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

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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–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 gap in 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. A drop of 3% (v/v) H2O2 was placed on a glass slide onto which bacterial colonies were added. Presence of catalase was observed by the formation of oxygen bubbles [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.

**Catalase Test**

A drop of 3% (v/v) H2O2 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 37°C 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 the isolates by streaking. These were incubated at 37°C for 4 days and examined daily. Change in colour from pink to red indicated urease positive [11].

Methyl Red Reaction Test
To the four-day-old culture, drops of methyl red solution were added. They were shaken and examined. The appearance of red colour 

Voges-Proskauer Test
To the culture above, 0.6ml 5% α- naphthol solution was added. 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 isolates 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 3).

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

<table>
<thead>
<tr>
<th>Sampling Site</th>
<th>Mean Bacterial Count (cfu/g) ± S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.5×10^5 ± 1.83</td>
</tr>
<tr>
<td>B</td>
<td>4.1×10^6 ± 0.21</td>
</tr>
<tr>
<td>C</td>
<td>8.4×10^6 ± 0.99</td>
</tr>
<tr>
<td>D (Control Site)</td>
<td>1.10×10^3 ± 0.03</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Code</th>
<th>Shape</th>
<th>Spore</th>
<th>Gram</th>
<th>Oxid</th>
<th>MR</th>
<th>Ure</th>
<th>Ind</th>
<th>Cit</th>
<th>Glu</th>
<th>Lac</th>
<th>VP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus sp.</td>
<td>A1</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>A2</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>A4</td>
<td>Rod</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B2</td>
<td>A5</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>B3</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>B4</td>
<td>Rod</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pentoeaagglomerans</td>
<td>B5</td>
<td>Rod</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>B6</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C2</td>
<td>C3</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>C4</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>C5</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>C6</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Klebsiella sp.</td>
<td>D3</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C7</td>
<td>D4</td>
<td>Rod</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>D5</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>D6</td>
<td>Rod</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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

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

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, Pantoeaagglomerans, Pseudomonas sp., Bacillus subtilis, Enterobacter alvei, Bacillus sp. and Klebsiella sp. respectively were first subjected to blood haemolysis 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.

<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Isolate</th>
<th>Inference</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5</td>
<td>Pseudomonas aeruginosa</td>
<td>Positive</td>
<td>3</td>
</tr>
<tr>
<td>B5</td>
<td>Enterobacter cloacae</td>
<td>Positive</td>
<td>2</td>
</tr>
<tr>
<td>B6</td>
<td>Pantoeaagglomerans</td>
<td>Positive</td>
<td>2</td>
</tr>
<tr>
<td>A4</td>
<td>Pseudomonas sp.</td>
<td>Positive</td>
<td>2</td>
</tr>
<tr>
<td>C3</td>
<td>Bacillus subtilis</td>
<td>Positive</td>
<td>5</td>
</tr>
<tr>
<td>D5</td>
<td>Enterobacter alvei</td>
<td>Positive</td>
<td>1</td>
</tr>
<tr>
<td>A1</td>
<td>Bacillus sp.</td>
<td>Positive</td>
<td>1</td>
</tr>
<tr>
<td>C5</td>
<td>Klebsiella sp.</td>
<td>Positive</td>
<td>2</td>
</tr>
</tbody>
</table>

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 ×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 aerogenes 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 Bahabali 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 bacteria capable of producing biosurfactants [12,17,14,23,28]. 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.

REFERENCES


