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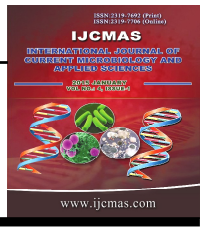


Original Research Article

RSM study for the production of rhamnolipid using *Catla catla* Fish fat

Santanu Sana[1], Munna Bhattacharya[1], Sriparna Datta[2]* and Dipa Biswas[2]

[1]Research Scholar, Department of Chemical Technology, University of Calcutta, Kolkata, India
[2]Professor, Department of Chemical Technology, University of Calcutta, Kolkata, India
**Corresponding author*

K e y w o r d s

Pseudomonas aeruginosa, Box-behnken design, RSM, Fish fat, Rhamnolipid

A B S T R A C T

Biosurfactant has great industrial importance due to its eco-friendly nature and its ability to function under extreme conditions of temperature, pH and salinity. There are reports on the production of biosurfactants from various substrates including waste oils but no such report on utilization of waste fat is found yet. A 3^3 full factorial response surface modeling (RSM) was employed with time, temperature and pH as variables to optimize biosurfactant production using *Pseudomonas aeruginosa* DSM 50071 isolated from oil contaminated soil using *Catla catla* fish fat. The responses selected were surface tension (ST) and optical density (OD_{600}) of culture media. The optimum conditions were found as 72 hours of incubation time, 37 ^{0}C temperature and pH 7.00 for maximum yield of 0.421 g/l with ST lowering

Introduction

Biosurfactants are microbiologically derived amphipathic heterogeneous group of surface active agents produced from hydrophobic or hydrophilic substances which includes glycerol, mannitol, glucose, n-paraffin, waste vegetable oils, etc (Abdel-Mawgoud *et al*., 2010).

Currently most of the surfactants are chemically synthesized from petroleum industry (Banat *et al*., 2000) and they are used in large number of industries. Biosurfactants have recently attracted the attention due to their biodegradable and environment friendly nature, biocompatibility, specific activity in extreme conditions of temperature, pH and salinity.

Rhamnolipid is the most popular type of biosurfactant belonging to the class of glycolipid type of biosurfactant. Rhamnolipid can be produced from *Pseudomonas aeruginosa* (Rodrigues *et al*., 2006) and *Burkholderia sp.* (Abdel-Mawgoud *et al*., 2010). Besides surface tension reducing property rhamnolipid also show antimicrobial activity against *Bacillus subtilis* (Haba *et al*., 2003), *Staphylococcus aureous*, *Salmonella typhimurium* (Benincasa *et al*., 2004), *Escherichia coli*, herpes simplex virus (HSV) (Remichkova *et al*., 2008), potato virus X and anticancer activity against human breast cancer cell line (Thanomsub *et al*., 2006).

Unfortunately biosurfactant cannot compete with synthetic surfactants due to their higher production cost. Hence attempts are being made to reduce the production cost of biosurfactants utilizing waste materials as media substrate such as waste vegetable oil (Haba *et al*., 2000), soybean soapstock waste (Nitschke *et al*., 2005), sunflower soapstock waste, various oil refinery wastes (Bednarski *et al*., 2004), potato process effluents and waste materials produced during the processing of cereals, pulses and molasses (Mukherjee *et al*., 2006). Animal fat and plant derived fatty substances may solidify or become viscous between the temperatures of 32 °F to 150 °F and hence create problem in pipes and sewers in cold countries during their disposal (Wright-Pierce, 2006). These fats can be used for the production of biosurfactant by microbial degradation which is also cheaper than vegetable oils. Till date only Deshpande and Daniels (1995) claimed that *Candida bombicola* can grow on animal fat and produce sophorolipid. So, animal fat could be a good alternative to vegetable oils remaining still unexplored as a substrate for production of biosurfactant.

Catla catla fish fat is a widely used, cheap fish in West Bengal and its fatty acid composition is already established by Menon *et al*. (2010). We, therefore, aimed to utilize it as a substrate for production of biosurfactant using microbes isolated from different fat contaminated sites of the city. A *Pseudomonas sp*. capable of rhamnolipid production was isolated. To obtain the maximum production of rhamnolipid, culture conditions were optimized by RSM using fat from a commonly used fish *Catla catla* as the carbon source.

Materials and Methods

Materials

Fish fat of *Catla catla* was procured from local market. Waste Frying Oil was collected from restaurants. Chemicals and solvents were of LR grade and purchased from local suppliers.

Isolation, identification and screening of best biosurfactant producing microorganism

A biosurfactant producing bacteria was isolated from fat contaminated soils of market place in Kolkata, and it was identified by 16S rRNA sequencing. Isolation and media optimization study were carried out using Bushnell Haas media containing (g/l): KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1, $MgSO_4$ 0.2, $CaCl_2$ 0.02 and $FeCl_3$ 0.05. The culture was maintained at 4 °C on Nutrient agar media and sub-cultured every two weeks. Throughout the study 20g/l fat was used as carbon source and 1% v/v bacterial suspension of OD_{600} - 0.5 was used as inoculum.

ST and OD_{600} measurement

After 7 days incubation the culture broth was centrifuged at 9000 × g (REMI, R24) for 20 minutes and the ST of the cell and fat free supernatant was measured (Garland et al. 2003) using digital tensiometer (Dataphysics DCAT-11, Germany) at 30 °C.

Bacterial growth was measured by observing OD_{600} after 20 times dilution in a colorimeter (CL 157, ELICO).

Optimization Studies

Selection of the parameters for RSM by varying one factor at a time

To find the optimum condition for maximum biosurfactant production, fish fat was under a wide variation of culture conditions like incubation time (12, 18, 24, 48, 72 and 120 hours), temperature (30°C, 33°C, 37°C, 40°C and 42°C) and pH (6, 6.5, 7, 7.5 and 8). From the results of one factor at a time 3 levels of time (48, 72, 96 hours), temperature (34°C, 37°C, 40°C) and pH (6, 7, and 8) were chosen.

Optimization of the culture conditions using Box-Behnken Design

Box-Behnken design (Annadurai *et al*., 2008) was used for screening media components to evaluate their effects and mutual interaction on ST reduction and OD_{600}. Three levels factorial design was used to optimize the physical conditions for biosurfactant production using the software Design of Experiments (DOE++, Trial Version 8.0.7.1, Stat-Ease, Minneapolis, USA). A total of 15 experiments were designed to evaluate the regression coefficient. The responses of the culture media i.e. ST and OD_{600} were taken as R_1 and R_2.

Extraction of the biosurfactant

The supernatant was acidified to pH 2 with concentrated HCl and kept overnight at 4°C.

Next day it was centrifuged at 15,000 × g for 30 minute. The precipitate was dissolved in de-ionized water and further extracted with two volumes of chloroform and methanol (2:1 v/v). The organic phase was evaporated in a rotary evaporator (EYELA, Rikakikai Co. Ltd., Tokyo, Japan) to isolate the crude biosurfactant.

Characterizations of crude biosurfactant

For identification of the Rhamnolipid biosurfactant, Molisch's test (Dubois *et al*., 1956), Orcinol-test (Chandrasekaran and Bemiller, 1980) and FTIR (Jasco, FT/IR-6300, USA) analysis were carried out in the 4000-400 cm^{-1} spectral region.

Study of biosurfactant production kinetics

The kinetic studies of the biosurfactant production were performed at optimized culture conditions up to 96 hours by measuring surface tension, biomass count and biosurfactant yield rate.

Result and Discussion

Isolation and Identification of fish fat degrading microorganism

From the large number of isolated bacterial strains, an aerobic, rod shaped Gram negative bacteria C2 was finally selected for its best fish fat (20 g/l) degrading efficiency (39%) and ST lowering capacity. 16S rRNA gene sequencing showed similarity of the isolated C2 strain with *Pseudomonas aeruginosa* DSM 50071 (Gene Bank Accession no. NR 026078).

Optimization of process parameters

Using C2 strain the two responses ST lowering and OD_{600} were observed at three different levels of time, temperature and pH (Fig. 1). The optimum condition for the best ST reduction and the OD_{600} were determined using the Box-Behnken design and compared with the predicted value as presented in Table 1.

The equation and the ANOVA tables obtained after regression analysis using the Design Expert software is as follows:

$$ST = +31.58 - 0.72*(A) + 0.21*(B) - 2.17*(C) - 0.61*(A)*(B) + 1.22*(A)*(C) - 0.64*(B)*(C) + 2.05*(A)^2 + 1.96*(B)^2 + 4.66*(C)^2$$

$$OD_{600} = +0.49 + 7.500e^{-3}*(A) + 0.021*(B) + 0.12*(C) + 0.038*(A)*(B) - 0.022*(A)*(C) - 0.074*(B)*(C) - 0.18*(A)^2 - 0.085*(B)^2 - 0.12*(C)^2$$

Where, A, B, and C are the coded levels of time (hr), temperature (°C) and pH.

From Table 2 we find that the model F-value of 39.12 is significant. Here A, C (time and pH), AC, A2, B2, C2 (quadratic components of three variables) have significant effect on the ST lowering capability. And the regression co-efficient R = 0.9929.

From Table 3 we find that the model F-value of 17.61 is significant. In this case C (pH), BC, A2, B2, C2 (squared variables) show significant effect on $OD_{600.}$ The coefficient of variation 16.29, signifies that the model shows dependability and the regression co-efficient R was 0.9846.

The effects of the interaction of any two variables (third having coded value 0) upon biosurfactant production were plotted in all the possible directions. The interaction between the three factors *i.e.* time, temperature and pH and their effect on ST lowering capability is shown in Fig. 2A, 2B, and 2C. Similarly Fig. 2D, 2E and 2F illustrated the interactive effect of the factors on the OD_{600}.

Effect of time

From Fig. 1a the range of incubation time for optimization study was found to be between 48 hrs to 96 hrs. The minimum ST of 31.30 mN/m and the OD_{600} were observed after 72 hour incubation time and at 37 °C temperature (Fig. 2A and 2D).

Sahoo *et al*. (2011) have optimized the rhamnolipid type biosurfactant production from *Pseudomonas aeruginosa* OCD_1 using n-Octadecane at 96 hour incubation.

Effect of temperature

The optimum temperature range for RSM was chosen from the Fig. 1b within the range 34 °C to 40 °C. The maximum ST lowering (31.30 mN/m) effect and OD_{600} was observed at 37 °C (Fig. 2B and 2E).

Effect of pH

The optimum pH range for the RSM was from pH 6.00 to 8.00 (Fig. 1c). The three dimensional plot of Fig. 2C and 2F represent

that the optimum pH is 7.00. This result is supported by Haba *et al*. (2000) using waste frying oil as substrate to produce rhamnolipid from *Pseudomonas aeruginosa* 47T2 NCIB 40044.

Characterization of biosurfactant

The Molisch's test and Orcinol test indicated the sugar moieties present in the biosurfactant may be rhamnose and hence the isolated biosurfactant could be rhamnolipid. FT-IR analysis also confirmed the presence of rhamnose and long hydrocarbon chains. Fig. 3 shows absorbance bands at 2921, 2852, 1461 cm^{-1} and 1375 $cm^{-1,}$ which may be due to the C-H stretching of $–CH_2$ and $–CH_3$ groups. C-O stretching bands rising from ester and carboxylic groups were found at 1162 cm^{-1} and 1054 cm^{-1}. The C-H and O-H deformation due to carbohydrates were found at 1461, 1375 and 1226 cm i.e., in the region 1461-1226. Similar results were also reported by Sing *et al*. (2013) and Sahoo *et al*. (2010).

Biosurfactant production kinetics

Fig. 4 shows the ST reduction, biomass and rhamnolipid production at 12 hours interval up to 96 hours under optimum culture condition. The ST dropped rapidly and reached its maximum of 31.57 mN/m after 72 hours incubation when the biosurfactant concentration reached 0.421 g/l. After 72 hours, the ST of the culture media slightly increased probably due to production of secondary metabolites which could interfere with the ST lowering property.

Table.1 Experimental plan for optimization of ST and OD600 using Box-Behnken design

Run	Factors			ST (mN/m)		OD_{600}	
	Time (hr)	Temp (°C)	pH	Exp	Predict	Exp	Predict
1	48.00	34.00	7.00	35.62	35.48	0.24	0.23
2	96.00	34.00	7.00	35.10	35.26	0.15	0.17
3	48.00	40.00	7.00	37.28	37.12	0.22	0.20
4	96.00	40.00	7.00	34.31	34.45	0.28	0.29
5	48.00	37.00	6.00	42.72	42.40	0.050	0.033
6	96.00	37.00	6.00	39.15	38.52	0.14	0.093
7	48.00	37.00	8.00	34.98	35.61	0.28	0.33
8	96.00	37.00	8.00	36.28	36.61	0.28	0.30
9	72.00	34.00	6.00	39.05	39.52	0.040	0.068
10	72.00	40.00	6.00	40.73	41.22	0.22	0.26
11	72.00	34.00	8.00	36.94	36.45	0.50	0.46
12	72.00	40.00	8.00	36.05	35.58	0.39	0.36
13	72.00	37.00	7.00	31.65	31.58	0.50	0.49
14	72.00	37.00	7.00	31.30	31.58	0.50	0.49
15	72.00	37.00	7.00	31.78	31.58	0.47	0.49

Legend: ST: Surface tension; mN/m: milinewton per meter; OD600: Optical density at 600 nm

Table.2 Analysis of variance for ST lowering capacity by P. aeruginosa DSM 50071

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	150.36	9	16.71	39.12	0.0004	significant
A-Time	4.14	1	4.14	9.70	0.0264	
B-Temperature	0.34	1	0.34	0.80	0.4108	
C-pH	37.82	1	37.82	88.57	0.0002	
AB	1.50	1	1.50	3.50	0.1202	
AC	5.93	1	5.93	13.88	0.0136	
BC	1.65	1	1.65	3.85	0.1068	
A^2	15.47	1	15.47	36.22	0.0018	
B^2	14.12	1	14.12	33.08	0.0022	
C^2	80.25	1	80.25	187.93	<0.0001	
Residual	2.14	5	0.43			
Lack of Fit	2.01	3	0.67	10.64	0.0871	not significant

Legend: CV 1.81; R^2 0.9860

Table.3 Analysis of variance for OD600 of P. aeruginosa DSM 50071

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	0.34	9	0.037	17.61	0.0028	significant
A-Time	$<0.45\times10^{-4}$	1	0.45×10^{-4}	0.21	0.6643	
B-Temperature	$<0.3403\times10^{-3}$	1	0.3403×10^{-3}	1.61	0.2609	
C-pH	0.12	1	0.12	58.39	0.0006	
AB	$<0.5625\times10^{-3}$	1	$<0.5625\times10^{-3}$	2.65	0.1642	
AC	$<0.2025\times10^{-3}$	1	$<0.2025\times10^{-3}$	0.96	0.3732	
BC	0.022	1	0.022	10.26	0.0239	
A^2	0.12	1	0.12	58.68	0.0006	
B^2	0.027	1	0.027	12.52	0.0166	
C^2	0.051	1	0.051	23.97	0.0045	
Residual	0.011	5	$<0.212\times10^{-3}$			

Lack of Fit	0.010	3	$<0.336\times10^{-3}$	13.01	0.0722	not significant

Legend: CV - 16.29; R^2 - 0.9694

Fig.1 Effect of variation of (a) time (b) temperature and (c) pH on ST lowering and OD600 in RSM study

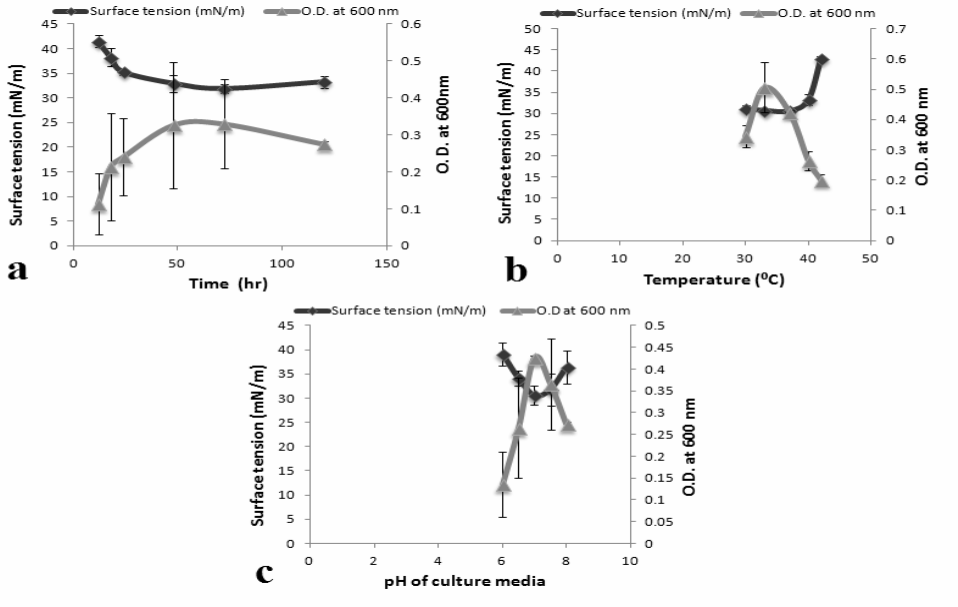
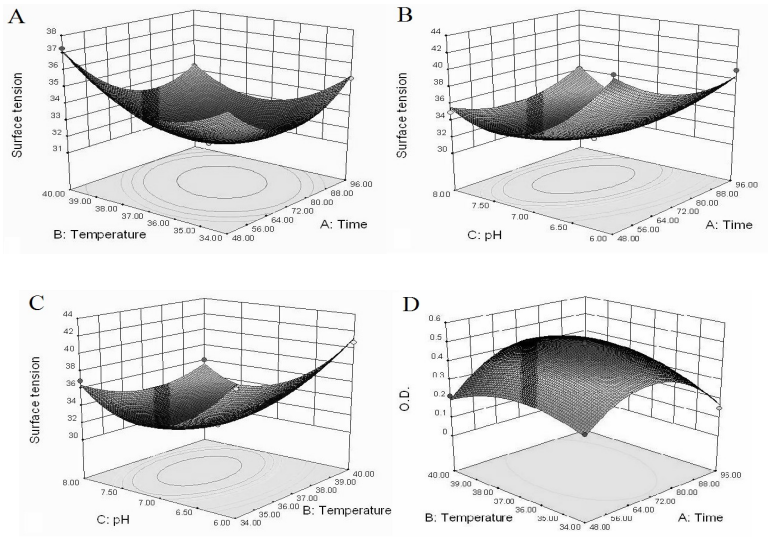


Fig.2 RSM graph showing effect of interaction between time, temperature and pH on ST lowering and OD600



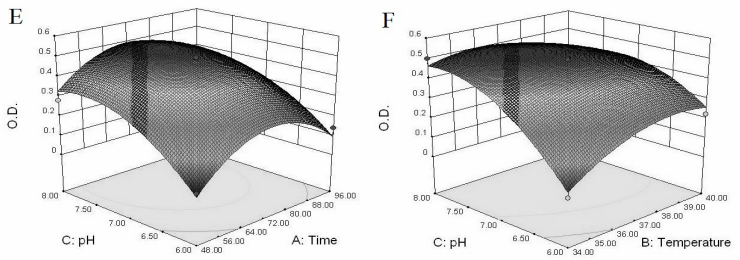


Fig.3 FT-IR spectrum of the extracted rhamnolipid

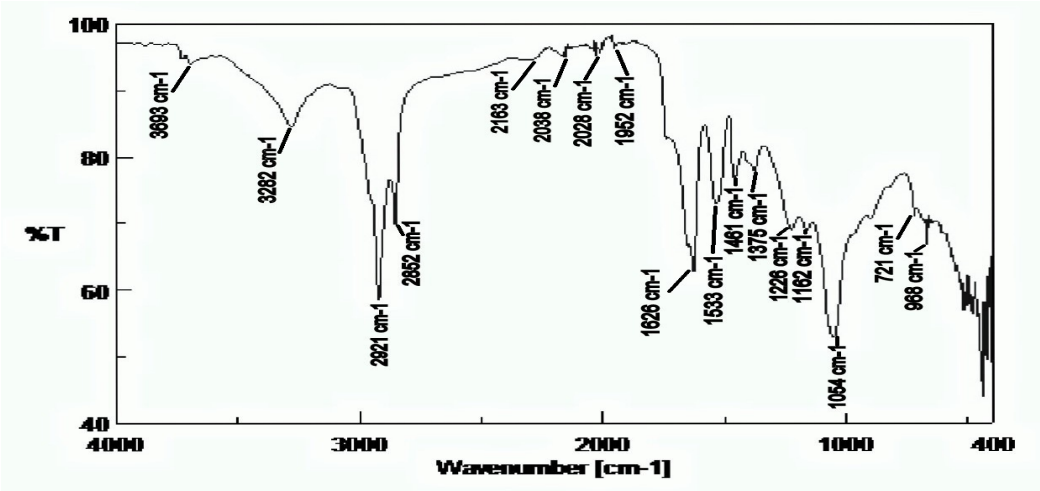
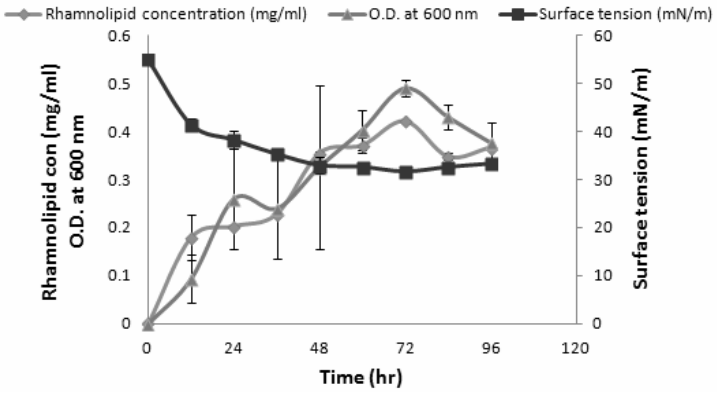


Fig.4 Biosurfactant production kinetics by the C2 strain at optimized culture conditions



This suggests that the rhamnolipid is a primary metabolite (Tabatabaee A *et al*, 2005) and it is produced during the exponential growth phase. This result is also supported by Abouseoud *et al*. (2007) during the growth of *Pseudomonas flourescens* Migula 1895-DSMZ on olive oil.

RSM is a useful statistical experimental

technique to optimize the factors and their interaction for any biotechnological processes. In this present study we have optimized the process parameters to find the condition for maximum rhamnolipid production from *Catla catla* fish fat by *Pseudomonas aeruginosa* C2 using shake flask method by RSM with minimum number of experiments and the optimum conditions for lowest ST of 31.3 mN/m were found as 72 hours incubation time at 37 °C temperature and pH 7.00. We are interested to use isolated biosurfactant for pharmaceutical application in our further work and also to utilize other cheap animal fats for the production of biosurfactant.

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