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Optimization of Keratinase Production and Enzyme Activity Using Response Surface Methodology with Streptomyces sp7

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Abstract

A two-step response surface methodology (RSM) study was conducted for the optimization of keratinase production and enzyme activity from poultry feather by *Streptomyces* sp7. Initially different combinations of salts were screened for maximal production of keratinase at a constant pH of 6.5 and feather meal concentration of 5 g/L. A combination of K_2HPO_4, KH_2PO_4, and NaCl gave a maximum yield of keratinase (70.9 U/mL) production. In the first step of the RSM study, the selected five variables (feather meal, K_2HPO_4, KH_2PO_4, NaCl, and pH) were optimized by a 2^5 full-factorial rotatable central composite design (CCD) that resulted in 95 U/mL of keratinase production. The results of analysis of variance and regression of a second-order model showed that the linear effects of feather meal concentration ($p < 0.005$) and NaCl ($p < 0.029$) and the interactive effects of all variables were more significant and that values of the quadratic effects of feather meal ($p < 1.72e\text{-}5$), K_2HPO_4 ($p < 4.731e\text{-}6$), KH_2PO_4 ($p < 1.01e\text{-}10$), and pH (p 7.63e-7) were more significant than the linear and interactive effects of the process variables. In the second step, a 2^3 rotatable full-factorial CCD and response surface analysis were used for the selection of optimal process parameters (pH, temperature, and rpm) for keratinase enzyme activity. These optima were pH 11.0, 45°C, and 300 rpm.

Index Entries: Poultry feather; keratinase; response surface methodology; central composite design.

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Introduction

Feathers are produced in large amounts as byproducts of poultry-processing plants, reaching millions of tons per year worldwide. Poultry feather constitutes the most abundant keratinous material in nature. The main component of feather is keratin, a mechanically durable and chemically unreactive protein, which renders it difficult to digest by most proteolytic enzymes. Recycling of feathers is a subject of interest because it is a potentially cheap and alternative protein supplement for use in animal feed *(1)*. Development of simple enzymatic treatment methods will improve the nutritional value of keratin waste *(2)*. Thus, the bioconversion of poultry waste from a potent pollutant to a value-added product offers considerable opportunities for developing new products using microbial technology *(3)*. Keratinolytic activity has been reported for several species, such as *Aspergillus species; Rhizomucor* species *(4); Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton gallinae, Microsporum canis, Microsporum gypseum (5); Streptomyces (6–8); Vibrio (9); Microbacterium (10);* and *Bacillus (11).*

The composition of fermentation media plays an important role in the production of primary and secondary metabolites. Designing an appropriate fermentation medium is of critical importance because medium composition influences product concentration, yield, and volumetric productivity *(12)*. The conventional method of media optimization involves changing one variable at a time, keeping the others at fixed levels. Being single dimensional, this laborious and time-consuming method often does not guarantee determination of optimal conditions *(13-15)*. In addition, carrying out experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required. Unlike conventional optimization, statistical optimization methods can take into account the interactions of variables in generating process responses *(12)*. Response surface methodology (RSM) *(16)*, an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization *(13)*. This method has been successfully applied for media optimization in different fermentation processes *(17-19)* as well as for establishing the conditions of enzymatic hydrolysis *(20)*, and sulfuric acid production *(21)*.

To develop a process for the maximum production of keratinase from poultry feather, standardization of media components is crucial. We have isolated a species of *Streptomyces* that is capable of rapidly degrading native feather. The purpose of the present study was to evaluate and optimize various salt combinations by conventional method. The effect of these selected process variables on the production of keratinase by *Streptomyces* sp. using RSM was studied. Initially a 2^5 full-factorial central composite design (CCD) and RSM was used for optimization of medium components for the maximal production of keratinases. Then a 2^3 full-factorial CCD and RSM was used for the optimization of keratinase enzyme activity conditions.

Table 1
Different Salt Media Combinations Tested for Keratinase Production by *Streptomyces*[a]

Salt combination	K_2HPO_4 (0.3 g/L)	KH_2PO_4 (0.4 g/L)	NaCl (0.5 g/L)	$MgSO_4$ (0.3 g/L)	$(NH_4)_2SO_4$ (0.4 g/L)	$CaCO_3$ (0.2 g/L)
SMC-1	+	−	+	+	−	−
SMC-2	−	+	+	+	−	−
SMC-3	−	−	+	+	+	−
SMC-4	−	−	+	+	−	+
SMC-5	+	+	+	−	−	−
SMC-6	+	−	+	−	−	+
SMC-7	−	+	+	−	−	+
SMC-8	+	−	+	−	+	−
SMC-9	−	+	+	−	+	−
SMC-10	−	−	+	−	+	+

[a]SMC, salt medium composition; +, present; −, absent.

Material and Methods

Isolation and Preparation of Inoculum

Soil samples were collected from dump yards of Food Corporation of India, a slaughterhouse, and poultry farms. Colonies exhibiting protease activity were grown in a medium containing trace salts and feathers as carbon and nitrogen source. The culture was incubated for 5 d at 30°C, 150 rpm.

Screening of Different Combinations of Salts for Feather Degradation and Keratinase Production

A set of experiments was conducted randomly to screen the combination of mineral salts that gave a maximal concentration of keratinase. Various combinations of salts (K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4$, $[NH_4]_2SO_4$, $CaCO_3$) were tested with a constant 5 g/L of feather meal at pH 6.5 (Table 1). Samples were drawn from the flasks at the end of 4 d, and keratinase production was estimated.

RSM Studies

A two-step RSM study was conducted. In the first step (first experimental design), keratinase production from poultry feather by *Streptomyces*sp7 was optimized, and in the second step (second experimental design), keratinase enzyme activity conditions were optimized. The statistical software package STATISTICA 6.0 (Stat Soft) was used for regression analysis of experimental data. Analysis of variance (ANOVA) for regression analysis and to plot contour plots was used to estimate the statistical parameters.

Experimental Design and Optimization for Maximum Production of Keratinase (First Experimental Design)

In the first step of the RSM studies, the best salt media combination obtained previously was further used for optimization of production media. The production medium used for RSM studies consisted of 0.3 g/L of K_2HPO_4, 0.4 g/L of KH_2PO_4, 0.5 g/L of NaCl, and 5 g/L of feather meal, at pH 6.5. The levels of five medium variables (feather meal, K_2HPO_4, KH_2PO_4, NaCl, and pH) were selected and each of the variables was coded x_1, x_2, x_3, x_4, and x_5, respectively, at five levels (–2, –1, 0, 1, and 2) by using Eq. 1:

$$x_i = \frac{X_1 - X_0}{\Delta X_1} \tag{1}$$

in which x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value. Table 2 presents the range and levels of experimental variables investigated in this study.

In these studies, a full-factorial rotatable CCD was used. According to this design, the total number of treatment combinations was $2^k + 2k + n0$, in which k is the number of independent variables, and $n0$ is the number of repetitions of the experiments at the center point. For this study, a 2^5 factorial design (32 points) with 10 star points and 6 replicates at the central points was employed to fit the second-order polynomial model given by Eq. 2, which resulted in 48 experiments (Table 3).

The keratinase production (U/mL) was considered the dependent variable or response (Yi). The quadratic model for predicting the optimal point is expressed according to Eq. 2:

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

The mathematical relationship of the independent variable and the response (keratinase production) was calculated by the second-order polynomial Eq. 3:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 \times x1 + \beta_2 \times x2 + \beta_3 \times x3 + \beta_4 \times x4 + \beta_5 \times x5 + \beta_{11} \times x1 \times x1 \\ & + \beta_{12} \times x1 \times x2 + \beta_{13} \times x1 \times x3 + \beta_{14} \times x1 \times x4 + \beta_{15} \times x1 \times x5 + \beta_{22} \times x2 \times x2 \\ & + \beta_{23} \times x2 \times x3 + \beta_{24} \times x2 \times x4 + \beta_{25} \times x2 \times x5 + \beta_{33} \times x3 \times x3 + \beta_{34} \times x3 \times x4 \\ & + \beta_{35} \times x3 \times x5 + \beta_{44} \times x4 \times x4 + \beta_{45} \times x4 \times x5 + \beta_{55} \times x5 \times x5 \end{aligned} \tag{3}$$

in which Y is the response, keratinase relative activity (%); β_0 is the intercept; β_1, β_2, and β_3 are linear coefficients; β_{11}, β_{22}, and β_{33} are squared coefficients; and β_{12}, β_{13}, and β_{23} are interaction coefficients.

Keratinase Assay

Keratinase activity was determined by a modified method using keratin azure *(22)* (Sigma) as substrate. Keratin azure was suspended in 10 m*M* carbonate buffer (pH 10.0) at a concentration of 4 mg/mL. The reaction mixture contained 1 mL of enzyme and 1 mL of keratin azure suspension.

Table 2
Experimental Range and Levels of Five Independent Variables Used in RSM in Terms of Actual and Coded Factors for Optimization of Keratinase Production[a]

Independent variable	Name of variable	Range and level		ΔX		
		–2	–1	0	1	2
$x1$ (g/L)	Feather meal	1	3	5	7	9
2						
$x2$ (g/L)	K_2HPO_4	0	0.1	0.3	0.5	0.7
0.2						
$x3$ (g/L)	KH_2PO_4	0.2	0.3	0.4	0.5	0.6
0.1						
$x4$ (g/L)	NaCl	0.1	0.3	0.5	0.7	0.9
0.2						
$x5$	pH	5.5	6	6.5	7	7.5
0.5						

[a]ΔX is a step increment in each variable value.

The reaction was carried out at 30°C, 300 rpm for 1 h. After incubation, the mixture was kept in ice for 15 min followed by centrifugation at 5000 rpm for 15 min to remove unutilized substrate. The supernatant was spectrophotometrically measured for the release of the azo dye at 595 nm. A mixture of enzyme and substrate was kept in ice before being carried out as a control. One unit of keratinase was defined as the amount of enzyme causing an increase of 0.1 absorbance between the sample and control at 595 nm in 1 h under the given conditions *(22)*. The protein content of the enzyme preparation was estimated by the Lowry method *(23)*.

Experimental Design for Optimization of Keratinase Activity (Second Experimental Design)

In the second step, experiments were conducted to optimize the keratinase enzyme activity by using RSM. The experimental design chosen for this study was a full-factorial CCD that helps in investigating linear, quadratic, and cross-product effects of three factors. The CCD used includes a 2^3 full-factorial design of eight experiments, six star point experiments, and six central point experiments for replication. A second-order polynomial function was fitted for the keratinase activity and is given by Eq. 4:

$$Y = \beta_0 + \beta_1 \times x1 + \beta_2 \times x2 = \beta_3 \times x3 + \beta_{11} \times x1 \times x1 + \beta_{12} \times x1 \times x2 + \beta_{13} \times x1 \times x3 + \beta_{22} \times x2 \times x2 + \beta_{23} \times x2 \times x3 + \beta_{33} \times x3 \times x3 \quad (4)$$

in which Y is the predicted response (keratinase enzyme activity); β_0 is the constant; $x1$ is the pH; $x2$ is the temperature (°C); $x3$ is the rpm; β_1, β_2, and β_0 are linear coefficients; β_{11}, β_{22}, and β_{33} are quadratic coefficients; and β_{12},

Table 3
Design Matrix of Full-Factorial CCD and Observed Results for Keratinase Production

Run no.[a]	x_1	x_2	x_3	x_4	x_5	Measured keratinase production (U/mL)	Predicted keratinase production (U/mL)
1	–1	–1	–1	–1	–1	60.57	65.19
2	1	–1	–1	–1	–1	95	84.23
3	–1	1	–1	–1	–1	49.23	48.2
4	1	1	–1	–1	–1	70.17	70.76
5	–1	–1	1	–1	–1	59.04	57.74
6	1	–1	1	–1	–1	63.17	66.21
7	–1	1	1	–1	–1	53.17	52.24
8	1	1	1	–1	–1	66.87	64.26
9	–1	–1	–1	1	–1	58.39	57.08
10	1	–1	–1	1	–1	63.77	66.81
11	–1	1	–1	1	–1	48.11	47.19
12	1	1	–1	1	–1	63.07	60.45
13	–1	–1	1	1	–1	55.22	51.3
14	1	–1	1	1	–1	53.36	50.47
15	–1	1	1	1	–1	50.4	52.91
16	1	1	1	1	–1	58.13	55.62
17	–1	–1	–1	–1	1	57.45	56.42
18	1	–1	–1	–1	1	62.16	62.75
19	–1	1	–1	–1	1	52.61	49.23
20	1	1	–1	–1	1	56	59.1
21	–1	–1	1	–1	1	58.87	57.94
22	1	–1	1	–1	1	56.34	53.72
23	–1	1	1	–1	1	59.49	62.27
24	1	1	1	–1	1	66.54	61.58
25	–1	–1	–1	1	1	59.15	58.23
26	1	–1	–1	1	1	57.88	55.26
27	–1	1	–1	1	1	55.37	58.15
28	1	1	–1	1	1	63.67	58.71
29	–1	–1	1	1	1	58.93	61.43
30	1	–1	1	1	1	50.42	47.91
31	–1	1	1	1	1	75	72.86
32	1	1	1	1	1	61.67	62.87
33	–2	0	0	0	0	65	62.53
34	2	0	0	0	0	61.58	71.57
35	0	–2	0	0	0	61.7	66.44
36	0	2	0	0	0	61.62	64.41
37	0	0	–2	0	0	48.3	51.95
38	0	0	2	0	0	44.77	48.64
39	0	0	0	–2	0	74.79	78.44
40	0	0	0	2	0	67.75	71.62

(Continued)

Table 3 *(Continued)*

Run no.[a]	x_1	x_2	x_3	x_4	x_5	Measured keratinase production (U/mL)	Predicted keratinase production (U/mL)
41	0	0	0	0	–2	59.1	63.84
42	0	0	0	0	2	59.53	62.31
43	0	0	0	0	0	82	84.75
44	0	0	0	0	0	88	84.75
45	0	0	0	0	0	86	84.75
46	0	0	0	0	0	86	84.75
47	0	0	0	0	0	86	84.75
48	0	0	0	0	0	88	84.75

[a]Experiments 1–32 were 2^5 full-factorial experiments, 33–42 were 2×5 star point experiments, and 43–48 were 6 central point experiments.

β_{13}, and β_{23} are interaction coefficients. pH, temperature, and rpm were chosen as the variables and are designated as x_1, x_2, and x_3, respectively. The levels of the three variables were coded x_1, x_2, and x_3 at five levels (–2, –1, 0, 1, and 2) by using Eq. 1 and are listed in Table 4. A total of 20 experiments was necessary to find the second-order polynomial model (Table 5).

Results and Discussion

Effect of Different Salts and Their Combinations on Keratinase Production

The effects of different salt combinations on keratinase production by *Streptomyces* sp7 were tested. Experiments conducted according to the combinations given in Table 1 with a constant pH, feather concentration, and salt combination (SMC-5) yielded maximal keratinase. The combination of K_2HPO_4, KH_2PO_4, and NaCl resulted in maximum amounts of keratinase (70.9 U/mL) production. From the results it is evident that the presence of K_2HPO_4 and KH_2PO_4 was significant in terms of keratinase production. A 3.5-fold increase in keratinase production was reported using similar salts *(24)*.

Optimization of Keratinase Production by RSM (First Experimental Design)

The results obtained in terms of keratinase production with reference to the experiments performed according to the CCD are given in Table 3. The coefficient of determination (R^2) was calculated as 0.911 for keratinase production (model summary in Table 6), indicating that the statistical model can explain 91.10% of variability in the response. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms

Table 4
Experimental Range and Levels of Five Independent Variables Used in RSM in Terms of Actual and Coded Factors for Optimization of Keratinase Enzyme Activity Conditions[a]

Independent variable	Name of variable	Range and level					
		–2	–1	0	1	2	ΔX
x_1	pH	5	7	9	11	13	2
x_2 (°C)	Temperature	30	35	40	45	50	5
x_3	rpm	150	200	250	300	350	50

[a]ΔX is a step increment in each variable value.

Table 5
Design Matrix of Full-Factorial CCD and Observed Results for Optimization of Keratinase Enzyme Activity Conditions

Run no.[a]	*x*1	*x*2	*x*3	Measured keratinase activity (%)	Predicted keratinase activity (%)
1	–1	–1	–1	73	78.6012
2	1	–1	–1	75	72.4788
3	–1	1	–1	67	62.4772
4	1	1	–1	99	102.8548
5	–1	–1	1	93	89.4772
6	1	–1	1	64	68.8548
7	–1	1	1	67	69.8532
8	1	1	1	100	95.7308
9	–2	0	0	69	68.9544
10	2	0	0	89	88.7096
11	0	–2	0	77	74.956
12	0	2	0	84	85.708
13	0	0	–2	82	80.956
14	0	0	2	84	84.708
15	0	0	0	76.36	78.168
16	0	0	0	79.21	78.168
17	0	0	0	78.97	78.168
18	0	0	0	78.9	78.168
19	0	0	0	77.9	78.168
20	0	0	0	78	78.168

[a]Experiments 1–8 were 2^3 full-factorial experiments, 9–14 were 2∗3 star point experiments, and 15–20 were 6 central point experiments.

Table 6
Model Coefficients Estimated by Multiple Linear Regression
(Significance of Regression Coefficients) for Keratinase Production

Model term	Parameter estimate	SE	Computed *t* value	*p* Value
(Constant)	84.745	1.858	—	0.000
x_1	2.260	0.739	0.176	0.005
x_2	–0.509	0.739	–0.040	0.497
x_3	–0.826	0.739	–0.064	0.273
x_4	–1.706	0.739	–0.133	0.029
x_5	–0.382	0.739	–0.030	0.610
x_1x_1	–4.423	0.848	–0.301	1.72e-5
x_1x_2	0.884	0.827	0.061	0.294
x_1x_3	–2.639	0.827	–0.184	0.004
x_1x_4	–2.326	0.827	–0.162	0.009
x_1x_5	–3.175	0.827	–0.221	0.001
x_2x_2	–4.830	0.848	–0.329	4.731e-6
x_2x_3	2.877	0.827	0.200	0.002
x_2x_4	1.776	0.827	0.124	0.041
x_2x_5	2.454	0.827	0.171	0.006
x_3x_3	–8.613	0.848	–0.586	1.01e-10
x_3x_4	0.419	0.827	0.029	0.616
x_3x_5	2.247	0.827	0.156	0.011
x_4x_4	–2.429	0.848	–0.165	0.008
x_4x_5	2.482	0.827	0.173	0.006
x_5x_5	–5.417	0.848	–0.369	7.63e-7

in the model. The value of the adjusted determination coefficient (Adj R^2 = 0.845) is also very high, advocating a high significance of the model *(13–16)*. 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 0.845, less than the R^2 value of 0.911. At the same time, a relatively lower value of the coefficient of variation (=11.12%) indicates a better precision and reliability of the experiments conducted *(25,26)*. By applying multiple regression analysis of the experimental data, the experimental results of the CCD design were fitted with a second-order full polynomial equation. The empirical relationship between keratinase production (Y) and the five test variables in coded units obtained by the application of RSM is given by Eq. 5:

$$\begin{aligned} Y = {} & 84.745 + 2.26 \times x1 - 0.509 \times x2 - 0.826 \times x3 - 1.706 \times x4 - 0.382 \times x5 \\ & - 4.423 \times x1 \times x1 + 0.884 \times x1 \times x2 - 2.639 \times x1 \times x3 - 2.326 \times x1 \times x4 \\ & - 3.175 \times x1 \times x5 - 4.83 \times x2 \times x2 + 2.877 \times x2 \times x3 + 1.776 \times x2 \times x4 \\ & + 2.454 \times x2 \times x5 - 8.613 \times x3 \times x3 + 0.419 \times x3 \times x4 + 2.247 \times x3 \times x5 \\ & - 2.429 \times x4 \times x4 + 2.482 \times x4 \times x5 - 5.417 \times x5 \times x5 \end{aligned} \quad (5)$$

in which Y, representing keratinase production (U/mL), is the response and $x1$, $x2$, $x3$, $x4$, and $x5$ are the coded values (g/L) of feather meal, K_2HPO_4, KH_2PO_4,

NaCl, and different pH values, respectively. ANOVA was conducted for the second-order response surface model and the results are given Table 6. The significance of each coefficient was determined by student's *t* test and *p* values, which are listed in Table 6. The larger the magnitude of the *t* value and the smaller the *p* value, the more significant is the corresponding coefficient, i.e., $p < 0.05$ at 5% sig-nificance level *(25,26)*. This implies that the linear effects of feather meal concentration ($p < 0.005$) and NaCl ($p < 0.029$), and the interactive effects of all variables are significant at the 5% significance level. The *p* values of the quadratic effects of feather meal, $x1*x1$ ($p <$ 1.72e-5); K_2HPO_4, $x2*x2$ ($p <$ 4.731e-6); KH_2PO_4, $x3*x3$ ($p <$ 1.01e-10); and pH, $x5*x5$ ($p <$ 7.63e-7) are more significant than the linear and interactive effects of process variables. Since the concentration of feather meal, K_2HPO_4, and KH_2PO_4 and the pH are also very significant at the quadratic level, a slight variation in these concentra-tions will alter the product formation rate. The model *F*-value of 13.77 and values of prob > *F* (<0.05) indicated that the model terms are significant. For poultry feather degradation and keratinase production, a statistically significant model can be written by the significant data obtained from the regression analysis.

The regression model developed can be represented in the form of contour plots. The yields (U/mL) of keratinase from poultry feather for different concentrations of test variables can also be predicted from the respective contour plots, as shown in Fig. 1. Each contour curve represents an infinite number of combinations of two test variables with the other three maintained at their respective zero level. These plots demonstrate that the production of keratinase was dependent on the linear effects of feather meal concentration and NaCl. The initial pH of the medium also greatly affected feather degradation and keratinase production. Figure 1C shows the keratinase production when pH and feather meal concentration varied at different levels by keeping the other variables at their respective zero levels. The elliptical nature of the contour plot shows the existence of a significant interaction relationship between pH and feather meal concentration. The optimum pH for keratinase production was 6.0 and an optimal feather meal concentration of 7 g/L for the isolate. Table 3 shows the production of keratinase at different combinations of five test vari-ables. From the results (Table 3) and the contour plots, it was clear that feather meal concentration had a significant effect on keratinase produc-tion. It was demonstrated that high feather concentrations may cause sub-strate inhibition or repression of keratinase production, resulting in a low percentage of feather degradation, which is previously reported in *Bacillus* sp. *(3)*. Members of *Streptomyces* and the related genera produce a large variety of extracellular enzymes, of which keratinases are of particular significance to the leather industry. The results of production medium optimization studies for keratinase from *Streptomyces* sp7 used in the present studies were good and compared well with those of studies of other microorganisms such as *Kocuria rosea* (17 U/mg) *(27)*, *Bacillus* sp. FK 46 *(3)*, and *Streptomyces* sp. SK_{1-02} *(22)* in which keratin azure was used as substrate. Other

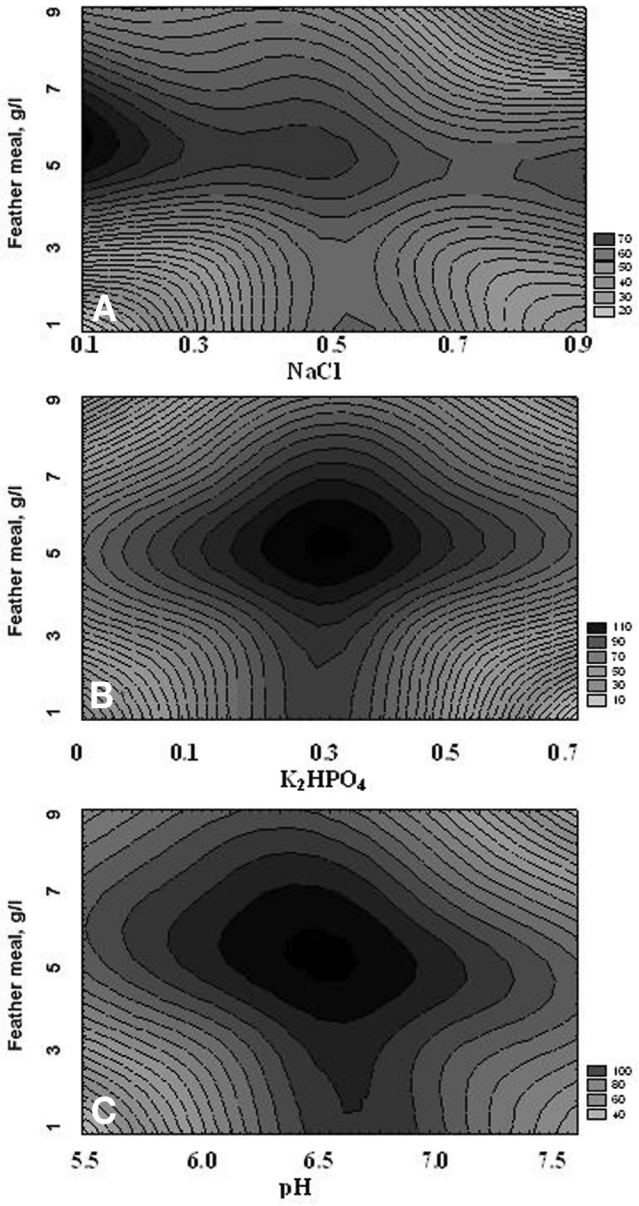

Fig. 1. Contour plots of keratinase production optimization studies. **(A)** Effect of feather meal and NaCl and their interaction on keratinase production; **(B)** effect of feather meal and K_2HPO_4 and their interaction on keratinase production; **(C)** effect of feather meal and pH and their interaction on keratinase production. Other values are held at zero level.

strains, *Streptomyces pactum* DSM 40530 (64.0 U/mL) *(7)*, *Fervidobacterium pennavorans* (13 U/mL) *(28)*, *Thermoanaerobacter keratinophilus* sp. (109 U/mL) *(29)*, *Bacillus licheniformis* (thermotolerant) (3.67 U/mL) *(30)*, *Streptomyces thermonitrificans* (47 U/mL) *(31)*, and *Chryseobacterium* sp. strain kr6 (96 U/mL) *(32)*, were also compared.

Process Optimization of Keratinase Enzyme Activity Conditions by RSM (Second Experimental Design)

Three variables, pH, temperature, and rpm, were optimized for better keratinase enzyme activity production by isolated *Streptomyces* sp7. Experiments were conducted per the design and RSM was performed with a full-factorial CCD, a second-order polynomial was fitted, and ANOVA of the data was conducted. Table 7 provides rhe coefficients of the regression model calculated. To characterize enzyme activity as a function of adjustable variables, the results of the factorial design were analyzed. The coefficient of determination (R^2) was calculated as 0.917 for keratinase enzyme activity, indicating that the statistical model can explain 91.7% of variability in the response. The significance of each coefficient was determined by student's *t*-test and *p* values, which are given in Table 7. The larger the magnitude of the *t* value and the smaller the *p* value, the more significant is the corresponding coefficient ($p < 0.005$) *(14,26)*. This implies that the first-order main effects of pH ($p < 0.001$) and temperature ($p < 0.029$) are highly significant, as is evident from their respective *p* values and their second-order interactive effects ($p <$ 9.14e-6). Second-order interactive effects of pH and rpm ($p < 0.028$) significantly affected keratinase activity. This suggests that the pH and temperature have a linear and second-order relationship with keratinase enzyme activity. The empirical relationship between keratinase enzyme activity (Y) and the five test variables in coded units obtained by the application of RSM is given by Eq. 6:

$$Y = 78.168 + 4.9388 \times x1 + 2.688 \times x2 + 0.938 \times x3 + 0.166 \times x1 \times x1 + 11.625 \times x1 \times x2 - 3.625 \times x1 \times x3 + 0.541 \times x2 \times x2 - 0.875 \times x2 \times x3 + 1.166 \times x3 \times x3 \quad (3)$$

in which Y, representing the keratinase enzyme activity (%), is the response and $x1$, $x2$, and $x3$ are the coded values of the test variables.

Figure 2 shows the contour plots of the test variables, and each contour curve represents an infinite number of combinations of two test variables with the other three maintained at their respective zero level. Keratinase was active in the alkaline condition, with optimal activity at pH 11.0, and activity declined as the pH increased above the optimum. The isolate *Strep-tomyces* sp7 secreted a protease that was characterized as keratinase by its activity on feather-based medium. The other alkaline keratinases reported with similar pH optima were *Bacillus pseudoformis (33)*, *B. licheniformis (34)*, *B. licheniformis* PWD-1 *(35)*, *Bacillus* sp. SCB-3 *(36)*, *Bacillus* sp. PS719 *(37)*, *Bacillus thermoruber (38)*, *Bacillus* sp. FK 28 *(39)*, *Bacillus* sp. No-AH-101, and *Bacillus halodurans (40,41)*. As shown in Table 5, maximum keratinase activ-ity (100%) was at 45°C, pH 11.0, and 300 rpm, whereas the keratinase from *S. pactum* was active at between 40 and 70°C *(7)* and pH 8.0, and a tempera-ture of 40°C was optimum for *Scopulariopsis brevicaulis (42)*. Keratinase from *Doratomyces microsporus* and *Streptomyces graminofaciens* showed an optimal activity at 50 and 40°C *(43,44)*.

Table 7
Model Coefficients Estimated by Multiple Linear Regression (Significance of Regression Coefficients) for Keratinase Enzyme Activity

Model term	Parameter estimate	SE	Computed *t* value	*p* Value
(Constant)	78.168	1.592	—	0.000
*x*1	4.938	0.998	0.452	0.001
*x*2	2.688	0.998	0.246	0.023
*x*3	0.938	0.998	0.086	0.370
*x*1*x*1	0.166	0.796	0.020	0.839
*x*1*x*2	11.625	1.411	0.753	9.14e-6
*x*1*x*3	–3.625	1.411	–0.235	0.028
*x*2*x*2	0.541	0.796	0.065	0.512
*x*2*x*3	–0.875	1.411	–0.057	0.549
*x*3*x*3	1.166	0.796	0.139	0.174

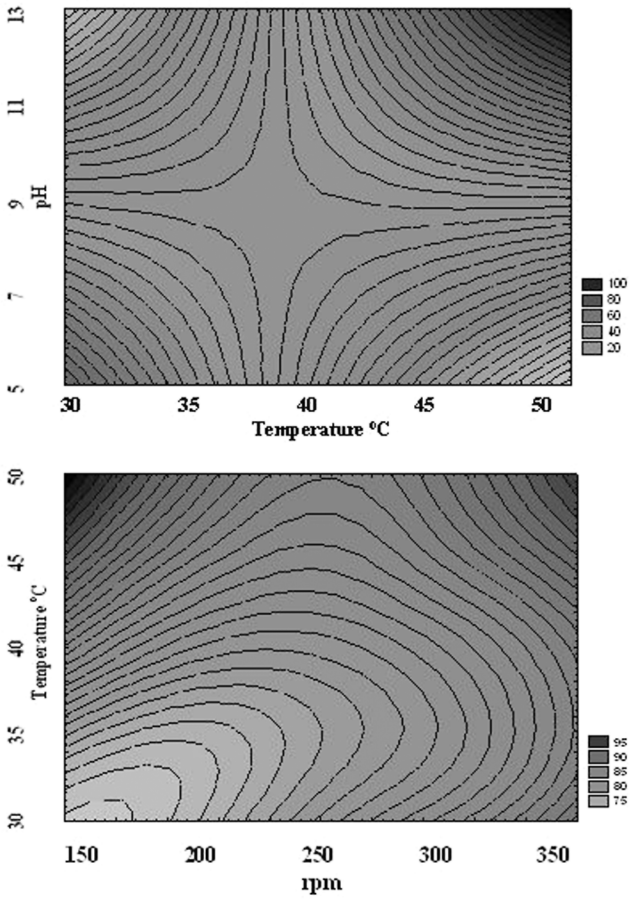

Fig. 2. Contour plots of keratinase enzyme activity optimization studies. **(A)** Effect of pH and temperature and their interaction on keratinase enzyme activity; **(B)** effect of temperature and rpm and their interaction on keratinase enzyme activity. Other variables are held at zero level.

Conclusion

The use of RSM with a full-factorial rotatable CCD for determination of optimal medium composition for keratinase production and optimal enzyme activity conditions was demonstrated. Use of this methodology will be successful for any process in which an analysis of the effects and interactions of many experimental factors is required. Rotatable central composite experimental design maximizes the amount of information that can be obtained while limiting the number of individual experiments. Thus, smaller and less time-consuming experimental designs could generally suffice for optimization of many fermentation processes.

The results of the present studies suggested that several factors influence keratinase production and its activity. The results of ANOVA and regression of the second-order model showed that the linear effects of feather meal concentration and NaCl and the interactive effects of all variables are more significant for keratinase production. The values of the quadratic effects of feather meal, K_2HPO_4, KH_2PO_4, and pH are more significant than the linear and interactive effects of process variables for keratinase production. The results of the second-order model and ANOVA demonstrated optimal keratinase activity conditions of pH (11.0), temperature (45°C), and rpm (300).

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