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Partial Purification and Characterization of Biosurfactant from *Pseudomonas Aeruginosa*

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Abstracts

Biosurfactant are an amphiphilic compound produced by various bacteria and fungi which reduces surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the surface areas of insoluble compounds leading to increased mobility. 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 aeruginosa. 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, Characterization, Rhamnolipid, Emulsification, Qualitative analysis, Quantitative analysis.

Introduction

Research in the area of biosurfactants has expanded quite a lot in recent years due to its potential use in different areas, such as the food industry, agriculture, pharmaceutics, the oil industry, petrochemistry and the paper and pulp industry amongst others. Biosurfactants are polymers totally or partially extracellular with an amphipathic configuration containing distinct polar and non-polar moieties which allow them to form micelles that accumulate at interface between liquids of different polarities such as water and oil. Many recent studies have focused on the use of microorganism in the production of biosurfactants due to their broad range of functional properties and diverse synthetic capabilites of microbes. They reduce the surface tension of aqueous media and the interfacial tension of liquid-liquid or solid-liquid systems **(Cameotra and Makkar, 1998)**.

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).**

Several structural homologs of rhamnolipids are produced by different strains of *Pseudomonas aeruginosa*. The type of rhamnolipid produced depends on the bacterial strain, the carbon source used and the process strategy **(Itoh *et al.*, 1971; Lang and Wullbrandt, 1999; De´ziel *et al.*, 2000)**. Rhamnolipids are formed by one or two rhamnose molecules linked to one or two fatty acids of saturated or unsaturated alkyl chain between C8 and C12 **(Rendell *et al.*, 1990)**. *Pseudomonas aeruginosa* has been reported to produce two main rhamnolipid homologs, L-rhamnopyranosyl-hydroxydecanoyl hydroxydecanoate (Rha-C10-C10) and 2-O-L-rhamnopyranosyl-L-rhamnopyranosyl-hydroxydecanoyl-hydroxydecanoate (Rha-Rha-C10-C10), when grown on olive oil waste water **(Mercade *et al.*, 1993)** or on frying oils **(Haba *et al.*, 2000).**

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. Biosurfactants can improve the bioavailability of hydrocarbons to the microbial cells by increasing the area of the aqueous hydrocarbon interface. This increases the rate of hydrocarbon dissolution and their utilization by microbes **(Tuleva *et al.*, 2002).** Among the best studied biosurfactants are rhamnolipids that belong to glycolipid class.

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Materials and methods

Screening of isolate for biosurfactant prodction

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).**

Lipase assay

Tributyrin agar (Appendix 1.3) plates was prepared using nutrient agar and Tributyrin(1%). The pH of the medium was adjusted to7.3-7.4 using 0.1 N NaOH. The isolates were streaked on the Tributyrin agar plates and incubated at 30ºC for 24-48 h. The plates were then examined for the zone of clearance around the colonies.

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 aeruginosa* 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 (10000 rpm 15 min) to remove the cells and clear sterile supernatant was obtained **(Abouseoud *et al.*, 2007).**

Biosurfactant Recovery

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

Qualitative tests

Oil displacement assay

30ml of distilled water was taken in a Petri-plate. 1ml 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).**

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)**

Quantitative test

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.

Biosurfactant Characterization

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

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.

Activity characterization

Foaming and emulsifying properties

The foam was produced by hand shaking a 5g/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).**

Result and discussion

Blood hemolysis

The present study revealed the β-hemolytic pattern on blood agar for screening biosurfactant activity of *Pseudomonas aeruginosa* (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); Samanta *et al.* (2012) and Sneha *et al.* (2012)** who used blood haemolysis test for screening of biosurfactant producing organisms.

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Fig-1: Blood hemolysis

Lipase assay

Zone of clearance was observed around the colonies of *Pseudomonas aeruginosa* indicating the lipase test positive. (Fig:2). **Ni'matuzah *et al.*, (2012)** in his study has screened the *Bacillus subtilis* for biosurfactant activity and production as in present investigation. Lipase enzyme can catalyse lipid hydrolysis reaction on interfacial oil and water. The hydrolysis of lipid might be due to formation of enzyme surfactant complex.

Fig-2: Lipase assay

Oil displacement assay and Drop collapse test

Pseudomonas aeruginosa 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 aeruginosa	0.36	0.59

Biosurfactant characterization

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	0.66
3	0.4	0.93
4	0.6	1.18
5	0.8	1.59
6	1.0	1.83
7	1.2	1.98

Activity characterization

Foaming and emulsifying properties

Total disappearance of the foam was observed at 1hr 35min. **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. Mustard oil was the best substrates for biosurfactant having higher emulsification activity followed by soyabean, palm and olive oil and least by coconut oil at 0h, 24h, 48h, 72h respectively. (Fig:3). Upon analysing the data statistically using two way ANOVA the differences in data found significant due to time and non-significant

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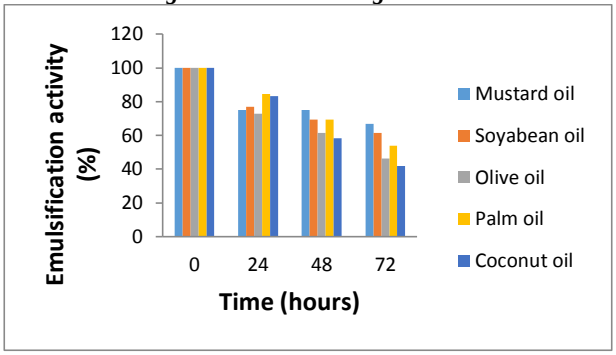
due to oil. The findings of the present study had revealed about the surface active nature of bacterial strains screened to show emulsification activity as a property of biosurfactant produced by them. Formation of emulsion usually results from the dispersion of liquid phase **(Desai and Banat, 1997).** 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-2: Emulsification activity (%) of Pseudomonas aeruginosa on selected vegetable oils



Due to oil- $F_{(cal)}=1.72<F_{(tab)}=3.26$, (NS)
Due to time- $F_{(cal)}=50.57>F_{(tab)}=3.49$, (S), SE=3.49, CD at 5% level of significance=5.74

Conclusion

In this study, biosurfactant produced from *Pseudomonas aeruginosa* was chemically characterized as glycolipid mainly consisting of lipid and carbohydrate. The emulsifying activity of the biosurfactant revealed that they could be used as emulsion forming agents for hydrocarbons and oils, giving stable emulsions. Future prospects of the present study include, biodegradation of oil, genetic characterization, biosurfactant production by immobilized cells or from precurssor supplements or by biotransformation so that the work can be carried upto pilot scale and eventually large scale production may be achieved industrially. This suggest that it is suitable for use in oil fields, biomedical application and removal of oil pollution.

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