Emulsification index/ability, those from Unguwar rogo have 5.3% and those from Minannata have 4.6%.

Table 4 shows the result of the biochemical tests carried out on isolate from dye effluent. The predominant bacterium isolated and identified was *Bacillus* spp.

Table 1. Physiochemical parameters of dye effluent.

Parameter	Site			Recommended limit (FEPA)
	Marina	Unguwar rogo	Minannata	
Colour	Dark Blue	Dark Blue	Dark Blue	Nil
Odor	Partially Agreeble	Partially Agreeble	PartiallyAgreeble	Nil
Temperature (°C)	31.00±0.58[a]	27.00±1.15[b]	30.00±1.15[a]	<40
pH	10.50±0.50[a]	10.40±0.23[a]	10.3±0.09[a]	6.0-9.0
Total hardness (mg/L)	1.10±0.06[a]	1.05±0.03[a]	0.70±0.12[b]	125
Total dissolved solid (mg/L)	442.00±1.15[b]	493.33±8.82[a]	501.00±0.57[a]	500
Total suspended solid (mg/L)	306.00±3.46[c]	328.00± 2.31[a]	631.00±0.58[a]	<200
Dissolved oxygen (mg/L)	2.20±0 .12[c]	8.10±0.06[a]	3.80±0.17[b]	<2.0
Biochemical oxygen demand (mg/L)	10.90± 0.11[a]	30.50± 0.14[c]	16.4000±.23[b]	15
Chemical oxygen demand (mg/L)	5.50±0.29[a]	5.30±0.17[a]	5.80±0.23[a]	40
Chromium content (mg/L)	1.16±0.09[b]	1.29± 0.02[a]	1.44±0.02[a]	<1.0

Values are mean±SEM. Means with different superscript in a row are significantly different (p<0.05); One-way ANOVA Followed by Duncan Multiple Range Test. Mg/L, Miligram/ L, Liter; FEPA, Federal Environmental Protection Agency.

Table 2. Bacterial colony count of the dye effluent.

Sample area	Colony count (×10^5 cfu/mL)
Marina	8.00±01.0[b]
Unguwar rogo	17.33±4.63[a]
Minannata	12.33±3.9[b]

Values are mean±SEM. Means with different superscript in a row are significantly different (p<0.05); One-way ANOVA Followed by Duncan Multiple Range Test.

DISCUSSION

In this study of biosurfactant production potentials of *Bacillus* species obtained from dye effluent, physicochemical characterization/analysis of the dye effluent indicates high concentration of dissolved chemicals. This is in agreement with findings of Srinivasan et al. (2014) who reported that dye effluent is rich in various parameters/physicochemical properties. Higher bacterial colony counts were recorded from Unguwar rogo areas. This might be due to availability of 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. This agrees with the findings of Adamu et al. (2015b) who suggested that difference in bacterial colony count could be due to availability of nutrients and favorable temperature of the effluent.

A total of nine bacteria were isolated and identified from this study. *Bacillus spp.* is the predominant from the three locations sampled (Marina, Unguwar rogo and Minannata). This might be due to the ability of *Bacillus* to survive in wide range of temperature, pH and having mechanistic enzymes dependent color removal strategy. This is in agreement with findings of Chen (2002) and Dave and Dave (2009) who reported that *Bacillus* has some enzymes system capable of color removal. The *Bacillus* identified in this study shows high hemolytic activity. This might be due extracellular secretions by catalytic enzymes. This agreed with the findings of Thavasi et al. (2011) and Elemba (2014) who suggested that the hemolytic ability could be attributed to extracellular secretions.

All the supernatant of three isolates were positive for drop collapse test, this is due to the reduction of surface-tension between the supernatant drop and hydrophobic oil surface this agreed with the findings of Tudunwada et al. (2007) who reported that drop collapses due to the reduction of surface-tension between supernatant drop and hydrophobic oil surface.

Also all the supernatant of the three isolate were positive for oil spreading test; this is due to the reduction of surface-tension between supernatant drop and hydrophobic oil surface. This also agreed with the findings of Tudunwada et al. (2007) who reported that oil spreading is due to the reduction of surface-tension

Table 3. Screening of biosurfactant producing organisms.

Isolate	Hemolysis	Emulsification index E_{24} (%)	Drop collapse		Oil spreading (oil displacement area)		
			Result	Time (s)	Result	Displacement (cm)	Time (min)
Bacillus lentus (MR)	+(2)	4.6	+	3	+	5.7	6
Bacillus brevies (UR)	+(3)	5.3	+	5	+	31.2	5
Bacillus lentus (MN)	+(2)	4.6	+	5	+	10.2	7

MR, Marina; MN, Minannata; UR, Unguwar Rogo.

Table 4. Morphological and biochemical characterization of isolates.

Coded isolate	Gram	MR	VP	H_2S	MOT	GLU	SUC	LAC	GAS	URA	CIT	Spore	IND	Organism
A1	G+rod and in chain	+	-	-	+	+	+	+	-	+	-	-	-	*B. spp.*
B3	G+rod and in chain	-	+	-	+	-	-	+	-	+	-	+	-	*B. spp.*
C1	G+rod and in chain	+	-	-	+	+	+	-	-	+	-	-	-	*B. spp.*

Gram, Gram reaction; MR, Methyl red; VP, voge'sproskauer; H_2S, Hydrogen sulphide production; MOT, Motility; GLU, Glucose; SUC, Sucrose; LAC, Lactose; GAS, Gas formation; URA, Ureas; CIT, Citrase; IND, Indole; B, Bacillus; P, *Pseudomonas;* E= *Escherichia.*

between supernatant drop and hydrophobic oil surface. Result of emulsification ability of the isolate revealed that all the three isolates have good emulsification ability. This is due to the stability of biosurfactant at different temperature and pH as agreed with the findings of Tabatabaee et al*.* (2005) and Techaoei et al*.* (2011) who reported that biosurfactant is stable at different temperature and pH.

Conclusion

This study indicates that *Bacillus* sp. isolated from Unguwar rogo could be a valuable source of biosurfactants. Although the composition was not determined, it can be suggested that the biosurfactants can be used in dye removal or decolorization of the effluents. Further studies need to be conducted in order to characterize the biosurfactants produced. Also molecular identification of the bacteria to the species level using 16s rRNA to know the type of species that produced this biosurfactant needs to be conducted

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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