PRODUCTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY PSEUDOMONAS FLUORESCENS

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ABSTRACT

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. These molecules have attracted considerable scientific attention due to lower toxicity, higher biodegradability. The present study deals with the screening, production and partial purification of a biosurfactant by Pseudomonas fluorescens. The properties of biosurfactant that was separated by acetone precipitation. The biosurfactant produced was a rhmnolipid-type in nature. It had a good foaming and emulsifying activities.

Keywords: Biosurfactant, Production, Rhamnolipid, Characterization.

I. INTRODUCTION

Biosurfactants are amphiphilic compounds that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and solid, and consequently, increase the surface areas of insoluble compounds leading to increased mobility, bioavailability and subsequent biodegradation and emulsification **(Ron and Rosernberg, 2001; Vasileva-Tonkova and Gesheva, 2007).** The biological function of this surface-active compound is related to hydrocarbon uptake, where spontaneous release occurs with hydrocarbons as substrates **(Guerra-Santos *et al.*, 1984; Makkar and Cameotra, 1998; Singh *et al.*, 2006).**

Biosurfactants are categorized by their chemical composition and microbial origin. One of the prevalent class is glycolipids constituting mono-, di-, tri- saccharides produced by *Rhodococcus erythropolis* used in oil spill cleanup operation **(Peng *et al.*, 2007)**. Sophorolipids are another class of biosurfactant produced by *Candida bombicola* having environmental application **(Daverey and Pakshirajan, 2010)**. Rhamnolipid produced by *Pseudomonas aeruginosa* too have application in bioremediation of oil contaminated sites **(Chen *et al.*, 2007)**. Lipopeptide produced by *Bacillus subtilis* strain have potential application in pharmaceutics, cosmetics and oil recovery are also reported. **(Wang *et al.*, 2008).**

Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity, high biodegradability, better environment compatibility **(Georgion *et al.*, 1990)** high foaming, selectivity and specific activity, efficient under broad range **(Velikonja, 1993)** and the ability to be synthesized from renewable feedstoks. These compounds reduce the surface and interfacial tensions in both water solutions and hydrocarbon mixtures, which makes them potential candidates for enhancing oil recovery **(Banat, 1995)** and de emulsification processes **(Banat, 1997)**.

II. MATERIALS AND METHODS

2.1 Screening of isolate for biosurfactant prodction

2.1.1 Blood hemolysis

Hemolytic activity was tested using blood agar plate containing 5% sheep blood. The isolate was streaked on blood agar and incubated at 30°C for 48-72 hrs **(Carillo *et al.*, 1996).**

2.2 Production of biosurfactant

Bushnell Haas broth was used as the production medium for the biosurfactant.100 ml of the Bushnell Haas broth was inoculated with 24-48h old culture of *Pseudomonas fluorescens* that was prepared in Nutrient broth medium (5ml). The inoculated flask was kept at room temperature in a shaking condition. The culture broth was centrifuged at 10000 rpm for 15 min) to remove the cells and clear sterile supernatant was obtained **(Abouseoud *et al.*, 2007).**

2.3 Biosurfactant Recovery

The biosurfactant was recovered from the cell free culture supernatant by cold acetone precipitation method **(Pruthi and Cameotra, 1995).**

2.4 Qualitative tests

2.4.1 Oil displacement assay

30ml of distilled water was taken in a Petri-plate. 1 ml of Mustard oil was added to the centre of the plate containing distilled water. Then 20µl of the surfactant was poked into the oil drop.The biosurfactant producing organism displaced the oil (increase in diameter) and spread in the water. **(Anandraj and Thivakaran, 2010).**

2.4.2 Drop collapse test

A drop of mustard oil was placed on the slide and then 10µl of the surfactant was added by piercing the drop using micropipette without disturbing the dome shaped of the oil. The drop collapsed within 1min was considered to be positive for the drop collapse test **(Das and Chandran, 2010).**

2.5 Quantitative test

2.5.1 Estimation of protein content in crude and partially purified surfactant

Protein content of the sample was measured by Lowry's method and bovine serum albumin (BSA) was used as standard.

2.6 Biosurfactant Characterization

The crude biosurfactant recovered was characterized on the basis of structural and activity characterization.

2.6.1 Structural characterization

Rhamnose test

The presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose test **(Dubois *et al.*, 1956)**. A volume of 0.5ml of surfactant was mixed with 0.5 ml of 5% phenol and 2.5ml of sulfuric acid and incubated for 15min before measuring absorbance at 490 nm.

2.6.2 Activity characterization

Foaming and emulsifying properties

The foam was produced by hand shaking a 5 g/l of crude biosurfactant solution for several minutes. The stability of the foam was monitored by observing it during 2h. The ability of the biosurfactant to emulsify some liquid hydrocarbons, such as mustard oil, olive oil, soyabean oil, coconut oil and palm oil was determined. The sterile biosurfactant (2ml) was added into each test tube (in a set of three) containing the substrate (2ml). The content of the tubes was vortexed at high speed for 2min and left undisturbed for 24h **(Cooper and Goldenberg, 1987).**

III. RESULT AND DISCUSSION

3.1 Blood hemolysis

The present study revealed the β-hemolytic pattern on blood agar for screening biosurfactant activity of *Pseudomonas fluorescens* (Fig:1). Similar study was conducted by **Anandraj and Thivakaran, (2010)** in which *Pseudomonas* was screened for biosurfactant producing activity on blood agar medium that showed an alpha hemolytic pattern. Blood haemolysis assay is used for priliminary screening of microorganism for the ability to produce biosurfactants. Therefore, those microorganisms which shows positive blood haemolysis are considered as potential biosurfactant producers. The approach to the screening method is valid because biosurfactants would cause lysis of erythrocytes. The assay also predicts about the surface activity of biosurfactant producing microorganisms. In comparision to the present study similar result with culture supernatant of *Pseudomonas aeruginosa* was observed alpha haemolytic activity by **Satpute *et al.* (2008); Nicholls *et al.*(2000); Anandraj and Thivakaran (2010); Samanta *et al.* (2012) and Sneha *et al.* (2012)** who used blood haemolysis test for screening of biosurfactant producing organisms.

Fig:1 Blood hemolysis

3.2 Oil displacement assay and Drop collapse test

Pseudomonas fluorescens showed drop collapse and oil displacement test positive for mustard oil. The drop collapse test and the oil displacement test were conducted for the primary screening of biosurfactant production. These qualitative tests are indicative of surface and wetting activities **(Youssef *et al.*, 2004).** The oil displacement test is an indirect measurement of surface activity of a surfactant sample tested against oil; a larger diameter represents a higher activity of the testing solution **(Rodrigues *et al.*, 2006).** The presence of biosurfactant results in displacement of oil and clearing zone formation. The diameter of clearing zone on the oil surface correlated to surface activity. Surfactant has a linear correlation between quality of surfactant and clearing zone diameter. A positive drop collapse test showed a preliminary indication of the biosurfactant activity of the bacterial cell that clearly indicated production of biosurfactant by the bacterial cell. The positive drop collapse assay also revealed about the extracellular production of the biosurfactant and its surface active nature. The study conducted by **Das and Chandran, (2010)** is in accordance with the present investigation.

Table:1 Estimation of Protein content in crude and partially purified biosurfactant

Isolate	Crude surfactant (mg/ml)	Partially purified surfactant (mg/ml)
Pseudomonas fluorescens	0.24	0.51

3.3 Biosurfactant characterization

3.3.1 Structural Characterization

The optical density increase with increasing the concentration of supernatant which confirmed that the rhamnose test was positive and seperated biosurfactant could be of glycolipid type. (Table:2). **Gujar and Hamde (2012)** also reported rhamnose test positive indicating biosurfactant could be of rhamnolipid type. **Abouseoud *et al.* (2007)** also reported rhamnose test was positive which indicates that the separated biosurfactant was of glycolipid type.

Table:2 Quantitative estimation of carbohydrate by rhamnose test.

S. No.	Concentration (ml)	Optical Density (490nm)
1	Blank	0
2	0.2	0.72
3	0.4	0.89
4	0.6	1.00
5	0.8	1.49
6	1.0	1.67
7	1.2	1.89

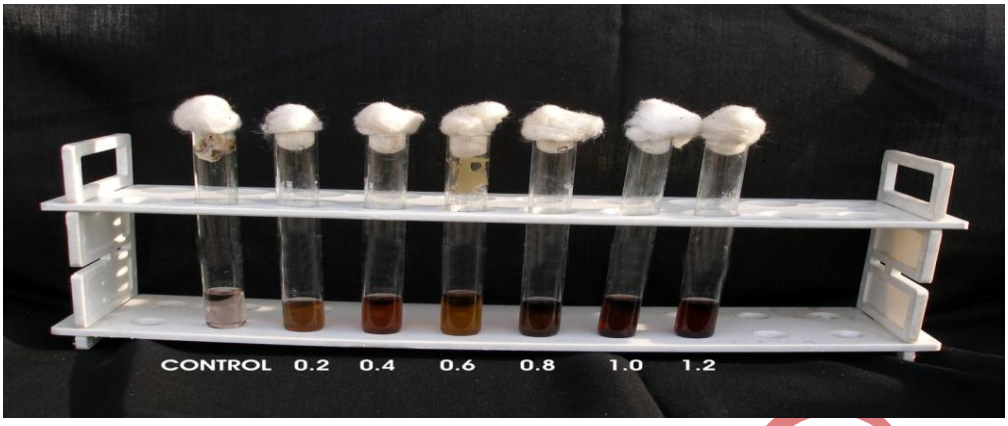

Fig:2 Rhamnose test

3.3.2 Activity characterization

Foaming and emulsifying properties

Total disappearance of the foam was observed at 1hr 18min. **Abouseoud *et al.*, (2007)** reported total disappearance of the foam was detected after 2h. **Gujar** and **Hamde (2012)** also reported total disappearance of the foam was detected after 2h. Emulsification activity gave indication on the presence of biosurfactant. Higher emulsification index indicated a higher emulsification activity of the tested biosurfactant. Palm oil was the best substrates for biosurfactant having higher emulsification activity followed by soyabean, olive and mustard oil and least by coconut oil at 0h, 24h, 48h, 72h respectively. (Fig:3).The findings of the present study had revealed about the surface active nature of bacterial strains screened to show emulsification activity as a property of biosurfactant produced by them. Formation of emulsion usually results from the dispersion of liquid phase **(Desai and Banat, 1997).** 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. Similar study was conducted by **Aparna *et al.*, (2011)** reported maximum emulsification activity of *P. aeruginosa* at 72h (80%). **Priya and Usharani (2009)** reported E_{24} 40% at 24 h. 72% were also reported **Sneha *et al.*, (2012).**

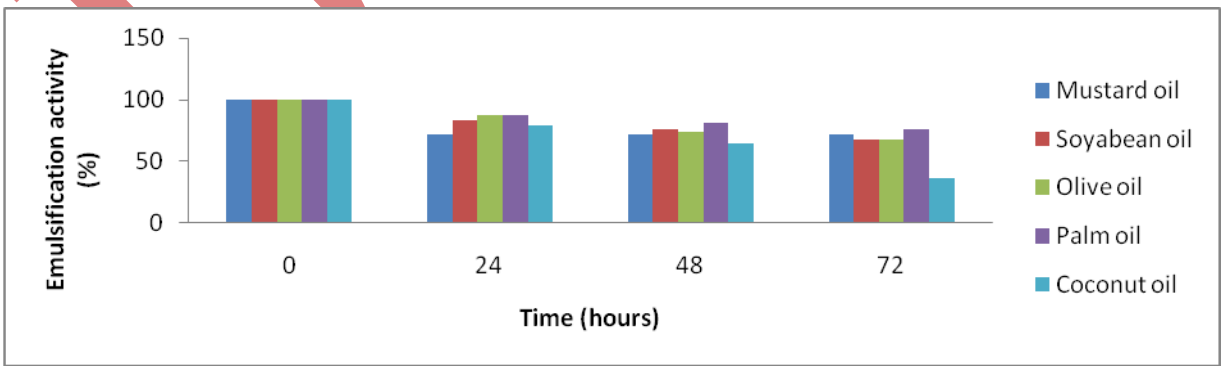

Fig:3 Emulsification activity (%) of *Pseudomonas fluorescens* on selected vegetable oils

IV. CONCLUSION

In this era of green technology biosurfactant have led considerable interest for present and future application. In this study, biosurfactant produced from *Pseudomonas fluorescens* was chemically characterized as glycolipid

mainly consisting of lipid and carbohydrate. 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. This has opened up a practically significant and commercially viable biotechnological approach to produce varities of biosurfactants having higher huge industrial application.

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