Research Journal of
Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (10): 705-716, 2007
ISSN 1816-4935
© 2007 Academic Journals Inc.

Optimised Production of Biosurfactant by *Serratia marcescens* DT-1P

[1]Rajkumar Bidlan, [1]N. Deepthi, [2]N.K. Rastogi and [1]H.K. Manonmani
[1]Department of Fermentation Technology and Bioengineering,
[2]Department of Food Engineering, Central Food Technological Research Institute,
Mysore-570020, Karnataka, India

Abstract: The cultural conditions for the production of biosurfactant by *Serratia marcescens* DT-1P were optimised by Response Surface Methodology (RSM). A study of complex interactions among glycerol (1-5% w/v), inoculum (1-10 OD_{600}), pH (4-8), temperature (20-40°C) and $(NH_4)_2SO_4$ (0.5-2.0%) were carried out using central composite rotatable design of experiments. The optimum conditions, which were obtained i.e., glycerol (3.4% w/v), inoculum (1.0 OD_{600}), pH (5.8), temperature (26.6°C) and $(NH_4)_2SO_4$ (0.2%) yielded 6.0 mg L^{-1} biomass and glycerol (5% w/v), inoculum (10.0 OD_{600}), pH (8.0), temperature (20.0°C) and $(NH_4)_2SO_4$ (0.05%), yielded 24.48 mg % of biosurfactant. The surfactant was identified as arabinolipid.

Key words: Biosurfactant, response surface methodology, central composite rotatable design, *Serratia marcescens*, arabinolipid

INTRODUCTION

Biosurfactants are amphiphilic compounds of microbial origin with considerable potential commercial applications in various industries. Surfactants are widely used in petroleum, pharmaceutical, cosmetic and food industries (Patel and Desai, 1997) and most of these are synthesised chemically (Benat *et al*., 2000; Kosaric, 1984) mainly derived from petroleum (Patel and Desai, 1997). However, rapid advances in biotechnology and increased environmental awareness among people have provided further impetus for the surfactant production of microbial origin as a possible alternative to chemically synthesised surfactants. Biosurfactants have many advantages over the chemically synthesised ones. Some of these are biodegradability, generally low toxicity, biocompatibility, digestibility, easy availability of raw materials for surfactant production, acceptable production-economics, specificity, use in environmental conditions, effectiveness even at extreme temperatures, pH and salinity (Kosaric, 1984). Many microorganisms have been reported to produce biosurfactants (Patel and Desai, 1997) and most of these microbial surfactants have been used in oil industries, both for petroleum production and for incorporation in oil formulations (Van Dyke *et al*., 1991), oil spill bioremediation/dispersion, both inland and at sea, removal/mobilisation of oil sludge from storage tanks, enhanced oil recovery (Georgiou *et al*., 1992; Kairs and Khan, 1994a and b), for emulsion polymerisation for paints, paper coatings and industrial coatings, in cement, textile and fibre manufacturing (Layman, 1985), metal treatment, mining, water treatment, coal slurry- defoamers and as wood preservatives (Layman, 1985).

Surfactants have also been used in cost- effective, contaminant- specific treatments to reduce the concentration of individual or mixed environmental pollutants (Head, 1998). The ability of a surfactant to enhance the biodegradation of slightly soluble organic compounds depends on the increase in the

Corresponding Author: H.K. Manonmani, Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore-570020, India
Tel: +91-821-2513658 Fax: +91-821-2517233

bio-availability of these organic compounds (Begley *et al*., 1996). The bio-availability could be increased either by the addition of the surfactant during degradation or by applying the biosurfactant-producing microorganisms to the contaminated environment.

In present studies, *Serratia marcescens* DT-1P was screened for biosurfactant production. The effect of biomass and surfactant yield was found to be a function of substrate concentration, inoculum concentration, pH, temperature and $(NH_4)_2SO_4$ concentration. The purpose of the present research is to study and to optimise the effect of these variables on biomass production and surfactant yield using response surface methodology.

MATERIALS AND METHODS

Bacterial Strain

The biosurfactant producing bacterial strain *Serratia marcescens* DT-1P was obtained from the Culture Collection Centre maintained at CFTRI, Mysore, India. The culture was maintained on nutrient agar containing (g $L^{-1}$of distilled water) peptone 5.0, beef extract 3.0, NaCl 5.0 and agar-agar 20.0. Final pH of the nutrient agar was 7.2.

Biosurfactant Production Medium

The medium used for biosurfactant production contained (g in 1 L of distilled water) peptone 4.0, glycerol 10-15, KH_2PO_4 and K_2HPO_4 0.1 M from pH 4-8 and $(NH_4)_2SO_4$ 0.5-2. Medium was autoclaved at 15 lb in^{-2}, 121°C for 15 min.

Inoculum

Inoculum was prepared by transferring 50 mL of 24 h old seed culture to 1 L fermentor containing 450 mL medium. The medium used for inoculum production contained (g L^{-1} of distilled water) peptone 4.0, glycerol 30.0, KH_2PO_4 3.0, K_2HPO_4 7.0 and $(NH_4)_2SO_4$ 1.0. The pH of the medium was 7.0. The growth was allowed to proceed at 200 rpm at ambient temperature (26-30°C) for 48 h, after which the cells were harvested by centrifuging at 10,000 rpm for 15 min at 4°C. The cells were washed once with 0.1 M-phosphate buffer, pH 7.0, resuspended in minimum known volume of the same buffer. This suspension was used as inoculum for optimization and validation experiments.

Experimental Design

A CCRD (Cochran and Cox, 1957) with five variables was used to study the response pattern and to determine the optimum combination of variables. The variables optimized were substrate concentrations (1-5% w/v), inoculum level (1-10 OD_{600}), pH (4-8), temperature (20-40°C) and $(NH_4)_2SO_4$ concentrations (0.05-0.2%) at 5 levels viz., -2, -1, 0, +1 and +2. The concentration of peptone was maintained constant (Table 1).

The CCRD shown in Table 2 was arranged to allow for fitting an appropriate regression model using multiple regression programme. The CCRD combines the vertices of the hypercubes whose co-ordinates are given by a 2n factorial design to provide for the estimation of curvature of the model (Cochran and Cox, 1957). Six replicates (treatments 27-32) at the centre of the design were used to allow estimation of pure error sum of squares. Experiments were randomised to minimise the effect of unexplained variability in the observed responses due to extraneous factors.

Statistical Analysis

A second order polynomial was used to fit the experimental data given in Table 2. The model proposed for the response (Y_1 and Y_2) was:

Table 1: Variables and their levels for CCRD

Variables	Symbols	-2	-1	0	1	2	Mean	SD
Substrate conc. (%)	x_1	1.0	2.000	3.00	4.000	5.0	3.00	1.000
Inoculum level (%)	x_2	1.0	3.250	5.50	7.750	10.0	5.50	2.250
pH	x_3	4.0	5.000	6.00	7.000	8.0	6.00	1.000
Temp. (°C)	x_4	20.0	25.000	30.00	35.000	40.0	30.00	5.000
$(NH_4)_2SO_4$ (g L^{-1})	x_5	0.5	0.875	1.25	1.625	2.0	1.25	0.375

Table 2: Treatment schedule for five-factor CCRD and response in terms of biomass yield

Exp. No.	Substrate conc. ((%) x_1	Inoculum level (%) x_2	pH x_3	Temp. (°C) x_4	$(NH_4)_2SO_4$ (g L^{-1}) x_5	Biomass yield (g L^{-1})	Biosurfactant yield (g L^{-1})
1	-1	-1	-1	-1	1	3.68	1.92
2	1	-1	-1	-1	-1	3.20	5.76
3	-1	1	-1	-1	-1	2.24	2.20
4	1	1	-1	-1	1	2.84	2.00
5	-1	-1	1	-1	-1	2.52	4.88
6	1	-1	1	-1	1	4.10	6.84
7	-1	1	1	-1	1	2.12	4.72
8	1	1	1	-1	-1	3.68	9.20
9	-1	-1	-1	1	-1	2.04	2.82
10	1	-1	-1	1	1	3.16	4.88
11	-1	1	-1	1	1	2.40	3.20
12	1	1	-1	1	-1	2.56	7.60
13	-1	-1	1	1	1	3.52	5.68
14	1	-1	1	1	-1	3.44	6.12
15	-1	1	1	1	-1	3.48	2.50
16	1	1	1	1	1	3.88	5.48
17	-2	0	0	0	0	2.40	2.20
18	2	0	0	0	0	4.00	6.36
19	0	-2	0	0	0	4.50	5.32
20	0	2	0	0	0	2.90	4.40
21	0	0	-2	0	0	1.50	2.52
22	0	0	2	0	0	3.48	6.04
23	0	0	0	-2	0	3.30	3.64
24	0	0	0	2	0	2.80	3.60
25	0	0	0	0	-2	4.24	3.50
26	0	0	0	0	2	5.20	2.64
27	0	0	0	0	0	4.30	3.50
28	0	0	0	0	0	3.95	3.00
29	0	0	0	0	0	4.00	2.88
30	0	0	0	0	0	4.50	2.99
31	0	0	0	0	0	3.90	3.40
32	0	0	0	0	0	4.40	3.28

$$y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_{11}xi^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{44}x_4^2 + a_{55}x_5^2 + a_{12}x_1x_2 + a_{13}x_1x_2 + a_{14}x_1x_2 + a_{15}x_1x_5 + a_{23}x_2x_3 + a_{24}x_2x_4 + a_{25}x_2x_5 + a_{34}x_3x_4 + a_{35}x_3x_5 + a_{45}x_4x_5.$$

The co-efficients for the fitted polynomial for biomass yield and surfactant yield are provided in Table 3.

Optimisation of the polynomials for the yield of surfactant and biomass was performed by a non-linear mathematical optimisation procedure of the Quattro Pro Software Package [Quattro Pro. Version 4.0 Borland International Inc., USA] (Joglekar and May, 1987). The optimum conditions were verified by conducting experiments under these conditions. Responses were monitored and results were compared with model predictions.

The fitted polynomial equation was expressed as surface plots to visualise the relationship between the response and experimental levels of each factor and to deduce the optimum conditions.

Period of Incubation vs Surfactant Production

Serratia marcescens DT-1P was cultivated in shake flasks and the effect of incubation period on surfactant production was studied. Flasks in triplicates were withdrawn on 4th, 8th, 12th and 15th day. Surfactant and biomass yields were estimated.

Table 3: Estimated coefficients of the fitted second order polynomial representing the relationship between the response and the process variable

	Biomass yield			Biosurfactant yield		
Coefficients	Estimated coefficients	Standard error	t-value	Estimated coefficients	Standard error	t-value
a_0	4.210	0.133	31.730[a]	3.083	0.167	18.458[a]
a_1	0.336	0.068	4.946[a]	1.178	0.085	13.784[a]
a_2	-0.236	0.068	-3.473[b]	-0.160	0.085	-1.872[c]
a_3	0.358	0.068	5.265[a]	0.920	0.085	10.762[a]
a_4	-0.038	0.068	-0.552[ns]	0.028	0.085	0.331[ns]
a_5	0.186	0.068	2.737[b]	-0.337	0.085	-3.938[a]
a_{11}	-0.279	0.061	-4.538[a]	0.368	0.077	4.757[a]
a_{22}	-0.154	0.061	-2.503[c]	0.513	0.077	6.632[a]
a_{33}	-0.456	0.061	-7.428[a]	0.368	0.077	4.757[a]
a_{44}	-0.316	0.061	-5.149[a]	0.203	0.077	2.623[b]
a_{55}	0.101	0.061	1.648[ns]	0.065	0.077	0.845[ns]
a_{12}	0.036	0.083	0.436[ns]	0.210	0.105	2.006[c]
a_{13}	0.129	0.083	1.548[ns]	-0.015	0.105	-0.143[ns]
a_{14}	-0.104	0.083	-1.248[ns]	-0.012	0.105	-0.119[ns]
a_{15}	-0.021	0.083	-0.256[ns]	-0.788	0.105	-7.521[a]
a_{23}	0.101	0.083	1.217[ns]	-0.078	0.105	-0.740[ns]
a_{24}	0.174	0.083	2.089[c]	0.035	0.105	0.334[ns]
a_{25}	-0.249	0.083	-2.991[b]	-0.365	0.105	-3.486[b]
a_{34}	0.231	0.083	2.781[b]	-0.780	0.105	-7.450[a]
a_{35}	-0.096	0.083	-1.157[ns]	0.400	0.105	3.8200[a]
a_{45}	0.021	0.083	0.256[ns]	0.423	0.105	4.035[a]

[a]Significant at 0.1% level, [b]Significant at 1% level, [c]Significant at 5.0% level, [ns]Not significant even at 5% level

Large-Scale Production of Surfactant

Serratia marcescens DT-1P was cultivated in 25 L reactor, containing 15 L of optimised medium. Mixing and airflow were through sparger. Temperature was maintained at 30±2°C. Optimised medium containing (g% w/v) peptone 0.4, glycerol 5, KH_2PO_4 and K_2HPO_4 to pH 8.0 (0.1 M) and $(NH_4)_2SO_4$ 0.05 was sterilized at 15 lb in^{-2}, 121°C for 15 min, cooled and inoculated at optimised level of inoculum (OD_{600} = 10.0). The reactor was kept in operation for 8 days. Samples were drawn at regular intervals and analyzed for biosurfactant yield as described.

Analyses

Biomass Yield and Biosurfactant Production

The culture broth was centrifuged and the cell pellet was washed twice with phosphate buffer (0.1 M, pH 7.0). The cell mass was dried at 80°C to constant weight.

The culture broth was centrifuged at 10,000 rpm at 4°C for 10 min and the biosurfactant was precipitated using 2 volumes of ethanol after setting the pH to 2.0 with 1:1 HCL. The precipitate was centrifuged and dried at room temperature. The concentration was expressed as g % or g L^{-1}. All the experiments were done in triplicates and the average values are reported.

Analysis of Biosurfactant

Estimation of Total Carbohydrates (Sugars)

Five milligrams of surfactant was dissolved in 1 mL distilled water and total sugars were analyzed by phenol-sulphuric acid method (Dubois *at al*., 1956).

Analysis of Carbohydrate

Twenty milligrams of biosurfactant was dissolved in 0.28 mL distilled water and hydrolysed by 0.72 mL of concentrated sulphuric acid in a boiling water bath for 6-8 h. Sulphuric acid (Selvendran and O'Neill, 1987) was diluted to 10% level and neutralised with solid barium

carbonate, followed by filtration through Amberlite IR 120 H^+. The samples were then converted to alditol acetate by treatment with sodium borohydride and analyzed by Gas Chromatography (Sawardekar, 1967).

Estimation of Total Lipids

Five gram of the surfactant was dissolved in 1 mL water and extracted thrice in chloroform. All the chloroform fractions were pooled and evaporated to dryness. The weight of the lipid fraction was estimated.

Analysis of Lipids

Ten milligrams of surfactant was taken in test tube and 1 mL of chloroform, 0.85 mL methanol and 0.15 mL concentrated sulphuric acid were added. Hydrolysis was carried out at 100°C in sealed tubes for 12 h 0.5 mL water was added to the hydrolysed samples and vortexed well. The chloroform layer was taken out after separation and analyzed by Gas Chromatography (Brandl *et al*., 1988).

RESULTS AND DISCUSSION

Diagnostic Checking of the Models

Two responses were measured in the experiments biosurfactant yield (y_1) and biomass yield (y_2) (Table 3). The significant terms were omitted based on students t-ratio (Khuri and Cornell, 1987). The responses under different combinations as defined in the design (Table 1 and 2) were analyzed using the analysis of variance (ANOVA) appropriate to the experimental design. The ANOVA for the data obtained using CCRD is presented in Table 4. It is evident that the first and second order terms were found to be significant and lack of fit was not significant. The lack of fit measures the failure of the model to represent data in experimental domain at points, which are not included in the regression. The values of coefficients of determination (R^2) also suggest that the model is a good fit. The R^2 is proportion of variability in response values explained or accounted for by the model (Duvnjak *et al*., 1983; Laycock *et al*., 1991).

Optimisation

In order to deduce the workable optimum conditions, a non- linear mathematical optimization procedure of Quattro pros software (Quattro pros version 4.0, Borland International Inc., UK) was used for optimization of polynomials (Table 5).

Table 4: Analysis of variance for the fitted second order polynomial model and lack of fit for biomass yield as per CCRD

		Biomass yield			Biosurfactant yield		
Source of variation	df	Sum of squares	Mean sum of squares	F-value	Sum of squares	Mean sum of squares	F-value
Regression							
First order terms	5	7.972	1.594	23.718[a]	56.991	11.398	154.245[a]
Second order terms	15	14.110	0.941	13.994[a]	41.960	2.797	37.855[a]
Total	20	22.082			98.951		
Residual							
Lack of fit	6	0.881	0.147	2.185[ns]	1.560	0.260	3.518[ns]
Pure error	5	0.336	0.067		0.369	0.074	
Total error	11	1.217			1.929		
Grand total	31	23.299			100.880		
Coefficient of Determination (R^2) =			0.948			0.981	

[a]Significant at 5% level, [ns]Not significant

Table 5: Feasible optimum conditions and predicted and experimental value of response at optimum condition

	Substrate conc. (%)	Inoculum level (%)	pH	Temp. (°C)	$(NH_4)_2SO_4$ (g L^{-1})
Maximum biomass	x_1	x_2	x_3	x_4	x_5
Coded value	0.489	-2.000	-0.143	-0.674	2.000
Uncoded	3.489	1.000	5.857	26.631	2.000
	Predicted value			Experimental value	
Biomass (Y_1)	6.010			5.36	
Yield (Y_2)	5.412			2.0	
Maximum biosurfactant yield	x_1	x_2	x_3	x_4	x_5
Coded value	2.000	2.000	2.000	-2.000	-2.000
Uncoded	5.000	10.000	8.000	20.000	0.500
	Predicted value			Experimental value	
Biomass (Y_1)	1.823			1.92	
Yield (Y_2)	21.844			24.48	

Response Surface Plotting

The effect of glycerol concentration, inoculum level, pH, temperature and $(NH_4)_2SO_4$ concentration on responses such as biomass yield and surfactant production are reported (Table 3) by the coefficients of second order polynomials. The responses based on these coefficients are shown in Fig. 1 and 2, with one variable kept at optimum level (Table 5) and varying the other two within the experimental range. In general, exploration of the response surfaces indicated a complex interaction between the variables.

Effect of Cultural and Environmental Conditions on Biomass Yield

At the optimum level of pH, temperature and inoculum concentration (Table 5), maximum biomass yield (6.0 g L^{-1}) was obtained at a substrate (glycerol) concentration of 3.5% (coded value 0.49) and $(NH_4)_2SO_4$ level of 2.0 g L^{-1} (coded value 2.0). For all the levels of substrate concentration, biomass increased with an increase in $(NH_4)_2SO_4$ concentration, which is due to increase in nitrogen concentration (Fig. 1a). Maximum biomass yield of 6.0 g L^{-1} was obtained at lowest inoculum level of 1.0% (coded value -2.0) and highest $(NH_4)_2SO_4$ concentration of 2.0 g L^{-1} (coded value 2.0) (Fig. 1b) which is because of increase in nitrogen concentration and the less number of initially added cells having the advantage of growing well with less competition and with good availability of carbon and nitrogen.

At optimum level of inoculum, substrate and $(NH_4)_2SO_4$, maximum biomass yield was obtained at pH 5.86 (coded value -0.14) and temperature 26.6°C (coded value -0.67). For all the levels of temperatures, the biomass increased with increase in pH up to 5.86 and then decreased with further increase (Fig. 1c). At the optimum level of pH, the biomass yield increased up to 26.6°C and a further increase in temperature decreased the biomass yield. This indicates that the isolate can grow to the full potential at these experimental conditions. This would be of advantage in tropical countries where the room temperature would vary from 24-26°C and a good yield can be obtained without the expenditure of energy for the maintenance of temperature. At optimum levels of substrate concentration, inoculum level and temperature, the maximum biomass yield was obtained at a highest $(NH_4)_2SO_4$ concentration of 2.0 g L^{-1} (coded value 2.0) and pH 5.85 (coded value -0.14) (Fig. 1d).

Effect of Cultural and Environmental Conditions on Biosurfactant Yield

Serratia marcescens DT-1P was able to produce surfactants on glycerol compared to other carbon sources (data not shown). Substrate (glycerol) is a simple fatty acid precursor, which can be easily utilized. Also glycerol has been used as a carbon source for surfactant production by *Pseudomonas* sp. (Babu *et al*., 1996) and it was found to be a better carbon source than n-alkanes.

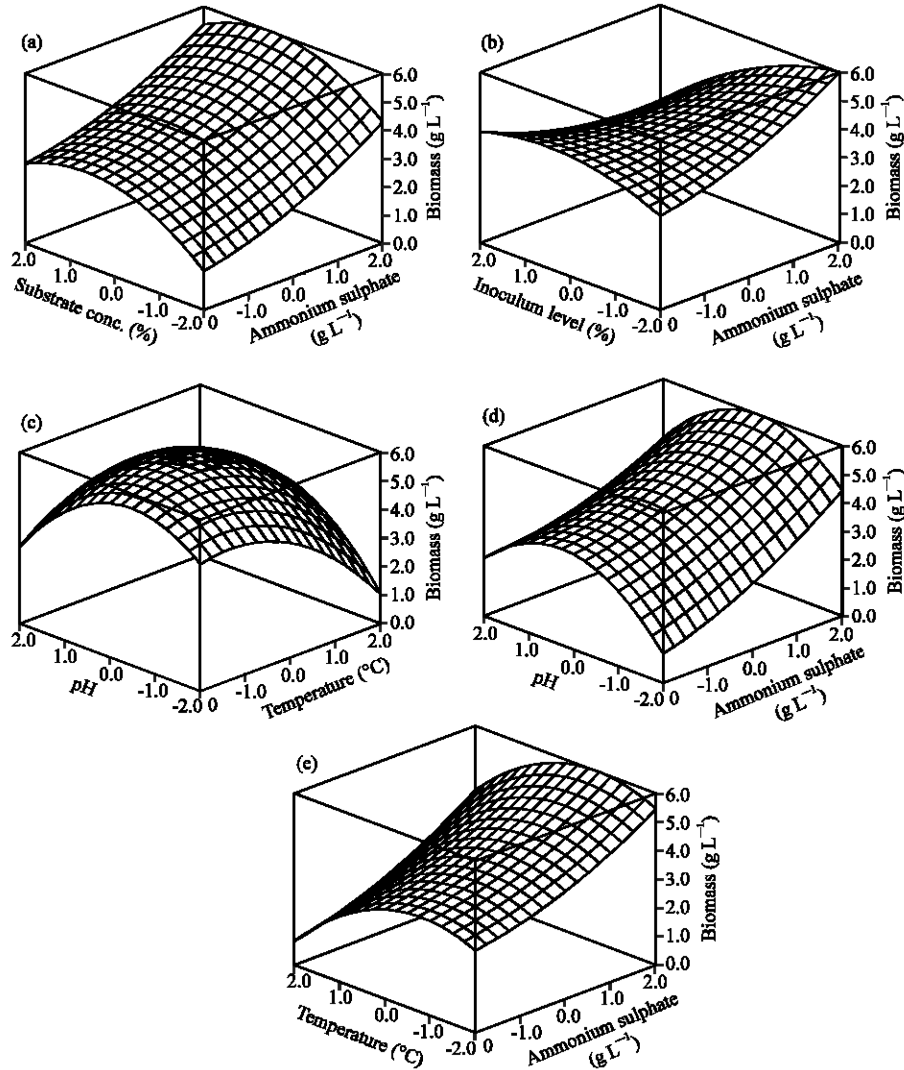

Fig. 1: Interactions between different parameters with biomass build-up

Substrate at 5% level yielded maximum surfactant (coded value 2.0) and $(NH_4)_2SO_4$ concentration of 0.5 g L^{-1} (coded value -2.0) (Fig. 2a) was found to be optimum. The surfactant production increased with increase in substrate concentration. Glycerol supported good growth and also surfactant production. Growth was however maximum at substrate level of 3.5% and surfactant production was maximum at 5% level. Surfactant production reached maximum at $(NH_4)_2SO_4$ concentration of 0.5 g L^{-1} and did not show much variation with increase in $(NH_4)_2SO_4$ concentration. Although at higher $(NH_4)_2SO_4$ level, the growth increased, surfactant production did not show significant changes. Thus the growth is not a linear function of substrate concentration but a linear function of $(NH_4)_2SO_4$ concentration. However, surfactant production is observed to be a linear function of substrate concentration, but not $(NH_4)_2SO_4$ concentration. Glycerol has been used for surfactant production by *Pseudomonas aeruginosa* GS 3 at 3% level (Patel and Desai, 1997). Type of carbon source plays a major role in biosurfactant production. Different carbon sources such as molasses

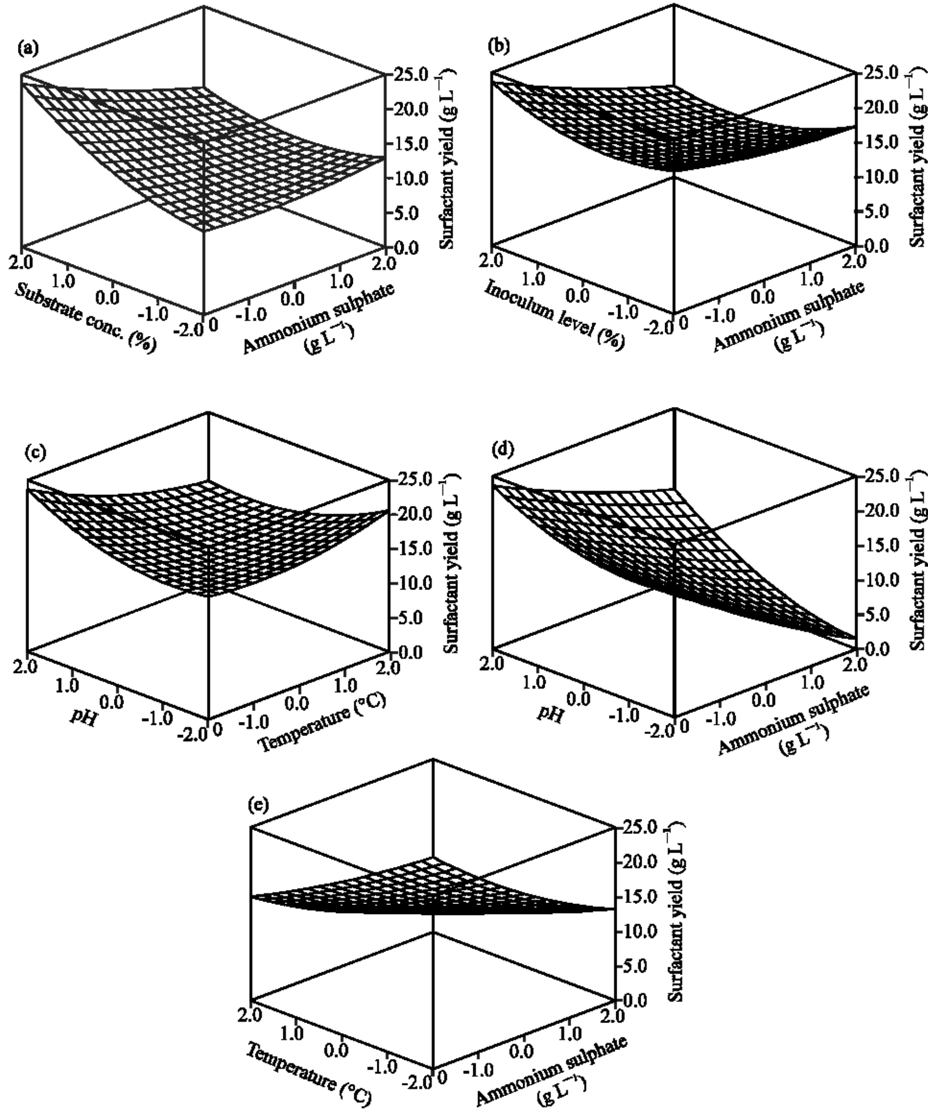

Fig. 2: Interactions between different parameters with biosurfactant production

(Patel and Desai, 1997), glucose (Laycock *et al*., 1991), corn steep liquor (Babu *et al*., 1996), distillery and whey wastes (Syldatk *et al*., 1974), n-alkanes (Yamaguchi *et al*., 1976; Syldatk and Wagner, 1987) etc. have been used for biosurfactant production. Agricultural and food industry wastes such as rice hull hydrolysate, starch waste liquors, whey, domestic wastes and potato processing wastes have been used for surfactant production (Kosaric, 1984). Municipal sludge waste has also been proposed to be utilized for surfactant production (Kosaric, 1984). The type of carbon source used has been shown to play a key role in the type of biosurfactant produced. When n-alkane was used as a carbon source, a rhamnolipid was produced by *Pseudomonas* sp. (Syldatk *et al*., 1974; Yamaguchi *et al*., 1976). The hydrocarbon chain length has also been shown to play a key role in the type of biosurfactant produced (Kosaric, 1984). In a similar study, glycerol was found to be the best carbon source for the production of rhamnolipid by *Pseudomonas* sp. (Laycock *et al*., 1991) than n-alkanes.

Growth of the bacterial strain was found to increase with an increase in biosurfactant production for all the levels used. Growth and biosurfactant increased with incubation time and reached maximum on day 8, beyond which both growth and biosurfactant production decreased (Table 6). Biosurfactants have been produced under growth associated, growth limiting stages as well as by resting cells and by the addition of precursors. The growth- associated production of biosurfactants could be by optimized medium composition, optimized environmental influences such as pH, temperature, aeration, agitation etc., by induction using lipophillic substances, by the addition of reagents which cause a change of cell wall permeability such as penicillin, ethambutol, EDTA etc., by the addition of reagents causing detachment of cell- wall bound biosurfactants into the medium such as alkanes, kerosene, EDTA etc. The microorganisms produce biosurfactants under growth limiting conditions such as nitrogen limitation, growth limiting environmental conditions such as pH or temperature. The resting cells produce biosurfactants as resting free cells, resting immobilized cells, resting immobilized cells with simultaneous product removal (Syldatk and Wagner, 1987).

Nitrogen limitation has been shown to play an important role in surfactant production. The nature of nitrogen source also effects biosurfactant production (Arino *et al*., 1996). Effects of different amino acids such as aspartic acid, aspargine, glycine and glutamic acid in mineral salts medium as well as yeast extract, peptone, bactopeptone and nutrient broth were evaluated for biosurfactant production by *Arthrobacter paraffineus* ATCC 19558 (Arino *et al*., 1996). Yeast extract yielded 6 times more surfactant than control. Aspargine could serve as a single nitrogen source. In present studies, among different organic nitrogen sources tested, peptone gave higher surfactant yield (data not shown). Similar observation has been made by Duvnjak *et al*. (1983). Among the inorganic salts used by these authors, the microorganisms were found to prefer ammonium to nitrate forms of nitrogen. Urea utilized both as sole source of nitrogen and in combination with an inorganic nitrogen salt, yielded relatively high surfactant concentration in the broth (Duvnjak *et al*., 1983). This is similar to our observation where $(NH_4)_2SO_4$ in combination with peptone gave higher surfactant compared to individual ones (data not shown).

At the optimum conditions of substrate concentration and $(NH_4)_2SO_4$ levels, surfactant production increased with increase in pH (Fig. 2c). At pH 8.0, (coded value 2.0), maximum surfactant production was obtained. Any change in pH to lower levels caused an appreciable drop in the biosurfactant production. Maximum surfactant production was observed at incubation temperature of 20°C (coded value -2.0) and increase in temperature did not show much variation in the surfactant yield (Fig. 2c). Growth and biosurfactant production were found to be independent as far as pH was concerned. The optimum pH required for biomass production was only 5.8 (Fig. 1c), however, pH 8.0, was needed for surfactant production (Fig. 2c). Culture conditions such as pH, temperature and ionic strength have been shown to influence biosurfactant production, Temperature played a crucial role in biosurfactant production from *Arthrobacter paraffineus* ATCC 19558 (Duvnjak *et al*., 1983), *R. erythropolis* (Arino *et al*., 1996) and *Pseudomonas* sp. (Syldatk and Wagner, 1987). In present studies, *Serratia marcescens* DT-1P was found to produce maximum surfactant at 20°C, pH 8.0. A slight change in these conditions reduced the surfactant yield.

The optimum conditions for biomass production did not yield to highest biosurfactant production. Similarly, optimum conditions required for higher surfactant production did not yield highest biomass. It is clear that the conditions for both these parameters were independent of each other.

The optimum conditions, which were obtained i.e., glycerol (3.4% w/v), inoculum (1.0 OD_{600}), pH (5.8), temperature (26.6°C) and $(NH_4)_2SO_4$ (0.2%) yielded 6.0 mg L^{-1} biomass and glycerol (5% w/v), inoculum (10.0 OD_{600}), pH (8.0), temperature (20.0°C) and $(NH_4)_2SO_4$ (0.05%), yielded 24.48 mg % of biosurfactant.

Verification of Results

The stability of the model equation for predicting the optimum response values were tested using the recommended optimum conditions. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to validate experimentally and predict the value of the responses using model equations. The experimental values were found to be in agreement with the predicted values (Table 5) with biosurfactant production by *Serratia marcescens* DT-1P.

Time Course of Biosurfactant Production

The optimized conditions were used to study the effect of incubation time on biosurfactant production. The biosurfactant production started on early stationary phase and was maximum by 8 days and decreased thereafter. The maximum yield of surfactant was 22.56 g L^{-1} (Table 6).

Surfactant Production in 25 L Reactor

In large batch of 15 L optimized medium in a 25 L reactor, *Serratia marcescens* DT-1P grew well and the biosurfactant production reached maximum by 8th day of incubation. The biosurfactant yield was 20 g L^{-1}. This was marginally less than that in shake flasks (Table 7).

Characterization of the Surfactant

The surfactant produced by *Serratia marcescens* DT-1P was found to be glycolipid containing arabinose sugar and C 18:1 lipid moiety (Data not shown).

The environmental conditions play a key role in the physiology of a microorganism (Syldatk and Wagner, 1987; Georgiou *et al*., 1992). The surfactant production from *Serratia marcescens* DT-1P was found to be effected by the interaction of the environmental factors i.e., the substrate concentration, inoculum level, pH, temperature and incubation time were found to influence biosurfactant production in both qualitative and quantitative way. Important quantitative factors were use of simple carbon and nitrogen sources. For quantitative factors it was difficult to establish a unique pattern of how a particular component affects biosurfactant synthesis. A complete characterization of these qualitative and quantitative factors was achieved by establishing optimal conditions by RSM technique that yielded high rhamnolipid biosynthesis.

Table 6: Incubation period and surfactant production

Incubation period (days)	Surfactant yield (g L^{-1})
0	0.00
2	12.00
4	16.72
6	18.32
8	22.56
10	16.28
12	14.84
14	10.76
16	10.32

Table 7: Biosurfactant production in fermentor

Incubation time (days)	Biomass (g L^{-1})	Biosurfactant (g L^{-1})
1	0.50	0.92
2	0.51	3.11
3	0.86	6.38
4	0.99	8.11
5	1.21	9.42
6	1.86	12.11
7	1.92	16.84
8	2.81	20.00

Serratia marcescens DT- 1P has been shown to produce rhamnolipid that can be effectively used in many industries. The surfactant can be produced with the medium containing substrate (glycerol) = 5%, inoculum level = 10%, pH = 8.0, temperature = 20°C and $(NH_4)_2SO_4$ = 0.5 g L^{-1}, the conditions optimized by RSM technique. But the maximum biomass can be obtained at substrate (glycerol) = 3.49%, inoculum level = 1%, pH = 5.9, temperature = 26.6°C and $(NH_4)_2SO_4$= 2.0 g L^{-1} With the usefulness of biosurfactants in many fields, especially in personal and health care as well as therapeutic agents, these optimized production processes will be beneficial in getting maximum yields at a given set of conditions.

ACKNOWLEDGMENTS

The students thank CSIR for providing fellowships. Thanks are also due to DBT for funding the project and Director, CFTRI, for his constant encouragement during the study. Authors would like to extend their gratitude towards Mrs. Mahadevamma and Mr. Rachhappaji for their support and help in the biochemical analysis.

REFERENCES

Arino, S., R. Marchal and J.P. Vandecasteele, 1996. Identification and purification of a rhamnolipid biosurfactant by a *Pseudomonas* sp. Applied Environ. Microbiol., 45: 162-168.

Babu, S., A.N. Vaidya, A.S. Bal, E. Kapur, A. Jueo and P. Khanna, 1996. Kinetics of biosurfactant production by *Pseudomonas aeruginosa* strain BS2 from industrial wastes. Biotech. Lett., 18: 263-268.

Begley, J., B. Croft and R.J. Swannel, 1996. Current research in to bioremediation of contaminated land. Land Contam. Reclm., 4: 199-208.

Brandl, H., A. Richard, R.Gross, W. Lenz and R.C. Fuller, 1988. *Pseudomonas oleovorans* as a source of Poly (β-hydroxyalkonates) for potential applications as biodegradable polyesters. Applied Environ. Microbiol., 54: 1977-1982.

Benat, I.M., R.S. Makkar and S.S. Cameotra, 2000. Pottential commercial applications of microbial surfactants. Applied Environ. Microbiol., 53: 495-508.

Cochran, W.G. and G.M. Cox, 1957. Experimental Design. 2nd Edn., New York, Wiley.

Dubois, M., K.A. Gilies, J.K. Hamilton, P.A. Rebers and F. Smith, 1956. Colorimetric method for determination of sugar and related substances. Anal. Chem., 28: 350-356.

Duvnjak, Z., D.G. Cooper and N. Kosaric, 1983. Production of surfactant by *Arthrobacter paraffineus* ATCC 19558. Biotechnol. Bioeng., 24: 165-175.

Georgiou, G., S.C. Lin and M. Sharma, 1992. Surface active compounds from microorganisms. Biotechnol., 10: 60-65.

Head, I.M., 1998. Bioremediation: Towards a credible technology. Microbiology, 144: 599-608.

Joglekar, A.M. and A.T. May, 1987. Product excellence through design of experiments. Cereal Food World, 32: 857-868.

Kairs, J.M. and M.I. Khan, 1994a. Microbial enhanced oil recovery (MEOR). Part I importance and mechanism of MEOR. Enzyme Microb. Technol., 16: 170-172.

Kairs, J.M. and M.I. Khan, 1994b. Microbial enhanced oil recovery (MEOR). Part II Microbes and the subsurface environment for MEOR. Enzyme Microb. Technol., 16: 258-259.

Khuri, A.L. and J.A. Cornell, 1987. Introduction to Response Methodology. In: Response surface Design and Analysis. New York, Marcel Dekker, pp: 21-45.

Kosaric, N., 1984. Biosurfactants in Biotechnology Vol. 6. Rehm, H.J., G. Reed, A. Puhler and P. Stadler (Eds.),VCH, Weinheim, pp: 659-717.

Laycock, M.V., P.D. Hildebran, P. Thibaurt, J.A. Walter and J.L.C. Wright, 1991. A potent peptidolipid biosurfactant and phytopathogenic mediator produced by a proteolytic strain of *Pseudomonas fluorescens*. J. Agric. Food Chem., 39: 483-489.

Layman, P.L., 1985. Industrial surfactants set for strong growth. Chem. Eng. News, 23: 23-48.

Patel, R.M. and A.J. Desai, 1997. Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. Lett. Applied Microbiol., 25: 91-94.

Sawardekar, J.S., J.H. Slonekar and A. Jeanes, 1967. Quantitative determination of monosaccharides as their alditol acetates by Gas Liquid Chromatography. Anal. Chem., 37: 1602-1604.

Selvendran, R.R. and M.A. O'Neill, 1987. Isolation and analysis of cell walls from plant material. Methods Biochem. Anal., 32: 25-153.

Syldatk, C., S. Lang, U. Matulovic and F.Z. Wagner, 1974. Production of four interfacial active rhamnolipids from n-alkanes or glycerol by resting cells of *Pseudomonas* sp. Dsm Z. Naturfurch, 40C: 51-60.

Syldatk, C. and F. Wagner, 1987. Production of Biosurfactants. In: Surfactant Science Series, 25: Biosurfactants and Biotechnology. Kosaric, N., W.L. Cairns and N.C.C. Gray (Eds.), pp: 89-120. Marcel Dekker, New York, Biotechnol. Lett., 5: 95-100.

Van Dyke, M.I., H. Lee and J.T. Trevors, 1991. Application of microbial surfactants. Biotechnol. Adv., 9: 241-252.

Yamaguchi, M., A. Sato and A. Yukayama, 1976. Microbial production of sugar lipids. Chem. Ind., 17: 741-742.