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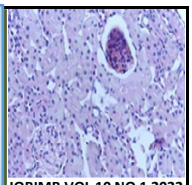

Isolation and Screening of Biosurfactant-producing Bacteria from Hydrocarbon-contaminated Soil in Kano Metropolis, Nigeria

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ABSTRACT

Biosurfactants are surface-active biomolecules produced by microorganisms that have different applications in solving many environmental problems. This study was carried out to screen biosurfactant-producing bacteria isolated from hydrocarbon-contaminated soil of Kano Metropolis, Kano State- Nigeria. Soil samples were collected and processed. Biosurfactant-producing bacteria were enumerated, isolated and characterized using cultural, morphological and biochemical characteristics. Blood haemolysis, oil drop collapse and oil displacement tests were employed for the screening of the bacterial isolates for the potential to produce biosurfactant. The viable aerobic heterotrophic bacterial count of the samples ranges from 1.0 to 8.4×10^6 cfu/g. Eight bacterial genera were biochemically identified as *Pseudomonas aeruginosa, Enterobacter cloacae, Pantoea agglomerans, Pseudomonas* sp., *Bacillus subtilis, Enterobacter alvei, Bacillus* sp. and *Klebsiella* sp. *Bacillus subtilis* had the highest frequency of occurrence of 5(27%) while *Bacillus* sp. and *Enterobacter alvei* have the least occurrences of 1(6%) each. The eight identified bacterial isolates were all positive for the haemolysis test, drop collapse and oil displacement test.

INTRODUCTION

Petroleum hydrocarbon pollution of soil is a worldwide environmental crisis [1]. The rapid expansion of the industrial sector has led to a surge in pollution and other environmental hazards. All forms of life, both on land and in water, are vulnerable to the consequences of petroleum pollution, but microorganisms, in particular, are at risk. The first stage of this impact is the transfer of hydrocarbons from the oil phase to the surface of the microbial cell, which occurs when the oil comes into contact with the microbial cell and is subsequently absorbed through the cell membrane [2]. Despite extensive research in this field [2], the mechanisms of n-alkane transport into the bacterial cell and hydrocarbon assimilation in microbial cells remain poorly known [3]. Several bacterial populations have been documented as showing resistance to oil transport, and even fewer have been shown to efficiently break down hydrocarbons. One method involves the consecutive steps of oil adhesion, pseudo-solubilization, and degradation of hydrocarbons to generate microscopic droplets of oils. Using active transport or diffusion at the interface between the cells and the hydrocarbons, microorganisms can take up substrates [4]. This is because the size of the hydrocarbon droplets is smaller than the cells.

A biosurfactant is an emulsifier that acts to lower the surface tension of a solution. Extracellular vesicles and intracellular organelles are both possible [5]. There are numerous publications on bacterial biosurfactants, but their chemical makeup determines the scope of their activity [2]. Mono- and di-rhamnolipids of the rhamnolipid type were found to be produced

by a strain of *Pseudomonas aeruginosa* [6]. There is a substantial association between the type of surfactant and the hydrocarbon that is degraded, as demonstrated by the fact that rhamnolipid and its generating microbes selectively destroyed hexadecane. Phenanthrene breakdown by different chemical surfactants [2] has been the subject of various research.

Another study showed that adding biosurfactant trehalose-5,50–dicorynomycolates to the artificial surfactant FinasolOSR-5 improved its oil breakdown capacity [7]. Bacteria that made glycolipids were also effective in breaking down polycyclic aromatic hydrocarbons (PAHs). The biodegradation of 2, 4-dichlorophenolindophenol (2, 4-DCPIP) was sped up by the addition of surface-active glycolipids to the hydrocarbon sites [8]. Soil-contaminated locations saw nearly full removal of PAHs in less than a month when glycolipids were present [8]. Biofilm is a bacterial biosurfactant that interacts with an interface to change the wettability and other properties of the surface. After 28 days of incubation, the marine bacteria *Pseudomonas aeruginosa*, which was isolated from oil-polluted sea water, was able to degrade hexadecane, octadecane, heptadecane, and nonadecane [2, 3]. The formation of a biosurfactant demonstrates the bacteria's capacity for destruction. *Pseudomonas aeruginosa* was shown to be capable of degrading a variety of hydrocarbons, including 2-methylnaphthalene, tetradecane, and pure [9].

Despite recent advancements in biosurfactant science, it has remained difficult to obtain efficient biosurfactant-producing microbial strains. The existence of biosurfactant-producing bacteria and fungi in a variety of settings has been described in previous research [5, 9, 14, 15, 22]. All of the described species have been tested in the lab, however, the varying success rates due to biosurfactant generation have rendered much of the reported success more theoretical. There is a significant propensity for the growth of biosurfactant generating strain in hydrocarbon impacted soil since the emulsification process is a mechanism of hydrocarbon biodegradation. In this context, the purpose of this paper was to isolate and screen biosurfactant-producing bacteria from hydrocarbon-contaminated soil in the hopes of discovering a powerful and quick biosurfactant-producing strain.

MATERIALS AND METHODS

Sampling

The soil sampling sites were shown in **Fig. 1** where sampling sites (hydrocarbon contaminated soil) were presented by A, B, C and D on the map along the road from Kabuga Bayero University Kano old site to Bayero University Kano new site campus (coordinates: 12° 0' 0.0000" N and 8° 31' 0.0012" E). Soil samples collected from D sampling site served as a control sample. The soil samples were collected from surface area (0-10) cm. The samples from each site were bulked and about 200g of each was properly labelled and transported immediately to the Postgraduate Microbiology Laboratory Bayero University Kano, Nigeria.

Bacteriological Analysis

Enumeration of Bacterial Loads in the Soil Samples

To prepare a stock solution for serial dilution, one gram of soil was added to nine milliliters of distilled water, and the mixture was thoroughly shaken before one milliliter was transferred to a test tube containing nine milliliters of sterile distilled water.

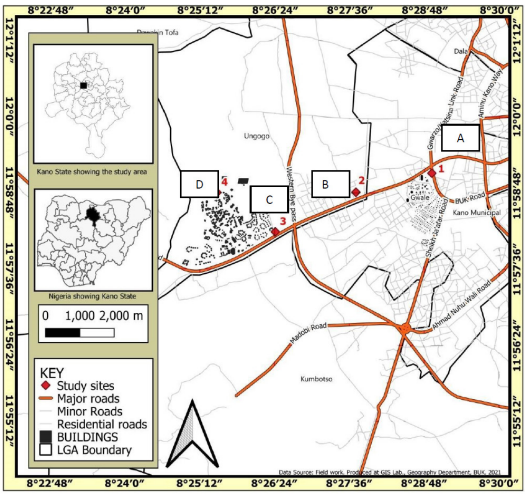

Fig. 1. Map of sampling area (A, B, C and D).

Aseptically, 0.1 ml of the dilution 105 suspensions was plated onto a Nutrient Agar (NA) plate, and the plate was incubated at 300C for 24 hours. Counts were multiplied by the final dilution to get the results, which were then expressed as cfu/g (colony forming units per gram) [10].

Isolation and Characterization of Bacterial Isolates

Distinct colonies obtained from the previous culture plates were subcultured on freshly sterilized nutrient agar to obtained pure bacterial isolates. The pure isolates were stored for characterization and further analysis.

Gram's Staining and Microscopy

Gram staining was performed according to Harley and Prescott's [10] instructions. The bacterial isolate was smeared using a drop of water on a clean, grease-free glass slide. As soon as the smudge had dried, it was touched to a flame. Smears were fixed, then stained with crystal violet or another primary dye for one minute, and finally rinsed in water. After one minute, the slide was covered with Lugol's iodine and washed. The stain was quickly removed with ethanol and washed off with water. Then, safranin was applied, left on for 30 seconds, and washed off with water. The slide's reverse side was dusted with cotton wool and left to dry naturally. Slides were analyzed with a microscope equipped with a 100x oil immersion objective lens. Gram-positive bacteria are blue or purple, and Gram-negative bacteria are red or pink.

Spore Staining

A smear was made on a slide and heat fixed. Malachite green (5%) solution was applied and heated until steam rises and allowed to cool and washed gently with cold water. The smear was counterstained with 0.5% safranin for 30 seconds and washed with water. The slide was blot dried and was examined under an oil immersion objective lens for the presence of spores. Spores stained green while vegetative cells stained red [11].

Catalase Test

A drop of 3% (v/v) H_2O_2 was placed on a glass slide onto which bacterial colonies were added. Presence of catalase was observed by the formation of oxygen bubbles [10].

Oxidase Test
An oxidase reagent (1% Tetramethylparaphenylene diamine dihydrochloride) was placed on the Whatman filter paper and a bacterial colony was smeared on the paper. The presence of the enzyme oxidase was observed by the appearance of a purple colour [10].

Triple Sugar Iron Test
Triple sugar iron slants were inoculated with the isolates using a sterile transfer needle. Using the needle, the butt was stabbed then the needle was withdrawn, and the surface was streaked. The inoculated slants were incubated at 37ºC for 24 hours after which they were examined for gas production, hydrogen sulphide production, glucose, lactose and sucrose fermentation [11].

Urease Production Test
Slants of urea medium in universal bottles were inoculated with a loopful of the isolates by streaking. These were incubated at 37ºC for 4 days and examined daily. Change in colouration from pink to red indicated urease positive [11].

Methyl Red Reaction Test
In a prepared glucose phosphate medium in a test tube, a loopful of the isolates was inoculated and incubated at 37ºC for 4 days. To the four-day-old culture, drops of methyl red solution were added. They were shaken and examined. The appearance of red colour on the surface of the reagent layer showed a positive methyl red reaction [11].

Voges-Proskauer Test
To the culture above, 0.6ml 5% α- naphthol solution was added and shaken. The test tubes were sloped and examined after 15 minutes. A red colouration indicated a positive VP reaction [11].

Indole Production Test
A loopful of the isolate was inoculated in a sterile nutrient broth. Incubation was done at 370C for 48 hours. After incubation, 0.5ml Kovac's reagent was added and shaken. This was examined after one minute. Red colouration in the reagent layer indicated indole production [11].

Citrate Utilization Test
To a sterile Simon's citrate medium, a loopful of the 24-hour old isolate was inoculated aseptically and incubated at 370C for 24 hours. The medium was examined daily for turbidity for 3 days. Turbidity indicated citrate utilization [11].

Screening of the Isolates for the Production of Biosurfactant
The isolates were screened in the mineral salt medium for biosurfactant production using the following screening tests.

Hemolytic Activity Analysis
Isolates were screened on blood agar plates containing 2% (v/v) sheep blood and incubated at 37°C for 48h. Hemolytic activity was detected by the presence of a clear zone around bacterial colonies [12].

The Collapse of a Drop
On an oil-coated solid surface, droplets of the cell-free supernatant were placed. 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 a surfactant, it spreads or even collapses [13].

Oil Displacement Assay
Ten (10) µL of crude oil was added to the surface of 40 ml of distilled water in a Petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant was gently placed in the centre of the oil layer. If a biosurfactant is present in the supernatant, the oil is displaced, and a clearing zone is formed [14].

Bacteriological Analysis
The enumeration of viable aerobic heterotrophic bacterial counts was shown in **Table 1**. The highest mean count of 8.4×10^6 cfu/g was observed at Site C while the lowest bacterial count was recorded 4.1×10^6 cfu/g at Site B. The results of morphological and biochemical characteristics of the isolates were shown in **Table 2**. A total of eighteen (18) bacterial isolates were identified from the soil samples of hydrocarbon-contaminated soil. And eight bacterial genera made the eighteen identified isolates that have different frequencies of occurrences each (**Table 2**).

The eight bacterial genera are *Pseudomonas aeruginosa, Enterobacter cloacae, Pentoeaagglomerans, Pseudomonas* spp., *Bacillus subtilis, Enterobacter alvei, Bacillus* sp. and *Klebsiella* spp. (**Table 3**). *Bacillus subtilis* had the highest percentage frequency of occurrence (5%) and it was found to be the most predominant specie among the isolates isolated from the hydrocarbon-contaminated soil. *Enterobacter alvei* and *Bacillus* sp. had the least percentage frequency of occurrence (1%) of the eight genera identified (**Table 3**).

Table 1. Mean aerobic heterotrophic bacterial counts from the sampling sites of hydrocarbon contaminated soils.

Sampling Site	Mean Bactierial Count (cfu/g) ±S.D
A	$6.5\times10^6\pm1.83$
B	$4.1\times10^6\pm0.21$
C	$8.4\times10^6\pm0.99$
D (Control Site)	$1.10\times10^7\pm0.03$

Table 2. Morphological and biochemical characteristics of the bacterial isolates.

Code	Shape	Spo	Gra	Cat	Oxd	MR`	Ure	Ind	Cit	Glu	Lac	VP	Organism
A1	Rod	+	+	+	-	-	-	-	+	+	-	+	*Bacillus* sp.
A2	Rod	+	+	+	+	-	-	-	+	+	+	+	*Bacillus subtilis*
A4	Rod	-	-	+	-	-	-	-	+	-	-	-	*Pseudomonas* sp.
A5	Rod	+	+	+	+	-	-	-	+	+	+	+	*Bacillus subtilis*
B2	Rod	-	-	+	+	-	-	-	+	-	-	-	*Pseudomonas aeruginosa*
B3	Rod	-	-	+	-	-	-	-	+	+	-	+	*Enterobacter cloacae*
B4	Rod	-	-	+	-	+	-	-	-	-	+	+	*Pentoeaagglomerans*
B5	Rod	-	-	+	-	-	-	-	+	+	-	+	*Enterobacter cloacae*
B6	Rod	-	-	+	-	+	-	-	-	-	+	+	*Pentoeaagglomerans*
C2	Rod	+	+	+	+	-	-	-	+	+	+	+	*Bacillus subtilis*
C3	Rod	+	+	+	+	-	-	-	+	+	+	+	*Bacillus subtilis*
C4	Rod	-	-	+	-	-	+	-	+	+	-	+	*Klebsiella* sp.
C5	Rod	-	-	+	-	-	+	-	+	+	-	+	*Klebsiella* sp.
C6	Rod	+	+	+	+	-	-	-	+	+	+	+	*Bacillus subtilis*
D3	Rod	-	-	+	-	-	-	-	+	-	-	-	*Pseudomonas* sp.
D4	Rod	-	-	+	+	-	-	-	+	-	-	-	*Pseudomonas aeruginosa*
D5	Rod	-	-	+	-	+	-	-	+	+	-	+	*Enterobacter alvei*
D6	Rod	-	-	+	+	-	-	-	+	-	-	-	*Pseudomonas aeruginosa*

Key: Spo-spore; Gra-Gram Reaction, Cat-Catalase, Oxd-Oxidase, MR-Methyl Red Reaction, Ure-Urease, Ind-Indole, Cit-Citrase, Glu-glucose, Lac-Lactose, VP-Voges-Proskauer test

Table 3. Frequency of occurrence of the identified bacterial isolates.

S/No.	Identified Isolates	Frequency of Occurrence	Percentage Frequency of Occurrence (%)
1	*Pseudomonas aeruginosa*	3	17
2	*Enterobacter cloacae*	2	11
3	*Pantoea agglomerans*	2	11
4	*Pseudomonas* spp.	2	11
5	*Bacillus subtilis*	5	27
6	*Enterobacter alvei*	1	6
7	*Bacillus* sp.	1	6
8	*Klebsiella* spp.	2	11

Screening of Bacterial Isolates for Biosurfactant Production
Screening of the isolates for the production of biosurfactant was carried out based on blood haemolysis, oil drop collapse, oil displacement and emulsification tests. The eight identified genera of bacterial isolates (A5, B5, B6, A4, C3, D5, A1 and C5) as *Pseudomonas aeruginosa, Enterobacter cloacae, Pentoeaagglomerans, Pseudomonas* sp., *Bacillus subtilis, Enterobacter alvei, Bacillus* sp. and *Klebsiella* sp. respectively were first subjected to blood haemolysis test and all the eight isolates were β-haemolytic positive and the result was presented in the **Table 4**.

The eight species were also subjected to oil drop collapse, and they were all positive as shown in **Fig. 2**. Plate I shows the image of the negative (control using water) and positive (crude biosurfactant produced by *Pseudomonas* sp.) of oil drop collapse test. Oil displacement test was carried out for the eight species and were all positive (**Fig. 3**). Plate II shows the image of a negative (control using water) and positive (crude biosurfactant produced by *Pseudomonas* sp.) of oil displacement test.

Table 4. Result of the blood haemolysis test.

Isolate Code	Isolate	Inference
A5	*Pseudomonas aeruginosa*	Positive
B5	*Enterobacter cloacae*	Positive
B6	*Pentoeaagglomerans*	Positive
A4	*Pseudomonas* sp.	Positive
C3	*Bacillus subtilis*	Positive
D5	*Enterobacter alvei*	Positive
A1	*Bacillus* sp.	Positive
C5	*Klebsiella*sp.	Positive

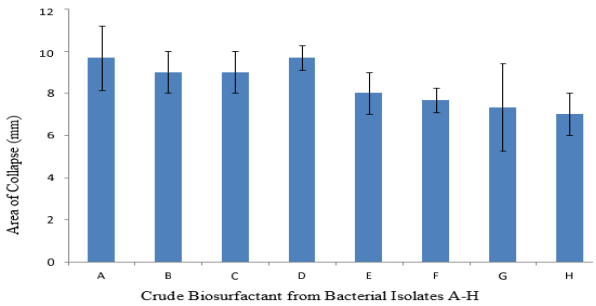

Fig. 2. Result of oil drop collapse test for the bacterial isolates (A-H). *Pseudomonas* spp., *Bacillus subtilis, Enterobacter alvei, Enterobacter alvei, Bacillus* sp. and *Klebsiella* spp. respectively. Key: The letters A, B, C, D, E, F, G and H stand for *Pseudomonas aeruginosa, Enterobacter cloacae, Pantoea agglomerans, Pseudomonas* spp., *Bacillus subtilis, Enterobacter alvei, Enterobacter alvei, Bacillus* sp. and *Klebsiella* spp. respectively.

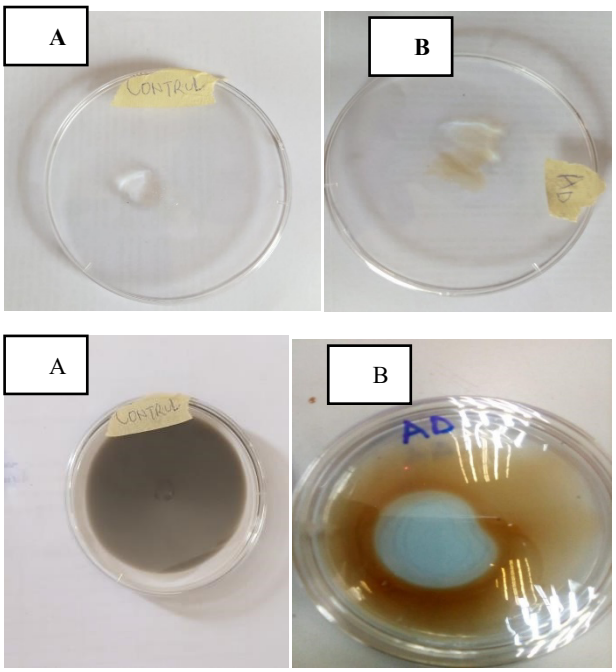

Fig. 3. Result of Oil Displacement Test of the Bacterial Isolates (A-H). Plate I: An image of oil drop collapse results: (A) A negative control of oil drop collapse test produced by crude biosurfactant of the bacterial isolate (B) A positive control of oil drop collapse test produced by crude biosurfactant of the bacterial isolate (upper plates). Plate II: An image of oil displacement test result: (A) A negative control test result of oil displacement test produced by crude biosurfactant of the bacterial isolate (B) A positive control test result of oil displacement test produced by crude biosurfactant of the bacterial isolate (lower plates).

DISCUSSION

In this study, the ability of bacterial species isolated from hydrocarbon-contaminated soil to produce biosurfactants was assessed. The total heterotrophic bacterial count of the soil samples ranged from 1.10 to 8.4 $\times 10^6$ cfu/g (**Table 1**). This indicated a high bacterial density in the sampled soils despite being contaminated with petroleum hydrocarbons. The result is similar to that of Ndubuisi-Nnaji [15], who analyzed soil samples of an automobile workshop in Uyo Metropolis, Nigeria. The findings of Akeredolu and Akinnibosun [16] have also reported a high bacterial load in soil contaminated with petroleum products in Benin City, Nigeria.

This result is further supported by the work of Hazim and Al-Ani [17] who reported high soil bacterial counts despite the toxicity of the hydrocarbon contaminants. In a study by Eze et al. [18] involving microbiological and physicochemical characteristics of soil contaminated with used petroleum products in Umuahia, Abia State Nigeria; high bacterial populations have also been reported. Results of this study showed that eight different bacterial species were isolated and identified. *Bacillus subtilis* were the predominant organism with a 27% occurrence rate respectively. These isolates are among the frequently reported bacteria isolated from hydrocarbon-contaminated soil.

The occurrence of *Bacillus* spp. in this study corresponds with that of Ndubuisi-Nnaji [15] and Eric et al. [19] who reported the identification of *Bacillus* spp. from oil polluted soil samples. The presence of *Bacillus* in soil could be a result of its ability to survive unfavorable environmental conditions because of its ability to form spores. The work of Akeredolu and Akinnibosun [16] revealed the presence of *Bacillus subtilis* in soil contaminated with petroleum products. Al-Dhabaan [20] also reported the isolation and identification of *Bacillus subtilis* and other *Bacillus* spp from oil-polluted soil in Dhahran Saudi Arabia. Recently, Saidu et al. [21] also reported the presence of *Bacillus* spp. in hydrocarbon-contaminated soil in the mechanic village in Dutse, Jigawa State, Nigeria. Adamu et al. [22] have reported the occurrence of *Bacillus* spp. and their biosurfactant production ability in harsh environments.

Pseudomonas aeruginosa isolated in this study made up about 17% of the identified bacteria. *Pseudomonas* spp. are widely distributed in soil contaminated with hydrocarbons due to their ability to degrade petroleum hydrocarbons within a short period. In a study that assessed soil contaminated with petroleum products in Benin City, Nigeria; the occurrence of *Pseudomonas aeruginosa* has been reported [16]. Findings in this study are also supported by the work of Al-Dhabaan [20] and Gamez et al. [23] both of which identified *Pseudomonas aeruginosa* from oil-contaminated soil. *Pseudomonas aeruginosa* was also isolated from oily polluted soil samples [18] and petroleum-contaminated soil in Suame, Ghana [19].

Enterobacter spp. was identified among the bacterial isolates in the present study. Despite being a member of the Enterobacteriaceae, this study identified *Enterobacter cloacae* and *Enterobacter alvei* in the hydrocarbon-contaminated soil. Their occurrence is not without precedence as Jemil et al. [24], isolated and identified Enterobacter cloacae from soil contaminated by natural gases. Similarly, *Klebsiella* spp. were also isolated and identified albeit at a lower rate. Our findings are supported by the work of Hazim and Al-Ani [17], who isolated *Klebsiella* spp. together with other bacterial species from soil contaminated with petroleum products. It is also in conformity with the findings of Eric et al. [19], who isolated *Klebsiella* spp. in soil contaminated with used petroleum products in Umuahia, Abia State, Nigeria. In addition, the present study identified *Pantoea agglomerans* another member of the Enterobacteriaceae family. And its presence is in agreement with the finding of Bahobail et al. [25], who show multiple degradation capabilities of *Pantoea agglomerans* isolated from petroleum hydrocarbon polluted soil. This finding is also in conformity with the findings of Gonzalez et al. [26], who isolated *Pantoea agglomerans* from producer surfactant pasture rhizosphere in Tanzania.

In this study, the bacterial species were screened for the ability to produce biosurfactants. All the isolates screened were positive for blood haemolytic activity. The organisms produced transparent clear zones on blood agar plates which serve as a sign of biosurfactant production ability. This technique has been used by various authors for the screening of soil bacteria capable of producing biosurfactants [12,27,14,23,28]. Mulligan [29], suggested the use of the blood agar lysis method as a preliminary screening method for biosurfactant production.

Confirmatory detection of biosurfactants produced by the bacterial isolates was also made possible through the use of techniques including oil spreading test and drop collapse assay. Many authors have advocated the use of these confirmatory techniques for screening microorganisms with the potential to produce biosurfactants [12,14,30]. The result of oil drop collapse and oil displacement test in this study were all positive in their individualistic response. This agreed with the findings of Joshi et al. [31] and Ibrahim et al. [14] that made a similar observation. The work of Ibrahim et al. [28] using biosurfactants produced by *Rhodococcus erythropolis* AQ5-07 isolated from Antarctica with waste canola as substrate also demonstrated the ability of biosurfactants to cause drop collapse and oil displacement at the water-oil interface.

CONCLUSION

Bacterial species have the different capacities in degrading petroleum hydrocarbons and secretion surface-active molecules that facilitate the rate of hydrocarbon degradation. Contaminated soil is one of the major reservoirs of biosurfactant producers and hydrocarbon degraders. The soil samples used in this study harbor a substantial bacterial population among which *Pseudomonas aeruginosa, Enterobacter cloacae, Pantoea agglomerans, Pseudomonas* sp., *Bacillus subtilis, Enterobacter alvei, Bacillus* sp., *Klebsiella* sp. and *Bacillus subtilis* made part of the bacterial community in the soil. All the isolates were hemolytic on blood agar, and their crude surfactants were able to cause the collapse of oil drop and dispersion of oil molecule; thus, signifying their ability to produce biosurfactants.

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