Journal of Global Biosciences ISSN 2320-1355
Vol. 3(2), 2014, pp. 477-483 http://mutagens.co.in
Date of Online: 26, March- 2014

STATISTICAL OPTIMIZATION OF KERATINASE PRODUCTION BY *BACILLUS THURINGIENSIS*

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Abstract

This study presents optimum parameters for keratinase production by *Bacillus thuringiensis* 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 thuringiensis* were: pH 10, temperature50°C and mannitol (1%). By optimizing with coded factor the maximum keratinase production observed by the model was 63.01 U/ml.

Key words: Keratinase %, RSM (CCD) Optimization, *Bacillus thuringiensis*.

INTRODUCTION

Keratins are valuable but unavailable fibrous animal proteins. They are components of a range of by-products occurring especially abundantly in slaughterhouses and meat and poultry plants: skin remains, bristle, animal hair, horns and hooves, feathers, etc. Keratin waste is classified as category 3 materials in regulation (EC) 1774/2002 of the European Parliament and Council of 3rdOctober 2002 laying down health rules concerning animal by-products not intended for human consumption. It is not suitable for consumption but does not transmit diseases to humans or animals and is obtained from carcasses of animals suitable for human consumption. The high number of disulfide bonds in the structure of a-keratin makes it insoluble and resistant to enzymatic lysis (proteases) (FilipelloMarchisio, 2000). This is a major obstacle in native keratin processing. Animal remains rich in a-keratin, such as animal skin and hair, human hair, horns, or claws, are in nature relatively quickly biodegraded by keratinolytic microorganisms represented by some bacteria and fungi. They use native keratin as the sole source of C, N, S and energy.(Burtt and Ichida, 1999).

Screening for non-pathogenic microorganisms with keratinolytic activity may prevent the need for isolation and purification of the enzymes. Their biomass could autolytically contribute to the protein and amino acids content of the fermented feather meal. The upgrading of the nutritional value of feathers should yield an enhanced protein feedstuff that may reduce the use of soybean and fish meal in livestock diets. (Bertsch and Coello, 2005).

Further, the industrial application and hence the market demand of keratinases as compared to other industrial enzymes is still in the stage of infancy and effort should be directed to reduce the keratinase production cost. 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 (Ramnani and Gupta,2004). Therefore, the present study was focused on statistical optimization of protease production using central composite design. Furthermore, wealso explored the possible industrial application

MATERIALS AND METHODS

OPTIMIZATION OF SIGNIFICANT VARIABLES FOR KERATINASE PRODUCTION USING CENTRAL COMPOSITE DESIGN (CCD)

To find the optimal cultivation conditions for keratinase production, CCD with five coded levels was used for locating the true optimum conditions of Mannitol (carbon source), pH (10) and Temperature (50 ºC). 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 center 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 Eq. 2 using a multiple regression technique.

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

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

KERATINASE PRODUCTION BY OPTIMIZED PARAMETERS

After 96 hours of incubation on optimized medium mannitol (carbon source) , pH (10) and Temperature 50 ºC,the culture medium was centrifuged at 5000 rpm for 15 minutes. The supernatant was used as crude enzyme source for keratinase assay.keratinase activity was assayed as per the method of Burtt andLchida (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 hour with constant agitation (900 rpm). This mixture was agitated until the Azo-casein 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 trichloro acetic 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. 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 0.01unit increase in the absorbance at 450nm as compared to the control after 15minutes 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

Table 1.The coded values and the levels of variables used in the central composite design for TS2.

Factors	Lower limit	Central point	Upper limit
pH	9	10	11
Temperature	45	50	55
Mannitol	0.5	1	1.5

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. 8a. the activity of the model can be seen in different between observed and predicted values. The co-efficient and analysis of variance are presented in the Table. 8b. the model F–value of 12.12 implies the model is significant. There is only a 0.03% chance that a “Model F-Value” this large could occur due to noise. Values of “probe >F-value less than 0.05 indicates model terms are significant. In this case B, 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 0.9160 indicating that 17.2 % of variability in the response could be explained by the model.

Table 2.ANOVA for model used in keratinase production by *Bacillus thuringiensis* (TS2)

Terms	***Bacillus thuringiensis* (TS2)**
F Value	12.12
P>F*	0.0003
Mean	156.70
R^2	0.9160
Adjusted R^2	0.8404
Co-efficient variance %	7.29
Adequate precision	9.618

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

Table 3. Central composite design for *Bacillus thuringiensis* (TS2)

Run	Factor 1 A:pH	Factor 2B:Temperature	Factor 3 C.Mannitol	Actual Keratinase U/ml	Predicted Keratinase U/ml
1	9.00	50.00	0.16	47.32	48.51
2	10.00	45.00	0.50	43.17	44.54
3	7.32	50.00	1.00	56.43	57.26
4	9.00	50.00	1.00	63.01	64.06
5	9.00	50.00	1.00	63.01	63.89
6	8.00	55.00	1.50	55.76	56.41
7	9.00	50.00	1.00	63.01	63.47
8	9.00	50.00	1.00	63.01	64.56
9	9.00	41.59	1.00	57.23	58.29
10	9.00	50.00	1.00	63.01	64.39
11	9.00	58.41	1.00	58.87	59.11
12	10.68	50.00	1.00	52.86	53.22
13	8.00	45.00	0.50	48.86	49.94
14	8.00	45.00	1.50	43.84	44.28
15	10.00	45.00	1.50	54.77	55.54
16	10.00	55.00	0.50	52.97	53.98
17	9.00	50.00	1.84	56.43	57.21
18	8.00	55.00	0.50	51.32	52.35
19	9.00	50.00	1.00	63.01	64.39
20	10.00	55.00	1.50	44.58	45.00

Regression Equation for the level of keratinase production in terms of coded factor:

(Y1)= +60.57+0.46 *A+3.31*B +1.98* C-1.36 *A*B+1.65*A*C-2.58*B*C-6.62*A^2-5.22*B^2-4.58*$C^2$by optimizing the above equation the following conditions were obtained the maximum keratinase production predicted by the model was 6 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. 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 10 and temperature 50^0C. The data observed by the varying concentration of starch and varying pH keeping temperature constant at 50^0C is plotted in Fig. 1b. It shows that on initial increase in pH with simultaneous increase in mannitol concentration resulted in an increase keratinase production. However on increase beyond this limit has affected the keratinase production.

Fig. 1c shows that RS plots illustrating the effect of the temperature and mannitol keeping pH is constant at 10. The plot revealed that the keratinase production was low at lower limit and increasing temperature resulted in an increasing keratinase production whereas increasing mannitol concentration less production of keratinase.

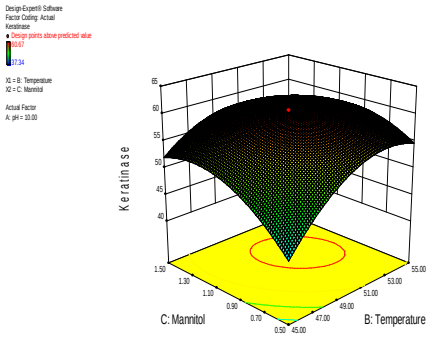

Fig . 1a. Interaction between mannitol and temperature.

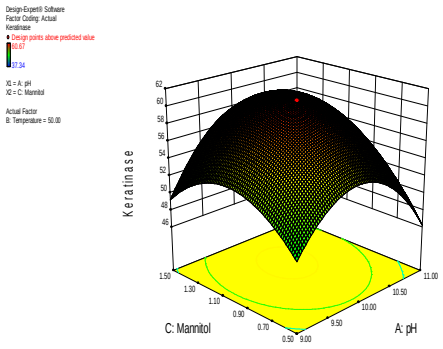

Fig. 1b.Interaction between mannitol and pH

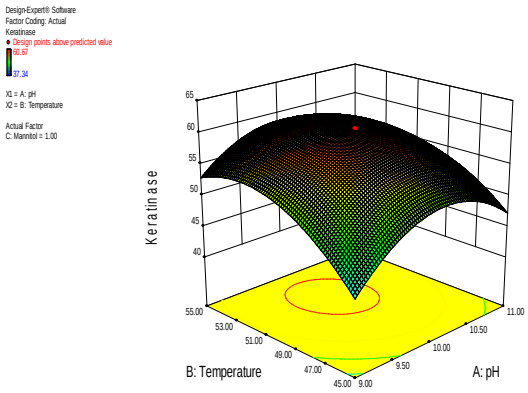

Fig.1c . Interaction between temperature and pH

Fig. 1a, 1b and 1c. Response surface plots showing interaction between variables on the production of keratinase by *Bacillus thuringiensis*

DISCUSSION

The use of statistical model to optimize culture medium components and conditions as 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 was selected using the Central composite design (CCD). Response surface methodology (RSM), a statistical approach, has an advantage over the traditional methods, of dealing with one variable at a time because the latter is a time consuming process and does not account for the combined interactions among various physicochemical parameters.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 variables at fixed level. These figures are helpful in understanding both linear and interaction effect of two variables. The 3D 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.

The Plackett – Burman experimental design proved to be a valuable tool for the rapid evaluation of the effect of the various medium component (Plackett and Burman, 1946) 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 successful applied to the production of keratinase by Zauari et al. (2010) in *Bacillus pumilus* AI, whereas the maximum production was 87.73 U/ml. the present study shows that the maximum keratinase enzyme production 63.01 U/ml was obtained by *Bacillus thuriengiensis.*

The three factors namely pH, temperature and mannitol were used for RSM optimization in *Bacillus thuriengiensis* TS2. The factors like glucose soybean and incubation time were used for RSM optimization by Tiwary and Gupta (2010a). The variable used by Zauari*et al.* (2010) were feather meal, soy peptone, sodium chloride, potassium chloride and potassium dihydrogen phosphate and the factors like sucrose, yeast extract and feather keratin was used by Xian et al. (2010) and Similarly, the factors like glucose soybean and incubation time were used for RSM optimization by Tiwary and Gupta (2010). The variable used by Zauari*et al.* (2010) were feather meal, soy peptone, sodium chloride, potassium chloride and potassium dihydrogen phosphate and the factors like sucrose, yeast extract and feather keratin was used by Xian et al. (2010).

Twenty run experimental setups was used in RSM in our study for the production of enzyme keratinase. Similar run experimental setup was used keratinase production by Tiwary and Gupta, (2010b). Seven experimental setup for maximizing the production of keratinase using RSM were demonstrated by Xian et al. (2010). Similar work for keratinase production using response surface methodology was performed by Xian et al. (2010); Tiwary and Gupta, (2010) and Zauari et al.(2010).

WhereasSivakumar et al. (2011)three factors namely pH, temperature and starch were used for RSM optimization in *Bacillus cereus.* 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. The maximum keratinase enzyme production was 63.01 U/ml by *Bacillus cereus* TS1.The above result favor to correlation to this study.Similarly the medium components play an important role in protease beta keratinase production by bacteria (Mukherjee et al., 2008; Rai and Mukherjee 2009; Rai et al., 2010). Therefore the designing an appropriate fermentation medium is critical importance in optimizing the product yield. Since this design is preliminary optimization techniques which test only two levels of each factor, it cannot provide the optimal quantity acquire for the optimum enzyme production it provides indication of how each factor tends to effect of bacterial growth and enzyme production (Kim et al., 2001).

The value of beta keratinase field obtained by batch culture 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 is 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 (Rai*et al.*, 2010). Oxygen transfer condition and especially the dissolved oxygen tension were reported amongst the vital factors for microbial enzyme synthesis (Calik et al., 1998; Rai and Mukherjee, 2009).

As a useful statistical technique, RSM has widely and successfully been applied to the optimization of medium components. At present, large research was aimed to isolate feather degrading microorganism and investigated the characterization of feather degrading enzyme for socio–economic importance (Wu *et al.*, 2007 and Matsui et al., 2009).

CONCLUSION

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 surfacemethodology is an efficient method and easy to handle large number of design parameters and maximum the keratinase yield with low cost of production.

ACKNOWLEDGEMENT

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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