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RESEARCH ARTICLE

BIOCHEMICAL CHARACTERIZATION AND ANTIMICROBIAL PROPERTIES OF RHAMNOLIPIDS PRODUCED BY PSEUDOMONAS FLUORESCENS USING MUSTARD OIL AS CARBON SOURCE

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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 production and partial purification and characterization of a biosurfactant by Pseudomonas fluorescens. The properties of biosurfactant that was separated by acetone precipitation. The identification of the ability of pseudomonas fluorescens to produce biosurfactants and evaluation their antimicrobial potential are the aim of this work.

INTRODUCTION

Biosurfactants are valuable microbial amphiphilic molecules with effective surface-active and biological properties applicable to several industries and processes. Microbes synthesize them, especially during growth on water-immiscible substrates, providing an alternative to chemically prepared conventional surfactants. These molecules could be widely used in cosmetic, pharmaceutical, and food processes as emulsifiers, preservatives, and detergents, and in bioremediation processes. They can be produced from various substrates, mainly renewable resources such as vegetable oils, distillery and dairy wastes. Surfactants, or surface active agents, can be classified into two main groups: synthetic surfactants and biosurfactants. Synthetic surfactants are produced by organic chemical reactions, while biosurfactants are produced by biological processes, being excreted extracellularly by microorganisms such as bacteria, fungi, and yeast. When compared to synthetic surfactants, biosurfactants have several advantages, including low toxicity, high biodegradability, low irritancy, and compatibility with human skin (Banat *et al.*, 2000; Cameotra and Makkar, 2004). 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). Biosurfactants can retain their properties even under extreme conditions of pH, temperature, and salinity. Due to these superior characteristics, biosurfactants have potential use in petroleum, petrochemical, cosmetics, and pharmaceutical industries (Desai and Banat, 1997). Biosurfactants can aggregate at interfaces between fluids having different polarities, such as water and oil, leading to the reduction of interfacial tension. Because of their efficiency in lowering interfacial tension, biosurfactants have been employed for the enhancement of oil production.

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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 fluorescens* 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 hydrocabons

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 marscens*, *Proteus vulgaris*, *Klebsiella sp.*
Gram positive bacteria: *S. aureus*

RESULTS 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 fluorescens* using Mustard oil was 24.835 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 fluorescens* 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 fluorescens* 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	*Pseudomonas fluorescens*	0.25	0.53	16

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 fluorescens*. 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 (Fig. 1). A similar kind of study was conducted by Abouseoud *et al.* (2008).

Effect of pH

Effect of pH on emulsification index (%) of partially purified biosurfactant from *Pseudomonas fluorescens* 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 fluorescens* 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 96h while there was record of gradual dropping in the emulsification index of the partially

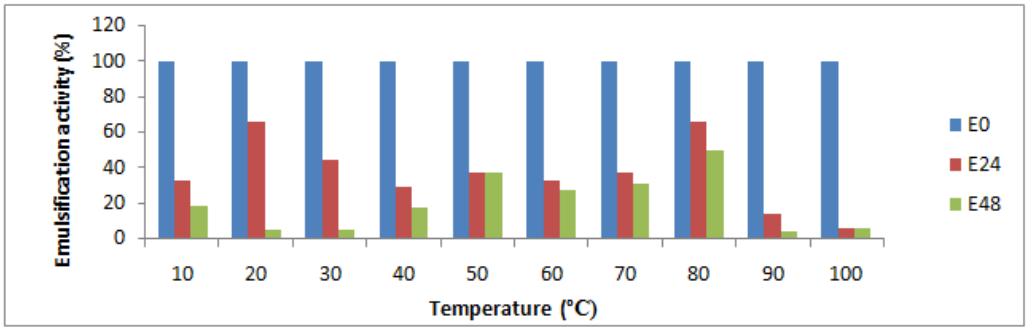

Fig. 1. Effect of temperature on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas fluorescens* on Mustard oil

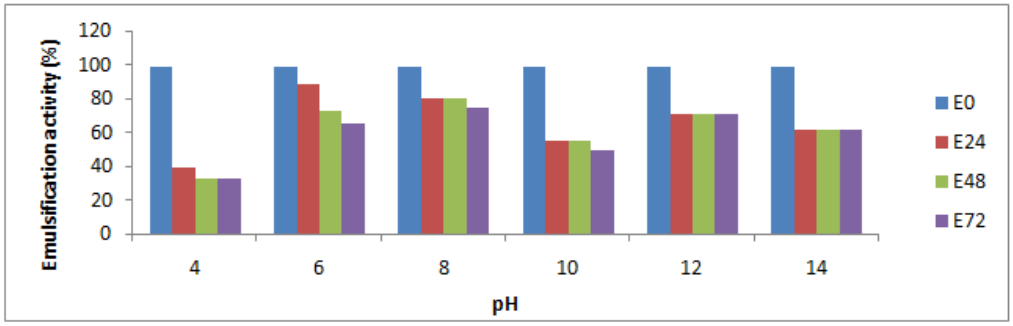

Fig. 2. Effect of pH on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas fluorescens* on Mustard oil

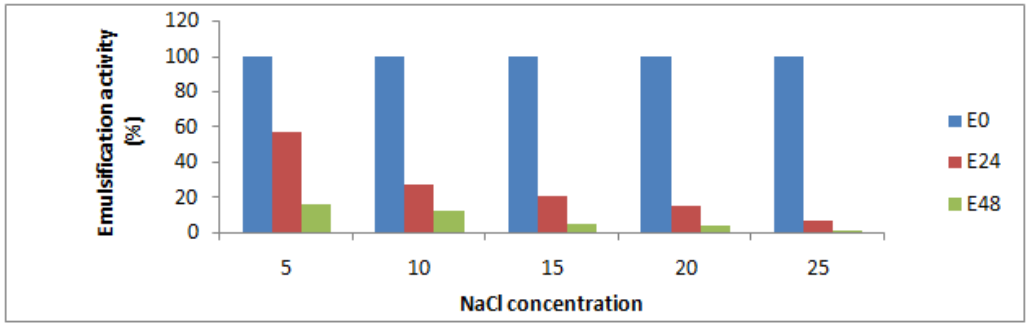

Fig. 3. Effect of NaCl concentration (%) on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas fluorescens* on Mustard oil

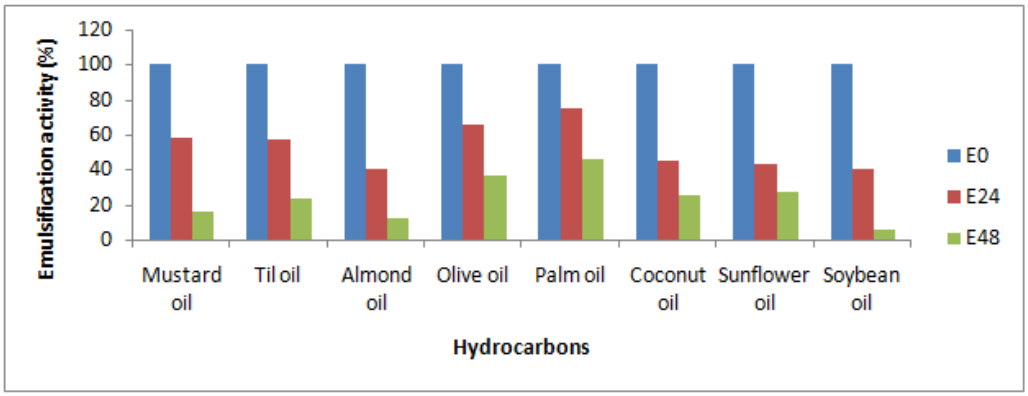

Fig. 4. Effect of different hydrocarbon on stability of emulsification activity of partially purified biosurfactant produced by *Pseudomonas fluorescens*

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

Bacterial strain	Conc. of partially purified biosurfactant (µg/ml DMSO)	Zone of inhibition in (mm)			
		Salmonella typhi	*Klebsiella pneumoniae*	*Serratia marscesens*	*S. aureus*
Pseudomonas fluorescens	50	13	15	20	22

purified biosurfactant at pH 4, 6, 8 and 10. The study revealed highest emulsification 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 96h. 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 (Fig. 2). 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).

Effect of NaCl concentration

Effect of NaCl conc. (%) on emulsification index (%) of partially purified biosurfactant from *Pseudomonas fluorescens* 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 fluorescens* was recorded 100% at 0h of incubation. The emulsification activity was found to decline 24h incubation onwards. Optimum stability of biosurfactant was observed at 5% NaCI concentration. Little change was observed in increased NaCI 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.

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 fluorescens*. At 0h the emulsification observed was 100% at allhydrocarbons *i.e.* Mustard oil, Til oil, Almond oil, Olive oil, Palm oil, Coconut oil, Sunflower, Soyabean oil. The highest emulsification was observed with Til oil (Fig. 4). The emulsification index generated for five different vegetable oils (Mustard oil, Olive oil, Soy bean oil, Sunflower oil and Coconut oil) and the stability of the emulsification was analysed from 0 – 48h 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 biosurfatants reaches Critical Micelle Concentration (CMC).

The CMC is defined as the minimum concentration of biosurfatant 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 aquous solution, the polar head groups of a micelle are oriented outwards towards aquous 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 olso possible. Vegetable oil contains 65-90% unsaturated fatty acids that are oleic acid (42-62%), linolic acid (21-34%) and linolinic acid (0-1%).

Determination of antibacterial activity of partially purified biosurfactant

The partially purified biosurfactant obtained from *Pseudomonas fluorescens* 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 13 mm, 15 mm, 20 mm, 22 mm from partially purified biosurfactant *Pseudomonas fluorescens.*

In a similar research conducted by Gomaa (2012) antimicrobial activity of biosurfactant from *Bacillus lichemniformis* 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 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 bactariel cell membrane permeability disturbances caused by the biosurfactant.

Conclusion

In this study, biosurfactant produced from *Pseudomonas fluorescens* 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.

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