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Studies on biosurfactant production by *Pseudomonas aeruginosa* R2 isolated from oil contaminated soil sample

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A biosurfactant producing strain was isolated from soil sample obtained from coconut oil mill and identified as *Pseudomonas aeruginosa* R2 based on physiological and biochemical test together with 16s rRNA sequence analysis. Primary screening for biosurfactant producer was carried out by observing hemolysis on Superimposed blood agar and zone of clearance on Tributyrin agar. Optimization of culture conditions involved use of various vegetable oils as carbon source and different organic as well as inorganic compounds as nitrogen source, of which 1 per cent coconut oil and 0.4 per cent ammonium nitrate at pH 7 when kept at 30^0C for 120 rpm/96 hours showed maximum biosurfactant yield. The biosurfactant was partially purified using chloroform and ethanol mixture (2:1) and quantitatively estimated by Anthrone assay which was found out to be 1.7 g/L. The biosurfactant could reduce the surface tension up to 35 mN/m with 70 per cent emulsification index (E24) in 36hrs. TLC analysis of biosurfactant demonstrated rhamnose as a sugar moiety and FT-IR results confirmed it to be rhamnolipid type of biosurfactant. It also exhibited antimicrobial activity and showed stability on exposure to high temperature (up to 100^0C). Emulsification activity found with the biosurfactant against hydrocarbons shows its possible application in bioremediation of environments polluted with oils.

Key words : Biosurfactant, Rhamnolipid, Emulsification activity, CTAB- Cetyl-trimethylammonium bromide, SIBA- Superimposed blood agar.

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INTRODUCTION

Microbial surfactants are low molecular weight surface-active metabolites which facilitate the diffusion of insoluble substrates like hydrocarbons into the cell (Panesar *et al.,* 2011) reducing the surface tension and interfacial tension in both aqueous solutions and hydrocarbon mixtures (Desai and Banat 1997; Zhang and Miller, 1995) They are made up of diverse group of chemical structures such as glycolipids, lipopeptides, lipoproteins, fatty acids, neutral lipids and phospholipids (Banat *et al.,* 2010). The features that make them commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability, greater environmental compatibility, better foaming properties and stability at extremes of pH, salinity and temperature (Desai and Banat, 1997). They possess anti-bacterial, antifungal and antiviral activity. They also exhibit biomedical properties like inhibition of fibrin clot and display anti-adhesive action against several pathogenic microorganisms (Banat *et al.,* 2000; Cameotra and Makkar, 2004; Gautam and Tyagi, 2005; Rodrigues *et al.,* 2006).

The genus *Pseudomonas* is capable of using different substrates such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils to produce rhamnolipid type of biosurfactant (Desai and Banat, 1997; Koch *et al.,* 1991). Unlike chemical surfactants, which are mostly derived from petroleum feedstock, biosurfactant molecules can be produced by using cheaper agro based substrates and waste materials (Abouseoud *et al.,* 2008).

The present study focuses on the screening, isolation and identification of the biosurfactant producing bacterium from soil obtained from coconut oil mill. We report optimization of media and growth conditions for biosurfactant production by *Pseudomonas aeruginosa* strain R2. The properties and initial chemical characterization of the biosurfactant are also presented.

RESEARCH METHODOLOGY

Sample collection, enrichment and screening of biosurfactant producer :

Soil sample from the vicinity of coconut oil mill was collected and 1 g of it was inoculated in Nutrient Broth (100ml)

containing 1 per cent coconut oil. The inoculated media was kept on rotary shaker at 120 rev min^{-1} for 7 days at 30^0C for enrichment and enriched sample was used for isolation on Nutrient Agar with 1per cent coconut oil. The obtained isolates were screened for their biosurfactant activity by observing hemolytic activity on SIBA (Abouseoud *et al.,* 2008), zone of clearance on Tributyrin agar (Sangcheol *et al.,* 2008) and positive CTAB test (Siegmund and Wagner, 1991).

Isolates showing all the three above tests positive were then inoculated in Nutrient Broth (100ml) with 1 per cent coconut oil and incubated at 30^0C (120 rev min^{-1}) for 7 days. The surface tension of the cell free supernatant was measured by using Du Nouy ring-type Tensiometer (Zhang and Miller, 1992). The values recorded are mean of three measurements. All measurements were made on cell free supernatant obtained by centrifuging the cultures at 8000 rpm for 20 minutes.

Identification of the potential biosurfactant producer :

The promising isolate was identified using Bergey's Manual of Determinative Bacteriology, 8th edition and the strain was confirmed by 16s rRNA analysis (Enzene Bioscience, Bangalore).

Screening of media for the biosurfactant production :

The given isolate was inoculated in the following media (i) Medium E (Najafia *et al.,* 2011) (ii) Mineral Salt Medium (MSM) (Abouseoud *et al.,* 2008), (iii) Biosurfactant Production Liquid Medium (BPLM) (Nie *et al.,* 2010) and (iv) Medium SM (Makkar and Cameotra, 1998).The enriched broth was then subjected to centrifugation of 8000 rpm for 20 minutes and the cell free supernatant was tested for surface tension reducing ability. Screening and selection of the optimum medium for maximum biosurfactant production was done on the basis of growth and maximum surface tension reduction (mN/m- milli Newton per meter).

Optimization of biosurfactant production :

To optimize the culture conditions for biosurfactant production, the strain was cultured under the conditions presented in Table A. The culture was inoculated in the selected production medium with 1 per cent inoculum adjusted to $O.D_{520}$ 0.65 (Joshi *et al.,* 2008) and incubated. It was subjected to centrifugation and extraction. The surface tension of the centrifuged broth was measured and the crude biosurfactant extract was analyzed for its emulsification activity.

Extraction of biosurfactant :

Biosurfactant was recovered from the culture broth after the removal of cells by centrifugation at 8000 rpm for 20 minutes. It was then precipitated by acidification of the supernatant to pH 2.0 using 6M HCl followed by centrifugation at 12,100 rpm for 20 minutes. The obtained precipitate was dissolved in 1M sodium carbonate (pH 8.6). From which biosurfactant was extracted with chloroform-ethanol (2:1) three times. The organic solvent was evaporated using a rotary vacuum evaporator, for further characterization the colorless viscous product was dissolved in minimum amount of methanol (Tayebe *et al.,* 2009).

Determination of emulsification activity :

E_{24} of culture sample was determined by adding 2ml of kerosene to 2ml of cell free supernatant, vortexing for 2 minutes, and leaving it to stand for 24hrs. The emulsification index, E_{24} (%) was the ratio of height of the emulsion layer (mm) by the total height of the mixture (mm) (Abouseoud *et al.,* 2008).

Characterization of biosurfactant :

Rhamnose content :

Carbohydrate content of the biosurfactant was quantified by Anthrone assay method (Thitima *et al.,* 2010) using rhamnose as a standard.

TLC :

Acid hydrolysis was carried out by adding sufficient amount of 1N HCl to the produced compound (glycolipid) and incubated for 3hrs at 90^0C. The sugar moiety was identified by thin layer chromatography using silica gel plates (Sifour *et al.,* 2007).

Table A: Culture conditions for the optimum biosurfactant production

Factors	Ranges
Time	24,48,72,96,120,144 and 168 hours
pH	3,4,5,6,7,8 and 9
Temperature	30, 37, 45 and 55°C
Carbon sources	Glucose, Glycerol, Mannitol, Coconut oil, Olive oil, Soyabean oil, Mustard oil and Sesame oil
Concentration of optimum carbon source	0.5, 1,1.5, 2,2.5, 3,3.5 and 4 per cent
Nitrogen sources	Yeast extract, Peptone, Ammonium chloride,Ammonium nitrate, Sodium nitrate, Potassium nitrate and Ammonium sulphate
Concentration of optimum nitrogen source	0.1, 0.2, 0.3, 0.4, 0.5 ,0.6, 0.7 and 0.8 per cent

FT-IR analysis :

Infrared spectroscopy was performed on FT-IR spectrophotometer (Make: Shimadzu; Model IR PRESTIGE-21, Accessory: ATR).

Temperature stability :

Determination of the effect of different temperatures on the stability of the biosurfactant was carried out by subjecting the cell free broth at a temperature range of 4, 25, 37, 55, 75 and 100^0C for 15 minutes which was then cooled to room temperature. The surface tension and E24 values of each treatment were measured (Abouseoud *et al.,* 2008).

Antimicrobial activity testing :

Antibacterial activity of the crude biosurfactant was determined by Agar cup diffusion method (Greenwood, 1995). The investigated pathogenic microorganisms were *Escherichia coli, Staphylococcus aureus strain 6538p, Shigella dysente riae, Proteus mirabilis, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes and Salmonella typhi*. A loopful of the given test strain was inoculated in 10 ml of Brain Heart infusion broth and incubated at 37^0C for 24 hours in order to activate the bacterial strain activity. Sterile 20 ml of Luria Bertanii Agar (Himedia) was melted and cooled and 0.2 ml test strain (0.25 OD_{620nm}) was seeded and poured into a 9cm diameter aneubra Petri plate. After solidification of the medium, agar cups were punched in the plate with the help of a cup borer. The cups were then filled with 200ml of biosurfactant preparation (crude biosurfactant dissolved in sterile phosphate buffer saline, pH 7) and the other cup was filled with only sterile phosphate buffer saline marking as a control, the plates were kept at 4^0C for 30 minutes and later incubated at 37^0C for 24hrs.The inhibitory zone against the particular test bacterial strain determined the antibacterial activity of the biosurfactant (zone size measured in mm). The mean value obtained for three individual replicates was used to calculate the zone growth of inhibition of each plate.

RESEARCH FINDINGS AND ANALYSIS

The experimental findings of the present study have been presented in the following sub heads:

Isolation, screening and identification of the biosurfactant producer :

Screening of the biosurfactant producing organisms from soil sample obtained from coconut oil mill resulted in seven isolates. Only one isolate was selected for further studies based on hemolytic activity on SIBA, zone of clearance on Tributyrin agar and maximum surface tension reduction upto 45 mN/m in Nutrient Broth. It also gave a positive CTAB test proving that it is capable of producing an extracellular anionic rhamnolipid (Siegmund and Wagner, 1991). The cultural, morphological and biochemical test were used to identify this promising isolate as *Pseudomonas aeruginosa* using Bergey's Manual of Determinative Bacteriology, 8th edition and the strain R2 was confirmed with 16s rRNA sequencing. This strain was selected for optimization for biosurfactant production and its characterization.

Screening of media for the biosurfactant production :

Amongst the four tested media, SM medium suggested by Makkar and Cameotra with slight modification (Mukherjee *et al.,* 2008) was selected as the standard production media as it supported growth of *Pseudomonas aeruginosa* strain R2 and showed maximum surface tension reduction as shown in Table 1.

Optimization of biosurfactant production :

Time course of growth and biosurfactant production :

The maximum biosurfactant production by this isolate was observed during its stationary phase *i.e.* at 96 hrs, lowering the surface tension of the media upto 45 mN/m (Table 2) with an E24 value of 44 per cent. The production of

Table 1: Screening of media for the biosurfactant production

Medias screened	Surface tension reading of the uninoculated media (mN/m)	Surface tension reading of the inoculated media (mN/m)
Medium E	66 mN/m	55 mN/m
MSM	70 mN/m	49 mN/m
BPLM	68 mN/m	47 mN/m
Medium SM	70 mN/m	40 mN/m

Table 2: *Optimisation* of incubation period for biosurfactant production

Time in hours	24	48	72	96	120	144	168	192
Surface tension mN/m	60	58	50	45	48	52	55	55
E24 (%)	0	10	20.12	44	40	18.55	15.86	16

rhamnolipid is typical of a secondary metabolite and increased considerably in the stationary phase in case of *Pseudomonas aeruginosa* (Rashedi, 2006).The increase in surface tension after 96 hrs relates to poor biosurfactant activity due to the rapid inactivation of the existing enzymes and accumulation of end products (Hua Yin *et al.,* 2009).

pH and temperature:

Pseudomonas aeruginosa strain R2 demonstrated optimum yield of biosurfactant at pH 7 and 30^{0}C (Table 3 and 4). The important characteristics of most organisms are their strong dependence on pH for their cell growth and production of secondary metabolite. *Pseudomonas aeruginosa* was able to give maximum biosurfactant yield at pH 7 (Rashedi, 2005). However, at low pH 3 there was no growth obtained. The pH of culture medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. Rhamnolipid production in *Pseudomonas* spp. was at maximum in the range from pH 6 to 6.5 and decreased sharply above pH 7 (Tayebe *et al.,* 2009). In case of *Pseudomonas* a change in temperature caused alteration in the composition of biosurfactant (Duvnjak *et al.*, 1982).

Carbon sources :

Carbon substrate is an important limiting factor affecting the production of microbial biosurfactant the type of carbon substrates used for production has been reported to influence both quality and quantity of biosurfactant (Das *et al.,*2009).This study envisaged the supplementation of different vegetable oils as carbon source. It was observed that coconut oil (Table 5) at the concentration of 1 per cent (Table 6) was more effective for increasing biosurfactant production as compared to other sources used. Water soluble carbon sources such as glycerol, glucose and mannitol were used for rhamnolipid production by *Pseudomonas species* however, it was inferior to that obtained with water immiscible substrates such as n-alkanes, coconut oil and olive oil (Tayebe *et al.,* 2009).

Nitrogen sources :

Seven different organic and inorganic nitrogen sources (0.3%) were tested of which ammonium nitrate (NH_4NO_3) was optimum for biosurfactant production by *Pseudomonas aeruginosa* strain R2 as shown in Table 7. Inorganic nitrogen sources such as NH_4Cl and $(NH_4)_2SO_4$, when added in the

Table 3: Effect of pH on biosurfactant production

pH ranges	3	4	5	6	7	8	9
Surface tension mN/m	58	50	45	42	35	38	39
E24 (%)	40	53	56	58	71	66	60

Table 4: Effect of temperature on biosurfactant production

Temperature (^{0}C)	30	37	45	55
Surface tension mN/m	40	44	46	46
E24 (%)	66	64	60	60

Table 5: Effect of different carbon sources on biosurfactant production

Carbon sources (1%)	Coconut oil	Olive oil	Soybean oil	Glucose	Mannitol	Glycerol	Mustard oil	Sesame oil
Surface tension mN/m	52	56	65	70	66	70	72	70
E24 (%)	63.55	32.77	-	60	-	-	-	-

(key - = no emulsification index)

Table 6: Effect of different concentrations of coconut oil on biosurfactant production

Parameters	0.5%	1%	1.5%	2%	2.5%	3%	3.5%	4%
Surface tension mN/m	38	37	40	36	37	38	39	40
E24 (%)	15.38	18.75	62	71	68	36	22	13

Table 7 : Effect of different nitrogen sources on biosurfactant production

Nitrogen sources (0.3%)	Peptone	Yeast extract	Ammonium nitrate	Potassium nitrate	Sodium nitrate	Ammonium chloride	Ammonium sulfate
Surface tension mN/m	41	41	38	40	39	49	59
E24 (%)	55.55	16.66	68.57	55.88	58.82	-	-

(key - = no emulsification index)

production media was shown to lower the growth and production of biosurfactant (Misun *et al.,* 2008). Tayebe *et al*. (2009) observed nitrate form to be the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1. From Table 8 it is observed that 0.4g/100ml of NH_4NO_3 was found to give maximum biosurfactant production by *Pseudomonas aeruginosa* strain R2. Whereas 0.3 g/100 ml of NH_4NO_3 was found to be the optimum nitrogen source for the production of biosurfactant by *P. putida* (Misun *et al.,* 2008).

Characterisation of biosurfactant :

Quantification of the biosurfactant production by Rhamnsose content :

The yield of biosurfactant produced by *Pseudomonas aeruginosa* strain R2 was quantified and found out to be 1.7g/l. The yield produced by *Pseudomonas fluorescens* gave a yield of 2g/l (Orathai *et al.,* 2008).

TLC and infrared spectroscopy analysis :

Preliminary chemical characterization of the biosurfactant by TLC suggested that it belongs to the class of Glycolipids. After the extract was subjected to acid hydrolysis it was observed as brown colored spots with orcinol and sulfuric acid reagent and as purple coloured spots with molisch reagent (Sifour *et al.,* 2007). Supporting this result, FT-IR spectrum of the biosurfactant produced by *Pseudomonas aeruginosa* strain R2 showed the presence of rhamnolipid structure composed of rhamnose rings and long hydrocarbon chains. As seen in Fig. 1 it is clearly indicated by the absorbance bands at the wave numbers of 3329.14 cm^{-1}. 2908.65 cm^{-1}, 1637.50 cm^{-1}, 1215 cm^{-1} 1080.92 cm^{-1}. Similar absorbance bands of FT-IR spectrum of the pure rhamnolipid produced by *Pseudomonas aeruginosa* BSZ-07 were observed (Zhang and Miller, 1992). A characteristic band for rhamnolipid produced by *Pseudomonas fluorescens* was found in the region of 3000-2700 cm^{-1} (Abouseoud *et al.,* 2008).

Temperature stability :

The stability of the obtained biosurfactant was tested over a wide range of temperatures. Rhamnolipids produced by *Pseudomonas aeruginosa* strain R2 was shown to be thermostable in nature. Heating the supernatant to 100^0C caused no significant effect on its surface tension reducing ability. The surface tension reduction and emulsification activity were quite stable at temperatures used (surface tension = 40 mN/m; E24 = 60-64%). The biosurfactant produced

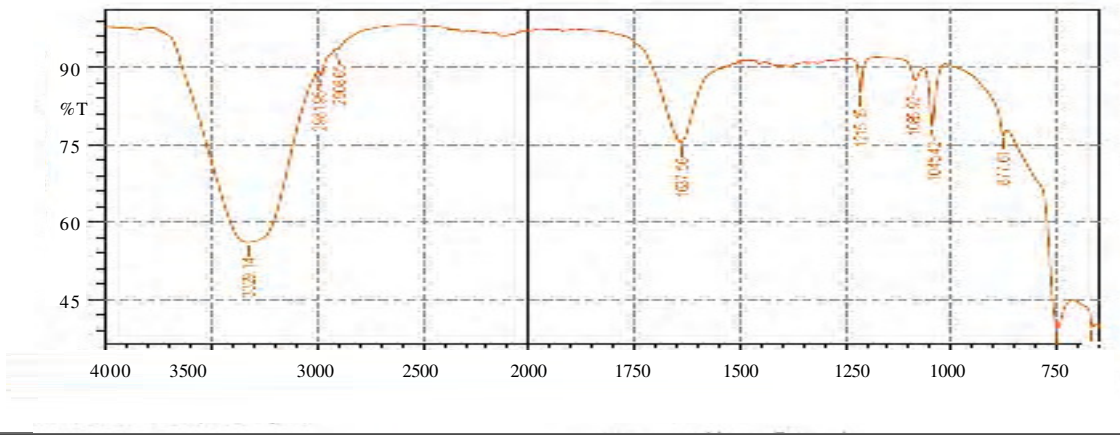

Fig. 1: Shows the FT-IR spectrum of the biosurfactant produced by *Pseudomonas aeruginosa* strain R2

Table 8: Effect of different concentration of ammonium nitrate on biosurfactant production

Parameters	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%	0.7%	0.8%
Surface tension mN/m	38	37	40	36	37	38	39	40
E24 (%)	15.38	18.75	62	71	68	36	22	13

Table 9: The antimicrobial activity of the crude biosurfactant

Test cultures	E.C.	S.a. 6538p	S.d.	P.m.	P.v.	S.p.	S.a.	S.t.
Zone size (mm)	18	22	Not inhibited	11	11	Not inhibited	20	26

Key: E.c = Escherichia coli; S.a 6538p = Staphylococcus aureus 6538p; S.d = Shigella dysenteriae;
P.m = Proteus mirabilis; P.v = Proteus vulgaris; S.p = Streptococcus pyogenes;
S.a=Staphylococcus aureus; S.t = Salmonella typhi.

by *P. fluorescens* was thermostable while the synthetic surfactants such as SDS exhibited a significant loss of emulsification activity beginning at 70^{0}C (Abouseoud *et al.,* 2008).

Antibacterial activity :

The biosurfactant was also tested for its antibacterial activity. As shown in Table 9, it was effective against *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Proteus vulgaris, Proteus mirabilis* and *Staphylococcus aureus 6538p* but was found having no activity against *Shigella dysenteriae* and *Streptococcus pyogenes*. Biosurfactants having antimicrobial activity are daptomycin, a cyclic lipopeptide from *Streptomyces roseosporus*, viscosin, a cyclic lipopeptide from *Pseudomonas*, rhamnolipids produced by *P. aeruginosa* (Das *et al.,* 2009). Rhamnolipid type of biosurfactant produced from soybean oil waste had antimicrobial activity against several bacteria and fungi, namely *Bacillus cereus, S. aureus, Micrococcus luteus* and antifungal activity (Mukherjee *et al*., 2006; Rodrigues *et al.,* 2006).

The present study is an attempt to find economically cheaper sources for the large scale production of microbial biosurfactants. *Pseudomonas aeruginosa* strain R2 screened from the environment gave maximum biosurfactant production (1.7 g/l) with ammonium nitrate and coconut oil as the sole source of nitrogen and carbon, respectively in the production medium SM, which was adjusted at pH 7 and incubated at 30^{0}C for 96 hours (120 rev min^{-1}). Satisfactory emulsification activity of the biosurfactant against kerosene along with its utilization of various other hydrocarbons indicated its diverse applicability in bioremediation. Purification and structural characterization of the biosurfactant stated it to be a rhamnolipid which was thermostable in nature. Having antibacterial activity against pathogenic strains of bacteria it could be taken up as a new approach to antibiotics and this could prove very beneficial for medical and pharmaceutical sectors. Further medium induction and bioprocess optimization should lead to substantially higher biosurfactant production from *Pseudomonas aeruginosa* strain R2 which could potentially have industrial application.

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