



INTERNATIONAL JOURNAL OF ENGINEERING SCIENCES & RESEARCH TECHNOLOGY

Determination of Antimicrobial Activity and Production of Biosurfactant by *Pseudomonas Aeruginosa* Using Mustard Oil as Carbon Source

Priyam Vandana* and Jyotsna Kiran Peter

* Department of Microbiology & Fermentation Technology, Sam Higginbottom Institute of Agriculture Technology & Sciences, Allahabad, U.P., India

Abstracts

Biosurfactants are amphiphilic compounds produced by microorganisms as secondary metabolite. Biosurfactants are microbially produced surface active agents and occur in nature as chemical entities such as glycolipids, phospholipids and lipopeptides. The present study deals with the production and partial purification and characterization of a biosurfactant by *Pseudomonas aeruginosa*. The identification of the ability of *Pseudomonas fluorescens* to produce biosurfactants and evaluation their antimicrobial potential are the aim of this work.

Keywords: Biosurfactant, Production, Emulsification activity, Characterization, Antimicrobial activity.

Introduction

Biosurfactants are amphiphilic compounds and are mainly classified into four categories based on the hydrophilic part: glycolipid type, fatty acid type, lipopeptide type and polymer type. Biosurfactants production is an important area of research, owing to the large number of potential applications, especially as substitutes for synthetic surfactants in oil and other industries (Banat *et al* 2000; Mulligan 2005). They can be used as emulsifiers, de-emulsifiers, wetting agents, foaming agents, functional food ingredients and detergents (Kosaric 1992). The major factors restricting the commercial viability of biosurfactants are the low yield and high production cost (Mukherjee *et al* 2006; Fiechter 1992).

Some of the advantages of biosurfactants over synthetic ones include lower toxicity, biodegradability, selectivity, specific activity at extreme temperatures, pH and salinity, the possibility of their production through fermentation, their potential applications in environmental protection and management, crude oil recovery, as antimicrobial agents in health care and food processing industries (Banat *et al.*, 2000; Kosaric, 1992).

Microorganisms are able to synthesize biosurfactants from crude oil, pure hydrocarbons and different nonhydrocarbon layers such as carbohydrates, acids and alcohols (Bordoloi and Onwar, 2007). Among microorganisms only bacteria are suitable agents for handling such processes; while fungi, yeasts and seaweeds cannot be proper executive species due to their morphological characteristics and special nurturing

<http://www.ijesrt.com>

conditions. *Pseudomonas* are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants to enhance the uptake of such immiscible hydrophobic compounds (Al-Tahhan *et al* 2000; Rahman *et al* 2002).

Materials and methods

Production of biosurfactants

Bushnell Haas broth was used as the production medium for the biosurfactants. 100 ml of the Bushnell Haas broth was prepared. The sterilized medium was seeded with 24-48h old starter culture of *Pseudomonas aeruginosa* that was prepared in Nutrient broth medium (5ml) under aseptic condition. The inoculated flask was kept in a shaking incubator maintained at 35°C for 10 days interval at 160 rpm. After 10 days the broth contained the biosurfactant that was further extracted at two sub stages *viz.* crude extraction of biosurfactants and solvent extraction of biosurfactants (partially purified biosurfactants).

Crude extraction from production medium

The broth culture was centrifuged at 10,000 rpm for 30 minutes at 4°C and supernatant was collected. The pellet was discarded that contained the bacterial cell fractions. The supernatant was collected as crude biosurfactant and was further purified through solvent extraction method.

Cold Acetone precipitation:

Biosurfactant was partially purified by cold acetone precipitation. Three volumes of chilled acetone was added to the crude biosurfactant solution and allowed to stand for 10 h at 4°C. The precipitate was collected by centrifugation at 10,000rpm for 20 min and the resulting

pellet served as partially purified biosurfactant it was further allowed to be evaporate to dryness to remove residual acetone after which it was dissolved in sterile water (Ilori *et al.*, 2005).

Characterization of biosurfactant

Preliminary characterization of the biosurfactant was done by determining the following

Determination of dry weight of surfactant

Sterilized petriplate was taken and the weight of the plate was measured in grams. The sediment was poured on the plate and placed in the hot air oven for drying at 100°C for 30 minutes. After drying the plates were weighed (Anandraj and Thivakaran, 2010). The dry weight of the biosurfactants was calculated using the following formula:

Dry weight of biosurfactants = Weight of the plate after drying - weight of the empty plate

Determination of glycolipid

Partially purified biosurfactant was screened for ability to produce anionic glycolipids on blue agar plates containing CTAB and methylene blue in MSM to detect extracellular glycolipid production (Seigmund and Wagner, 1991). Dark halos formed around colonies were considered to produce glycolipid.

Penetration assay

The penetration assay is a simple, quantitative technique for screening large amounts of potential isolates (Maczek *et al.*, 2007). The phenomenon by which silica gel is entering the hydrophilic phase from hydrophobic phase in much faster if biosurfactants are present. This assay relies on the contacting of two insoluble phases which leads to a colour change.

Biochemical characterization of Biosurfactant

Chemical composition of the biosurfactant was analysed following standard methods. Carbohydrate content of the biosurfactant was determined by anthrone reagent method using 620 nm (Spiro, 1966). D-glucose used as standard. Protein content was determined by the Lowery *et al.* (1951) using bovine serum albumin as a standard. Lipid content was estimated adopting the procedure of Folch *et al.* (1956).

Determination of stability of emulsification property of partially purified biosurfactant

The stability of biosurfactant included determination of emulsification activity at different pH, temperature (°C), different concentrations of sodium chloride (%) and different hydrocarbons.

Effect of temperature

To observe the effect of different temperature on emulsification index, emulsification activity of the partially purified biosurfactants was performed by adding 2 ml of the Mustard oil to 2 ml of the aqueous solution of the partially purified biosurfactant and vortexed at high speed for 2 min. The emulsion stability was determined at 0 hour and after 24 h, 48h at varying temperatures *viz.* 10, 20, 30, 40, 50, 60, 70, 80, 90, 100°C. The emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height (cm) and multiplying by 100. (Anandraj and Thivakaran, 2010).

Effect of pH

To observe the effect of different pH on emulsification index, emulsification activity of the partially purified biosurfactants was performed by adding 2 ml of the Mustard oil to 2 ml of the aqueous solution of the partially purified biosurfactant and vortexed at high speed for 2 min. The emulsion stability was determined at 0 hour and after 24 h, 48h, 72h at varying pH *viz.* 4, 6, 8, 10, 12, 14. The emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height (cm) and multiplying by 100. (Anandraj and Thivakaran, 2010).

Effect of salt concentration

To observe the effect of salt concentration on emulsification index, emulsification activity of the partially purified biosurfactants was performed by adding 2 ml of the Mustard oil, to 2 ml of the aqueous solution of the partially purified biosurfactant and vortexed at high speed for 2 min. The emulsion stability was determined at 0 hour and after 24 h, 48h at varying NaCl conc. *viz.* 5, 10, 15, 20, 25 %. The emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height (cm) and multiplying by 100. (Anandraj and Thivakaran, 2010).

Effect of different hydrocarbons

To observe the effect of different hydrocarbons on emulsification index, emulsification activity of the partially purified biosurfactants was performed by adding 2 ml of the Mustard oil, Til oil, Coconut oil, Palm oil, Almond oil, Sunflower oil, Olive oil and Soyabean oil to 2 ml of the aqueous solution of the partially purified biosurfactant and vortexed at high speed for 2 min. The emulsion stability was determined at 0 hour and after 24 h, 48h. The emulsification index (E_{24}) was calculated by dividing the measured height of emulsion

layer by the mixture's total height (cm) and multiplying by 100.

Antimicrobial activity of the partially purified biosurfactant

The antimicrobial activity was evaluated by agar well diffusion method (Bauer *et al.*, 1966). Nutrient agar plate was prepared. After solidification of the Nutrient agar medium test bacteria was swab inoculated onto the medium and well of 5mm diameter was made using a well borer under aseptic condition. Another nutrient agar plate was swab inoculated without well that served as organism control. An uninoculated nutrient agar plate served as medium control. The wells were filled with known quantity (50µg/ml) of partially purified biosurfactants dissolved in DMSO. All the plates were allowed to incubate at 37°C for 24h to determine the zone of inhibition. Among all the wells one well for each test bacteria one was filled with same quantity of DMSO used as a treatment control. The diameter of the clear zone formed around the colony was measured using a scale in mm. (Gomma, 2012).

The bacteria tested were procured from the Microbial Culture Collection Bank (MCCB), Department of Microbiology and Fermentation Technology, Jacob School of Biotechnology and Bioengineering, SHIATS, Allahabad.

Name of the test bacteria:

Gram negative bacteria: *Salmonella typhi*, *Serratia marcescens*, *Proteus vulgaris*, *Klebsiella sp.*

Gram positive bacteria: *S. aureus*

Result and discussion

Production, partial purification and characterization of partially Purified biosurfactant

Submerged production of biosurfactants was conducted in 250 ml capacity Erlenmeyer Flasks in rotary shaker incubator at 160 rpm at 35°C for 10 days. The downstream processing of the biosurfactant involved harvesting at crude level and then partially purified using solvent purification method. The yield after solvent purification of partially purified biosurfactant was measured in terms of g/l of BH broth medium.

Determination of dry weight of surfactant

Yield of partially purified biosurfactants from *Pseudomonas aeruginosa* using Mustard oil was 18.943 g/l.

Determination of glycolipid

Glycolipid determination was done for characterizing the biosurfactant producing microbe. CTAB agar plate method is a semi quantitative assay for the detection of extra cellular glycolipids or other anionic surfactants.

Dark halos formed around colonies indicating the biosurfactant production on mineral salt medium plate containing CTAB and methylene blue. *Pseudomonas aeruginosa* showed positive results for glycolipid production as the dark blue colour halos were formed around the wells. A similar kind of study was conducted by Nishanthi *et al.*, 2010 in which colonies were surrounded by the blue dark colour halos.

Penetration assay

The penetration assay is a simple, quantitative technique for screening large amounts of potential isolates. *Pseudomonas aeruginosa* showed positive result for penetration assay. The silica gel is entering the hydrophilic phase and the upper phase will change red to cloudy white which results in mixing of the two distinct phases within 15 minutes. This assay relies on the phenomenon that silica gel is entering the hydrophilic phase from hydrophobic paste much more quickly if biosurfactants are present. A similar kind of study was conducted by Nishanthi *et al.*, 2010 in which the penetration ability of the isolates BPB7 and BPB13 resulted in mixing of two distinct phases within 15 minutes.

Biochemical characterization of Biosurfactant

Table:1 Determination of carbohydrate/ protein/ lipid contents of the partially purified biosurfactant

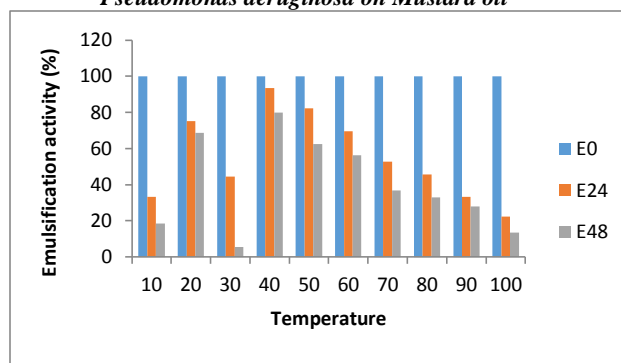
S. No	Name of isolate	Carbohydrate, protein and lipid estimation (µg/ml)		
		Carbohydrate (mg/ml)	Protein (mg/ml)	Lipid (µl/ml)
1	<i>Pseudomonas aeruginosa</i>	0.23	0.49	14

Determination of stability of emulsion for abiotic stress tolerance from partially purified biosurfactant

Effect of temperature

Effect of varying temperature *viz.* 10, 20, 30, 40, 50, 60, 70, 80, 90, 100°C was examined on stability of the emulsification index generated for emulsification of mustard oil by partially purified biosurfactant obtained from *Pseudomonas aeruginosa*. At 0h the emulsification observed was 100% at all temperatures *i.e.* 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C. At high temperature the % emulsification tend to decline while at lower temperature the emulsification stability was retained and the highest emulsion activity was observed at 40°C upto 48h. (Fig:1). A similar kind of study was conducted by Abouseoud *et al.*, 2008.

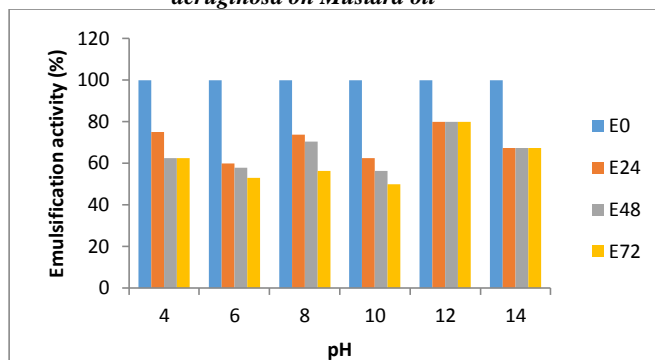
Fig:1 Effect of temperature on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas aeruginosa* on Mustard oil



Effect of pH

Effect of pH on emulsification index (%) of partially purified biosurfactant from *Pseudomonas aeruginosa* was determined for Mustard oil using a range of pH as 4, 6, 8, 10, 12 and 14 and the stability of the emulsion formed was routinely analyzed from 0 to 72 h at 24 h interval. Emulsification activity of partially purified biosurfactant from *Pseudomonas aeruginosa* was recorded 100% at 0h of incubation. It was found that the emulsification was stable at pH 12 and 14 throughout the observation period *i.e.* from 24h to 72h while there was record of gradual dropping in the emulsification index of the partially purified biosurfactant at pH 4, 6, 8 and 10. The study revealed highest emulsification stability towards extreme alkaline pH *i.e.* 12 and 14. It is also found that the emulsion was stable at a wide range of pH and the stability persisted upto 72h. These results indicate that increase pH has a positive effect on emulsification activity and stability. This could be caused by a better stability of fatty acid surfactant micelles in the presence of NaOH and the precipitation of secondary metabolites at higher pH values. The effect of pH on surface activity has been reported for biosurfactants for different microorganisms by **Abouseoud *et al.*, 2008**; **Abu-Ruwaida *et al.*, 1991**. (Fig:2).

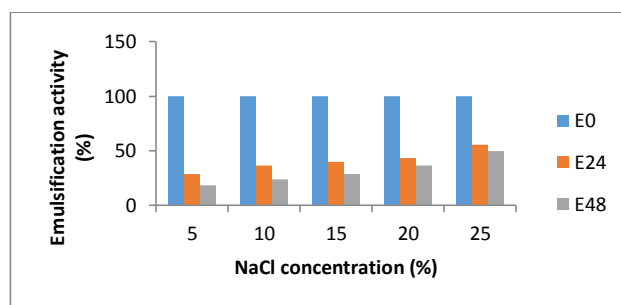
Fig:2 Effect of pH on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas aeruginosa* on Mustard oil



Effect of NaCl concentration

Effect of NaCl conc. (%) on emulsification index (%) of partially purified biosurfactant from *Pseudomonas aeruginosa* was determined for Mustard oil using a range of NaCl conc. (%) as 5%, 10%, 15%, 20% and 25% and the stability of the emulsion formed was routinely analyzed from 0 to 48 h. Emulsification activity of partially purified biosurfactant from *Pseudomonas aeruginosa* was recorded 100% at 0h of incubation. The emulsification activity was found to decline 24h incubation onwards. Optimum stability of biosurfactant was observed at 25% NaCl concentration. Little change was observed in increased NaCl concentration up to 25% (w/v) (Fig:3). Stability of emulsion in the presence of salt has been reported by **Ilori *et al.*, 2005** as one of the properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2.

Fig:3 Effect of NaCl concentration (%) on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas aeruginosa* on Mustard oil

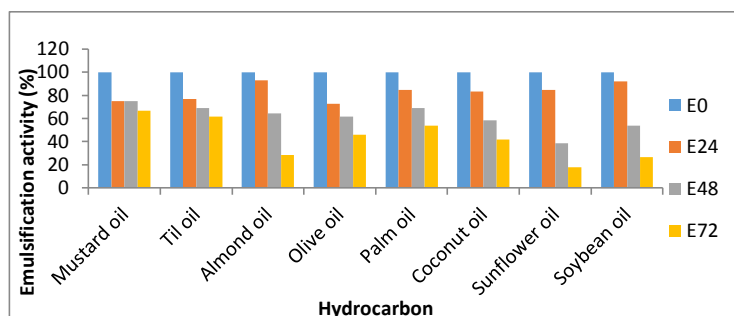


Effect of hydrocarbons

Effect of different hydrocarbons *viz.* Mustard oil, Til oil, Almond oil, Olive oil, Palm oil, Coconut oil, Sunflower, Soyabean oil was examined on stability of the

emulsification index generated for emulsification by partially purified biosurfactant obtained from *Pseudomonas aeruginosa*. At 0h the emulsification observed was 100% at all hydrocarbons *i.e.* Mustard oil, Til oil, Almond oil, Olive oil, Palm oil, Coconut oil, Sunflower, Soybean oil. The emulsification index generated for seven different vegetable oils (Mustard oil, Olive oil, Til oil, Palm oil, Soybean oil, Sunflower oil and Coconut oil) and the stability of the emulsification was analysed from 0 – 72h of incubation. In a study conducted by **Chopade et al. (2010)**, marine bacteria were examined for emulsification activity (EA) and emulsification stability (ES) of wide variety of hydrocarbons and vegetable oils. As biosurfactants monomers are added to the solution the surface or the interfacial tension decreases until the biosurfactant reaches Critical Micelle Concentration (CMC). The CMC is defined as the minimum concentration of biosurfactant required to initiate micelle formation. Above CMC no further reduction in surface or interfacial tension is observed. At CMC surfactant monomers begin to spontaneously associate into structural aggregates such as micelle, vesicles or continuous bilayers. These layers are produced as a result of numerous weak chemical interactions such as hydrophobic, vander wals and hydrogen bonding. Since, no chemical bond are formed these structures are fluid like and are easily transformed from one state to another. The aggregate structure depends on the polarity of the solvents in which the surfactants has been dissolved. In an aqueous solution, the polar head groups of a micelle are oriented outwards towards aqueous phase and hydrophobic tails will associate in the core of the micelle within oil in water micelle. In contrast, in oil the polar head groups associates towards centre of micelle with the hydrophobic tails oriented outwards within water in oil micelle. The formation of microemulsion is also possible. Vegetable oil contains 65-90% unsaturated fatty acids that are oleic acid (42-62%), linolic acid (21-34%) and linolinic acid (0-1%).

Fig:4 Effect of different hydrocarbon on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas aeruginosa*



Determination of antibacterial activity of partially purified biosurfactant

The partially purified biosurfactant obtained from *Pseudomonas aeruginosa* was examined for the antibacterial activity against selected bacterial pathogens namely, *Salmonella typhi*, *Klebsiella pneumonia*, *Serratia marscesens* and *S. aureus*. Antibacterial activity was examined on Nutrient agar plates using well diffusion assay. 50 µl of biosurfactant was dissolved in 1ml of DMSO. The zone of inhibition was recorded after 24h of incubation. *Salmonella typhi*, *Klebsiella pneumonia*, *Serratia marscesens* and *S. aureus* was inhibited as 13mm, 15mm, 20mm, 22mm from partially purified biosurfactant *Pseudomonas aeruginosa*.

In a similar research conducted by **Gomaa (2012)** antimicrobial activity of biosurfactant from *Bacillus lichemiformis* was examined against pathogens namely, *S. aureus*, *E. coli*, *S. typhimurium*, *proteus vulgaris*, *Klebsiella pneumonia* *Listeria monocytogens* with zone of inhibition in range of 10-25mm diameter. In an another research of **Hamouda and Baker, (2000)** two surfactant lipid preparations were investigated to determine the antimicrobial action of lipopeptide against gram negative bacteria. **Shai and Avrahami (2004)** studied the antimicrobial activity of active peptides against *E. coli*. The antibacterial action of biosurfactant might be an attribute of bacteriel cell membrane permeability disturbances caused by the biosurfactant.

Table:2 Determination of antibacterial activity of partially purified biosurfactant against selected bacterial pathogens

Bacterial strain	Conc. of partially purified biosurfactant ($\mu\text{g/ml}$ DMSO)	Zone of inhibition in (mm)			
		<i>Salmonella typhi</i>	<i>Klebsiella pneumoniae</i>	<i>Serratia marcescens</i>	<i>S. aureus</i>
<i>Pseudomonas aeruginosa</i>	50	15	10	15	20

Conclusion

In this study, biosurfactant produced from *Pseudomonas aeruginosa* was characterized as glycolipid. The result from the study reports that even from the cheapest carbon source like mustard oil at very less concentration of 2%, a good biosurfactant can be produced. The study directly highlights the application of biosurfactant in treatment of diseases, environmental pollution control, health care and cosmetic industry and oil industry. Biosurfactants have led considerable interest for present and future application due to non-toxic and ecofriendly nature.

Acknowledgement

I am very thankful to my family for their encouragement and full support. I extend my sincere thanks to my advisor for her assistance and kind support throughout my dissertation. The authors offer gratuitous thanks to Hon'ble Vice Chancellor, Most Rev. Prof. R.B. Lal, SHIATS, Naini, Uttar Pradesh, India for provision of research conductance. Heartfelt thanks to Head, Department of Microbiology and Fermentation Technology, JSBB, SHIATS, Allahabad, Uttar Pradesh, India for the kind cooperation towards the research.

References

1. **Abouseoud, M., Maachi R., Amrane A., Boudergua S. and Nabia A. 2008.** Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. *Journal of Microbiology and Biotechnology*. 223:143-151.
2. **Abu-Ruwaida, A.S., Banat I.M., Haditirto, S., Salem, A. and Kadri M. 1991.** Isolation of biosurfactant producing bacteria, product characterization and evaluation. *Acta Biotechnol.* 11: 315-324.
3. **Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A. and Maier, R.M. 2000.** Rhamnolipid-induced removal of

lipopolysaccharide from *Pseudomonas aeruginosa* : effect on cell surface properties and interaction with hydrophobic substrates. *Appl. Environ. Microbiol.* 66:3262.

4. **Anandraj, B. and Thivakaran, P. 2010.** Isolation and production of biosurfactant producing organism from oil spilled soil. *Journal of Bioscience Technology*. 16(3):175-181.
5. **Banat, I.M., Makkar, R.S. and S.S. Cameotra. 2000.** Potential commercial applications of microbial surfactants. *Appl. Environ. Microb.* 53: 495-508.
6. **Bauer, R. W., Kirby, M. D. K., Sherris, J. C. and Turck, M. 1966.** Antibiotic susceptibility testing by standard single disc diffusion method. *American Journal of Clinical Pathology*. 45: 493-496.
7. **Bordoloi, N.K. and Onwar, B.K. 2007.** Microbial surfactant-enhanced mineral oil recovery under laboratory condition, *Colloids and Surfaces*. 63:73-82.
8. **Chopade, A. B., Satpute, S.K., Banat, I. M., Dhakephalkar, P.K. and Banpurkar, A. G. 2010.** Biosurfactants, bioemulsifiers and expolysaccharides from marine microorganisms. *Biotechnology Advances*. 28: 436-450.
9. **Fiechter, A. 1992.** Biosurfactants: moving towards industrial application. *Trends Biotechnol.* 10: 208.
10. **Folch, J. M., Lees, M. and Stanly, H. S. 1956.** A simple method for the isolation and quantification of total lipids from animal tissues. *J. Biol. Chem.* 226:497-509.
11. **Gomaa, E. Z. 2012.** Antimicrobial activity of a biosurfactant produced by *Bacillus licheniformis* strain M104 grown on whey. *African Journal of Microbiology Research*. 6(20): 4396-4403.
12. **Ilori, M. O., Amobi, C. J. and Odocha, A. C. 2005.** Factors affecting Biosurfactant production by oil degrading *Aeromonas spp.* isolated from a tropical environment. *Chemosphere*. 6:110-116.
13. **Kosaric, N. 1992.** Biosurfactants in industry. *Pure Appl. Chem.* 64 (11): 1731-1737.
14. **Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951.** Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
15. **Maczek, J., Junne S. and Gotz P. 2007.** Examining biosurfactant producing bacteria-an

- example for an automated search for natural compounds. Application Note CyBio AG.
16. **Mukherjee Das, S.P. and R, Sen. 2006.** Towards commercial production of microbial surfactants. *Trends Biotechnol.* 24:509.
 17. **Mulligan, C.N. 2005.** Environmental applications for biosurfactants. *Environ. Pollut.* 133:183.
 18. **Nisanthi, R., Kumaran, S., Palan, P., Chellaram, C., Prem Anand, T. and Kannan, V. 2010.** Screening of biosurfactant from hydrocarbon degrading bacteria. *Journal of Ecobiotechnology.* 2(5): 47-53.
 19. **Rahman, K.S.M., Rahman, T.J., McClean, S., Marchant, R. and Banat, I.M. 2002.** Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost raw materials. *Biotechnol. Prog.* 18:1277.
 20. **Shai, Y. and Avrahami, D. 2004.** A new group of antifungal and antibacterial lipopeptides derived from non-membrane active peptides conjugated to palmitic acid. *The Journal of Biological Chemistry.* 279 (13):12277–12285.
 21. **Siegmund, I. and Wagner, F. 1991.** New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnology Techniques.* 5(4): 265-268.
 22. **Spiro, R.G. 1996.** Analysis of sugar found in glycoproteins. *Methods. Enzymol.* 8: 7-9