Optimization of Alkaline Protease Production from *Bacillus* sp. by Response Surface Methodology

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Abstract. High yields (1939 U/ml) of an alkaline protease were obtained in batch fermentation of a *Bacillus* sp. using a response surface methodology. The interaction of four variables, viz., starch, peptone, incubation time, and inoculum density, suggested inoculum density to be an insignificant variable. However, incubation time had a profound effect on protease yields at all the concentrations of carbon and nitrogen used. The response surface raised and flattened with increase in time of incubation, and maximum protease production up to 1939 U/ml was obtained after 96 h of incubation. The model equation obtained was validated experimentally at maximum starch (15 mg/ml) and peptone (7.5 mg/ml) concentration with increased incubation time up to 144 h in the presence of minimum inoculum density (1%). An overall 2.6-fold increase in protease production was obtained as compared with mean observed response (750 U/ml) at zero level of all variables.

The 'one-at-a-time-approach' is the most frequently used operation in biotechnology to obtain maximum cell density, high yields of the desired metabolic product, or enzyme levels in a microbial system. This approach is not only time consuming, but ignores the combined interactions among various physicochemical parameters [4]. On the contrary, the response surface methodology (RSM), which includes factorial design and regression analysis, helps in evaluating the effective factors and building models to study interaction and select optimum conditions of variables for a desirable response [3, 4]. Recently, a number of statistical experimental designs with response surface methodology (RSM) have been employed for optimizing enzyme production from microorganisms [1, 3, 17].

It is a well-known fact that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources [6–10], metal ions [17], and physical factors such as pH, temperature, inoculum density [13], dissolved oxygen [5, 12], and incubation time [13, 14, 16]. We have earlier reported the effectiveness of an SDS-stable alkaline protease from a newly isolated *Bacillus* sp. RGR-14 for use in detergent formulations [14]. Here, we report optimization of this protease from *Bacillus* sp.

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as a result of the interaction between four variables with response surface methodology.

Materials and Methods

Chemicals. Casein for protease assay was from Sigma (St. Louis, MO, USA). All other analytical reagents and media components used were of highest purity grade available commercially in India.

Microorganism and inoculum preparation. *Bacillus* sp. RGR-14, which produces an SDS-stable alkaline protease [14], was used in the present study. It was identified to be closely related to *Bacillus* sp. TSG437 strain on the basis of 16S rRNA homology by MIDILABS Inc., (Newark, DE, USA). The 6-h-old seed culture [prepared in nutrient broth (pH 7) at 37°C under shaking (200 rpm)] was used to inoculate production flasks.

Selection of best carbon and nitrogen source. For selection of the best source of carbon and nitrogen for protease production, various simple and complex carbon (glucose, starch, pectin, wheat bran, carboxy methyl cellulose, xylan, chitin) and inorganic and complex nitrogen sources (ammonium chloride, soybean meal, casamino acids, casein, peptone, yeast extract, cornsteap solids, cornsteap liquor, corn glutan meal, skim milk) were supplemented individually in the previously reported medium [14] for protease production from *Bacillus* sp. All these sources were supplemented in the production medium at a final concentration of 1% wt/vol and 0.5% wt/vol for carbon and nitrogen, respectively, and protease yield was determined after 48 h of incubation at 37°C under shaking (200 rpm).

Experimental design and protease production. The next stage in medium formulation was to determine the optimal levels of four variables, viz. starch (A), peptone (B), inoculum density (C), and

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

| Variables | Range of levels | | | | | |
	Actual	Coded	Actual	Coded	Actual	Coded
Starch (mg/ml)	5	−1	10	0	15	+1
Peptone (mg/ml)	2.5	−1	5	0	7.5	+1
Incubation time(h)	24	−1	48	0	72	+1
Inoculum (%)	1	−1	2	0	3	+1

incubation time (D) on protease production. For this purpose, the response surface approach by using a set of experimental design (FCCCD: Face Centered Central Composite design) was adopted for improving total protease production. The statistical software package 'Design-Expert® 6.0.1', StatEase, Inc., (Minneapolis, MN, USA) was used to analyze the experimental design.

Each factor in the design was studied at three different levels (−1, 0, +1). For a 2^4 FCCCD with four factors, including six center points, a set of 30 experiments was carried out. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form are listed in Table 1. Upon completion of experiments, the average maximum protease yield was taken as the dependent variable or response (Y). A second-order polynomial equation was then fitted to the data by the multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a four-factor system, the model equation is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11}A^2$$
$$+ \beta_{22}B^2 + \beta_{33}C^2 + \beta_{44}D^2 + \beta_{12}AB + \beta_{13}AC$$
$$+ \beta_{14}AD + \beta_{23}BC + \beta_{24}BD + \beta_{34}CD$$

with Y, predicted response; β_0, intercept; β_1, β_2, β_3, β_4, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, squared coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34}, interaction coefficients.

Design Expert Software, using the above model to obtain the optimum concentration of the medium components, was then used to generate response surface graphs.

The final basal minimal medium (pH 7) used for protease production contained (g/L): starch (variable), peptone (variable); KH_2PO_4, 1; K_2HPO_4, 3. The production medium (50 ml in a 250-ml Erlenmeyer flask) was inoculated with the inoculum density ($A_{550nm} \cong 0.250$–0.300) designated for each set and incubated at 37°C under shaking (200 rpm) in a New Brunswick Scientific Shaker (Edison, NJ, USA). After proper incubation time designated for each set, the cell-free supernatant was obtained by centrifugation at 14,000 rpm for 30 min at 4°C in a cold centrifuge (Sorvall® RC 5C Plus), and the protease production was determined in the cell-free supernatant.

Protease assay. The protease was assayed at 60°C in glycine NaOH buffer (0.05 M, pH 10.0) by using casein as substrate, as described previously [14]. One unit of protease was equivalent to the amount of enzyme required to release 1 μg of tyrosine/ml/min under standard assay conditions.

Results and Discussion

Effect of various carbon and nitrogen sources on protease production. Among the various carbon sources

Table 2. Effect of various carbon and nitrogen sources on protease production by *Bacillus* sp. after 48 h at 37°C under shaking (200 rpm).

Nutrient source	Protease yield (U/ml)
Carbon source (1%):	
Starch	390 ± 27
Pectin	325 ± 31
Wheat bran	284 ± 14
Carboxymethyl cellulose	169 ± 19
Chitin	157 ± 12
Glucose	24 ± 4
Nitrogen source (0.5%):	
Peptone	687 ± 38
Casein	481 ± 42
Soybean meal	451 ± 49
Yeast extract	449 ± 33
Casamino acids	390 ± 27
Cornsteep solids	365 ± 42
Corngluten meal	356 ± 28
Skim milk	342 ± 20
Cornsteep liquor	249 ± 14
Ammonium chloride	24 ± 4

studied, the *Bacillus* sp. produced maximum protease in starch (390 U/ml), followed by pectin (325 U/ml) and wheat bran (284 U/ml) (Table 2). Glucose drastically inhibited protease production. Among nitrogen sources, peptone (687 U/ml) favored maximum protease production, followed by casein (481 U/ml), skim milk (451 U/ml), and yeast extract (449 U/ml). Thus, starch and peptone were selected as the source of carbon and nitrogen, respectively, for design of response surface methodology.

Optimization by response surface methodology. The results of FCCCD experiments for studying the effects of four independent variables, viz., starch, peptone, incubation time, and inoculum density, on protease production are presented in Table 3 along with the mean predicted and observed response. The regression equation obtained after the analysis of variance (ANOVA) indicated the R^2 value of 0.79 (a value of $R^2 > 0.75$ indicates the aptness of the model), which ensured a satisfactory adjustment of the quadratic model to the experimental data and indi-

Table 3. Experimental designs used in RSM studies by using four independent variables with six center points showing observed and predicted values of protease production

Run order	A (Starch)	B (Peptone)	C (Inoculum density)	D (Incubation time)	Mean observed response	Predicted response
1.	−1	−1	−1	−1	271.60	331.28
2.	−1	−1	−1	+1	391.60	585.78
3.	−1	−1	+1	−1	410.60	199.29
4.	−1	−1	+1	+1	316.30	135.34
5.	−1	+1	−1	−1	318.30	220.71
6.	−1	+1	−1	+1	467.40	334.96
7.	−1	+1	+1	−1	368.80	469.32
8.	−1	+1	+1	+1	198.40	265.12
9.	+1	−1	−1	−1	296.60	157.52
10.	+1	−1	−1	+1	301.10	207.52
11.	+1	−1	+1	−1	705.40	846.78
12.	+1	−1	+1	+1	552.60	578.33
13.	+1	+1	−1	−1	321.60	511.50
14.	+1	+1	−1	+1	282.30	429.25
15.	+1	+1	+1	−1	1847.90	1581.36
16.	+1	+1	+1	+1	1223.40	1172.66
17.	−1	0	0	0	194.60	394.31
18.	+1	0	0	0	707.20	761.20
19.	0	−1	0	0	164.52	368.49
20.	0	+1	0	0	560.62	610.36
21.	0	0	−1	0	456.60	337.10
22.	0	0	+1	0	273.60	646.81
23.	0	0	0	−1	750.97	610.32
24.	0	0	0	+1	702.20	734.45
25.	0	0	0	0	750.01	610.32
26.	0	0	0	0	750.30	610.32
27.	0	0	0	0	748.70	610.32
28.	0	0	0	0	756.30	610.32
29.	0	0	0	0	747.77	610.32
30.	0	0	0	0	747.77	610.32

cated that 79% of the variability in the response could be explained by the model. An adequate precision of 9.263 indicates an adequate signal as it measures the signal-to-noise ratio. The coefficients of the regression equation were calculated using Design Expert, and the following regression equation was obtained.

$$Y = 610.32 + 183.44*A + 120.94*B + 154.86*C$$
$$- 38.55*D - 32.57*A^2 - 120.90*B^2$$
$$- 118.37*C^2 + 162.68*D^2 + 116.14*AB$$
$$+ 205.31*AC - 51.13*AD$$
$$+ 95.15*BC - 35.06*BD - 79.61*CD$$

with Y, protease production (response); A, starch; B, peptone; C, incubation time; and D, inoculum density.

The three-dimensional response surface curves were then plotted to understand the interaction of the medium components and the optimum concentration of each component required for maximum protease production. Analysis of variance (ANOVA) showed that the factor D (inoculum density) was insignificant. It was, therefore, taken as minimum (1%) for further interpretation of the response surfaces. The response surfaces shown in Figs. 1–3 show the relative effect of the two variables (starch and peptone), with varying incubation time at constant inoculum density (1%). The coordinates of the central point within the highest contour levels in each of these figures corresponded to the optimum concentrations of the respective components.

Figure 1 shows the response for the interactive factors, starch (A) and peptone (B), when the incubation time (C) was kept at the minimum (24 h). Maximum enzyme yield in these conditions was predicted to be 520 U/ml, corresponding to maximum levels (+1) of both starch (15 mg/ml) and peptone (7.5 mg/ml). However, the curve also suggested that the response did not vary much as a function of peptone concentration.
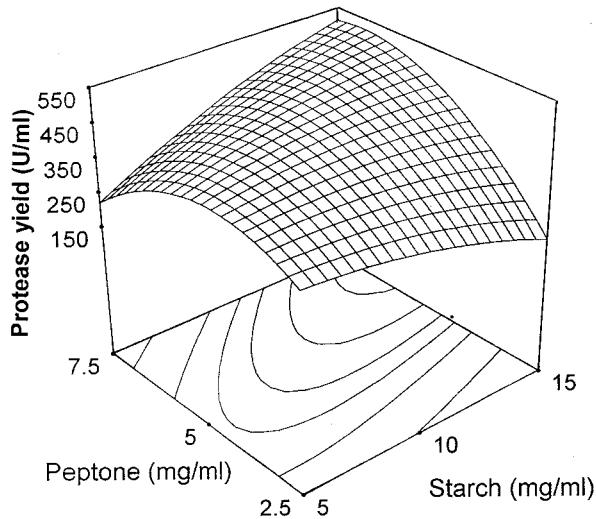
Fig. 1. Response surface curve of protease production from *Bacillus* sp. showing interaction between starch and peptone after 24 h of incubation.
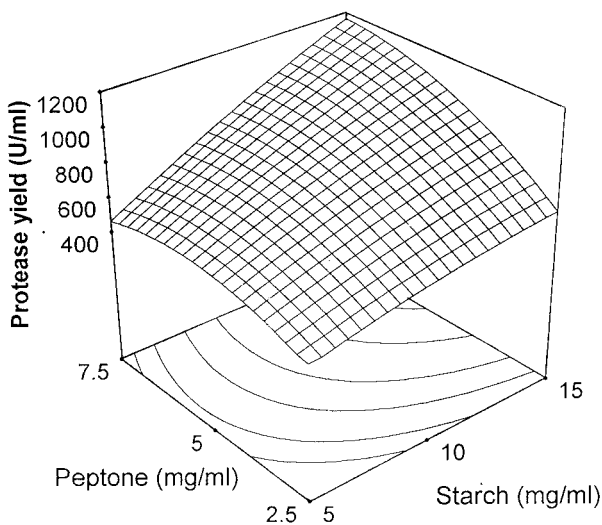

Fig. 2. Response surface curve of protease production from *Bacillus* sp. showing interaction between starch and peptone after 48 h of incubation.
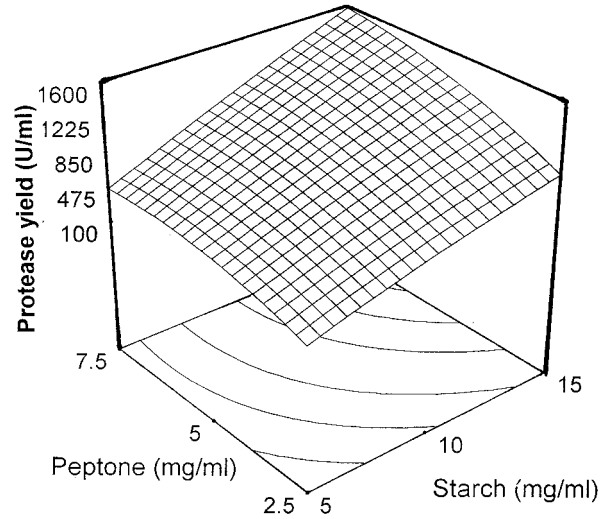

Fig. 3. Response surface curve of protease production from *Bacillus* sp. showing interaction between starch and peptone after 72 h of incubation.

Table 4. Validation of the response surface model.

Incubation time (h)	Predicted response (U/ml)	Mean observed response (U/ml)
72 (+1 level of the model)	1581	1848
96 (validation)	1758	1939
120 (validation)	1718	1710
144 (validation)	1425	1450

With an increase in incubation time, the enzyme production further increased to 1165 U/ml after 48 h (Fig. 2) and 1581 U/ml after 72 h (Fig. 3). However, the response curves did not show curvature; rather, they were flattened with more and more points moving towards higher enzyme units (Figs. 2 and 3). Thus, the surface plots suggested demand for higher concentrations of both starch and peptone along with longer time of incubation to obtain maximum protease production. This confirms earlier reports on protease production, where it has been observed that these complex carbon and nitrogen sources are better substrates for protease production than simpler sugars, such as glucose, which cause catabolite repression [6, 8, 9].

Validation of the model. The model indicated that the selected concentrations of starch and peptone were limiting and thus did not result in the appropriate curved surface in the response surface graph. Therefore, further increase in their concentrations, along with increase in time interval, should be worked out for validation. But, owing to experimental limitations (increased starch concentrations lead to high viscosity of the medium), the model was validated only with increased time interval. The validation experiments showed that there was an increase in enzyme production (1939 U/ml) after 96 h (Table 4), which was closer to the predicted response (1758 U/ml) (Fig. 4). However, the major decline in enzyme production was obtained after 144 h, and protease production after 120 and 144 h was 1710 U/ml and 1450 U/ml, respectively (Fig. 4; Table 4). This could be because of expected carbon and nitrogen limitation. During a batch culture, the consumption of substrates or
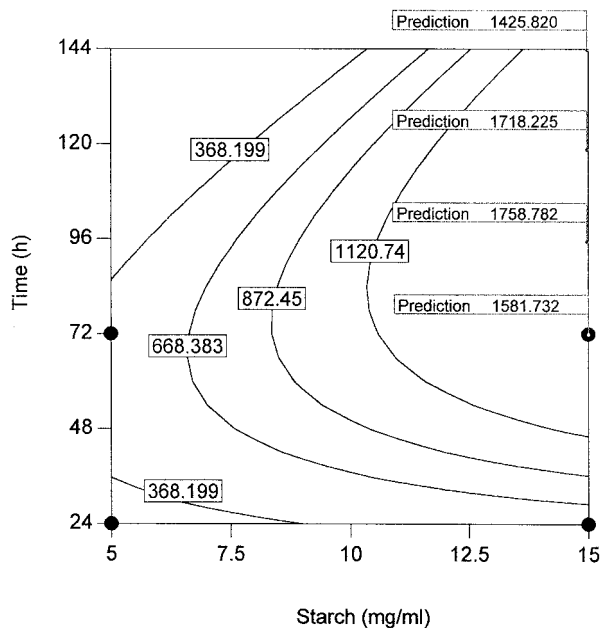
Fig. 4. Contour plot of protease production from *Bacillus* sp. as a function of starch concentration and incubation time

depletion of nutrients has profound effects on protease production. Several factors, such as autodigestion of proteases and proteolytic attack by other proteases, have also been suggested to be involved in cessation of enzyme synthesis [2, 15]. An overall 2.6-fold (1939 U/ml) increase in protease production was achieved compared with the mean observed response (750 U/ml) in the basal medium (with all factors at their central levels) after optimization by using response surface methodology. Such high enzyme yields for protease over a longer time of 96 h have not been reported earlier in bacterial batch fermentations. However, controlled fed-batch fermentations and chemostat cultures [8, 11, 12] have so far been used successfully for improving protease production for a long time of incubation from a number of microorganisms. In a similar report, the production of an alkaline protease from *Rhizopus oryzae* was enhanced up to 2.5-fold after optimizing concentrations of Tween-80 and metal ions in the medium by using a multifactorial approach [1].

Thus, a multifactorial statistical approach that considers the interaction of independent variables provides a basis for the model to search for the nonlinear nature of the response in a short-term experiment.

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