SCREENING OPTIMIZATION AND PRODUCTION OF BIOSURFACTANTS FROM BACILLUS AND PSEUDOMONAS SPECIES

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ABSTRACT

Biosurfactants are surface active compounds produced by microorganisms. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures. Oil contaminated soil were collected from different automobile shop. **Bacillus** and **Pseudomonas sp** were isolated from the four soil samples and these isolates were screened for biosurfactants activity using petrol, diesel and kerosene by Emulsification capacity. Surface tension measurement, Oil spreading techniques, Drop collapse method and Haemolytic activity. Biosurfactant production by the isolated bacteria using different pH, temperature and concentration of carbon source was studied. Effective production of biosurfactant in both **Bacillus** and **Pseudomonas sp** using Mineral salt broth with optimized condition.

**Keywords:** Biosurfactants, Rhamnolipid, Lipopeptide, Emulsification, Haemolytic activity.

INTRODUCTION

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively (Karanth et al., 1999). Biosurfactant producing microorganisms were naturally present in oil contaminated soil. Oil contaminated environment contain large amount of hydrocarbons, i.e., aliphatic and aromatic hydrocarbons. Microorganisms exhibit emulsifying activity by producing biosurfactants and utilize the hydrocarbons as substrate often mineralizing them or converting them into harmless products.

The enormous market demands for surfactants are currently met by numerous synthetic mainly petroleum based, chemical based surfactants. These compounds are usually toxic to the environment and non-degradable. Tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternative to chemical surfactants (Banat et al., 2000).

Biosurfactants have gained more importance in the fields of enhanced oil recovery, environmental bioremediation, food processing, and pharmaceuticals owing to their unique properties: higher biodegradability, lower toxicity, and effectiveness at extremes of temperature, pH and salinity. Biosurfactants can be produced by microbial fermentation processes using cheaper agro based substrates and waste materials. In various industrial processes, they are potentially useful surface-active agents for emulsion polymerization, wetting, foaming, phase dispersion, emulsification and de-emulsification (Desai et al., 1997).

Biosurfactants are mainly categorized mainly by their chemical composition and microbial origin. Generally their structures include a hydrophilic moiety consisting of amino acids or peptides, mono-di or polysaccharides and hydrophobic moiety comprising unsaturated or saturate fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides, lipoproteins, phospholipid, fatty acids, polymeric biosurfactant and particulate biosurfactants (Manneerat et al., 2005).

The most prevalent bacterial hydrocarbon degraders and surfactant producers, belong to the genera are **Pseudomonas**, **Achromobacter**, **Flavobacterium**, **Micrococcus**, **Bacillus**, **Arthrobacter**, **Klebsiella**, **Acinetobacter**, **Aeromonas**, **Alkaligenes**, **Streptococcus sp**, **Corynebacterium sp**, **Moraxella sp**, and **proteobacteria** (Mishra et al., 2001)

Among the different classes of biosurfactants rhamnolipid and surfactin are best studied. Rhamnolipid is produced by **Pseudomonas aeruginosa**, a gram-negative, motile, non spore forming bacteria. Surfactin is cyclic lipopeptide commonly used as an antibiotic. Surfactin's structure consists of a peptide loop of seven amino acids(L-asparagine, glycine, two L-Leucine, L-valine, and two D-Leucins) and an hydrophobic fatty acid chain thirteen to fifteen carbon long (Peypoux et al., 1999).

MATERIALS AND METHODS

The soil samples were collected from polluted sites and isolate the bacteria **Bacillus** and **Pseudomonas** from oil contaminated soil by gram staining and by various biochemical test. Screening of bio surfactants using the following methods.

**Screening for biosurfactant activity**

The supernatant was subsequently subjected to the preliminary screening methods by using 3 different oil namely petrol, Diesel and kerosene.

**Oil spreading techniques**

The 50ml of distilled water was added to a large petri dish (15 cm diameter) followed by the addition of 20µl of oil to the surface of water, 10µl of supernatant of culture broth (Rodrigues et al., 2006).

**Emulsification capacity (E24) test**

E24 of culture samples was determined by adding 2ml of oil to the same amount of culture, mixing with a vortex for 2min and leaving to stand for 24 hours. The E24 index is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm) (Sarubbo, 2006).

\[ E24 = \frac{\text{Height of emulsion formed}}{\text{Total height of the solution}} \times 100 \]

**Drop collapse method**

For drop collapse method 2µl of oil was added to each well of a 96 well containing micrometer plate lid. The lid was equilibrated for 1 h at room temperature. MSM cultures of bacterial isolates was centrifuged and then 5µl of the drop on the surface of oil was evaluated after 1 min (Jain et al., 1991).

**Haemolytic activity**

Isolates were screened on blood agar plates containing 5% sheep blood and incubated at 37°C for 48 h. Hemolytic activity was detected as the presence of clear zone around bacterial isolates (Piazza et al., 2006).

**Surface tension measurement**

Surface tension of biosurfactant was measured by drop weight method. A burette is held vertically and liquid drops are allowed to
from its lower end at a very slow rate about 8 drops per minute. A clean dry beaker is weighted and placed under flow of burette. 50 drops of the liquid are collected and weighted the beaker. Again repeat the same for 3 times. The average mass of one drop is calculated and then surface tension of liquid containing biosurfactant calculated by \( \text{surface tension}(T) = \frac{Mg}{3.8r} \)

### Process optimization and biosurfactant production

Optimization of biosurfactant production involves changing one variable at a time, while keeping the others at fixed levels, however this method is laborious and time consuming.

### Effect of PH

In 100ml Erlenmeyer flasks containing 50ml mineral salt medium were adjusted at different PH values from 6-8. 0.5ml of overnight culture was inoculated and incubated for 7 days at 30°C. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration by UV-VIS spectrophotometer measured at 620 nm.

### Effect of temperature

In 100ml Erlenmeyer flasks containing 50ml sterile mineral salt medium were inoculated with over night cultures of Bacillus, Pseudomonas sp and incubated at different temperature ranges from 35-39°C for one week. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration by UV-VIS spectrophotometer measured at 620 nm.

### Effect of kerosene concentration

From an overnight culture broth of Bacillus and Pseudomonas sp were inoculated in 50ml mineral salt medium. The kerosene (Carbon source) concentration is adjusted between 0.1 - 0.5%. Cultures were incubated for one week at 30°C. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration by UV-VIS spectrophotometer measured at 620nm.

### Biosurfactant Production

Isolates were grown in 500ml Erlenmeyer flasks containing 100ml mineral salt medium adjusted to \( \text{pH} \) 7.0 was used as culture medium. The flasks were incubated at 37°C on a shaker incubator for 7 days. To isolate the biosurfactant, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22µm pore size filter (milipore).

Biosurfactant was obtained by adjusting the supernatant \( \text{pH} \) 2.0 using 6N Hcl and keeping it at 4°C overnight. The precipitate thus obtained was pelleted by centrifugation for 20 min, dried and weighed.

For further purification the crude surfactant was dissolved in distilled water at \( \text{pH} \) 7.0 and dried at 60°C. The dry product was extracted with Chloroform: Methanol (65:15) filtered and the solvent evaporated.

### RESULT AND DISCUSSION

Four isolates of bacillus and pseudomonas have highest E24 value was observed in diesel (Priya & usharami, 2009). In case our study Bacillus and Pseudomonas have the ability to emulsifying oils. The highest E24 value was observed in petrol and Pseudomonas showed the better E24 value than bacillus. (Table –1 & Fig-1). In oil spreading techniques bacillus shows higher zone formation of 4.1mm, 3.6mm, 3.2mm in petrol, diesel, and kerosene respectively. Similarly our study focused Pseudomonas showed 3.9mm, 3.3mm, 2.8mm in petrol, diesel, and kerosene (Fig -2).

#### Table 1(A): Emulsification Index In Petrol

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Emulsified Layer (cm)</th>
<th>Total Liquid Column(cm)</th>
<th>% of E24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>0.6</td>
<td>2.9</td>
<td>21</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.8</td>
<td>2.7</td>
<td>30</td>
</tr>
</tbody>
</table>

#### Table 1(B): Emulsification Index In Diesel

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Emulsified Layer (cm)</th>
<th>Total Liquid Column(cm)</th>
<th>% of E24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>0.3</td>
<td>3.23</td>
<td>917</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.5</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

#### Table 1(C): Emulsification Index In Kerosine

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Emulsified Layer (cm)</th>
<th>Total Liquid Column(cm)</th>
<th>% of E24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>0.4</td>
<td>3.23</td>
<td>913</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.3</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

#### Chart-I
Surface tension reduction was measured by Kruss Hamburg Nr2215 Tensio meter. Results were compared to medium composition as negative control (Paviran et al., 2004). Since our study of surface tension reduction was measured by drop weight method and Pseudomonas shows 0.007 surface tension reduction per drop (Fig-3).

Blood agar method is often used for a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media (Schulz et al., 1991). From our results both the isolates will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies (Fig-4).

The pH ranges from 6, 7 and 8, temperature ranges from 35°C, 37°C, 39°C and kerosene concentration from 1%, 3% and 5% for optimization of biosurfactant production in Bacillus and Pseudomonas sp. Both the organisms have maximum growth rate observed at pH 7, temperature of 37°C in 0.3% kerosene concentration. Among the two isolates Bacillus shows better growth rate than Pseudomonas. Biosurfactant production from Bacillus and Pseudomonas sp can be achieved using Mineral salt broth (chart-1 & Fig-5).
Fig. 3: Surface tension measurement

Fig. 4: Hemolysis in Blood Agar

Fig. 5: Optimized Production of Biosurfactant
CONCLUSION

From the above observation, it was concluded that both bacterial isolates of bacillus and pseudomonas have the ability to secrete surface active agents it is gain more important in future for industrial and environmental applications.

REFERENCES