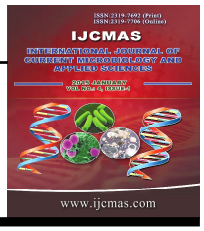
Int.J.Curr.Microbiol.App.Sci (2015) 4(1): 245-253

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 1 (2015) pp. 245-253**
http://www.ijcmas.com



Original Research Article

Production and characterization of biosurfactant from *Pseudomonas spp*

S.K.Arora[2], Jitesh Sony[1], Anayata Sharma[1]* and Mridul Taneja[1]

[1]Department of Biotechnology, D.A.V College, Abohar, India
[2]Principal, D.A.V College, Abohar, India
**Corresponding author*

A B S T R A C T

Biosurfactants are amphiphilic compounds produced by various bacteria and fungi which reduce surface and interfacial tension. The Bacterial strain *Pseudomonas spp* was used for the production of biosurfactant and biosurfactant activity was tested against different oils. The parameters for better growth of the bacterial strain were optimized and production of surfactant was carried out. The crude biosurfactant was extracted and the emulsification potency was assessed using different vegetable edible oils. Further, the rhamnolipid was detected from the extracted biosurfactant and was confirmed by Infrared spectroscopy. The results showed that strain showed high surfactant activity over the Kerosene oil, required mesophilic temperature and pH-7 for its better growth. The surfactant showed comparatively high emulsification index over Kerosene oil at the rate of 44%.

Keywords

Biosurfactant, *Pseudomonas*, Rhamnolipids, Biopreservatives

Introduction

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membranes by a variety of yeast, bacteria and filamentous fungi (Anandaraj and Thivakaran, 2010; Ghayyomi *et al.,* 2012) from various substances including sugars, oils and wastes. The amphiphiles that form micelles can be potentially used for surface chemical works are termed as surface active agents or surfactants (Jaysre *et al.,* 2011; Kigsley and Pekdemir, 2004). Soaps and detergents can be described as having similar characteristics as surfactants. A widely accepted role of biosurfactants is to enhance the uptake of insoluble substrates.

Three methods of uptake of hydrocarbons are mentioned in literature, i.e. the direct interfacial uptake of the hydrocarbon by hydrophobic cell membranes, interfacial uptake enhanced by emulsification by biosurfactants and solubilization (micelle transfer) of hydrocarbons by biosurfactants. Two of these methods are directly influenced by biosurfactants (emulsification and solubilization).

The direct uptake of hydrocarbons is enhanced in some cases of biosurfactants e.g. increase in growth of *Pseudomonas aeruginosa* culture on hydrocarbons was observed upon the exogenous addition of rhamnolipid, through an increase in cell

membrane hydrophobicity. The increased hydrophobicity has been attributed to the loss of lipopolysaccharide from the cell membrane, which is influenced by the rhamnolipid concentration (Priya and Usharani, 2009). Addition of rhamnolipid in the medium of biosurfactant-negative mutants of *Pseudomonas aeruginosa KY-4025* and *Pseudomonas aeruginosa* PG-201 restored their growth on n-paraffin and hexadecane, respectively, which otherwise showed poor growth on these hydrocarbons as compared to the parent strains.

Pseudomonas aeruginosa strains have different degrees of hydrophobicity of their cell membranes, and thus some are naturally superior in degrading hydrocarbons through direct uptake e.g. *Pseudomonas aeruginosa* ATCC 27853 and ATCC 15442 have been found to have naturally hydrophobic membranes. Another important role of biosurfactants is their role as antagonistic molecules against other organisms in the environment. Rhamnolipids, for example, have considerable antimicrobial activity. In the current study, Production and extraction of biosurfactant was carried out using *Pseudomonas spp.*, the parameter optimization for the better growth of the strain was carried out. The extraction and emulsification potency of the surfactant was performed in order to use as bioemulsifier in food.

Materials and Methods

5 gram of the oil spilled soil sample and uncontaminated soil sample were inoculated in the 50ml nutrient broth and inoculated at 25˚C for 72 hours. After incubation this medium was serially diluted from 10^{-1} and 10^{-6} in sterile water. From the dilutions 10^{-1} to 10^{-6}, 1ml was transferred to sterile plates containing Nutrient agar media. The plates were then inverted and incubated at 25˚C, for 48hrs. Control and replica plates were maintained. After incubation morphologically different colonies were selected for the further analysis.

Screening of biosurfactant producing organism

The potential biosurfactant producer was screened by oil spreading technique. Based on the surface tension reducing capacity of *Pseudomonas aeruginosa* on oils were used to screen the bio surfactants and it was calculated by using oil spreading technique.

Oil spreading technique

30ml of distilled water was taken in the Petriplates. 1ml of vegetable cooking oil was added to the center of the plates containing distilled water. Now add 20µl of the supernatant of the cultures isolated from soil to the center. The biosurfactant producing organism can displace the oil and spread in water. The diameters of the clear zone on the surface were measured and compared with the control using uninoculated medium.

Characterization biosurfactant producing organism

The screened biosurfactant producing organism was then characterized by using different tests. They are Gram staining, endospore staining, motility test, indole test, methyl red - Voges Proskauer test, citrate test, indole test, starch hydrolysis, catalase test.

Extraction of biosurfactants

The culture was inoculated in 50ml of nutrient broth with 1ml of petrol. The culture was incubated at 25˚C for 7 days with shaking condition. After incubation the

bacterial cells were removed by centrifugation at 5000rpm, 4˚C for 20 minutes. The supernatant was taken and the pH of the supernatant was adjusted to 2, using 1M H_2SO_4. Now add equal volume of chloroform: methanol (2:1). This mixture was shaken well for mixing and left overnight for evaporation. White colored sediments were obtained as a result i.e., the "Biosurfactant".

Dry weight of biosurfactant

Sterile Petriplate was taken and the weight of the plate was measured. Now the sediment was poured on the plates. They were placed on the hot air oven for drying at 100˚C for 30 minutes. After drying the plates were weighted. The dry weight of the biosurfactants was calculated by the following formula:

Dry weight of biosurfactant = (Weight of the plate after drying – weight of empty plate)

Characterization of biosurfactants

Preliminary characterization of the biosurfactant was done by thin layer chromatography method. Silica gel plates were prepared by mixing gel and then pouring it on to glass plates. A spot of crude biosurfactant was placed on silica plate. The biosurfactant was separated on the plate using chloroform: methanol: water (90:10:0.5). Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spots. Anthrone reagent was sprayed to detect glycolipid, biosurfactant as yellow spots.

Emulsification activity

The emulsification capacity was determined by adding 2ml of oil to the same amount of cell-free culture broth, mixed for 2 min on a vortex mixer and allowed to stand for 24hrs. E24 index is defined as percentage of the height of emulsified layer divided by the height of liquid column. The ability of *Pseudomonas aeruginosa* on the emulsification index on hydrocarbon was calculated by standard method:

E 24 = (height of the emulsified layer/ total height of the hydrocarbon)*100

Medium optimization

The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed at a specific set of conditions. Two factors were chosen aiming to obtain higher productivity of the biosurfactant: carbon source (C) & nitrogen source (N). The carbon sources were used as Glucose and Sucrose at a concentration of 20g/l. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, yeast extract and urea were employed at a concentration of 10g/l.

Effect of pH, temperature & sodium chloride on biosurfactant production

Effect of pH and temperature on production of biosurfactant was studied by adjusting the pH to 6, 7, 8 & 10 and temperature of the basal medium to 4˚C, 37˚C, and Room temperature which was 31˚C. Effect of NaCl on biosurfactant production was studied by varying the concentration of NaCl (w/v) added to the basal medium to the values of 4%, 6%, 8% and 10%. Biosurfactant activity was expressed as percentage relative activity.

Results and Discussion

Isolation and screening of biosurfactant producing organism

In the pour plates from the serial dilution

tubes the colonies were enumerated. Six different types of colonies were identified morphologically and were purified and stored at 4˚C for further analysis

Screening of biosurfactant producing organism

The six colonies were centrifuged and added to the oil containing plates. The biosurfactant producing organism can only able to displace the oil. Six colonies were tested positive and were named as P1, P2, P3, P4, P5 and P6

Physiological and biochemical characterization

First the organism was identified by different physical and biochemical tests. The results of it's were tabulated (Table 1). Comparing the results with Bergey's Manual, it showed that the isolated and screened organism were of *Pseudomonas sp*,

Extraction of biosurfactants and calculation of their dry weight

The culture inoculated in mineral salt medium with oil was centrifuged and the supernatant was taken mixed with Chloroform: Methanol. White sediment was retained while placed in the rotor. The dry weights were measured and estimated (Table 2).

Characterization of biosurfactants

The biosurfactant produced by using TLC plates. The sediment obtained was placed in TLC plate and the plates when sprayed with anthrone reagent it showed yellow spots in the plates. This shows the presence of Rhamnolipid biosurfactants in the organism

Emulsification activity

E24 index is defined as percentage of the height of emulsified layer divided by the height of liquid column. The ability of cultures on the emulsification index on hydrocarbon was calculated by standard method:

E 24 = (height of the emulsified layer/ total height of the hydrocarbon)*100

Emulsification values of the biosurfactant were measured and estimated (Table 3 and Fig. 1)

Medium optimization

The biosurfactant producing organisms were inoculated in medium containing different carbon sources and nitrogen sources such as glucose, sucrose and yeast, urea respectively. The growth of the culture was measured by checking their E24 Index. The results were tabulated (Table 4). The cultures P1 and P6 worked well in optimized medium and provided maximum biosurfactant production.

Effect of temperature, pH & sodium chloride on biosurfactant production

As a part of stability studies effect of pH, temperature & sodium chloride at various levels was studied on the biosurfactant production by cultures of interest. The growth of the culture was measured by checking their E24 Index. The results were tabulated (Table 5 to 7 and Fig. 2). The biosurfactant producing organisms were found to be *Pseudomonas* spices. The potential biosurfactant producer was screened by oil spreading technique. Based on the surface tension reducing capacity of *Pseudomonas aeruginosa* on oils were used to screen the bio surfactants and it was calculated by using oil spreading technique. The cultures P4 and P6 showed maximum surface tension reducing capacity on oil by displacing oil by 5mm.

Table.1 Characterization results

	P1	P2	P3	P4	P5	P6
Gram staining	–,rods	–,rods	–,rods	–,rods	–,rods	–,rods
Endospore staining	-	-	-	-	-	-
Catalase activity	+	+	+	+	+	+
Indole production	-	-	-	-	-	-
Citrate	-	-	-	+	+	+
MR	-	-	+	-	-	-
VP	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Starch	-	-	-	-	-	-
Glucose Fermentation	+	+	+	+	+	+
Fructose Fermentation	+	-	+	-	-	-
Sucrose Fermentation	+	+	+	+	+	+
Mannitol Fermentation	+	+	+	+	+	+
Lactose Fermentation	+	+	+	+	+	+

- Means negative results and + Means positive results

Table.2 Dry weight of biosurfactant

CULTURE	PLATE WEIGHT (g)	AFTER DRYING OF BIOSURFACTANT IN THE PLATE (g)	DRY WEIGHT OF BIOSURFACTANT (g)
P1	6.715	7.043	0.328
P2	6.715	7.112	0.397
P3	6.715	7.182	0.467
P4	6.715	7.083	0.368
P5	6.715	7.291	0.576
P6	6.715	7.151	0.436

Table.3 Emulsification activity of various Biosurfactant; Where T.L refers to Total height of liquid column and B.L refers to Height of bubble column or the Emulsified Layer in centimetres

CULTURE	KEROSENE OIL (K)		MUSTARD OIL (M)		E24 INDEX (%)	
	T.L	B.L	T.L	B.L	K	M
P1	1.4	0.5	1.2	0.1	37	8.3
P2	1.5	0.5	1.3	0.2	36	15.3
P3	1.4	0.6	1.0	0.1	42	10.0
P4	2.0	0.8	1.8	0.2	44	10.0
P5	1.4	0.3	1.4	0.1	21.4	7.1
P6	1.7	0.6	1.0	0.1	38	10.0

Table.4 The growth of biosurfactant producing organisms on different carbon sources

CULTURE	CARBON SOURCES				NITROGEN SOURCES				E24 INDEX (%)			
	GLUCOSE (G)		SUCROSE (S)		YEAST (Y)		UREA (U)		G	S	Y	U
	T.L	B.L	T.L	B.L	T.L	B.L	T.L	B.L				
P1	1.5	1.0	2.0	1.5	2.0	1.2	1.6	0.5	66.6	75.0	60.0	31.3
P2	1.6	0.5	1.5	0.1	1.5	1.0	1.5	0.2	31.25	6.6	66.6	13.3
P3	1.6	0.4	1.5	0.3	1.6	0.2	1.6	0.0	25.0	20.0	12.5	0.0
P4	2.0	0.1	1.2	0.9	1.7	0.1	1.2	0.0	5.0	75	5.8	0.0
P5	1.5	0.0	1.5	0.2	1.5	0.3	1.5	0.1	0.0	13.3	20.0	6.6
P6	1.5	1.0	1.5	1.0	1.6	0.8	1.5	1.0	66.6	66.6	50.0	66.6

Table.5 E24 Index values of various biosurfactant producing cultures grown on three temperature scales

CULTURES	TEMPERATURE						E24 INDEX		
	4˚C		ROOM TEMPERATURE (R.T)		37˚C		4˚C	R.T	37˚C
	T.L	B.L	T.L	B.L	T.L	B.L			
P1	2.2	0.0	2.0	0.1	2.1	0.1	0.0	5.0	4.7
P2	2.1	0.0	2.0	0.2	2.2	0.3	0.0	10.0	13.6
P3	2.0	0.0	2.0	0.1	2.1	0.1	0.0	5.0	4.7
P4	2.2	0.0	2.0	0.1	2.3	1.1	0.0	5.0	47.8
P5	2.0	0.0	1.8	0.1	2.2	0.3	0.0	5.5	13.6
P6	2.0	0.0	2.0	0.5	2.0	0.9	0.0	25.0	40.9

Where T.L refers to Total height of liquid column and B.L refers to Height of bubble column or the Emulsified Layer in centimetres

Table.6 E24 Index values of various biosurfactant producing cultures grown on four different pH scales

CULTURE	pH SCALE								E24 INDEX (%)			
	6		7		8		10		6	7	8	10
	T.L	B.L	T.L	B.L	T.L	B.L	T.L	B.L				
P1	2.0	0.2	2.2	0.4	2.2	0.4	2.0	0.6	10.0	18.1	18.1	30.0
P2	2.0	0.8	2.1	1.3	2.1	1.1	2.0	0.8	40.0	60.0	55.0	40.0
P3	2.0	0.5	2.1	0.9	2.1	0.8	2.1	0.5	25.0	42.8	40.0	23.8
P4	2.0	0.0	2.0	0.0	2.1	1.2	2.0	0.2	0.0	0.0	57.1	10.0
P5	2.0	0.3	2.1	0.5	2.1	0.7	2.1	0.6	15.0	23.8	33.3	28.5
P6	1.8	0.4	2.0	0.7	2.0	0.5	2.0	0.3	22.2	25.0	35.0	15.0

Table.7 E24 Index values of various biosurfactant producing cultures grown on four different pH scales

CULTURE	CONCENTRATION OF SODIUM CHLORIDE (%)								E24 INDEX (%)			
	4		6		8		10		4	6	8	10
	T.L	B.L	T.L	B.L	T.L	B.L	T.L	B.L				
P1	1.6	0.2	1.7	0.1	1.8	0.0	2.0	0.0	12.5	5.8	0.0	0.0
P2	1.8	1.0	1.9	0.4	1.6	0.1	2.0	0.0	55.5	21.0	6.3	0.0
P3	2.0	0.3	1.7	0.1	1.6	0.0	1.5	0.0	15.5	5.9	0.0	0.0
P4	2.0	1.0	2.0	0.1	1.6	0.0	2.0	0.0	50.0	5.0	0.0	0.0
P5	1.6	0.1	1.8	0.1	2.0	0.0	2.0	0.0	6.3	5.5	0.0	0.0
P6	2.0	0.5	1.8	0.4	2.0	0.2	1.4	0.1	25.0	22.0	10.0	7.1

Figure.1 Emulsification activity of Biosurfactant obtained from culture P5 and P6. From top Left to Right: Emulsification activity of Biosurfactant obtained from culture P5 with Kerosene oil and with mustard oil. From bottom Left to Right: Emulsification activity of Biosurfactant obtained from culture P6 with Kerosene oil and with Mustard oil

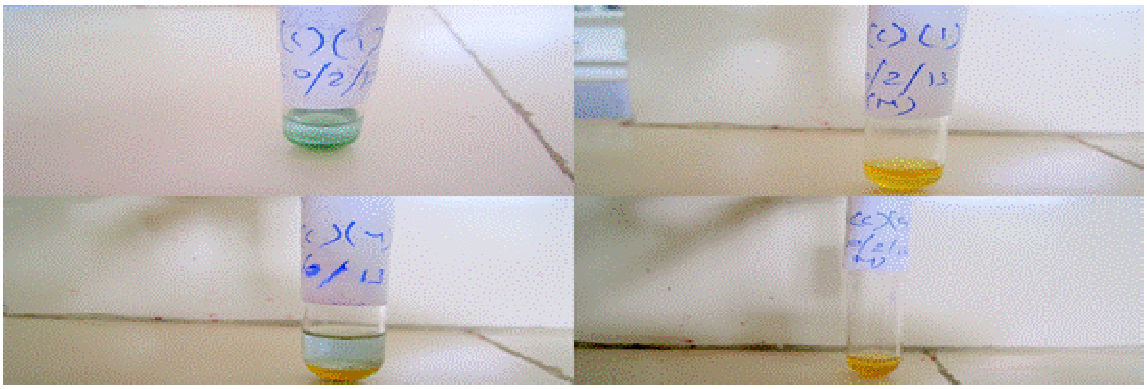
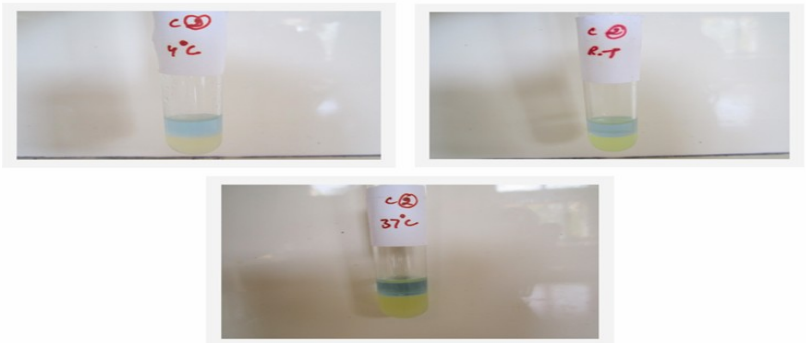


Figure.2 Results of Emulsification Activity of culture P4 grown on three temperature scales (4˚C, room temperature and 37˚C)



The *Pseudomonas sp*, inoculated in mineral salt medium with oil produced biosurfactants. It was extracted by centrifugation and sedimentation. The dry weight of the biosurfactant produced from culture P5 had maximum dry weight of 0.576 grams/ml.

The biosurfactants extracted was characterized by using TLC. The components were obtained as rhamnolipid i.e., a glycolipid while sprayed ninhydrine reagent on the TLC plate. The E24 index which is the measure of emulsification activity was calculated by standard method by using kerosene oil and mustard oil. The culture P4&culture P3 shows maximum activity with kerosene oil (44%) and (42%).

The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed at a specific set of conditions. Two factors were chosen aiming to obtain higher productivity of the biosurfactant: carbon source (C) & nitrogen source (N).

The carbon sources were used as Glucose and Sucrose at a concentration of 20g/l. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, yeast extract and urea were employed at a concentration of 10g/l. The cultures P1 and P6 show maximum biosurfactant production in all optimized medium. The growth was calculated in terms of E24 index. The applicability of biosurfactants in several fields depends on their stability at different temperatures and pH.

The cultures were subjected to various scales of pH and temperatures. The effect of salinity was checked by subjecting cultures to various concentration of Sodium Chloride (dissolved in culture medium). All the cultures show significant emulsification activity at temperature scale of 37˚C and room temperature. Therefore, it can be concluded that biosurfactants maintain their surface properties which is unaffected in different range of temperatures (30˚C and above).

The surface activity of the biosurfactant remained relatively stable at pH 7 & 8. The surface activity decreases at deviation from this pH scales. The effect of sodium chloride addition on biosurfactant produced from various cultures was studied. Maximum activity was observed at 4% NaCl concentration. Little changes were observed in increase in concentration of NaCl up to 8% (w/v). Significant retardation in activity was observed with further increase in concentration of NaCl.

From the stability studies it can be concluded that biosurfactants produced from the cultures under consideration have stability at alkaline pH and high salinity; such biosurfactants may be useful for bioremediation of spills in marine environment because of their stability in alkaline condition and in the presence of salt.

References

Anandaraj, B., Thivakaran, P. 2010. Isolation and production of biosurfactant producing organism from oil spilled soil. *J. Biosci. Technol.*, 1(3): 120–126.

Ghayyomi, J.M., Forghani, F., Deog-Hwan, Oh. 2012. Biosurfactant production by *Bacillus* sp. Isolated from petroleum contaminated soil of Sirri Island. Ame. J. Appl. Sci., 9(1).

Jaysre, R.C., Basu, S., Singh, P., Ghosal, T., Patra, P., Keerthi, Y., Rajendran 2011. Isolation of biosurfactant

producing bacteria from environmental sample. *Pharmacol. online* 3: 1427–1433.

Kigsley, U., Pekdemir, T. 2004. Evaluation of biosurfactants for crude oil contaminated soil washing. *Chemosphere*, 57: 1139–1150.

Priya, T., Usharani. 2009. Comparative study for biosurfactant production by using *Bacillus subtilis* and *Pseudomonas aeruginosa*. *Bot. Res. Int.*, 2(4): 284–287.