

**INTERNATIONAL JOURNAL OF SCIENCE FOR GLOBAL SUSTAINABILITY****Isolation and Screening of Biosurfactant Producing Bacteria from Petroleum Contaminated Soil**

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ABSTRACT

Biosurfactants are a diverse and heterogeneous group of microbial metabolites synthesized by bacteria, yeasts, and fungi. Biosurfactant release the surface tension between the two substances. This study was aimed at production of biosurfactant by *Bacillus subtilis* and Soil from petroleum contaminated site was used to isolate *Bacillus subtilis* which was identified morphologically by Gram's staining, biochemically by catalase, indole, and methyl red voges proskauer test and molecularly using 16s rRNA. The bacteria was screened for the potential to produce biosurfactant using hemolysis test, drop collapse test, oil displacement test and emulsification index. The screened bacteria can be produced biosurfact using petrol as carbon source.

1.0 INTRODUCTION

Many different microbial species of bacteria, yeast, and mold are capable of degrading hydrocarbons, and bacteria are the best described biosurfactant producer (Kaczorek *et al.*, 2008). Bushnell and Hass (1941) were the first to demonstrate the bacterial production of biosurfactants, using strain of *Corynebacterium simplex* and *Pseudomonas* grown in a mineral media containing kerosene, mineral oil, or paraffin. Since then, studies on the structure and mechanisms involved in the production and action of biosurfactants have been reported (Ilory *et al.*, 2008; Salihu Ibrahim *et al.*, 2020). The most reported genera of biosurfactant-producing bacteria include *Pseudomonas spp.*, *Acinetobacter spp.*, *Bacillus spp.*, and *Rhodococcus spp.*, among others.

Microorganisms that produce biosurfactants are isolated mainly from sites that are or were contaminated with petroleum hydrocarbons contamination (soils, effluents, and wastewater). Thus, have ability to grow on substrates considered potentially noxious to other non biosurfactant producing microorganisms. Biosurfactants play a physiologic role in increasing bioavailability of hydrophobic molecules, which are involved in cellular signaling and differentiation hence, facilitate the consumption of carbon sources present in soil (Katemi, 2012).

Biosurfactants have several advantages over synthetic surfactant which include high biodegradability, low toxicity, low irritancy and compatibility with human skin, selectivity, specific activity at extreme temperatures, pH, salinity etc. (Banat, 1995; Desai and Banat, 1997; Cameotra and Makkar, 1998; Banat *et al.*, 2010).

2.0 MATERIALS AND METHODS**2.1 Sample Collection**

The soil sample was collected using polythene at the depth of 10cm in the morning, from NNPC depot Gusau. NNPC depot is located at Gidan Ali, Gusau, Zamfara, latitude (12°13'9.40872" N) and longitude (6°37'34.65696" E) using aseptic microbiological sampling method.

2.2 Sample processing

The soil sample was serially diluted from 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵, 1mL from the last test tube was transferred to nutrient agar by pour plating method, the petri dish was incubated for 24hr and colony was observed.

2.3 Research Design

This study was design to utilize petroleum contaminated soil from NNPC depot Gusau for the production of biosurfactant and the optimum condition (pH, Temperature, Incubation

period and Substrate concentration) using one-factor at-a-time (OFAT) for the production of biosurfactant.

2.4 Sterilization

All glass wares such as pipettes, conical flask, test tubes, beakers, universal bottles were washed thoroughly with detergents dried and then sterilized using a hot air oven at 161°C for 1hr (Oyeleke and Manga, 2008).

2.5 Media Preparation

The media was prepared according to Hi media manufacturer's instruction.

2.5.1 Minimal Salt Media

This media was prepared using the following composition in g/l NaCl 0.1, KH_2PO_4 1.2, Mg_2SO_4 0.2, K_2HPO_4 1.8, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01, NH_4Cl 4.0. The composition was dissolved in 1 liter of distilled water in a conical flask and was heated on a hot plate at 40°C to enhance dissolution. The solution was pipette in to universal bottles and autoclaved for 15minutes at 121°C. Petrol (2%) was added into each bottle using a sterile pipette and was shaken thoroughly to enhance mixing (Francy *et al.*, 1991).

2.5.2 Nutrient Agar

This was prepared according to manufacturer's instructions. A nutrient agar powder of 2.8g was weighed and dissolved into 1000 ml of distilled water. The preparation was then heated using a hot plate to dissolve completely. A cotton wool was placed on the top of conical flask and wrapped with aluminum foil. Solution was autoclaved at 121°C for 15minutes; it was then allowed to cool and dispensed in to sterile petri-dishes.

2.5.3 Blood Agar

Blood agar was prepared by weighing 2.8g of nutrient agar powder into a sterile conical flask containing 1000 ml of distilled water. The preparation was heated using a hot plate to dissolve completely. Cotton wool was placed on the mouth of the conical flask and wrapped with aluminum foil. The solution was autoclaved at 121°C for 15minutes after which it was allowed to cool to ambient temperature and then 10% of fibrinated blood was added. The content of the flask was

shaken and dispensed immediately into sterile petri-dishes.

2.6 Smear Preparation for Gram Staining

About one drop of normal saline was placed on a clean, grease free microscopic slide. Then a loopful of the colonies isolated after incubation was picked using sterile wire loop, mixed with normal saline, then allowed to air dry and heat fixed by passing over the flame of a Bunsen burner (Oyeleke and Manga, 2008). The smear was flooded with crystal violet for 60 seconds and then washed with clean tap water. Iodine was poured and allowed for 30 seconds and washed with clean tap water. The smear was decolorized with ethanol, counter stained with safranin for 60 seconds. An oil immersion was added to the stained slide and viewed under a microscope, using x100 (Oyeleke and Manga, 2008). The bacteria isolated were identified on the basis of microscopic examination, cultural and morphological characteristics.

2.7 Biochemical Tests

2.7.1 Urease Test

The test organism was inoculated in bijou bottles containing 3mls of Christensen's modified urea broth and incubated at 37°C for 24hrs. Positive reaction is indicated by changing of color from yellow to pink (Oyeleke and Manga, 2008).

2.7.2 Indole Test

The test organism was inoculated in test-tubes containing 3ml of sterile tryptone water and 0.5ml of Kovac's reagent was added and shaken. Production of indole was confirmed by the formation of red ring colour on the surface of the medium, (MacFaddin, 2000).

2.7.3 Voges Proskauer (VP) Test.

A colony was picked using sterilized wire loop and was inoculated into the glucose peptone medium and incubated at 37°C for 48hrs. Aliquots of 0.2ml of 40% aqueous potassium hydroxide solution and 0.6ml of alpha-naphthol solution were added, and the tubes were sloped so as to increase the liquid air interface. Production of strong red color indicate a positive result (Oyeleke and Manga, 2008).

2.7.4 Methyl Red (MR) Test

Fresh culture was picked and inoculated into the glucose phosphate medium; it was then incubated at 37°C for 48 hours. Five (5) drops of methyl red reagent was added and shaken gently, formation of red colour indicates positive result (Oyeleke and Manga 2008).

2.7.5 Citrate Test

Using Simmons's citrate agar, the slope of the medium was prepared, and the slope was streaked with a saline suspension of the test organism and then stabbed. It was then incubated at 35°C for 48 hours. Bright blue formation in the medium indicates citrate test positive. (Jawetz, 1989).

2.7.6 Sugar Fermentation Test

The media used contain three sugars i.e. glucose, sucrose and lactose. Colonies from the slant were picked and sub-cultured into the nutrient agar medium in the Petri dishes, and incubation at 37°C for 24 hours to obtain a fresh culture. Colonies from the fresh culture were picked and streaked on the surface of the slope and then stabbed on the bottom of the test tubes by using straight line wire loop. The tubes were incubated at 37°C for 24 hours (Harley, 2005).

Some microorganisms ferment all the sugars present and produce sufficient acid, which changes the color of the indicator to yellow. While others ferment only one of the sugar producing little amount of the acid, indicated by yellow color at the bottom. Production of hydrogen sulfide (H₂S) was detected by blackening of the media along the stab line. Gas production was detected by the production of gas bubbles or cracks.

2.7.7 Motility Test

Motility agar was prepared and inoculated with a straight inoculating needle making a single stab down into the medium. The motility of the organism was examined after incubation at 37°C for 24 hours. Motility was indicated by the presence of diffused growth away from the line of inoculation (Hemraj *et al.*, 2013)

2.7.8 Starch Hydrolysis Test

This test shows whether an isolate is capable of utilizing starch or not. Each isolate was inoculated into starch hydrolysis medium (nutrient agar +

starch) and incubated for 24 hrs. After incubation, iodine solution was added to each growth and blue black colour was observed. Development of blue black colour signifies presence of starch, that is, the isolates could not utilize starch and so negative for this test. Absence of starch was indicated by failure to develop blue black coloration and so positive because the isolates have been able to utilize the starch (Hemraj *et al.*, 2013).

2.8 Molecular Identification of the Bacterial Isolates

2.8.1 DNA Extraction

Genomic DNA was extracted using DNA extraction kit from sigma aldrich from pure bacterial culture Genomic DNA was extracted. Genomic DNA was used as template for the amplification of the 16S ribosomal RNA (16S rRNA) coding region with the universal primers obtain from Merck, UK, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16srRNABact 1492-R (3'-GGTTACCTTGTTACGACTT- 5') (Weisburg *et al.*, 1991).

2.8.2 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was carried out using a reaction mixture of 50 µL containing 35.5µL of water, 5.0µL of PCR buffer 10 X, 1.5µL of MgCl₂ (50mM), 1.0µL of each primer (10mM), 4.0µL of deoxynucleoside triphosphate solution (2.5mM) and 1.0µL of Taq DNA polymerase (5.0 U). Amplification conditions were adjusted on a thermal cycler as follows: initial denaturation step at 94°C for 3 min; 30 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 30 s; and a final elongation step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1.2% agarose gel electrophoresis (Ahmed *et al.*, 2015). The gel was stained with ethidium bromide, visualized under UV light, and photographed. The amplified products with the 250bp size were then purified and sequenced (Abdelhaleem *et al.*, 2019).

2.8.3 DNA Sequencing

The microbial product was sent to Malaysia (MyTacq Laboratory) for sequencing and sequenced using Sanger sequencer. DNA sequencing was performed with the universal primer (forward). The sequence obtained was

assembled using MEGA7 software to get the consensus sequence for each strain and were compared with bacterial 16S rRNA gene sequences deposited in the GenBank database from the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990; Ahmed *et al.*, 2015).

2.9 Screening of Isolate for Biosurfactant Production

The isolates were screened for biosurfactant production using haemolysis test, Drop collapse test, Oil displacement assay and Determination of emulsification index.

2.9.1 Hemolytic Activity Analysis

Isolates were screened on blood agar plates containing 2% (v/v) sheep blood and incubated at 37°C for 48hrs. Hemolytic activity was detected by the presence of a clear zone around bacterial colonies (plaza *et al.*, 2006; youssef *et al.*, 2004).

2.9.2 drop Collapse Test

Drops of the cell free supernatant was placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable whereas if the drop contains

surfactant it spreads or even collapse due to the presence of biosurfactant (Yusef *et al.*, 2004).

2.9.3 Oil Displacement Assay

Crude oil of 10µl was added to the surface of 40 ml of distilled water in a petridish to form a thin oil layer. Then, 10µl of culture or culture supernatant was gently place on the center of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone was formed due to the presence of biosurfactant (Morikawa *et al.*, 1993).

2.9.4 Determination of Emulsification Index (E24%)

Emulsification index of cell free broth was determined by adding 2 ml of oil to 2ml of cell free broth, mixed for 2 min, and allowed undisturbed for 24hr. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (Cooper and Goldenberg, 1987). The total height of liquid, as given by the expression:

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

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.

3.0 RESULT AND DISCUSSION

From this study the isolation of the bacteria reveals the following results.

Table 1: Biochemical Identification of the Isolates Using Bergey's Manual

CODE	Gram Reaction	Shape	Cell Arrangement	Spore Formation	Motility
S ₁	Positive	Rod	Scattered	Positive	Negative

Key: S₁ Isolate 1

Table 2 Biochemical Characterization of the Bacteria Isolated from NNPC Depot Gusau

Isolates	GLU	SUC	LAC	H ₂ S	Oxidase	Gas production	MR	VP	CIT	IND	CAT	URE	Starch hydrolysis
S ₁	+	+	+	-	-	-	-	+	+	-	+	-	+

Key;- GLU= Glucose, LAC= Lactose, SUC= Sucrose, H₂S= Hydrogen Sulphite, MR= Methyl red

VP= Voges proskauer, CIT= Citrate, CAT= Catalase, URE= Urease
 + = Positive
 - = Negative

Identification of the Isolates

The isolate was identified to be *Bacillus sp.* according to morphological and biochemical test.

Table 3 The Identified Isolate According to the Morphological and Biochemical Characteristics

CODE	IDENTIFIED ORGANISMS
S ₁	<i>Bacillus sp.</i>

Key:- S₁ Isolate 1

Electrophoregram and Phylogenetic Analysis of The *Bacillus subtilis*

The result obtained for electrophoregram and phylogenetic analysis of 16SrRNA for biosurfactant producing bacteria are presented in figure 4.1 and 4.2 respectively.

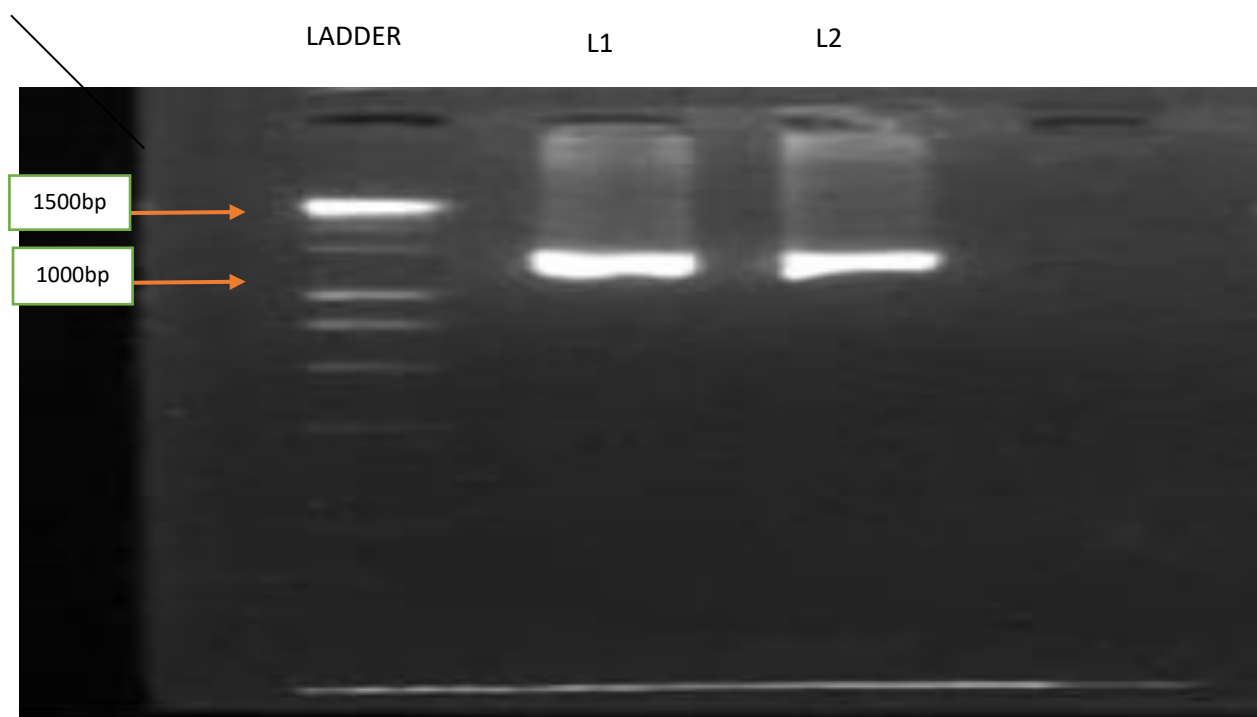


Figure 1: Agarose gel electrophoresis, lane 1 and lane 2 indicates extracted DNA (1500) of *Bacillus subtilis* and *Bacillus spp* respectively.

Screening Test for Biosurfactant Production

The results obtained for the screening test (haemolysis test, oil displacement test, and drop collapse tests and emulsification index) for the production of biosurfactant are presented below

Table 4: Screening Test for the Production of Biosurfactant

Isolate	β -haemolytic activity (mm)	Oil displacement assay	Drop collapse test.	Emulsification Index E ₂₄ %
S ₁	8.00	++	+	40.0

Key: += Very low zone of inhibition (Oil displacement assay), Presence of emulsion (Drop collapse test) += No zone of inhibition (Oil displacement assay), No emulsion (Drop collapse test)

++ = moderate zone of inhibition

4.0 DISCUSSIONS

In this study the potential of *Bacillus subtilis* for the production of biosurfactant in solid state fermentation was examined. Several authors have reported using some other species of bacteria such as *Rhodococcus*, *Pseudomonasaeruginosa*, *candida bombicola*, *candida lipolytica* (Banat *et al.*, 2010) for the production of biosurfactant. However use of *Bacillus subtilis* for the production of biosurfactants was underreported especially in Nigeria. From the isolation and identification both biochemically and molecularly *Bacillus subtilis* have been identified according to bergey's manual (Brenner *et al.*, 2005). This was supported by molecular analysis, the sequence shows that its *Bacillus subtilis*. Isolate from petroleum contaminated soil shows ability to produce biosurfactant this is in conformity with the finding of Waites *et al.* (2008) who isolated *Bacillus spp* from soil.

The screening procedure for the production of biosurfactant shows that the zone of inhibition, drop collapse test, emulsification index and oil displacement test were positive is in line with the findings of Khanna and Pattnaik (2019) who screen the bacteria for biosurfactant production and found that were positive for haemolysis test, drop collapse test, oil displacement and emulsification index in a research conducted on Production and functional characterization of food compatible biosurfactant.

From the stability test result, the biosurfactant produce by the strain of *Bacillus subtilis* shows the highest emulsification index of 45% at 2% w/v of Nacl concentration which indicates it is stability at that percentage. Similarly it shows the emulsification index of 45% at the temperature of 20^oC this shows that its stable at acidic environment, this agrees with the finding of Ibrahim, 2020 who conducted the research on Biosurfactant Production and Growth Kinetics Studies of the Waste Canola Oil-Degrading Bacterium *Rhodococcus erythropolis* AQ5-07 from Antarctica.

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