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Production of Alkaline Protease from *Bacillus licheniformis* through Statistical Optimization of Growth Media by Response Surface Methodology

BKM Lakshmi and KPJ Hemalatha*

Department of Microbiology, Andhra University, Visakhapatnam, Andhra Pradesh, India

***Corresponding author:** KPJ Hemalatha, Department of Microbiology, Andhra University, Visakhapatnam, Andhra Pradesh, India, Tel: 91-891-2844687; E-mail: hemalathakpj@gmail.com

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Abstract

An alkaline protease producing bacterial strain *Bacillus licheniformis* (MTCC NO. 7053), which was obtained from IMTECH, Chandigarh used for protease production studies. Three significant variables effecting protease production like rice husk (variable 1); inoculum size (variable 2); KNO_3 (variable 3) have been identified under preliminary optimization studies. These variables are selected for alkaline protease production in current study. By using these eloquent variables, the basal media was subjected to statistical optimization using response surface methodology. The superlative growth media components and bacterial growth conditions for ultimate protease production were as follows: rice husk (3%); inoculum size (2%); KNO_3 (0.75%); salt solution, 5% (v/v)-{($MgSO_4.7H_2O$, 0.5% (w/v); KH_2PO_4 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01% (w/v) at 37°C and 160 rpm for 72 h production. The alakaline protease activity was notably increased with statistically optimized medium (185.4 ± 0.23 U/ml) when compared to unoptimized basal medium (132 ± 0.76 U/ml). This shows that model was satisfactory and also indicates the adequacy of the model.

Keywords: Alkaline protease; *Bacillus licheniformis*; Media formulation; Response surface methodology

Introduction

In general enzymes are considered as "green chemicals" due to their eco-friendly nature and wide range of applications ranging from industrial sector to house-hold products. In the midst of proteases from different sources, microbial proteases exemplify largest group of commercial enzymes and account for relatively 60% of the total world enzyme sale leading industrial enzyme market worldwide. Proteases from microbes are classified as acidic or aspartic proteases, neutral proteases and alkaline or basic proteases depending on their maximum activity at pH [1]. Amidst of these, alkaline proteases had extensive applications in textile, pharmaceuticals, laundry detergent, food processing, leather and paper industries [2]. Media components of bacterial growth media have play a great role in extracellular alkaline protease production and it varies from one microorganism to another. Therefore the required media components with their concentrations have to be optimized accordingly [3-6]. As industrial fermentation techniques and methods are moving towards well optimized techniques apart from normal traditional practices, statistical optimization methods could be used [7].

Extracellular protease production in microorganisms is also strongly influenced by media components, e.g. variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose [8] and metal ions [9]. Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as aminoacids in the medium. Besides these, several other physical factors, such as aeration, inoculum density, pH, temperature and period of incubation, also affect the amount of protease produced [10]. In order to scale up protease production from microorganisms at the industrial level, biochemical and process engineers use several strategies to obtain high yields of protease in a fermenter. Controlled batch and fed-batch fermentation using simultaneous control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability [11] and chemostat cultures have been successfully used for improving protease production using a number of microorganisms.

Research efforts have been directed mainly toward the evaluation of the effects of various carbon and nitrogenous nutrients as cost effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation. In addition, no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme yield. Production of an enzyme exhibits a characteristic relationship with regard to the growth phase of that organism. The synthesis of protease in Bacillus species is controlled by numerous complex mechanisms operative during the transition state between exponential growth and the stationary phases [12].

The implementation of statistical methods for screening of growth media and optimization of notable factors leads to the identification and association between prominent factors. The important basic steps in statistical approach are to privilege and disclose the suitable range of preferred control factors (variables) in the starting experiments. In general, several research workers pick the ranges of test values for variables based on their experience. Hence selection of starting test range is the important step for obtaining the correct optimum response. Development of the cost effective fermentation process is very challenging. Response surface methodology (RSM) is an efficient mathematical approach widely applied to evaluate and understand the interactions between different parameters. It is relevant to select optimum conditions of variables and at the same time verifies a predicted model and desirable response or multiple responses.

Response surface methodology (RSM) is focused to detect optimal operating conditions of system or a region of the factor space by satisfying the operating parameters. It was used to study how the responses were influenced by various factors by maintaining their variation in a particular number of experiments. Hence it was thought that it will meet the scope of the current study as it provides better idea regarding the interactions of key media components which influences protease production. Therefore it was aimed to carry out the medium formulation in cost effective manner using RSM as it proved to be efficient in previous works. The current work explains the use of central composite design (CCD) for ultimate protease production by determining optimal conditions. Central composite design enhances the obtained information by minimizing the number of individual experiments required.

Materials and Methods

Microorganism

The microorganism (*Bacillus licheniformis*, MTCC NO. 7053) was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh.

Stock cultures of *Bacillus licheniformis* was maintained by keeping the culture in30% glycerol at -20oC. Alkaline protease production was carried out by using basal media which contains glucose, 0.5% (w/v); peptone, 0.75% (w/v); salt solution, 5% (v/v) - {($MgSO_4.7H_2O$, 0.5% (w/v); KH_2PO_4 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01% (w/v) at 160 revolution per minute (rpm). Preliminary bacterial growth studies and production kinetics of protease was performed with this basal medium.

Inoculum preparation

Bacterial cells (*Bacillus licheniformis*) from a 48 h aged culture in sterile inoculation media were used as inoculum for alkaline protease production. The inoculum medium is same as of basal medium. At first, the cultures were grown at 37°C for 24 h in an orbital shaking incubator until optimal growth was achieved. Optimum growth was monitored by using absorbance (optical density) and that culture was used for inoculation of production flasks.

Growth conditions

The alkaline growth medium (pH 10.0) used for protease production contains rice husk (variable); inoculum size (variable); KNO3 (variable); salt solution, 5% (v/v) - {($MgSO_4.7H_2O$, 0.5% (w/v); KH_2PO_4 0.5% (w/v)}; and $FeSO4.7H_2O$, 0.01% (w/v). The operating growth conditions were maintained at a temperature of 37°C and 72h incubation period in an orbital shaker incubator at 160 rpm.

Protease assay

The protease activity was performed according to Mc Donald and Chen method [13]. One unit enzyme activity is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per min under the assay conditions. The range of concentration 50-250 µg of tyrosine was used as standard.

Protein estimation

For total protein estimation, broth was harvested at 10,000 rpm at 4°C for 10 minutes. Appropriate aliquots from this supernatant were taken and total protein was estimated by Lowry method (1951) [14], using crystalline bovine serum albumin as standard.

Experimental design and optimization

The main aim of this experimental design is to upgrade the growth media composition for eloquent alkaline protease production by *Bacillus licheniformis* by optimizing the various levels of topmost factors which were selected under preliminary optimization studies [15]. In the preliminary study, it was revealed that rice husk (%)- X1, nitrogen source KNO3 (%)- X2 and inoculum size (%) – X3 were the major three independent variables which incomparably increases the alkaline protease yield (Y). An effect of these variables in different proportions on response was by a statistical method, response surface methodology (RSM).

Variables classify include

X1 =% r w /v rice husk concentration

X2 = % w/v Potassium nitrate (KNO3)

X3 = % v/v Inoculum size

The final response variable

Y1 = Protease activity

Central composite design (CCD) was used to determine the optimal concentrations of the above mentioned three variables. They were able to influence the alkaline protease production (Y) by *Bacillus licheniformis* at pH 10.0 and 37°C. Central composite design (CCD) actually has three groups of design points: two-level factorial or fractional factorial, axial and central points. It was used to estimate the coefficients of a quadratic model [16]. The three eloquent factors with respective coded levels consist of 17 experimental runs and they were used for analysis of experimental data for better estimation of the experimental error with extra information about yield of experimental region. The data of minimum and maximum range of investigated variables and full experimental plan with coded values were listed in Table 1.

Variables	Range and levels		
	-1	0	1
Rice husk (%) X1	1	2	3
Nitrogen source (KNO3) (%) X2	0.25	0.375	0.75
Inoculum size (%) X3	0.5	1.5	2

Table 1: Experimental range and levels of the independent variables.

All the statistical analysis including response surface plots were performed using Design expert Software® (Minneapolis. MN 55413-2726, USA). All the experiments were performed in triplicates.

The multiple regression analysis of the data was performed with the statistical package (Stat-Ease Inc., Minneapolis, MN, USA). The following second–order polynomial equation defines the predicted response (Y) interms of the independent variables (X1, X2 and X3) was:

$$Y = bo + b1X1 + b2X2 + b3X3 + b11X2 + b22X2 + b33X2 + b12X1X2 + b13X1X3$$

Where

bo = intercept term

b1, b2, b3 = linear coefficients

b11, b22, b33 = squared coefficients and

b12, b23, b13 = interaction coefficients

X1X2 and X1X3 are a combination factors exemplifies interaction between individual factors. Finally, response is a function of levels of factors and response surface graphs indicates individual and combinational effects of variables to determine their optimal levels for ultimate alkaline protease production. These predictions were validated using defined optimized medium and the experiments were carried out thrice.

Results and Discussion

Statistical optimization of *Bacillus licheniformis* using response surface methodology

Central composite design (CCD) results for growth media optimization of *Bacillus licheniformis* consists of predicted and experimental data for studying the effects of three selected independent variables namely rice husk, potassium nitrate and inoculum size on alkaline protease production were presented in Table 2.

S. No	Factor 1 A Rice Husk (%)	Factor 2 (X2) B. Nitrogen Source	Factor 3 (X3) C. Innoculum Size (%)	Response (Y) Protease Activity (U/ml) Observed	Response (Y) Protease activity(U/ml) Predicted
1	3	0.75	2	185.4 ± 0.23	184
2	2	0.375	1.5	109 ± 0.37	110
3	2	0.75	0.5	142.1 ± 0.29	140
4	1	0.375	0.5	81 ± 0.12	80
5	3	0.375	2.5	160 ± 0.25	161
6	2	0.375	1.5	90.7 ± 0.19	90
7	2	0.375	1.5	91 ± 0.25	90
8	1	0.25	1.5	65 ± 0.11	64
9	2	0.25	0.5	98 ± 0.16	97
10	3	0.375	0.5	177.6 ± 0.32	176
11	2	0.375	1.5	112 ± 0.27	110
12	3	0.25	1.5	146 ± 0.08	144
13	2	0.25	2.5	56 ± 0.34	57
14	2	0.375	1.5	89 ± 0.17	90
15	2	0.75	2.5	109 ± 0.29	108
16	1	0.375	2.5	73 ± 0.14	72
17	1	0.75	1.5	60 ± 0.27	61

Table 2: Experimental design along with observed and predicted protease activity.

The data was followed in accordance with mentioned second order polynomial function.

The analysis of variance (ANOVA)(Table 3) indicates that the model terms X1,X2,X3 were significant (" probe>F" less than 0.005).

Significance of the model was implied by the Model F-value of 17.11, which lies in the significant range. There is only a chance of 0.06% that this large F-value could occur due to noise. Values of "Probe>F" less than 0.0500 also indicates the model terms are significant. In this experimental model A, B, C, A^2 model terms are significant. The model terms with values greater than 0.1000 are not significant. The "Lack of Fit F-value" of 1.74 indicates that it is not significant relative to the pure error. Non-significant lack of fit is good. Analysis of variance (ANOVA) data includes regression equation analysis and multiple correlation coefficients which indicate that the model can explain variation in response. It also shows that the interaction between the variables, AB significantly influences the alkaline protease production than AC and BC. These results confirmed a satisfactory adjustment of the simplified quadratic model to the experimental data.

Source	Squares	df	Square	Value	Probe> F	
Model	24348.7	9	2705.41	17.11	0.0006	significant
A-Rice husk	15348.9	1	15348.9	97.05	<0.0001	significant
B-Potassium nitrate	1323.24	1	1323.24	8.37	0.0232	significant
C-Inoculum size	1006.16	1	1006.16	6.36	0.0397	significant
AB	670.14	1	670.14	4.24	0.0785	significant
AC	26.22	1	26.22	0.17	0.696	
BC	15.29	1	15.29	0.097	0.7649	
A^2	1566.99	1	1566.99	9.91	0.0162	significant
B^2	444.39	1	444.39	2.81	0.1376	
C^2	77.34	1	77.34	0.49	0.5069	
Residual	1107.04	7	158.15			
Lack of Fit	627.04	3	209.01	1.74	0.0965	significant
Pure Error	480	4	120			
Cor Total	25455.8	16				

Table 3: ANOVA for Response Surface Quadratic model.

Std. Dev.	12.58	R-Squared	0.9565

Mean	107.88	Adj R-Squared	0.9006
C.V. %	11.66	Pred R-Squared	0.8989
PRESS	15302.23	Adeq Precision	13.721

Table 4: Regression analysis.

The present model required small adjustment of R2 value, which corrects the correlation value of the large sample size and number of terms in the predictive model. In the present study, the adjusted R2 value is lesser than the actual R2 value (Figure 1). This is because, there are many terms in the model and the sample size is not very large.

Factor	Coefficient Estimate	df	Standard Error	95% CI (Low)	95% CI (High)	VIF
Intercept	98.16	1	5.62	84.86	111.45	
A-Rice husk	46.27	1	4.7	35.17	57.38	1.12
B-Potassium nitrate	47.86	1	16.55	8.74	86.99	6.97
C-Inoculum size	-11.82	1	4.68	-22.89	-0.74	1.14
AB	18.94	1	9.2	-2.82	40.69	1.19
AC	-2.55	1	6.26	-17.34	12.25	1.05
BC	-2.75	1	8.83	-23.63	18.14	1.21
A^2	19.45	1	6.18	4.84	34.06	1.02
B^2	-32.86	1	19.6	-79.21	13.49	7.01
C^2	4.44	1	6.35	-10.57	19.46	1.03

Table 5: Coded factors.

The "Pred R-Squared" and "Adj R-Squared" values are 0.8989 and 0.9006 respectively. "Adeq Precision" is a measure of signal to noise ratio. A ratio greater than 4 is desirable (Figure 2). The ratio of 13.721 indicates adequate signal and this model can be used to navigate design space. The interaction among the coded factors was shown in the following Table 5. The maximum alkaline protease activity of 184 U/ml was predicted by this model (Figure 3). The proposed medium composition was repeated thrice and it was proved that experimental values were in close agreement with predicted values indicate model authenticity (Figure 4). The *Bacillus licheniformis* produced 185.4 ± 0.23U/ml protease with specific activity 19.8U/mg under experimental cultural conditions with three significant variables which is higher than basal medium (132 ± 0.76 U/ml). The calculated recommended concentrations of the optimized independent variables by the Design expert software showing the highest desirability near to 1.0 (Figure 5).

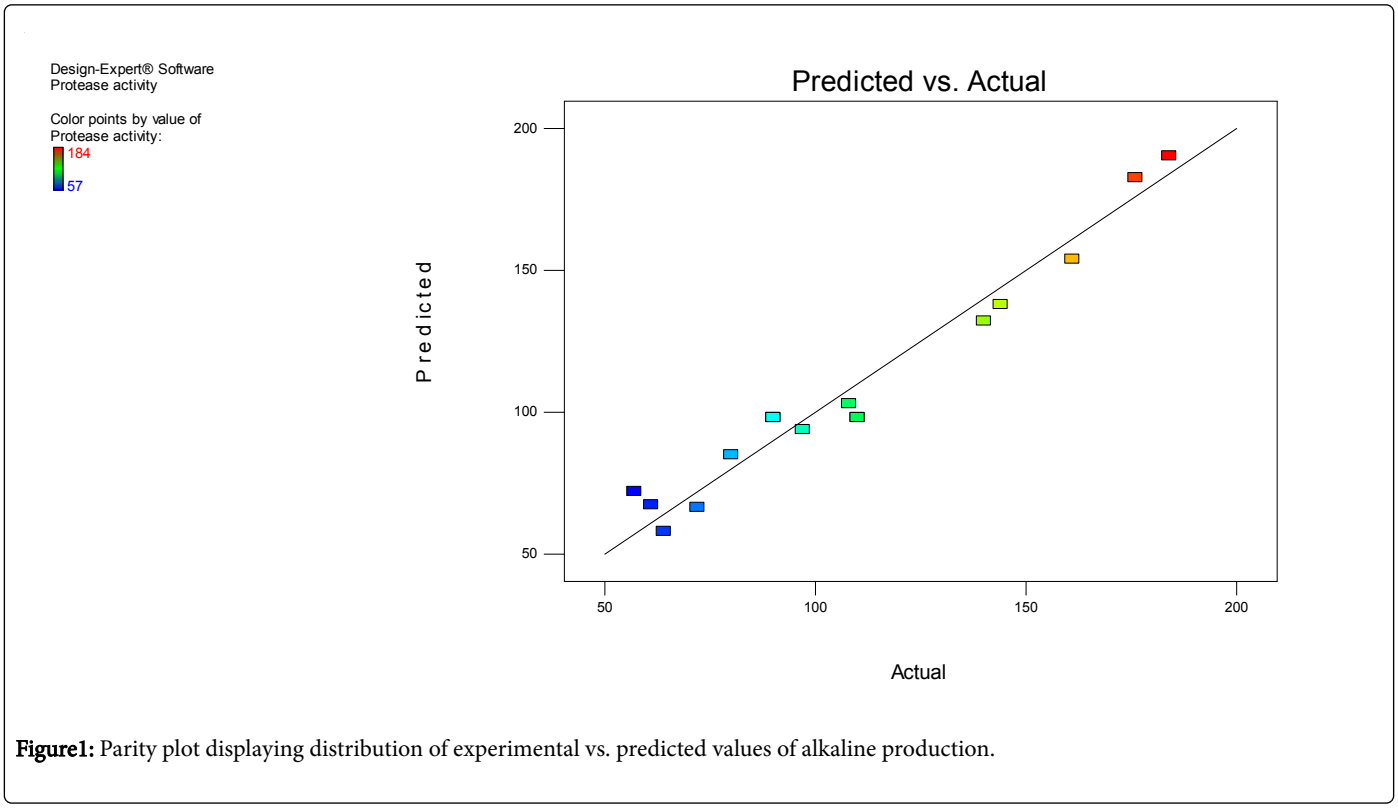

Figure1: Parity plot displaying distribution of experimental vs. predicted values of alkaline production.

The extensive grid and feasibility searches provided superlative conditions with respectively desired function response. The highest desirability indicates significance of the formulated medium using this method [17]. This statistical method (RSM) employed to study the different physicochemical factors on alkaline protease production from *Bacillus mojavenisis*. The predicted model of RSM for protease production was validated in a 14 L bioreactor and the response surface approach involving face centered composite design (FCCCD) was implemented for enhancing protease production by the *Bacillus* sp. RGR-14 by [18,19] also used the FCCCD for enhanced production of protease and biosurfactant by *B. licheniformis* RG1. The effects of four variables soy bean meal, maltose, Tween 80 and initial pH on protease

production were evaluated using RSM by Tari et al. [20,21] employed response surface methodology to produce protease of 518 U by *Bacillus subtilis* DM-04 through submerged fermentation [22] carried out the optimization of growth medium for protease production by Haloferaxlucentensis VKMM 007 with the help of RSM.

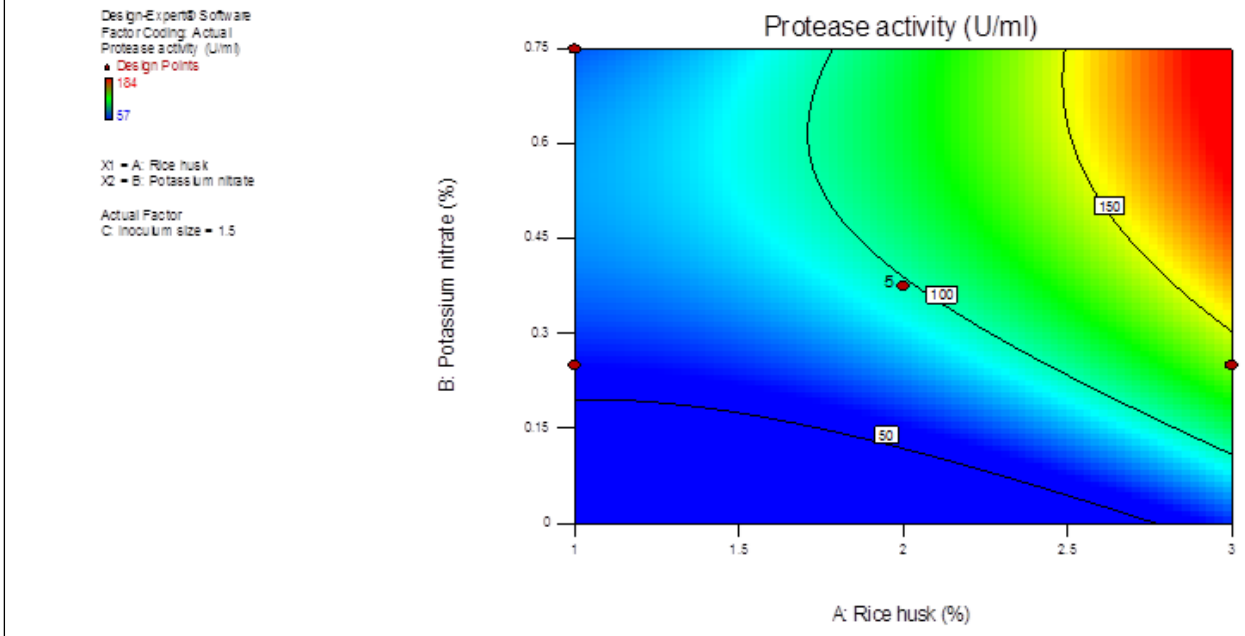

Figure 2: Contour plot displaying the effect of optimized components on alkaline protease production.

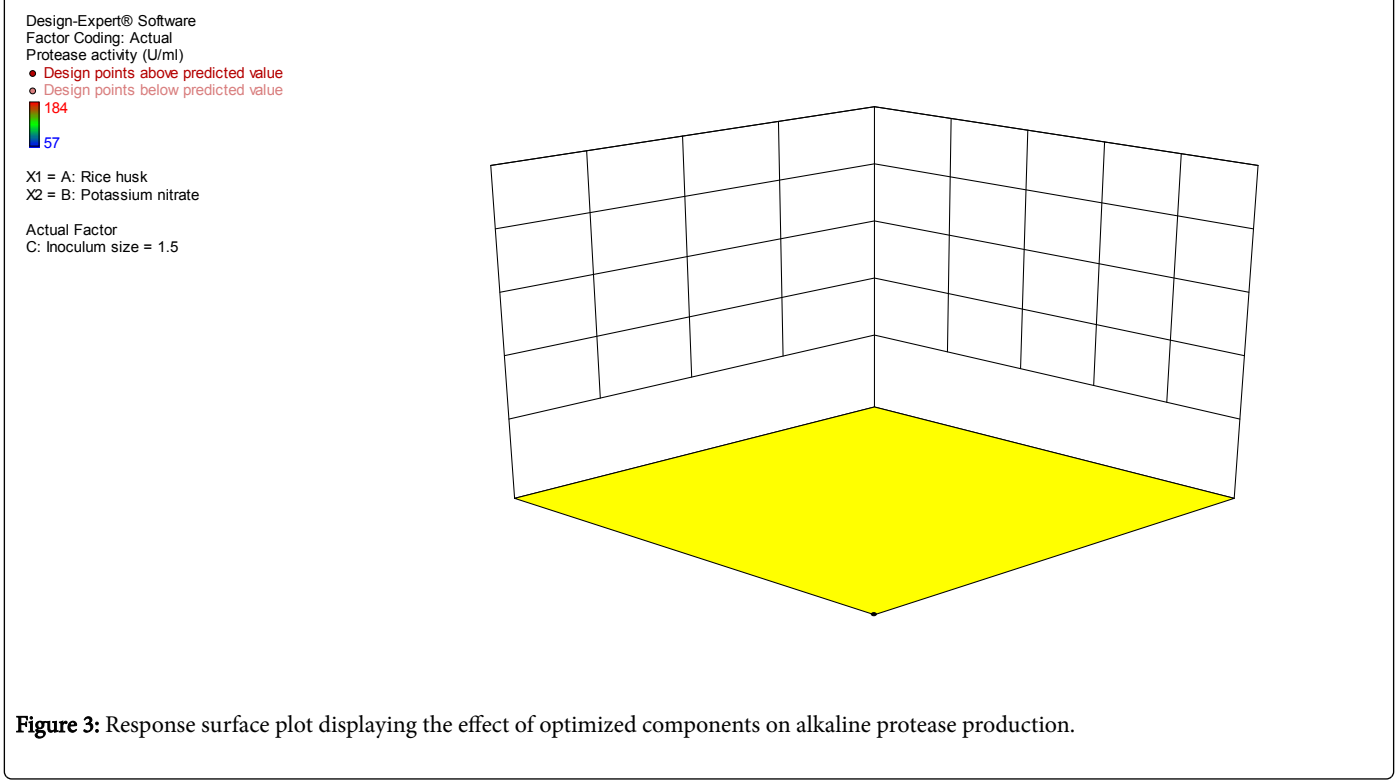

Figure 3: Response surface plot displaying the effect of optimized components on alkaline protease production.

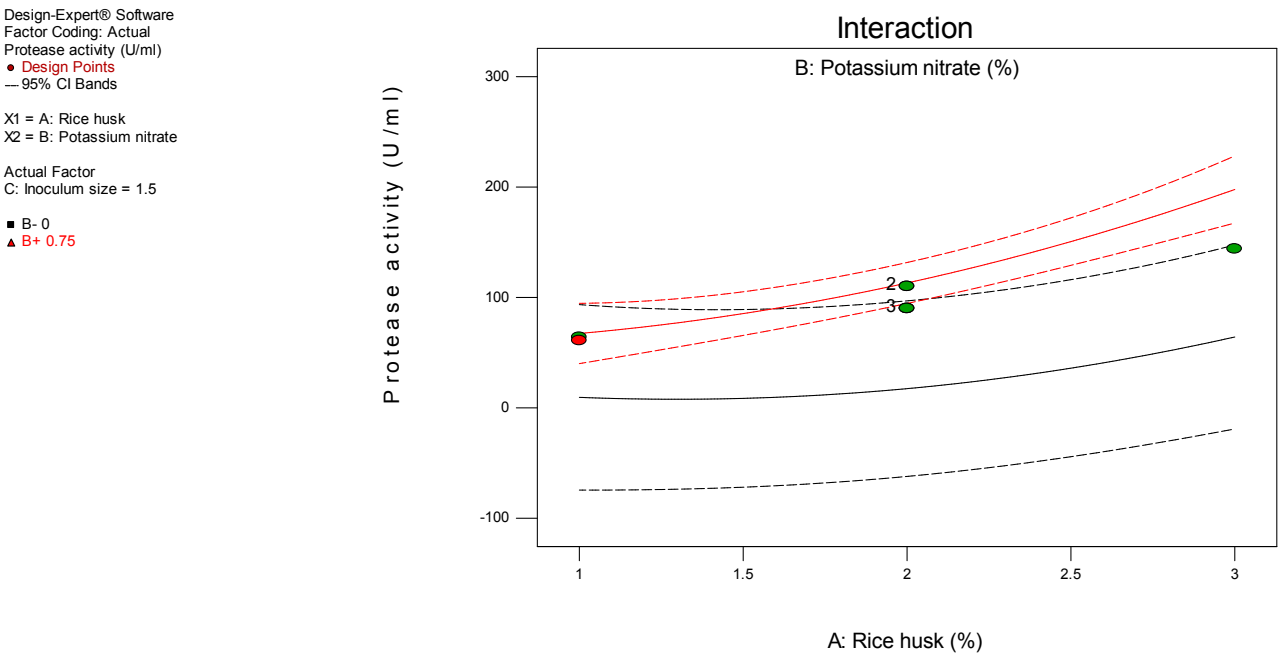

Figure 4: Response for the interaction factors.

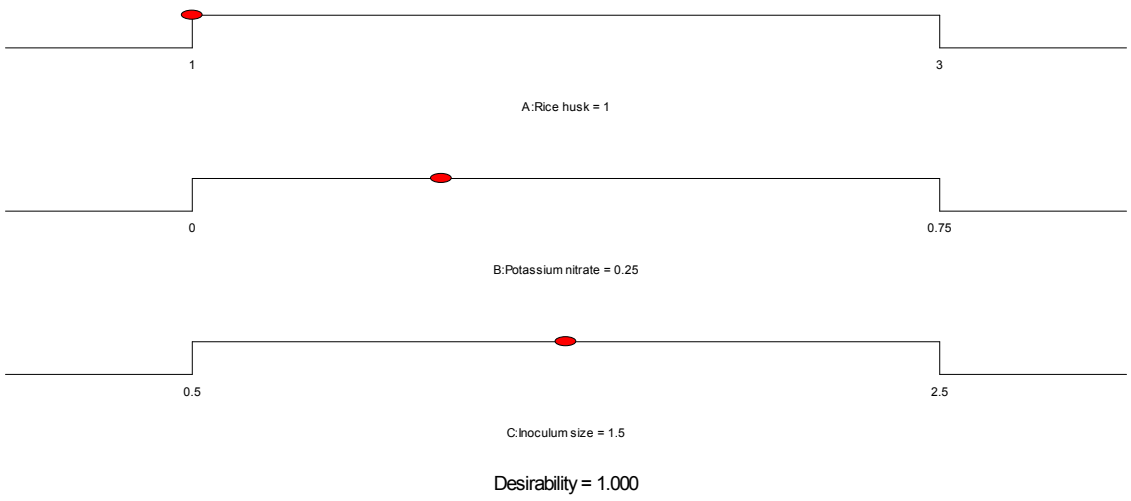

Figure 5: Desirability of the optimized media (Desirability=1.00).

Conclusion

Optimization of medium by classical methods is extremely time consuming and expensive, when large numbers of variables are evaluated. To overcome this difficulty, central composite design can be employed to optimize the medium components. Response surface methodology is a statistical approach used to study the effects of various factors which influence the final responses by varying them accordingly in a limited number of experiments. Hence it was thought

to fit the scope of this study. The central composite design method allows study of cultural conditions by supporting the change in concentration of media components. Due to the increasing economic relevance of alkaline protease enzymes, this study was conducted in an attempt to optimize a variety of fermentation parameters, including medium compositions and culture conditions, for maximal alkaline protease production. The significant combination of media components and culture conditions for ultimate alkaline protease production was found to be rice husk (3%); inoculum size (2%); KNO_3 (0.75%); salt solution, 5% (v/v) - {($MgSO_4.7H_2O$, 0.5% (w/v); KH_2PO_4 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01% (w/v) at 37°C and 160rpm for 72h production. The protease activity increased significantly with an optimized medium (185.4 ± 0.23U/ml) when compared to unoptimized basal medium (132 ± 0.76U/ml). Moreover, the cost of the medium was low since rice husk is a cheap by-product of the rice industry. The optimized medium developed in this work would offer advantages in terms of lower cost of production and improved yields for the large-scale production of protease. The optimized medium established in this work might result in a significant reduction in the cost of medium constituents.

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