ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Statistical Screening and Optimization of the Medium Components for Production of Novel Flavolipid Biosurfactant by *Flavobacterium* sp. MTCC 2495

Karadi R V[a], Lokesh KN *[b], Channarayappa [b], Marikunte Venkata Rangana [C], and Siva Kiran RR[b]

[a] K.LE's University College of Pharmacy, Hubli-580031, Karnataka, India.
[b] M.S.Ramaiah Institute of Technology, Bangalore-560054, Karnataka, India.
[c] Connexios Life Sciences Pvt Ltd Bangalore- 560078, Karnataka, India.

ABSTRACT

The design of experiments were used for screening and optimization of the medium constituents for production of flavolipid biosurfactant by *Flavobacterium* sp MTCC 2495 , which was carried out in basal Mineral Salt Medium (MSM). The important medium constituents of MSM like, carbon and nitrogen sources of Solution A and the trace elements of Solution B were screened by means of high resolution Plackett-Burman foldover design (Resolution IV) and further, their optimal concentration were determined by Central Composite Design (CCD). The outcome obtained by CCD model were used to constitute Modified Mineral Salt Media (MMSM) which consist of critical media constituents like, 10.85 g/l of sucrose and 3.11 g/l peptone of Solution A and 0.03g/100ml $FeSO_4.7H_2O$ of Solution B**.** Batch growth kinetic profile of *Flavobacterium* MTCC 2495 in MMSM medium has shown R^2 value of 0.97, compared to R^2 value of 0.86 for MSM, the biomass yield was increased quantitatively by 29 %, determined by regression model. The multivalent ion (ferrous sulphate) has shown inductive effect on flavolipid production at lower 0.01 to 0.05 g/100ml.

Keywords: Flavolipid**,** *Flavobacterium* MTCC 2495, Plackett-Burman fold-over design, Modified Mineral Salt medium (MMSM), multivalent ion, inductive effect.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Biosurfactants are amphilphiles which has both hydrophilic and hydrophobic moieties. They categories into different types, based on chemical composition and microbial origin viz Rhamnolipids (glycolipid type) from *Pseudomonas* sp., Surfactin (Lipopeptides type) obtained from B*. subtilis,* etc [1,2] Their potential applications are reported in the environmental protection, crude oil recovery, health care, and food-processing industries [3-5].

Flavolipid biosurfactant produced by *Flavobacterium* sp MTN 11, has strong emulsifier activity and reported its bioremediation potential for hexadecane and cadmium from soil sample [6]. Few species of *Flavobacterium* degrade organic contaminants, such as pentachlorophenol, nylon oligomers, polyaromatics, and pesticides [7-9]. Many biosurfactant producers were screened from hydrocarbon contaminated soil samples and other organic contaminated sites [10, 11]

Media formulation is an essential stage in the design of successful laboratory experiments, pilot-scale development and manufacturing process. The constituents of a medium must satisfy the elemental requirement for cell biomass and metabolite production, Cell growth and the metabolic products formation are strongly influenced by medium compositions such as carbon source, nitrogen source, growth factors, and inorganic salts, inducers, inhibitors etc. Thus, it is difficult to search for the major factor and to optimize them for biotechnological processes [12]. Environmental factors and growth conditions such as pH, temperature, agitation oxygen availability also effects on biosurfactant production through their effects on cellular growth or activity.

There are many ways to approach the problem of optimization and design of a fermentation process, one could determine the nutritional requirements of the organism and design a medium based upon the optimum combination of each nutrient, i.e., glucose, amino acids, vitamins, minerals, etc. This approach has two drawbacks. First, it is very time-consuming to study each nutrient and determine its optimum level, let alone its interaction with other nutrients. Secondly, although knowledge of the optimal nutritional requirements is useful in designing a media, this knowledge is difficult to apply when economics dictate the use of commercial substrates such as corn steep liquor, soy bean meal, etc., which are complex mixtures of many nutrients. In order to find the optimum conditions, it would have been necessary to repeat the one-variable-at-a-time process at each step to verify that the true optimum was reached. This requires numerous sequential experimental runs, a time-consuming and ineffective strategy, especially when many variables need to be optimized. Because of the complexity of microbial metabolism, interaction between the variables is inevitable, especially when using commercial substrates which are a complex mixture of many nutrients. Therefore, since it is both time-consuming and inefficient, the one-variable-at-a-time approach is not satisfactory for fermentation development. Fortunately, there are a number of statistical methods which will find the optimum quickly and efficiently [13].

The objective of our work was to assess the biosurfactant producing ability of *Flavobacterium* sp MTCC 2495, which reported with bio-degradation of metaphors (methyl parathion) by hydrolytic activity, used in microbial biosensors for detection of pesticide residues [14]. The screening of important nutritional constituents for flavolipid production was performed by 2^{k-p} factorial Plackett-Burman foldover design (PB) of resolution IV category. The optimization of cellular growth of the Flavobacterium sp. MTCC2495 was achieved using a 2^{6-2} fractional factorial central composite design and surface modeling method. The effect of trace elements on flavolipid production was also studied.

MATERIALS AND METHODS

2.1. Strains and Culture – Conditions:

Flavobacterium sp. MTCC 2495 was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India and sub-cultured in Modified Wakimoto Medium (MWM) containing: 0.5 g/l Ca $(NO_3)_2.4H_2O$, 2.0 g/l Na_2PO_4. H_2O, 5.0 g/l Peptone, 15.0 g/l sucrose, 0.5 g/l $FeSO_4$ and 15g/l agar. The culture was incubated at 30°C for 24 hours in orbital shaker incubator. The cultures were maintained by sub-culturing every 2 weeks. Few stock cultures were also maintained in deep freezer with cryoprotectant, such as glycerol 10% v/v for future use.

2.2. Inoculum Preparation:

Inoculum was prepared in 250 ml Erlenmeyer flasks containing 50 ml of MWM media (without agar) of pH 7.0. The media was autoclaved at 121ºC (15lbs) for 20 min and inoculated with *Flavobacterium sp* MTCC 2495. The inoculated Erlenmeyer flasks were kept on an orbital shaker (Remi make) at 200 rpm for 24 h and were used as Inoculum. The inoculum size of 2 % (contains 10^6 cell/ml) was used throughout the study for subsequent inoculations unless otherwise specified.

2.3. Basal Media for Optimization Process:

For production of flavolipid biosurfactant by *Flavobacterium* sp. MTCC 2495, Mineral Salt Media (MSM) amended with 10g/l glucose as sole carbon and energy source was used as basal media for preliminary optimization studies, which has Solution A consisting of 2.5 g/l $NaNO_3$, 0.4 g/l $MgSO_4.7H_2O$, 1.0 g/l NaCl, 1.0 g/l KCl, 0.05 g/l $CaCl_2.2H_2O$, 10 ml H_3PO_4 (85.0%), adjusted to pH 7.2 with KOH pellets. One milliliter of Solution B was added, which consist of 0.05 g/l00ml $FeSO_4.7H_2O$, 0.015 g/l00ml $ZnSO_4.7H_2O$, 0.015g/l00ml $MnSO_4.H_2O$, 0.03g/100ml H_3BO_3, 0.015g/l00ml $CoCl_2.6H_2O$, 0.015g/l00ml $CuSO_4.5H_2O$, and 0.01g/100ml $NaMo_2O_4.2H_2O$ [6].

2.4. Cell Growth Characterization:

The cell growth characteristics was measured on dry weight basis, pre weighed filter membrane, Sterlitech® (Cellulose acetate filter membrane, 47mm in diameter, 0.45µm in pore size) was used for filtration, a fixed volume of fermentation broth was filtered and left to dry at 105°C for 24 hrs. The weight of membrane filter before and after drying was determined.

2.5. Cell Growth and Biosurfactants Surface-Activity Determination:

The surface tension of cell free supernatant was measured by using a du Nouy ring type tensinometer (Kruss, K10T) and compared with surface tension of the distilled water without biosurfactant. The surface measurement was carried out at 25 ± 1 ºC after dipping the platinum ring in the solution for a while in order to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. For calibration of the instrument, the surface tension of the pure water was measured before each set of experiment.

2.6. Selection of Carbon and Nitrogen Source for Process Optimization:

The conventional One-factor-at-a-time method (OFAT) was used for selection and optimization of media of constituents for Flavolipid production, which were carried out by shake flask studies. The nutritional media components required for production of flavolipids by *Flavobacterium sp.* MTCC 2495 were studied in 250 ml Erlenmeyer flasks at 200 rpm containing 50 ml of Basal MSM medium. The optimized parameter obtained based on highest biomass and reduced Surface tension of cell free supernatant. Effect of different carbon sources was studied, using glucose, sucrose, maltose, fructose and galactose at fixed concentration (15 g/l). All the carbon sources were separately autoclaved. The effect of inorganic nitrogen sources was studied using sodium nitrate, ammonium chloride, calcium nitrate and ammonium sulfate at 0.5 g/l and organic nitrogen sources such as yeast extract, peptone, corn-steep liquor, and beef extract at 2g/l. The carbon and nitrogen sources which significantly affected on flavolipids production were further studied by statistical methodologies.

2.7. Statistical Methodology:

The Statistical techniques those are useful for screening, modeling and analysis of processing problem which is a response of interest, influenced by several independent variables in less time span with adequate accuracy.

2.7.1. Plackett-Burman fold-over design (P-B design):

The screening of the most significant processing parameters affecting flavolipid production was studied by P-B fold-over design which is regarded as high resolution category IV design matrix, used for screening of statistically significant independent variables [15]. Totally, eleven (n) variables including five nutritional (sucrose, peptone, calcium nitrate, ferrous sulphate and manganese sulphate), two physical (temperature and pH), and four dummy

ISSN: 0975-8585

variables designated as F3, F6, F9, F11 were studied in 24 experimental runs, [n+1] (+ foldover) experiments. The identification of coded factors value and their concentrations are shown in Table 1. Each variable was represented at two levels, high and low, denoted by +1 and -1 signs, respectively. The difference between the two values was taken large enough to ensure that the peak area for highest biomass production.

Table 1: Coded factors for Plackett-Burman foldover design.

	levels		
Factors	-1	1	unit
F1: Temperature	28	33	°C
F2: pH	6.5	7.5	
F3: Dummy (I)	**	**	**
F4: sucrose **	10	20	g/l
F5: Peptone **	2	4	g/l
F6: Dummy (II)	**	**	**
F7: Calcium Nitrate **	0.5	1	g/l
F8: Ferrous sulphate ##	0.02	0.06	g/100ml
F9: Dummy (III)	**	**	**
F10: Manganese sulphate ##	0.02	0.06	g/100ml
F11: Dummy (IV)	**	**	**

The Four factors F3, F6, F9 and F11 designated as "dummy variables"
** **Solution A components Expressed in g/l.**
Solution B components expressed in g/l00ml.

The number of positive and negative signs per experiment or trial are (n+1)/2 and (n-1)/2, respectively, with each column having equal number of positive and negative signs. The design matrix applied to this study is shown in Table 2. The effect of each variable or factor is the difference between the average of the measurement made at the high level of the factor and the average of the measurement made at the high level of that factor and the average of the measurements made at the low level of that factor, which was determined by the following Eq. (1):

$$E_{Xi} = \frac{2\left(\sum P_{i+} - \sum P_{i-}\right)}{N} \qquad \textbf{(1)}$$

Where, $E_{(Xi)}$ is the concentration of tested variable, and P_{i+} and P_{i-} represents biosurfactant production from the trials where the variables X_i being measured were added to the production medium at high and low concentration , respectively and N is the number of experiments carried out.

The significant level (*P*- value) of the effect of each concentration and the square root of the variance of an effect i.e. standard error (S.E) was determined by Student's t-test as shown in Eq.(2):

ISSN: 0975-8585

$$T_{(Xi)} = \frac{E_{(Xi)}}{S.E} \qquad (2)$$

Where, $E_{(Xi)}$ is the effect of variable *Xi.*

Table 2: Plackett-Burman Fold- over design (Resolution IV) experimental design and results

Run No	F1	F2	(F3)	F4	F5	(F6)	F7	F8	(F9)	F10	(F11)	Biomass gdw/l Observed	Biomass gdw/l predicted
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	4.9	5.05
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	3.55	4.06
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	3.55	3.25
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	3.44	3.42
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	2.2	2.46
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	3.92	3.36
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	3.52	3.17
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	2.43	2.37
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	4.12	3.41
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	3.33	3.09
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	4.28	4.50
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	3.62	3.42
13	-1	1	-1	1	1	1	-1	-1	-1	1	-1	1.52	1.88
14	-1	-1	1	-1	1	1	1	-1	-1	-1	1	2.15	2.87
15	1	-1	-1	1	-1	1	1	1	-1	-1	-1	3.78	3.68
16	-1	1	-1	-1	1	-1	1	1	1	-1	-1	3.32	3.51
17	-1	-1	1	-1	-1	1	-1	1	1	1	-1	4	4.47
18	-1	-1	-1	1	-1	-1	1	-1	1	1	1	3.92	3.57
19	1	-1	-1	-1	1	-1	-1	1	-1	1	1	3.91	3.76
20	1	1	-1	-1	-1	1	-1	-1	1	-1	1	4.42	4.56
21	1	1	1	-1	-1	-1	1	-1	-1	1	-1	4.02	3.52
22	-1	1	1	1	-1	-1	-1	1	-1	-1	1	3.88	3.84
23	1	-1	1	1	1	-1	-1	-1	1	-1	-1	2	2.43
24	1	1	1	1	1	1	1	1	1	1	1	3.5	3.51

2.7.2. CCD design:

The response surface methodology was used to develop an empirical model of process and to get more precise estimate of optimum operating conditions obtained by interaction of factors involved. In 2^3 factorial central composite designs, the influence of all significant experimental variables and their interaction effects on the response are investigated. Three significant variables (short listed from PB design) of MSM medium are set as factors for CCD design. Each variable in the design was studied at five different levels, with four cube points, six replicates and four star points for one factor an axial distance to the center of ±α, where as the other factor is at level 0, total 16 experiments was employed, shown Table 3. Biomass production was taken as response (Y), the experimental observed values and predicted biomass response was tabulated. Multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variable.

ISSN: 0975-8585

The analysis of experimental results was performed based on the first order polynomial model to calculate the coefficient value of each selected constituents using following equation Eq. (3).

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_1 \beta_4 AD + \beta_2 \beta_3 BC + \beta_2 \beta_4 BD + \beta_3 \beta_4 CD \quad \textbf{(3)}$$

Where Y is the response, β0 is intercept, $\beta_1, \beta_2, \beta_3, \beta_4$ are linear coefficients $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}$ are squared coefficient $\beta_1\beta_2, \beta_1\beta_4, \beta_2\beta_3, \beta_2\beta_4, \beta_3\beta_4$ are interaction coefficients and A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD, and CD are independent variables.

Table 3: Experimental design and results of the Central composite design

Run No.	Peptone (g/l)	Ferrous sulphate (g/100ml)	Sucrose (g/l)	Biomass gdw/l	
				observed	predicted
1	2	0.02	10	3.72	3.761
2	2	0.02	20	2.2	2.656
3	2	0.04	10	3.82	3.870
4	2	0.04	20	2.5	2.810
5	4	0.02	10	4.22	4.198
6	4	0.02	20	2.9	3.138
7	4	0.04	10	4.43	4.262
8	4	0.04	20	3	3.247
9	1.31	0.03	15	2.7	2.329
10	4.68	0.03	15	3.1	3.064
11	3	0.013	15	3.98	3.695
12	3	0.046	15	4	3.878
13	3	0.03	6.59	4.98	5.178
14	3	0.03	23.4	4	3.395
15	3	0.03	15	5.28	5.185
16	3	0.03	15	5.02	5.185

2.7.3. Software and data analysis:

Experimental designs matrix were generated by using statistical software package, Stat Soft®, STATISTICA version 6.0. Statistical analysis of experimental design was also performed by using this software. The quality of the first order polynomial model was expressed by the coefficient of determination (R^2 value) and its significance was validated by *F*-test. The 2D interaction contour surface plot of significant variable was generated. The critical experimental values and highest predicted response for the model was also obtained by this software.

2.8. Validation of the experimental model:

Validation of polynomial model was performed by using optimal points of peptone, sucrose and ferrous sulphate in production medium. Flavolipid production was carried out in shake flask studies and validated the obtained response to the predicted response of polynomial model at the critical values. Batch growth kinetic profiling of the strain *Flavobacterium* sp MTCC 2495 was performed in MSM (before optimization) and optimized MSM (after optimization) medium separately. The growth kinetic was validated by using R^2 value (regression coefficient) and regression equation was used to compare the biomass yield in respective medium.

2.9. Effect of iron on biosurfactant production:

The effect of trace element on flavolipid production was studied, by culturing the *Flavobacterium* sp MTCC 2495 in MSM with different concentration of ferrous sulphate. The shake flask studies was carried in two different set up, one set up with higher (0.1-0.5g/100ml) and another set up with low concentration (0.01-0.05g/l00ml) of ferrous sulphate, kept in lab scale fermenter (Sartorious B-lite) at 300 rpm . The biomass (gdw/l), surface activity ST (mN/m) was recorded in triplicates and data interpretation was carried out.

RESULTS

3.1. Screening of significant independent variables by P-B fold-over design:

In the present investigation, among 11(n=11) variables, which were expected to play a critical role in enhancing flavolipid production, three factors i.e. peptone (F5), ferrous sulphate (F8), and sucrose (F4) significantly affected flavolipid biosurfactant production. None of four dummy variables (F3, F6, F9, F11) included in the design exhibited any impact on flavolipid production, which makes the design significant, towards screened independent variables. Based on experimental data, the Pareto chart of standardized effects were plotted for identifying the factors that are important in flavolipid production. The selected factors main effects are ranked in order according to their level of significance. The chart also show a vertical line to indicate the statistical significance (P=0.05). If selected variable is significant in the process, the variable bar crosses the vertical line or vice-versa. The standardized effects of dummy variables or error (F3, F6, F9, and F11) were neglected with provided “ignore” option of Statistica-6, software. It is evident from the Pareto chart Fig. 1 that the most important variables are F5 (peptone), F8 (Ferrous sulphate) and F4 (sucrose), having standardized effects (absolute value) 4.05, 2.8 and 2.69 respectively. Further confirmation about the significant factors was obtained from ANOVA. The observed lowest p-value (0.0009) and highest F-value (16.4) that is (F>P value) was observed with peptone (F5), followed by ferrous sulphate and sucrose as shown in Table 4. The Half-Normal probability plot of effects is very useful for separating random noise from ‘real effects based on their distribution on the plot as shown in Fig.2 It is evident from that among selected factors peptone (F5), ferrous sulphate (F8), and sucrose (F4) were positioned outlier with better factor confidence levels.

ISSN: 0975-8585

In the P-B Fold-over design the 1st and 20th experimental runs shows the highest activity in terms of biomass production, the observed biomass was 4.90gdw/l and 4.42gdw/l respectively. Fig.3 presents the relationship between the observed and predicted biomass concentration for P-B design, most of the points are nearby the line of adjustment which means that the values determined experimentally are similar to those determined by model. The P-B foldover design signified the importance of peptone (organic nitrogen source) over calcium nitrate (inorganic nitrogen source) on cell growth.

Table 4: ANOVA results of P-B (foldover design)

Factors	SS	df	MS	F	p
Temperature(F1)	0.29482	1	0.294817	0.89657	0.357786
pH(F2)	0.00027	1	0.000267	0.00081	0.977634
sucrose(F4)	2.38140	1	2.381400	7.24208	0.016062
Peptone(F5)	5.39602	1	5.396017	16.40984	0.000927
Calcium Nitrate(F7)	0.01402	1	0.014017	0.04263	0.839035
Ferrous sulphate(F8)	2.60042	1	2.600417	7.90813	0.012524
Manganese sulphate(F10)	0.00602	1	0.006017	0.01830	0.894087
Error	5.26125	16	0.328828		
Total SS	15.95420	23			

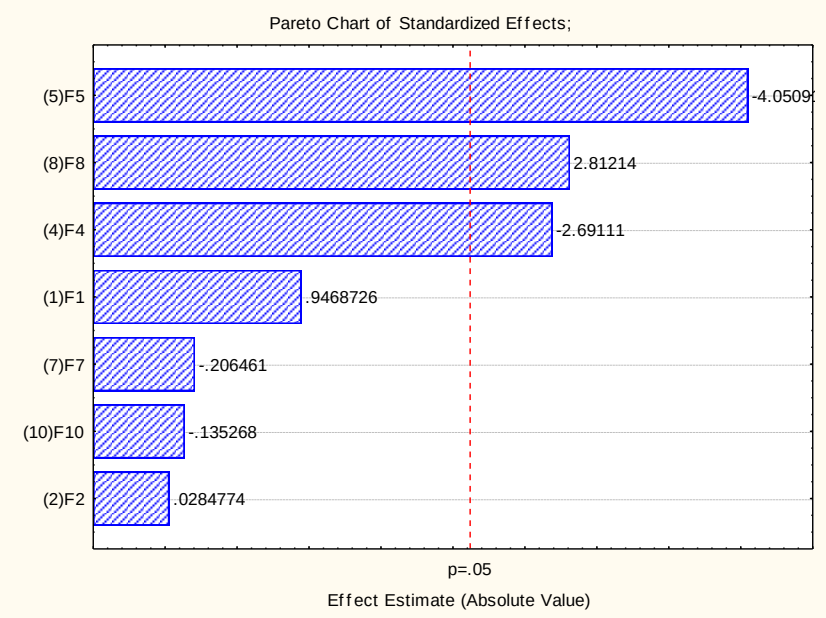


Fig. 1: Pareto chart of standardized effects of independent variables on *Flavobacterium* sp MTCC 2495(biomass yield).

ISSN: 0975-8585

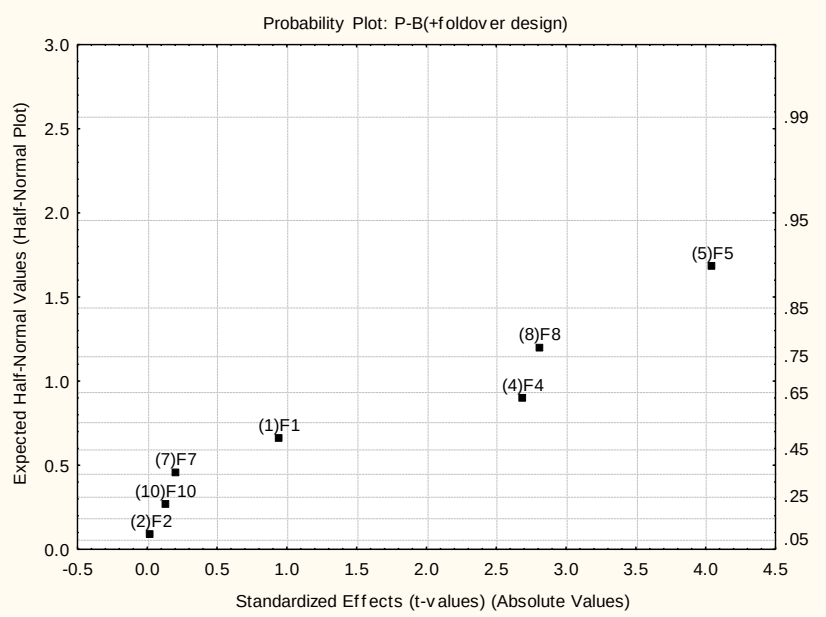


Fig. 2: The Half –normal probability plot of effects on biomass production (gdw/l).

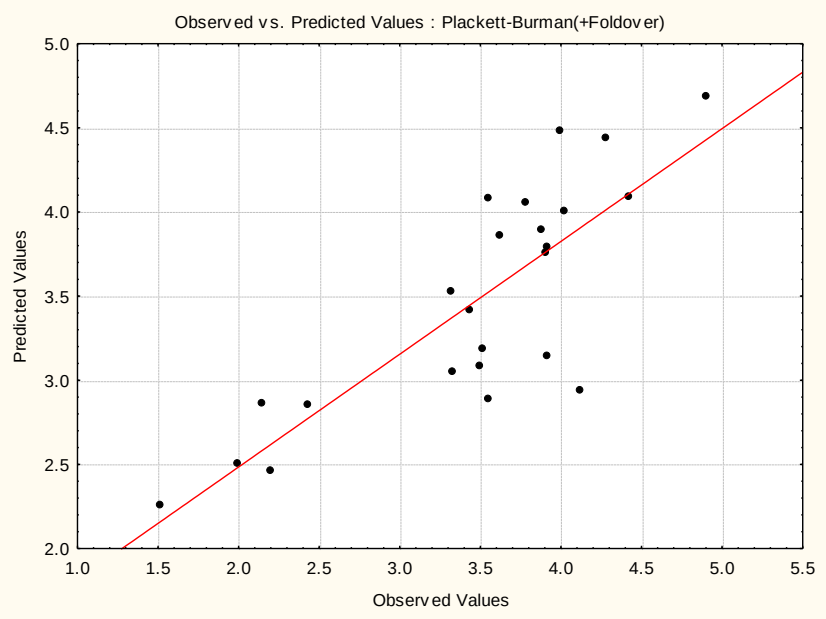


Fig.3: Plot of observed v/s. predicted biomass concentration gdw/l values for *Flavobacterium* sp MTCC 2495. The biomass concentration is response variable of interest. The expected biomass concentration values are determined by the model equations determined for P-B Foldover design.

3.2. Optimization of screened variables and their interaction analysis:

The optimum levels of significant factors (sucrose, peptone, ferrous sulphate) and the effect of their interaction on flavolipid production were determined by factorial central composite design (CCD). The observed and predicted biomass concentration for factorial CCD

ISSN: 0975-8585

design as shown positive co-relation, most of the points are nearby the line of adjustment which means that the values determined experimentally are similar to those determined by model as shown in Fig. 4

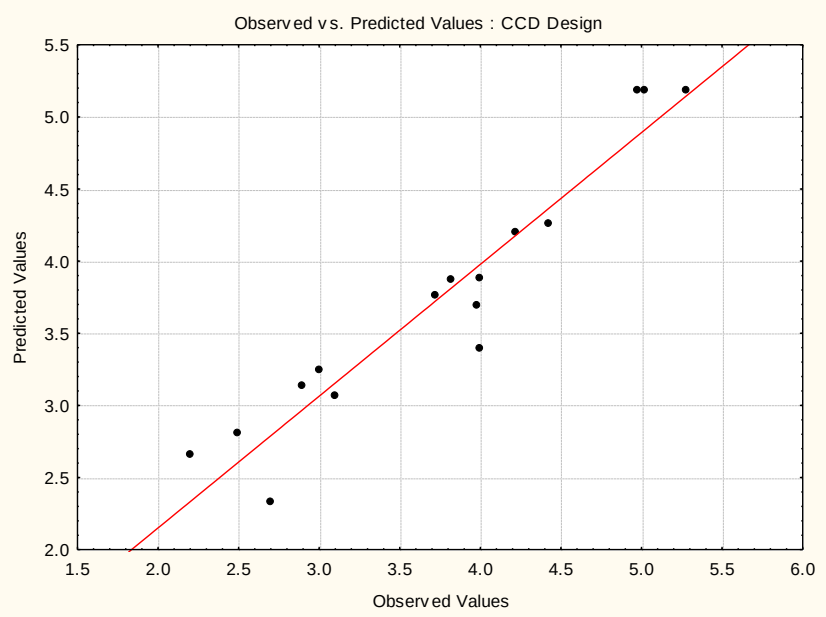


Fig.4: Plot of observed V/s. predicted biomass concentration gdw/l values for *Flavobacterium* sp MTCC 2495. The biomass concentration is response variable of interest. The expected biomass concentration values are determined by the model equations determined for Central composite design (CCD).

The Second order regression equation provided the levels of biosurfactant production as a function of initial values of sucrose, peptone, ferrous sulphate which can be predicted by the following equation Eq (4).

$$Y = -7.66 + 4.93X_1 - 0.78X_1^2 + 0.49X_2 - 0.02X_2^2 + 123.39X_3 - 2966.21X_3^2 + 1.13X_1X_3 + 0.23X_2X_3$$

(4)

Where, Y represents biomass yield, gdw/l and X_1, peptone (2-4g/lwith 3g/l as central value), X_2, sucrose (10-20 g/l with 15g/l as central value) and X_3 (ferrous sulphate 0.020- 0.040 g/l00ml with 0.030 as central value).The model adequacy was checked and it was found to be adequate, the goodness of fit of the model was expressed by the coefficient of determination R^2, which was calculated to be 0.92, indicating that 92% of the variability in the response could be explained by the model. The *P*-value obtained for the significant variables was 0.0008. This proves that the model equation as expressed in Eq. (4) provides a suitable model to describe the response of the experiment pertaining to cell growth. Fig. 5(A-C) shows the surface response plot of the model equation. From equations derived by differentiation of Eq. (4), we can obtain the critical values of the model, which was 3.11 g/l of peptone, 0.003 g/l of ferrous sulphate and 10.85 g/l of sucrose. The model predicted a maximum response for biomass concentration of 5.42 g/l for this point. In order to confirm the predicted results of the model, experiments using the medium representing this maximum point were performed and a value

ISSN: 0975-8585

of 5.31 g/l (triplicate experiments were carried out and corresponded standard deviation within ± 0.077) was obtained. Thus, the optimum medium composition amended with 10.85 g/l of sucrose as sole carbon and energy source for growing Flavobacterium sp. MTCC 2495 in a medium consisting of Solution A: 3.11 g/l peptone, 0.4 g/l $MgSO_4.7H_2O$, 1.0g/l NaCl, 1.0 g/l KCl, 0.05 g/l $CaCl_2.2H_2O$, 10 ml H_3PO_4 (85.0%), adjusted to pH 7.2 with KOH pellets. One milliliter of Solution B was added, which consist of 0.03 g/100ml $FeSO_4.7H_2O$, 0.015g/100ml $ZnSO_4.7H_2O$, 0.03 g/100ml H_3BO_3, 0.015g/100ml $CoCl_2.6H_2O$, 0.015g/100ml $CuSO_4.5H_2O$ and 0.01g/100ml $NaMo_2O_4.2H_2O$. The basal media composition of MSM was modified by incorporating the optimum medium components such as: sucrose, peptone, ferrous sulphate in the basal medium. Thus obtained optimized medium was designated as "Modified Mineral Salt Medium" (MMSM). The batch growth kinetic profile of *Flavobacterium* MTCC 2495 was compared separately in basal MSM and MMSM medium by shake flash studies as shown in Fig.6 (A-B). It was observed that in MMSM for same time intervals, a higher biomass concentration and reduced surface activity of fermentation (broth due to biosurfactant production) was observed. The optimization procedure allowed an increase of 29 % in the biomass production, using Modified Mineral Salt Medium. For MMSM medium, the goodness of fit was i.e., R^2 value was 0.97, compared to R^2 value of 0.85 for Mineral salt medium. So on this basis, we justified that the optimized medium i.e., modified mineral salt medium was preferred medium for biosurfactant production by *Flavobacterium* sp MTCC 2495.

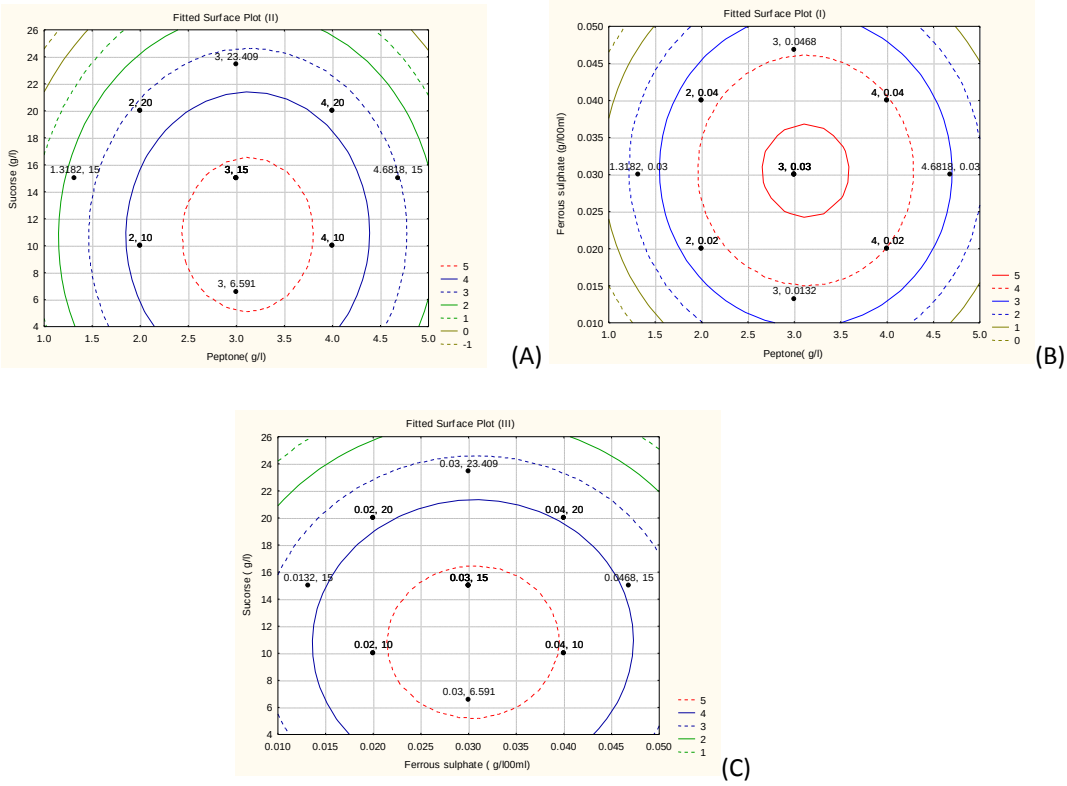
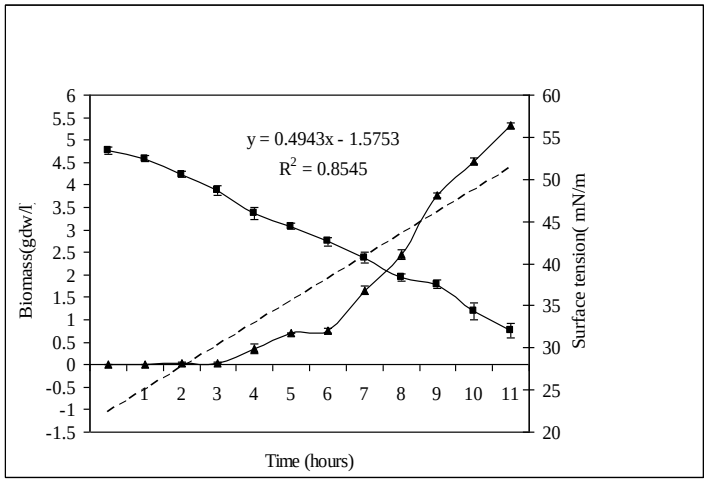


Fig 5(A-C): Contour graphs showing interaction between variables (peptone, ferrous sulphate and sucrose). Plot (A): Ferrous sulphate and peptone. Plot (B): Sucrose and Peptone. Plot(C): sucrose and ferrous sulphate.

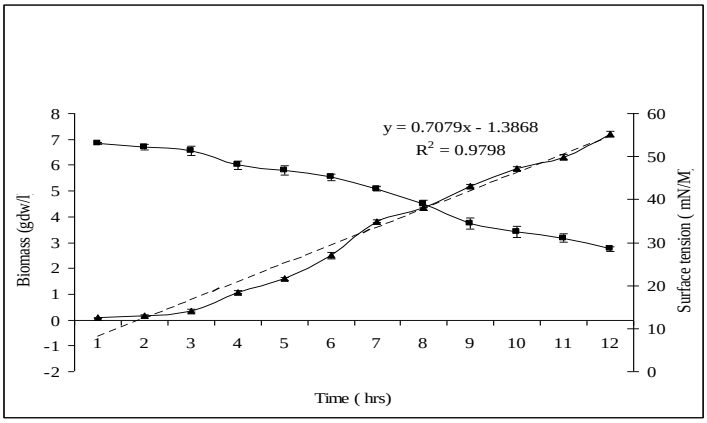
ISSN: 0975-8585

3.3. Effect of trace element on flavolipid biosurfactant production:

The outcome of PB fold-over design inferred that ferrous sulphate significantly affects on yield of biomass compared to manganese sulphate. The factorial CCD model was used to find the optimal concentration of ferrous sulphate which was found to be 0.003 g/l00ml as in Solution-B. The effect of multivalent cation that is inductive or inhibitory effect on flavolipid biosurfactant was studied separately in lab scale fermenter using the ferrous sulphate in higher (0.1 to 0.3 g/l00ml) and lower concentrations (0.01 to 0.05 g/100ml). The relation between the biomass and surface tension is represented as shown in Fig.7 (A, B). At higher concentration of ferrous sulphate exhibited exponential decline in biomass and increases surface activity, where as the lower concentration of ferrous sulphate has increase the biomass yield and significantly decrease the surface tension of broth, further increase in concentrate tend to decline the activity. This study justify that the lower concentration of iron has inductive effect on flavolipid biosurfactant production.



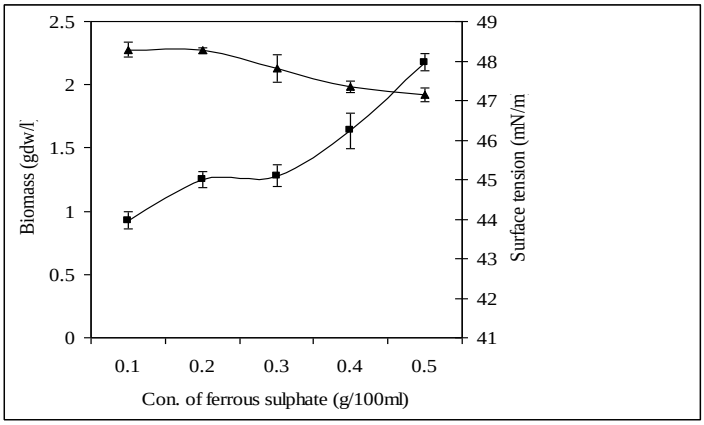
(A)



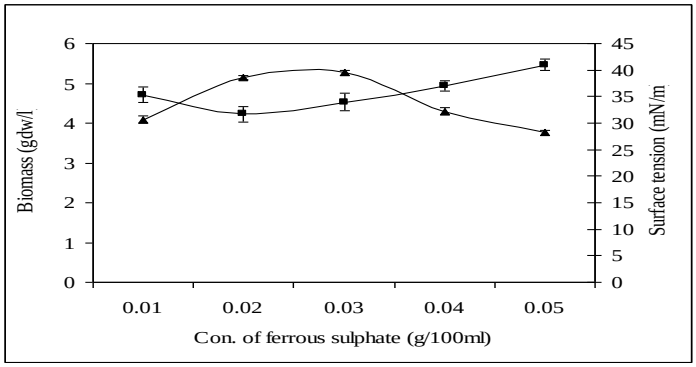
(B).

Fig 6 (A, B): Growth pattern for *Flavobacterium* sp MTCC 2494: A variation of Biomass concentration ▲ (gdw/l and surface tension ■ (mN/M), in time (hrs). (A) Growth profile of *Flavobacterium* sp MTCC 2495 in Mineral salt media (MSM) with R^2 value of 0.85. (B) Kinetic growth pattern of *Flavobacterium* sp MTCC 2495 in Modified Mineral Salt media (MMSM) with R^2 value of 0.97.

ISSN: 0975-8585



(A)



(B)

Fig 7 (A-B) : Effect of iron (ferrous sulphate) on biomass concentration (g/l) and surface tension (mN/m) for *Flavobacterium* sp MTCC 2495 (A) Inhibitory effect of ferrous sulphate on biomass concentration (B) Inductive effect of iron sulphate on biomass (g/l), when used in concentration of 0.01 to 0.03 g/l on biomass production.

DISCUSSION

Flavolipid biosurfactant produced by *Flavobacteium* sp MTCC 2495 were found to be growth associated, further the cell growth and production was enhanced by media optimization using response surface methodology. Growth associated biosurfactant production has been described for the production of rhamno-lipid by *Pseudomonas* sp and for glycoprotein AP-6 by *P. fluorescens* 378 [16, 17]. In our study, direct relation exists between flavolipid production (indicated by decrease in surface tension) and cell growth during the fermentation process.

The primary metabolic pathway (de novo) involved in synthesis of hydrophilic and hydrophobic moieties by hydrocarbons and carbohydrates respectively. The pathways for the synthesis of these two groups of precursors are diverse and utilize specific sets of enzymes [18].

Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol were used for rhamnolipid production by *Pseudomonas* spp. Biosurfactant product, however, was inferior to that obtained with water-immiscible substrates such as *n*-alkanes and olive oil [19, 20]. In the present study, during the production of flavolipid from *Flavobacterium* MTCC 249,

hydrophilic carbon source i.e., sucrose (disaccharides) was preferred against other carbon sources.

In general, the mechanisms, namely, induction, repression, nitrogen and multivalent ions, operate in the regulation of biosurfactant production [1]. In our study the sucrose has shown inductive effect on flavolipid biosurfactant production, compared to other hydrophilic carbon sources like glucose, maltose, fructose and galactose. During media preparation particularly sterilization, may affect the suitability of carbohydrates for individual fermentation process. It is often best to sterilize sugars separately because they may react with ammonium ions and amino acids to form black nitrogen containing compounds due to Millard or Caramelization reaction, which will partially inhibit the growth of many micro-organisms [21]. In the present work, in the MMSM medium sucrose was used as sole carbon source instead of glucose. Sucrose when autoclaved it undergoes hydrolysis to form glucose and fructose that is nutritional quality of medium is maintained and there was no need for separate sterilization (membrane filtration) due to caramelization or Millard reaction.

Medium constituents other than carbon source also affect the production of biosurfactants. Among the inorganic nitrogen salts, ammonium salts and urea were preferred nitrogen sources for biosurfactant production, the nitrogen not only causes overproduction of biosurfactant but also change the chemical composition of the biosurfactant produced [22,23]. In our study for production of flavolipid biosurfactant, the Modified Mineral salt medium (MMSM) with peptone 3.11g /l was responsible for overproduction of flavolipid biosurfactant than inorganic nitrogen source i.e., calcium nitrate alone. The P-B design (+fold over design) results has clearly indicated peptone as preferred nitrogen source over the calcium nitrate. Significantly the basal MSM was devoid of organic nitrogen source. But the optimized MMSM contains optimal proportion of peptone as nitrogen source which is responsible for overproduction of flavolipid biosurfactant.

Medium optimization by the classical method of changing one independent variable while fixing all the others at a certain level or one-factor-at-a-time method can be extremely time consuming and expensive for a large number of variables [12].The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels. Being single dimensional, this laborious and time consuming method often does not guarantee determination of optimal conditions. On the other hand carrying out experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required [24]. In the first screening, it is recommended to evaluate the result and estimate the main effects according to a linear model. After this evaluation, the variables that have the largest influence on the result are selected for new studies. Thus, a large number of experimental variables can be investigated without having to increase the number of experiments to the extreme [25].

The approach used in this study (P-B design followed by factorial CCD design) allowed the screening and determination of the medium compositions that give the highest biomass concentration for *Flavobacterium* sp MTCC 2495. In both cases, suitable models were found to

describe the response of the experiments pertaining to cell growth, as the values obtained experimentally are in accordance with the expected values determined by the models. The models were validated by comparing the observed and predicted values in the optimum point, and a deviation of about 0.077 was found. The optimization procedure allowed an increase in biomass concentration by 29%.

The limitation of multivalent cations also causes overproduction of biosurfactants Guerra-Santos et al. [26] demonstrated that by limiting the concentrations of salts of magnesium, calcium, potassium, sodium, and trace elements, a higher yield of rhamnolipid can be achieved in *P. aeruginosa* DSM 2659. Iron limitation stimulates biosurfactant production in *P. fluorescens* [27] and *P. aeruginosa* [26] whereas addition of iron and manganese salts stimulates biosurfactant production in both *B. subtilis* [28].

In present work, the multivalent ion, ferrous sulphate has shown inductive effect on flavolipid biosurfactant production, when it is used in lower concentration (0.01-0.03 g/l00ml) as Solution B, at higher concentration ferrous sulphate possess inhibitory effect on the flavolipid production. Many media which containing the iron and most of the calcium, manganese and zinc present in the medium tend to form insoluble white precipitate of meal ions. The problem of insoluble metal precipitate (phosphates or sulphates) may be eliminated by incorporating low concentration of chelating agents such as ethylene diamine tetracetic acid (EDTA), citric acid etc [29] . In the present work, both MSM and MMSM, medium are supplemented with EDTA, 1 g/l00ml (as solution B), which inhibited the formation of insoluble precipitate formation.

CONCLUSION

In conclusion, using the method of design of experiment, PB fold-over design and factorial CCD design, it was possible to screen critical medium constitutes and formulate novel medium which supported cell growth and biosurfactant yield. The present work, not only gives significant information about the formulation of novel medium for flavolipid biosurfactant production but also signifies the influence of critical factors on the production. The chosen method of optimization of medium composition was efficient, relatively simple, and time and material saving for process development.

ACKNOWLEDGMENTS

Authors are grateful to the dean, Dr. F V Manvi and Principal Dr A D Taranhalli of KLE's University College of Pharmacy, Belgaum for funding and providing necessary facilities for the research work. Authors want to extend their warm regards to M.S. Ramaiah institute of technology for giving valuable inputs on design of experiments and also acknowledge valuable suggestions from Dr. Marikunte Venkatrangana of Conexious Life Sciences pvt. Ltd. Bangalore.

ISSN: 0975-8585

REFRENCES

[1] Desai AJ, Patel RM, Desai JD. J Sci Ind Res 1994; 53:619–629.
[2] Rosenberg E. Crit Rev Biotechnol 1986; 3:109–132.
[3] Banat IM. Bioresource Technol 1995;51:1–12.
[4] Fiechter A. Trends Biotechnol 1992; 10:208–217.
[5] Klekner V, Kosaric N. Biosurfactants for cosmetics. In N. Kosaric (ed.), Biosurfactants: production, properties, applications. Marcel Dekker, Inc., New York, N.Y. 1993; . 329–372.
[6] Adria A, Bodour,Claudia Guerrero-Barajas, Beth VJiorle,Mark E, Malcomson, Amanda K Paull, Arpad Somogyi, Long N Trinh et al. Applied and environmental micro 2004; 70:114–120.
[7] Lo KV, Zhu CM, Cheuk W. Environ Technol 1998; 19:91–96.
[8] Negoro S. Appl Microbiol Biotechnol 2000; 54:461–466.
[9] Wang SY, Vipulanandan C. J Environ Eng 2001; 127:748–754.
[10] Jennings EM, Tanner RS. The Sixth International Petroleum Environmental Conference Proceedings 1999; 267-275.
[11] Plaza GA, Zjawiony I, Banat IM. Journal of Petroleum Science and Engineering 2006;*50*: 71-77.
[12] Peter F Stanbury, Allan Whitaker, Stephen J Hall. Principle of fermentation technology. Butterworth-Heinemann (Elsevier) 1994; p.93-117**.**
[13] Henry C Vogel, Celeste L. Todaro. Fermentation and biochemical engineering .Handbook principles, process design and equipment. 2nd edition.Noyes publications. 1997. 160-180 .
[14] Jitendra Kumar, Sandeep Kumar Jha, D'Souza SF. Biosensors and Bioelectronics 2006; 21: 2100–2105.
[15] Box GEP, Draper NR. Empirical Model Building and Response surfaces. Experimental Design: A chemometric approach, John Wiley & sons, New York, NY. 1987; p.11.
[16] Robert M, Mercade ME, Bosch MP, Parra JL, Espuny MJ, Manresa MA, J Guinea. Biotechnol Lett 1989; 11:871–874.
[17] Persson A E, Oesterberg M, Dostalek. Appl Microbiol Biotechnol 1988; 29:1–4.
[18] Syldatk C, Wagner F. Production of biosurfactants. In N Kosaric, W L Cairns, N C C Gray (ed.), Biosurfactants and biotechnology. Marcel Dekker, Inc., New York 1987; p 89–120.
[19] Robert M, Mercade ME, Bosch MP, Parra JL, Espuny MJ, Manresa M, Guinea J. Biotechnol Lett 1989; 11:871–874.
[20] Syldatk C, Lang S, U Matulovic, F Z Wagner. Z Naturforsch 40C, 1985;61–67.
[21] Solomons GL. Materials and methods in fermentation. Academic press, London.
[22] Peypoux F, Michel G. Control biosynthesis of Val-7 and Leu-7. Surfactins Appl. Microbiol Biotechnol 1992; 36:515–517.
[23] Duvnjak Z, Cooper DG, Kosaric N. N Kosaric (ed.), 1983; p 66–72.
[24] Sen R. J Chem Technol Biotechnol 1997; 68:263–70.

[25] Montgomery DC. Response surface methods and other approaches to process optimization. In: Montgomery DC, editor. Design and analysis of experiments. New York: John Wiley and Sons; 1997. p. 427–510.
[26] Guerra-Santos, L H O Kappeli, A Flechter. Appl Microbiol Biotechnol 1986; 24:443–448.
[27] Persson A, Molin G, Weibull C. Appl Environ Microbiol 1990; 56:686–692.
[28] Cooper DG, MacDonald CR, Duff SJB, Kosaric N. Appl Environ Microbiol 1981; 42:408–412.
[29] Gaunt DM, Trinci A J, Lynch JM. Trans Br Mycol Soc 1984; 83: 575-581.