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Statistical Optimization of Alkaline Protease Production Using Isolated Strain by Submerged Fermentation

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Authors' contributions

This work was carried out in collaboration between both authors. Authors TP and UG designed the study. Author TP performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors UG and TP managed the analyses of the study. Author TP managed the literature searches. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: Optimization of alkaline protease production using a newly isolated strain *Alternaria* sp. by submerged fermentation. The production of inexpensive proteolytic enzymes not only solves environmental problems, but also promotes the economic value and utilization of waste treatment.
Study Design: Response Surface Methodology (RSM) was employed to optimize the environmental parameters to enhance protease production.
Place and Duration of Study: Department of Food technology and Biochemical Engineering, Jadavpur University, Kolkata, West Bengal, India between July 2014 and September 2014.
Methodology: The isolated culture *Alternaria* sp. was grown on modified Czapek-Dox media. The statistical design RSM was utilized to optimize the parameters: Volume of medium, temperature, time, age of inoculum and agitation showed significant influence on enzyme production. The data on alkaline protease production was processed by Analysis Of Variance (ANOVA). The

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mathematical relationship of independent variables and second order polynomial equation was used for the analysis of protease production.
Results: RSM was employed to optimize environmental factors for production of alkaline protease. The highest specific activity was obtained using 40 ml of medium, inoculated at 30℃ for 9 days at 120 rpm using 7 days old culture. However the maximum biomass production was obtained with 40 ml medium, 30℃ temperature, 5 days of fermentation, 140 rpm agitation using 7 days old culture and it was 7.76 mg/ml which was very close to 7.78 mg/ml predicted by Box-Behnken design (RSM).
Conclusion: The specific activity which was found to be 200 U/mg optimized by one-factor at a time, was later on calculated as 615 U/mg optimizing the same with the statistical approach. Thus it can be concluded that the optimization of Alkaline protease production gave significant higher specific activity when carried out with the process parameters non-individually.

Keywords: Alkaline protease; casein; Czapek-Dox; RSM; Box-Behnken.

1. INTRODUCTION

Proteases are complex enzymes responsible for the hydrolysis of protein molecules [1]. These enzymes account to 60% of total enzyme sales in the world [2]. Alkaline proteases are the most important group of enzymes exploited commercially for applications in meat tenderization, detergents, cheese-making, de-hairing, baking, waste management and silver recovery [3,4].

Alkaline proteases are produced by a wide range of microbes, including bacteria, molds, yeasts and mammalian tissues [5]. Two third of the commercial proteases are obtained from microbial sources [6]. The various microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce these enzymes [7]. Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe [8]. *Aspergillus clavatus* ES1 has been recently identified as a producer of an extracellular bleaching stable alkaline protease [9].

Biosynthesis of extracellular protease is greatly influenced by media composition especially carbon and nitrogen sources and other factors such as temperature, pH, incubation time, agitation and inoculum density [10].

Proper design and optimization of various process parameters are required for successful operation of submerged fermentation. Optimization of parameters by traditional “One-variable-at–a-time” method has some limitation such as time consuming, requirement of more experimental data sets, and interactions among variables are not considered [11].

Owing to these disadvantages, it has been substituted by statistical optimization such as response surface methodology (RSM). This statistical approach is an efficient tool for designing experiments, building models, evaluating the effects of factors and optimizing different parameters for desirable responses [12].

In the present study statistical optimization was carried out to determine the influence of variables on bio-catalytic property of the enzyme and for achieving maximum enzyme production under the best possible economic condition.

2. MATERIALS AND METHODS

2.1 Microorganisms

Alternaria sp. isolated from soil sample collected from local poultry farm was maintained on Potato Dextrose Agar (PDA) media and stored at 4℃.

2.2 Optimization of Protease Production

A loop full of culture was added into 50 ml of modified basal medium (pH 9.0) containing glucose 30%, casein 1%, KCl 0.5%, $FeSO_4$ 0.01%, $MgSO_4$ 0.5, K_2HPO_4 1% into 250 ml Erlenmeyer flask. The medium was incubated at 30℃ for 7 days at 120 rpm. After fermentation, the medium was centrifuged at 4000 rpm for 10 minutes and the culture filtrate was used as a crude enzyme.

2.3 Protease Assay

Protease activity was measured using the method described by Kembhavi et al. [13]. Briefly, a 0.5 ml of suitably diluted culture supernatant was mixed with 0.5 ml of 100 mM Tris-Hcl buffer (pH 8.0) containing 10 g/L casein. The reaction mixture was incubated for 30 min at 37℃ and stopped with adding 0.5 ml of 20% trichloroacetic acid. The mixture was allowed to stand at room temperature for 15 min and then filtered through Whatman no.1 filter paper. The activity of the filtrate was estimated spectrophotometrically at 280 nm. The proteolytic unit was defined as the amount of enzyme that released 1 µg of tyrosine per minute under the assay condition.

2.4 Protein Assay

Protein estimation was done by the method of Lowry et al. [14], with bovine serum albumin (BSA) as standard.

2.5 Fungal Biomass Measurements

Culture media were filtered using Whatman No. 1 filter paper and dried at 70℃ overnight [15].

2.6 Optimization by Response Surface Methodology

RSM was employed to study the interaction of independent variables (volume of medium, temperature, time, age of inoculum and agitation) and the responses (specific Activity of enzyme and biomass). A set of 46 experiments was generated, with three different levels (-1, 0 and +1) minimum, central and maximum value.

2.7 Statistical Analysis

Analysis of variance (ANOVA) was applied to optimize the parameters using the Box-Behnken design (Design-Expert® 7.0 Stat-Ease Inc., Minneapolis, USA). The design gave the second order multiple regression polynomial equation as:

$$Y = \beta_0 + \sum_{i}^{n} \beta_i X_i + \sum_{ii}^{n} \beta_{ii} X_i^2 + \sum_{ij}^{n} \beta_{ij} X_i X_j$$

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{15} AE + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{34} CD + \beta_{35} CE + \beta_{45} DE$$

Where, Y is Response variable, β_0 is Intercept, β_1 β_2, β_3, β_4, β_5 are linear coefficients, β_{11}, β_{22}, β_{33}, β_{44}, β_{55} are Squared coefficients, β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35}, β_{45} are interaction coefficients, A, B, C, D, E, A^2, B^2, C^2, D^2, E^2, AB, AC, AD, AE, BC, BD, BE, CD, CE, DE are variable factors according to the regression equation.

2.8 Validation of the Model

The second-order polynomial multiple regression equation obtained from the experimental data can be used to predict the response at any levels of the variables within the range of the experimental design. In order to determine the accuracy of the model, the five factors (volume of medium, temperature, time, age of inoculum, and agitation) were subsequently validated using the predicted values given by the statistical method. It showed significant similarity between the predicted and observed values obtained using the design, which showed the statistics to be true.

Table 1. Experimental range and levels of the five independent variables used in RSM in terms of actual and coded factors

	Range of levels					
Variable	**Actual**	**Coded**	**Actual**	**Coded**	**Actual**	**Coded**
Volume of medium	20	-1	30	0	40	+1
Temperature	20	-1	30	0	40	+1
Time	5	-1	7	0	9	+1
Age of inoculum	5	-1	7	0	8	+1
Agitation	110	-1	120	0	130	+1

3. RESULTS AND DISCUSSION

3.1 Experimental Design

A statistical design approach using RSM was employed to study the interactive effects of process parameters on protease production. Response surface methodology the most accepted statistical technique for bioprocess optimization can be used to examine the relationship between a set of practicable experimental factors and the observed results. A total of 46 experiments with diverse combinations of the selected parameters were performed. The variables selected for optimization, i.e., volume of medium, temperature, time, age of inoculum and agitation were coded as A, B, C, D and E respectively. The goodness-of-fit of the model was checked by determining the coefficient of determination (R^2) and adjusted R^2. The R^2 value is always between 0 and 1. The model is stronger and predicts better response when R^2 value is closer to 1 [16]. Smaller the P-value, more significant is the consequent coefficient.

This regression equation was evaluated statistically for analysis of variance and the results are predicted in Tables 2 and 3. A large R^2 value indicates that the regression has accounted for a large proportion of the total unpredictability in the observed value of Y which favors the regression equation model. In this case, the value of determination coefficients (R^2 = 99.58%; R^2 = 99.64%) for specific activity and biomass respectively, demonstrated a high correlation between the experimentally observed and predicted values.

From Tables 2, 3 the F value was found to be 295.28, 344.52 for specific activity and biomass respectively. The above value implied that the model is significant. There is only 0.01% chance that a model F value so large could occur due to noise. These P-values for the model and for lack of fit (3210.50 and 0.025) for specific activity and biomass respectively, also suggested that the obtained experimental data was accurately fitted by the model. The $p<0.05$ suggested that model terms are significant. The adjusted R^2 value (99.24% in specific activity and 99.35% in biomass) in the present study advocated for a high significance of the model. These results show that the response equation provided by the model is appropriate for the BBD experiment.

The results obtained by implementing Box-Behnken design were then analyzed by standard ANOVA which gave the following regression equation:

$$Specific\ activity = +200.00 + 115.06\,A - 35.81B + 40.25C - 39.50D + 84.75E + 6.00AB + 127.00AC - 100.75AD + 76.50AE - 6.25BC + 46.00BD - 18.00BE - 4.25CD + 25.00CE - 40.00DE + 57.85A^2 + 108.69B^2 + 72.10C^2 - 48.06D^2 + 52.60E^2$$

$$Biomass = +6.04 + 0.52\,A + 0.11B + 0.075C + 0.59D + 0.37E + 0.092AB - 1.12AC + 0.14AD + 0.55AE - 0.80BC + 0.015BD + 0.040BE - 0.40CD + 0.31CE + 0.76DE - 0.38A^2 - 0.11B^2 - 1.50C^2 - 1.21D^2 + 0.73E^2$$

Table 2. ANOVA for response surface quadratic model (Response – Specific Activity)

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	7.584E+005	20	37919.23	295.28	<0.0001	Significant
A-Volume of medium	2.118E+005	1	2.118E+005	1649.51	<0.0001	
B-Temperature	20520.56	1	20520.56	159.79	<0.0001	
C-Time	25921.00	1	25921.00	201.85	<0.0001	
D-Age of inoculums	24964.00	1	24964.00	194.39	<0.0001	
E-Agitation	1.149E+005	1	1.149E005	894.88	<0.0001	
AB	144.00	1	144.00	1.12	0.2998	
AC	64516.00	1	64516.00	502.38	<0.0001	
AD	40602.25	1	40602.25	316.17	<0.0001	
AE	23409.00	1	23409.00	182.28	<0.0001	
BC	156.25	1	156.25	1.22	<0.0001	

Source	Sum of squares	df	Mean square	F-value	P-value	
BD	8464.00	1	8464.00	65.91	<0.0001	
BE	1296.00	1	1296.00	10.09	0.0039	
CD	72.25	1	72.25	0.56	0.4602	
CE	2500.00	1	2500.00	19.47	0.0002	
DE	6400.00	1	6400.00	49.84	<0.0001	
A^2	29211.09	1	29211.09	227.47	<0.0001	
B^2	1.031E+005	1	1.031E+005	802.80	<0.0001	
C^2	45373.19	1	45373.19	353.32	<0.0001	
D^2	20160.03	1	20160.03	156.99	<0.0001	
E^2	24150.09	1	24150.09	188.06	<0.0001	
Residual	3210.50	25	128.42			
Lack of Fit	3210.50	20	160.52			not significant
Pure Error	0.000	5	0.000			
Cor Total	7.616E+005	45				

R^2 value 0.9958, Adjusted R^2 value 0.9924, Predicted R^2 value 0.9831

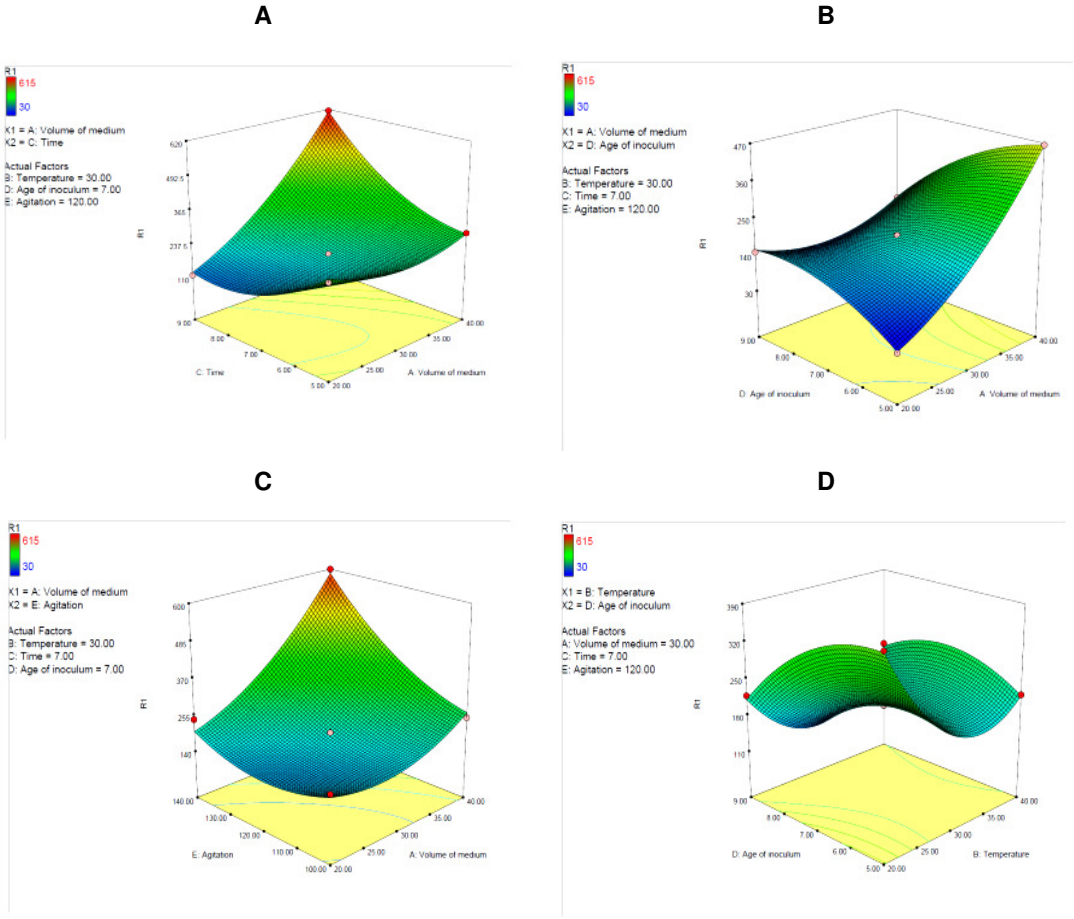

Fig. 1. (A-D) represents the 3D curves obtained for the interaction effects of the physical parameters for specific activity

The optimum level of each variable and the effect of their interaction on the production of the alkaline protease and biomass were studied by plotting 3D curves against any two independent variables, while keeping the other variable at its respective central values. (Fig. 1 A-D) represents the various interactions between the process parameters and their optimum values. The plots

are a representation of the specific activity varying with the chosen parameters in pairs. Thus the plot could be said to follow a quadratic model of fit. The multiple regression equations obtained from the ANOVA confirms the presence of different interactive plots as obtained. The predicted results showed the maximum specific activity of 615 U/mg was achieved by carrying out the fermentation in 40 ml medium at 30℃ for 9 days with 120 rpm agitation and using 7 days old culture and was seen to be in close agreement with the observed value of 614 U/mg. Mechanical agitation is known to be an essential factor in fermentation processes because of its efficacy in mixing the contents of the medium, uniform air distribution and prevention of cell clumping [17]. A good correlation coefficient of 0.9996 using Box-Behnken design and alkaline protease production of 112.90 U/ml by *Shewanella oneidensis* MR-1 strain through response surface methodology was also seen [18]. From the 3D plots in (Fig. 2 A-D), it is clearly indicated that the mutual interaction is highly prominent among parameters. The maximum biomass production of 7.76 mg/ml was very close to 7.78 mg/ml predicted by Box-Behnken design with 40 ml medium at 30℃ for 5 days with 140 rpm agitation and using 7 days old culture.

3.2 Validation of the Experimental Model

The model was validated with regard to all of the five variables within the design space. In order to determine the accurateness of the model, a random set of five experimental combinations were prepared and tested for the major effect on protease production. Validation of the model and regression equation were achieved by taking the optimum values of A, B, C, D, E. The second-order polynomial regression equation can be used to control the enzyme production. The values were in close agreement with the statistically predicted ones, confirming the model's legitimacy and applicability of the statistical model.

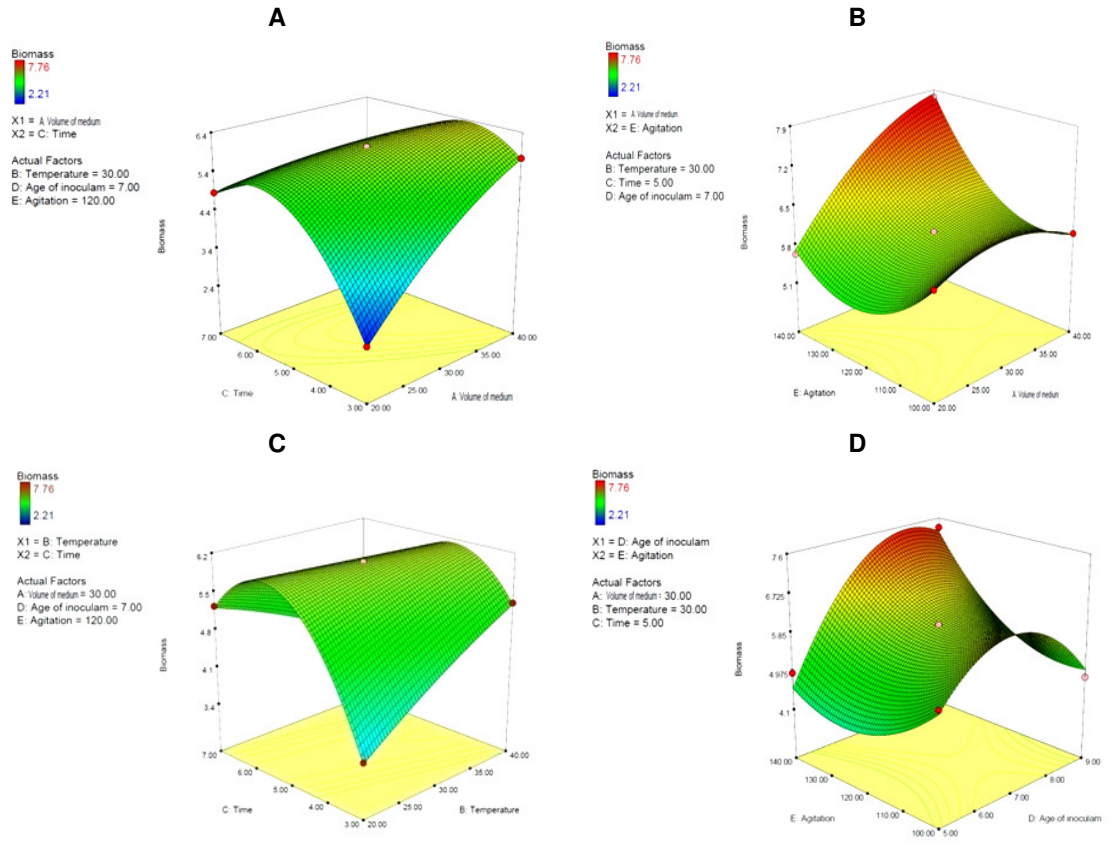

Fig. 2. (A-D) represents the 3D curves obtained for the interaction effects of the five physical parameters for biomass

Table 3. ANOVA for response surface quadratic model (Response – Biomass)

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	67.66	20	3.38	344.52	<0.0001	Significant
A-Volume of medium	4.26	1	4.26	434.15	<0.0001	
B-Temperature	0.18	1	0.18	18.42	0.0002	
C-Time	0.090	1	0.090	9.17	0.0057	
D-Age of inoculums	5.58	1	5.58	568.40	<0.0001	
E-Agitation	2.23	1	2.23	226.85	<0.0001	
AB	0.034	1	0.034	3.47	0.0744	
AC	4.97	1	4.97	506.43	<0.0001	
AD	0.084	1	0.084	8.56	0.0072	
AE	1.20	1	1.20	122.11	<0.0001	
BC	2.58	1	2.58	262.34	<0.0001	
BD	9.000E-004	1	9.000E-004	0.092	0.7646	
BE	6.400E-003	1	6.400E-003	0.65	0.4271	
CD	0.63	1	0.63	64.36	<0.0001	
CE	0.38	1	0.038	39.15	<0.0001	
DE	2.28	1	2.28	232.20	<0.0001	
A^2	1.23	1	1.23	125.22	<0.0001	
B^2	0.097	1	0.097	9.86	<0.0001	
C^2	19.52	1	19.52	1987.58	<0.0001	
D^2	12.73	1	12.73	1296.81	<0.0001	
E^2	4.66	1	4.66	474.14	<0.0001	
Residual	0.25	25	9.820E-003			
Lack of Fit	0.25	20	0.012			not significant
Pure Error	0.000	5	0.000			
Cor Total	67.91	45				

R^2 value 0.9964, Adjusted R^2 value 0.9935, Predicted R^2 value 0.9855

4. CONCLUSION

The study demonstrated the applicability of RSM for optimization of variables for better yield of alkaline protease by *Alternaria* sp. Finally the overall 3.1 fold increase in specific activity was achieved when the environmental parameters were optimized by RSM. The statistical model was successfully validated.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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