APPLICATION OF RESPONSE SURFACE METHODOLOGY IN MEDIUM COMPONENTS OPTIMIZATION TO ENHANCE SERRATIOPEPTIDASE PRODUCTION BY *STREPTOMYCES HYDROGENANS*MGS13

Jyothi Vanama
Haritha Chintada
GirijaSankar Guntuku
Prabhakar Tadimalla
Pharmaceutical Biotechnology Division, A. U. College of Pharmaceutical Sciences, Andhra University, Andhra Pradesh, India

Abstract

For enhanced production of Serratiopeptidase by an actinomycete strain, *Streoptomyces hydrogenans* MGS13, optimization of fermentation medium was initially carried out by conventional method of 'one-factor-at-a-time'. Later it was optimized by applying response surface methodology. Interactions were studied with four variables viz. levels of dextrose, soya bean meal and inoculum & pH using Central Composite Design. This model was validated by conducting the experiments under the optimized conditions, which resulted in the improved Serratiopeptidase production of 254.56 IU/mL (Predicted response 278.087 IU/mL), thus proving the validity of the model. *Streptomyces hydrogenans* MGS13 strain isolated from mangrove soil sediment was taken up for this study. This study demonstrates the ability of the strain, *Streptomyces hydrogenans* MGS13 for the Serratiopeptidase production and the application of response surface methodology with improved Serratiopeptidase production. The statistical experimental design is simple and less time consuming & is adequate to economize the fermentation. This is the first report on the application of response surface methodology for Serratiopeptidase production by an actinomycete isolate.

Keywords: *Streptomyces hydrogenans* MGS13, central composite design (CCD), serratiopeptidase

1. Introduction

Optimization through factorial design and response surface analysis is a common practice in biotechnology and various research workers have applied this technology for the optimization of culture conditions (Chen 1996; Rao *et al*., 1993). Recent research efforts have focused on medium optimization and scale up for enzyme production. The medium parameters play a vital role in enhancing the enzyme production; the determination of optimal values for processing parameters such as pH, temperature, aeration (Harris *et al*., 1990), feeding rates (Bazaraa & Hassan 1996) etc. Traditionally medium optimization is done by 'one-factor-at-a-time' technique (Gokhade, 1991). Single variable optimization methods are tedious and may lead to misinterpretation of results, especially if the interaction effects between factors are overlooked (Wenster-Botz, 2000). This method is not only time consuming but also laborious and also often leads to incomplete understanding of the system behaviour, and resulting in confusion and lack of ability to predict the results.

Limitations and drawbacks of the single factor optimization can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the factors in the fermentation process (Elibol, 2004). Response surface methodology not only deals with experimental strategies but also deals with mathematical methods and statistical inference for constructing and exploring an appropriate functional relationship between process variables and set of design variables. Basically this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model. Using the mathematical model the levels of the variables giving the maximum response can then calculated (Maddox and Richert, 1977). Statistical methods have been applied for optimization of enzyme production (Dey *et al.*, 2001; Francis *et al.,* 2002; Ahuja *et al*., 2004; Kunamaneni *et al*., 2005). No defined medium has been established for the optimum production of enzymes from different microbial sources. Each organism has its own specific conditions for enzyme production. The use of reliable statistical approach is essential to develop better strategies for the optimization of fermentation process (Ghaly *et al*., 2005).

Serratiopeptidase (E.C.3.4.24.40) belongs to a group of alkaline extracellular metalloprotease which hydrolyses specifically insulin B chain (Morihara *et al*., 1968). Serratiopeptidase production is influenced by media components, especially carbon and nitrogen sources and physical factors such as pH, temperature, inoculum level and incubation time. Response surface methodology has been used to enhance enzyme production by optimizing the seed and induction conditions by *S.marcesens* (Venil 2009).

In the present study, a new actinomycete strain, *Streptomyces hydrogenans* MGS13 has been subjected to special conditions using response surface methodology for enhancing the Serratiopeptidase production with optimum medium factors and conditions.

Materials and methods:

Actinomycetes strain and growth conditions

The novel actinomycete strain *Streptomyces hydrogenans* MGS13 was isolated from Koringa mangrove forest soil sediments and the culture was maintained at 4°C and subcultured monthly. Nutrient medium containing (g/L): soya bean meal- 13.5g; glucose – 13.5; glycerol -2.44mL; $CaCO_3$– 0.9 g; tryptone -15.6 g; KH_2PO_4 – 2.24 g; was prepared for the production of Serratiopeptidase from *Streptomyces hydrogenans* MGS13. The pH of the medium was adjusted to 7.0 with 1N HCl or 1N NaOH and was autoclaved at 121°C for 15 minutes.

Production of Serratiopeptidase enzyme:

50 mL of nutrient broth was inoculated with 10% inoculum and was incubated at 28°C for 4 days. After incubation the crude enzyme was recovered by centrifugation of the culture broth at 8000 ×g for 10 minutes at 4°C. The cell free supernatant was assayed for Serratiopeptidase activity.

Serratiopeptidase assay:

Assay was done as per the procedure of IP 2010; one unit of Serratiopeptidase is defined as the amount of enzyme required to liberate one µm of free tyrosine per minute under the specified assay conditions.

Optimization by response surface methodology:

Response surface methodology is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multi variable equations simultaneously. The medium for maximum Serratiopeptidase production has been first optimized by 'one-variable-at-a-time' approach. The medium composition that resulted in the highest enzyme titre was considered as the basal medium and used for optimization by response surface methodology (RSM) using Central Composite Design (CCD) (Box and Wilson, 1951; Box and Hunter, 1957).

According to this design, the total number of experimental combinations is $2^K + 2K + n0$, where '*K*' is the number of independent variables and *n*0 is the number of repetitions of the experiments at the center

point. For statistical calculation, the variables X_i have been coded as x_i according to the following transformation:

$$x_i = X_i - X_0 / \delta X$$

Where x_i is dimensionless coded value of the variables X_i, X_0 the value of the X_i at the center point, and δX is the step change. The levels of four independent variables, dextrose (D), soya bean meal(S), pH (P) and inoculum level (I) chosen for this study were optimized by the experimental plan.

Table 1: Levels of the four components used in the Central Composite Design

Variable	Medium parameter	Level of the component				
		-2	**-1**	**0**	**1**	**2**
D	Dextrose (%w/v)	0.5	1.0	1.5	2.0	2.5
S	Soya bean meal (%w/v)	0.5	1.0	1.5	2.0	2.5
P	pH	5.0	6.0	7.0	8.0	9.0
I	Inoculum level (%v/v)	5	7.5	10	12.5	15

The statistical software package 'DESIGN-EXPERT®9.0' Trial version, Stat-Ease, Inc., Minneapolis, USA was used for analyzing the experimental design. Each factor in the design was studied at five different levels (-2, -1, 0, +1, +2) as shown in Table 1.

A 2^K –factorial design with axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components. All the variables were taken at a central coded value considered as zero. The minimum and maximum levels of variables were investigated and the full experimental plan with respect to their values in actual and coded form is listed in Table 2. Upon completion of experiments, the average maximum Serratiopeptidase production was taken as the dependent variable or response(Y). A second order polynomial equation was then fitted to the data by a multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a four factor system the model equation is,

$$Y=\beta_o +\beta_1 A +\beta_2 B+\beta_3 C+\beta_4 D+\beta_{11} A^2+\beta_{22} B^2+\beta_{33} C^2+\beta_{44} D^2+\beta_{12} AB+\beta_{13} AC+\beta_{14} AD +\beta_{23} BC+\beta_{24} BD +\beta_{34} CD$$

Where Y, predicted response; β_o, intercept; $\beta_1, \beta_2, \beta_3, \beta_4$, linear coefficients, $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}$, squared coefficients; $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$, interaction coefficients.

Design Expert Software, using the above model to optimize experimental components, was used to generate response surface graphs

Table 2: Experimental and predicted values of Serratiopeptidase yield recorded in the experimental setup of RSM

Std	Run	D	S	P	I	Serratiopeptidase activity (IU/mL)	
						Actual	**Predicted**
1	20	-1	-1	-1	-1	54.21	59.212
2	21	1	-1	-1	-1	67.2	83.035
3	16	-1	1	-1	-1	79.26	95.351
4	12	1	1	-1	-1	111.89	124.009
5	27	-1	-1	1	-1	54.87	53.131
6	23	1	-1	1	-1	94.76	118.334
7	10	-1	1	1	-1	59.45	74.925
8	18	1	1	1	-1	142.67	144.963
9	22	-1	-1	-1	1	56.32	63.001
10	1	1	-1	-1	1	89.34	102.304
11	7	-1	1	-1	1	163.31	168.175
12	24	1	1	-1	1	201.60	212.313
13	3	-1	-1	1	1	85.42	101.740
14	11	1	-1	1	1	189.54	182.423
15	15	-1	1	1	1	199.43	192.570
16	2	1	1	1	1	254.65	278.087
17	6	-2	0	0	0	56.32	47.108
18	9	2	0	0	0	184.65	156.448
19	30	0	-2	0	0	66.21	49.157
20	8	0	2	0	0	201.32	180.960
21	14	0	0	-2	0	134.87	111.442
22	26	0	0	2	0	185.12	171.135
23	13	0	0	0	-2	112.43	86.812
24	19	0	0	0	2	235.52	223.725
25	28	0	0	0	0	210.43	219.038
26	5	0	0	0	0	221.23	219.038
27	29	0	0	0	0	220.54	219.038
28	25	0	0	0	0	219.93	219.038
29	17	0	0	0	0	221.12	219.038
30	4	0	0	0	0	220.98	219.038

Table 3:ANOVA for Response Surface Quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	1.294E+005	14	9245.54	23.25	< 0.0001	significant
A-D	*17932.85*	*1*	*17932.85*	*45.09*	*< 0.0001*	
B-S	*26058.18*	*1*	*26058.18*	*65.53*	*< 0.0001*	
C-pH	*5344.94*	*1*	*5344.94*	*13.44*	*0.0023*	
D-I	*28117.89*	*1*	*28117.89*	*70.71*	*< 0.0001*	
AB	*23.38*	*1*	*23.38*	*0.059*	*0.8117*	
AC	*1712.30*	*1*	*1712.30*	*4.31*	*0.0556*	
AD	*239.63*	*1*	*239.63*	*0.60*	*0.4497*	
BC	*205.78*	*1*	*205.78*	*0.52*	*0.4830*	
BD	*4765.83*	*1*	*4765.83*	*11.98*	*0.0035*	
CD	*2008.83*	*1*	*2008.83*	*5.05*	*0.0401*	
A^2	*23571.27*	*1*	*23571.27*	*59.27*	*< 0.0001*	
B^2	*18534.58*	*1*	*18534.58*	*46.61*	*< 0.0001*	
C^2	*10362.96*	*1*	*10362.96*	*26.06*	*0.0001*	
D^2	*6971.34*	*1*	*6971.34*	*17.53*	*0.0008*	
Residual	5965.03	15	397.67			
Lack of Fit	*5874.97*	*10*	*587.50*	*32.62*	*0.0006*	*significant*
Pure Error	*90.06*	*5*	*18.01*			
Cor Total	1.354E+005	29				

Results

The results of CCD experiments for studying the effects of four independent variables, dextrose, soya bean meal, pH and inoculum level are presented in the Table 2 along with the mean predicted and observed response. The regression equations obtained after the analysis of variance (ANOVA) give the level of Serratiopeptidase produced as a function of the initial values of dextrose, soya bean meal, pH and inoculum level.

The results obtained were subjected to analysis of variance on Stat-Ease package, with the regression model, for the prediction of Serratiopeptidase production.

Final equation in terms of actual factors:

Y = + 219.038 + 27.335*D + 32.95*S +14.92*P + 34.22*I + 1.208*D*S + 10.345*D * P + 3.8700* D * I - 3.58625* S * P + 17.2597* S * I + 11.20500* P * I - 29.31500* D^2 - 19.43* P^2 - 15.94* I^2

Where Y, enzyme production (IU/mL); D, dextrose concentration (%w/v); S, soya bean meal concentration (%w/v); P, pH; I, inoculum level (%v/v).

The coefficient of determination (R^2) was calculated to be 0.9559 for Serratiopeptidase production. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.00, the stronger the model is and the better it predicts the response (Haaland 1989). When expressed as a percentage, R^2 is interpreted as the percent variability in the response explained by the statistical model. This implied that the sample variation of 95.59% for Serratiopeptidase production was attributed to the independent variables and only 4.41% of the total variation was not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data. The purpose of statistical analysis is to determine which experimental factors generate signals, which are large in comparison to the noise. Adequate precision measures signal to noise ratio (Haaland, 1989). A ratio greater than 4 is desirable. An adequate precision of 16.380 for Serratiopeptidase production indicated for adequate signal. The predicted R^2 of 0.7491 for Serratiopeptidase yield is in reasonable agreement with the Adjusted R^2 of 0.9148. This indicated a good agreement between the experimental and predicted values for the Serratiopeptidase production. The adjusted R^2 corrects the R^2 value for the sample size and for the number of terms in the model. 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. This should be a caution signal that too many terms are present in the model (Haaland, 1989). In this case the adjusted R^2 was very close to the R^2 value. The coefficients of regression equation were calculated using Design Expert.

The model F-value 23.25 for Serratiopeptidase production implied that the model is significant as shown in Table 3. Values of 'Prob> F' less than 0.0500 indicated that the model terms are significant. For Serratiopeptidase production A, B, C, D, BD, CD, A^2, B^2, C^2, D^2 are significant model terms, A being dextrose concentration (%), B being soya bean meal (%), C being pH and D being inoculum level (%) respectively. The 'Lack of Fit F-value' of 32.62 for Serratiopeptidase production, implied the lack of fit is significant.

The three dimensional response surface graphs were plotted by statistically significant model to understand the interaction of the medium components and their optimum values required for maximum Serratiopeptidase production. Analysis of variance (ANOVA) showed that the factor DS, DP, DI, SP were insignificant, and D (dextrose concentration), S (soya bean meal), p(pH) and I (inoculum level), SI, PI, D^2, S^2, P^2, and I^2

were significant model terms. The interactive effect of four variables at constant & two variables at zero level are depicted in below figures.

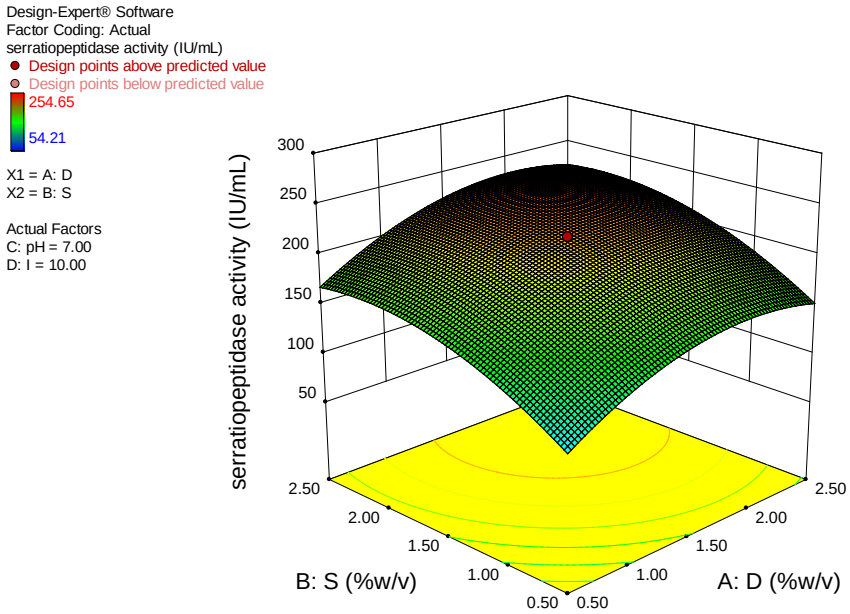

Fig. 1: Response surface graph for Serratiopeptidase production showing interaction between dextrose and soya bean meal, at constant pH and inoculum level at zero.

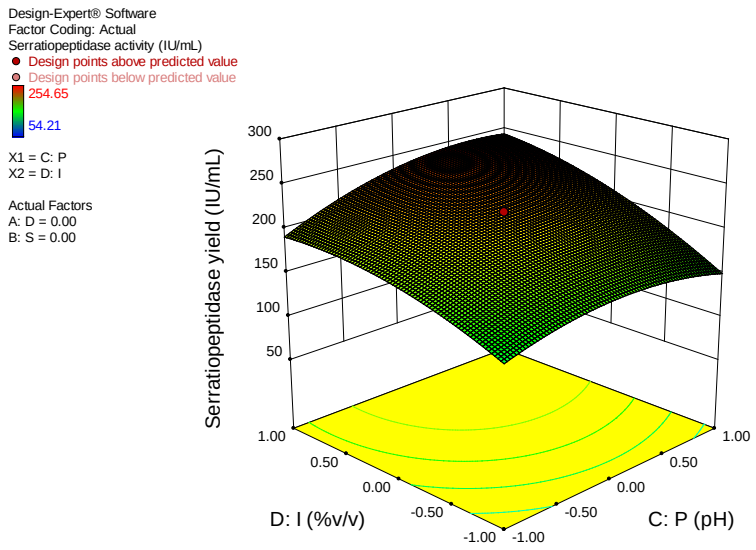

Fig. 2: Response surface graph for Serratiopeptidase production showing interaction between pH and inoculum level, at constant dextrose and soya bean meal concentration.

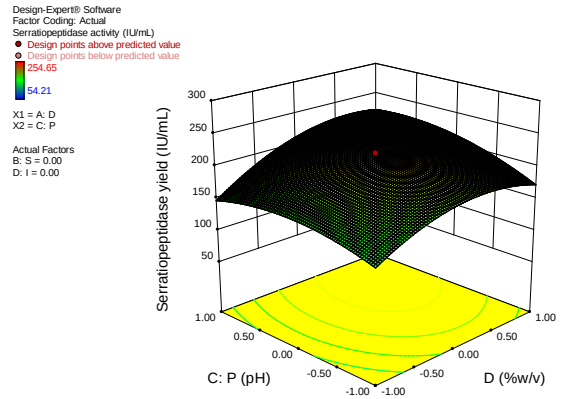

Fig. 3: Response surface graph for Serratiopeptidase production showing interaction between dextrose and pH, at constant inoculum level and soya bean meal concentration.

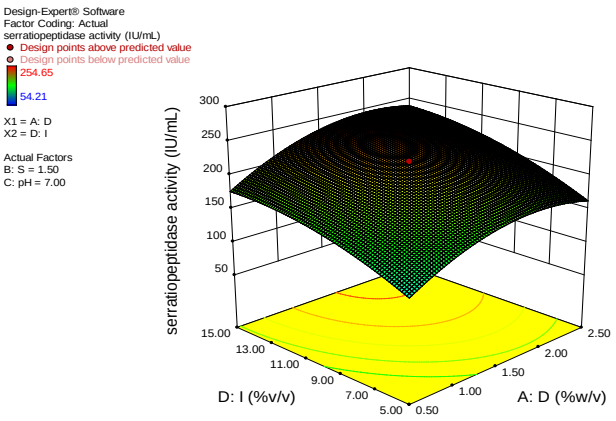

Fig. 4: Response surface graph for Serratiopeptidase production showing interaction between dextrose and inoculum level, at constant pH and soya bean meal concentration.

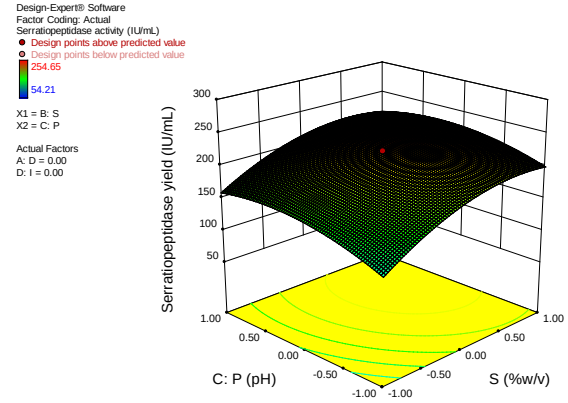

Fig. 5: Response surface graph for Serratiopeptidase production showing interaction between soya bean meal and pH, at constant inoculum level and dextrose concentration.

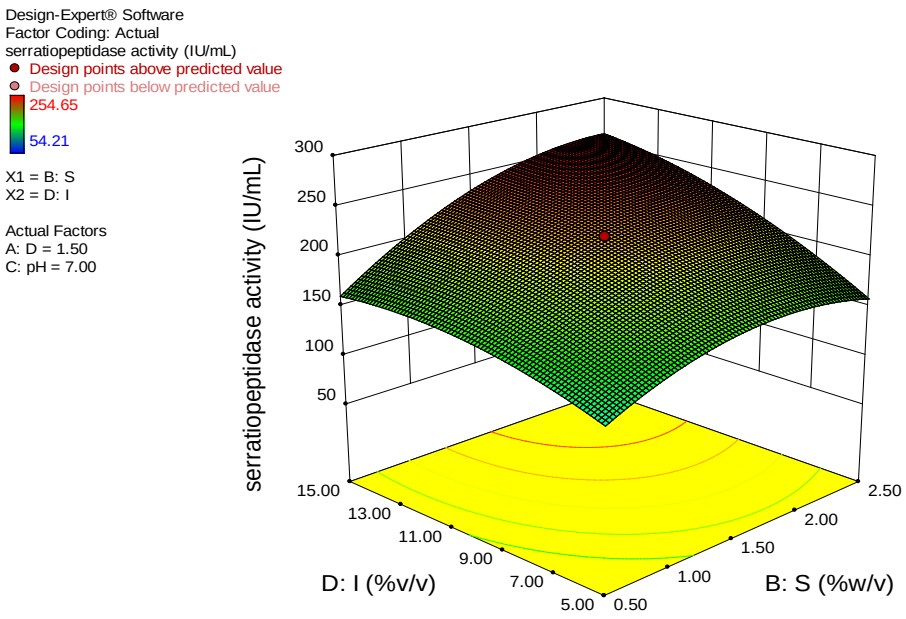

Fig. 6: Response surface graph for Serratiopeptidase production showing interaction between soya bean meal and inoculum level, at constant pH and dextrose concentration.

Fig.1 shows the response plot obtained as a function of dextrose *vs* soya bean meal, while all other variables are maintained at zero level. An increase in Serratiopeptidase yield by 235.67 IU/ml was observed at 2.09 (%w/v) and 2.07 (%w/v) concentrations of dextrose and soya bean meal respectively. **Fig. 2** shows the response plot obtained as a function of pH *vs* inoculum level, while all other variables are maintained at zero level. An increase in Serratiopeptidase yield by 245.5 IU/ml was observed at pH 8.5 and inoculum level at 14.5% concentration. **Fig. 3** shows the response plot obtained as a function of dextrose *vs* pH, while all other variables are maintained at zero level. An increase in Serratiopeptidase yield by 235.155 IU/mL was observed at pH 8.2 and dextrose concentration at 2.06 (%w/v).**Fig. 4** shows the response plot obtained as a function of dextrose *vs* inoculum level, while all other variables are maintained at zero level. An increase in Serratiopeptidase yield by 241.055 IU/mL was observed at dextrose concentration at 2.03 (%w/v) and 14.5% inoculum level. **Fig. 5** shows the response plot obtained as a function of dextrose *vs* pH, while all other variables are maintained at zero level. An increase in Serratiopeptidase yield by 235.155 IU/mL was observed at pH 8.2 and dextrose concentration at 2.06 (%w/v). **Fig. 6** showed the response plot obtained as a function of soya bean meal *vs* inoculum level, while all other variables are maintained at

zero level. An increase in Serratiopeptidase yield by 259.91 IU/mL was observed at 14.5% inoculum level and soya bean meal concentration at 2.20 (%w/v).

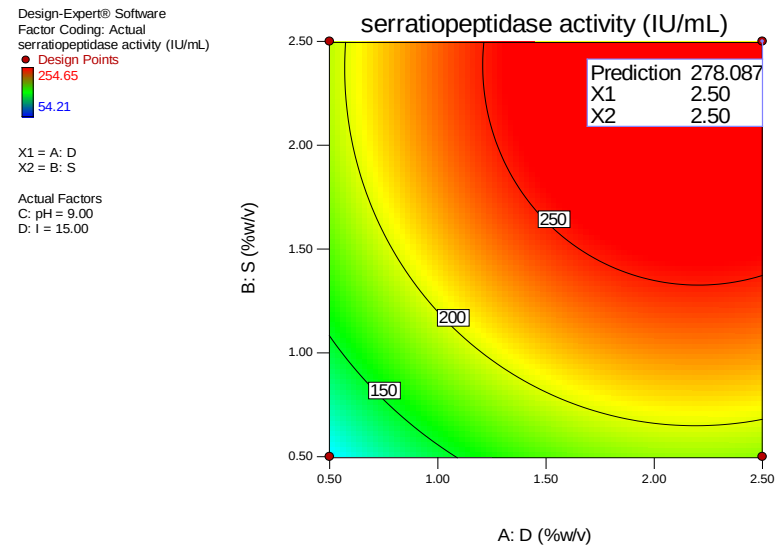

Fig. 7: Contour plot showing the maximum Serratiopeptidase yield at optimum values of the various variables by *Streptomyces hydrogenans*MGS13.

The maximum experimental response for Serratiopeptidase production was obtained as 254.65 IU/mL whereas the predicted value is 278.087 IU/mL indicating the strong agreement between them. The optimum values of the tested variables are when the dextrose concentration was 2.5%, Soya bean meal 2.5%, pH 9.0 and inoculum level 15%. Increasing the dextrose concentration to 4% led to a slight decline in Serratiopeptidase production, however on increasing the concentration of soya bean meal beyond (4%w/v), Serratiopeptidase production declined significantly and decline of Serratiopeptidase yield was observed when pH less than 7.0 and inoculum level less than 10%. This is very clear from the one factor plot of varying dextrose concentration with constant soya bean meal and vice versa. At constant dextrose concentration and soya bean meal, varied concentrations of pH and inoculum level on Serratiopeptidase production influenced significantly. The model was also validated by repeating the experiments under the optimized conditions, which resulted in the Serratiopeptidase production of 254.65 IU/mL (predicted response- 278.087 IU/mL), thus providing the validity of the model. Similar response surface methodology has been used to optimize Serratiopeptidase production value by 279.05 IU/mL by *Serratia marcescens* SB08 was observed at a

concentration of yeast extract 3 g/L, pH 6.0, incubation time 51.0 h and agitation 100 rpm (Venil CK, 2009).

Discussion

Numerous studies were carried by various researchers to optimize the production of various microbial secondary metabolites by applying response surface methodology. The response surface methodology, a smaller and less time consuming experimental design, could generally satisfy the optimization of many microbial processes. Central Composite Design, a response surface methodology maximizes the amount of information that can be obtained, while considering the interaction of independent variables and limiting the numbers of individual experiments required (Chauhan and Gupta, 2004; Elibol, 2004; Abdel-Fattah *et al.,* 2005). This study is an attempt that has demonstrated the application of a multifactorial statistical approach for determining the fermentation conditions that lead to the maximum yield of Serratiopeptidase production from *Streptomyces hydrogenans* MGS13, a novel isolate of actinomycete strain producing Serratiopeptidase enzyme.

By applying response surface methodology, in Serratiopeptidase production by *Streptomyces hydrogenans* MGS13, optimum pH, inoculum level, dextrose and soya bean meal concentrations are found to be positive factors playing significant role. In this study, maximum Serratiopeptidase production of 254.65, when dextrose and soya bean meal concentrations at 2.04 (%w/v) and 2.09 (%w/v) was supplemented at optimum levels. Production medium contains complex nutrients such as vitamins, lipids and other substances which might be necessary for growth and production of secondary metabolites. Soya bean meal and dextrose are the key nutrient materials which controls the biosynthesis of the Serratiopeptidase enzyme. This fact has also been suggested previously other enzyme production experiments on nitrogen repression effects (Crueger and Crueger, 1984; Frankena *et al*., 1986; Kole *et al*; 1988; Giesecke *et al*., 1991). Similar studies have been conducted on modelling to study pH and dextrose concentration on other enzyme production experiments (Tijskens *et al*., 2001; Vohra and Satyanarayana, 2002).

Validation of the model

A validation of the model and regression equation was done by taking D (2.04%w/v), S (2.09%w/v), P (pH 8.0) and I (12.5%) in the experiment. The predicted response for Serratiopeptidase was 278.087 IU/mL and the actual response was 254.65 IU/mL, which thus proves the validity.

Conclusion

This study using the method of factorial design and response surface analysis; it was possible to determine optimal cultural medium conditions to improve Serratiopeptidase yield. Response surface graphs and contour plots are very helpful in visualizing the main effects and interaction of effects. The optimum operational conditions obtained in this experiment give a basis for further study with batch or fed-batch cultivation in a bioreactor for scale up production of targeted secondary metabolite from *Streptomyces hydrogenans* MGS13. Thus, a multifactorial statistical approach that considers interaction of variables provided a basis for the model to search for a non-linear nature of the response in a short term experiment.

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