ISSN : 2348- 8069 Int.J.Adv.Res.Biol.Sci.2014; 1(1):149-161

International Journal of Advanced Research in Biological Sciences

www.ijarbs.com

Research Article

Production and biodegradation potentials of biosurfactant from *Pseudomonas aeruginosa* isolated from oil polluted water samples

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Abstract

Biosurfactants have been used for gene transfection, as ligands for binding immoglobulins, as adjuvants for antigens and also as inhibitors for fibrin clot formation and activators of fibrin clot lysis. Some biosurfactants are a suitable alternative to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents. These has been increasing interest in the effect of biosurfactants on human and animal cells and cell lines. When colonies of different morphology where tested for haemolytic activity the zone of clear zone was between 6mm and 12mm.The most potential strain was biochemically identified as *Pseudomonas aeruginosa* which was designated as *Ps.aeruginosa* SBS1001.BATH assay showed of 76% adherence to oil.D_{610} value which represented emulsification activity was found to be 1.4.The growth optimization experiments showed that the organism exhibited maximum growth at 30^0C, pH extract as nitrogen source preferred. When optimized conditions were used for mass cultivation in shake flasks 0.6mg/ml of biosurfactant was produced. The present study is on biosurfactant production by *Pseudomonas aeruginosa* SBS 1001 strain isolated from Cuddalour harbour. The rate of degradation of crude oil was surprisingly high (i.e) 85% degradation in 120 hrs and 50% reduction in 48hrs.The FT-IR analysis of the purified biosurfactant revealed that it is a rhamnolipid.

Keywords: Biosurfactants, *Pseudomonas aeruginosa* , haemolytic activity, rhamnolipid, FT-IR analysis.

Introduction

Oil pollution is a great hazard to soil and aquatic environments. Crude oil is a complex mixture of many compounds such as alkanes, aromatics, resins and asphaltenes. Biosurfactants or microbial surfactants are surface metabolites that is produced by bacteria, yeast and fungi having very different chemical structures and properties (Ron and Rosenberg, 2001) .These biosurfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic domains that find application in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Gautam and Tyagi, 2006).

Nowadays, biosurfactants are used in industries as a cosmetic and special chemical substances, food, pharmaceutics, agriculture, cleansers, enhanced oil recovery and bioremediation of oil-contaminated sites (Makkar and Cameotra, 2002). They are

potential alternatives of chemically synthesized surfactants in a variety of application because of their advantages such as lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelle concentration, easy of production, ability to be synthesized from renewable resources, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Mukherjee and Das et al.,2006).In recent years, the biosurfactants have been placed on the environmental impacts of chemical surfactants and new surfactants for use in any field.

Surfactants are amphiphilic molecules that tend to lower the interfacial tension. Biosurfactants are microbially produced surface-active compounds. They are amphiphilic molecules with both hydrophilic and hydrophobic regions causing them to aggregate at interfaces between fluids with different polarities such as water and hydrocarbons (Georgiou *et al.,* 1990;Fiechter, 1992;Banat, 1995 and Karanth *et al*., 1999) .

Biosurfactants are produced and excreted by a wide variety of microbes under specific growth conditions (Mukherjee *et al*.,2006). Glycolipids and lipopeptides are low molecular weight biosurfactants that effectively lower surface and interfacial tensions.

Microbiologically derived surfactants or biosurfactants are heterogeneous group of surface active molecules produced by a wide variety of bacteria, yeast and filamentous fungi, which either adhere to cell surface or are excreted extracellularly in the growth medium. Having both hydrophobic and hydrophilic moieties, biosurfactants are able to reduce surface tension and interfacial tension between two fluids at the surface and interface respectively. These are also able to form microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbon (Desai and Banat,1997).Microbial surfactants are complex molecules, comprising a wide variety of chemical structures, such as glycolipids, lipopeptides, fatty acids, polysaccharides protein complexes, peptides, phospholipids and neutral lipids (Banat and Makkar *et al.,*2000).

Most microbial surfactants are complex molecules, comprising different structures that include lipopeptides, glycolipids, polysaccharide protein complex, fatty acids and phospholipids (Nitschke and Pastore, 2006). The different types of biosurfactants include lipopeptides synthesized by many species of *Bacillus* and other species, glycolipids synthesized by *Pseudomonas* species and *Candida* species, phospholipids synthesized by *Thiobacillus thiooxidans*, polysaccharide lipid complexes synthesized by *Acinetobacter* species, or even the microbial cell surface (Mousa *et al*., 2006).

Surface active compounds produced by microorganisms are divided into two main types. These that reduce surface tension at air water interface (biosurfactants) and those that reduce the interficial tension between immiscible liquids, or at the solid-liquid interfaces (bioemulsifires). Biosurfactants are amphipathic compounds excreted by microorganisms that exhibit surface activity (Nitschke and Pastore, 2006). There are many complex molecules included in biosurfactants, e.g. glycolipids, lipopeptides, fatty acids, polysaccharide protein complexes, peptides, phospholipids and neutral lipids.

Various types of biosurfactants are synthesized by a number of microbes particularly during their growth on water immiscible substrates. A majority of biosurfactants are produced by bacteria. Among them, the *Pseudomonas* species is well known for its capability to produce rhamnolipid biosurfactants with potential surface-active properties when grown on different carbon substrates. Rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*, in particular offer special advantages because of their potent emulsifying activity and low critical micelle concentration (Cooper *et al*., 1981).

Aim and Objective

To isolate hydrocarbanoclastic bacteria from Cuddalour harbour. To identify the potential Biosurfactant producing strain. To optimize the

biomass production using various parameters like pH, temperature, salinity, carbon sources and nitrogen sources. To produce biosurfactant in large scale. To study the biodegradation of crude oil. Characterization of biosurfactant using Fourier Transform Infrared Spectroscopy (FTIR).

Materials and Methods

Collection of Water Samples

Water samples from Cuddalour harbour were collected using pre-sterilized plastic bottles contaminated with oil pollutants due to continuous operation of fishing boats.

Isolation of biosurfactant producing microbes

Water sample were serially diluted, processed and suitable dilutions were spreaded on the surface of BH agar (Bushnell Hass Agar) containing 1% crude oil and incubated at 35^{o}C for 2-3 days. Morphologically different colonies were picked and pure cultures obtained using quadrant streak method and strains were stored in the same medium in slants at 4°C for further study.

Identification of the potential strain

The potential strain was identified based on the result obtained in the following screening procedures.

Screening for biosurfactant production
Haemolytic activity (Mulligan *et al*., 1984)

The isolated strains were inoculated to the BHA broth and incubated for over night. The blood agar plates were prepared using defibrinated goat blood. Then the medium in the plates were punchered with the help of gel puncture. 0.1ml of overnight cultures were inoculated in individual well and incubated at 37ºC for 24-48 hrs. The plates were visually inspected for zone of clearance around the wells which is an indication of biosurfactant production. The diameter of the clear zone was taken as an indicator of biosurfactant production. The strain showed the maximum zone of clearance was taken as the most potential biosufactant producing strain.

Oil spreading technique (Anandaraj and Thivakaran, 2010)

30ml of distilled water was taken in the pertiplates. 1 ml of crude oil, petrol, diesel, kerosine and engine oil was added to the centre of the plates containing distilled water. Now 20µl of the supernatant of the culture was added to the water to the centre. The biosurfactant producing organism can displace the oil and spread it in the water

Bacterial adhesion to hydrocarbons (BATH Assay) (Rosenberg *et al*., 1980)

The strains which are positive in haemolytic assay are selected for BATH assay

The optical density of cells in the Bushnell Hass Broth was determined initially at 610 (or) 660nm. To 10ml of suspension containing known density of cells to 0.7ml of crude oil was added and mixed thoroughly in a 20ml glass tube on a reciprocating shaker for 15 min. The mixture was taken out and allowed to stand for 30 minutes for the separation of oil and water phases. The aqueous phase was carefully removed by a Pasteur pipette and optical density was measured again. The percentage of bacteria adhered to the oil was calculated by using the formula.

% of bacterial adherence = [1-(OD shaken with oil / OD original)] X 100

Estimation of emulsification activity (Rosenberg *et al.,* 1979)

5ml of supernatant was taken in a 30ml screw capped test tube. Emulsification activity was tested against crude oil. To the above solution 5mg of hydrocarbon was added and shaken well for 20 min in a shaker at 150rpm and the mixture was allowed to stand for 20 min. The optical density of the mixture was measured at 610nm and the results were expressed as D_{610}

Optimization of biomass production

Based on the haemolytic activity and BATH assay the potential biosurfactant producing potential strain was selected for the optimization studies for maximum biomass production. Biomass was estimated gravimetrically. Broth culture was taken and allowed to stand for 20 minutes. 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 mg/ml (dry weight). Biomass production was estimated at various parameters such as temperatures (25°, 30°, 35° and 40°C), different carbon source (glucose, sucrose, maltose, starch and cellulose) and nitrogen sources (peptone, ammonium nitrate, beef extract and yeast extract) different incubation periods (0-96hrs) and pH (6.0, 7.0, 8.0, 9.0, 10.0 and 11.0). The impact of NaCl concentration on biomass production was also evaluated using various concentrations (0.5%, 1.0%, 1.5%, 2.0% and 2.5%) in BH broth with 1% crude oil as substrate (Fig.3-7).

Mass scale culture and biosurfactant production (Li *et al*., 1984)

Using optimized parameters together, mass scale was done is shake flasks. After mass cultivation in shake flasks, the broth culture was centrifuged at 6000rpm for 20 min and extracted thrice with chloroform and methanol (2:1vol/vol). The purified biosurfactant was checked again for emulsification activity.

Biodegradation of crude oil in shake flasks (Parsons *et al*., 1984)

The potential bacterial strain was used in the biodegradation study with crude oil in shake flask (150rpm) at room temperature for 10 days in BHB. Rate of biodegradation was estimated using 1.0% crude oil. After incubation period the culture in the flask was centrifuged at 10,000 rpm and cell free aqueous solution was collected. Then hydrocarbon content in the solution was washed three times using dimethyl chloride. The extract was stored at dark at a low temperature (5°C). The solvent was evaporated to dryness in a rotatry evaporator at 30°C under reduced pressure. Made up to n-hexane and the fluorescence of the sample was measured at 310nm excitation and at 374nm emission wavelength in a Varian fluorescence spectrophotometer. Crude oil was used as standard and from the values % of degradation was calculated. The concentrate was extracted using 10ml of n-hexane (Fig.7).

Characterization of biosurfactant: fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is widely used method for identifying the types of chemical bonds (functional groups). Therefore it 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 (Table.2; Figure 8).

Results and Discussion

Isolation of hydrocarbonoclastic bacteria

Water sample were collected from the Cuddalour harbour showed density in the range of $1.12x10^4$ CFU/ml to 2.82×10^4 CFU/ml.

Screening for biosurfactant production

Haemolytic activity

The morphologically different colonies were tested for haemolytic activity on blood agar for the determination of biosurfactant production.

Maximum zone of clearance observed was 12 mm and minimum was 6 mm

BATH assay

The strains showing activity in the haemolytic assay were considered for the BATH assay, to ascertain and conformation of the biosurfactant production in which 76% of adherence was observed as the maximum. The strain which showed the maximum activity in haemolytic assay (12mm) and BATH assay with 76% was conformed as the highly potential strain for the biosurfactant production and selected for the further study.

Identification of potential biosurfactant producing microbes

The selected bacteria was identified based on its morphological, cultural and various sugar assimilation tests. On Bushnell Hass Agar plate the colony was found to be white, shiny and round. The sugar assimilation results obtained are shown in Table 1.

Optimization of Biomass production and mass scale culture

Optimization studies for biomass production were carried out and different incubation periods (0-96hrs), pH (6-11), temperature (25-40^{o}C), salinity (0.5-2.5%) using crude oil as a substrate (1%) were tested. Incubation period seems to be the important factor for the biomass production and maximum growth was observed at 96hrs. Growth in different pH values showed maximum at pH 8 and minimum at 6 . Maximum growth at temperature 30^{o}C and minimum at 25^{o}C were observed. Higher growth was observed at 2% salinity and minimum at 0.5% . Likewise regarding substrate the maximum growth was observed in glucose and minimum in cellulose and nitrogen source the maximum growth was obtained yeast extract was maximum and minimum in ammonium nitrate (Figures 1-5).

Estimation of biodegradation and emulsification activity

In the optimal conditions, 0.6 mg/ml of biosurfactant was observed. Emulsification activity of D_{610} was found to be 1.4. Crude oil degradation was studied for 6 days and degradation activity was increased on incubation time and maximum degradation was observed in 120hrs (85%) where as 50% degradation activity was observed at 48h itself by *Pseudomonas aeruginosa*. (Figure.6,7)

Biosurfactants are surface-active compounds produced by microorganisms. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures. In recent years, interest in biosurfactants has generated due to their possible applications in environmental protection, crude oil drilling, and in the pharmaceutical and food processing industries (Wong et al., 2005). Petroleum related industry was found to be one of the industries that have a great potential in producing a microorganism that may produce biosurfactants. Likewise petroleum contaminated areas like ports, harbours, garages, water-service stations which are constantly exposed to oil become rich in hydrocarbonoclastic bacteria.

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.

The study focused on using the minimal media according to Abu-Ruwaida *et al.* (1991) supplemented with crude oil as carbon and energy source for isolation of biosurfactant bacteria. In the present study the sampling area was found to harbour 2.82x10^{4}CFU/ml of hydrocarbonoclastic bacteria (i.e.) Uppanar estuary seems to be rich in oil degrading bacteria. This showed that the area might have exposed to oil pollutants regularly. The Cuddalore harbour activities along with more than

Table.1 Biochemical characteristics of *Pseudomonas aeruginosa*

Test	**Reaction**
Gram's staining	Rod, Negative
Morphological characteristics	Small, Circular, Smooth
Spore	Non-spore forming
Motility	Motile
Pigment	Green
Indole	-
Type	Aerobic
Citrate (Simmon's)	+
Growth at 42°C	+
Growth on Centrimide agar	+
Fluorescein	+
Oxidation of: Glucose (OFBM)	+
Fructose	+
Galactose	+
Mannose	+
Rhamnose	-
Assimilation of: Glucose	+
Maltose	-
D-Trehalose	-
D-Mannitol	d

Fig. 1 Effect of pH on growth

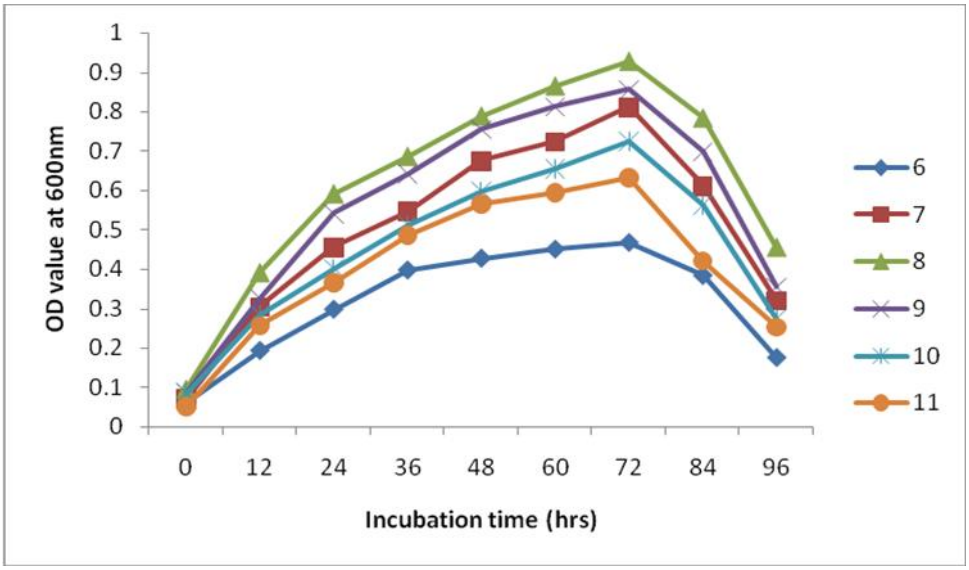

Fig.2 Effect of temperature on growth

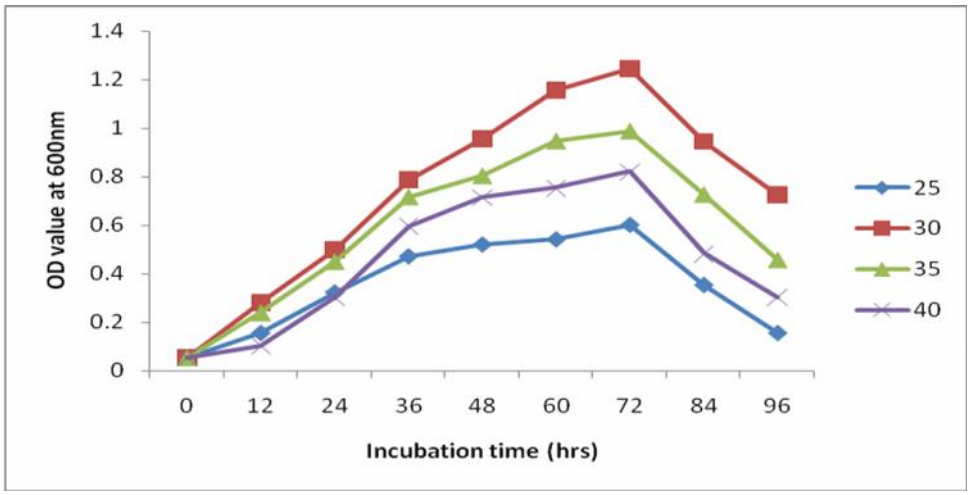

Fig.3 Effect of NaCl Concentration on growth

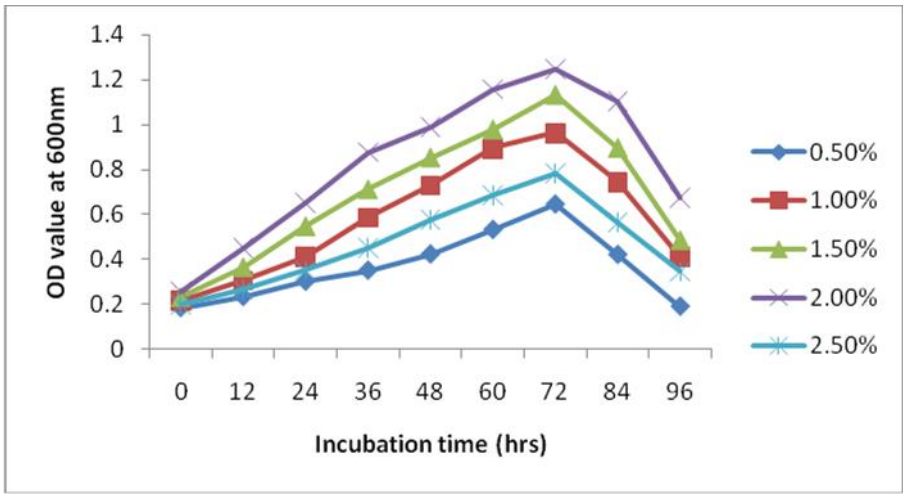

Fig. 4 Effect of carbon sources on growth

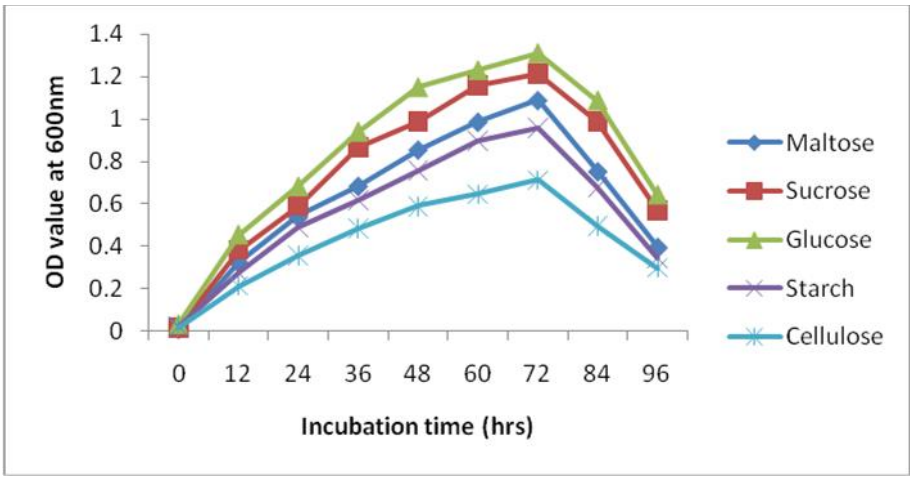

Fig. 5 Degradation of crude oil with respect to time

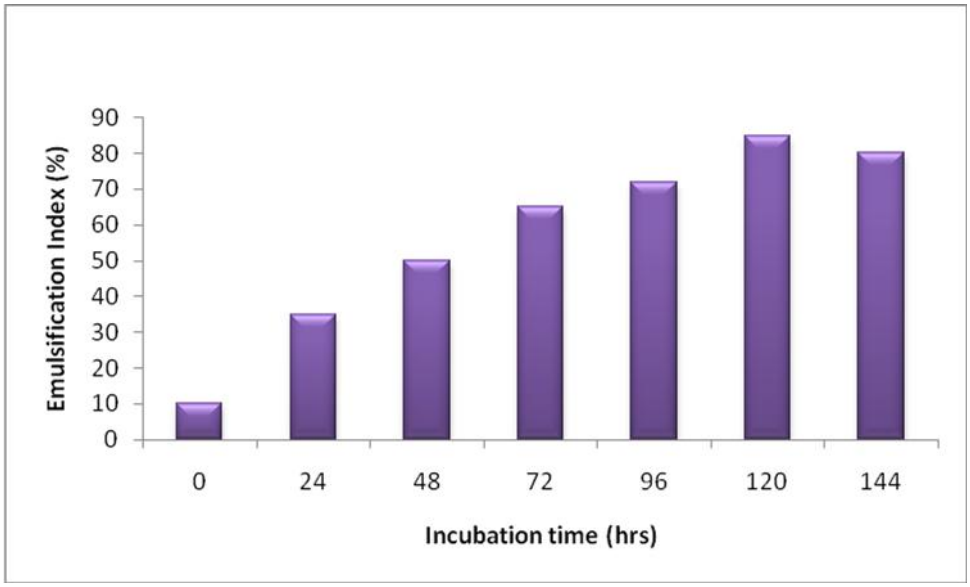

Fig. 6 Emulsification Activity of potential strain

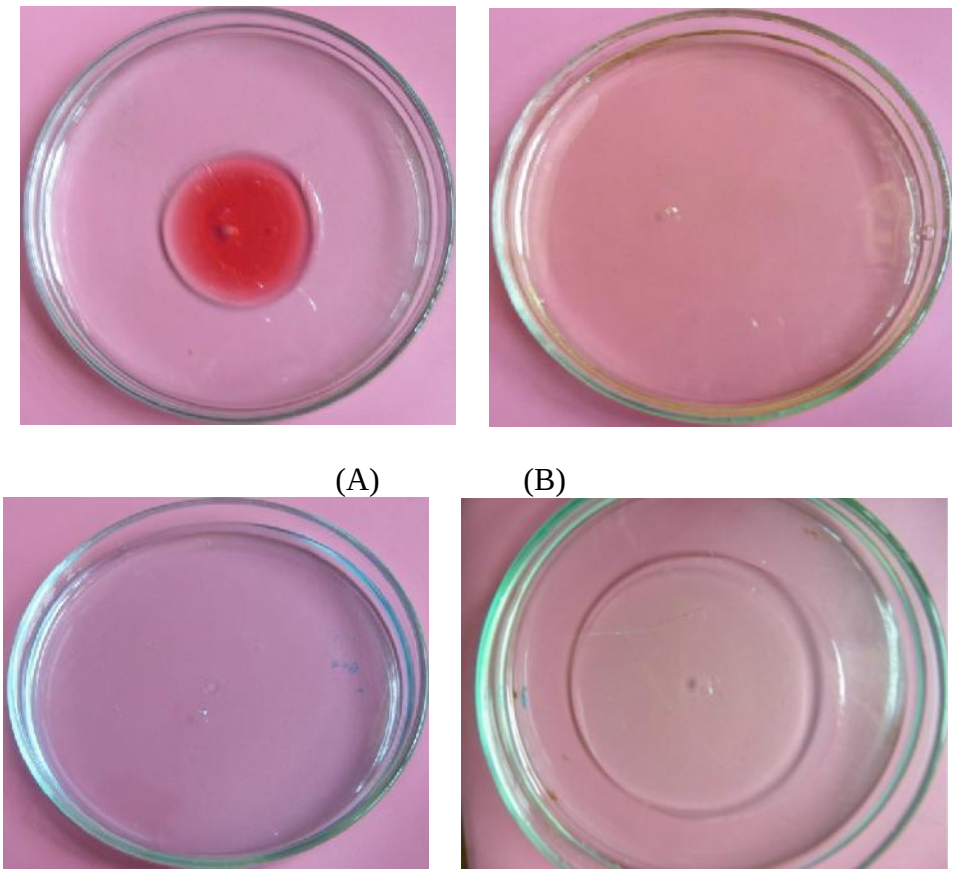

Fig. 7 oil spreading test

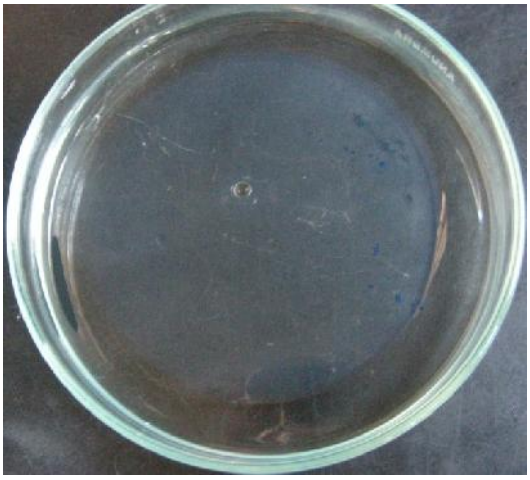

Fig. 8 Characterization of Biosurfactant using FTIR

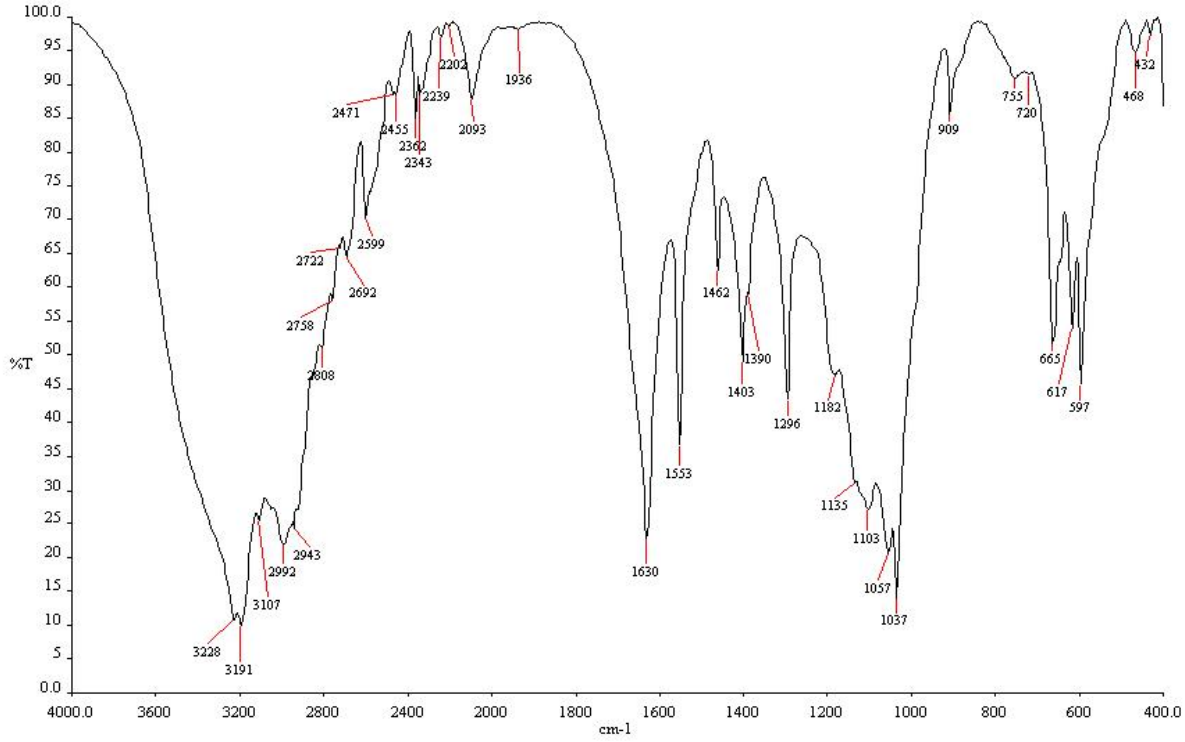

Table.2 Characterization of Biosurfactant using FTIR

3228	C - H
3191	C - H
3107	C-H
2992	C - H
2943	C - H
2808	C - H
2758	C - H
2722	C - H
2692	C Ξ C
2599	C Ξ C
2471	CΞC
2455	C Ξ C
2362	C Ξ C
2343	C Ξ C
2239	C Ξ C
2202	C Ξ C
2093	C Ξ C/C Ξ N
1936	C Ξ C
1630	N - H
1553	N - H
1462	N - H
1403	C - H
1390	C - H
1296	O - H
1182	C - F
1135	C - F
1103	C - F
1057	Phosphates
1037	Phosphates
909	Silicates
755	C - Br
720	C - Br
665	Sulfates
617	Sulfates
597	C - Br

250 mechanized boats which are involved in regular fishing activities might have contributed to oil pollution due to crude oil and diesel. Through the untreated sewage entering into this estuaring area various oil pollutants along with other pollutants might be contaminating not only this estuarine area as well as nearby coastal waters also.

When the morphologically different colonies were tested for hemolytic activity the zone of clearance was ranged from 6mm-12mm and the one with 12mm was identified biochemically as *Pseudomonas aeruginosa.* Pseudomonads in general not only ubiquitous but also bestowed with bioremediation potential to various pollutants. The reason might be their rich enzyme system with is capable of breaking unusual substrates also.

Eric Deziel, *et al.,* 1996 used *Pseudomonas aeruginosa*, for their studies on biosurfactant production. The *Pseudomonas* sp, inoculated in mineral salt medium with oil produced biosurfactants. The present study endorsed once again that the haemolytic activity could be considered as one of the major criteria for biosurfactant production. It correlates with the studies of Carrillo *et al.,* 1996 and Rashedi, *et al.,* 2005 used blood haemolysis test for screening biosurfactant producing organisms.

Biosurfactant production are sometimes detected by hemolytic activity (HA) (Yonebayashi, H., 2000). Palmisano *et al.,* 2001 recommended oil spread technique while youssef *et al.,* 2007 supported surface tension measurement for identifying oil degrading potential. It also easy to perform and to standardize and less time-consuming than surface tension measurements, makes it applicable for large scanning studies. The bacterial lipopeptide iturin A is able to cause hemolysis of human erythrocytes in a dose dependent manner (Francisco, 2005). Haemolytic activity has been used for the isolation of glycolipid biosurfactants (Mulligan *et al.,* 2001). Lai used soil collected from automobile workshops for isolation of the biosurfacter producing organisms. The culture supernatant displaces oil by 5mm. The oil displacemen method was also followed by (Kingsley Urum *et al.,* 2004) for the screening of biosurfactant micro organisms. Urum *et al.,* 2004 selected a strain (C_3) among five isolates based on screening by oil displacement method.

In the present study morphologically different colonies were tested for haemolytic assay and maximum size of zone is 12mm and minimum size is 6mm was observed which was further undergone form emulsification activity, oil spreading test and BATH assay. In the present experiment emulsification analysis was based on the large increase in turbidity of a mixture of water and oil obtained from the emulsion of the hydrocarbon in the aqueous phase. Emulsification of the insoluble substrate (oil) is an important step before degradation. Emulsification of hydrocarbons was used as a criterion for biosurfactant production by many workers (Rosenberg *et al.,* 1979 and Juwarkar and Khirsagar, 1991). In the present study on providing the optimal conditions, 0.6 mg/ml of biosurfactant was produced and emulsification activity of D_{610} = 1.4 was observed.

In the present investigation crude oil degradation by *Pseudomonas aeruginosa* was studied for 6 days and degradation activity was observed in 120hrs (85%) at temperature 30°C and pH 8, whereas 50% degradation activity was observed at 48hrs itself. Thavasi *et al* (2007) reported biodegradation of crude oil by *Candida maritime* showed maximum degradation of crude oil (85.15%), followed by *Bacillus megaterium* (78.5%), *Corynebacterium kutscheri* (76.4%) and *Lactobacillus delbrueckii* (71.6%) and similar results were obtained with bacterial strains namely *Rhodococcus rhodochrous* KUCC 8801 (93.1%), *Rhodococcus* sp. ISO1 (81.1 %), *Acinotebacter calcoaceticus* IRO7 (91.2%) and *Pseudomonas putida* IR32 (47.6%) with 5 days of incubation in another study (Sorkhoh *et al.,* 1990). The present study proved that the strain isolated might be equally good.

Biosurfactants can enhance the mobility of heavy metals by reducing the interfacial tension between the metals and soil and by forming micelles. One more attractive characteristic is that they are natural products, may be less toxic to biodegrading bacteria and can be degradable themselves. Therefore, they present effective and nontoxic candidates for the remediation of the contaminated sites. The present study resulted in one such candidates species. Thus the present study proved the bioremediation potential of *Pseudomonas aeruginosa* isolated from the Cuddalore harbour area, against crude oil, which is the major oil pollutant in coastal waters.

Conclusion

Biosurfactants are attracting attention in recent years because of the several advantages they offer over chemical surfactants and also their ability to be produced from renewable and cheaper substrates. Internet in application of biosurfactants to commercial products is on the rise. Not only do biosurfactants provide highly valued surface

properties they have a high degree of biodegradability and environmental compatility that synthetic surfactant lack. Even with potential for several application, production costs must be lowered, and high yield mutant strains need to be developed for these compounds to be profitable on a commercial scale. It is evident from this investigation that isolated bacterium degraded crude oil. *Psedomonas sps* can be used for bioremediation of crude oil pollutant from the environment. Since growth of the bacterial isolate on crude oil has been associated with the production of biosurfactant, We can conclude that the crude oil metabolizing bacterium is able to secrete surfactants which further enhance the hydrocarbon degradation. *Pseudomonal aeruginosa* ill help in developing strategies for removing crude oil from polluted areas. The results of present study showed that the isolated *Pseudomonas aeruginosa* could produce extracellular biosurfactants utilizing both hydrocarbons and sugars. The surface tension of the culture medium was lowered to different extent depending on the carbon source. Glucose was a better carbon source than all other carbon source for biosurfactant production. The emulsifing activity of the biosurfactant revealed that they could be used as emulsion forming agents for hydrocarbons and oils giving stable emulsion. The products exhibited a high level of thermal and pH stability and demonstrated a high level of toleranceto ionic strength. These observations show clear perspectives for the use of the products in extreme environmental conditions in bioremediation, pharmaceutical formulations and other industrial fields. The production of surface active compounds or biosurfactants by microorganisms has been a subject of increasing interest in recent years especially due to the potential applications in enhanced oil recovery. The study provided many valuable information about the bioremediation aspects of oil pollutantion along with characterization of product involved (i.e) the biosurfactant.

References

Abu-Ruwaida, A.S., Banat, I.M., Haditirto, S. and Khamis, A., 1991.Nutritional requirements and growth characteristics of a biosurfactant producting Rhodococcus bacterium.J.Microbiol.Biotechnol., 7:53-61.

Anandaraj, B. and Thivakaran, P., 2010. Isolation And Production of Biosurfactant Producing Organism From Oil Spilled Soil. J. Biosci. Tech., 1(3):120-126.

Banat, I.M., Makkar, R. and Cameotra, S., 2000. Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol ., 53: 495–508.

Banat, I.M., Makkar, R. and Cameotra, S., 2000. Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol ., 53: 495–508.

Carrillo, P.G., Mardaraz, C., Pitta-Alvarez, S.J. and Giulietti, A.M., 1996.Isolation and selection of biosurfactant-producting bacteria.J.World Microbiol.Biotechnol., 12: 82-84.

Cooper, D. and Goldenberg, B., 1987. Surface-active agents from two *Bacillus* species. Appl .Environ. Microbiol., 53: 224–9.

Desai, J.D. and Banat, I.M., 1997.Microbial production of surfactants and their commercial potential. Microbiol and Mol Biol ., 61: 47–64.

Eric, D., Gilles, P., Richard, V., Francois, L. and Jean-Guy, B., 1996.Biosurfactant production by a soil Pseudomonas strain growing on polycyclic aromatic hydrocarbons.Appl. and Environ.Microbiol., 62(6) :1908-1912.

Fiechter, A., 1992. Biosurfactants: Moving Towards Industrial Application. Tre. Biotechnol., 10:208-217.

Gautam, K.K. and Tyagi, V.K., 2006. A Review of Microbial Surfactant.. J. of Oleo Sci., *55*(4): 155-166.

Georgiou, G., Lin, S.C. and Sharma, M.M., 1990. Surface active compounds from microorganisms. Biotechnol., 10: 60-65.

Karanth, N.G.K., Deo, P.G. and Veenanadig, N.K., 1999. Microbial Production of Biosurfactants and their Importance. Cur.Sci., 77: 116 – 126.

Makkar, R.S. and Cameotra, S.S., 2002. An update on the Use of Unconventional Substrates for Biosurfactant Proction and Their New Applications. Appl. Biotechnol., *58*, 428-434.

Moussa, T.A.A., Ahmed, A.M. and Abdelhamid, S.M.S., 2006. Optimization of cultural Biotechnol. Adv. 9: 241 252.

Mukherjee, S., Das, P. and Sen, R., 2006. A Review of Towards Commercial Production of Microbial Surfactants.Tre.Biotechnol., *24*(11): 509-515.

Mulligan, C.N., 2005.Environmental applicationfor biosurfactants. Environ.Pollut.,133: 183–98.

Nitschke, M. and Pastore, G.M., 2006. Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Biores. Technol. 97: 336341.

Palmisano, M.M., Nakamura, L.K., Duncan, K.E. and Istok, C.A., 2001.Bacillus sonorensis species a close relative of Bacillus licheniformis, isolated from soil in the sonoran desert, Arizona.Int.J.Syst.Evol.Microbiol., 51:1671-1679.

Ron, E.Z. and Rosenberg, E., 2001. A Review of Natural Roles of Biosurfactnats. Environ. Microbiol., *3*(4): 229-236.

Rosenberg, E., Rachal, L., Ariel, K., Ran, T., Ellik, A. and Eliora, Z., 1992. Petroleum bioremediation-a multiphase problem. Biodegra., 3(3):37-350.

Wong, J.W.C., Fang, M., Zhao, Z. and Xing, B., 2005.Effect of a surfactants on solubilization and Degradation of phenanthene under Thermophilic conditions.J. of Environ.Qua., 33(6) : 2015-2025.

Wong, J.W.C., Fang, M., Zhao, Z. and Xing, B., 2005.Effect of a surfactants on solubilization and Degradation of phenanthene under Thermophilic conditions.J. of Environ.Qua., 33(6) : 2015-2025.

Yonebayashi, H., Yoshida, S., Ono, K. and Enomoto, H., 2000.Screening of microorganisms for microbial enhanced oil recovery process.J.Sekiyu Gakkaishi., 43(1) : 59-69.