Biosurfactants are surface-active agents produced by hydrocarbanoclastic bacteria isolated from oil contaminated sites. In the present study a potential oil degrading bacterium was isolated from Cuddalore harbor waters and identified as Pseudomonas aeruginosa by using both biochemical and 16S rRNA sequencing. Growth optimization of the strain was done against varying physiochemical parameters viz., Temperature, pH, salinity, incubation period, carbon source and nitrogen source. Mass scale cultivation was done using optimum parameters viz., 35°C, pH, 1.5 % salinity, sucrose as carbon source and ammonium nitrate as nitrogen source. At 72hrs of incubation period, maximum growth was observed. 1.35mg/ml of biosurfactant was obtained in shake flask and 2.5mg/ml was obtained in laboratory scale fermentor. FTIR study proved that it was a rhamnolipid.

1. INTRODUCTION
Pollution of the sea by crude oil, mostly caused by stranding of tankers, is one of the urgent and serious environmental issues over the world. Ship operations also produce wastes that are collected in the lowest part of the hull, called the bilge area. This oil-containing bilge waste must be managed properly to avoid environmental pollution (Olivera et al., 2003). Biosurfactants are found as extracellular compounds or localized on the cell surface of microorganisms. For the latter case, the microbial cell itself is a biosurfactant and adheres to hydrocarbon. Those biosurfactants are capable of increasing the bioavailability of poorly soluble polycyclic aromatic hydrocarbons such as phenanthrene (Gilewicz et al., 1997 and Olivera et al., 2003) and resins (Venkateswaran et al., 1995). Therefore, the use of biosurfactants should be a promising means to emulsify the polluted oils prior to biodegradation. Several reports are available on the production of biosurfactants on water-immiscible substrates, especially hydrocarbons and water-soluble substrates such as carbohydrates. For instances, Pseudomonas aeruginosa which produces rhamnolipids, Candida (formerly Torulopsis) bombicola, one of the few yeasts to produce biosurfactants, which produces high yields of sophorolipids from vegetable oils and sugars and Bacillus subtilis, which produces a lipopeptide called surfactin (Mulligan, 2005 and Canter, 2004). Biosurfactants show many advantages over chemical surfactants as regards biodegradability, low toxicity, and effectiveness at extreme temperatures, pH, or salinity (AbuRuwaida et al., 1991). When microorganisms grow in environment rich in hydrocarbon, they undergo many adaptations. One such adaptation is biosurfactant production; it influences the uptake of hydrocarbons as substrates (Marin et al., 1996 and Johnsen et al., 2005). The low solubility and high hydrophobicity of many hydrocarbon compounds make them highly unavailable to microorganisms. Biosurfactant production helps the hydrocarbon degrading bacterium to gain better access to their hydrophobic substrates as it brings about changes like reduction of surface tension of the environment around the bacterium, reduction of interfacial tension between bacterial cell wall and hydrocarbon molecules, membrane modifications like increasing the hydrophobicity of cell wall by reducing the lipopolysaccharide content of cell wall, enhancing the dispersion of hydrocarbon by encapsulation of the hydrocarbon into micelles, etc (Zhang and Miller, 1992; Desai and Banat, 1997; Barkay et al., 1999 and Al-Tahan et al., 2003) and resins (Venkateswaran et al., 1995).
In the present study, a crude oil degrading and biosurfactant producing organism, *Pseudomonas aeruginosa* was isolated from Cuddalore fishing harbor area. Since microbial growth on crude oil has been associated with the production of surfactants, the present study finding out if crude oil metabolizing bacteria might be able to produce surfactants.

2. MATERIALS AND METHODS

2.1 Isolation of Hydrocarbonoclastic bacteria (HB)

The hydrocarbonoclastic bacterium *Pseudomonas aeruginosa* was isolated from Cuddalore fishing harbor area and identified using biochemical test and 16S rRNA sequencing.

2.2 Optimization of Growth of potential strain

The potential oil degrading bacterium was examined for the optimization studies for maximum biomass production.

**Biomass production was estimated at various parameters such as temperature (25, 30, 35, 40, 45 and 50°C), pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0 11.0 and12.0), different carbon sources viz. glucose, sucrose, maltose, glycerol, crude oil etc., different nitrogen sources (yeast extract, beef extract, peptone, ammonium sulphate, ammonium nitrate and potassium nitrate) and varying incubation periods (0-120hrs).** The impact of NaCl concentration on biomass production was also evaluated using various concentration (0, 0.5, 1.0, 1.5, 2.0 and 2.5%) in BH broth.

2.3 Mass scale culture

Using optimized parameters mass scale culture was done in both shake flasks and laboratory scale fermentor. Biomass was estimated gravimetrically. broth culture was taken and allowed to stand for 20 min. When the oil phase gets separated, the bottom phase with cells was siphoned out.
and filtered through a 0.45µm sized Millipore filter paper. Then the paper with cells was dried at 80°C in a hot air oven and weighed. Biomass was quoted in terms of g/L (dry weight).

2.4 Extraction of biosurfactant production
After mass cultivation, the broth culture was centrifuged at 6000rpm for 20 min. And extracted thrice with chloroform and methanol (2:1vol/vol). The solvents were removed by rotary evaporation and the residue was purified in a silica gel (60-120mesh) column and the elutions were made with chloroform and methanol ranging from 20: 1 to 2: 1 vol/vol in a gradient manner with 10 fractions. The fractions were pooled and the solvents were evaporated and the resulting residue was dialyzed against distilled water and lyophilized. The purified product was used for emulsification activity.

2.5 CHARACTERIZATION OF BIOSURFACTANT: (FTIR)
Fourier transform infrared spectroscopy (FTIR) is widely used method for identifying the types of chemical bonds (functional groups). Therefore can be used to elucidate some components of unknown mixture. The molecular characterization was performed using one milligram of freeze dried partially purified biosurfactant which was ground with 100 mg of KBr and pressed with 7500 kg for 30 sec to obtain translucent pellets. Infrared absorption spectra were recorded on a Thermo Nicolet, AVATAR 330 FTIR system with a spectral resolution wave number accuracy of 4 and 0.01 cm⁻¹, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as a background reference.

3. RESULTS
The oil degradating microbe was isolated from Cuddalore fishing harbor area and identified using biochemical test and 16S rRNA sequencing as Pseudomonas aeruginosa (Fig.1).

3.1 Optimization of growth of potential strain
Optimization studies for biomass production was carried out using various parameters like pH (5-12), temperature (25-50°C), salinity (0.0-2.5% NaCl), carbon sources (glucose, sucrose, maltose, glycerol, crude oil-0.1%) and nitrogen sources (0.5%-yeast extract, beef extract, peptone, ammonium nitrate, ammonium sulphate and potassium nitrate) and incubation period (0-120 hrs). Growth in different pH showed the maximum biomass at pH 9 and minimum at 12 (Fig.2). Likewise regarding temperature 35°C was seemed to be ideal and 50°C was least favorable (Fig.3). When varying sodium chloride concentration was tested highest growth was observed at 1.5% salinity and minimum at 0.0% (Fig.4). Regarding carbon sources, when five different substrates were tried, sucrose resulted in the maximum growth whereas the minimum was observed in maltose (Fig.5). When different nitrogen sources tested maximum growth was observed in yeast extract and minimum was in ammonium nitrate (Fig.6). Irrespective of the parameters tested at 72hrs of incubation period, maximum growth was observed.
Fig: 8 OD value and biomass at different incubation periods (Fermentor study)

Fig: 9 FTIR spectra analysis of biosurfactant

Table 1: Characterization of Biosurfactant using FTIR

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Characterization</th>
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<tbody>
<tr>
<td>2954</td>
<td>CH₃</td>
</tr>
<tr>
<td>2924</td>
<td>CH₂</td>
</tr>
<tr>
<td>2953</td>
<td>C - H</td>
</tr>
<tr>
<td>2362</td>
<td>C (\cong) C</td>
</tr>
<tr>
<td>2344</td>
<td>C (\cong) C</td>
</tr>
<tr>
<td>1793</td>
<td>Open chain acid anhydride</td>
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<td>1773</td>
<td>Aryl carbonate</td>
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<tr>
<td>1744</td>
<td>Alkyl carbonate</td>
</tr>
<tr>
<td>1735</td>
<td>6 membered ring Lactone</td>
</tr>
<tr>
<td>1718</td>
<td>Carboxylic acid</td>
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<td>Aromatic ring stretch</td>
</tr>
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<tr>
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<tr>
<td>722</td>
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<td>579</td>
<td>C - Br</td>
</tr>
<tr>
<td>420</td>
<td>C-I</td>
</tr>
</tbody>
</table>

3.2 Mass scale cultivation

In the optimal conditions, in shake flask maximum OD value and biomass (1.3 OD and 1.9g/L) and minimum was (0.056 OD and 0.34g/L) (Fig.7). In fermentation condition the maximum OD value and biomass (1.6 OD and 2.3g/L) and minimum was (0.09 OD and 0.4g/L) (Fig.8).

3.3 Estimation of Biosurfactant production

After mass cultivation, the broth culture was purified using chloroform and methanol method. In shake flask condition 1.35mg/ml of biosurfactant was obtained and 2.5mg/ml was observed in fermentation study. Compared with Erlenmeyer’s conical flask tests, maximum biosurfactant production was achieved earlier and maximum quantity was observed in fermentation cultivation.

3.4 FTIR analysis of biosurfactant

Consistent with this finding is the infrared spectra (IR) analysis of the organic extract of Pseudomonas aeruginosa surface active compounds and of the referent rhamnolipids. As seen from Fig. 9. bands characteristic of rhamnolipids (appearance of carbonyl absorption arising from ester and carboxylic groups) were observed at 1735 and 1718 cm⁻¹. The absorption bands of higher frequencies (1735 cm⁻¹) were assigned to the ester groups while those at about 1718 cm⁻¹ originate from carboxylic groups. In the region 3000-2700 cm⁻¹ several C-H stretching bands of CH₂ and CH₃ groups were also observed (Table-1).

4. DISCUSSION

Petroleum hydrocarbons are important energy resources used by industry and in our daily life. At the same time petroleum is a major pollutant of the environment. Biosurfactants have advantages over their chemical counterparts in lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH, and salinity.

In the present study, the oil degradating microbe was isolated from Cuddalore fishing harbor area. Different biosurfactants have been isolated from various marine bacteria: glucose lipid produced by Alcaligenes sp. (Poremba et al., 1991) and Alcanivorax borkumensis (Abraham et al., 1998); trehalose tetraester and trehalose diester produced by Arthrobacter sp. SI 1 (Schulz et al., 1991); and polymeric biosurfactants produced by Pseudomonas nautica (Husain et al., 1997) and an yeast, Yarrowia lipolytica (Zinjarde and Pant, 2002).

In the present study the potent oil degradating bacterial isolate Pseudomonas sp. was purified and identified on the basis of morphological, physiological and biochemical characteristics were carried out according to Bergy's Manual of Determinative Bacteriology (Hensyl, 1994) and using the phylogenetic technique by 16S rDNA for complete identification of the isolate Pseudomonas sp. to give Pseudomonas aeruginosa. Under certain conditions many microbes can be induced to produce extracellular biosurfactants. In this connection, the effect of different operating factors on biosurfactant production is of paramount importance. Many environmental factors, such as pH, temperature, salinity, type of nutrients, etc., can influence the physicochemical properties including carbon nitrogen sources, divalent cations, and specific substrate availability (Adamczak and Bednarski, 2000).

In the present study Pseudomonas aeruginosa was tested in different pH showed that the maximum biomass at pH 9. Regarding temperature 35°C seemed to be ideal and
when varying sodium chloride concentrations were tested highest growth was observed at 1.5% salinity. Regarding carbon sources, when five different substrates were tried, sucrose resulted in the maximum growth whereas the minimum was observed in maltose. When different nitrogen sources tested maximum growth was observed in yeast extract and minimum was in ammonium nitrate. Irrespective of the parameters tested at 72hrs of incubation period, maximum growth was observed. Miscellization of biosurfactant or modification of the molecular area at the air-water interface can be improved with monovalent cation (Thimon et al., 1992). This phenomenon might be a common feature of active compounds produced by marine bacteria. The concentration of salts in aquatic environments ranges from less than 0.05% (w/v dissolved salts) to saturated salt up to 3.0% and above and NaCl is a major component of seawater (Cameotra and Makkar, 1998). NaCl activated biosurfactant activity of many strains, which were isolated from seawater or petroleum reservoirs (Yakimov et al., 1995).

Biosurfactant isolated in the present study was lipid containing amphiphatic molecules that could be extracted using an appropriate organic solvent system. Various type of organic solvent could be used and they were applicable either singly or in combination for biosurfactant extraction (Kuyukina et al., 2001). In the present study, chloroform and methanol (2:1vol/vol) solvent system was used. Based on the amount of total biosurfactant recovered indicated that the mixture of chloroform and methanol at the ratio 2:1 (2.5mg/ml). Mixtures of solvents were commonly used to facilitate adjustment of the polarity between the solvent as the extraction agent and the biosurfactant to be extracted (Desai and Banat, 1997 and Kuyukina et al., 2001).

In the present study the Infrared spectrum chart of purified biosurfactant by Pseudomonas aeruginosa strain was characterized by the appearance of carbonyl absorption arising from ester and carboxylic groups, (i.e.) component was identified as rhamnolipid. Rashedi, et al., 2005 also reported rhamnolipid from Pseudomonas aeruginosa in TLC plate. There are many complex molecules included in biosurfactants, e.g. glycolipids, lipopeptides, fatty acids, polysaccharide protein complexes, peptides, phospholipids and neutral lipids. For instance, the biosurfactant obtained from Streptococcus thermophilus A was a multicomponent biosurfactant, consisting of protein and polysaccharides (Rodrigues, 2006).

Hence the present study evidence that crude oil degrading Pseudomonas aeruginosa strain was indeed involved in the biosurfactant production. Multifunction of biosurfactants is known. Because their chemical structures and surface properties are so different, their role in mechanisms of assimilation/uptake and in hydrocarbon biodegradation is difficult to generalize. However their role had been proved as they result in oil degradation. The present study also proved the same.

**5. CONCLUSION**

The study proved that the Pseudomonas aeruginosa strain isolated from Cuddalore harbor water is a potential producer of rhamnolipid biosurfactant. This seems to be an ideal strain for industrial scale production.

**REFERENCES**

Screening for biosurfactants among crude oil degrading marine microorganisms from the North Sea.

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