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Process Parameter Optimization for the Production of Gentamicin using *Micromonouspora Echiniospora*

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***Abstract*—**The process parameters, temperature, pH and substrate concentration, were optimized for the production of gentamicin using *Micromonouspora echinospora*. Experiments were carried out according to central composite design in response surface method. The optimum conditions for the maximum production of gentamicin were found to be: temperature – 31.7°C, pH – 6.8 and substrate concentration – 3%. At these optimized conditions the production of gentamicin was found to be – 1040 mg/L. The R^2 value of 0.9465 indicates a good fitness of the model.

***Keywords*—**Gentamicin, Micromonouspora echinospora, response surface method, optimization, central composite design.

I. Introduction

GENTAMICIN is an aminoglycoside antibiotic, and can treat many types of bacterial infections, particularly Gram negative infection. Gentamicin is one of the few heat-stable antibiotics that remain active even after autoclaving, which makes it particularly useful in the preparation of certain microbiological growth media. Gentamicin is a basic and water-soluble antibiotic, first invented by Weinstein et al [1] from soil fungus M*icromonospora purpurea*. There are some studies on the gentamicin production [2-7].

Response surface methodology (RSM) is an advanced tool, now a days commonly applied involves three factorial designs giving number of input (independent) factors and their corresponding relationship between one or more measured dependent responses. RSM is widely used for multivariable optimization studies in several biotechnological processes such as optimization of media, process conditions, catalyzed reaction conditions, oxidation, production, fermentation, etc., [7-13]. Optimization of process parameters for enhancing the production of gentamicin has not been attempted so far. Hence this work is aimed to find out the optimum conditions of process parameter by response surface methodology for the production of gentamicin by *Micromonospora echinospora subs pallida*.

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II. Materials and Methods

Micromonospora echinospora subs pallida (MTCC 708) obtained from MTCC, Chandigarh, is used for the batch studies. The Growth medium consist of: Beef extract - 3g/L, Glucose - 1g/L, Yeast extract - 5g/L, $CaCO_3$ - 4g/L, Soluble starch -24g/L, Agar -15g/L. The production medium was optimized in our earlier study (Rajasimman and Subathra, 2009a), which consists of Starch – 8.9 g/L, Soya bean meal – 3.3 g/L, K_2HPO_4 – 0.8 g/L, $CaCO_3$ – 4 g/L, $FeSO_4$ – 0.03 g/L.

Response surface methodology is used in this study. The experimental variables at different levels used for the production of Gentamicin by *Micromonouspora echinospora subs pallida* using CCD is given in Table 1. A total of 20 runs are used to optimize the medium. The average from two replicated values of each run is taken as dependent variables or response or yield (production of gentamicin).

TABLE I
EXPERIMENTAL VARIABLES AT DIFFERENT LEVELS USED FOR THE PRODUCTION OF GENTAMICIN BY *MICROMONOUSPORA ECHINOSPORA SUBS PALLIDA* USING CCD

Variable	Levels					
	Code	-1.68	-1	0	+1	+1.68
Temperature	A	25	30	35	40	45
pH	B	5	6	7	8	9
Substrate Concentration	C	1%	2%	3%	4%	5%

The experimental design is carried out using Design Expert 7.1.5 (Stat Ease, USA). Central composite design (CCD) is used to identify the optimum operating condition in order to obtain maximum gentamicin production (Y_1) as response. The collection of experiments provides an effective means for optimization through these process variables. Besides, the design permits the estimation of all main and interaction effects. On the other hand, the purpose of the center points is to estimate the pure error and curvature. A second-degree quadratic polynomial can be used to represent the function in the range of interest.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,\, i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

where X_1, X_2, X_3, X_4, . . ., X_k are the input variables which affect the response Y and β_0, β_i, β_{ii} and β_{ij} are the constants. A second-order model is designed such that variance of Y is constant for all points equidistant from the center of the

design. Production of Gentamicin was found by following the procedure given by Wang et al. [14].

Cell suspension was prepared from slant culture obtained from MTCC Chandigarh. The cell suspension was then added to the 50 ml of growth medium in a 250 ml Erlenmeyer flask. The medium was sterilized at 121°C for 20 minutes in an autoclave. The inoculated flask was kept in a rotary shaker at 150 rpm at 28°C. Growth period of *micromonospora echinospora* was two days. The grown medium was used for the production of gentamicin. Experiments were carried out according to the CCD given in Table 2.

III. Results and Discussion

Experiments were performed according to the CCD experimental design given in Table 2 in order to search for the optimum combination of process parameters for the maximum production of gentamicin. The Model F-value of 19.14 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The Lack of Fit F-value of 11.32 implies the Lack of Fit is significant. There is only a 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise.

TABLE II
CENTRAL COMPOSITE DESIGN MATRIXES ALONG WITH PREDICTED AND EXPERIMENTAL VALUES OF GENTAMICIN PRODUCTION

Run No	X_1	X_2	X_3	Gentamicin, mg/L	
				Experimental	Predicted
1	1.00000	1.00000	-1.00000	650	654.20
2	0.00000	-1.68179	0.00000	825	826.54
3	0.00000	0.00000	1.68179	890	890.94
4	-1.68179	0.00000	0.00000	900	893.09
5	0.00000	0.00000	0.00000	910	911.04
6	0.00000	0.00000	0.00000	910	911.04
7	1.68179	0.00000	0.00000	675	645.96
8	0.00000	0.00000	0.00000	910	911.04
9	-1.00000	-1.00000	-1.00000	890	922.50
10	0.00000	0.00000	0.00000	910	911.04
11	0.00000	1.68179	0.00000	790	752.42
12	1.00000	1.00000	-1.00000	600	654.20
13	-1.00000	-1.00000	1.00000	880	847.40
14	-1.00000	1.00000	1.00000	880	912.90
15	0.00000	0.00000	0.00000	910	911.04
16	1.00000	-1.00000	1.00000	725	750.84
17	0.00000	0.00000	-1.68179	925	887.62
18	0.00000	0.00000	0.00000	910	911.04
19	1.00000	1.00000	1.00000	740	733.30
20	1.00000	-1.00000	-1.00000	815	807.94

The Fisher F-test with a very low probability value (P_{model} > F = 0.0001) demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). The coefficient of determination (R^2) was calculated to be 0.9451 for gentamicin production. This implies that 94.51% of experimental data of the gentamicin production was compatible with the data predicted by the model (Table 1) and only 5.49% of the total variations are not explained by the model. The R^2 value is always between 0 and 1, and a value >0.75 indicates aptness of the model. For a good statistical model, R^2 value should be close to 1.0. 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 = 0.8957) is also high to advocate for a high significance of the model. Here in this case the adjusted R^2 value is 0.8957, which is lesser than the R^2 value of 0.9451. The Pred R^2 of 0.7050 is in reasonable agreement with the Adj R^2 of 0.8957. The value of CV is also low as 3.93 indicate that the deviations between experimental and predicted values are low. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this work the ratio is 11.99, which indicates an adequate signal. This model can be used to navigate the design space. The mathematical expression of relationship to the gentamicin production with variables are shown below

$$Y_1 = 911.04 - 73.55X_1 - 22.06X_2 + 0.99X_3 - 50.14X_1^2 - 43.07X_2^2 - 7.71X_3^2 - 20.76\,X_1X_2 + 4.51X_1X_3 + 34.05X_2X_3$$

The results of multiple linear regressions conducted for the second order response surface model are given in Table 2. The significance of each coefficient was determined by Student's *t*-test and *p*-values, which are listed in Table 3. The larger the magnitude of the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case X_1, X_2, X_1^2, X_2^2, X_2 X_3 are significant model terms. Values greater than 0.10 indicate the model terms are not significant. This implies that the linear effect of temperature ($p < 0.0001$) is more significant than the other factors, i.e., ($p < 0.05$). Table 2 also indicate that the interaction between, pH and substrate concentration and interactive effects of temperature and pH ($p < 0.05$) had very significant influence on gentamicin yield by the *micromonouspora echinospora subs pallida* used in this study.

TABLE III
ANALYSIS OF VARIANCE (ANOVA) FOR RESPONSE SURFACE QUADRATIC MODEL

Source	Coefficient factor	F	P-value Prob> F
Model	911.04	19.14	< 0.0001[a]
X_1	-73.55	58.40	< 0.0001[a]
X_2	-22.06	5.31	0.0440
X_3	0.99	0.011	0.9198
X_1* X_2	-20.76	2.46	0.1482
X_1* X_3	4.51	0.12	0.7407
X_2* X_3	34.05	6.71	0.0269
X_1^2	-50.14	33.13	0.0002
X_2^2	-43.07	24.44	0.0006
X_3^2	-7.71	0.78	0.3967
Residual			
Lack of fit		11.32	0.0058
Pure Error			
Cor Total			

[a] – significant variable

R^2 - 0.9451; Adj R^2- 0.8957; C.V. % - 3.93; Pred R^2 - 0.7050; Adeq Precision – 11.99

Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels are more helpful in understanding both the main and the interaction effects of these two factors. These plots can be easily obtained by calculating from the model, the values taken by one factor where the second varies with constraint of a given Y value. The response surface curves were plotted to understand the

interaction of the variables and to determine the optimum level of each variable for maximum production. The response surface curves for gentamicin production are shown in Figures 1 - 3. Figure 1 shows that increase in temperature up to 35°C, leads to increase in gentamicin production, beyond which the production decreases. From Figure 2 it was observed that increase in substrate concentration increases gentamicin production. From all the Figures, it was observed that the lower and higher levels of all the variables did not result in higher gentamicin yields. The studies of the contour plot also reveal the best optimal values of the process conditions lies within the range; temperature: 30-35, pH: 6.5 –7 and substrate concentration: 2.6-3.2%.

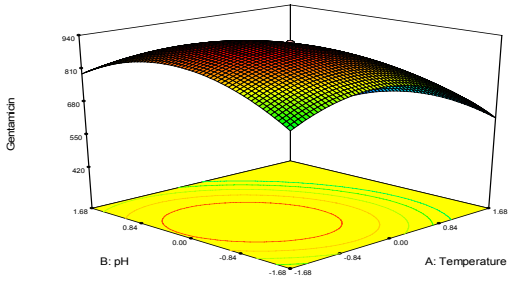

Fig. 1. The 3D plot showing the effects of temperature, pH and their mutual interaction on gentamicin production

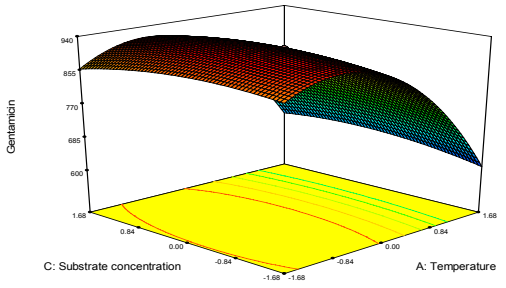

Fig. 2. The 3D plot showing the effects of temperature, substrate concentration and their mutual interaction on gentamicin production

The optimum values obtained by substituting the respective coded values of variables are: temperature: 31.7°C, pH: 6.8 and substrate concentration: 3%. The optimized results for the three test variables are verified by carrying out shake flask experiments. The maximum concentration of gentamicin obtained experimentally was found to be 1040 mg/L. This is obviously in close relation with the model prediction. After optimization the gentamicin production was enhanced by 130 mg/L experimentally.

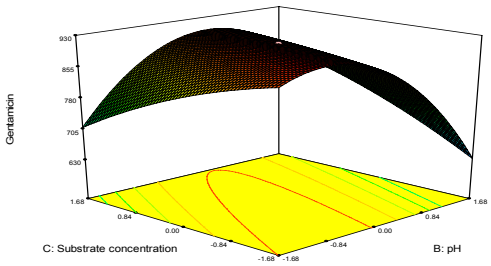

Fig. 3. The 3D plot showing the effects of pH, substrate concentration and their mutual interaction on gentamicin production

IV. Conclusion

Response surface methodology was applied for the optimization of production medium components for the production of gentamicin. The model developed for CCD had R^2 values of 0.9286 for gentamicin production. The optimum values obtained by substituting the respective coded values of variables are:temperature – 31.7°C, pH – 6.8 and substrate concentration – 3%. 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 950 mg/L. The analysis of the data shows that optimized values of medium components give more production of gentamicin (1020 mg/L) in comparison with the conventional optimization methods.

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