Global Journal of Biotechnology & Biochemistry 6 (4): 197-202, 2011
ISSN 2078-466X

Statistical Optimization of Keratinase Production by *Bacillus cereus*

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Abstract: This study presents optimum parameters for keratinase production by *Bacillus cereus* TS1 using response surface methodology (RSM) based on central composite design (CCD) model. Statistical testing was performed for analysis of variance (ANOVA) for quadratic regression equations of both linear and interaction effect of variables. Optimum conditions for keratinase production by *Bacillus cereus* were: pH 9, temperature 50°C and starch- 1%. By optimizing with coded factor the maximum keratinase production observed by the model was 60.67 U/ml.

Key words: Keratinase · RSM (CCD) Optimization · *Bacillus Cereus* · ANOVA

INTRODUCTION

Increase in poultry industry all over the world resulted in the generation of millions of tones of chicken feathers waste [1]. Keratin, a hard to degrade insoluble animal protein, represents 90% of this keratinous waste [2]. Feathers are poultry products rich in protein (mainly keratin), generated in very large amounts as a waste product from poultry processing industry. Industrially, a great part of the feather waste is cooked under high pressure and temperature, producing a feather meal that can be incorporated into poultry food stuff as a protein supplement. However feather meal has two important nutritional limitations namely amino acid imbalance and poor digestibility and most animal protein (feathers) is currently disposed by incineration [3]. Hydrothermal treatment achieves limited and varying nutritional improvement; sustains losses of essential amino acids such as lysine, methionine and tryptophan and causes the formation of non-nutritive amino acids [4]. Considering the thermo energetic cost of conventional processing of feather against the backdrop of its limited nutritional improvement, investigation into alternative technology with prospects for nutritional enhancement, environmental friendliness or compatibility, bioresources optimization and cost effectiveness seems justifiable.

Response surface methodology, an experiment strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization. This method has been successfully applied for media optimization in different fermentation processes 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 [5].

Optimization of the fermentation process parameters through a statistical approach, such as Plackett Burman 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. Furthermore, the possible industrial application of an alkaline keratinase purified from this strain in laundry detergent formulations and in leather industry as a dehairing agent was also explored [6].

Production using central composite design optimization of medium by the classical method involves changing one independent variable (i.e., nutrient, pH, temperature) while unchanging all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables and also may result in wrong conclusions (Ref.). Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses [7], this method has been successfully applied in many areas of biotechnology such as bioconversion of cheese whey to mycelia of *Ganoderma lucidum*, optimization of neomycin production, enzyme production, enzyme kinetics and

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bacteriocin production. With respect to protease production, it was utilized for example for Bacillus species. Extracellular protease production by microorganisms is greatly influenced by physical factors such as pH, temperature and incubation time and by others factors such as media composition and presence of metal ions (Ref.).

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 [8]. Therefore, this study was mainly focused on statistical optimization of keratinase production using central composite design for high yield with low cost.

MATERIALS AND METHODS

Isolation and Identification of Microorganism: The soil sample was collected from the feather dumping site at Sivakasi, Tamilnadu, India. The selected isolate shows a clear zone around the colony in skim milk agar plates. Then biochemical, carbohydrate test, FAME analysis and 16S rDNA sequences were performed.

Keratinase Assay: To test the keratinolytic activity of keratinase on azokeratin, 5mg of azokeratin was added to a 1.5ml centrifuge tube along with 0.8ml of 50mM potassium phosphate buffer (pH 7.5) kept at 37°C for 1h with constant agitation (900rpm). This mixture was agitated until the azokeratin was completely suspended. 0.2ml aliquot of supernatant of crude enzyme (From where) was added to the azokeratin, mixed and incubated for 15 min at 50°C with shaking. The reaction was terminated by adding 0.2ml of 10% trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity.

The absorbance of the filtrate was measured at 450nm with a UV-160 spectrophotometer (Company). A control sample was prepared by adding TCA to a reaction mixture before the addition of enzyme solution. The unit of keratinase activity was defined as 0.01 unit increase in the absorbance at 450nm as compared to the control after 15min of reaction [9].

Optimization of Keratinase Production by Central Composite Design (RSM): Response surface methodology was used to optimize the conditions for the extracellular production of keratinase by *Bacillus cereus*. Central composite design was used consisting of three factors at two level patterns. The pH, temperature and starch were taken for optimization by a rotatable central composite design (CCD) resulting in 20 experimental runs. The treatment combinations were allocated into block. The first block contained the factorial runs accompanied by four central runs. The second block contained the axial runs accompanied by two central runs. The modeling and statistical analyses were performed using Design Expert, version 8.0.4.1 software.

RESULTS

Statistical Condition for Optimization of Keratinase Production by *Bacillus cereus* TS1 Using RSM: Keratinase production by *Bacillus cereus* was optimized using CCD and RSM. The quadric model in the version of design expert 8.0.4.1 software keratinase production of this bacterium was optimized by varying concentration of the medium components especially starch (carbon source) and the two factors (pH and temperature). The optimization of condition was performed using CCD with fixed central points of pH 9, temperature 50°C and starch 1%. The coded values and the levels of variables used in the central composite design were shown in the Table 1. RSM helps in evaluation of relationship between dependent (keratinase production) variable and independent variable (media components and factors like pH, temperature) observed and predicted values of the keratinase production as shown in the Table 2. The activity of the model can be seen between observed and predicted values. The co-efficient and analysis of variance are presented in the Table 3. The model F value of 3.62 implies the model is significant. There is only a 2.87%

Table 1: The coded values and the levels of variables used in the central composite design for *Bacillus cereus* TS1

Factors	Lower limit	Central point	Upper limit
pH	8.0	9	10.0
Temperature	45.0	50	55.0
Starch	0.5	1	1.5

Table 2: ANOVA for model used in keratinase production by *Bacillus cereus* (TS1)

Terms	*Bacillus cereus* (TS1)
F Value	3.6200
P>F*	0.0287
Mean	76.6200
R^2	0.7651
Adjusted R^2	0.5500
Co-efficient variance %	8.3500
Adequate precision	5.0100

*P>F value less than 0.05 indicate that the model term is significant

Table 3: Central composite design for *Bacillus cereus* TS1 fermentation processes

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A: pH	B: Temperature	C: Starch	Actual Keratinase U/ml	Predicted Keratinase U/ml
1	11	55	1.5	47.23	49.41
2	9	55	0.5	55.70	57.21
3	11.68	50	1	42.78	44.69
4	10	50	1	60.67	61.89
5	9	55	1.5	46.54	48.00
6	10	50	1.0	60.67	61.87
7	11	45	0.5	38.56	40.58
8	10	41.59	1.0	40.12	42397
9	9	45	1.5	39.03	40.33
10	9	45	1.0	41.78	43.27
11	10	58.41	1.0	47.92	49.24
12	10	50	0.5	60.67	61.05
13	11	55	1.5	48.23	50.14
14	11	45	1	46.26	49.36
15	10	50	1	60.67	61.03
16	10	50	1.84	53.78	55.37
17	10	50	1	37.86	40.21
18	8.32	50	0.16	60.67	61.25
19	10	50	1	59.15	60.41
20	10	50	1	60.67	61.48

chance that a "Model F-Value" this large could occur due to noise. Values of "probe >F-value less than 0.0500 indicate model terms are significant. In this care A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The fit of the model was checked by the co-efficient of determination R^2 was calculated to be 0.7657 indicating that 14.5% of variability in the response could be explained by the model.

Regression Equation for the Level of Keratinase Production in Terms of Coded Factor: (Y1)=+63.17-0.75*A+1.23*B+1.31*C-1.85*A*B+0.47*A*C-1.32*B*C-4.00*A^2-2.80*B^2-4.98*C^2.

By optimizing the above equation the following conditions were obtained, the maximum keratinase production predicted by the model was 64.06 Revise with Table 3 U/ml. The excellent correlation between predicted and actual values of this experiment justifies the validity of the response model and the existence of an optimum point.

Response surface and contour plot figures obtained by the analysis of the experimental data of CCD showed a relationship between two variables at time while maintaining third variable at fixed level. These figures are helpful in understanding both linear and interaction effect of two variables. The 3-D response surface plot described by the regression model were drawn to illustrate the combined effects of the independent variables and combined effects of each independent variables upon the response variable. Fig. 1a shows the interaction of pH and temperature with the fixed coded values of starch in g/L, an increasing pH with simultaneous increase in temperature let to an initial increase in keratinase production until they reached the optimal keratinase production which shows that the pH 9 and temperature 50°C. The data observed by the varying concentration of starch and varying pH keeping temperature constant at 50°C was plotted (Fig. 1b). It shows that initial increase in pH with simultaneous increase in starch concentration resulted in an increase keratinase production. However the increase beyond this limit has affected the keratinase production.

Fig. 1c shows that RS plots illustrating the effect of the temperature and starch keeping pH constant at 9. The plot revealed that the keratinase production was low at lower limit and increasing temperature resulted in an increasing keratinase production where as increasing starch concentration resulted in less production of keratinase.

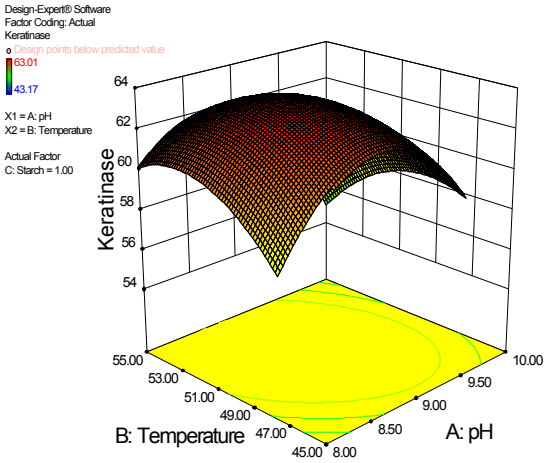

Fig. 1a: Interaction between temperature and pH.

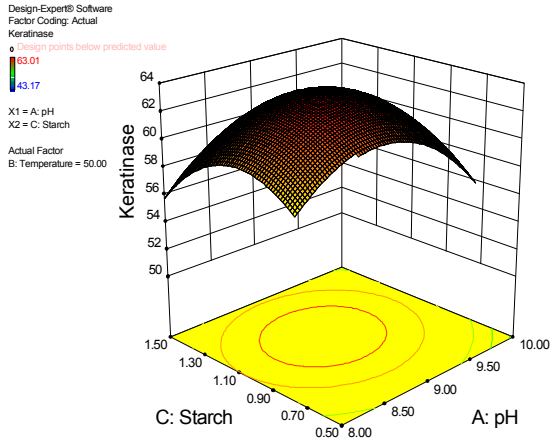

Fig. 1b: Interaction between starch and pH.

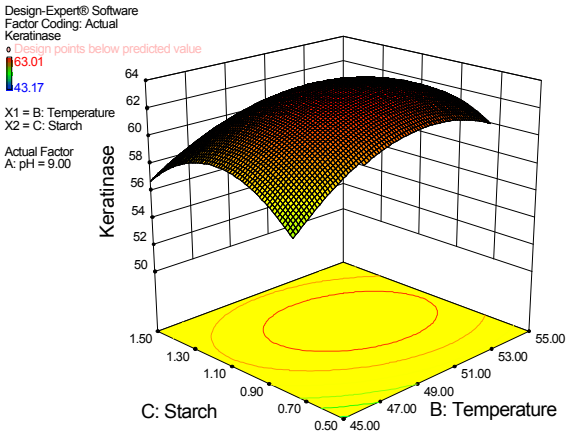

Fig. 1c: Interaction between starch and temperature.

Fig. 1: Response surface plots showing interaction between variables on the production of keratinase by *Bacillus cereus*

DISCUSSION

Response Surface Methodology (CCD): The use of statistical model to optimize culture medium components and conditions is increased in a present day biotechnology due to its easy applicability, reliability and validity. In the present study the significant variables necessary for the enhancement of keratinolytic enzyme production were selected using the central composite design (CCD). The RSM applied to the optimization of keratinase production in this 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 successful applied to the production of keratinase by Zauari *et al.* [10]. In *Bacillus pumilus* AI, whereas the maximum production was 87.73 U/ml. the present study showed that the maximum keratinase enzyme production was 63.01 Revise with Table 3 U/ml by *Bacillus cereus* TS1.

The three factors namely pH, temperature and starch were used for RSM optimization in *Bacillus cereus*. The factors like glucose, soybean and incubation time were used for RSM optimization by Tiwary and Gupta Revise authors names [11]. The variable used by Zauari *et al.* [10] were feather meal, soy peptone, sodium chloride, potassium chloride and potassium dihydrogen phosphate and the factors like sucrose, yeast extract and feather keratin were used by Xian *et al*. [12].

Twenty run experimental set up was used in RSM in our study for the production of enzyme keratinase. Similar run experimental setup was used in keratinase production by Tiwary and Gupta [13]. Seven experimental setups for maximizing the production of keratinase using RSM were demonstrated by Xian *et al*. [12]. Similar work for keratinase production using response surface methodology was performed by Zauari *et al*. [10], Xian *et al*. [12] and Tiwary and Gupta, [13]. The medium components play an important role in protease beta keratinase production by bacteria [14]. Therefore designing an appropriate fermentation medium is of critical importance in optimizing the product yield. Since this design is a preliminary optimization technique which tests only two levels of each factor, it cannot provide the optimal quantity acquired for the optimum enzyme production it provides indication of how each factor tends to effect of bacterial growth and enzyme production [15].

The value of beta keratinase field obtained by batch culture is slightly higher than observed highest experimental value in shake flask study as well as the prediction value of the protease yield by RSM. A slight variation in the experimental condition may lead to discrepancy of beta keratinase yields in a shake flask and in a bioreactor may be due to slight variation in experimental conditions [15]. Oxygen transfer condition and especially the dissolved oxygen tension were reported amongst the vital factors for microbial enzyme synthesis [16, 17].

As a useful statistical technique, RSM has widely and successfully been applied to the optimization of medium components. At present, large research aims to isolate feather degrading microorganism and investigate the characterization of feather degrading enzyme for socio-economic importance [18].

Using RSM based on CCD model, by optimizing with coded factors the maximum keratinase production observed by the model was 61.89 U/ml. Response surface methodology is an efficient method and easy to handle large number of design parameters and maximum the keratinase yield with low cost of production.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Department of Microbiology, Ayya Nadar Janaki Ammal College and Sivakasi for providing the facilities to carry out this work successfully.

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