ABSTRACT
Aim of the study is to compare the antibacterial activity of the biosurfactants from *Pseudomonas aeruginosa* and *Bacillus subtilis* of the oil contaminated soil samples which were collected from the oil spilled places of Vaniyambadi and Ambur areas of Vellore District, Tamil Nadu, India. The bacterial strains were isolated from the soil through serial dilution and spread plate technique and the Biosurfactant activity of the strains were tested through Emulsification index and Drop collapsing test. After the confirmation, the biosurfactants were extracted from the strains and tested against the pathogens (*Penicillium mitalicum*, *Trycophyton sp.*, *Aspergillus flavus*, *Trichoderma viride* and *Candida albicans*). This study clearly demonstrates that, the biosurfactants of *Pseudomonas aeruginosa* have shown antibacterial activity against most of the used test pathogens when compared to biosurfactants of *Bacillus subtilis*.

KEYWORDS: Biosurfactants, antibacterial activity, soil pollution, emulsification index, drop collapsing test.

1. INTRODUCTION
Biosurfactants are produced by diverse microorganisms to fulfill various natural functions. They exhibit a broad spectrum of chemical structures such as glycolipids, phospholipids, polysaccharide-lipid complexes, lipoproteins-lipoproteptides, hydroxylated and cross linked fatty acids and complete cell surface (*Urum and Pekdemir, 2004*). Among these biosurfactants glycolipids have much higher yield when compared to other classes of biosurfactants (*Kitamoto et al. 2002*). Rhamnolipid belongs to the class of glycolipids and it is
produced by the *Pseudomonas sp.*, *(Sharma et al. 2007)*. Rhamnolipids has been used in bioremediation of hydrocarbons, removal of heavy-metals and cleaning of oil spills *(Mulligan 2004)*. The amphiphilic membrane-active biosurfactant surfactin, fengycin, iturin, mycosubtilins, and bacillomycins are produced by *Bacillus subtilis* strains, which can be used in biotechnological and biopharmaceutical applications *(Sriram et al, 2011)*. These biosurfactants also have antiviral, antitumor, haemolytic, blood anticoagulant and fibrinolytic activities *(Kim et al, 2006)*. This present study aimed at the evaluation of antibacterial activity of biosurfactants of the strains *Pseudomonas aeruginosa* and *Bacillus subtilis* isolated from oil contaminated soil. Biosurfactants from the strains were isolated and it was dried and measured. These isolated biosurfactants were further used for the evaluation of antibacterial activity.

2. MATERIALS AND METHODS

2.1. Collection of soil sample
The oil contaminated soil samples were collected from the oil spilled places of Vaniyambadi and Ambur areas of Vellore District, Tamil nadu, India.

2.2. Isolation of bacteria from the soil
The bacterial strains were isolated from the soil through serial dilution technique and using nutrient agar as a media. Staining techniques and biochemical tests *(Koneman et al, 1998)* were performed to identify the isolated bacterial strains. These two strains were further confirmed through hydrolysis test and growth of the streaked culture on cetrimide agar.

2.3. Screening for Biosurfactant Activity
Biosurfactant activity of the isolated bacteria was detected by using emulsification stability test and Drop collapsing test using four different oil sources namely Petrol, Diesel, Kerosene and Tween 20.

2.3.1. Emulsification Test
The emulsifying capacity was evaluated by an emulsification index $E_{24}$ *(Iqbal et al, 1995)*. The emulsifying activity of the culture supernatant was estimated by adding 3 ml of the supernatant and adding equal volume of oil of interest to the same tube. The tube was vortexed for 10 seconds to 1 minute. Then held stationary for 1 minute and then visually examined for turbidity of stable emulsion. Emulsifying power was measured by vortexing equal volumes of the centrifuged culture with the oil for 1 minute and determining the
percentage of volume to settle for 24 hours and the height of the emulsion was measured
(Sarubbo, 2006).

\[ E_{24} (\%) = \left( \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \right) \times 100 \]

2.3.2. Drop collapsing test
2 µl of oil was added to each well of plate lid. The lid was equilibrated for 1 hour at room
temperature and then 5 microliter of the culture supernatant was added to surface of oil. The
shape of the drop on the oil surface was inspected after 1 minute. Biosurfactant producing
cultures giving flat drops were scored as ‘positive’. Those cultures that have rounded drop
were scored as ‘negative’ indicative of the lack of biosurfactant production
(Youssef et al., 2004).

2.4. Extraction of Biosurfactants
The bacterial cultures of Pseudomonas aeruginosa and Bacillus subtilis inoculated in 50 ml
of NA broth. The culture was incubated at 25\(^{\circ}\)C for 7 days with shaking condition. After
incubation the bacterial cells were removed by centrifugation at 5000rpm, 4\(^{\circ}\)C for 20
minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using
1MH2SO4. Then add equal volume of chloroform: methanol (2:1). This mixture was shaken
well for mixing and left overnight for evaporation. White colored sediment was obtained as a
result i.e., the “Biosurfactants” (Anandarajan and Thivakaran, 2010).

2.5. Dry weight of Biosurfactants
Sterile petriplate was taken and the weight of the plate was measured. Now the sediment was
poured on the plates. They were placed on the hot air oven for drying at 100\(^{\circ}\)C for 30
minutes. After drying, the plates were Weighted (Anandarajan and Thivakaran. The dry
weight of the biosurfactants was calculated by the following formula

\[ \text{Dry weight of biosurfactants} = (\text{Weight of the eppendorff after drying} – \text{Weight of the empty eppendorff}) \]

2.6. Antibacterial activity assay
Antibacterial activity of the biosurfactants was determined by disc diffusion method on
Muller Hinton agar (MHA) medium. Minimum inhibitory concentration of the biosurfactants
was determined against five bacteria namely, Salmonella typhimurium, Enterococcus
faecalis, Staphylococcus aureus, Escherichia coli and Klebsiella sp. The MHA medium was
weighed as 3.8 gms and dissolved in 100 ml of distilled water and add 1gm of agar. Then the medium was kept for sterilization. After sterilization the media was poured in to sterile petriplates, these petriplates were allowed to solidify for twenty minutes. After the medium was solidified, the above mentioned inoculums were spread on the solid plates with sterile swab. The white powder of biosurfactants was dissolved in 100% DMSO, from this 20 µl of the sample with different concentrations [Concentration: 1000 µg, 500 µg, 250 µg, 125 µg, 62.5 µg] were added to the disc. Here, DMSO has no inhibitory effects; hence it is used as a negative control and streptomysin as a positive control. Then the discs were placed in MHA plate. The plates were incubated at 37ºc for 24 hours. After the incubation period the zone of inhibition was observed around the disc and it was measured (Cao et al., 2009).

3. RESULTS AND DISCUSSION

The microbial derived Biosurfactants have some of the potential applications in pollution and environmental control, hydrocarbon degradation heavy metal removal, hexa-chloro cyclohexane degradation and antimicrobial activity (Singh, et al., 2007). In this present study the bacterial strains such as Pseudomonas aeruginosa (Fig 1) and Bacillus subtilis (Fig 2) were isolated.

Table 1 shows the Emulsification index (E24%) of the bacterial isolates. The confirmed bacterial strains Pseudomonas aeruginosa and Bacillus subtilis were screened for the biosurfactant activity. Biosurfactant play a role in emulsifying hydrocarbons. Out of seven isolated bacterial strains, based on the growth five bacterial strains were selected for the screening of biosurfactant production. These 5 bacterial isolates have shown better results against different oils. In this study S9, S10, S12 were Pseudomonas aeruginosa and S14, S15 were Bacillus subtilis. This two bacterial strains have the ability of emulsifying oils. S9 have shown the best of 62.85% , 68.57% against Kerosene and Tween 20 respectively. Same way S12 shown best of 62.86% against Diesel.
Table 2 shows the drop collapsing test for the biosurfactant production from bacterial isolates. Biosurfactant production of bacterial strains were further screened by using the qualitative drop collapsing test against different oils. Here, based on the flat drop appearance(+) and round drop appearance (-), the biosurfactant production was confirmed. Same way S9 and S14 have given the flat drop appearance against different used oils. This two test results confirms that *Pseudomonas aeruginosa* and *Bacillus subtilis* have biosurfactant activity. *Saravanan and Vijaykumar (2012)*, also confirmed the biosurfactant activity of microbes using drop collapsing test.

Table 3 shows the dry weight of the biosurfactants isolated from bacterial strains. Through centrifugation the supernatants (Fig 3) were collected from the strains and the biosurfactants were extracted and dried. The resulted powder was measured; it shows that *Pseudomonas aeruginosa and Bacillus subtilis* have the yield of 1.401 gm and 1.381 gm respectively. The *Pseudomonas aeruginosa* showed higher biosurfactant yield when compared to *Bacillus subtilis*. According to *Priya T and Usharani G (2009)* study, *Pseudomonas aeruginosa* have higher biosurfactant activity when compared to *Bacillus Subtilis*. *Chart 4* is the graphical representation of the dry weight of the isolated biosurfactants.

### Table 1: Screening of Emulsification Index (E_24) of Bacterial Isolates

<table>
<thead>
<tr>
<th>S.NO</th>
<th>SAMPLE SOURCES</th>
<th>Control</th>
<th>S9</th>
<th>S10</th>
<th>S12</th>
<th>S14</th>
<th>S15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Petrol</td>
<td>57.14</td>
<td>57.14</td>
<td>62.85</td>
<td>57.14</td>
<td>60.00</td>
<td>57.14</td>
</tr>
<tr>
<td>2.</td>
<td>Diesel</td>
<td>57.14</td>
<td>60.00</td>
<td>57.14</td>
<td>57.14</td>
<td>62.86</td>
<td>60.00</td>
</tr>
<tr>
<td>3.</td>
<td>Kerosene</td>
<td>57.14</td>
<td>62.85</td>
<td>57.14</td>
<td>57.14</td>
<td>60.00</td>
<td>57.14</td>
</tr>
<tr>
<td>4.</td>
<td>Tween 20</td>
<td>57.14</td>
<td>68.57</td>
<td>57.14</td>
<td>57.14</td>
<td>57.14</td>
<td>57.14</td>
</tr>
</tbody>
</table>

### Table 2: Drop collapsing test for the bacterial isolates

<table>
<thead>
<tr>
<th>S.NO</th>
<th>SAMPLE SOURCES</th>
<th>Control</th>
<th>S9</th>
<th>S10</th>
<th>S12</th>
<th>S14</th>
<th>S15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Petrol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Diesel</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Kerosene</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Tween 20</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+ ) – Presence, (-) – Absence.
Table: 3 Dry weight of the biosurfactants isolated from *Pseudomonas aeruginosa* and *Bacillus subtilis*

<table>
<thead>
<tr>
<th>S.No:</th>
<th>Sample Weight (gms)</th>
<th>Weight of eppendrof (gms)</th>
<th>Weight of eppendrof + Dry weight of Biosurfactants (gms)</th>
<th>Dry weight of Biosurfactants (gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1.019</td>
<td>2.420</td>
<td>1.401</td>
</tr>
<tr>
<td>2.</td>
<td><em>Bacillus subtilis</em></td>
<td>1.019</td>
<td>2.400</td>
<td>1.381</td>
</tr>
</tbody>
</table>

Chart 4: Weight of biosurfactants present in the *Pseudomonas aeruginosa* and *Bacillus subtilis*

Table 4: Antibacterial activity of the biosurfactants isolated from *pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>S.No</th>
<th>Microorganisms</th>
<th>Zone of Inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1000µg</td>
</tr>
<tr>
<td>1</td>
<td><em>Salmonella typhimurium</em></td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td><em>Enterococcus faecalis</em></td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td><em>Staphylococcus aureus</em></td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td><em>Escherichia coli</em></td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td><em>Klebsiella sp.</em></td>
<td>9</td>
</tr>
</tbody>
</table>
Antibacterial activity of the biosurfactants isolated from *Pseudomonas aeruginosa*

**Fig 5: Enterococcus faecalis**  **Fig 6: Staphylococcus aureus**

**Fig 7: Escherichia coli**

**Fig 8: Klebsiella sp.**  **Fig 9: Salmonella typhimurium**

### Table 5: Antibacterial activity of biosurfactants isolated from *Bacillus subtilis*

<table>
<thead>
<tr>
<th>S.No</th>
<th>Microorganisms</th>
<th>Zone of Inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1000µg 500µg 250µg 125µg 62.5µg DMSO Streptomycin 10µg</td>
</tr>
<tr>
<td>1</td>
<td><em>Salmonella typhimurium</em></td>
<td>9         9         8         8         8         -     11</td>
</tr>
<tr>
<td>2</td>
<td><em>Enterococcus faecalis</em></td>
<td>-         -         -         -         -         -     16</td>
</tr>
<tr>
<td>3</td>
<td><em>Staphylococcus aureus</em></td>
<td>-         -         -         -         -         -     15</td>
</tr>
<tr>
<td>4</td>
<td><em>Escherichia coli</em></td>
<td>8         8         8         8         8         -     9</td>
</tr>
<tr>
<td>5</td>
<td><em>Klebsiella sp.</em></td>
<td>-         -         -         -         -         -     16</td>
</tr>
</tbody>
</table>
Antibacterial activity of biosurfactants isolated from *Bacillus subtilis*

Table 4 shows the antibacterial activity of the biosurfactants isolated from *Pseudomonas aeruginosa*. In this study the antibacterial activity of the isolated biosurfactants were tested against five pathogens like *Salmonella typhimurium*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella Sp*. The biosurfactants showed inhibition against all the above mentioned organism except *Enterococcus faecalis*. According to Toribio et al (2010), the antimicrobial effect of biosurfactant produced by Pseudomonas aeruginosa may be due to the presence of Rhamnolipids. Table 5 shows the antibacterial activity of the biosurfactants isolated from *Bacillus subtilis*. The isolated biosurfactants were tested against the same five pathogens like *Salmonella typhimurium*, *Enterococcus faecalis*, *Staphylococcus*
aeruginosa, Escherichia coli and Klebsiella Sp. Here, the biosurfactant showed inhibition against Salmonella typhimurium and Escherichia coli only. According to Ghribi et al (2011), the antibacterial activity is due to the presence of Surfactin and iturin produced by Bacillus subtilis. This study clearly demonstrates that Pseudomonas aeruginosa produced biosurfactants have higher antibacterial activity when compared to Bacillus subtilis.

4. CONCLUSION
The antibacterial activity of the biosurfactants isolated from microbes reveals that this biosurfactants can be used in the pharmaceuticals for the production of novel drugs, cosmetics and production of antifungal agents. It is hoped that this study would lead to the establishment of new and more potent antimicrobial drugs.

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REFERENCES


