Research Article

Biosurfactant producing abilities of some bacteria isolated from bitumen contaminated soils

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Abstract

Bio-surfactants produced by bacteria are surface-active compounds required in the degradation of hydrocarbons. They are complex groups of surface-active molecules produced by microorganisms that stick to the specific cell covering or are secreted extracellularly in the growth medium. This study is aimed at determining the bio-surfactant-producing abilities of bacteria that are isolated from bitumen-polluted soil.

Isolation of bacteria from bitumen-polluted soil samples was carried out using standard methods. The bio-surfactant producing ability of the isolated bacteria was investigated by several assays, including drop collapse test, tilting glass slide test, emulsification index, and foaming activity. Characterization of the isolated bacteria was carried out using 16S rRNA, and the extracted genome from each isolate was sequenced, showing the gene annotation of the isolates. Crystal violet biofilm analysis was carried out to determine the biofilm-producing ability of the isolated bacteria. The extracted bio-surfactant was characterized with Fourier Transform Infrared Spectroscopy (FTIR) spectra and Scanning Electron Microscopy (SEM). Whole-genome sequencing analysis was done on two best Bio-surfactant-producing bacteria.

Characterization of the bacteria isolates by 16S rRNA showed their homology in the phylogenetic tree in which Lysinibacillus sphaericus belong to the clade of Lysinibacillus and Bacillus. The biofilm analysis revealed that all the isolates were biofilm producers, with one high producer, three moderate producers and one weak producer. The SEM spectra revealed the structure of the product produced, and FTIR confirmed their chemical nature, indicating rhamnolipids.

The bio-surfactant results indicated that two isolates, Lysinibacillus sphaericus and Pseudomonas sp. were the best Bio-surfactant-producing isolates.

Introduction

Bio-remediation points to the application of microbes to debase contaminants that pose a human and environmental threat [1]. Bio-remediation methods typically require many diverse microorganisms operating in likeness or progression to achieve the bio-remediation process. In situ and ex situ (extraction and treating in different place) remediation methods are used. The versatility of microorganisms to degrade a wide collection of pollutants makes bio-remediation a technology that can be used in various soil conditions [1].
choice to chemically-synthesized surfactant for environmental clean-up. As microbes typically produce bio-surfactants, researchers have actively been studying them for over a decade [2]. According to Habe and Omori [3], the natural remediation of Poly Aromatic Hydrocarbon-polluted soil should be cost-effective and time-efficient. The biological technique has several benefits which include: complete degeneration of the contaminants, cheaper treatment cost, increased safety, and minor soil disruption.

Till the Critical Micelle Concentration (CMC) is attained, the concentration of surface-active chemicals determines the activity of bio-surfactants [4]. The emulsification of the hydrocarbon in the medium is frequently observed when microorganisms grow on it. This has been attributed to the generation of surface-active chemicals in most situations [4].

The biochemical process of bioremediation has been adopted to reduce environmental pollution and economic loss 2011). It represents the primary mechanism by which petroleum and other hydrocarbon pollutants can be removed from the environment. It is non-invasive and cheaper than other remediation technologies, and a complex process that depends on nature and the amount of hydrocarbon present.

In recent times, Pseudomonas aeruginosa is one of the well-known bio-surfactant producing microbes, and they are ubiquitous. Bio-surfactants are used to improve petroleum hydrocarbon degradation efficiency because of advantages such as wide application range, low toxicity, good environmental capability, strong foaming ability and high environmental tolerance [5]. The significance of this research work is to obtain different strains of microorganisms that are capable of bioremediation of biodegraded hydrocarbons.

Bitumen-contaminated soils were selected for this study because it has a larger rate and it needs more action from the bacteria to degrade its component. Bitumen takes about 40 days for total biodegradation depending on the strain of bacteria used. Whereas, petroleum, kerosene and other crude oil fractions take a shorter period. And since the study requires a long time to be carried out effectively, bitumen-contaminated soils were chosen.

New strains of bacteria that have not been recorded before were obtained and recorded in this review.

Methods

Preparation of Minimal Salt Medium (MSM)

Minimal salt Medium (MSM) is a medium with the most negligible but necessary nutrients for the growth of bacteria in which carbon source is absent. Bitumen serves as the carbon source during the inoculation of these isolates, which serves as a selective medium for their growth [6]. The composition of MSM was as follows: 0.2g of MgSO₄, 0.2g of CaCl², 1.0g of KH₃P, 1.0g of K₂HP, 1.0g of NH₄N, 0.05g of FeCl₂ in 1 liter of distilled water and shaken briskly while 2% agar was added for solidification. This was then placed on a hot plate for proper homogenization before autoclaving at 121°C for 15mins [6].

Isolation of bitumen degrading bacteria

Ten grams of the bitumen contaminated soil samples were introduced into 100ml of a minimal salt medium, and bitumen was introduced as a carbon source. This was inoculated at 37°C for 48 hours. After 48 hours, 50μl was introduced into the already prepared nutrient agar plates separately and spread, then incubated for 24 hours; distinct colonies were picked and streaked to get a pure colony [7].

DNA isolation

The isolated bacteria were cultured by streaking on plates containing MSM and was incubated for two days. Visible colonies were produced on the plates. A single colony was inoculated in 1-3 ml of the MSM and allowed to grow in a shaker bath up to the mid-log phase. The cells were collected by centrifugation of 1.5 ml of the culture for 2 min at 8000 rpm in the micro-centrifuge. If the growth of the culture was poor after centrifugation, a second 1.5 ml aliquot of the culture could be centrifuged in the same tube.

The cell was suspended in pellet 500 μl of TE buffer and 30 μl of Sodium Dodecyl Sulfate (SDS) together with 5 μl of proteinase K. The tube was mixed by inversion and incubated at 37°C for 1 hour to allow cell lysis. 100 μl of 5M NaCl was added and vortexed for few seconds. 80 μl of CTAB was added, mixed and heated for 10 min at 65°C. An equal volume (about 800 μl) of chloroform: isoamyl alcohol was added, vortexed for a few seconds and centrifuged for 5 min at 11000 rpm. The aqueous upper phase was collected in a new tube, and an equal volume of phenol:chloroform: isoamyl alcohol was mixed by vortex and centrifuged for 5 min at 11000 rpm. The upper aqueous phase was recovered in a fresh tube, two μl of RNAs was added and incubated for 30 min at 37°C. An equal volume of isopropanol was added, DNA precipitated for 5 min at room temperature and centrifuged for 5 min at 11000 rpm. The supernatant was discarded, and the pellet was washed with 70% ethanol and centrifuged again for 5 min at 11000 rpm. The pellet was dried under vacuum and solubilized in 10-20 μl of sterile TE buffer solution.

Molecular identification of bacteria isolates

The genetic identity of bacterial isolates was determined by isolating and amplifying DNA sequence data from 16S rRNA. The 16S rRNA sequence has been established as the primary bacterial barcode (Schoch, et al. 2012).

Phylogenetic characterization

The amplified products were sequenced in line with the Big Dye Terminator sequencing procedure in an Abl 377 programmed DNA Sequencer (Applied Biosystems, USA) 16S rRNA sequence reads obtained after sequencing were assembled into contig with Geneious alignment tool. Each contig sequence was a blast on NCBI (National Center for Biotechnology Information) database for identification; some other sequences were searched and downloaded based on each query sample genus name and query covered.
Alignment of all sequences, including the query sequence, was conducted with the Muscle Alignment tool. A phylogenetic tree was constructed using Neighbor-Joining and the maximum likelihood base on genetic distance model with a bootstrap value of 1000 and 100 numbers of replicates to indicate the evolutionary process analyzed over time. Geneious tree builder version 9.0.5 was used to edit and to determine the evolutionary relatedness and diversities.

Fourier transform infrared spectroscopy

To understand the overall chemical nature of the extracted bio-surfactant, Fourier transforms infrared (FTIR) spectroscopy was employed. The technique helps to explore the functional groups and the chemical bonds present in the crude extract [8]. The analysis was done using Shimadzu FTIR Spectrophotometer (Model 8400S). Samples were prepared by homogeneous dispersal of 1 mg of the bio-surfactant sample in potassium bromide pellets (Merck, USA). IR absorption spectra were obtained using a built-in plotter. IR spectra were collected over the range of 450–4500 cm$^{-1}$ with a resolution of 4 cm$^{-1}$. The spectral data were the average of 50 scans over the entire range covered by the instrument. The spectrum was studied to interpret the chemical nature of the bio-surfactant fraction [8].

Screening of bio-surfactant producing isolates

Bacteria were cultivated aerobically in 500 ml Erlenmeyer flask with 100 ml of mineral salt medium containing (gl$^{-1}$) 1.0 K$_2$HPO$_4$, 0.2 MgSO$_4$.7H$_2$O, 0.05 FeSO$_4$.7H$_2$O, 0.1 CaCl$_2$.2H$_2$O, 0.001 Na$_2$MoO$_4$.2H$_2$O, 30 Sodium Chloride and crude oil (1.0%, w/v). Flasks holding purified mineral salt medium were introduced with a loopful bacterial culture grown in crude oil containing nutrient agar plates [9]. The culture flasks were shaken for seven days at 200 rpm and 30°C. After seven days of incubation, culture broth from each flask was centrifuged at 6000 rpm and 4°C for 15 minutes and the supernatant was filtered through 0.45μm pore size filter paper (Millipore). This cell-free culture broth was used for drop collapse assay, oil spreading assay, emulsification assay and surface tension measurement [10]. All the screening experiments were carried out in triplicates (except otherwise stated).

Drop collapse test

Two liters of crude oil were poured into the wells of a polystyrene 96–well microplate cap and allowed to dry for 24 hours at 22°C. The center of the oil–coated well was used for drop collapse assay, oil spreading assay, emulsification assay and surface tension measurement [10]. The experiments were carried out with three independent replicates.

Emulsification index

In a 30 ml screw–capped test tube, 5 ml of 50 mM Tris buffer was added to 1 ml of cell-free supernatant (pH 8.0). The ability of crude oil to emulsify was investigated [12]. Both layers received 5 mg of crude oil, which was vortexed for 1 minute before the emulsion mixture was allowed to resolve for 20 minutes. The emulsified mixture’s optical density was measured at 610 nm [12]. The negative control was buffered, while the positive control was crude oil with Triton X–100.

\[
\text{Emulsification index} = \frac{\text{Emulsification layer height/total height}}{100}
\]

\[
\text{Emulsification Index} = \frac{\text{Emulsification layer height}}{\text{Total height}}
\]

Tilting glass slide test

In the tilting glass slide test which was developed by Persson and Molin [13], a single colony is picked up from the Bushnell Haas agar plate and transferred on the surface of a sterile glass slide near one of the edges. It is then mixed with a droplet of 1% saline. The slide is gradually tilted to the other side and was examined for the flow of a water droplet over its surface. Bio-surfactant production is implied if water flows over the surface.

Phenol sulfuric acid assay

The presence of carbohydrate groups in the bio-surfactant molecule was assayed using this standard method. A volume of 0.5 ml of culture supernatant was mixed with 0.5 ml of 5% phenol solution and 2.5 ml of sulfuric acid and incubated for 15 min before measuring absorbance at 490 nm [14].

Surface tension measurement

Bacterial strains that exhibited positive results for bio-surfactants production were then evaluated for surface tension reduction and stable emulsion formation. Strains were grown in MSM broth with crude oil 2% (v/v) and incubated at 200 rpm for seven days. For surface tension measurements, 5ml of broth supernatant was transferred to a glass tube submerged in a water bath at constant temperature (28°C). Surface tension was calculated by measuring the height reached by the liquid after freely ascending through a capillary tube. As a control, broth without inoculation was used. The experiments were carried out with three independent replicates [10].

Foaming activity

Foaming ability was determined by growing the cultures in 50 ml nutrient broth in a 250 ml Erlenmeyer flask. It was incubated at 30°C for 96 hours on a shaker incubator [15]. The 10 ml of culture was shaken vigorously for 2 min in a graduated cylinder, and the equation detected foaming activity.

\[
\text{Foaming} = \frac{\text{Height of foam}}{\text{Total height}} \times 100
\]

Bacterial adhesion to hydrocarbon assay

Cell hydrophobicity was measured by bacterial adherence to hydrocarbons according to a method described by Rosenberg, et al. 1980. The cell pellets were washed twice and suspended in a buffer salt solution (16.9 g/l K$_2$HPO$_4$ and 7.3g/l KH$_2$PO$_4$) and diluted using the same buffer solution to an optical density
(OD) of ~ 0.5 at 610 nm [10]. To the cell suspension (2 ml) in test tubes (10 ml volume with 10 x 100 mm dimension), 100 μl of crude oil was added and vortex-shaken for 3 min. After shaking, crude oil and aqueous phases were allowed to separate for 1 hour. OD of the aqueous phase was then measured at 610 nm in a spectrophotometer [10]. From the OD values, the percentage of cells attached to crude oil was calculated using the following formula.

\[
\text{% of bacterial cell adherence} = \left(1 - \left( \frac{\text{OD shaken with oil}}{\text{OD original}} \right) \right) \times 100
\]

Where: \(\text{OD shaken with oil}\) is the OD of the mixture containing cells and crude oil

\(\text{OD original}\) is the OD of the cell suspension in the buffer solution (before mixing with crude oil).

**Results**

**Contaminated soil sample**

The 16S rRNA (ribosomal RNA) gene sequencing of the isolates were identified as *Brucella intermedia* (6A1), *Pseudomonas aeruginosa* (8AA1), *Kocuria indica* (LB1P4), *Lysinibacillus sphaericus* (LD15A), *Pseudomonas sp.* (LC1PC5) with NCBI blast. The neighbor-joining analysis showed that isolate LD15A was close to the clade of *Lysinibacillus* and *Bacillus*; 8AA1 belonged to the clade of *Ochrobacterium*, LC1PC5 was close to the *Pseudomonas aeruginosa*, while LB1P4 belonged to the clade of *Kocuria* (Figure 1). The accession numbers of the five isolates as presented in Table 1 depict 6A1 as *Brucella intermedia* (MW677449), 8AA1 as *Pseudomonas aeruginosa* (MW677446), LB1P4 as *Kocuria indica* (MW677447), LC1PC5 as *Pseudomonas sp.* (MW677450), and LD15A as *Lysinibacillus sphaericus* (MW677448). Figure 2 shows the annotation of LC1PC5 (*Pseudomonas sp.*) RBS Kozak, T7 trans RBS and Casp-3, Thromb Tag and others were found in the DNA. They are a nucleic acid motif that functions as the protein translation initiator in most eukaryotic mRNA transcripts. Regarded as the optimum sequence for initiating translation in eukaryotes, the sequence is an integral aspect of protein regulation and overall cellular health and has implications in human disease.

Gene annotation was carried out to help in the identification

**Table 1: Molecular Characterization (16S rRNA) of the bacterial Isolates.**

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Isolate Identity</th>
<th>Ascension Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A1</td>
<td><em>Brucella intermedia</em></td>
<td>MW677449</td>
</tr>
<tr>
<td>8AA1</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>MW677446</td>
</tr>
<tr>
<td>LB1P4</td>
<td><em>Kocuria indica</em></td>
<td>MW677447</td>
</tr>
<tr>
<td>LC1PC5</td>
<td><em>Pseudomonas sp.</em></td>
<td>MW677450</td>
</tr>
<tr>
<td>LD15A</td>
<td><em>Lysinibacillus sphaericus</em></td>
<td>MW677448</td>
</tr>
</tbody>
</table>

**Figure 1: Phylogenetic Relationship of Isolated Bacteria from Bitumen-Contaminated Soil Sample.**

**Citation:** Temitope KK, Ayanfemi AA, Rebecca OK, Hannah AY, Lawrence EJ, et al. (2021) Biosurfactant producing abilities of some bacteria isolated from bitumen contaminated soils. Open J Environ Biol 6(1): 026-034. DOI: https://dx.doi.org/10.17352/ojeb.000023
Figure 2: Gene Annotation of *Pseudomonas* sp.

Figure 3: Gene Annotation of *Lysinibacillus sphaericus*.

Citation: Temitope KK, Ayanfemi AA, Rebecca OK, Hannah AY, Lawrence EJ, et al. (2021) Biosurfactant producing abilities of some bacteria isolated from bitumen contaminated soils. Open J Environ Biol 6(1): 026-034. DOI: https://dx.doi.org/10.17352/ojeb.000023
of gene location and coding regions. It also helps to give an insight on what these genes do in the body by establishing structural aspects and relating them to functions of different proteins.

Figure 3 shows the annotation of LD15A (Lysinibacillus sphaericus), RBS Kozak, T7-transl-en-RBS and Casp-3, Thromb Tag and others were found in the DNA. They are a nucleic acid motif that functions as the protein translation initiator in most eukaryotic mRNA transcripts. Regarding the optimum sequence for initiating translation in eukaryotes, the sequence is an integral aspect of protein regulation and overall cellular health.

Brucella intermedia was positive for the drop collapse test, negative for the tilting glass slide test and positive for the sulphuric acid test. Pseudomonas aeruginosa was negative for the drop collapse test and tilting glass slide test but positive for the sulphuric acid test. Kocuria indica was negative for the drop collapse test but positive for both the tilting glass slide test and sulphuric acid assay. Lysinibacillus sphaericus is positive for the three tests showing a better activity than other isolates. Pseudomonas sp. was negative only for the tilting glass slide test (Table 2).

Table 3 showed that Pseudomonas aeruginosa had the highest surface tension while Lysinibacillus sphaericus had a low foaming activity with a moderate surface tension of 42 cm.

Table 2: Drop Collapse, Tilting Glass Slide and Sulphuric Acid Tests of Isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Drop collapse</th>
<th>Tilting glass slide</th>
<th>Sulphuric Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella intermedia</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Kocuria indica</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Lysinibacillus sphaericus</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Consortium</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Table 3: Test on Foaming Activity and Surface Tension of Isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Foaming activity (cm)</th>
<th>Surface tension (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella intermedia</td>
<td>1.63±0.02 ab</td>
<td>40.00±1.00 a</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>1.20±0.10 ab</td>
<td>47.00±1.00 b</td>
</tr>
<tr>
<td>Kocuria indica</td>
<td>1.00±0.10 ab</td>
<td>45.00±1.00 b</td>
</tr>
<tr>
<td>Lysinibacillus sphaericus</td>
<td>0.33±0.06 a</td>
<td>42.00±2.00 a</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>1.60±0.10 ab</td>
<td>40.00±1.00 a</td>
</tr>
<tr>
<td>Consortium</td>
<td>2.50±0.10 b</td>
<td>40.67±1.53 a</td>
</tr>
</tbody>
</table>

Table 4: Biofilm Forming Ability of Isolates by Crystal Violet Biofilm Test.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Crystal violet Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kocuria indica</td>
<td>0.85±0.003 e</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.22±0.002 b</td>
</tr>
<tr>
<td>Lysinibacillus sphaericus</td>
<td>0.28±0.001 c</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>0.11±0.001 a</td>
</tr>
<tr>
<td>Brucella intermedia</td>
<td>0.29±0.002 d</td>
</tr>
<tr>
<td>Consortium</td>
<td>0.10±0.003 e</td>
</tr>
</tbody>
</table>

Kocuria indica showed a surface tension of 45 cm with foaming activity of 1 cm. Brucella intermedia and Pseudomonas sp. showed the lowest surface tension.

The Biofilm-producing ability of the isolates, as shown in Table 4, revealed a significant difference in their biofilm-producing ability. Kocuria indica produced the highest biofilm (0.85), while Pseudomonas sp. produced the least (0.11).

Table 5 showed the classification of bacteria isolates based on their biofilm-forming ability. Kocuria indica was a strong biofilm production ability having a yield greater than 4x OD<sub>600</sub> (0.412). Three isolates were moderate biofilm producers, and these include Pseudomonas aeruginosa, Lysinibacillus sphaericus and Brucella intermedia. Only Pseudomonas sp. was a weak producer.

The emulsiﬁcation index of the isolates, as shown in Figure 4, revealed a signiﬁcant difference in their ability to produce an emulsion. For Brucella intermedia, Pseudomonas aeruginosa, Kocuria indica, Lysinibacillus sphaericus, Pseudomonas sp., and Consortium, the Emulsiﬁcation Index obtained were 43.20%, 35.00%, 31.30%, 44.12%, 41.86% and 29.54%, respectively.

Figure 5 showed the percentage of cells bound to the hydrocarbon phase. For Brucella intermedia, the percentage of cells bound to the hydrocarbon phase was high on the 5th (62.2%) day, but its percentage dropped a little on day 10 (61.5%) and by 0.1% on day 15 (61.6%). Pseudomonas aeruginosa had a low percentage of cells (8.86%) bound to the hydrocarbon phase on the 5th day, equivalent to the increase in turbidity of the aqueous phase. On day 10, it increased to 9.8% and dropped to 9.3% on day 15, showing that the isolates Pseudomonas aeruginosa had a small number of bio-surfactants producing microbes. Kocuria indica had the highest percentage at day 5 (49.7%), then it dropped at day 10 to 49.2% and increased to 49.6% on day 15. Lysinibacillus sphaericus had its highest percentage on days 5 and 15, which is (64.8%). For Pseudomonas sp., the percentage of cell-bound to hydrocarbon assay increases gradually from day 5 to day 15 (70.1 to 70.4 to 70.6%). The Consortium had a constant percentage (66%) from day 5 to day 15.

The C-H bending band of Brucella intermedia was observed in the region 1435 cm<sup>-1</sup> with a peak height of 6.658 cm. The product also contains residual water vapor, observed in the region 1839 cm<sup>-1</sup> with a peak height of 7.6072 cm. The NH<sub>3</sub> amino acid group was observed at two regions; 3222 cm<sup>-1</sup> and 3276 cm<sup>-1</sup> at the peak heights of 1.2827 cm and 1.1747 cm, respectively (Figure 6). The H-O-H stretching vibration of a cluster of water molecules of crystallization of Pseudomonas aeruginosa was observed in the region 1636 cm<sup>-1</sup>. Residual water vapor was also
a constituent of the product, which was proved at 1804 cm\(^{-1}\) with a peak height of 5.9052 cm. C-O-C polysaccharides were observed at peak 1127 cm\(^{-1}\) with a height of 8.7747 cm. Benzene ring ortho-substituted and meta-substituted with condensed ring system were observed at the peak of 736 cm\(^{-1}\) and a peak height of 17.7787 cm (Figure 7).

The C=O Ester Fatty acid group of the consortium was proved from the band at 1742 cm\(^{-1}\). There is the presence of a double conjugate bond (C=C) with a peak at 2010 cm\(^{-1}\) and a peak height of 12.3985 cm. It contains ash in coal at the peak of 993 cm\(^{-1}\) and a peak height of 9.9490 cm. C-H of the aromatic ring was proved at 2990 cm\(^{-1}\) with a peak height of 8.9966 cm (Figure 8).

The C=O Ester Fatty acid group of \textit{Kocuria indica} was proved from the band at 1742 cm\(^{-1}\) with a peak height of 6.7274 cm. The C-H bending aliphatic band was also observed at peak 1434 cm\(^{-1}\) and a peak height of 6.7639 cm. Residual water vapor was also a constituent of the product, observed at the region 1827 cm\(^{-1}\) with a peak height of 7.0581 cm (Figure 9).

The C=O Ester Fatty acid group of \textit{Kocuria indica} was proved from the band at 1742 cm\(^{-1}\) with a peak height of 6.7274 cm. The C=H bending aliphatic band was also observed at peak 1434 cm\(^{-1}\) and a peak height of 6.7639 cm. Residual water vapor was also a constituent of the product, observed at the region 1827 cm\(^{-1}\) with a peak height of 7.0581 cm (Figure 9).

The stretching band of \textit{Pseudomonas} sp. was observed at 1432 cm\(^{-1}\) with a peak height of 11.7264 cm. Benzene ring ortho-substituted and meta-substituted with condensed ring system were observed at the peak of 732 cm\(^{-1}\) and a peak height of 19.3488 cm. The ash in coal was observed at the region 992 cm\(^{-1}\) with a peak height of 13.1187 cm (Figure 10).

The stretching band of \textit{Lysinibacillus sphaericus} was proved
were isolated from the bitumen-contaminated soil samples. The bacterial isolates obtained in his work belong to the genus *Bacillus*, *Lysinibacillus* *sphaericus*, *Kocuria indica*, *Lysinibacillus sphaericus*, *Lactobacillus sp.*. This may be due to the source of industries and high levels of production [18-25]. Rhamnose, produced by *Lysinibacillus sphaericus*, *Pseudomonas sp.*, *Brucella intermedia*, *Kocuria indica* and *Pseudomonas aeruginosa* exhibited high homology with *Lysinibacillus and Bacillus, Ochrabacterium, Ochrabacterium, Kocuria* and *Pseudomonas aeruginosa*, respectively, which correlate with the work of Saisa-ard, et al. (2014).

The Fourier Transform Infrared Spectroscopy (FTIR) spectra of the samples range from 500-4000cm⁻¹. The peak of LD15A (*Lysinibacillus sphaericus*) in the region of 1137cm⁻¹ indicates C–O–C stretching in the rhamnose. A similar result was reported by Thenmozhi, et al. (2011). Rhamnolipids produced by *Lysinibacillus sphaericus* were the most studied biosurfactants due to their potential applications in a wide variety of industries and high levels of production [18-25]. Rhamnose, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxy-decanoic acid, is the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa*. The findings of this study were in accordance with Rahman, et al. (2007), who reported bio-surfactant production by *P.aeruginosa* DS10-129 characterized by FTIR technique belonged to rhamnolipid type. Rhamnolipids produced from *Lysinibacillus sphaericus* were characterized, and its ability for dissolution of hydrophobic pesticides was evaluated. It produced 1.6g/L of an anionic bio-surfactant that reduced surface tension. The Bio-surfactant was found stable over a wide range of pH, temperature, salt concentration and was identified as rhamnolipids reported by Vivek, et al. (2019).

**Discussion**

A total of 5 different bacterial were isolated from the bitumen-contaminated soil samples. The 5 bacterial isolates were identified as *Brucella intermedia*, *Pseudomonas aeruginosa*, *Kocuria indica*, *Lysinibacillus sphaericus* and *Pseudomonas sp.*. This result is different from what Amao, et al. [16] reported. Three of the bacterial isolates obtained in his work belong to the genus *Bacillus*, one was *Lactobacillus planetarium*, while four others were *Klebsiella Pneumoniae*. This may be due to the source of samples used in the two studies.

Dans and Chandran (2011) described the drop collapse test according to which the degree of collapse of the culture supernatant describes the surfactant concentration. Of the six samples, two samples show no complete collapse, while for the four other samples, the drops turned flat. The tilted glass slide test, developed by Persson and Molin [13], was positive for three isolates.

**Isolate** *Lysinibacillus sphaericus* followed by *Brucella intermedia* exhibited the highest emulsification capacity; they gave 44.12±0.02 and 43.20±0.02 of E24%, respectively in this study which agrees with the work of Sidkey, et al. (2016). Dussan and Numpaque (2012) had reported that *Pseudomonas sp.* is a useful candidate for bio-surfactant production. Sarubbo, et al. (2016) concluded that the emulsification index (E24) provides a rapid and reliable measure of the quantity of Bio-surfactant. *Lysinibacillus sphaericus* had a low foaming activity which is less than 0.5, which correlates with the work of El-sheshtawy (2011). The percentage of cells bound to the hydrocarbon phase showed a characteristic feature of Bio-surfactant-producing microbes. *Pseudomonas sp.* showed the highest percentage, while *Pseudomonas aeruginosa* showed the lowest percentage.

*Brucella intermedia*, *Lysinibacillus sphaericus* and *Pseudomonas sp.* are the best known bacterial groups for biosurfactant-producing genera (Suwansukho, et al. 2008), and they were also found in our screening. The majority of the isolated strains belonged to the genus *Bacillus, Brucella* and *Pseudomonas*. These are frequently isolated from hydrocarbon–contaminated environments. Many strains belonging to these genera have been demonstrated to be efficient hydrocarbon degraders and bio-surfactant-producing bacteria [17]. The isolates *Lysinibacillus sphaericus*, *Pseudomonas sp.*, *Brucella intermedia*, *Kocuria indica* and *Pseudomonas aeruginosa* exhibited high homology with *Lysinibacillus and Bacillus, Ochrabacterium, Ochrabacterium, Kocuria* and *Pseudomonas aeruginosa*, respectively, which correlate with the work of Saisa-ard, et al. (2014).

The Fourier Transform Infrared Spectroscopy (FTIR) spectra of the samples range from 500-4000cm⁻¹. The peak of LD15A (*Lysinibacillus sphaericus*) in the region of 1137cm⁻¹ indicates C–O–C stretching in the rhamnose. A similar result was reported by Thenmozhi, et al. (2011). Rhamnolipids produced by *Lysinibacillus sphaericus* were the most studied biosurfactants due to their potential applications in a wide variety of industries and high levels of production [18-25]. Rhamnose, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxy-decanoic acid, is the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa*. The findings of this study were in accordance with Rahman, et al. (2007), who reported bio-surfactant production by *P.aeruginosa* DS10-129 characterized by FTIR technique belonged to rhamnolipid type. Rhamnolipids produced from *Lysinibacillus sphaericus* were characterized, and its ability for dissolution of hydrophobic pesticides was evaluated. It produced 1.6g/L of an anionic bio-surfactant that reduced surface tension. The Bio-surfactant was found stable over a wide range of pH, temperature, salt concentration and was identified as rhamnolipids reported by Vivek, et al. (2019).

**References**


