0273-2289/1559-0291 (Online)/06/134/0143/$30.00

Optimization of Critical Medium Components for the Maximal Production of Gentamicin by *Micromonospora echinospora* ATCC 15838 Using Response Surface Methodology

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Received November 11, 2005; Revised December 19, 2005;
Accepted December 30, 2005

Abstract

Optimization of the fermentation medium components for maximum gentamicin production by *Micromonospora echinospora* ATCC 15838 was carried out. Response surface methodology was applied to optimize the medium constituents. A 2^4 full-factorial central composite design was chosen to explain the combined effects of the four medium constituents, viz. starch, soyabean meal, K_2HPO_4, and $CoCl_2$ and to design a minimum number of experiments. A second order model was developed and fitted using least square method. The R^2 value of the model was 0.9723, which shows that model is best fit for the present studies. The results of analysis of variance and regression of a second order model showed that the linear effects of starch ($p < 0.001697$) and $CoCl_2$ ($p < 7.99E\text{-}13$), and cross product effects of starch and soyabean meal ($p < 0.029876$) and soyabean meal and $CoCl_2$ ($p < 0.008909$) were more significant, suggesting that these were critical variables having the greatest effect on the production of gentamicin in the production medium. The optimized medium consisting of 9 g/L starch, 3 g/L soyabean meal, 0.9 g/L K_2HPO_4, and 0.01 g/L $CoCl_2$ predicted 850 mg/L of gentamicin which was almost 110% higher than that of the unoptimized medium. The amounts of starch, soyabean meal, and K_2HPO_4 required were also reduced with RSM.

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Index Entries: Gentamicin; production medium; rotational central composite design ($\alpha = 2$); response surface methodology; optimization.

Introduction

Microbiologically produced secondary metabolites are extremely important to health and nutrition of humankind. The best-known secondary metabolites are the antibiotics. As a group, they have tremendous economic importance. Many scientists have studied production of antibiotics by fermentation by using different microorganisms *(1–3)*. Fermentation by *Micromonospora* species, *echinospora* and *purpurea*, produces a family of aminocyclitol antibiotics called gentamicin *(4)*. Gentamicin is a broad spectrum, basic, and water-soluble antibiotic, first reported by Weinstein et al. *(5)*. Among the clinically more important species of Gram-negative organisms responsive to gentamicin are both indole-positive and indole-negative *Proteus, Pseudomonas, Escherichia coli, Aerobacter, Klebsiella, Salmonella*, and *Shigella (6)*. Gentamicin is highly active against Gram-negative bacteria and *Mycobacterium tuberculosis (7)*. In addition to its use as antibacterial agent, the potential anti viral properties of some gentamicin conjugates recently have been demonstrated *(8)*.

The composition of fermentation media plays an important role in the production of secondary metabolites. Designing an appropriate fermentation medium is of critical importance because of medium composition aspects, product concentration, yield, and volumetric productivity *(9)*.

The classical method of experimental optimization for the production of gentamicin by *Micromonospora echinospora* 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 about the effect 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. To develop a process for the maximum production of gentamicin, standardization of media components would be crucial.

Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different physiological and nutritional parameters *(10–16)*. A prior knowledge and understanding of these parameters are necessary for achieving a more realistic model.

In the present study, based on the results obtained by the classical approach, parameters found significantly affecting gentamicin production from *M. echinospora* were taken into account. A 2^4 full-factorial central composite design (CCD) and RSM were used for optimization of medium components for the maximal production of gentamicin. The regression analysis was performed to obtain the optimum medium concentration.

Materials and Methods

Growth and Maintenance of the Microorganism

M. echinospora subsp. *pallida* ATCC 15838 was used as the production strain. These are maintained in agar slants contained the following components: 10 g/L yeast extract, 10 g/L glucose, 3 g/L $CaCO_3$, and 15 g/L agar.

Inoculation medium contained: 3 g/L beef extract, 1 g/L glucose, 24 g/L soluble starch, 5 g/L yeast extract, and 4 g/L $CaCO_3$. The pH was adjusted to 7.6 before sterilization. The inoculum medium (50 mL per 250- mL Erlenmeyer flask) was inoculated with *M. echinospora* (one slant for each flask) under aseptic conditions. The inoculated flasks were kept on a rotary shaker at 200 rpm at 27° ± 2C for 72 h to obtain good growth.

Selection of Production Medium Components

In an earlier study, the production medium components were optimized by one factor at a time method, by keeping the other factors at constant level *(17)*. It was found that starch, soyabean meal, K_2HPO_4, $FeSO_4$, and $CoCl_2$ had significant effects on the production of gentamicin from *M. echinospora*.

Estimation of Gentamicin

At specified intervals, production of the antibiotic was determined by an agar disc technique using *Staphylococcus aureus* MTCC 737 as the assay organism *(18)*. Growth was measured as the weight of cell mass obtained from a culture by vacuum filtration (Whatman No. 1 filter disc) and drying at 100°C for 4 h. The residual sugar was determined by method of Frank. A. Loewus *(19)*.

Experimental Design and Optimization

The optimum concentrations of production medium components for the gentamicin production by *M. echinospora* 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 *(20)*. According to this design, the total number of treatment combinations was $2\wedge k + 2k + n0$ 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, four critical components of the production medium were selected and further evaluated for their interactive behaviors by using a statistical approach. The levels of four medium variables viz. 10 g/L starch ($x1$), 5 g/L soyabean meal ($x2$), 1 g/L K_2HPO_4 ($x3$), and 0.01 g/L $CoCl_2$ ($x4$) 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 variables Xi were coded as xi according to the following transformation.

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

Variables	Range and levels					
	–2	–1	0	1	2	Δx
Starch, g/L, *x*1	5	7.5	10	12.5	15	2.5
Soyabean meal, g/L, *x*2	3	4	5	6	7	1
K_2HPO_4, g/L, *x*3	0.5	0.75	1	1.25	1.5	0.25
$CoCl_2$, g/L, *x*4	0.005	0.075	0.01	0.0125	0.015	0.0025

Where ΔX is step increment in each variable values

The range and levels of the variables in coded units for RSM studies are given in Table 1.

$$x_i = X_i - X_0/\Delta X \tag{1}$$

where x_i is the dimensionless coded value of the variable X_i, X_0 the value of the X_i at the center point, and ΔX the step change.

The behavior of the system was explained by the following quadratic model 2.

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \tag{2}$$

where Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} the interaction effect. The full quadratic equation for four factors is given by model 3.

$$\begin{aligned} Y = \beta_0 + \beta_1 x1 + \beta_2 x2 + \beta_3 x3 + \beta_4 x4 + \beta_{11} x1{*}x1 + \beta_{12} x1{*}x \\ 2 + \beta_{13} x1{*}x3 + \beta_{14} x1{*}x\,4 + \beta_{22} x2{*}x2 + \beta_{23} x2{*}x3 + \beta_{24} x2{*}x\,4 \\ + \beta_{33} x3{*}x3 + \beta_{34} x3{*}x4 + \beta_{44} x4{*}x4 \end{aligned} \tag{3}$$

Several experimental designs have been considered for studying such models, and CCD was selected *(21)*. For this study, a 2^4 factorial design with eight star points and six replicates at the central points were employed to fit the second order polynomial model, which indicated that 30 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 production 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 gentamicin production as a function of the initial values of starch, soyabean meal, $CoCl_2$, and K_2HPO_4. The application of RSM yielded the following regression equation, which is empirical relationship between gentamicin concentration (Y) and the test variables in coded unit.

Table 2
Design of Experiments by Central Composite Design (CCD) for RSM Studies

Run number	$x1$	$x2$	$x3$	$x4$	Coefficients assessed by	Measured value, mg/L	Predicted value, mg/L
1	–1	–1	–1	–1	Full-factorial 2^4 design (16 experiments)	313	319.875
2	1	–1	–1	–1		194	200.875
3	–1	1	–1	–1		262	269.2084
4	1	1	–1	–1		209	215.7084
5	–1	–1	1	–1		293	299.875
6	1	–1	1	–1		227	233.875
7	–1	1	1	–1		230	236.7084
8	1	1	1	–1		229	236.2084
9	–1	–1	–1	1		527	506.375
10	1	–1	–1	1		442	421.875
11	–1	1	–1	1		558	537.7084
12	1	1	–1	1		539	518.7084
13	–1	–1	1	1		507	486.875
14	1	–1	1	1		476	455.375
15	–1	1	1	1		526	505.7084
16	1	1	1	1		560	539.7084
17	–2	0	0	0	Star points (8 experiments)	399	412.4165
18	2	0	0	0		314	327.4165
19	0	–2	0	0		334	347.5831
20	0	2	0	0		368	381.2499
21	0	0	–2	0		375	388.4173
22	0	0	2	0		376	389.4173
23	0	0	0	–2		169	127.9165
24	0	0	0	2		550	617.9165
25	0	0	0	0	Central points (6 experiments)	405	403.3333
26	0	0	0	0		398	403.3333
27	0	0	0	0		409	403.3333
28	0	0	0	0		401	403.3333
29	0	0	0	0		399	403.3333
30	0	0	0	0		408	403.3333

Table 3
Model Summary and Analysis of Variance (ANOVA) for the Quadratic Model

Source of variations	Sum of squares	Degrees of freedom	Mean square	*F*-value	Probability (*p*)
Regressions	392642.0	14	28045.85	37.62608	4.09E-09
Residual	11180.7	15	745.38		
Total	403822.7				

R = 0.98605919, R^2 = 0.97231273, Adjusted R^2 = 0.94647127, CV = 6.09

Results and Discussion

The RSM is an effective and sequential and stepwise procedure *(21)*. The lead objective of the RSM was to run rapidly and efficiently along the path of improvement toward the general vicinity of the optimum. It is appropriate when the optimal region for running the process has been identified. The four independent variables, starch, soyabean meal, K_2HPO_4, and $CoCl_2$, in the fermentation medium were chosen to optimize the production of gentamicin by *M. echinospora.*

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 0.9723 for gentamicin production (Model summary, Table 3), indicating that the statistical model can explain 97.23% 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 *(22)*. In this case, the value of the determination coefficient (R^2 = 0.9723) indicates that only 2.77% 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 = 0.9464) is also very high to advocate for a high significance of the model *(23,24)*. 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 0.9464, which is lesser than the R^2 value of 0.9723. At the same time, a relatively lower value of the coefficient of variation (CV = 6.09%) indicates a better precision and reliability of the experiments carried out *(21–24)*.

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 gentamicin production (Y) and the four test variables in coded units obtained by the application of RSM is given by Eq. 4.

$$Y = 403.3333 - 21.2500*x1 + 8.4167*x2 + 0.2500*x3 + 122.5000*x4 - 8.3542*x1\text{^}2 + 16.3750*x1*x2 + 13.2500*x1*x3 + 8.6250*x1*x4 - 9.7292*x2\text{^}2 - 3.1250*x2*x3 + 20.5000*x2*x4 - 3.6042*x3*x3 + 0.1250*x3*x4 - 7.604*x4\text{^}2 \quad (4)$$

Table 4
Model Coefficients Estimated By Multiple Linear Regressions
(Significance of Regression Coefficients)

Model term	Parameter estimates	S.E.	Computed *t*-value	*p*-value
Intercept	403.3333		36.18678	0.000000
*x*1	–21.2500	0.042963	–3.81307	0.001697[a]
*x*2	8.4167	0.042963	1.51027	0.151750
*x*3	0.2500	0.042963	0.04486	0.964811
*x*4	122.5000	0.042963	21.98122	7.99 e-13[a]
*x*1*x*1	–8.3542	0.044024	–1.60256	0.129876
*x*1*x*2	16.3750	0.042963	2.39912	0.029876[a]
*x*1*x*3	13.2500	0.042963	1.94127	0.071253
*x*1*x*4	8.6250	0.042963	1.26366	0.225640
*x*2*x*2	–9.7292	0.044024	–1.86633	0.081670
*x*2*x*3	–3.1250	0.042963	–0.45785	0.653625
*x*2*x*4	20.5000	0.042963	3.00347	0.008909[a]
*x*3*x*3	–3.6042	0.044024	–0.69138	0.499888
*x*3*x*4	0.1250	0.042963	0.01831	0.985630
*x*4*x*4	–7.6042	0.044024	–1.45869	0.165272

[a]Significant at $p < 0.05$ S.E., standard error.

where *Y* is gentamicin production in mg/L, is response and *x*1, *x*2, *x*3, and *x*4 are the coded values of the test variables, starch (g/L), *x*2 is soyabean meal (g/L), *x*3 is $CoCl_2$ (g/L), and *x*4 is K_2HPO_4 (g/L)

The results of multiple linear regressions conducted for the second order response surface model are given in Table 4. The significance of each coefficient was determined by Student's *t*-test and *p*-values, which are listed in Tables 3 and 4. The larger the magnitude of the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient *(22–24)*. This implies that the linear effects of starch ($p < 0.001697$) and K_2HPO_4 ($p < 7.99$ E-13), and interactive effects of starch and soyabean meal ($p < 0.029876$) and soyabean meal and K_2HPO_4 ($p < 0.008909$) are more significant than the other factors, i.e., ($p < 0.05$). These suggest that the concentrations of starch and K_2HPO_4 have a direct relationship with the production of gentamicin and interactive effects of starch, soyabean meal, and K_2HPO_4 in this particular complex production medium. This is in good accordance with the previous work in which gentamicin production was mainly affected by starch and K_2HPO_4 concentrations *(17)*.

The model F-value of 37.62, and values of prob > F (<0.05) indicated that the model terms are significant. For gentamicin production, *x*1 (starch), *x*4 ($CoCl_2$), *x*1**x*2 (interaction of starch and soyabean meal), and *x*2*–*x*4 interaction (soyabean meal and $CoCl_2$) are significant. The statistically

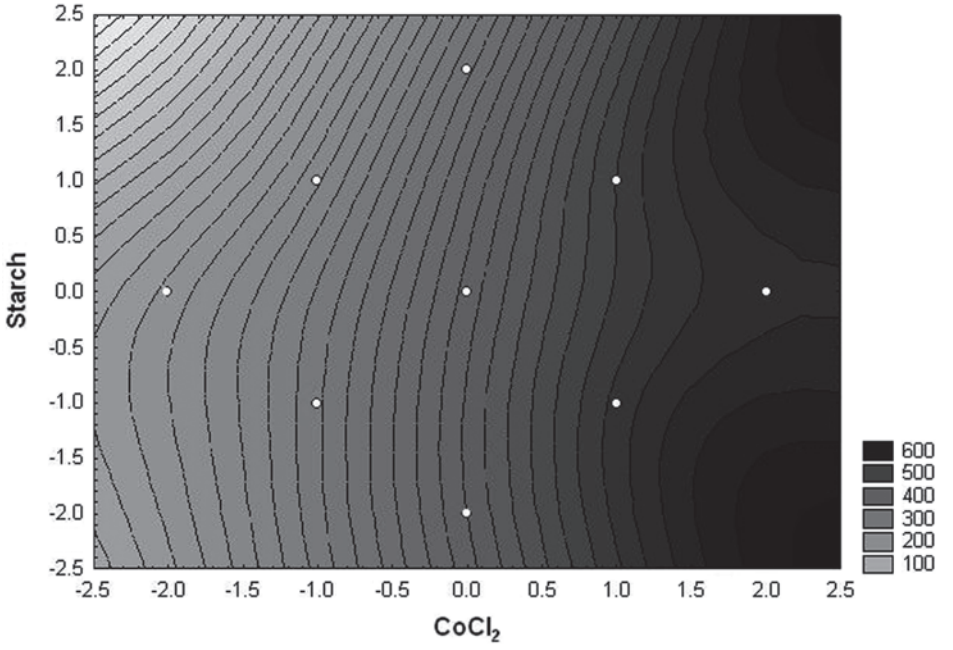

Fig. 1. Contour plot of gentamicin concentration (mg/L): the effect of starch and $CoCl_2$ on gentamicin production. Other variables are held at 0 level.

significant model of the optimization studies is given by the following equation.

$$\text{Gentamicin (mg/L)} = 403.3333 - 21.2500 * x1 + 122.5000 * x4 + 16.3750 * x1*x2 + 20.5000*x2*x4$$

RSM proved to be a powerful tool in optimizing the fermentation medium for the production of gentamicin from *M. echinospora* ATCC 15838. In the present study, the experimental results clearly showed that the gentamicin production was dependent mainly on the starch and $CoCl_2$.

The regression model developed can be represented in the form of 2D and 3D surface and contour plots. The yields of gentamicin for different concentrations of variables can also be predicted from the respective contour plots as shown in Figs. 1–4 *(20–23)*. Each contour curve represents an infinite number of combinations of tow test variables with the other two maintained at their respective 0 level. These plots demonstrate that the production of gentamicin is dependent on linear effects of $CoCl_2$ and soyabean meal. It is also evident from the contours plots that the variables starch, soyabean meal, K_2HPO_4, and $CoCl_2$ had linear effects on maximum gentamicin production by *M. echinospora* at their respective levels.

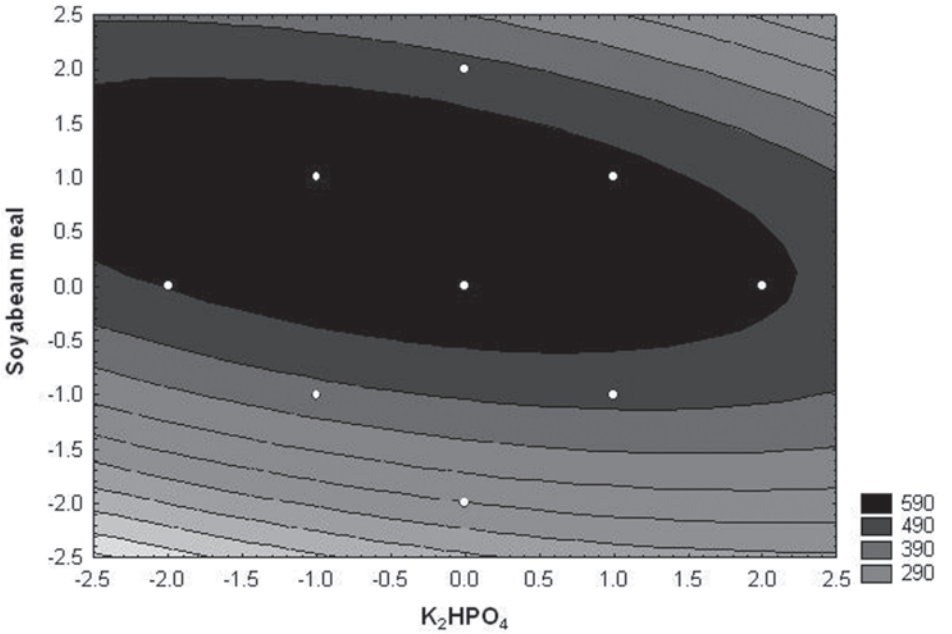

Fig. 2. Contour plot of gentamicin concentration (mg/L): the effect of soyabean meal and K_2HPO_4 on gentamicin production. Other variables are held at 0 level.

A numerical method given by Myers and Montgomery was used to solve the regression Eq. 4 *(21)*. The optimal values of the test variables in coded unit are as follows:

$$x1 = -11.79,\ x2 = -8.95,\ x3 = -0.175, \text{ and } x4 = -8.054$$

with the corresponding $Y = 850$ mg/L. The natural values obtained by substituting the respective values of x_i in Eq. 1 are: 9 g/L starch, 3 g/L soyabean meal, 0.9 g/L K_2HPO_4, and 0.01 g/L $CoCl_2$. The regression model fitted for the present CCD predicts that the maximum concentration of gentamicin can be obtained using the optimal concentrations of four test variables calculated previously is 850 mg/L, with a variation of 808 and 893 mg/L in the confidence limits of 95%.

The optimized results for the four test variables were verified by carrying out shake flask experiments. The maximum concentration of gentamicin obtained experimentally was found to be 880 mg/L. This is obviously in close relation with the model prediction. After optimization the gentamicin production was enhanced by almost 110% experimentally. The comparison of gentamicin production by *M. echinospora* ATCC 1588 before and after optimization is shown in Table 5. After optimization the amounts of starch, soyabean meal, and K_2HPO_4 were reduced to 1, 2, and 0.1 g/L, respectively. The reduction in the production medium concentra-

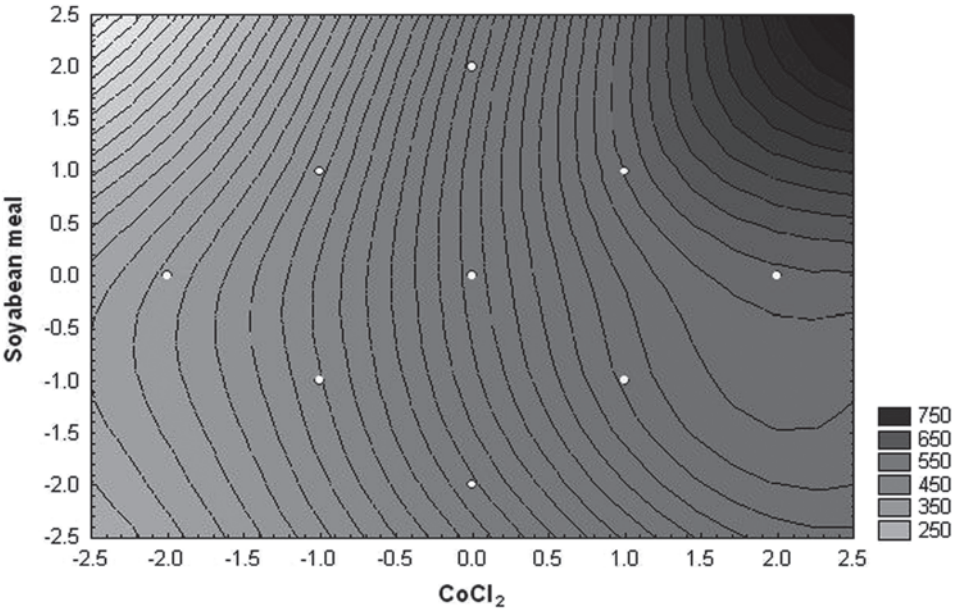

Fig. 3. Contour plot of gentamicin concentration (mg/L): the effect of soyabean meal and $CoCl_2$ on gentamicin production. Other variables are held at 0 level.

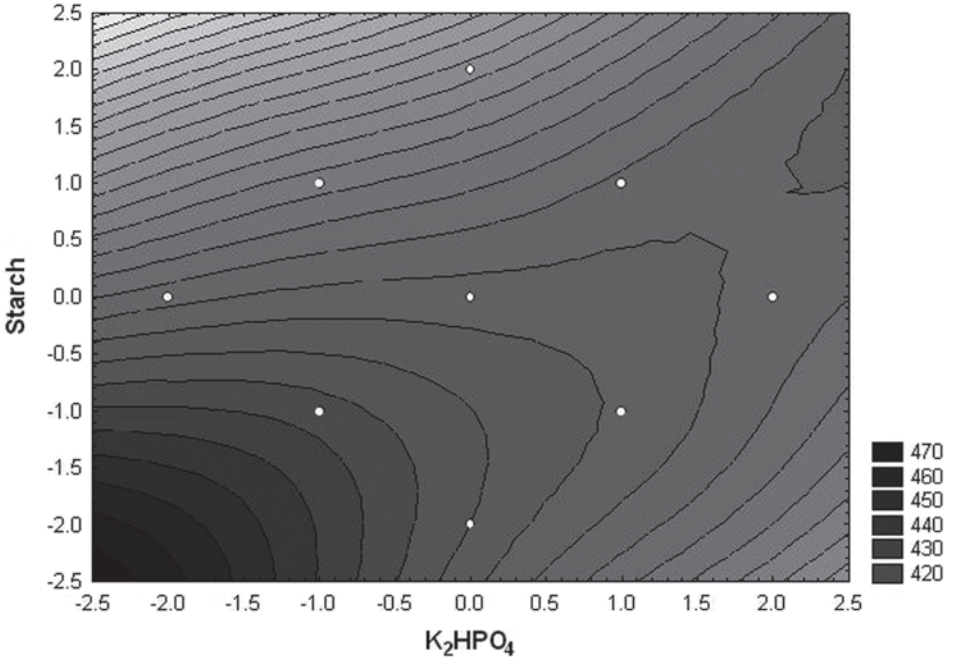

Fig. 4. Contour plot of gentamicin concentration (mg/L): the effect of starch and K_2HPO_4 on gentamicin production. Other variables are held at 0 level.

Table 5
Verification of the Effect of the Optimized Variables on Gentamicin Production

Variables	Concentration of Variables (g/L)		Gentamicin Production (mg/L)		
	Before optimization	After optimization	Before optimization	After optimization	
				Predicted	Experimental
Starch	10	9	403	850	880
Soyabean meal	5	3			
K_2HPO_4	1	0.9			
$CoCl_2$	0.01	0.01			

tions and at the same time increase in the yield of gentamicin will enhance the cost benefit of the process during large-scale production *(16)*.

Conclusion

RSM for the optimization of medium components for the production of gentamicin was applied. The CCD was good design for the optimization of variables in the present work. The model developed for CCD had R^2 values of 0.9723. The analysis of the data shows that optimized values of medium components could give a 110% more production of gentamicin in comparison with the conventional optimization methods. Besides this, starch, soyabean meal, and K_2HPO_4 are used in lesser concentration when compared with the values of before optimization. Such an approach could be quite efficient and useful for many gentamicin-producing industries in reducing the production medium cost.

Acknowledgments

The authors are thankful to Dr. J. S. Yadav director, I I C T, for his cooperation and one of the authors, M. Himabindu is thankful to Council of scientific and industrial research, New Delhi, for providing financial support to carry out this work and awarding junior research fellowship. The authors are grateful to Prof. Dr. Lakshmimanga, Osmania for her advice concerning the statistical analysis.

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