Optimization of Protease Production from *Aspergillus Oryzae* Sp. Using Box-Behnken Experimental Design

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Received 2 July 2006; Accepted 3 August 2006

Abstract: Protease production by *Aspergillus oryzae* was optimized in shake-flask cultures using Box-Behnken experimental design. An empirical model was developed through response surface methodology to describe the relationship between tested variable (peptone, glucose, soyabeanmeal and pH). Maximum enzyme activity was attained with Peptone at 4 g/L; temperature at 30 °C glucose at 6 g/L; 30 °C and pH at 10. Experimental verification of the model showed a validation of 95%, which is more than 3-fold increase compare to the basal medium

Keywords: Box-Behnken design, *Aspergillus oryzae*, medium optimization and protease

Introduction

Proteolytic enzymes are the most important group of enzymes produced commercially^{1,2}. Of these, proteases produced by alkalophilic microorganisms are investigated not only in scientific areas such as protein chemistry and protein engineering but also find wide application in laundry detergents, leather preparation, protein recovery or solubilization, meat tenderization, biscuit and cracker industry^{3,4}. The use of these enzymes as detergent additives in the 1960s stimulated their commercial development and led to a considerable expansion of fundamental research into these enzymes. Since then, there has been renewed interest in the discovery of proteases with novel properties⁵. With the renewed emphasis on biotechnology, microbial enzymes have received increasing attention and processes that

involve versatile utilization of microbial metabolic machinery for the production of enzymes are currently being studied with great interest. Unlike animal and plant proteases, microbial proteases can be produced in large quantities and genetic manipulation to increase activity is easier. Proteases are produced by a wide range of microorganisms including bacteria, fungus, yeast and also mammalian tissues. Currently, a large proportion of commercially available proteases are derived from *Bacillus* strains⁶⁻⁸ although potential use of fungal proteases is being increasingly realized⁹. Fungal proteases offer a distinct advantage over bacterial enzymes in terms of ease of down stream processing. Thirty to forty percent of the production cost of industrial enzymes is estimated to be the cost of the growth medium¹⁰. To develop a bioprocess for industrial purpose, it is important to optimize highly significant factors affecting this process. Optimization process involving one-variable-at a-time method (OVAT) is a time-consuming technique and it neglects the interaction between variables and it does not guarantee attaining optimal point¹¹. Box-Behnken optimization design abolishes these disadvantages, besides it creates empirical model equations that correlate the relationship between variables and response(s)¹².

In the present investigation, the objectives were to better understand the relationships between the factors (Peptone, glucose, soyabean meal and pH) and the response (enzyme activity), and to determine optimum conditions for protease production from *Aspergillus oryzae* using Box-Behnken experimental design.

Experimental

Chemicals: Casein for protease assay was from Qualigens Mumbai India. All other analytical reagents and media components used were of highest purity grade available commercially in India

Microorganism: The microorganism used in this study, *Aspergillus oryzae* 637 was obtained from National Collection of Industrial Microorganisms (NCIM), Pune India.

Production medium and enzyme preparation

A 5% (v/v) fungal suspension was transferred from an overnight nutrient broth seed culture to the production basal medium composed of (g /L): peptone, 5; yeast extract 3; glucose 2, malt extract 2 and pH 8. For the purpose of the experimental design, the production medium was prepared in different formulae as illustrated in Table 1. After 48 h the cells were removed by centrifugation and the supernatants were used for measurement of protease activity. In the present work, the effects of peptone, glucose, soya bean meal and pH on protease activity was evaluated. Levels of these factors were optimized for maximum protease production (the response) using one of the response surface methodologies, the Box-Behnken statistical design (Box & Behnken 1960). Table 1 represents a 29-trial experimental design, where each variable was tested in three different coded levels: low (–1), middle (0) and high (+1). The coded values correspond for Peptone: –1 (3 g/L), 0 (4 g/L), +1 (5 g/L). For Glucose: –1 (4 g/L), 0 (6 g/L), +1 (8 g/L). For Soya bean meal: –1 (1.5g/L), 0 (2g/L), +1 (2.5g/L). For pH: –1 (9), 0 (10), +1 (11). Once the protease activity (U/mL) was measured, a second-order polynomial model was fitted to the response data obtained from the design. The polynomial equation is in the following form:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{22}X_{22} + \beta_{33}X_{23} + \beta_{44}X_{24},$$

Where Y is the predicted response, β_0 is the model constant; X_1 , X_2 , X_3 and X_4 are independent variables; β_1 , β_2 , β_3 and β_4 are linear coefficients and β_{11} , β_{22} , β_{33} and β_{44} are the quadratic coefficients.

Table 1.

Trial No.	Peptone (g/L) Var 2	Glucose (g/L) Var 1	Soyabean meal (g/L)	pH	Resulting Protease activity (U/mL)
1	4	6	2.5	11	972
2	4	6	2	10	1016
3	4	8	1.5	10	946
4	4	4	1.5	10	926
5	3	8	2	10	912
6	4	8	2.5	10	952
7	4	6	2	10	1012
8	5	4	2	10	892
9	5	8	2	10	896
10	4	4	2	11	918
11	4	6	2.5	9	964
12	3	4	2	10	870
13	5	6	1.5	10	904
14	4	6	1.5	11	942
15	5	6	2	11	906
16	4	6	1.5	9	964
17	4	4	2.5	10	924
18	4	8	2	9	928
19	3	6	2	9	886
20	4	4	2	9	914
21	5	6	2	9	906
22	3	6	2.5	10	910
23	3	6	2	11	902
24	4	8	2	11	992
25	5	6	2.5	10	930
26	3	6	1.5	10	966
27	4	6	2	10	1006
28	4	6	2	10	1012
29	4	6	2	10	994

Data analysis and optimization

Microsoft Excel 2000 was used to fit the quadratic response surface model to the experimental data through the multiple regression analysis. Optimization of the culture conditions in terms of peptone, glucose, soya bean meal and pH was analyzed using the predictive polynomial model. Using Statistica version 6.0 software, three-dimensional plots were created by holding two variables of the model as constants. All data are the mean of triplicates.

Results and Discussion

Application of Box-Behnken design and data analysis

A preliminary experiment was carried out to monitor the growth of *A.oryzae* and enzyme production in the basal production medium that is malt extract-2, glucose-6, yeast extract-2, peptone-3, salt solution 50mL, pH 10 and temperature 30°C at 48 hours. We have obtained the maximum protease activity (950 U/mL)¹³. The maximum protease activity was attained after 48 h. Further experiments were carried out to obtain a quadratic model consisting of 24 trials plus three replicates at the center point (trials 9, 18 27,28 and 29). The design of this experiment is given in Table 1 together with the experimental results. Enzyme activity was measured at 48 h. Regression analysis was performed to fit the response function (protease activity) with the experimental data. The analysis of variance for the four variables (peptone, glucose, soya bean meal and pH) indicated that enzyme activity could be well described by a polynomial model with a relatively high coefficient of determination ($R^2 = 0.88$). The statistical analysis of the full model in Table 2 & 3 shows that glucose, soya bean meal and pH each had a significant effect on protease synthesis. The probability value of the coefficient of linear effect of peptone (χ^2) was very high, 0.98. In addition, the interaction coefficients of this variable with other variables were high, which indicates the insignificance of these coefficients. On the other hand, the effect of peptone shows high significance ($P = 0.000002$) and all other must be included in the mode (Fig 1) I. Non-significant interaction coefficients are eliminated and the reduced model can be expressed as follows:

$$Y = 898.4222 + 15.1667X_1 - 1.0X_2 + 0.3333X_3 + 5.8333X_4 + 23.1833X_1^2 + 35.0583X_2^2 + 8.8083X_3^2 + 15.4333X_4^2$$

Where X_1 , X_2 , X_3 and X_4 represent codified values for Peptone, Glucose, Soya bean meal and pH, respectively. Although the elimination of non-significant coefficients does not enhance the coefficient of determination of the polynomial model, it is worth mentioning that the lack of fit is insignificant in the reduced model. Accordingly, it can be assumed that the model accurately represents data in the experimental region (Strobel R & Sullivan G 1999) this was confirmed by the residual analysis of the data (data not shown). For more description of the relationship between bioprocess variables and the response partial-effects are illustrated in three-dimensional plots.

Table 2. ANOVA table

	Glucose	Peptone	Soya bean meal	pH
P value	0.000003	0.0000002	0.066808	0.00090
F value	25.769	49.211	3.102	10.163

Table 3. Critical values

	Observed minimum	Critical value	Observed maximum
Glucose (g/L)	4.0	6.32	8
Peptone (g/L)	3.0	3.99	5
Soya meal(g/L)	1.5	2.00	2.5
pH	9	10	11

The main results of this study are presented in Figures, which represents the expected protease response and correlation between variables in three dimensional plots. Figure 3a shows effects of peptone and glucose due to the significant interaction between them.

In Figure 3b it is obvious that maximum protease activity was attained at moderate levels of glucose (6 g /L) as reported by (Banerjee *et al* 1999) and soya bean meal (2 g/L) as reported by (Srinivasan and Dhar 1990). In Figure 3c illustrates that increasing peptone value to 4 at moderate level of soya bean meal led to maximum protease activity. Other three dimensional plots presenting the effect of protease activity with respect to pH and glucose (Fig 3 d), peptone and pH (Fig 3 e), soya meal & pH(Fig 3 f) respectively.

Pareto Chart of Standardized Effects; for Protease activity
4 3-level factors, 1 Blocks, 29 Runs; Protease activity

Figure 1. Pareto Chart of standard effects

Figure 2. Predicted vs. observed values

Figure 3(a). Effect of Glucose & Peptone on protease activity

Figure 3(b). Effect of glucose & soya meal on protease activity

Figure 3(c). Effect of peptone & soya meal on protease activity

Figure 3(d). Effect of pH & glucose on protease activity

Figure 3(e). Effect of peptone & pH on protease activity

Figure 3(f). Effect of Soya meal & pH on protease activity

Validation of optimum point

The adequacy of the model was examined by an additional experiment using the derived optimal conditions. The predicted value was 1009 U l⁻¹ and in the experimental value was 992 ± 20 U/mL. This is approx. 95% of the predicted value, which indicates that the generated model is an adequate prediction of the enzyme activity.

Conclusions

The response surface methodology allowed the development of an empirical polynomial model for the production of protease by an *Aspergillus oryzae* strain. The model was able to foresee accurately the protease activity by changing provided culture conditions. Application of such models is of great importance for industrial bioprocess.

Acknowledgements

I would like to thank all the faculty members for their valuable suggestions during the work.

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