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Isolation and Characterization of Biosurfactants Producing Bacteria from Oil Polluted Soil

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Abstract
Bio-surfactants are structurally diverse group of surface-active molecules, synthesized by micro-organisms. Oil polluted soils were collected from four oil spilled areas of different automobile workshops in Owerri Imo State. The samples were enriched in nutrient agar and serially diluted and pour plated in a nutrient broth plates. *Pseudomonas aeruginosa* and *Bacillus subtilis* were isolated and identified from these four samples. A, B, C, and D, were screened for biosurfactant production using haemolysis test and oil spreading techniques which showed the oil displacement in the plates. The biosurfactant was characterized by running the surfactant in TCL plates. Among nine isolates ($X^1 – X^9$), X^4 and X^8 were the best sources for the production of bio-surfactant for both *Bacillus subtilis* and *Pseudomonas aeruginosa.*
Key words: Bacteria, *Pseudomonas aeruginosa, Bacillus subtilis,* biosurfactant, oil polluted soil

1. Introduction

Surfactants are usually organic compounds that are *amphiphilic*, meaning they contain both hydrophobic groups (their tails) and hydrophilic groups (their heads). The hydrophilic (polar) end part of the biosurfactant is insoluble in water and may have a long chain of fatty acids, hydroxyl fatty acids or α-alkyl-β -hydroxy fatty acids. The hydrophilic (polar) end can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Jaysree *et al*., 2011).

Surfactant or surface active agents can be classified into two main groups; synthetic surfactant and bio-surfactant. Synthetic surfactant is produced by chemical reactions, while bio-surfactant is produced by biological processes, being excreted extracellularly by microorganisms such as bacteria, fungi and yeast (Jayrees *et al*., 2011). Chemically-synthesized surfactants have been used in the oil industry to aid clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment (Tabatabaee *et al*., 2005). When compared to synthetic surfactant, bio-surfactant have several advantages including high bio-degradability, low toxicity, low irritancy, ecological acceptability, compatibility with human skin and ability to be produced from renewable and cheaper substrates (Banat *et al.,* 2000) Therefore, it is reasonable to expect diverse properties and physiological functions of bio-surfactants such as increasing the surface area and bio-availability of hydrophobic water-insoluble substrates, metal binding, bacterial pathogenesis, quorum sensing, and bio-film formation (Priya & Usharani, 2009). Unlike synthetic surfactants, microbial-produced compounds are easily degraded and particularly suited for environmental applications such as bioremediation and dispersion of oil spills (Mohan *et al.*, 2006). The aim of this study is to isolate and screen bacterial species from different hydrocarbon polluted sites for bio-surfactants production.

1.1 Materials and methods

1.1.1 Sampling: Soil samples (A-D) were collected from oil spilled surfaces of different automobile workshops in Owerri Imo-State, Nigeria. The samples were collected in sterile polythene bags and were taken to the laboratory for analysis. The pH of the samples during collection was 7.

1.1.2 Isolation and enumeration of bacterial isolates from the sample

5g of the oil spilled soil samples were inoculated in 50ml of nutrient broth and incubated at 25˚C for 72 hours. After incubation the medium was serially diluted from 10^{-1} to 10^{-6} in sterile water. From the dilutions (10^{-1} to 10^{-6}) 1ml was transferred to sterile petri-dish and over that 20mls of nutrient agar was poured. The plates were then inverted and incubated at 25˚C for 48 hours. Control and replica plates were maintained.

1.1.3 Bacteriological isolation techniques

After incubation, the different discrete colonies formed on the plate that had between 30 and 300 colony forming unit (cfu) were streaked on nutrient agar slant and incubated at ambient temperature (37˚C) for 24 hours to obtain their pure cultures. These pure cultures, were sub-cultured on nutrient agar slant, incubated at 37˚C for 24 hours and stored at 4˚C for bio-surfactants production screening.

1.1.4 Oil spreading technique

Each of the bacterial species was screened for bio-surfactants production using the oil spreading techniques (Anandaraj & Thivakaran, 2010; Priya & Usharani, 2009). The procedure is as follows: the bacterial isolates were streaked on nutrient agar slant and incubated for 24 hours at 37˚C. After 24 hours, two loops of culture were inoculated in 50ml of nutrient broth in a 50ml Erlenmeyer flask and incubated at 37˚C for 48 hours. After the inoculum development, 50ml of distilled water was added to a large petri dish (25cm in diameter) followed by the addition of 20µl of crude oil to the surface of the distilled water and 20µl of the supernatant of the cultures isolated from the soil.

1.1.6 Blood haemolysis test

The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. The plates were incubated for 48-72 hours at 37˚C. The bacterial colonies were then observed for the presence of clear zone around the colonies. These clear zones indicate the presence of bio-surfactants producing bacteria.

1.1.7 Charaterization of biosurfactants producing isolates

The bio-surfactants producing bacteria were characterized by cultural and biochemical tests. They are gram staining, spore staining, motility test, oxidase test, indole test, catalase test, citrate test, coagulase test, methyl red and vogues proskauer test.

1.1.8 Analytical method

Thin layer chromatography

Preliminary characterization of biosurfactant was done by TCL method. A portion of the crude biosurfactant was separated on a silica gel plate using a developing solvent system with different colour developing reagents. Ninhydrin reagent (0.5g ninhydrin in 100ml anhydrous acetone) was used to detect lipopeptide biosurfactant as red spots and anthrone reagent (1g anthrone reagent in 5ml sulfuric acid mixed with 95 ml ethanol) to detect glycolipid biosurfactant as yellow spots (Yin *et al*., 2008).

1.1.9 Result

Table 1: Mean total viable heterotrophic bacteria count

SAMPLES	TVBC ($_C$fu/ml x10^5)
A	35
B	66
C	31
D	97

The Table 1 showed the bacterial count ranged from 31 X 10^5 to 97 X 10^5 cfu/ml. It showed highest bacterial count from workshop D and least from workshop C.

Table 2: Identification of the bacterial culture isolates, morphological and biochemical identification

Tests	***Bacillus subtilis***	***Peudomonas aeruginosa***
Morphology	Large round, thick, opaque colonies with dull irregular edges	Large round, bluish colonies with irregular edges which show swarming phenomenon
Shape	Rod	Rod
Grams reaction	G +ve	G –ve
Spore reaction	-	-
Motility	-	-
Oxidase	+	+
Methyl red	+	+
Voges proskauer	-	-
Citrate	+	+
TSI (H_2S)	+	+
Glucose	A/G	A/G
Maltose	A	+ A/G
Lactose	A	- A/G
Sucrose	A/G	-

Key - = negative + =Positive A/G = Acid/gas
A = Acid +AG =Positive Acid and gas
-AG =Negative Acid and gas

Table 3: Oil displacement method

S/N	**Culture isolates**	**Zone formation (mm)**
1	X^1	No Zone
2	X^2	No Zone
3	X^3	No Zone
4	X^4	11
5	X^5	No Zone
6	X^6	No Zone
7	X^7	No Zone
8	X^8	7
9	X^9	No Zone

In Table 3, nine different cultures were tested for the ability to display the zones of clearance. There were only two colonies out of the nine, which are the colony X^4 and X^8 that have the ability to display zones of displacement in oil.

Table 4: Blood haemolysis test

Culture isolates	**Zone formation (mm)**
X^1	α-haemolysis
X^2	α-haemolysis
X^3	α-haemolysis
X^4	β- haemolysis
X^5	α- haemolysis
X^6	α- haemolysis
X^7	α- haemolysis
X^8	β- haemolysis
X^9	α- haemolysis

In Table 4, Blood haemolysis test was also conducted on the different 9 isolates, it was discovered that isolates X^1 ,X^2, X^3 ,X^5, X^6,X^7 and X^9 displays α - haemolysis while X^4 and X^8 displayed β-haemolysis. β-haemolysis is one of the characteristics also of biosurfactant producing microorganisms.

1.1.10 Discussion

Bacillus subtilis and *Peudomonas aeruginosa* were isolated from the four different oil contaminated soil. *Bacillus subtilis* is a gram positive motile, endospore forming bacteria and it hydrolysis starch, casein and gelatin (Table 4). *Peudomonas aeruginosa* is a gram negative, rod shaped, motile bacteria and its biochemical characters are shown in Table 4. Anandaraj & Thivakaran, (2010); Priya & Usharani, (2009); Jaysree *et al*., (2011); Tabatabaee *et al*., (2005) had also used *Peudomonas aeruginosa* and *Bacillus subtilis* for their studies on biosurfactant production. The biosurfactants extracted were characterized by using TLC. Ninhydrin reagent was

used to detect lipopeptide biosurfactant as red spots from *Bacillus subtilis* and anthrone reagent to detect glycolipid biosurfactant as yellow spots from *Peudomonas aeruginosa* (Yin *et al*., 2008). The components were obtained as rhamnolipid i.e., a glycolipid from *Peudomonas aeruginosa* while sprayed with anthrone reagent on TLC plate. Anandaraj & Thivakaran, (2010); Priya & Usharani, (2009) also reported rhamnolipid from *Peudomonas aeruginosa* and surfactin from *Bacillus subtilis* in TLC plate. The result of this research work is in line with those of Tabatabaee *et al*., 2005 and Jaysree *et al*., 2011 who confirmed the isolation of biosurfactant producing micro organisms from oil polluted samples. Also that biosurfactant producing micro organisms can displace oil on water oil interface and show β- haemolysis on blood agar. In my study among the nine isolates obtained, the cultures X^4 and X^9 were found to produce the biosurfactant. This was screened by the oil displacement method and blood haemolysis test. The culture supernatants displaced oil by 11mm and 7mm respectively. The oil displacement method and blood haemolysis test were also followed by Anandaraj & Thivakaran (2010); Priya & Usharani, (2009); Jaysree *et al*., (2011); Tabatabaee *et al*., (2005).

Conclusion and recommendation

Biosurfactants are surfactants that are produced extracelluarly or as part of the cell membrane by bacteria, yeast and fungi. The main commercial use of biosurfactant is in oil industry, foods, cosmetics, pharmacology and environmental technology because of their ability to stabilize emulsions. The features that make them commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability and greater environmental compatibility. Production and characterization of biosurfactants produced by these bacterial isolates is recommended.

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