academicJournals

Vol. 12(48), pp. 6776-6784, 27 November, 2013
DOI: 10.5897/AJB2013.13224
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Keratinase production by *Bacillus megaterium* RS1 using the statistical tool central composite design

T. Shankar, R. Rama, P. Thangamathi and T. Sivakumar*

Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi-626124,Tamilnadu, India.

Accepted 21 November, 2013

Keratinase producing bacterium *Bacillus megaterium* RS1 was obtained from feather dumping site of Rajapalayam. The optimal level of the key variables (starch, feather meal, calcium chloride) was used to determine the effect of their interactions on keratinase production using the statistical tool [(Central composite design (CCD) of response surface methodology (RSM)]. The second-order quadratic model with the optimum conditions [(starch (1%); feather meal (3%) and calcium chloride (0.02%)] was used. The nearness of the coefficient of determination (R^2 = 1.0000) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The maximum keratinase production was 142.9 U/ml.

Key words: Keratinase, Central composite design (CCD), response surface methodology (RSM), *Bacillus megaterium* RS1 starch, feather meal, calcium chloride.

INTRODUCTION

The incremental intensification in poultry industry all over the world resulted in the generation of millions of tonnes of chicken feather waste (Williams et al., 1990). Keratin a hard to degrade insoluble animal protein represents 90% of this keratinous waste (Bockle et al., 1995). This group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases called keratinases and these belong to the extracellular enzymes (Rao et al., 1998). They are classified into various groups based on whether they are acidic, neutral or alkaline conditions. The protein chains are packed tightly either in α-helix (α-keratins) or in β-sheet (β-keratins) structures, which fold into a final 3-dimensional form (Kim, 2007). Microbial keratinase is an enzyme capable of degrading the insoluble structural protein found in feathers, hair, nail and wool. Keratin is a substrate by which sensitivity and resistance are closely linked to them fundamentally permitted of collagenase activity. High cysteine content is the most important property that differentiates keratins from other structural protein such as collagen and elastin (Sivakumar et al., 2012). These are poorly susceptible to digestion by enzymes such as trypsin, pepsin and papain (Gupta and Ramani, 2006). Keratinases from microbial source have many applications in feed, fertilizers, detergent, leather and pharmaceutical industries. Many organisms produce keratinase such as *Chrysoporium*, *Aspergillus*, *Alternaria*, *Trichurus*, *Curvularia*, *Cladosporium*, *Fusarium*, *Penicillium* (fungi), Streptomyces, *Vibrio*, *Mycobacterium* and *Bacillus* sp. (Vijay Kumar et al., 2011). Response Surface Methodology (RSM) is a statistical technique for the modelling and optimization of multiple variables, which determine optimum process conditions by combining experimental designs with interpolation by first or second polynomial equations in a sequential testing procedure (Ferreira et al., 2009). RSM has already been successfully applied for the optimization of enzymatic hydrolysis of other bioprocesses. Response surface methodology (RSM) is a useful tool which integrates mathematical and statistical approaches to analyze the effects of defined independent variables on the response without the need for prior knowledge of a predetermined

*Corresponding author. E-mail: sivasadhana@yahoo.co.in.

Table 1. Independent variables and their coded levels for the central composite design used for keratinase production by *Bacillus megaterium* RS1.

Variable	-α	Low value	Coded variable	High value	+α
Starch	0.159104	0.5	1	1.5	1.8409
Feather meal	1.31821	2	3	4	4.68179
Calcium chloride	0.00318207	0.01	0.02	0.03	0.0368179

relationship between the response function and the variables. RSM is now considered as a standard statistical approach for designing experiments, building models, evaluating the effects of many factors and finding the optimal conditions for desirable responses and reducing the number of required experiments (Coninck et al., 2000). Response surface methodology was used to optimize the conditions for the extracellular production of keratinase (Siva et al., 2012a).

Optimization of the fermentation process parameters through a statistical approach, such as 'central composite design' and response surface methodology (RSM), has been well appreciated for a significant improvement in yield as well as a decrease in the production cost of the enzyme (Sivakumar et al., 2012b). Therefore, this study was mainly focused on statistical optimization of keratinase production using central composite design for high yield with low cost. In this work, RSM was adopted to determine the optimal conditions for the production of keratinase from *Bacillus megaterium* RS1 and the interacttions among the factors that influence the response of the keratinase production were determined.

MATERIALS AND METHODS

Optimization of significant variables for keratinase production using CCD

To find the optimal cultivation conditions for keratinase production, CCD with five coded levels was used for locating the true optimum conditions of starch (carbon source), feather meal (substrate concentration) and calcium chloride (metal ions). For the three factors, this trial was essentially a full 2^3 factorial design with six axial points (α = 1.68) and six replication of the centre points, resulting in a total number of 20 experiments. The levels of the variables and the experimental design are shown in Table 1. The results of CCD were expressed as the following second-order polynomial (Equation 2) using a multiple regression technique.

$$Y = \beta_o + \Sigma \beta i \chi i + \Sigma \beta ii \chi i^2 + \Sigma \beta ij \chi i \chi j$$

Where, Y is the predicted response, β0 the intercept term, βi the linear coefficients, βii the quadratic coefficients, βij the interactive coefficients, xi and xj the coded independent variables (Song et al., 2007).

Keratinase production by optimized parameters

After 48 h of incubation on optimized medium [starch (carbon source), 1% yeast extract (nitrogen sources); 0.5%, calcium chloride (metal ions); 0.2%, PEG (surfactants); 0.02%, inoculum concentration (2%), 3% feather meal (substrate concentration) at pH 7.0, 40°C) the culture medium was centrifuged at 5000 rpm for 15 min. The supernatant was used as crude enzyme source for keratinase assay. Keratinase activity was assayed as per the method of Burtt and Lchida (1999) using Azocasein. About 5 mg of Azocasein was added to a 1.5 ml centrifuge tube along with 0.8 ml of 50 mM potassium phosphate buffer (pH- 7.5) at 37°C for 1 h with constant agitation (900 rpm). This mixture was agitated until the Azocasein was completely suspended. Then, 0.2 ml aliquot of supernatant (crude enzyme) was added to the Azocasein, mixed and incubated for 15 min at 50°C with shaking. The reaction was terminated by adding 0.2 ml of 10% of trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity. The absorbance of the filtrate was measured at 450 nm with a UV-160 spectrophotometer. A control sample was prepared by adding TCA to a reaction mixture before the addition of enzyme solution. The unit of keratinase activity was measured at 0.01 unit increase in the absorbance at 450 nm as compared to the control after 15 min of reaction. Standard curve was performed with tyrosine and the enzyme activity was expressed in units.

Statistical analysis

Experimental designs and the polynomial coefficients were calculated and analyzed using a trial version of Design-Expert software (version 8.0.4, Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

RESULTS

Central composite design (CCD) and response surface methodology (RSM)

The optimal level of the key variables (starch, feather meal and calcium chloride) and the effect of their interacttions on keratinase production were further explored using the CCD of RSM. The design matrix and the corresponding experimental data to determine the effects of three independent variables are shown in Table 1. The mutual interactions between every two of the three variables which were significant under the optimum condition, the predicted maximum keratinase production were calculated as 142.9 U/ml. By applying multiple regression analysis to the experimental data (Table 2), the following second order polynomial equation was established:

Terms of coded factors

$$\text{Keratinase} = +142.89 - 7.11*A + 8.78*B - 6.11*C - 2.94*A*B - 0.34*A*C - 1.64*B*C - 27.01*A^2 - 0.16*B^2 - 13.68*C^2$$

Table 2. Variance analysis of response surface quadratic model for keratinase production by *Bacillus megaterium* RS1.

Source	Sum ofsquares	Df	Meansquare	F-value	p-value prob > F
Model	24797.15	9	2755.24	81468.99	< 0.0001[c]
A-starch	690.31	1	690.31	20411.50	< 0.0001[c]
B-feather meal	1052.89	1	1052.89	31132.53	< 0.0001[c]
C-calcium chloride	596.58	1	596.58	17640.15	< 0.0001[c]
AB	69.03	1	69.03	2041.17	< 0.0001[c]
AC	0.91	1	0.91	26.94	< 0.0001[c]
BC	21.45	1	21.45	634.29	< 0.0001[c]
A^2	10304.33	1	10304.33	3.047E+005	< 0.0001[c]
B^2	12850.99	1	12850.99	3.800E+005	< 0.0001[c]
C^2	4851.80	1	4851.80	1.435E+005	< 0.0001[c]
Residual	0.34	10	0.34		
Lack of fit	0.34	5	0.068		
Pure error	0.000	5	0.000		
Cor total	24797.49	19			

R^2 = 1.0000; Adj R^2 = 1.0000; CV% = 0.20; [c]Model terms are significant.

Terms of actual factors

Keratinase = 320.02917 + 220.81347* Starch + 198.90234* feather meal + 5420.42848* calcium chloride - 5.87500* starch* feather meal 67.50000* starch* calcium chloride 163.75000* feather meal* calcium chloride - 108.03198* starch^2 - 30.16134* feather meal^2 - 1.36808e + 005* calcium chloride^2 2

Where, Y1 was the keratinase production, X1 the starch, X2 the feather meal and X3 the calcium chloride.

The model F-value of 81468.99 implies the model is significant. The "model F-value" is 0.01% occurence due to blare. Values of "Prob > F" less than 0.0500 indicate that the model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "pred R-squared" of 0.9999 is in reasonable agreement with the "adj R-squared" of 1.0000. "Adeq precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 768.125 indicates an adequate signal. This model can be used to navigate the design space.

In the present study, all these results showed a good agreement between the experimental and predicted values and implied that the mathematical models were suitable for the simulation of keratinase production (Tables 3 and 4).

DISCUSSION

The enlightening of microbial keratinase production is the aim of these investigations, being the production capacity of the organism depending on the successful selection of growth conditions and substrate. The strain *B. megaterium* RS1 showed higher keratinase production on mutual interactions between every two of the three variables which is an inexpensive and readily available substrate. Thus, the utilization of such substrate may result in a cost effective process. The three-dimensional response surfaces (Figure 1a, b, c) and contour plots are shown in Figure 2a, b, c (keratinase production) which depicts the interactions between the two variables by keeping the other variables at their zero levels. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. A circular contour plot of response surfaces indicates that the interaction between the cor-responding variables can be ignored, while an elliptical or saddle nature of the contour plot suggests that the inte-raction between the corresponding variables is significant (Shankar and Isaiarasu, 2012). The second-order quadratic model with the optimum conditions (starch - 1%; feather meal- 3% and calcium chloride - 0.02%) resulted in a maximum titre of 142.9 U/ml of keratinase at 48 h. The nearness of the coefficient of determination (R^2 = 1.0000) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. Likewise, model of RSM was employed in the optimization of major keratinase producing conditions such as starch, feather meal and calcium chloride.

Optimization has one of the most important criteria when it comes to developing any new microbial process. The box-Brinker design experiment is used to determine the maximum keratinase production at the most adequate pH, temperature and catalyst concentration (Anbu et al., 2005). Response surface methodology, an experiment strategy for seeking the optimum conditions for a multi-variable system, is a much more efficient technique for optimization. This method has been successfully applied

Table 3. Central composite design for keratinase production by *Bacillus megaterium* RS1.

Standard	Run	Factor 1	Factor 2	Factor 3	Keratinase (U/ml)
		Starch (%)	Feather meal (%)	$CaCl_2$ (%)	
15	1	1.00	3.00	0.02	142.9
3	2	0.50	4.00	0.01	98.1
12	3	1.00	4.68	0.02	72.7
20	4	1.00	3.00	0.02	142.9
8	5	1.50	4.00	0.03	62.5
1	6	0.50	2.00	0.01	71.5
10	7	1.84	3.00	0.02	54.9
9	8	0.16	3.00	0.02	78.8
2	9	1.50	2.00	0.01	63.7
7	10	0.50	4.00	0.03	83.4
5	11	0.50	2.00	0.03	63.1
6	12	1.50	2.00	0.03	54.2
18	13	1.00	3.00	0.02	142.9
4	14	1.50	4.00	0.01	78.8
17	15	1.00	3.00	0.02	142.9
19	16	1.00	3.00	0.02	142.9
11	17	1.00	1.32	0.02	43.2
14	18	1.00	3.00	0.04	76.1
13	19	1.00	3.00	0.00	100.5
16	20	1.00	3.00	0.02	142.9

Table 4. The matrix of the CCD experiment and the corresponding experimental data by *Bacillus megaterium RS1.*

Standard	Run	Factor 1	Factor 2	Factor 3	Actual value	Predicted value
		Starch (%)	Feather meal (%)	Calcium chloride (%)		
15	1	1.00	3.00	0.02	142.9	142.8
3	2	0.50	4.00	0.01	98.1	98.2
12	3	1.00	4.68	0.02	72.7	72.5
20	4	1.00	3.00	0.02	142.9	142.8
8	5	1.50	4.00	0.03	62.5	62.6
1	6	0.50	2.00	0.01	71.5	71.5
10	7	1.84	3.00	0.02	54.9	54.7
9	8	0.16	3.00	0.02	78.8	78.6
2	9	1.50	2.00	0.01	63.7	63.8
7	10	0.50	4.00	0.03	83.4	83.4
5	11	0.50	2.00	0.03	63.1	63.3
6	12	1.50	2.00	0.03	54.2	54.2
18	13	1.00	3.00	0.02	142.9	142.8
4	14	1.50	4.00	0.01	78.8	78.8
17	15	1.00	3.00	0.02	142.9	142.8
19	16	1.00	3.00	0.02	142.9	142.8
11	17	1.00	1.32	0.02	43.2	43.0
14	18	1.00	3.00	0.04	76.1	75.9
13	19	1.00	3.00	0.00	100.5	100.3
16	20	1.00	3.00	0.02	142.9	142.8

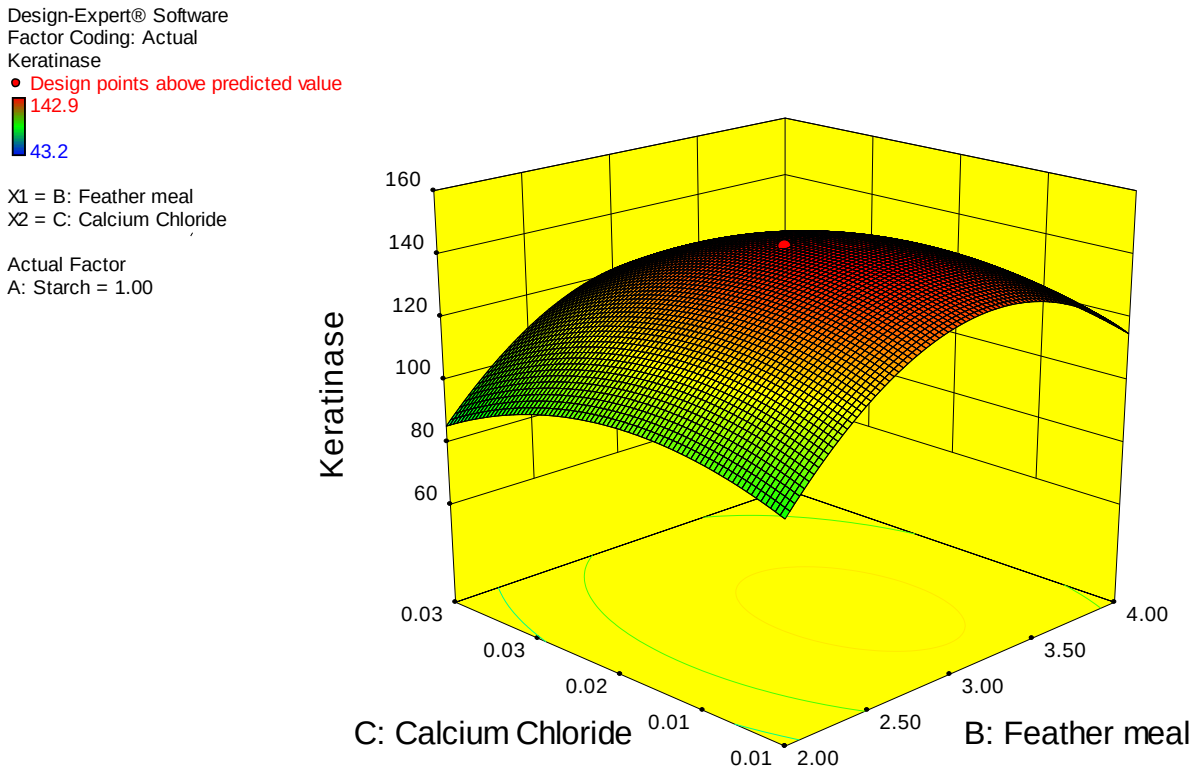

Figure 1a. Interaction between calcium chloride and feather meal.

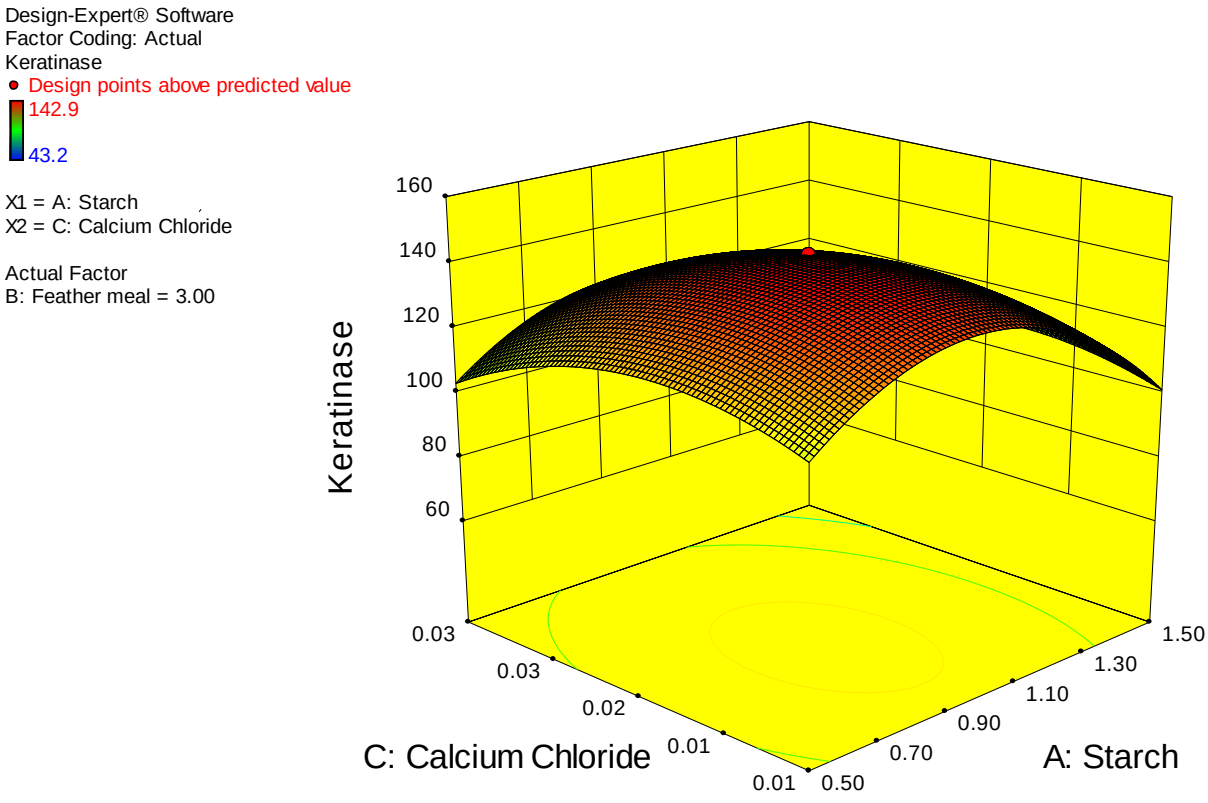

Figure 1b. Interaction between calcium chloride and starch.

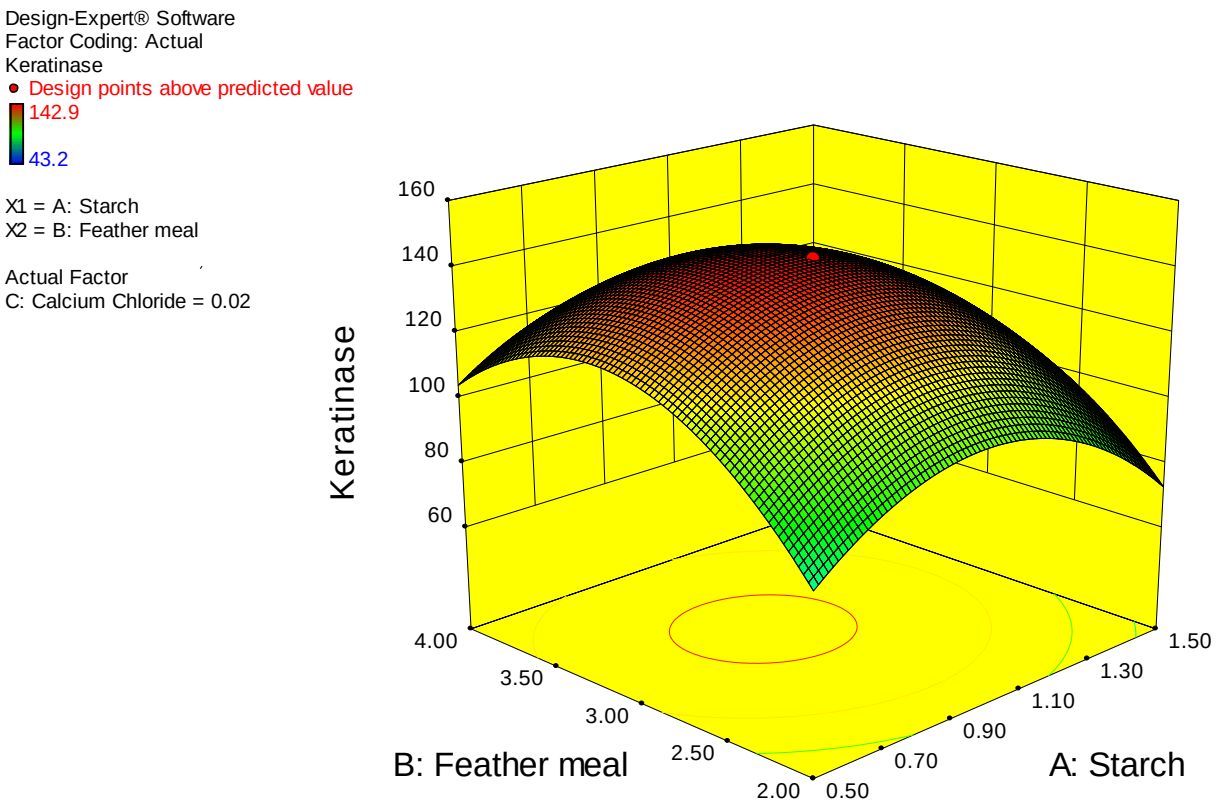

Figure 1c. Interaction between feather meal and starch.

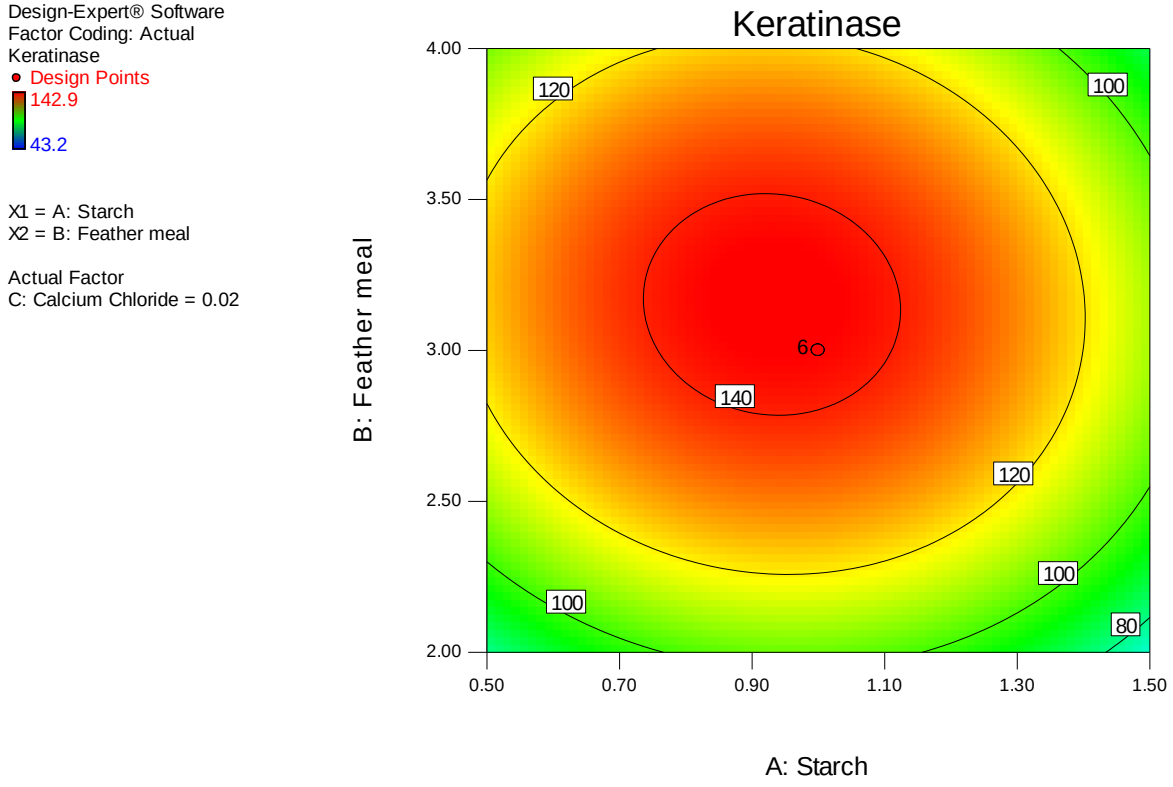

Figure 2a. Contour plot for feather meal and starch.

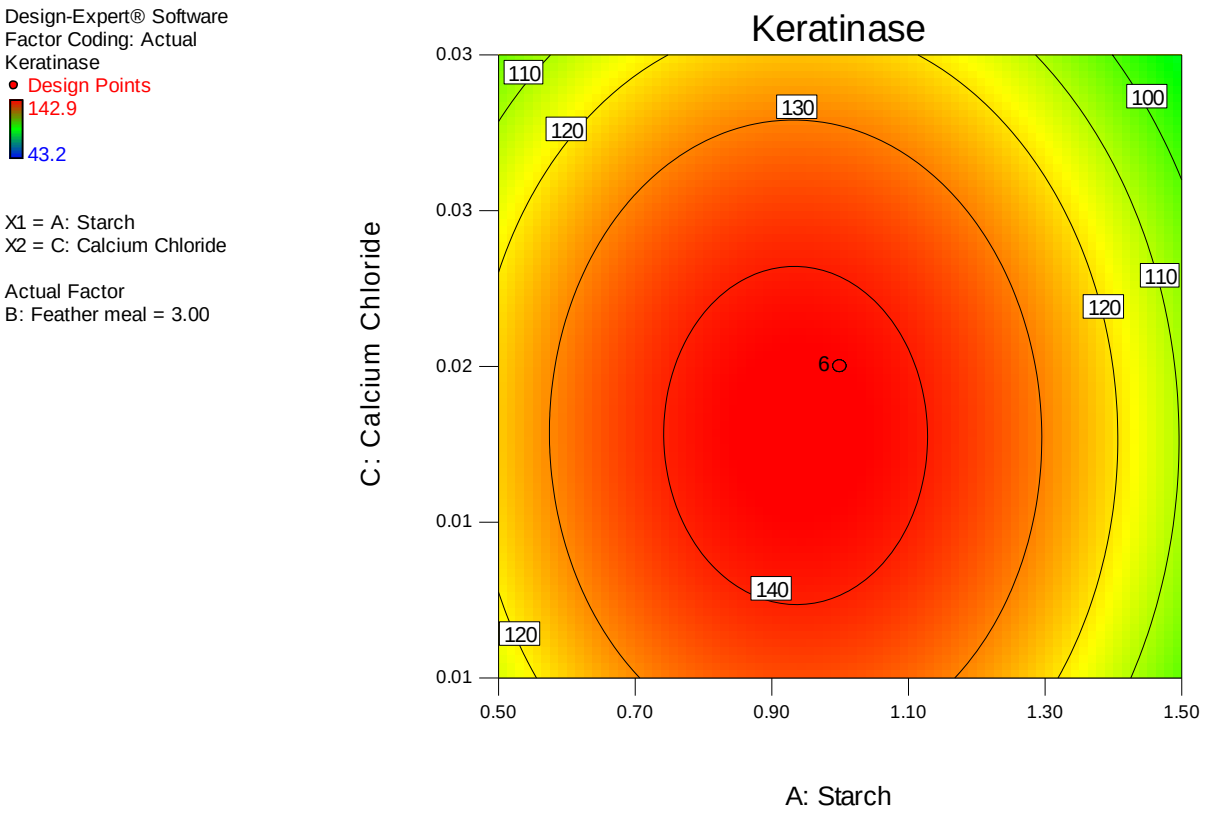

Figure 2b. Contour plot for calcium chloride and starch.

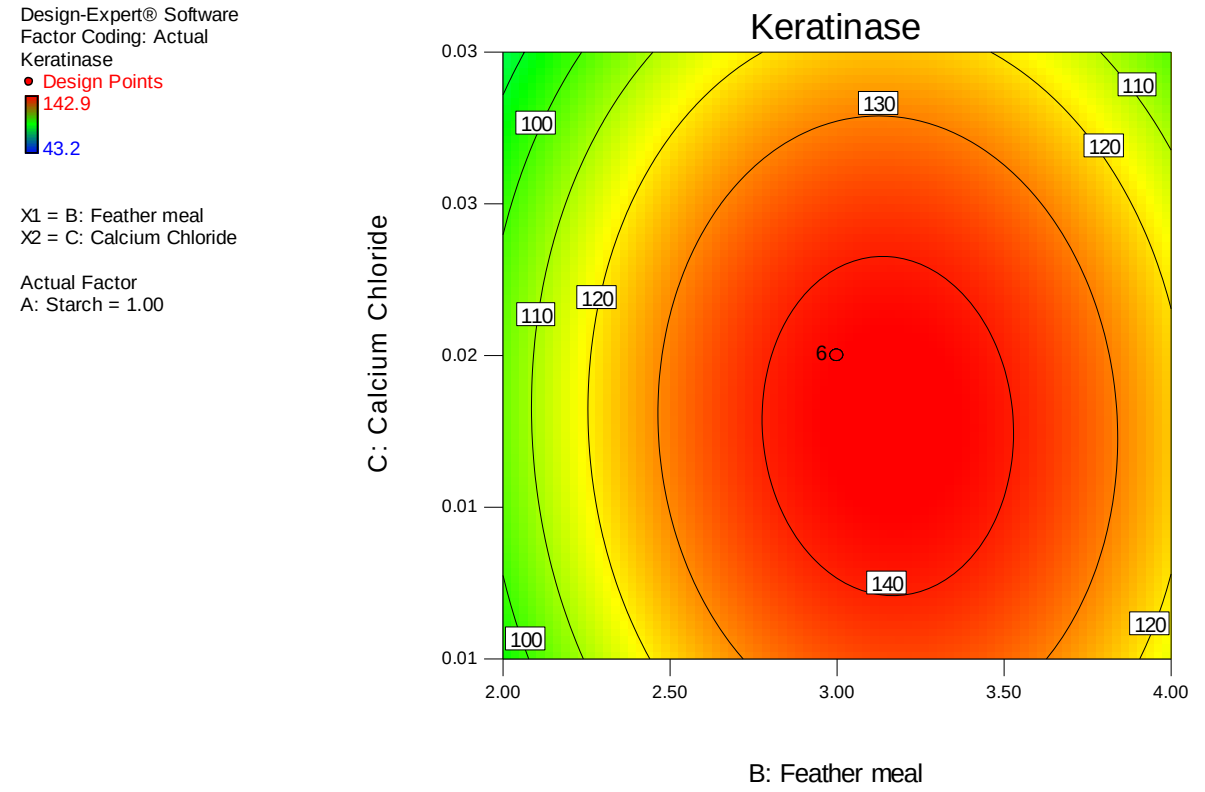

Figure 2c. Contour plot for calcium chloride and feather meal.

for media optimization in different fermentation process as well as for establishing the conditions of enzymatic hydrolysis and sulfuric acid production. To develop a process for maximum production of keratinase from poultry feather, standardization of media components is crucial. Isolated species of *Streptomyces* sp. is capable of rapidly degrading native feather. The production of keratinase was achieved by *Streptomyces* sp. using CCD and RSM (Tatineni et al., 2008). A two-step RSM study is conducted for the optimization of keratinase production and enzyme activity from poultry feather by *Streptomyces* sp 7. Initially, different combinations of salts are 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 $NaCl_2$ gives 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, $NaCl_2$ and pH) are optimized by a 2^5 full-factorial rotatable central composite design (CCD) that has resulted in 95 U/ml of keratinase production. The results of analysis of variance and regression of a second-order model show that the linear effects of feather meal concentration ($p < 0.005$) and $NaCl_2$ ($p < 4.731$ e-6), KH_2PO_4 ($p < 1.01$e-10) and pH (p 7.63e-7) are more significant than the linear and interactive effects of the process variables. These optima are pH 11.0, temperature of 45°C at 300 rpm (Radhika et al., 2007).

The RSM applied to the optimization of keratinase production in the investigation suggested that the importance of verity of factors at different levels, the central composite design (CCD) exploited in the present study enabled as to study and explore the culture conditions, which would support a 3.4 fold increase in keratinase production. The high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for enzyme production. RSM was successfully applied to the production of keratinase by Zauari et al. (2010). On the other hand, *Bacillus pumilus* AI showed the maximum production was 87.73 U/ml. Likewise, Sivakumar et al. (2012c) stated that the maximum keratinase enzyme production was 63.01 and 60.67 U/ml obtained by *Bacillus cereus* TS1 and *Bacillus thuriengiensis* TS2, respectively. There were three factors namely pH, temperature and starch used for RSM optimization in *B. cereus* TS1 and pH, temperature, mannitol were used for RSM optimization in *B. thuriengiensis* TS2. In this context, the present study similar to that of Matsui et al. (2009) having large research aimed to isolate feather degrading microorganism and investigated the characterization of feather degrading enzyme for socioeconomic importance.

Conclusion

The present work of the optimum cultural conditions for keratinase production by *B. megaterium* RS1 species was studied by RSM using central composite design with three variables (starch, feather meal and calcium chloride) for maximizing the production of keratinase.

ACKNOWLEDGEMENT

The facilities provided by Department of Microbiology of Ayya Nadar Janaki Ammal College, Sivakasi to carry out this study are gratefully acknowledged.

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