Statistical Optimization of Keratinase Enzyme Production from Streptomyces *Griseus NCIM-2622*

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Abstract

The Microorganism Streptomyces griseus NCIM -2622 was selected to study the effect of medium components enhancing keratinase production. These parameters were statistically optimized in two steps: Preliminary screening by Plackett– Burman design to select the most significant nutrients such as $FeSO_4.7H_20$, Yeast extract, NaCl and NH_4Cl and the second level optimization by Response Surface Methodology.

To study the responses from the experimental design and the effects of each variable were calculated and the interaction between the four factors and the interaction effect on enzyme production were clearly demonstrated by 3D contour plots. The final optimized medium provided a maximum yield of 66,92 IU / ml of keratinase at 72 h of incubation.

Keywords: Keratinase, Optimization, Contour plots, Response Surface Methodology, Plackett– Burman design.

Introduction

Keratinases are the versatile extraordinary proteases having capability to hydrolyze insoluble; hard to degrade keratin and keratinous waste obtained from poultry and leather industries[1] into valuable products like rare amino acids like serine, cysteine and proline[2], peptides, fertilizers, glues, films, foils etc.[3] It is also used in detergent formulations for the elimination of horny epithelial cells adhering to the pharmaceutical and cosmetic textile fiber industries and become an alternative resourceful low-cost, ecofreindly material for biotechnological applications[4,5]. Microorganisms generally produce keratinases when they act on the keratin substrate in nature[6]. Various studies have been reported including keratinases from fungi Microsporum[7], Trichophyton[8] as well as from bacteria like *Bacillus Fervidobacterium*, *Bacillus licheniformis*, *Bacillus pumilus*[9], *Chryseobacterium sp.*, *Streptomyces*[10].

Bacillus species are the predominant one. Nurturing conditions are crucial for successful enzyme production, physicochemical parameters such as pH, temperature and media compositions are imperative in developing this process. Medium optimization by the old method is immensely time consuming and costly when a large number of variables are examined; it involves changing one independent variable while all others variables remain constant at a fixed level[2]. To overcome this complexity, the methodology of experimental factorial design and response surface may be used to optimize the medium components. The statistical approaches like Placket Burman method and central composite design have been previously used by several researchers for media ingredient optimization.

Material and Methods

Keratinase Production and Enzyme assay: The basic media used to stimulate the production of keratinase consists of glucose 1%, peptone 1%, KH_2PO_4 0.9%, K_2HPO_4 0.3% Feather 0.5%. This production media was inoculated with 1% of *Streptomyces griseus-NCIM-2622* culture and incubated at 37 ˚C at 180 rpm for 3days. Keratinase activity was determined by using the cell free extract of chicken feather powder as substrate. In the assay conditions, an increase of 0.01 absorbance was considered as 1 unit of keratinase per ml in 1 hour at 280nm.

Screening of Variables by Plackett Burman Design: Keratinase production by *Streptomyces griseus NCIM-2622* was optimized by statistical design experiments in two steps (Design Expert® 8.0.2.0 Stat-Ease, Inc., Minneapolis, MN, USA). In Plackett–Burman Design eleven variables namely concentrations of eleven nutrients (Yeast extract, ammonium chloride, potassium di-hydrogen phosphate, sodium chloride, potassium chloride, magnesium sulphate, ferrous sulphate, peptone, sodium nitrate, urea and di-potassium hydrogen phosphate) were used.

Plackett–Burman Design does not consider the interaction effects among the variables and it was used to study the significance of process variables. The software for PBD is Minitab-16, this software is widely used for PBD and RSM in bioprocesses optimization because of its convenience and power in statistical analysis and experimentation. The experiments were repeated thrice and the mean values of production of enzymatic hydrolysis of substrate were taken as response. After fixing the minimum and maximum values of nutrient concentrations, a set of 12 experiments was conducted as designed by Plackett–Burman Design.

Results and Discussion

Optimization of Keratinase Production for the Selected Nutrients by Response Surface Methodology using *S.griseus* with GFP as Substrate: Plackett Burman design was used to identify the most important components which affect the production of keratinase enzymes. The experimental designs and the results obtained for the twelve experiments with the eleven components using P.B design are given in table 1. The twelve run of experimental Plackett-

Burman design showed a wide variation in keratinase activity from 14.66 IU / ml to 27.99 IU / ml which reflected the importance of optimization in achieving the higher yield. The results are shown in Pareto chart of fig. 3. The variables $FeSO_4$, yeast extract, NaCl and NH_4Cl were found to be the most significant components affecting the production of keratinase from the Pareto chart and were considered for further optimisation using CCD.

Response surface methodology (RSM) through central composite design (CCD) was used to further optimize the selected nutrients for the production of keratinase enzyme by submerged fermentation using *S. griseus* NCIM (2622). All of the insignificant variables from the PB design are neglected and the optimal values of the four most significant variables are used to further optimize using RSM CCD to obtain maximum production of keratinase.

For this study, 2^4 full factorial central composite design with sixteen star points, eight axial points and six replicates at the centre points to estimate the experimental error and to have a satisfactory orthogonality for coefficient estimation (all factors at level 0) were employed to fit the second order polynomial model which indicated that 30 experiments were required for this procedure. The coded and actual values of the significant variables are shown in table.2. The predicted and observed responses along with design matrix are presented in table 3. and the results were analysed by ANOVA. The following second order polynomial equation was obtained which describes the correlation between keratinase and the four variables.

$$Y = 66.92 - 0.11\ A + 0.78b + 0.80\ C + 2.52\ D - 2.61\ AB - 0.61\ AC - 1.33\ AD - 0.61\ BC - 0.50\ BD + 0.20\ CD - 3.63\ A^2 - 3.21\ B^2 - 5.71\ C^2 - 7.88\ D^2 \quad (1)$$

where Y is the keratinase activity (IU/ml) respectively and A, B, C and D are $FeSO_4.7H_2O$, yeast extract, NaCl and NH_4Cl respectively. The ANOVA for the response surface is shown in table 4. The independent variables are fitted to the equation of the second order model and examined for fitness goodness. F-test verifies the statistical significance of equation. The results demonstrated that the model is highly significant and is evident from Fischer's F-test with a low probability value (P model >F less than 0.05).

For keratinase the model F-value of 114.41 implies that the model is significant. Such a large F-value could occur due to noise and only 0.01 per cent is likely to get such a high F-value. Values of Prob>F greater than 0.1 indicate that the model terms are not significant. The linear effects, interactive effects and squared effects in the present work are significant model terms for the production of keratinase.

The fit of the model was checked by the coefficient of determination R^2. The coefficient of determination R^2 for keratinase was calculated as R^2= 0.9907 which is almost equal to 1 indicating that the model could explain 99.07 percent variability of the response and that the model could not explain only about 0.93 percent of the total variation.

This shows that the model was very much suitable for keratinase production using *Streptomyces griseus NCIM-2622* by SmF. The predicted R^2 value of keratinase activity was found to be 0.9466 and was in reasonable agreement with the adjusted R^2 value of 0.9821. Normally a regression model having an R^2 value higher than 0.90 is considered to have a very high correlation[11].

The interactive effects of these variables on keratinase production are studied by plotting the 3D response surfaces with the vertical axis representing enzyme activity (response) and two horizontal axis representing the coded values of two independent variables while keeping other variables at their constant level. The three dimensional response surface curves of the calculated response (keratinase production) with the interactions between the variables are shown in figs. 2 to 7**.**

Fig. 2 to 4 show the effect of $FeSO_4.7H_2O$ on keratinase production. Keratinase activity increases with an increase in urea concentration up to a concentration of 0.399 per cent w / v $FeSO_4.7H_2O$ and thereafter the enzyme activity decreases with an increase in the concentration of $FeSO_4.7H_2O$. The metal ion Fe^{3+} stimulated enzyme production. However, the effect of transition metal ions depended on the type keratinase [12].The same trend has been observed in the study of the effect of yeast extract on the production of keratinase in SmF.

The results are shown in figures 3 to 6. As yeast extract concentration was increased from 0.20 % w/v to 0.5015 % w/v, the keratinase activity was found to increase and the maximum activity of the enzyme was found at a concentration of0.5015 % w/v of yeast extract. For further increase in the concentration of yeast extract , the keratinase activity was found to decrease.

Hence 0.5015 % w/v of the yeast extract concentration was found to be the optimum concentration and was used for further studies. Maximum keratin hydrolyzing activity was in the presence of 2% yeast extract concentrations. Because higher concentration of yeast extract provides required concentrations of aminoacids, proteins and vitamins are essential for improved cell growth and synthesis of enzymes such as proteases[13].

Figures 3, 5 and 7 show the effect of NaCl on keratinase activity. As NaCl concentration was increased from 0.01 % w/v to 0.11015 % w/v, the keratinase activity was found to increase and the maximum activity of the enzyme was found at a concentration of 0.11015% w/vof NaCl. Previous studies have also indicated a requirement for high concentration of sodium ions observed for alkaline protease production[14].

The effect of NH_4Cl on keratinase production was shown in the figures 3, 6 and 7. As the concentration of NH_4Cl increased from 0.40 per cent w / v to 0.804 per cent w / v, keratinase activity increased and maximum enzyme activity was found at a concentration of 0.804 per cent w / v of NH_4Cl. On further increase in the concentration of NH4Cl, keratinase activity decreased. Ammonium chloride was found to support the maximum production of keratinase in *Bacillus pumilus* FH915.

The maximum expected yield was indicated by the limited surface in the surface response plot and the optimum values were achieved by the solution of the second order polynomial equation. The optimum concentrations of the four independent variables in the encoded units are A (-0.1010), B (0.1414), C (0.0606) and D (0.1818) and the corresponding uncoded values for maximum keratinase production are A- (0.399 per cent w / v), B- extract (0.5015 per cent w / v), C- (0.11015 per cent w / v) and D-NH_4Cl (0.804 per cent w / v). The predicted values of the regression equation are in close agreement with those of the experimental values. Fig. 8 shows that the experimental keratinase activity values were well aligned with the expected response values.

Experimental Validation of the Model: Experimental validation of the model shall be tested by performing a batch experiment under optimal conditions of operation. The experiments are repeated three times and the results are compared. The keratinase activity 67.123 IU / ml from the experiments was very close to the actual response 66.92 IU / ml predicted by the regression analysis which demonstrated the validity of the model.

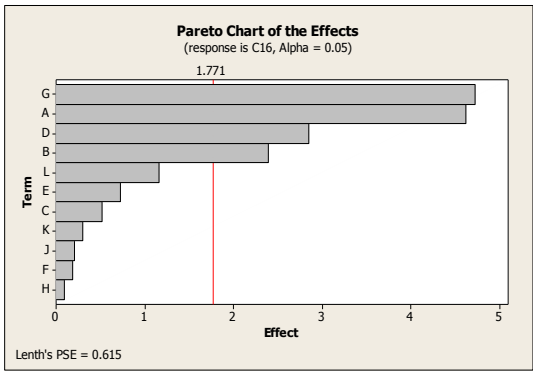

Fig. 1: Pareto graph showing the effect of the media components on the activity of keratinase

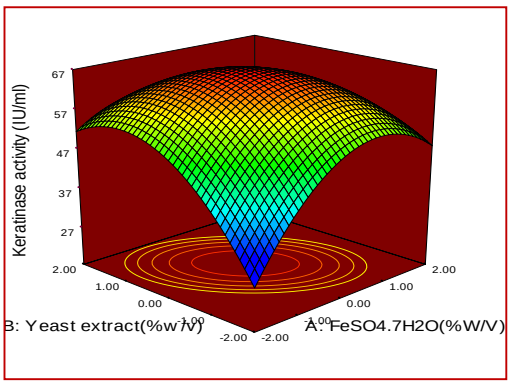

Fig. 2: Interactive effect of yeast extract and $FeSO_4.7H_2O$ on the activity of keratinase

Table 1
Plackett-Burman experimental design matrix for the screening of important variables for the production of keratinase using *S. Grayus*

A	B	C	D	E	F	G	H	J	K	L	Keratinase activity(IU/ml)
-1	-1	1	1	1	-1	1	1	-1	1	-1	25.98
-1	1	1	-1	1	-1	-1	-1	1	1	1	22.99
1	-1	-1	-1	1	1	1	-1	1	1	-1	23.99
-1	1	1	1	-1	1	1	-1	1	-1	-1	24.31
1	1	-1	1	1	-1	1	-1	-1	-1	1	19.22
-1	-1	-1	1	1	1	-1	1	1	-1	1	22
-1	1	-1	-1	-1	1	1	1	-1	1	1	27.99
1	-1	1	-1	-1	-1	1	1	1	-1	1	26
-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	23.92
1	-1	1	1	-1	1	-1	-1	-1	1	1	18.63
1	1	1	-1	1	1	-1	1	-1	-1	-1	16.99
1	1	-1	1	-1	-1	-1	1	1	1	-1	14.66

Table 2
Ranges and levels of independent variables used in RSM for the production of keratinase enzyme

S.N.	Variables (%w/v)	Code	Levels				
			-2	-1	0	+1	+2
1.	$Feso_4.7H_20$	**A**	0.2	0.3	0.4	0.5	0.6
2.	Yeast extract	**B**	0.2	0.35	0.5	0.65	0.8
3.	NaCl	**C**	0.01	0.06	0.11	0.16	0.21
4.	NH_4Cl	**D**	0.4	0.6	0.8	1.0	1.2

Table 3
Experimental conditions (coded values) and observed response values of 2^4 central composite design for keratinase enzyme production

Run No	Independent Variables				Keratinase Activity(IU/ml)	
	A	B	C	D	Exp.	Pred.
1	0	0	-2	0	43.646	42.4983
2	-1	1	-1	-1	44.739	46.0252
3	1	-1	-1	-1	45.000	45.9296
4	1	1	1	-1	42.500	43.2347
5	-1	1	-1	1	51.300	52.3371
6	0	0	0	-2	32.063	30.3568
7	-1	-1	1	1	49.000	49.7770
8	0	0	0	2	41.384	40.4523
9	-1	-1	-1	-1	37.000	37.0320
10	-1	-1	1	-1	39.000	40.6651
11	0	0	0	0	66.920	66.9200
12	0	0	0	0	66.920	66.9200
13	1	-1	-1	1	47.988	48.9205
14	-2	0	0	0	54.330	52.6023
15	1	-1	1	-1	46.770	47.1160
16	0	0	0	0	66.920	66.9200
17	-1	-1	-1	1	44.831	45.3511
18	1	1	-1	-1	43.870	44.4761
19	1	1	-1	1	45.870	45.4598
20	0	-2	0	0	53.780	52.5136
21	0	2	0	0	56.990	55.6185
22	0	0	0	0	66.920	66.9200
23	0	0	0	0	66.920	66.9200
24	2	0	0	0	53.086	52.1758
25	1	1	1	1	43.660	45.0111
26	-1	1	1	-1	46.780	47.2306
27	0	0	0	0	66.920	66.9200
28	1	-1	1	1	50.931	50.8996
29	0	0	2	0	47.173	45.6828
30	0	0	0	0	66.920	66.9200

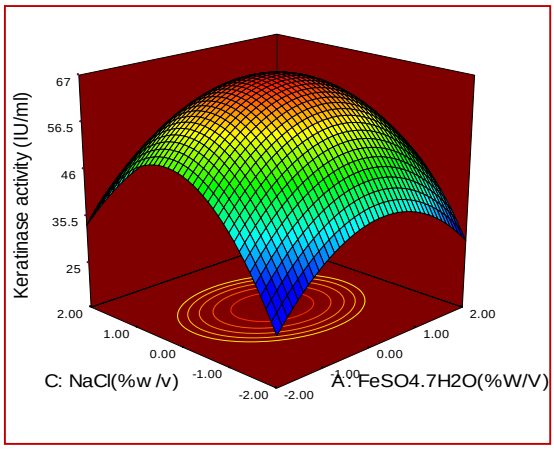

Fig. 3: Interactive effect of NaCl and $FeSO_4.7H_2O$ on keratinase activity

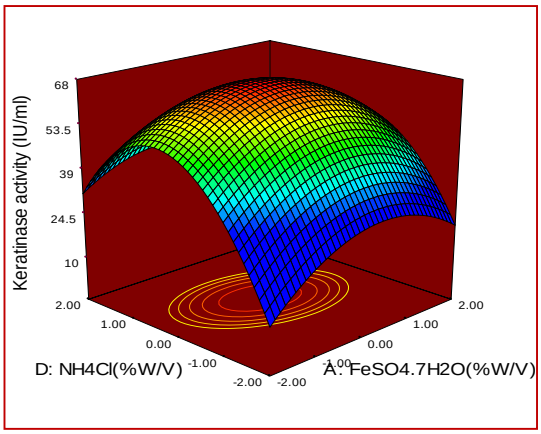

Fig. 4: Interactive effect of NH_4Cl and $FeSO_4.7H_2O$ on keratinase activity

Table 4
Analysis of variance (ANOVA) for quadratic surface response model

Source	Coeff Estimate	Sum of Squares	df	Mean Square	F Value	P value Prob> F
Model	66.92	2782.5	14	198.75	114.41	< 0.0001
A	-0.106625	0.27	1	0.27	0.16	0.6975
B	0.776208	14.46	1	14.46	8.32	0.0113
C	0.796125	15.21	1	15.21	8.76	0.0098
D	2.52388	152.88	1	152.88	88	< 0.0001
AB	-2.61169	109.13	1	109.13	62.82	< 0.0001
AC	-0.611687	5.99	1	5.99	3.45	0.0831
AD	-1.33206	28.39	1	28.39	16.34	0.0011
BC	-0.606938	5.89	1	5.89	3.39	0.0853
BD	-0.501813	4.03	1	4.03	2.32	0.1486
CD	0.198187	0.63	1	0.63	0.36	0.5565
A*A	-3.63274	361.97	1	361.97	208.37	< 0.0001
B*B	-3.21349	283.24	1	283.24	163.05	< 0.0001
C*C	-5.70736	893.46	1	893.46	514.32	< 0.0001
D*D	-7.87886	1702.67	1	1702.67	980.14	< 0.0001
Residual		26.06	15	1.74		
Lack of Fit		26.06	10	2.61		
Pure Error		0	5	0		
Corr Total		2808.55	29			
Std. Dev. – 1.32, R^2- 0.9907, Mean – 50.57, Adj R^2- 0.9821, C.V.% - 2.61, Pred R^2 - 0.9466, Adeq Precision – 39.232, PRESS – 150.09						

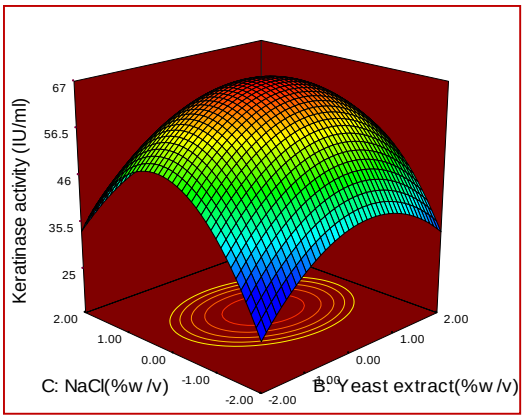

Fig. 5: Interactive effect of NaCl and yeast extract on keratinase activity

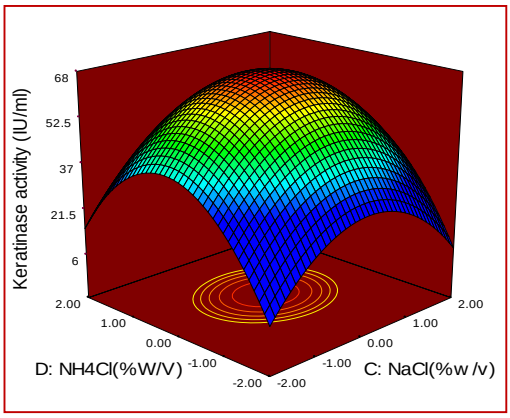

Fig. 7: Interactive effect of NH_4Cl and NaCl on keratinase activity

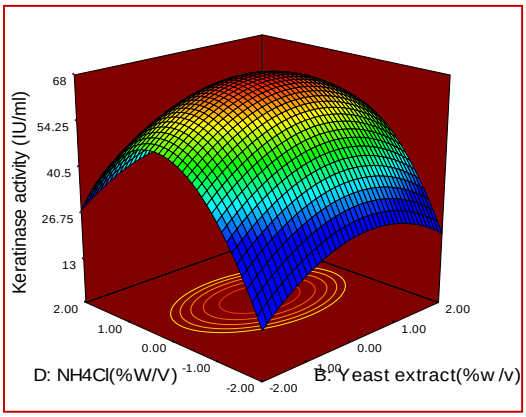

Fig. 6: Interactive effect of NH_4Cl and yeast extract on keratinase activity

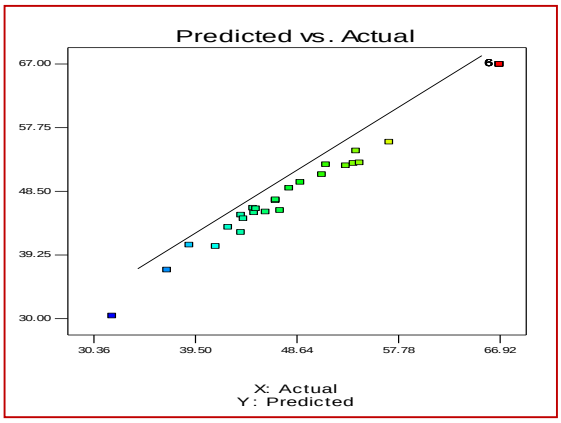

Fig. 8: Predicted Vs. Actual (Experimental) Value

Conclusion

The present study shows that the microorganism *Steptomyces griseus* NCIM-2622 is a potential producer of keratinase.The surface response methodology used in this study provides information on the growth requirements of the organism and also helps to predict optimized conditions for keratinase production. The optimum nutrient composition is A.

$FeSO_4.7H_2O$ (0.399 per cent w / v), B – yeast extract (0.5015 per cent w / v), C – NaCl (0.11015 per cent w / v) and D – NH_4Cl (0.804 per cent w / v). Maximum keratinase activity of 66,92 IU / ml was achieved at 72 hours of incubation.

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