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RESEARCH ARTICLE

CHARACTERISATION AND OPTIMISATION OF BIOSURFACTANT PRODUCED BY *PSEUDOMONAS FLUORESCENS* FROM OIL CONTAMINATED SOIL

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ABSTRACT

The biosurfactant producing strain *Pseudomonas fluorescens* was isolated from Dharmapuri District, Tamil Nadu. India. The biosurfactant produced by *Pseudomonas fluorescens* was able to reduce the surface tension of media to 34.64 mN/m. Using FT-IR spectroscopy, the chemical structure of the purified biosurfactant was identified as lipopeptide. To enhance the biosurfactant production, optimization was employed by central composite design (CCD) in response surface methodology (RSM). In the optimization study, glucose as carbon source, yeast extract as a nitrogen source, pH and salinity (NaCl gL^{-1}) were assigned as a factor. The maximum emulsification index of *Pseudomonas fluorescens* was obtained under the optimal condition as glucose 20.28 gL^{-1}; yeast extract 2.51 gL^{-1}, pH at 7.01 and NaCl 5.37 gL^{-1}. The optimised production of biosurfactant yield was approximately increased to 2.2 folds. The results from this study showed that the biosurfactant produced by *Pseudomonas fluorescens* may have potential application in bioremediation.

KEYWORDS: Biosurfactant, *Pseudomonas fluorescens*, Lipopeptide, RSM, Emulsion index

INTRODUCTION:

Biosurfactant are a diverse group of surface active molecules/chemical compounds synthesized by microorganisms [1]. Biosurfactant are classified according to their molecular structure into mainly glycolipids (e.g., rhamnolipids and sophorolipids), lipopeptides (e.g., surfactin), polymers biosurfactant (e.g., smulsan and alasan), fatty acids (e.g., 3-(3-hydroxyalkanoyloxyl) alkanoic acids) [2]. These compounds are metabolic products produced during the growth of microorganisms on water-soluble and water immiscible substrates [3]. Biosurfactants are environmental friendly and have potential industrial and environmental applications.

When compared to synthetic surfactant, biosurfactants have several advantages, including high biodegradability, low toxicity, low irritancy, and compactability with human skin [4]. The current study gives special attention to the influence of nutritional requirement and optimal environmental condition on the production of *Pseudomonas fluorescence* isolated oil contaminated soils of Dharmapuri district. An insight into characterisation of extracted biosurfactant has been studied through FT-IR spectroscopy.

MATERIALS AND METHODS:

SAMPLING AREA AND SAMPLING:

Around ten oil contaminated soil samples were collected from different location of Dharmapuri district, Tamil Nadu. India.

PRODUCTION OF CULTURE MEDIUM:

Mineral salts medium (MSM) used to enrich and isolate for biosurfactant producing microorganisms**.** The composition of the mineral medium used was as follows (gL^{-1}): 4 g NH_4NO_3, 0.1g KCl, 5g KH_2PO_4, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, and 0.01 g $FeSO_4 \cdot 7H_2O$ and supplemented with 1 ml trace element solution containing (L^{-1}): 0.75 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $COCl_2 \cdot 6H_2O$, 0.075 g $CuSO_4 \cdot 5H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 0.15 g H_3BO_3, and 0.06 g $NaMoO_4 \cdot 2H_2O$. The pH of culture media was adjusted to 7.

ISOLATION AND IDENTIFICATION OF BIOSURFACTANT PRODUCING MICROORGANISM:

The soil samples were collected from different location of Dharmapuri district, under aseptic condition by using sterile sampling bottle. Accordingly, A few grams of the soil sample were transferred to 100ml of Mineral salt medium (MSM) in a 250ml Erlenmeyer flask. The flasks were incubated at 30°C on a rotatory shaker at 200rpm for 7days. The isolates were screened for biosurfactant

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production. Control and replica plates were maintained. The biosurfactant producing isolates were determined by qualitative studies and identified by biochemical tests. All these results were compared with Bergey's Manual of Determinative Bacteriology to determine the genus [5]. The biosurfactant production of the isolates was evaluated by rapid tests *viz*., hemolytic activity [6], drop collapse test [7] and oil displacement test [8] were used for screening of biosurfactant producers. The surface tension was measured using Du-Nouy ring tensiometer (Krüss, GmbH, Hamburg, Germany). The distilled water and un-inoculated medium was used as negative control and Tween-20 was used as positive control. The measurements were repeatedly taken thrice and the average value was used to express the surface tension of the sample.

BIOSURFACTANT PRODUCTION:

The biosurfactant producing isolate was transferred to 5ml of nutrient rich broth medium containing 1% yeast extract, 1.5% nutrient broth (Hi media) and 1% ammonium sulfate and incubated at 37°C for 12h as seed culture at 180rpm. The optical density of the culture was 0.8 at 600nm. 5ml of culture was transferred to 500ml of minimal salt medium (MSM) in 2000 ml Erlenmeyer flask and incubated on a rotary shaker (180rpm) incubator at 37°C. At different intervals the samples were collected and were monitored for biosurfactant production and surface tension.

EXTRACTION OF BIOSURFACTANT:

The cells were removed by centrifugation at 12500 rpm for 30 min. The pH of cell free supernatant was lowered to 2 using 6M hydrochloric acid solution. The acidified supernatant was leftover night at 4°C for precipitation. The precipitate was dissolved and extracted with the solvent (2:1v/v chloroform to methanol ratio) and then extracted using the separating funnel. The organic phase was transferred to rotatory evaporator to remove the solvents.

CHARACTERIZATION OF BIOSURFACTANT -FOURIER TRANSFORM INFRARED SPECTROSCOPY:

The biosurfactant isolated from Pseudomonas fluorescence was subjected to Fourier transform infrared spectroscopy (FT-IR) analysis to identifying chemical bonds or the functional groups present. One milligram (freeze dried) partially purified biosurfactant was ground with 100 mg KBr pellet and pressed with 7500 kg for 30 seconds to obtain translucent pellet. For this study, AVATAR-NICOLAT FTIR system was used with a spectral resolution and wave number accuracy of 4 and 0.01cm^{-1}, respectively. All measurements consisted of 500 scans and a KBr pellet was used as background reference.

OPTIMIZATION OF CULTURE MEDIUM:

RSM is an empirical statistical modelling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. In this regard, carbon source, nitrogen source, pH and salinity (NaCl gL^{-1}) were considered as independent variables for the emulsion index (E_{24}) in the culture media. The specific codes for each independent variable and range of the variables used for this experiment are given in Table 2. The experiment was performed using central composite design (CCD). In CCD, a total 30 treatment combinations were generated using designer expert 7.0 software (Stat-Ease Inc. Minneapolis, USA).

From the experimental data according to this design, a second-order polynomial regression model equation was derived.

$Y= \beta_0+ \beta_1A+ \beta_2B+ \beta_3C+ \beta_4D+ \beta_{11}A^2+ \beta_{22}B^2+ \beta_{33}C^2+ \beta_{44}D^2+ \beta_{12}AB+ \beta_{13}AC+ \beta_{14}AD+ \beta_{23}BC+ \beta_{24}BD+ \beta_{34}CD.$

Where Y: predicted response (Emulsion Index, %), β0: intercept , A: Carbon source, B: Nitrogen source, C: pH, D: Salinity, β_1, β_2, β_3 and β_4 are the linear coefficients; β_{11}, β_{22}, β_{33}, andβ_{44} are the squared coefficients; β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are the interaction coefficients; A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD, CD are the interaction between the variables as significant terms.

STATISTICAL ANALYSIS:

This data was analysed by analysis of variance (ANOVA) technique to find out which factors had the most effective interactions for higher biosurfactant production [9].

3. RESULTS AND DISCUSSION:

ISOLATION AND SCREENING OF BIOSURFACTANT PRODUCING MICROORGANISM:

The soil samples screened for biosurfactant producers were collected from oil contaminated soils of Dharmapuri district, Tamilnadu. Totally twelve strains were isolated. Among them, *Pseudomonas fluorescens* had highest biosurfactant production and surface activity was selected for further study (fig1). The isolate showed β-hemolytic activity (fig 2), Oil displacement test (8.0mm) and reduction in surface tension (38.2 mN/m) in Mineral salt medium. The isolate was examined based on colony morphological and biochemical characteristics presented in table1. Morphological observation revealed that the colony

was circular and convex with an entire margin. The isolate was characterized as Gram negative and rod shaped bacterium. The biochemical test was carried out according to Bergey's Manual [5] clearly identified the strain to be *Pseudomonas fluorescens.*

BIOSURFACTANT PRODUCTION:

Biosurfactant production of the isolate was preliminary screened by haemolytic activity, the drop collapse test, oil displacement test and measuring the surface tension of the solution. In drop collapse test a flat drop was observed and in oil displacement test, a clear diameter of 8.0mm^2 was observed and the area was 50.25mm^2.The surface tension of cell-free culture, decreased from 58.9 to 35.23 mN/m. Surface tension measurement would be the best method for quantifying the biosurfactant production. The surface tension remained invariable even after 72h. In the middle of logarithm phase (17 h) the surface tension (35.23 mN/m) was lowest.

EXTRACTION OF BIOSURFACTANT:

The partially purified product was considered as the crude biosurfactant. For further purification, the crude biosurfactant was dissolved in 0.05 M sodium bicarbonate. After filtration, the pH of this solution was adjusted to 2.0 mL using 6 M HCl and then the solution was kept at 4°C for 24 hours. The precipitate was finally collected by centrifugation at 12500 rpm for 15 min, freeze-dried, and stored in airtight container for FT-IR spectroscopy.

FOURIER TRANSFORM INFRA-RED ANALYSIS:

The molecular composition and structural analysis of the purified biosurfactant was evaluated by FT-IR analysis. The spectrum was presented in fig3. The peak at 3405.72 cm^{-1} show the presence of amide N-H stretch; wavenumber 1656.81 cm^{-1}, resulting from the stretching mode C=O bond and wavenumber 1546.76 cm$^{-1,}$ resulting in the deformation mode of N-H bond combined with C–N stretching mode. Three other sharp absorbance peak is seen at 2956, 2923 and 2852 cm^{-1}. Wave number 3000 cm^{-1} to 2800 cm^{-1}, C–H stretching mode suggests the presence of an aliphatic chain. Peaks at 1236 and 1112 cm^{-1} are probably because of C-O-C vibration in esters. FT-IR analysis confirmed the biosurfactant produced by *Pseudomonas fluorescens* as lipopeptide derivative compound.

OPTIMIZATION OF BIOSURFACTANT PRODUCTION:

The RSM is used as a statistical design to determine the significance of growth parameters and their interaction on the biosurfactant production. In the present study, optimization of parameters for better growth conditions of the strain with the help of RSM for designing the experiment is to achieve the highest rate of biosurfactant production. The selected variables, glucose as carbon source, yeast extract as a nitrogen source, pH and salinity (NaCl concentration gL^{-1}) as input parameters. The coded values of each parameter are presented in Table 2. The model was built with the result of the 30 treatments (runs) presented, actual and predicted value of the % E_{24} in Table3. A second-order polynomial equation was used to determine the influence of individual input parameter on the production of biosurfactant through multiple regression analysis. After regression analysis, the second order response model was obtained which is given in equation 1.

Emulsification Index = +67.54+0.89A+0.14B-0.11C-2.22D+ 0.34AB+2.66AC+0.44AD+ 2.46BC-0.36BD + 0.31 C D-7.09A^2 -7.14 B^2 -7.29C^2 -5.27D^2 –Eq. (1).

Where, A: glucose, B: yeast extract, C: pH and D: salinity and A_2, B_2, C_2, D_2, AB, AC, AD, BC, CD were identified as significant terms.

After regression analysis, the results were analysed using ANOVA. The results were given in table 3. The ANOVA analysis results showed that glucose, yeast extract, pH and NaCl had a significant effect on biosurfactant production. The P value was used as a tool to determine the significance of each of the coefficient, which in turn is necessary to understand the pattern of mutual interaction between test variables. Smaller the magnitude values of P, more the significant of the corresponding coefficient. The low value of the coefficient of variation (2.50 %) indicates the very high degree of precision and a good reliability of the experimental values. The value of P <0.0001 indicates the model term that is significant. The lack of fit F-value 2.71 implies there is 14.09 % chance that the lack of fit F value this large could occur due to noise. The fit of the model was expressed with the coefficient of determination R^2 which was found to be 0.9949 and could be indicating that 99.49 % of variability in the response could be explained by this model. The adjusted R^2 value of the model was found to be 0.9901 and predicted R^2 value was 0. 9740. The predicted and experimental value plot of E_{24} showed that actual values were nearer to the straight line (data not shown). The ANOVA result showed that the yeast extract variable had significant (P<0.05) effect on the production of biosurfactant produced by *Pseudomonas fluorescens*. The adequate precision which measures the signal to noise ratio was 37.629 and indicates an adequate signal. The ratio of > 4 is desirable. The optimal concentration of four components was found as glucose 20.28 gL^{-1}; yeast extract 2.51 gL^{-1}, pH at 7.01and NaCl 5.37 gL$^{-1.}$ The quadratic component of glucose and yeast extract

had the highest significant effect ($p<0.0001$) on biosurfactant production. It has been seen as a possibility that glucose and yeast extract has tremendous potential to support microbial growth and biosurfactant production. The ANOVA results showed that the pH value of the medium had significant effect ($p<0.05$) on biosurfactant production at pH 7.01. *Pseudomonas fluorescens* is pH dependent with the optimum production to occur in the particular range where the bacterial strain is most active for biosurfactant production. The strain was able to produce biosurfactant in a pH range of 6-8, although the maximum yield of the biosurfactant was obtained at pH 7.01 The ANOVA results showed that the salinity (NaCl gL^{-1}) value of the highest had significant effect ($p<0.001$) on biosurfactant production at salinity (NaCl gL^{-1}) 5.37. The quadratic component of salinity (NaCl gL^{-1}) had the significant effect on biosurfactant production. Thus, the appropriate combination of glucose, yeast extract, pH and salinity (NaCl gL^{-1}) could enhance the production of biosurfactant by strain *Pseudomonas fluorescens.* Hence our results show that application of RSM enhances the biosurfactant production with the combination of inputs.

Table1. Biochemical test for *Pseudomonas fluorescens*

Tests	Results
Citrate	Positive
Indole	Negative
MR	Negative
VP	Negative
Oxidase	Positive
Catalase	Positive
TSI	Positive
Nitrate reduction	Positive
Gelatin liquefaction	Positive
Starch hydrolysis	Negative

Table2. Specific coded values of Carbon source (glucose g/L), Nitrogen source (Yeast Extract (g/L), pH and Salinity (NaCl g/L) values

Sr. No	Independent variable	Coded values				
		-2	-1	0	1	2
1	glucose (gL^{-1})	10.0	15.0	20.0	25.0	30.0
2	Yeast extract (gL^{-1})	0.0	1.75	2.5	3.25	4.0
3	pH	5	6	7	8	9
4	Salinity (NaCl gL^{-1})	0.0	3.0	6.0	9.0	12.0

Table 3: Central Composite Design (CCD) matrix of independent variables and their corresponding experimental and predicted yields of emulsification activity (E_{24} %)

Run No	Media Components (Coded values)				Emulsification index (E_{24}) %	
	Carbon	Nitrogen	pH	Salinity	Experimental	Predicted
1	0	0	-2	0	37.89	38.81
2	1	1	-1	-1	33.42	33.58
3	-1	1	1	-1	42.15	42.55
4	0	0	0	2	43.21	43.94
5	1	1	1	-1	44.78	44.91
6	0	0	0	0	70.21	68.85
7	1	1	-1	1	33.57	33.87
8	-1	-1	-1	-1	49.10	48.41
9	0	0	2	0	37.14	37.45
10	-1	-1	1	1	31.45	30.77
11	1	-1	1	-1	42.81	42.44
12	1	-1	-1	-1	43.21	42.30
13	-1	-1	1	-1	38.31	37.22

14	-1	1	-1	-1	42.63	42.54
15	-1	1	-1	1	37.12	36.97
16	-1	1	1	1	35.36	35.46
17	0	0	0	0	70.24	68.85
18	0	-2	0	0	35.45	37.93
19	2	0	0	0	40.41	40.81
20	0	0	0	0	65.61	68.85
21	0	0	0	-2	49.56	50.10
22	-2	0	0	0	37.82	38.69
23	0	0	0	0	69.62	68.85
24	1	-1	-1	1	44.12	43.21
25	1	1	1	1	43.54	43.71
26	0	0	0	0	68.72	68.85
27	-1	-1	-1	1	44.37	43.45
28	0	0	0	0	68.82	68.85
29	0	0	2	0	35.12	33.92
30	1	-1	1	1	42.58	41.85

Table 4: Analysis of variance (ANOVA) of main effects of factors for production of biosurfactant by *Pseudomonas fluorescens*

Factors	DF	Sum of Squares(SS)	Mean sum of squares (MSS)	F-Ratio	P-Value
Model	14	3888.34	277.74	208.90	<0.0001
Residual	15	19.94	1.33		
Error	5	3.10	0.62		
Corrected total	29	3908			
R-square=0.9949; Adj. R-square=0.9901; predicted R-square=0.9740					

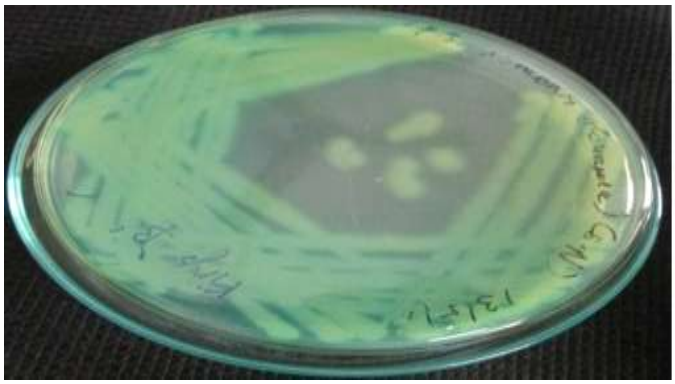

Figure 1: *Pseudomonas fluorescens* culture on King's B media.

Figure 2: Blood agar plate of *Pseudomonas fluorescens*

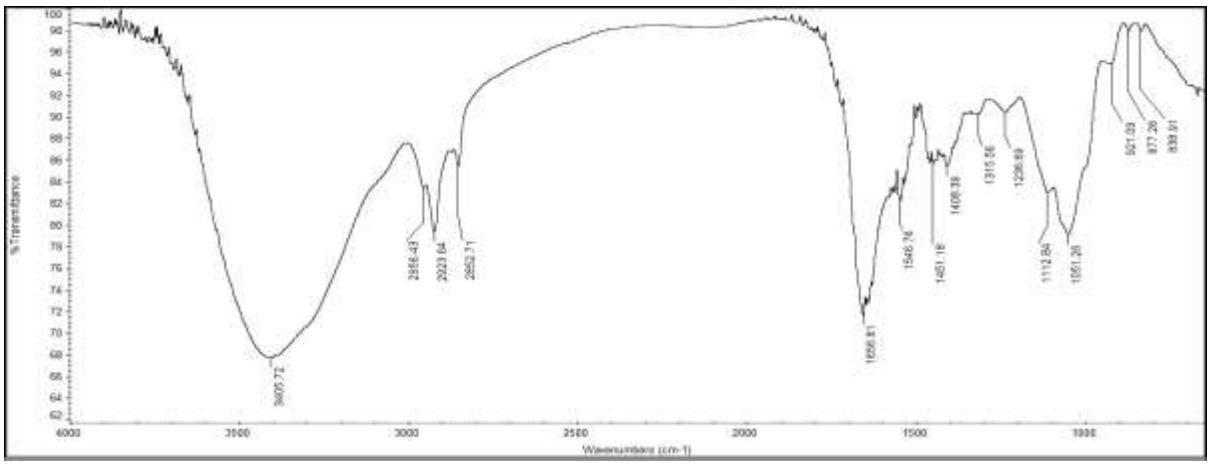

Figure 3: Fourier transform infra-red spectrum of biosurfactant produced by *Pseudomonas fluorescens*

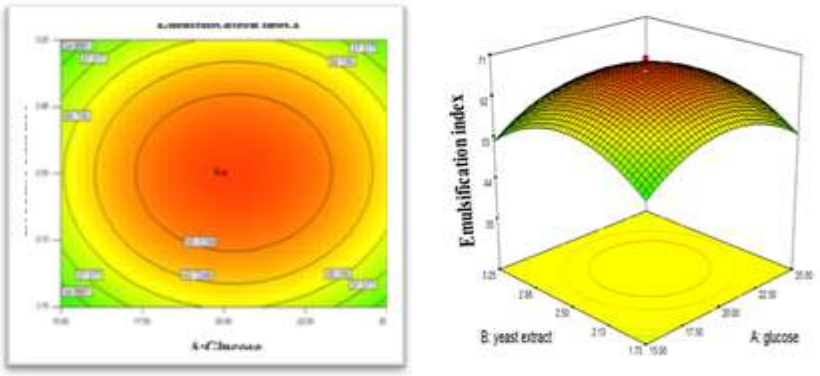

Figure 4A:

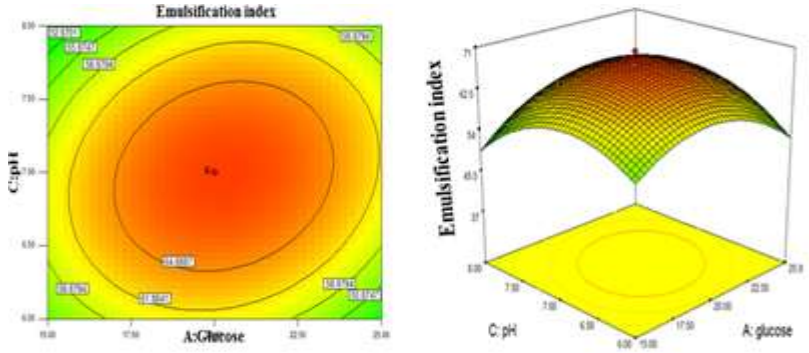

Figure 4B:

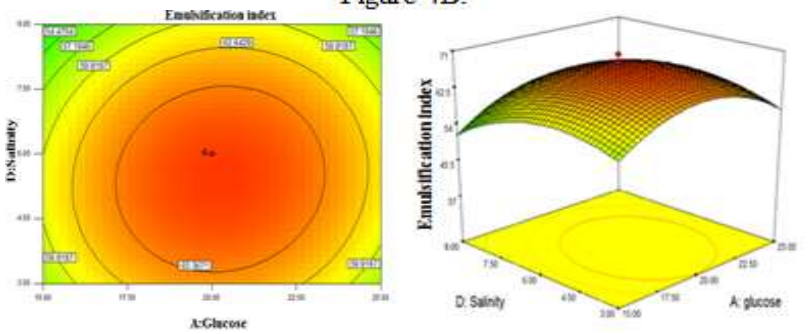

Figure 4C:

Figure 4: Two and three dimensional contour plots for the maximum emulsification index (maximum biosurfactant production).
A). Emulsification index as function of glucose and yeast extract.
B). Emulsification index as function of glucose and pH.
C). Emulsification index as function of glucose and salinity.

CONCLUSION:

In the present investigation indigenous strain of *Pseudomonas fluorescens* was is a potent biosurfactant producing strain. The characterization study FT-IR confirmed as lipopeptide. The optimisation of a variable could increase the biosurfactant production at 20.28 gL^{-1} glucose, 2.51 gL^{-1} yeast extract, pH 7.01 and salinity 5.37 gL^{-1} of NaCl. The production yield is approximately 2.2 fold increased than the original production. The conclusion of this study represented *Pseudomonas fluorescens* is the novel strain and used for bioremediation of oil contaminated soils.

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