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Optimization Of Culture Conditions For Enhanced Production Of Antimalarial Artemisinin By *Streptomyces* Sp. Using Response Surface Methodology

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ABSTRACT

Optimization of media components for maximal biomass production of a *Streptomyces* sp. involved in the bioconversion of arteannuin B to artemisinin, a potential antimalarial drug has been studied by Response Surface Methodology (RSM). A 2^4 full-factorial central composite design was chosen to explain the combined effects of the five medium constituents, viz. malt extract, yeast extract, K_2HPO_4, $MgCl_2$ and NaCl. A second order full polynomial equation was developed by applying multiple regression analysis to the experimental data. The coefficient of determination (R^2) of the model was 0.8630. The linear effects of malt extract ($p < 0.004156$), yeast extract ($p < 0.001355$) and $MgSO_4$ ($p < 0.001355$) and multiple effects of K_2HPO_4 ($p < 0.005575$) were found to be more significant than other factors, i.e., ($p < 0.05$) from the results of analysis of variance and regression of a second order model. The optimized media composition for maximal biomass production consisted of malt extract 19.1732 (g/l); yeast extract, 6.3287 (g/l); K_2HPO_4, 0.941 (g/l); $MgSO_4$, 0.60895 (g/l) and NaCl, 0.53667 (g/l). Our studies further indicate that the increase in biomass has increased the protein concentration and bioconversion activity of the crude extract of *Streptomyces* sp.

INTRODUCTION

Artemisinin (qinghaosu) is a potential antimalarial drug effective against the multi-drug resistant forms of the malarial parasite (WHO,2005). It is derived from Artemisia annua L., an annual herb native of China. Artemisinin isolated in its pure form in 1972 is a sesquiterpene lactone with an unique endoperoxide bridge (Christen and Veuthey 2001, Van et al., 1990). *Artemisia annua* L., is the only natural source of artemisinin but it produces very low quantities of artemisinin as compared to its biogenetic precursors artemisinic acid and arteannuin-B (Jung et al., 1990, Roth and Acton, 1987). The concentration of artemisinin in Artemisia annua varies widely, depending on plant material and growth conditions: yields generally range between 0.01% and 0.8% of the dry weight (Abdin et al., 2003, Wang et al., 2003). This short-fall in the production of artemisinin can be overcome by alternate approaches like biotransformation (Vikas Dhingra et al., 2000). Arteannuin-B, a sesquiterpene with an unusual α-methylene-γ-lactone is a biogenetic precursor of artemisinin that is produced in higher quantities in the plant [Singh et al., 1986). Earlier, studies were carried out on the biotransformation of arteannuin-B to artemisinin with crude leaf homogenates of Artemisia annua (Nair and Basile, 1993). The enzyme involved in the bioconversion was also purified from leaves of Artemisia annua (Vikas Dhingra and Lakshmi Narasu M, 2001). However, studies on biotransformation of arteannuin B to artemisinin using microbial cell free extracts have not been carried out extensively. In the present study we report the isolation of a *Streptomyces* sp. with the ability to convert arteannuin B to artemisinin and we also report the optimization for maximal biomass production.

We have isolated a Streptomyces sp. with the ability to convert arteannuin-B to artemisinin from soil samples. An intracellular enzyme produced by this Streptomyces sp. was found to convert arteannuin B to artemisinin. Hence it is desirable to obtain the maximal biomass of the *Streptomyces* sp. to develop an efficient bioconversion system.

The composition of growth media plays an important role in the production of primary and secondary metabolites. Designing an appropriate growth medium is of critical importance in order to maximize the yields of any desired product (Akknazarova and Kafarov, 1982). The classical method of experimental optimization for the maximal production of *Streptomyces* sp. biomass involves changing one variable at a time keeping the others constant. In addition, it is not practical to carry out experiments with every possible factorial combination of the test variables because of the large number of experiments required. This does not consider the effects of interactions of various parameters. Besides this, it is a tedious, cumbersome, and time-consuming process especially when a large number of parameters are taken into account. An alternative and more efficient approach is the use of statistical method. Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different physiological and nutritional parameters (Cladera-Olivera et al., 2004). A prior knowledge and understanding of these parameters is necessary for achieving a more realistic model.

In the present study, based on the results obtained by the classical approach, parameters found significantly affecting the maximal biomass production of Streptomyces sp. were taken into account. A 25-1 fractional-factorial central composite design (CCD) and RSM were used for optimization of medium components for the maximal production of biomass. The regression analysis was performed to obtain the optimum medium concentration.

MATERIALS AND METHODS

Microorganism

Soil samples collected from various industrial areas in and around Hyderabad, India have been used for the isolation of microorganisms. These are subsequently purified and screened for their ability to convert arteannuin B to artemisinin. One Actinomycete species with bioconversion ability was identified as a Streptomyces sp. by its morphological, physiological and biochemical characteristics, according to the Bergey's Manual of Determinative Bacteriology (8th Edn.).

Culturing of *Streptomyces* sp.

Streptomyces sp. was cultured in malt-yeast extract medium containing malt extract 1% (w/v), yeast extract 0.4% (w/v), K2HPO4 0.1% (w/v), MgSO4 0.1% (w/v) and NaCl, 0.05% (w/v) adjusted to a pH of 7.2. Cultivation of Streptomyces sp. was carried out for a period of 72h at a temperature of 300C in an orbital shaker at 120 rpm.

Estimation of the biomass of Streptomyces sp.

The 72h old culture of Streptomyces sp. was centrifuged at 12000rpm for 10 min and the pellet was weighed for the estimation of biomass.

Assay of bioconversion activity

Preparation of the crude cell free extract

On completion of an incubation period of 72 h, the culture of Streptomyces sp was harvested by centrifugation at 12000rpm for 10 min and the pellet obtained was homogenized in 50mM Tris buffer (pH-7.2) containing β-mercaptoethanol, EDTA, Glycerol and PMSF. The supernatant obtained on centrifugation of the homogenate at 12000rpm for 30 min at 40C was used for the bioconversion assay.

Estimation of protein concentration

The concentration of protein in the crude cell free extract was estimated by Lowry method (Lowy et al., 1951) with bovine serum albumin (BSA) as the standard.

Evaluation of enzyme activity

Bioconversion activity was assayed by incubating 2 ml of crude extract with 100µg of the precursor, arteannuin-B, and the co-factors: ATP (0.1mM), Mg+2 (1.0mM) & Mn+2 (1.0mM) in Tris-HCl buffer system (pH-7.2) for a period of 180 min at 30°C. A control was run parallel to

this with all the other components except the substrate. The reaction was stopped by adding ethanol and chloroform in 1:1 proportion and artemisinin was extracted from the reaction mixture with hexane. The hexane fraction was collected separately and allowed for evaporation. The residue was dissolved in methanol and was further tested for the presence of artemisinin by TLC (thin layer chromatography). TLC was performed on silica gel plates with a mobile phase of ethyl acetate and hexane in the ratio of 2:8 and artemisinin was detected by spraying iodine vapors.

Quantification of artemisinin by HPLC

HPLC was performed for quantitative determination of artemisinin produced by bioconversion of arteannuin B (Gupta MM and VermaRK, 1997). The chromatographic system consisted of a Shimadzu – LC-10AT VP Series with a Phoenomenex column (250 X 4.6 mm, C18, ODS with particle size of 5 µm) and a UV-VIS detector (Shimadzu UV-Visible SPD-LC 10A VP Series) controlled by Spinchrom CFR (version 2.2) software. Filtered samples of standard artemisinin (Sigma- Aldrich) (1mg/ml), standard arteannuin B (1mg/ml) and the methanolic extract of the test sample were injected on to the column and eluted with a mobile phase containing of TFA (1% in water) and acetonitrile in the ratio of 30 : 70 at a flow rate of 1 ml/min. Artemisinin was monitored at 220nm in the UV-VIS detector.

Selection of growth medium components for RSM studies

A typical microbiological media consists of a carbon source, nitrogen source and mineral salts. Screening studies carried out on an array of carbon sources, nitrogen sources and mineral salts to identify the compounds that significantly influence the growth of Streptomyces sp. have resulted in the selection of malt extract, yeast extract, K2HPO4, MgSO4 and NaCl for optimization studies.

Experimental Design and Optimization

The optimum concentrations of growth medium components for the maximal production of Streptomyces sp. biomass were determined by means of RSM. The RSM consists of a group of empirical techniques devoted to the evaluation of relationships existing between a cluster of controlled experimental factors and measured responses according to one or more selected criteria (Khuri AI and Cornell JA, 1987). According to this design, the total number of treatment combinations was 2^k + 2*k + n0 where k is the number of independent variables and n0 is the number of repetitions of the experiments at the center point. Based on the best results of one at a time approach, five critical components of the growth medium were selected and further evaluated for their interactive behaviors by using a statistical approach. The levels of five medium variables viz. Malt extract, 10 g/l (x1); yeast extract, 4 g/l (x2); K_2HPO_4, 1 g/l(x3); MgSO4, 1 g/l (x4) and NaCl, 0.5 g/l (x5) were selected and each of the variables were coded at five levels –2, –1, 0, 1, and 2 by using Eq. 1.

For statistical calculations, the centre variables Xi were coded as xi according to the following transformation. The range and levels of the variables in coded units for RSM studies are given in Table 1.

$$xi = Xi - X_0/\Delta X \quad (1).$$

where xi is the dimensionless coded value of the variable Xi, X0 the value of the Xi at the center point, and ΔX the step change.
The behavior of the system is explained by the following quadratic model

$$Y = \beta_0 + \sum \beta_i * x_i + \sum \beta_{ii} * x_i^2 + \sum \beta_{ij} * x_{ij} \quad (2)$$

where Y is the predicted response, β0 the intercept term, βi the linear effect, βii the squared effect, and βij the interaction effect. The full quadratic equation for four factors is given by model 3.

Y = β0 + β1 x1 + β2 x2 + β4 x3 + β4 x4 + β5 x5 + β11 x1*x1 + β12 x1*x2 + β13 x1*x3 + β14 x1*x 4 + β15 x1*x5 + β22 x2*x2 + β23 x2*x3 + β24 x2*x 4 + β25 x2*x5 + β33 x3*x3+ β34 x3*x4+ β35 x3*x5 + β44 x4*x4+ β45 x4*x5 + + β55 x5*x5 ---3

Several experimental designs have been considered for studying such models, and CCD was selected . For this study, a 25-1 factorial design with ten star points and six replicates at the central points were employed to fit the second order polynomial model, which indicated that 32 experiments were required for this procedure. STATISTICA 6.0 (Stat Soft, Inc, Tulsa, OK) software was used for regression and graphical analysis of the data obtained.

In order to search for the optimum combination of major components of the growth medium, experiments were performed according to the CCD experimental plan (Table 2). The results of CCD experiments for studying the effect of three independent variables are presented along with the mean predicted and observed responses in Table 3. The regression equations obtained after the analysis of variance (ANOVA) gave the level of Streptomyces sp. biomass as a function of the initial values of malt extract, yeast extract, K_2HPO_4, $MgSO_4$, and NaCl.

Effect of biomass on the protein concentration and bioconversion activity of the crude cell free extract of *Streptomyces* sp.

This factor has been investigated by estimating the protein concentration and bioconversion activity of the crude cell free extract of the biomass obtained during each run of the CCD design. The biomass obtained during each run was used for the preparation of crude cell free extract and the protein concentration of the extract was estimated by Lowry et al method. The crude extract was further assayed for the bioconversion activity and the artemisinin was quantified by the standard protocol that has been specified in earlier sections.

RESULTS AND DISCUSSION

Artemisinin and its derivatives are a class of compounds of great significance in the treatment of drug-resistant malaria. An approach to enhance the production of artemisinin is the microbial bioconversion of its precursor arteannuin B to artemisinin to the later. Media optimization for maximal biomass production of a Streptomyces sp. with this desired biotransformation ability is reported in the present paper.

Under the experimental conditions mentioned earlier, crude cell free extract of the 72h old culture of Streptomyces sp. was incubated with the precursor arteannuin B to assay the bioconversion activity. Artemisinin produced by bioconversion was qualitatively detected on silica gel plates by TLC. The Rf values of artemisinin and arteannuin B were found to be 0.5 and 0.4 respectively. HPLC analysis of the standard artemisinin (1mg/ml) (Figure 1-A), standard arteannuin B (1mg/ml) (Figure 1-B) and the test samples have been carried out. The retention times of standard samples of artemisinin and arteannuin B were 6.9-7.1min and 5.7–5.9 min respectively. The HPLC chromatogram of the experimental sample with the crude cell free extract of Streptomyces sp. has shown the elution of arteannuin B and artemisinin at the retention times of 5.880min and 7.093min respectively (Figure 1-C) which is complementary to the standards of arteannuin B and artemisinin. Quantification of artemisinin produced has shown a 17.64% bioconversion of arteannuin B to artemisinin on molar basis with a specific activity of 0.11units/mg under initial conditions of study.

The RSM is an effective and sequential and stepwise procedure (Khuri AI and Cornel JA, 1987). The lead objective of the RSM was to run rapidly and efficiently along the path of improvement towards the general vicinity of the optimum. It is appropriate when the optimal region for running the process has been identified. The five independent variables, malt extract, yeast extract, K_2HPO_4, $MgSO_4$, and NaCl in the growth medium were chosen for optimizing the maximal production of Streptomyces sp. biomass. Experiments were performed according to the CCD experimental design given in Table 2 in order to search for the optimum combination of components of the medium.

The coefficient of determination (R^2) was calculated as 0.8630 for biomass production (Model summary, Table 3), indicating that the statistical model can explain 86.30% of variability in the response. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response. In this case, the value of the determination coefficient (R^2 = 0.8630) indicates that only 13.70% of the total variations are not explained by the model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj R^2 = 0.6141) is also very high to advocate for a high significance of the model (Cochran WG et al., 1957). If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2. Here in this case the adjusted R^2 value is 0.6141, which is lesser than the R^2 value of 0.8630. At the same time, a relatively lower value of the coefficient of variation (CV = 13.89%) indicates a better precision and reliability of the experiments carried out.

By applying multiple regression analysis on the experimental data, the experimental results of the CCD design were fitted with a second order full polynomial equation. The empirical relationship between biomass production (Y) and the five test variables in coded units obtained by the application of RSM is given by equation 4.

Y= 8.33+1.13*x1+1.33*x2-0.40*x3-1.30*x4+0.3175*x5-0.3517*x1x1+0.0712*x1x2-0.2387*x1x3-0.1712*x1x4-0.0172-0.4104*x2x2-0.215*x2x3-0.3425*x2x4-0.025*x2x5-0.9754*x3x3+0.055*x3x4+0.0775*x3x5-0.4417*x4x4-0.215*x4x5-0.4329*x5x5 ----- (4)

where *Y* is biomass production in U/100ml, is response and *x*1, *x*2, *x*3, *x*4, and *x5* are the coded values of the test variables, malt extract, 10 g/l (*x*1); yeast extract, 4 g/l (*x2*); K_2HPO_4, 1 g/l(*x3*); $MgSO_4$, 1 g/l (*x4*) and NaCl, 0.5 g/l (*x5*).

ANOVA has been conducted for the second order response surface model and the results are given Table 3 and 4. The significance of each coefficient was determined by Student's *t*-test and *p*-values, which are listed in Table 3 and 4. The larger the magnitude of the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient. This implies that the linear effects of malt extract ($p < 0.004156$), yeast extract ($p < 0.001355$) and $MgSO_4$ ($p < 0.001355$) and multiple effects of K_2HPO_4 ($p < 0.005575$) are more significant than the other factors, i.e., ($p < 0.05$). The model F-value of 3.467, and values of prob > F (<0.05) indicated that the model terms are significant. The regression model developed can be represented in the form of 2D and 3D surface and contour plots. The yields of maximal production of *Streptomyces* sp biomass for different

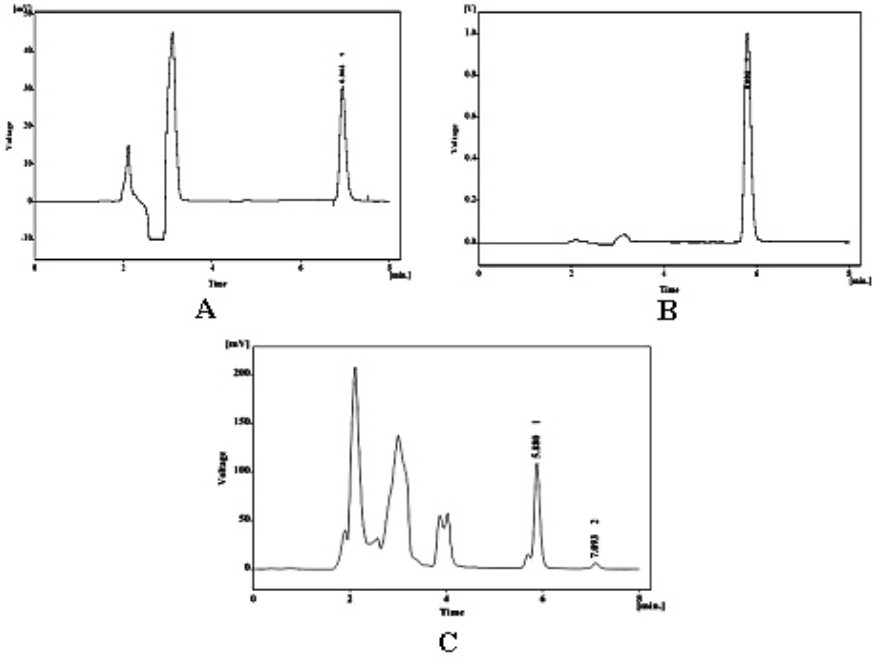

Figure 1. HPLC chromatograms of standard artemisinin, standard arteannuin B and the test sample incubated with the crude cell free extract. Figures A, B and C correspond to HPLC chromatograms of standard artemisinin, standard arteannuin B and test sample incubated with the crude cell free extract of Streptomyces sp. respectively. Peak 1 of fig-A corresponds to the standard artemisinin eluting at the retention time of 6.943min. Peak 1 of fig-B shows the elution of standard arteannuin B at the retention time of 5.803min. Peaks 1 and 2 of fig-C correspond to the elution of arteannuin B and artemisinin at the retention times of 5.880min and 7.093min respectively.

Table 1. Range and Levels of the Variables in Coded Units for RSM Studies.

Ingredient	**-2**	**-1**	**0**	**+1**	**+2**	**∆X**
Malt Extract	2	6	10	14	18	4.0
Yeast Extract	2	3	4.0	5	6	1
K_2HPO_4	0.5	0.75	1.0	1.25	1.5	0.25
$MgSO_4$	0.5	0.75	1.0	1.25	1.5	0.25
NaCl	0.3	0.4	0.5	0.6	0.7	0.1

Table 2. Design of experiments by Central Composite Design (CCD) for RSM studies.

RUN	**Factors**				
	X1	**X2**	**X3**	**X4**	**X5**
1	-1	-1	-1	-1	+1
2	+1	-1	-1	-1	-1
3	-1	+1	-1	-1	-1
4	+1	+1	-1	-1	+1
5	-1	-1	+1	-1	-1
6	+1	-1	+1	-1	+1
7	-1	+1	+1	-1	+1
8	+1	+1	+1	-1	-1
9	-1	-1	-1	+1	-1
10	+1	-1	-1	+1	+1
11	-1	+1	-1	+1	+1
12	+1	+1	-1	+1	-1
13	-1	-1	+1	+1	+1
14	+1	-1	+1	+1	-1
15	-1	+1	+1	+1	-1
16	+1	+1	+1	+1	+1
17	-2	0	0	0	0
18	+2	0	0	0	0
19	0	-2	0	0	0
20	0	+2	0	0	0
21	0	0	-2	0	0
22	0	0	+2	0	0
23	0	0	0	-2	0
24	0	0	0	+2	0
25	0	0	0	0	-2
26	0	0	0	0	+2
27	0	0	0	0	0
28	0	0	0	0	0
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30	0	0	0	0	0
31	0	0	0	0	0
32	0	0	0	0	0

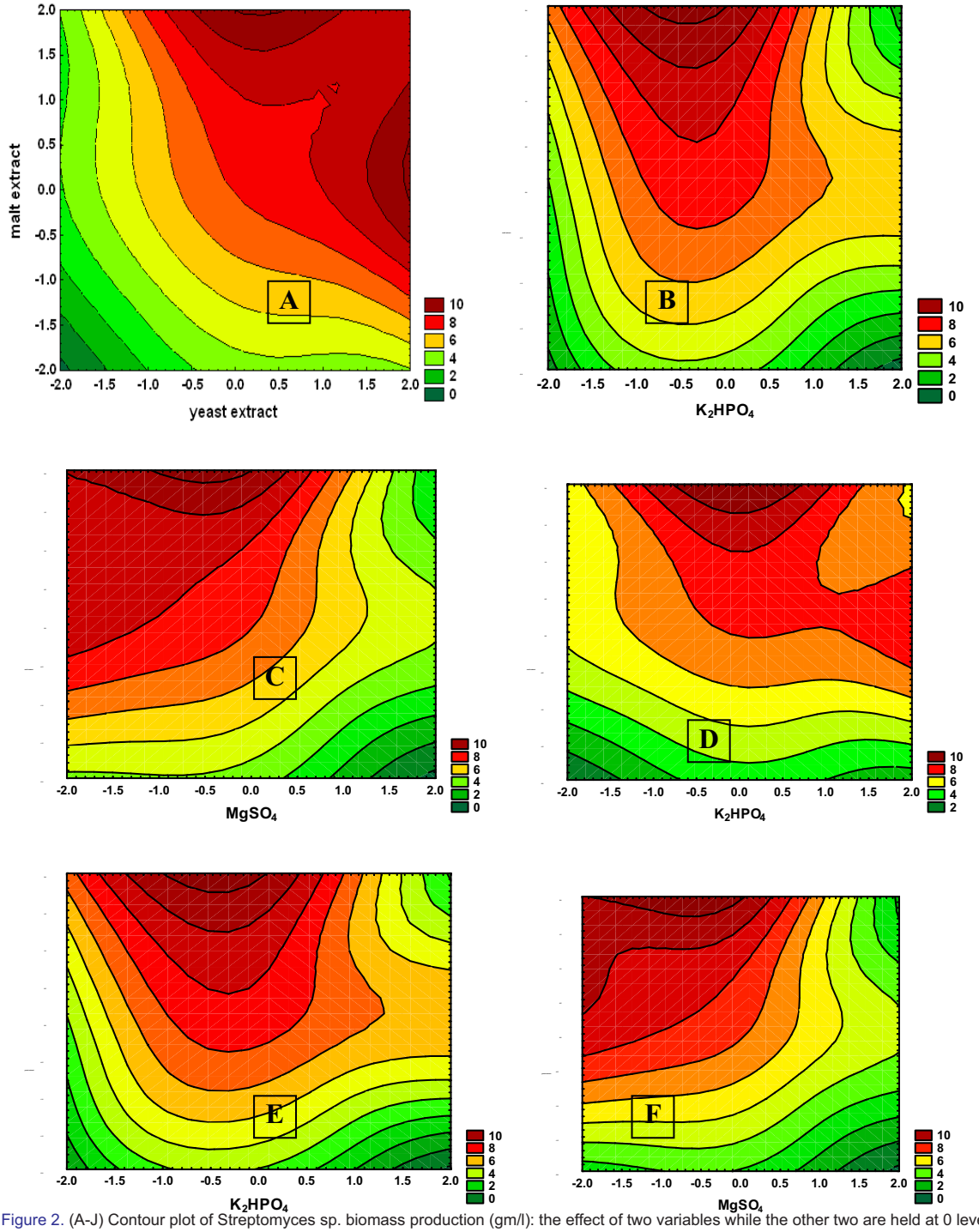

Figure 2. (A-J) Contour plot of Streptomyces sp. biomass production (gm/l): the effect of two variables while the other two are held at 0 levels.

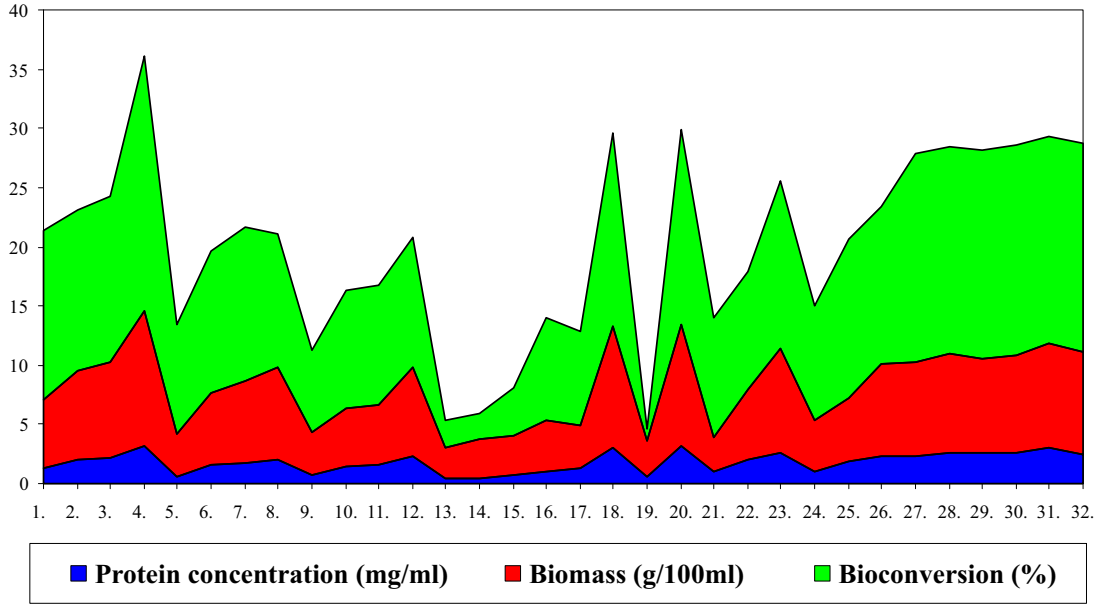

Figure 3. Comparative analysis of the biomass, protein concentration of crude cell free extract and percent bioconversion. .

Table 3. The predicted and measured values of the CCD experiments.

Flask No	Weight of Biomass Gms/100ml predicted	Weight of Biomass Gms/100ml measured	Protein Concentration (mg/ml)	Percent Bioconversion
1.	4.672348	5.75	1.29	14.32
2.	6.651515	7.5	1.97	13.62
3.	7.264015	8.15	2.12	14.01
4.	11.34818	**11.38**	3.21	**21.56**
5.	3.407348	3.6	0.56	9.2
6.	6.661515	6	1.65	12.01
7.	7.554015	6.93	1.76	13
8.	8.653182	7.8	2.07	11.24
9.	2.310682	3.5	0.77	7
10.	4.664848	5	1.39	9.98
11.	4.777348	5.15	1.53	10.02
12.	7.316515	7.46	2.32	11
13.	2.920682	2.6	0.44	2.34
14.	3.809848	3.26	0.49	2.12
15.	3.832348	3.32	0.7	4.01
16.	5.726515	4.36	1.01	8.69
17.	4.66697	3.56	1.32	8.02
18.	9.190303	**10.25**	3.05	**16.29**
19.	4.02197	2.99	0.6	1.07
20.	9.365303	**10.35**	3.13	16.37
21.	5.238636	2.82	1.02	10.1
22.	3.628636	6	1.99	9.88
23.	9.175303	**8.75**	2.63	14.23
24.	3.96197	4.34	1.01	9.66
25.	5.968636	5.32	1.87	13.46
26.	7.238636	7.84	2.29	13.31
27.	8.335455	**7.96**	2.33	17.65
28.	8.335455	**8.36**	2.58	17.52
29.	8.335455	**7.99**	2.59	17.63
30.	8.335455	**8.29**	2.61	17.71
31.	8.335455	**8.78**	3.02	17.55
32.	8.335455	**8.68**	2.42	17.58

Table 4. Model Summary .

R	R^2	Adjusted R^2	Standard error of the Estimate
0.929024	0.863086	0.61415	1.538066

Table 5. Analysis of Variance (ANOVA) for the Quadratic Model.

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*- value	Sig Probability (*p*)
Regression	164.0392	20	8.201961	3.46711	0.01914
Residual	26.02213	11	2.365648		
Total	190.0614	31			

concentrations of variables can also be predicted from the respective contour plots as shown in Figure 2 (A-J). Each contour curve represents an infinite number of combinations of two test variables with the other two maintained at their respective 0 level.

A numerical method given by Montgomery, 2001 was used to solve the regression equation 4. The optimal values of the five test variables in coded units were observed to be x1 = 2.2933, x2 = 2.3287, x3 = -0.2327, x4=-1.5642 and x5 = 0.3677. The predicted value of Y (biomass production, gm/l) at these values of x's was 16.2834. The real values of the five test variables were obtained by substituting the respective coded values in equation 1 and found to be malt extract (x2), 19.1732; yeast extract (x2), 6.3287; K_2HPO_4 (x3), 0.941; $MgSO_4$ (x4), 0.60895 and NaCl (x5), 0.53667.

The optimum conditions of selected parameters were predicted using RSM and the maximum predicted biomass production of 16.2834 gm/l could be achieved with the media consisting of malt extract (x2), 19.1732; yeast extract (x2), 6.3287; K_2HPO_4 (x3), 0.941; $MgSO_4$ (x4), 0.60895 and NaCl (x5), 0.53667. The validation of the optimized conditions was done in a 250 ml EM flask containing 100 ml of growth medium. The experiments were conducted in three similar flasks for the reproducibility of the biomass production using optimized conditions. The biomass production of 15.286 gm/l (average of three experiments) obtained by using optimized conditions show that an increase of 95% was achieved than that obtained before optimizing the experimental conditions. The experimental value of the biomass production was almost equal if we consider 95% of the confidence limits for the prediction of Y value at optimized conditions with shake flask results.

Apart from the estimation of biomass, the protein concentration and bioconversion activity of the crude extract of *Streptomyces* sp. has also been advocated for each run that has been conducted in the CCD design. A linear increase in the yield of protein concentration and bioconversion activity has been observed with the increase in biomass. A graphical representation of the relationship between the biomass and protein concentration and bioconversion activity of the crude extract has been given in the figure3

CONCLUSION

Media optimization studies for the maximal biomass production of *Streptomyces* sp. involved in the bioconversion of arteannuin B to artemisinin has been carried out by response surface methodology (RSM). A second order polynomial equation developed for the CCD design has yielded a R^2 value of 0.8630 indicating that the statistical model can explain a good variance of 86.30% in the response. Optimization of media has not only increased the yield of biomass but also has resulted in an increase in bioconversion activity of the crude extract of *Streptomyces* sp.

Table 6. Model Coefficients Estimated By Multiple Linear Regressions (Significance of Regression Coefficients).

Model term	Model term	Unstandardised co-efficients		Standardized co-efficients	Computed t - value	Sig (*p*-value)
		Parameter estimates (B)	Standard error	Beta		
(Constant)	(Constant)	8.335455	0.613476		13.58725	3.21E-08
VAR00001	x1	1.130833	0.313956	0.401844	3.601879	0.004156
VAR00002	x2	1.335833	0.313956	0.474691	4.254836	0.001355
VAR00003	x3	-0.4025	0.313956	-0.14303	-1.28202	0.226195
VAR00004	x4	-1.30333	0.313956	-0.46314	-4.15132	0.001613
VAR00005	x5	0.3175	0.313956	0.112824	1.011287	0.333607
VAR00006	x1x1	-0.3517	0.283984	-0.13973	-1.23846	0.241322
VAR00007	x1x2	0.07125	0.384517	0.020673	0.185298	0.856368
VAR00008	x1x3	-0.23875	0.384517	-0.06927	-0.62091	0.547309
VAR00009	x1x4	-0.17125	0.384517	-0.04969	-0.44536	0.664694
VAR00010	x1x5	-0.07125	0.384517	-0.02067	-0.1853	0.856368
VAR00011	x2x2	-0.41045	0.283984	-0.16307	-1.44534	0.176242
VAR00012	x2x3	-0.215	0.384517	-0.06238	-0.55914	0.587267
VAR00013	x2x4	-0.3425	0.384517	-0.09937	-0.89073	0.39214
VAR00014	X2x5	-0.025	0.384517	-0.00725	-0.06502	0.949327
VAR00015	x3x3	-0.97545	0.283984	-0.38754	-3.43489	0.005575
VAR00016	x3x4	0.055	0.384517	0.015958	0.143037	0.888848
VAR00017	x3x5	0.0775	0.384517	0.022486	0.201552	0.843946
VAR00018	x4x4	-0.4417	0.283984	-0.17549	-1.55538	0.148139
VAR00019	x4x5	-0.215	0.384517	-0.06238	-0.55914	0.587267
VAR00020	x5x5	-0.43295	0.283984	-0.17201	-1.52457	0.155587

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No potential conflict of interest was reported by the author.

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