Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus* sp.

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Abstract. PURPOSE: Optimization of the fermentation medium for maximum alkaline protease production was carried out using a new strain, *Bacillus* Sp. PE-11. **METHODS:** The carbon source (glucose), the nitrogen source (peptone) and salt solution were selected to optimize. A 2^3 full factorial composite experimental design and response surface methodology were used in the design of experiments and in the analysis of results. This procedure limited the number of actual experiments performed while allowing for possible interactions between the three components. **RESULTS AND DISCUSSION:** The optimum values for the tested variables for the maximum alkaline protease production were; glucose 7.798 (g/L), peptone 9.548 (g/L) and salt solution 8.757%. The maximum alkaline protease production was 4,98,123 PU/L. This method was efficient; only 20 experiments were necessary to assess these conditions, and model adequacy was very satisfactory, as the coefficient of determination was 0.941. **CONCLUSIONS**: In the work, we have demonstrated the use of a central composite factorial design by determining the conditions leading to the high yield of enzyme production. Thus, smaller and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries viz., detergent, food, pharmaceutical, leather, silk and for recovery of silver from used x-ray films (1-5). These proteases account for 30% of the total worldwide enzyme production (6).

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Pepsin was used in laundry detergents as early as 1913, but is now being replaced by a mixture of serine and metal microbial proteases that appear to be less degradable by soaps, alkaline conditions and high temperatures (7).

Alkaline proteases are produced by a wide range of microorganisms including bacteria, moulds, yeasts and also mammalian tissues. Among bacteria, *Bacillus Sp.* are specific producers of extracellular proteases (8-10). These are used as cleansing additives in detergents to facilitate the release of proteinacious materials in stains due to grime, blood, milk, etc. *Bacillus Sp.* grows in a pH range of 7.0 - 11.0 and produces extracellular alkaline proteases (8-10).

Alkaline proteases are generally produced by submerged fermentation. In addition, solid state fermentation processes have been exploited to a lesser extent for production of these enzymes (11-13). In commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly towards evaluating the effect of various carbon and nitrogen nutrient 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 optimum production of alkaline protease from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production. In our preliminary studies in the development of the production medium, glucose and peptone were found to be important factors in enhancing the alkaline protease formation. However, no systematic study to achieve optimum medium composition and process conditions has been reported for the production of alkaline proteases.

The conventional method of optimization involves varying one parameter at a time and keeping the others constant. This often does not bring about the effect of interaction of various parameters as compared to factorial design (14). Response surface methodology (RSM) is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments.

The present investigation is aimed at optimization of medium components (glucose, peptone and salt solution), which have been predicted to play a very significant role in enhancing the production of alkaline protease. Hence the use of experimental factorial design (15) and response surface methodology (16-17), already successfully applied in other fields, is well suited to the study of the main and interaction effects of the factors on the production of alkaline protease. Thus a 2^{3-1} full factorial central composite design and response surface methodology (18-24) was used in this study. Optimization of medium by the classical method involves changing one independent variable (glucose, peptone or salt solution) while maintaining all others at a fixed level is extremely time consuming and expensive when a large number of variables are evaluated. To overcome this difficulty, experimental factorial design and response surface methodology can be employed to optimize the medium components.

MATERIALS AND METHODS

Micro-organism

A new strain of *Bacillus sp.* PE-11 (25), isolated in our laboratory, was used as the producer of alkaline protease enzyme. It was grown at 37° C for 24 h and maintained on nutrient agar slants at 4° C and was subcultured at four week intervals.

Inoculum preparation

Inoculum was prepared by transferring 5ml suspension prepared from a 24 h old slant culture, into 250 ml Erlenmeyer flask containing 45 ml of sterile inoculum medium. The composition of the inoculum medium consisted of glucose 2.0 g/L, casein 0.5 g/L, peptone 0.5 g/L, yeast extract 0.5 g/L and salt solution 50 mL (salt solution containing KH_2PO_4 5 g/L, $MgSO_4.7H_2O$ g/L and $FeSO_4.7$ H_2O, 0.1 g/L). The flask was kept on a rotary shaker at 220 rpm at 37° C. A 10 mL aliquot per 100mL production medium served as its inoculum.

Shake flask experiments

Five milliliters of 24 hour aged inoculum (10^8 cells/mL) of *Bacillus PE-11* was added to 45 mL production medium in 250 mL Erlenmeyer flasks. The composition of production medium comprising: glucose 5.0 g/L, peptone 5.0 g/L and salt solution 50 ml (salt solution containing KH_2PO_4 5 g/L, MgSO4.7H_2O 2 g/L and $FeSO_4.7$ H_2O, 0.1 g/L). The range and levels of test variables are given in Table 1.

Table 1: Boundaries of experimental domain and spacing of levels expressed in coded and natural units.

Code unit	Experimental factor		
	Glucose(g/l)(x_1)	Peptone(g/l)(x_2)	% of salt solution (x_3)
-2	2	2	2.5
-1	4	4	5
0	6	6	7.5
1	8	8	10
2	10	10	12.5
Δx	2	2	2.5 %

Δx is the increment of the experimental factor natural values corresponding to one unit of the coded variable

The concentrations of glucose, peptone and salt solution in the production medium were varied according to the experimental design shown in the Table 2.

The pH value of the medium was adjusted before sterilization by adding 1N NaOH or 1N HCl. Five milliliter quantities of samples were withdrawn from each flask after 48 hours of fermentation and centrifuged at 2000 rpm for 10 min. The clear supernatant broth was used to determine the enzyme yield. All the experiments were carried out in triplicate and average values were reported.

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Analytical methods

Alkaline protease activity was determined by a modified procedure based on the method of Tsuchida *et. al* (26). One protease unit (PU) is defined as the amount of

enzyme that releases 1 μg of tyrosine per mL per min under the above assay conditions. All experiments were conducted in triplicate and the mean of the three is presented.

Table 2: Central composite design consisting of 20 experiments for the study of 5 experimental factors in coded units

Run no.	X_1	X_2	X_3	Coefficients assessed by
1	1	1	1	
2	1	-1	-1	
3	-1	1	-1	
4	1	1	-1	
5	1	-1	1	Fractional 2^{3-1} factorial design
6	-1	1	1	
7	-1	-1	1	
8	-1	-1	-1	
9	-2	0	0	
10	0	-2	0	
11	0	0	-2	
12	2	0	0	Star points (6 points)
13	0	2	0	
14	0	0	2	
15	0	0	0	
16	0	0	0	
17	0	0	0	Central points
18	0	0	0	
19	0	0	0	
20	0	0	0	

Experimental design and optimization by RSM

Response surface methodology (RSM) consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria. A prior knowledge and understanding of the process and the process variables under investigation are necessary for achieving a more realistic model. Based on the results obtained in preliminary experiments, glucose, peptone and salt solution were found to be major variables in the protease production. Hence, these variables were selected to find the optimized conditions for higher protease production using central composite design and response surface methodology.

The range and the levels of the experimental variables investigated in this study are given in the **Table 1**. The central values (zero level) chosen for experiment design were glucose 6 g/L, peptone 6 g/L and salt solution 75 mL/L.

In developing the regression equation the test factors were coded according to the equation.

$$x_i = (x_i - x^x{}_i)/\Delta x_i \text{ ---------- (1)}$$

Where x_i is the coded value of the i^{th} independent variable, X_i is the natural value of the i^{th} independent variable, $X^x{}_i$ is the natural value of the i^{th} independent variable at the center point and ΔX_i is the step change value.

$$Y = b_o + \sum_i b_i x_i + \sum_i \sum_y b_{ij} x_i x_j + \Sigma b_{ii} x^2{}_i + e \text{ ------ (2)}$$

Where Y is the measured response, b_o, intercept term, b_i, b_{ij}, and b_{ii} are, respectively the measures of the effects of variables X_i, X_iX_j, and X_i^2. The variable X_iX_j represents the first- order interactions between X_i and X_j ($i<j$).

Table 3: Observed responses and predicted values.

Run no.	Alkaline protease yield		Residual value
	Observed response	Predicted value	
1	453.000	479.687	-26.687
2	192.000	227.437	-35.437
3	223.000	267.687	-44.687
4	388.000	371.812	16.188
5	298.000	302.812	-4.812
6	343.000	357.062	-14.062
7	198.000	263.687	-65.687
8	184.000	206.812	-22.812
9	250.000	201.125	48.875
10	161.000	121.375	39.625
11	201.000	182.375	18.625
12	345.000	344.375	0.625
13	369.000	359.125	9.875
14	378.000	347.125	30.875
15	467.000	460.750	6.250
16	478.000	460.750	17.045
17	470.000	460.750	9.250
18	461.000	460.750	0.250
19	473.000	460.750	12.250
20	465.000	460.750	4.250

Several experimental designs have been considered to study such model, and we selected the central composite design proposed by Box *et al* (18). For this study, 2^{3-1} fractional factorial design with six star points and six replicates at the centre points were employed to fit the second order polynomial model which indicated that 20 experiments were required for this procedure. The 'statistica' software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables

were obtained by solving the regression equation and also by analyzing the response surface contour plots (24).

In all, the 20 fermentation experiments were conducted in triplicate, and the average values of alkaline protease yields were tabulated, as given **Table 3**, under column, observed response.

The predicted values were calculated by using the mathematical model derived from the coefficients of the model shown in **Table 4**, and the predicted values are tabulated in **Table 3**.

Table 4: Model coefficients estimated by multiples linear regression.

Factor	Coefficient	Computed t-value	p-value
Intercept	460.750	29.599	4.53E-11
X_1	35.812	3.671	0.0043*
X_2	59.437	6.092	0.0001*
X_3	41.187	4.221	0.0017*
X_{12}	20.875	1.513	0.1612
X_{13}	4.625	0.335	0.7444
X_{23}	8.125	0.589	0.5690
X_{11}	-47.000	-6.039	0.0001*
X_{22}	-55.125	-7.083	3.36E-05*
X_{33}	-49.000	-6.296	8.96E-05*

* Significant at $p < 0.01$

RESULTS AND DISCUSSIONS

The results of the second order response surface model fitting in the form of analysis of variable (ANOVA) are given in **Table 5**.

Table 5: Analysis of variance (ANOVA) for the five factorial design

Sources of variation	Sum of squares	Degrees of freedom	Mean square	*F* value	Prob (*P*) >*F*
Regress.	243508	9	27056.44	17.76457	5.07E-05
Residual	15230.56	10	1523.056		
Total	258738.5	19			

$R^2 = 0.941$, $R = 0.970$, Adjusted $R^2 = 0.888$

The fisher F-test with a very low probability value ($P_{model} > F = 5.07E-05$) demonstrate a very high significance for the regression model (19, 24). The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient ($R^2 = 0.941$) indicates that only 5.90% of the total variations are not explained by the model. The value of the adjusted determination coefficient (Adj. $R^2 = 0.888$) is also very high, which indicates a high significance of the model (19,24). A higher value of the correlation coefficient ($R = 0.970$) signifies an excellent correlation between the independent variables (18). At the same time a relatively lower value of the coefficient of variation (CV= 11.20) indicates improved precision and reliability of the conducted experiments (19,23).

The application of response surface methodology (22-24) yielded the following regression equation which is an empirical relationship between the logarithmic values of enzyme yields and test variables in coded unit:

$$Y = 460.750 + 35.812\,x_1 + 59.437\,x_2 + 41.187\,x_3 + 20.875\,x_1x_2 + 4.625\,x_1x_3 + 8.125\,x_2x_3 - 47.000\,x_1^2 + 55.125\,x_2^2 - 49.000\,x_3^2 \quad \text{-------- (3)}$$

Where Y is the response, that is, the enzyme concentrations expressed in logarithmic values, and x_1, x_2 and x_3 are the coded values of the test variables (glucose, peptone and salt solution respectively).

The significance of each coefficient was determined by student's t-test and p values, which are listed in **Table 4**. The larger the magnitude of the t- value and the smaller the p- value, the more significant is the corresponding coefficient (19,24). This implies that the quadratic main effects of glucose, peptone and salt solution are more significant than their respective first order effects. The second order main effects of both peptone and salt solution are significant, as is evident from their respective P-values ($P_{x1}^2 < 0.0001$, $P_{x2}^2 < 3.36E-05$ and $P_{x3}^2 < 8.96E-05$) with their first order main effects ($P_{x1} < 0.0043$, $P_{x2} < 0.0001$ and $P_{x3} < 0.0017$). These values suggest that the concentration of glucose and peptone have a direct relationship on the production of the enzyme. In **Table 3** each of the observed values for $Y_f(0)$ is compared with the predicted values, $Y_f(P)$, from the model. The comparison of the residuals showed an error variance Se^2 (=0.022), which indicates that none of the individual residual values exceed twice the square root of the residual variances (19). All of the above considerations indicate an excellent adequacy of the regression model (18-24).

Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels (zero, for instance), are more helpful in understanding both the main and the interaction effects of these two factors. These

plots can be easily obtained by calculating from the model the values taken by one factor where the second varies (from –2.0 to +2.0, step 0.5 for instance) with constraint of a given Y value. The yield values for different concentrations of the variables can also be predicated from the respective response surface plots (**Figure 1-3**). The maximum predicted yield is indicated by the surface confined in the response surface diagram.

Figure 1 shows the response surface plot obtained as a function of glucose concentration vs. peptone concentration, while all other variables are maintained at zero level. An increase in alkaline protease yield with increase in glucose concentration, versus peptone concentration was observed.

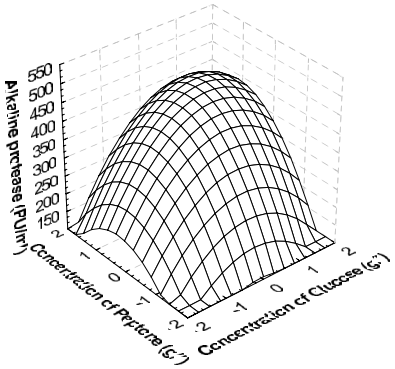

Figure 1: Response surface plot showing the effect on glucose concentration, peptone concentration and their mutual effect on the production of alkaline protease. Other variables are held at zero level.

Figure 2 shows the response surface plot obtained as a function of glucose concentration vs. salt solution concentration, while other variables are maintained at zero level. An increased alkaline protease yield with increase in glucose concentration vs. salt solution concentration was observed.

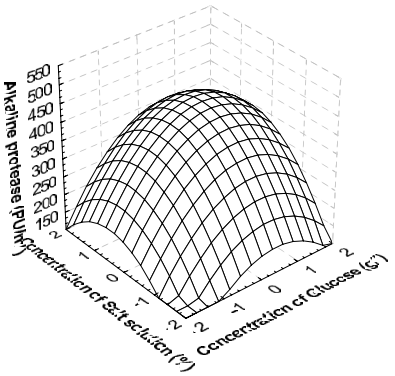

Figure 2: Response surface plot showing the effect on glucose concentration, salt solution concentration and their mutual effect on the production of alkaline protease. Other variables are held at zero level.

Figure 3 shows the effect of peptone concentration vs. the salt solution concentration, while other variables are maintained at zero level. An increase in alkaline protease yield with increased peptone concentration vs. salt solution concentration was observed.

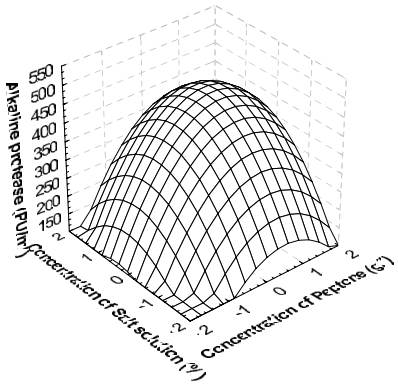

Figure 3: Response surface plot showing the effect on peptone concentration, salt solution concentration and their mutual effect on the production of alkaline protease. Other variables are held at zero level.

The alkaline protease production is predominantly influenced by glucose and peptone concentrations. Glucose and peptone are therefore the key nutrient materials which control the biosynthesis of the enzyme. At higher concentrations both nutrients may cause inhibition of enzyme synthesis. This fact was also suggested during other enzyme production experiments on carbon and nitrogen repression effects (27-30).

The multi-stage Monte-Carlo optimization method (31) was used to solve the regression equation (eqn. 2). The optimal values of the test variables in coded units are as follows; x_1 = 0.557, x_2= 0.682 and $x3$ = 0.503 with the corresponding Y= 501.345. The natural values obtained by substituting the respective values of x_1 in equation (1) are: glucose 7.798 g/L, peptone, 9.548 g/L, salt solution 8.757%. The modeled values predict that the maximum concentration of enzyme that can be obtained by using the above optimized concentrations of the variables is 5,01,345 PU/L. Verification of the predicted results were accomplished by using the optimized conditions in incubation experiments. An increase in enzyme production of about 4,98,123 PU/L was observed. This results therefore corroborate the predicted values, and the effectiveness of the model.

CONCLUSIONS

This work has demonstrated the use of a central composite factorial design by determining the conditions leading

to the optimum yield of enzyme production. This methodology could therefore be successfully employed to any process, where an analysis of the effects and interactions of many experimental factors are required. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. Isoresponse curves are very helpful in visualizing the main effects and interaction of the factors. Thus, smaller and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes.

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