INTRODUCTION

Microorganisms produce a wide variety of extracellular and intracellular components which possesses beneficial characteristics applicable to medical, agricultural and industries fields, etc. Biosurfactants are such compounds produced under suitable conditions by bacteria, fungi, and yeast. They are called the surface active agents as it alters the surface tension of hydrophobic substrates thus increasing its bioavailability.[1] It consists of hydrophilic and hydrophobic moieties which provide them with the ability to accumulate between two immiscible fluids thereby lowering the surface and interfacial tensions.[2]

The growth of microorganisms in medium containing hydrocarbons is due to the production of surface-active molecules which emulsifies the hydrocarbon. Biosurfactants are composed of different naturally occurring biomolecules such as lipoproteins, lipopeptides, fatty acids, phospholipids, polymeric biosurfactants, and fatty acids. Moreover, they have numerous advantages over chemical surfactants, for example, lower toxicity, biocompatibility, tolerance to different temperatures, pH values, biodegradability, and structural diversity. The diversity of biosurfactants increases their applicability in various fields. The chemical nature and physical properties can be manipulated by biological, genetic, and chemical methods.[3] However, it has not been extensively utilized in industries due to the high cost of production and some technical difficulties.

Many microbiologists have done a lot of research on the production of biosurfactant by Bacillus sp. under different aerobic and anaerobic conditions. It was found to be of great potential when produced with low-cost substrates.

ABSTRACT

Aim: The study was to isolate biosurfactant producing organisms from petroleum contaminated soil. Two isolates were selected based on the positive results obtained for the screening tests. The production was carried out using them in a combined ratio, and the product was characterized using Fourier transform-infrared spectrosocpy (FT-IR). Materials and Methods: Soil samples were collected from Palakkad and Ernakulam regions. Screening tests selected to identify biosurfactant are drop collapse test, oil spreading technique, foaming activity, emulsification index, and blood hemolysis. 16S rRNA sequencing was carried out to confirm the species. The MSM medium with 2% engine oil as carbon source was used as the production medium. The crude biosurfactant was obtained by acid precipitation followed by Chloroform: Methanol extraction. The dry cell weight was measured. FT-IR analysis of the extract was performed. Results and Discussion: The positive organisms were found to be Bacillus subtilis (B50) and Bacillus cereus (B55). The GenBank accession number for isolates is MF521625 and MF684782, respectively. The biosurfactant yield was found to be approximately 0.24 gm/100 ml of the medium which was considerably more than individual production. FTIR spectrum indicates the presence of lipopeptide. Conclusion: The crude biosurfactant was determined to be lipopeptide which has properties similar to that of surfactants. Thus, microbially produced surfactants are eco-friendly, non-toxic, and biodegradable which can be used for different applications.

KEY WORDS: 16S rRNA, Bacillus cereus, Bacillus subtilis, Chloroform: Methanol extraction, Engine oil, GenBank accessions
The present research work aims at the isolation of *Bacillus* sp., production of biosurfactant and studies its emulsifying properties.

**MATERIALS AND METHODS**

**Sampling areas:** Two sampling areas (Palakkad and Ernakulam) were selected for the study. Some of the automobile workshops were chosen for obtaining fuel oil contaminated soil samples from these places.

**Sampling Procedure**

Black colored soil samples were taken from under 15–20 cm depth using a sterile shovel in sterile containers.

**Enrichment Procedure**

About 1 g of soil sample was inoculated in 100 ml of MSM medium containing 2% engine oil as a carbon source and was incubated at 30°C for 24 h. 0.1 ml of overnight culture was spread on MSM agar plates and incubated for 24–48 h at 30°C.

**Isolation and Screening of Biosurfactant Producing Bacteria**

The cultures which are morphologically different were selected and identified by biochemical tests.

The isolates were grown in MSM medium incorporated with engine oil (2% w/v) for 48–96 hrs on a shaker at room temperature. After incubation, the broth was centrifuged at 10,000 rpm for 30 min, and the supernatant was collected which was used to study the surfactant property.

**Blood hemolysis**

This is the preliminary test for the production of biosurfactant. The pure cultures of isolates were single streaked on blood agar plates and incubated at 30°C for 48–72 h. The results were observed and recorded.

**Drop collapse test**

A glass slide coated with oil was taken, and a drop of the supernatant was placed over it. If it contains biosurfactant, the drop collapses or spreads, and if negative, the drop remains stable.

**Oil spreading technique**

About 40 ml Dist. H2O was taken in a Petri dish and 10 µl of diesel oil was poured to the surface of the water to form a thin layer. To this, 10 µl of culture supernatant was gently added to the center of the thin oil layer. The oil gets displaced, and a clear zone can be observed if the supernatant contains biosurfactant.

**Emulsification index (E24%)**

2 ml of culture supernatant was added to 2 ml of diesel oil and vortex for 2 min and was kept undisturbed for 24 h. The emulsification index was determined by the formula:

\[
E_{24\%} = \frac{\text{Height of emulsified layer (mm)}}{\text{Total height of liquid column (mm)}} \times 100
\]

**Foaming activity**

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

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

**Identification of Best Biosurfactant Producer**

The most efficient isolates were identified from the screening tests, the biomass of cultures and crude biosurfactant yield. The organisms were characterized by different biochemical and physiological tests. 16 S rRNA sequencing was performed in Yaazh Xenomics lab Coimbatore, for the confirmation of organisms.

**Determination of Biomass**

The selected isolates in the ratio 1:2 (*Bacillus subtilis* and *Bacillus cereus* respectively) were inoculated into 100 ml of MSM medium containing 2% engine oil as the major source of carbon and energy. It was then incubated at 30°C for 48–96 h. Following incubation, the culture was centrifuged at 10,000 rpm for 30 min, and the supernatant was collected which contains the biosurfactant. The pellet obtained after centrifugation of production medium was used for biomass determination. A mixture of petroleum ether and acetone (1:3 ratio) was thoroughly mixed with the pellet and further centrifuged at 3000 rpm for 20 min. This process was repeated till the unutilized oil was removed from the cell debris. The debris was again treated with acetone and then washed with deionized water and allowed to dry at 60°C O/N. The dry weight was measured.

**Extraction of Biosurfactant**

The pH of the supernatant was adjusted to 2 using 6 N HCl and refrigerated for 24 h. The chilled Chloroform and Methanol in the ratio 2:1 was added to the supernatant in equal volume and mixed vigorously to form an organic layer which contains biosurfactant. Using a separating funnel, this layer was separated, and dry mass was obtained by evaporation at 40°C. The dry weight was recorded in g/L.
Fourier Transform Infrared Spectroscopy (FT-IR) Analysis of Biosurfactant

The FT-IR was carried out for the extracted biosurfactant in the IR range of 400–4000/cm with a speed of 20 scans.

RESULTS AND DISCUSSION

Enrichment of Soil Sample

The soil samples collected were enriched to provide a favorable condition for the growth of biosurfactant producing microbes.

Isolation and Screening of Biosurfactant Producing Bacteria

A total of 75 isolates were incurred from soil samples taken from automobile workshops.

Blood hemolysis

A total of 17 isolates showed β-hemolysis which is considered as a positive result for biosurfactant production [Figure 1].

Drop collapse test

Among those 17 isolates, 13 showed a positive result for drop collapse test. The drop of supernatant got spread in 1 min, and the result was recorded [Figure 2].

Oil spreading technique

Eight isolates gave a positive result for oil spreading technique. The zone of clearance was recorded [Figure 3].

Emulsification index \((E^{24\%})\)

These isolates showed emulsification index range from 20% to 60%. The isolates which showed maximum index value were selected.

Foaming activity

Six isolates showed good foaming activity which indicates the presence of biosurfactant in the medium [Figure 4]. The results are shown in Table 1.

Among the screening tests, oil spreading technique was considered the most sensitive method for the identification of biosurfactant. Many researchers had reported such findings. The oil spreading technique was suggested to be a more suitable method to find the lower concentration of biosurfactants than drop collapse method.\(^{10}\)
Identification of Best Biosurfactant Producer

The most efficient biosurfactant producers were selected from the screening tests, the biomass of culture and yield of crude biosurfactant [Table 2]. They were identified as \textit{B. subtilis} B50 and \textit{B. cereus} B55, and the GenBank accession number is given as MF521625 and MF684782, respectively [Figures 5 and 6].

The two isolates which showed the best result for all the screening tests were selected identified and characterized. They were subjected to 16S rRNA sequencing to confirm the organism. The organism was found to be \textit{B. subtilis} and \textit{B. cereus}. The accession number given by GenBank for this organism is MF521625 and MF684782, respectively.

The biosurfactant production was carried out with a consortium of these organisms in a medium which was optimized and obtained a yield of 0.24 g/100 ml of the medium which was considerably more than individual production. FT-IR spectrum indicates the presence of lipopeptide [Figure 7].

<p>| Table 1: Screening results for biosurfactant production |</p>
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Blood Hemolysis</th>
<th>Drop collapse test</th>
<th>Oil spreading test</th>
<th>Emulsification index (E_{24}% )</th>
<th>Foaming activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>β</td>
<td>+ve</td>
<td>10 mm</td>
<td>35</td>
<td>+ve</td>
</tr>
<tr>
<td>B14</td>
<td>β</td>
<td>+ve</td>
<td>20 mm</td>
<td>32</td>
<td>+ve</td>
</tr>
<tr>
<td>B16</td>
<td>β</td>
<td>+ve</td>
<td>15 mm</td>
<td>38</td>
<td>+ve</td>
</tr>
<tr>
<td>B25</td>
<td>β</td>
<td>+ve</td>
<td>15 mm</td>
<td>39</td>
<td>+ve</td>
</tr>
<tr>
<td>B50</td>
<td>β</td>
<td>+ve</td>
<td>25 mm</td>
<td>60</td>
<td>+ve</td>
</tr>
<tr>
<td>B55</td>
<td>β</td>
<td>+ve</td>
<td>25 mm</td>
<td>63</td>
<td>+ve</td>
</tr>
</tbody>
</table>

<p>| Table 2: Selection of biosurfactant producer based on biomass and biosurfactant yield |</p>
<table>
<thead>
<tr>
<th>Isolates</th>
<th>Biomass (g/100 ml)</th>
<th>Biosurfactant (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.50</td>
<td>0.010</td>
</tr>
<tr>
<td>B14</td>
<td>1.20</td>
<td>0.055</td>
</tr>
<tr>
<td>B16</td>
<td>1.34</td>
<td>0.062</td>
</tr>
<tr>
<td>B25</td>
<td>0.39</td>
<td>0.021</td>
</tr>
<tr>
<td>B50</td>
<td>1.32</td>
<td>0.089</td>
</tr>
<tr>
<td>B55</td>
<td>1.5</td>
<td>0.15</td>
</tr>
</tbody>
</table>

CONCLUSION

In this present study, 75 cultures were isolated from soil samples of automobile workshops. From them, two strains were selected based on screening results for biosurfactant production. It was identified to be \textit{B. subtilis} B50 and \textit{B. cereus} B55. This study has shown that a combination of organisms can produce more yield of biosurfactant compound with various activities. This will be used for further studies on antimicrobial, antifungal, antioxidant, and larvicidal activities.
REFERENCES


