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Isolation and Screening of Spent Engine Oil Utilizing Bacteria for the Production of Biosurfactants

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Abstract

This study was conducted to isolate and screen spent engine oil utilizing bacteria for possible production of biosurfactants from spent engine oil polluted soil in Dutse Mechanic village, Jigawa State. Pour plating method through the use of sterile nutrient agar medium enriched with spent engine oil as a sole carbon source was employed to isolate the desired hydrocarbonoclastic bacteria. Emulsification assay, foaming activity and blood haemolysis tests were conducted to screen biosurfactant producing bacteria. Out of the five bacteria isolated and screened, only two (S1 and S2) showed positive results with diameter of clear zone, 2.2 and 3.1cm respectively. Foaming stability in S1 was higher than that of S2. S1 recorded higher emulsifying activity (61.9%) than S2 (45.0%). S1 recorded β blood haemolysis while S2 was γ haemolysis. The isolated and screened hydrocarbonoclastic bacteria that demonstrated tendency to produce biosurfactants in this study are possibly *Bacillus subtilis* and *Bacillus cereus* based on the morphological and biochemical attributes displayed. However, molecular identification of the bacterial isolates screened in this study is recommended with a view to confirming their identity further.

Keyword: Biosurfactants, Oil spreading assay, spent engine oil and hydrocarbonoclastic bacteria

1. Introduction

Surfactants are surface active compounds that have got ability to reduce the interfacial tension between two liquids, or a liquid and a solid (Rosen and Kunjappu, 2012). The authors reiterated further that surfactants are organic compounds that contain both hydrophobic and hydrophilic moieties. However, the expensive nature of surfactants and their established unfriendliness to the immediate environment where it is employed have made its utilization a huge concern over the years. Biosurfactants, which are the accepted alternative to surfactants are biological compounds produced extracellularly or as

part of the cell membranes by a diversity of bacteria, yeast and filamentous fungi originating from many substances including waste products and sugars (Cho *et al*., 2005). They are mostly tagged as lipid compounds whose attributes are associated with two ends that are present in the molecule; one end is hydrocarbon part which is hydrophobic end. The hydrophobic part of the molecules is described as a long-chain of fatty-acids, hydroxyl fatty acid or α-acyl hydroxyl-fatty acids. The other end known as hydrophilic is more of soluble in water and consists of carbohydrate, amino acid, cyclic peptide, phosphate and carboxylic acid or alcohol (Cho *et al*., 2005; Volchenko *et al*., 2007).

According to Banat (1995); Woo and Park (2004); Cho *et al*. (2005), interest in biosurfactants has been steadily increasing in recent years, as they have got many advantages over chemical surfactants ranging from lower toxicity, higher biodegradability and foaming, better environmental compatibility and effective properties at extreme temperature, pH levels and salinity. Owing to the advantages highlighted above, they have got protracted applications in petrochemical and oil industries, pharmacy, medical, cosmetics, food and pharmaceutical industries (Babu *et al*., 1996; Makkar and Cameotra, 2002).

Due to their versatile enzymatic prowess, biosurfactant producing microorganisms have been documented to be naturally present in oil polluted soil as such environment contains large quantity of oil most especially aliphatic and aromatic hydrocarbons that these microorganisms eat, consume, metabolize degrade and utilize for their metabolic activities. Microorganisms exhibit emulsifying activity by producing biosurfactants and utilize the hydrocarbons as substrate often mineralizing them or converting them into biologically inert products. Due to the establishment of bacteria versatility in the production of biosurfactants, this study was conducted with a view to isolating and screening spent engine oil degraders for the production of biosurfactants as they are indigenous in such environment.

2. Materials and Methods

2.1.Description of Sampling Site

Spent engine oil polluted soil samples were collected in the mechanic village Dutse (Lat 11^0 46’39’’N and Long 9^0 20’3’’E) Jigawa state, Nigeria. According to Britannica (2019), the undulating relief of the area is characterized by Sudan savanna agro-ecological zone.

2.2 Collection of spent engine oil polluted soil samples

Spent engine oil polluted soil samples were collected using a 22-cm hand-dug soil auger as done by Ayotamuno *et al.* (2006) from five (5) different sites within Dutse Mechanic village, Jigawa state. Five (5) grams of the different samples were collected in sterile polythene bags, labeled appropriately and immediately taken to the laboratory for further analysis.

2.3 Isolation of Spent Engine Oil Utilizing Bacteria

Two-fold serial dilution was carried out and pour plate method was adopted for the isolation of spent engine oil utilizing bacteria. One (1) mL of 10^{-2} dilution was inoculated on sterile petri dishes, after which the sterilized media was poured aseptically on the inoculated plates. The plates were incubated at 37^oC for 24hours. After incubation, morphologically different colonies observed on the plates were sub-cultured on nutrient agar plates to obtain pure culture of the organisms and were subsequently transferred into already sterilized nutrient agar slants. The slants were kept in the refrigerator at 4^0C as stock culture.

2.4 Screening of Biosurfactant Producing Bacteria

The methods chosen for the screening of hydrocarbonoclastic bacteria that can produce biosurfactants in this study were guided by Walter *et al*. (2013). The isolated colonies were taken and tested for the ability of the isolated bacteria to produce biosurfactants. The isolates were inoculated into 10ml of broth medium each and be incubated at 37^oC for 72 hours. The culture media was centrifuged at 3000 revolutions per minute (R.P.M.) for 30 minutes. The supernatants were collected and the cells discarded. The supernatant was used for the various biosurfactant screening tests or assays.

The following screening tests were conducted:

(a) **Oil Spreading Assay:** Oil spreading assay for oil displacement activity of surfactants was done according to the method described by Morikawa *et al.* (2000). The principle of this method is based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. The surface pressure of the biosurfactant naturally displaces the oil. In this method 10μL of kerosene oil was added to the surface of 50 mL

distilled water in a petri dish and allowed the oil to form a thin layer. Subsequently, 10µL cultured supernatant was gently placed on the center of the oil layer. A positive result indicated the displacement of the oil leading to the formation of a clear zone. The displaced diameter was measured after 30 seconds. This is also known as oil displacement activity. Measured area is expressed in biosurfactant (BS) unit. According to Thaniyavarn *et al.* (2003), one BS unit is defined as the amount of surfactant forming 1 cm^2 of oil displaced area.

(b) Emulsification Assay: Emulsification activity was calculated by emulsification index known as E_{24}. Emulsification assay was carried out by adding 2mL kerosene in 1mL cell free supernatant which was obtained after the centrifugation, and then it was vortexed for 5 minutes. The emulsification activity was observed after 24 hours and it was calculated by using the formula suggested by Cooper and Goldenberg (1987) below.

$$E_{24} = \frac{h_{emulsion}}{h_{total}} x\ 100\% \quad (1)$$

$Where\ h = \ Height$

(c) Foaming Activity: Isolated bacteria were grown separately in 250 mL Erlenmeyer flasks, each containing 100 mL of sterilized nutrient broth medium. The flasks were incubated at 37°C on a shaker incubator (200 rpm) for 72 hours. Foam activity was detected as duration of foam stability, foam height and foam shape in the graduated cylinder.

(d) Blood Haemolysis Test: This test was done to determine the ability of bacterial colonies to induce haemolysis when grown on blood agar. Fresh isolated colony was streaked on sterile blood agar plates and incubated at 30^0C for 72 hours. After then, the results were observed, and compared. Presence of clear zone indicated the biosurfactant producing bacteria. Ideally, there are three types of haemolysis α, β, and γ. α haemolysis was recorded when the agar under the colony became dark and greenish, β was recorded when it became lighten yellow and

transparent and when no any change then it was recorded as γ haemolysis.

2.5 Identification of Bacterial Isolates

The following methods were employed to identify the isolated spent engine oil utilizing bacteria;

Gram Staining: Gram staining is a method that differentiates bacteria in to two large groups; gram positive and gram negative. Distinct colonies of the bacteria were picked and transferred to other glass slides to form smears. Gram staining procedure was conducted according to Olutiola *et al.* (1991) by flooding the smear on the slide with crystal violet solution for sixty (60) seconds, and rinsed with water. All the cells turned purple and the smear was again flooded with Iodine for one (1) minute and the slide was rinsed with 75% alcohol until the crystal violet was completely washed off. It was subsequently counter stained with safaranin for one (1) minute and finally rinsed with water. The slides were finally viewed under an oil immersion objective.

Cell Morphology: The Gram stained cells were observed based on their respective shapes and colours as done by Cheesbrough (1991).

2.6 Biochemical Identification of Isolated Bacteria

The following biochemical tests were conducted with a view to identifying the isolated bacteria;

Catalase Test: Gas bubbles detected within 10 seconds after the addition of purified bacterial culture to 5mL of hydrogen peroxide solution was recorded as a positive catalase test as done by Cheesbrough (1991)..

Urease test. Slanted 2 mL of urea medium placed in bijou bottles were employed applied for the incubation of bacterial colony at room temperature. Red-pink colour in the medium was recorded as a positive test for urease induction as done by Cheesbrough (1991).

Indole test. Appearance of bright red and yellow color which composed after added 0.5 ml of Kovac's reagent to incubated bacterial culture at 35^{0}C for 24 hours on SIM media indicated a

positive and negative results respectively (Cheesbrough, 1991).

Simmons Citrate test: Simmons Citrate test was performed through the employment of sterilized Simmons Citrate Agar plates (TSBA, Himedia) in which bacterial isolates were streaked on and subsequently

incubated at 35^{0}C for 48 hours. A change in the colour of the media from green to bright blue was recorded as a positive reaction as done by Al-Dhabaan (2018).

Methyl red (MR) test. After the addition of methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and subsequent incubation at 35^{0}C for four (4) days, a change of colour to red indicated MR test positive result as reported by Al-Dhabaan (2018).

Nitrate Reduction Test: Nitrate broth was inoculated with the test bacteria aseptically. It was later incubated for 48 hours and one drop of sulfanilic acid and one drop of α-naphthylamine were added to each broth respectively. A change in colour to red indicated a positive nitrate reduction test while no change in colour indicated a negative result as established by Microbeonline (2019)

3. Results and Discussion

3.1 Results

3.1.1 Screening Assays

Oil Spreading Technique

The results obtained from the oil spreading assay of the isolates are depicted in Table 1 below.

Emulsification Assay

The isolated spent engine utilizing bacteria that showed positive results were tested for their abilities to emulsify spent engine oil. The results are shown in Table 2 below.

Table 1: Results of oil spreading assay

Isolates	Diameter of clear zone (cm)	Status
S1	2.2	+
S2	3.1	+
S3	0	_
S4	0	_
S5	0	_

Key: + = Positive, - = Negative, S1 = Sample 1, S2 = Sample 2, S3 = Sample 3, S4 = Sample 4, S5 = Sample 5

Table 2: E_{24} index of bacterial isolates

Isolates	Emulsified layer (cm)	Total liquid layer (cm)	E_{24} (%)
S1	**1.3**	**2.1**	**61.9**
S2	**0.9**	**2.0**	**45.0**

S1 S2

Figure 1. **Foaming activity of both bacterial isolates**

3.1.2 Identification of Bacterial Isolates

(a) Colony Morphology: The two (2) bacterial isolates that showed positive results for the production of biosurfactants are shown in Figure 3 below depicting their respective colony morphologies when streaked on nutrient agar

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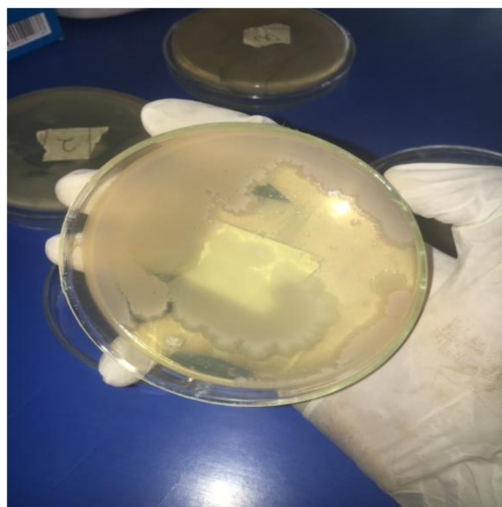

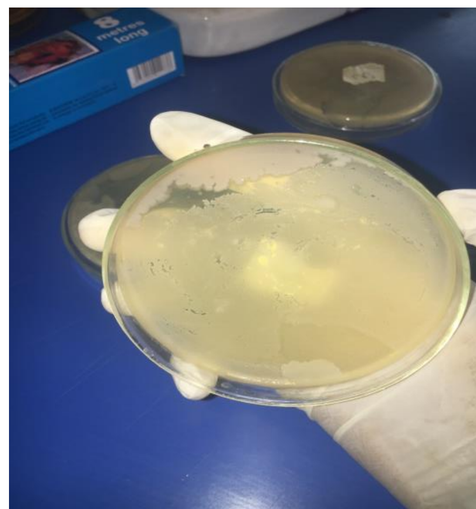

Figure 3. Appearance of Samples 1 and 2 after culture on Nutrient agar plates.

(b) Gram's staining: Both samples were observed to be gram positive *Bacillus* as shown in Table3.

Table 3: Cell and colony morphology of the Bacterial isolates

Isolates	Gram Staining	Cell Shape	Colony Shape	Pigment(Colony)	Oxygen Requirement
S1	+	*Streptobacilli* that occurred in long chains and clusters.	Circular, wet, smooth, concave	Coloured	Aerobic
S2	+	Bacilli appeared singly, in pairs and short chains	Circular, dry, smooth, flat and irregular with lobate margins	Coloured	Aerobic

Key: + = Positive, - = Negative, S1 = Sample 1, S2 = Sample 2

(c) Biochemical Identification: The results of the various biochemical tests done with a view to identifying the isolates that demonstrated production of biosurfactants are shown in Table 4 below

Table 4: Biochemical Tests for Biosurfactants Producing Isolates

Biochemical Tests	S1	S2
Indole	-	-
Catalase	+	+
Citrate	+	+
Methyl Red	-	+
Urease	-	+
Nitrate	+	-

Key: + = Positive, - = Negative, S1 = Sample 1, S2 = Sample 2.

3.2 Discussion of Results

Isolates S1 and S2 showed positive results in the oil spreading assay performed with clear zone diameter of 2.2cm and 3.1cm respectively (Table 1). These results are in agreement with the reports of Youssef *et al.* (2004); Plaza *et al*. (2006), vouching for the reliability of the oil spreading assay employed in this current study. Other bacterial isolates showed negative results, hence, they were not considered for further screening (Table 1). Isolate S1 (61.9%) recorded high emulsifying activity while S2 (45.0%) recorded appreciable emulsifying activity. These results are in line with the findings of Soni (2012).

Having carried out the blood haemolysis test as prescribed by Mulligan *et.al.* (1984), isolate S1 showed β haemolysis through the formation of yellow colour when streaked on blood agar and S2 recorded γ haemolysis following no observed change in colour on the plate.

It was observed that foaming stability in S1 was higher than S2 as depicted in Figure 1. However, S1 and S2 showed Gram positive results, and they appeared in rod and circular shape respectively (Figure 4). S1 appeared as *Streptobacilli* that occurred in long chains and clusters while S2 appeared also as *Bacilli* but occurred singly, in pairs and short chains under the microscope (Figure 4). As done by Al-Dhabaan (2018), biochemical tests were conducted to identify the two bacterial isolates that demonstrated capability for the production of biosurfactants. S1 recorded negative result for Indole test, positive for catalase, positive for citrate, negative for methyl red, negative for urease and positive for nitrate (Table 4). S2 showed negative result for Indole, positive for catalase, positive for citrate, positive for methyl red, positive for urease and negative for nitrate (Table 4).

It can be seen from the results presented in Table 4, that out of the various biochemical tests conducted on the two isolates that demonstrated biosurfactant production and cell morphological attributes, the isolated and screened bacteria are *Bacillus* spp. Out of the five (5) bacterial isolated and screened in this study, two (2) bacteria (S1 and S2) demonstrated biosurfactant producing capability. These results are in concord with the report of Nitschke and Pastore (2004) indicating that while *Bacillus* spp. occur in diverse habitats, they have equally got the highest biosurfactant producing ability.

Conclusion

Biosurfactant producing bacteria are novel in nature, hence, the need for strict observance of laboratory procedure when handling them. The isolated and screened hydrocarbonoclastic bacteria that demonstrated tendency to produce biosurfactants in this study are possibly *Bacillus subtilis* and *Bacillus cereus* based on their rod shaped appearance under the microscope after gram staining coupled with a positive test result for Catalase but may belong to two different species based on their differences in some biochemical test results conducted. The findings of this study show that biosurfactant producing organisms are present in Dutse. mechanic village. However, molecular identification of the bacterial isolates screened in this study is recommended with a view to confirming the identity of the bacteria.

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