

PROCESS OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY FOR EXTRACELLULAR ALKALINE PROTEASE PRODUCTION FROM *BACILLUS SUBTILIS*

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Abstract

Three microbial cultures *Bacillus subtilis* DSM 1970, *Bacillus subtilis* GCU-8 and *Bacillus licheniformis* DSM 1969 were screened for protease production by casein agar plate method. Among these *Bacillus subtilis* GCU-8 was found to be the most potent protease producer in wide pH range (5.0 to 8.0). Fermentation conditions were optimized for the production of alkaline protease using two statistical tools: Plackett Burman Model for linear regression study and Response Surface Model for interactive effects of significant factors on production. The alkaline protease was optimally produced after 48 hours of incubation at 37°C in fermentation media containing equal amounts of substrates (soybean meal and wheat bran, 7.5 g), MgSO₄.7H₂O, 0.10 g and yeast extract 0.55 g. The protease was purified to homogeneity by salt precipitation, ion-exchange chromatography and size exclusion chromatography. The homogeneity and molecular weights were checked by SDS-PAGE. The protease was 45 KDa protein, predominantly alkaline and optimally active at pH 8.0.

Introduction

Proteases catalyze hydrolysis of proteins into smaller peptides and free amino acids and can also catalyze peptide synthesis in organic solvents or in solvents with lower water content (Yossan *et al.*, 2006). Animals, plants and microorganisms are among major sources of proteases (Rao *et al.*, 1998; Banik & Parkash, 2004). However, microorganisms have been successful in attracting researcher's attentions for last many decades as they have broad biochemistry, feasibility of mass culturing and ease of genetic manipulations. Due to wide range of industrial applications, proteases take 60% of total world market of industrial enzymes (Parasanthi *et al.*, 2008). The 35% of this 60 % is contributed by alkaline proteases (Banik & Parkash, 2004; Guangrong *et al.*, 2008). The world market for enzymes is expected to grow 7.6 % per year (David *et al.*, 2009).

Mode of fermentation is very critical for optimum production. Solid state fermentation (SSF) is more advantageous than the submerged fermentation as it involves lower cost as slightly processed or unprocessed agricultural wastes are used as substrates (Prasanthi *et al.*, 2008). It requires less energy input, low initial capital cost, improved product recovery and less effluent generation. Therefore alkaline proteases are mostly produced by solid state fermentation using wheat bran and soybean meal as substrate (Rashbehari *et al.*, 2003).

Members of the genus *Bacillus* are widely used in industry in the large-scale production of enzymes, particularly proteases. *Bacillus subtilis* is a Gram-positive and catalase-positive bacterium commonly found in soil (Madigan & Martinko., 2005; Mukhtar & Haq, 2008; Mukhtar & Haq, 2012). These produce proteases and other hydrolases after the exponential growth phase, when the culture enters the stationary phase and begins the process of sporulation. The present study is focused on statistical optimization of medium constituents to find out the critical contribution of each factor. Both Plackett Berman model

(PBM) and Response Surface Methodology (RSM) were used to optimize the process for production of alkaline proteases. PBM determines the linear correlation factors while RSM interactions of various factors and their influence on the production (Carely *et al.*, 2004, Myers & Montgomery, 2009).

The objectives of a statistically designed optimization study were to confirm effects and interactions of variables, estimate specific curvature or quadratic effects, and determine optimal settings of the critical factors.

Materials and Methods

Culture maintenance: Two bacterial strains *Bacillus licheniformis* DSM 1969 [ATCC 21424] and *Bacillus subtilis* DSM 1970 [ATCC 21228] were donated by the German Resource Centre for Biological Materials (DSMZ). *Bacillus subtilis* GCU-8 was isolated from the soil samples. All the three cultures were grown at 37°C on agar slants containing (g/L); peptone: 5, meat extract: 3 and agar: 20. After 24 hrs of growth, the cultures were maintained at 4°C.

Screening of cultures for protease activity: All three strains of bacteria were screened for protease activity using casein agar plates containing (g/L) casein 10, agar 20 and Sodium Azide 0.2 (to prevent growth of contaminants) of three different pH (5, 7 and 8) and incubated at 37° C for 24 hrs (Chantawannakul *et al.*, 2002). Strain with significant casein digestion zones on alkaline casein agar plate was selected for further optimization.

Enzyme production and extraction: The enzyme was produced by solid state fermentation. Inoculum medium containing (g/L) peptone 5 and meat extract 3. The medium was prepared and autoclaved in 250mL shake flasks containing 25mL medium. Inoculated medium

was incubated at 37°C and 170 RPM for 24 hrs in shaking incubator. Fermentation medium containing constituents (soybean meal, wheat bran, calcium carbonate, ammonium sulfate and yeast extract) in amounts according to 1st (Plackett-Burman model) and 2nd level (RSM) factorial designs, was inoculated with 24 hrs old Inoculum having viable cell count 6.24×10^5 CFU/mL. All experiments were performed in duplicates and results shown are average values. Enzyme was extracted by filtration after adding 100 mL distilled water to each flask and 1 hr of shaking (Suresh & Chandrasekaran, 1999, Kumar *et al.*, 1999).

Selection of significant factors by Plackett-Burman Model (PBM): PBM can only be used to determine the linear correlation of factors not for their interactions (Plackett & Burman, 1946) in present study it was used for screening of nine factors effecting production of alkaline proteases. The factors studied were, Incubation period (A), moisture level (B), Inoculum size (C), wheat bran (D) and soybean meal (E), $(\text{NH}_4)_2 \text{SO}_4$ (F), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (G), yeast extract (H), incubation temperature (J). These factors were tested for low, medium and high values. 17 possible combinations of these factors were investigated in duplicates.

The factors were screened linearly using the approach given below:

$$Y = \beta_0 + \sum \beta_i x_i \quad (i=1-k)$$

In this equation Y is the target function, β_0 and β_i are the intercept and regression coefficient respectively.

The effect of each variable was tested by the equation:

$$E(X_i) = 2 (\sum M_i^- - M_i^+) / N$$

In this equation $E(X_i)$ = the effect of the tested variables

M_i^- and M_i^+ = the total production from the trials where the variable X_i measured at low and high levels, respectively, and N = the number of trials.

Optimization of significant factors by response surface methodology: Significant factors selected by PBM were optimized by Response Surface Methodology (RSM). These factors include inoculum size (% age, v/w), soybean meal and yeast extract.

Statistical analysis

Coded equation for significant factors was

$$Z = (X - X^0) \Delta X$$

where "Z" = coded value of independent variable, "X" = the corresponding real value; "X⁰" = real value of an independent variable at the centre point and "ΔX" = step change of real value at the variable for "Z" the value.

The relationship between the response and the independent variables was explained by using second order polynomial equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

In this equation Y = the predicted response, β_0 = the interception coefficient; β_i , β_{ii} and β_{ij} = the linear, quadratic and interception coefficients respectively. Software package Design-Expert version 7.1.6 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for multiple regression analysis of the equation and construction of response surface groups. The significance of regression equation was worked out by F-test and lack-of-fit and explained with the help of coefficient of determination R^2 , adjusted R^2 , predicted R^2 and coefficient of variance. The 2nd order fitted polynomial equation was explained in the form of three dimensional graphs to show the relationship between the response and experimental factors. The point optimization method was used to optimize the maximum response of each variable was optimized by point optimization method. The method was validated by optimized variables yielding maximum response. All the runs were performed in duplicates for 48 hours at 37°C and their mean values were used for analysis.

Analytical techniques

Protease assay: Protease in fermented broth was estimated by using method designed by Takami *et al.*, (1989). Tyrosine standard curve was used for calibration. One mL 1% casein in buffer was incubated with 100 μl enzyme at 40°C for 30 minutes, reaction was stopped by precipitating remaining protein by adding 1.5 mL 5% TCA. Precipitates separated by centrifugation at 3000 RPM supernatant was treated with Folin's reagent to determine the tyrosine released by protease. Casein agar plates were also used to measure the zones of proteolytic enzymes as Meraz *et al.*, (2006) used skimmed milk agar plates in 2005. One protease unit (PU) is the amount of enzyme required to release μg of tyrosine per ml per minute under the conditions of assay.

Total proteins estimation: Total soluble proteins of fermented broth were determined by using method of Lowry *et al.*, (1951). BSA standard curve was used for calibration.

Enzyme purification: Crude enzyme extract was precipitated by ammonium sulphate and then subjected to Ion-Exchange Chromatography using DEAE, followed by Size Exclusion Chromatography using Biogel G-100.

SDS-PAGE: SDS-PAGE of purified enzyme was carried out under reductive conditions. Sample was prepared in sample buffer (0.0625 M Tris-HCl, 2% SDS, 10% sucrose, pH 6.8) with 0.1 M beta-mercaptoethanol and subjected to electrophoresis. The gel was run at room temperature with a voltage of 60 mV for stacking and 120 mV for separating gel. Low molecular weight markers of

PAGE ruler SM601 Fermentas was used as molecular mass standard and molecular weight of protease was determined using Quantity One Software of Biorad. Molecular weight was determined from SDS-PAGE using medium range standard molecular weight markers.

Results and Discussion

Screening of cultures for protease activity: Bacterial cultures were screened for protease activity on casein agar plates at different pH. Figure 1 shows that *Bacillus subtilis* GCU-8 formed significant casein digestion zones in all ranges of pH (5, 7 and 8), thus showing the production of alkaline, acidic and neutral proteases at a time. However, *Bacillus licheniformis* DSM 1970 produced proteases at pH 5.0 and pH 8.0 while *Bacillus subtilis* DSM 1969 produced digestion zones at pH 8.0 only. The digestion zone by *Bacillus subtilis* GCU-8 at pH 8.0 was maximum (21 mm) as compared to 15 mm and 17 mm at pH 5.0 and 7.0, respectively. *Bacillus subtilis* GCU-8, therefore, was selected as a potent alkaline protease producer for further studies.

Selection of significant factors by PBM: Nine different reaction variables were investigated in the present study (Table 1) to evaluate the activity of proteases (U/g). The under study reaction variables include; Incubation time (A), Water added (B), Inoculum size (C), Wheat bran (D), Soybean meal (E), Ammonium sulphate (F), Magnesium sulphate (G), Yeast extract (H) and incubation temperature (J) with the average levels 48h, 82.5%, 2.55%, 7.5g, 7.5g, 0.506%, 0.12%, 0.55 and 37°C, respectively.

Out of these nine reaction variables inoculum size, soybean meal and yeast extract were found to be significant with p value < 0.05. These significant variables were further used for the optimization of Protease production. Their levels ranged from 0.1-6.670% (v/w), 0.0-15.0 (g/flask) and 0.1-1.31% (w/w) for Inoculum size, Soybean meal and Yeast extract. The protease activity (U/g) with respect to 20 experimental Runs is shown in Fig. 2 which described that the maximum protease U/g level i.e., 6387 U/g was achieved when reaction was carried out using inoculum size, soybean meal and yeast extract with the levels 2.55% (v/w), 7.5 (g/flask) and 0.55% (w/w), respectively.

The model F-value of 281.57 implies the model is significant as shown in the data of Tables 2-4. There is only a 0.01% chance that such a large F-Value could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A², B², C² are significant model terms. The three-dimensional response surface and contour presentations were plotted to study the interaction among various media formulation factors used and to find out the optimum level of each factor for maximum alkaline protease production from *Bacillus subtilis* GCU-8. However, to understand the interaction behaviors of parameters, the response surfaces were investigated for each couple of variables, keeping third one constant.

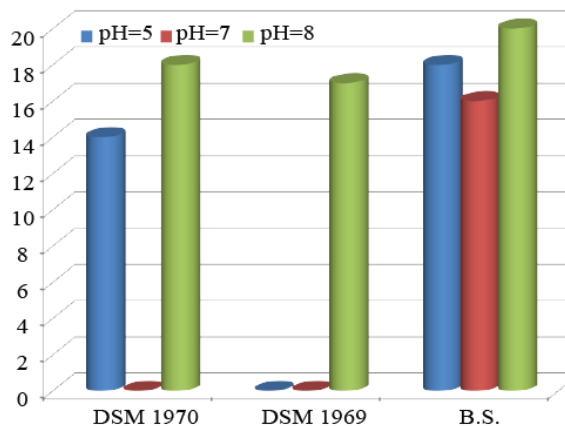


Fig. 1. Screening of bacterial cultures for protease activity at different pH.

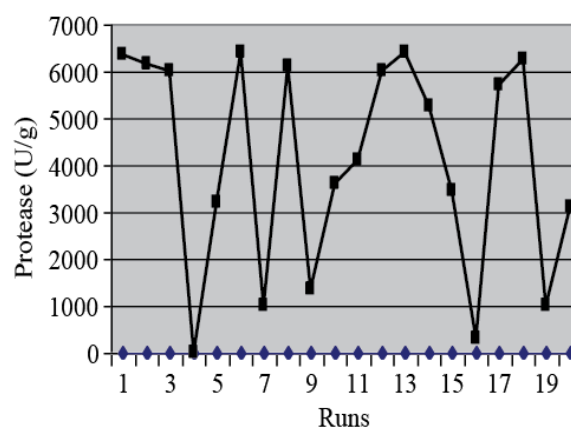


Fig. 2. Response surface optimization of significant variables.

Table 1. Selection of significant variables by PBM.

Sr. No.	Reaction variables	Average
1.	Incubation time (A)	48h
2.	Water added (B)	82.5%
3.	Inoculum size (C)	2.55%
4.	Wheat bran (D)	7.5g
5.	Soybean meal (E)	7.5g
6.	Ammonium sulphate (F)	0.506%
7.	Magnesium sulphate (G)	0.12%
8.	Yeast extract (H)	0.55%
9.	Incubation temperature (J)	37°C
	Observed Protease activity (U/g)	2105.75

It is clear from Fig. 3a that high protease units (7200 U/g) were produced at low concentrations of soybean meal and larger inoculum size. Figure 3b represents the interaction between yeast extract and inoculum size, response was high at low yeast extract concentration and larger inoculum size. Soybean meal and yeast extract interaction resulted in good response when soybean meal was in excess as compared to yeast extract (Fig. 3c). Soybean meal and inoculum size interact most significantly and results in higher response generation as compared to two other combinations. Therefore soybean meal and inoculum size were key factors.

Table 2. Reaction variables and their levels used for optimization study.

Reaction variables	Inoculum size (% age, v/w)	Soybean meal (g/flask)	Yeast extract (% age, w/w)	Response protease (U/g)
Range	0.1-6.670	0.0-15.0	0.1-1.31	289-6387
Average	2.55	7.5	0.55	4081.65

Table 3. ANOVA for response surface quadratic model.

Source	Sum of squares	Df	Mean square	F-value	P-value *Prob > F	Significance
Model	9.950E + 007	9	1.106E + 007	281.57	< 0.0001	Significant
A-Inoculum size	1.640E + 007	1	1.640E + 007	417.78	< 0.0001	Significant
B-Soybean meal	3.292E + 007	1	3.292E + 007	838.48	< 0.0001	Significant
C-Yeast extract	3.729E + 006	1	3.729E + 006	94.98	< 0.0001	Significant
AB	1.119E + 006	1	1.119E + 006	28.50	0.0003	Not-significant
AC	2.657E + 005	1	2.657E + 005	6.77	0.0264	Not-significant
BC	5.984E + 005	1	5.984E + 005	15.24	0.0029	Not-significant
A ²	3.190E + 007	1	3.190E + 007	812.40	< 0.0001	Significant
B ²	1.360E + 007	1	1.360E + 007	346.45	< 0.0001	Significant
C ²	6.085E + 006	1	6.085E + 006	154.98	< 0.0001	Significant

Table 4. Optimization of interaction significant factors for protease production.

Factor	Coefficient		Standard df	95% CI error	95% CI	
	Estimate				Low	High
Intercept	6204.65		1	80.81	6024.59	6384.72
A-Inoculum size	1095.94		1	53.62	976.47	1215.41
B-Soybean meal	1552.60		1	53.62	1433.14	1672.07
C-Yeast extract	522.56		1	53.62	403.09	642.03
AB	374.00		1	70.06	217.91	530.09
AC	182.25		1	70.06	26.16	338.34
BC	-273.50		1	70.06	-429.59	-117.41
A ²	-1487.72		1	52.20	-1604.02	-1371.42
B ²	-971.54		1	52.20	-1087.84	-855.24
C ²	-649.80		1	52.20	-766.10	-533.50

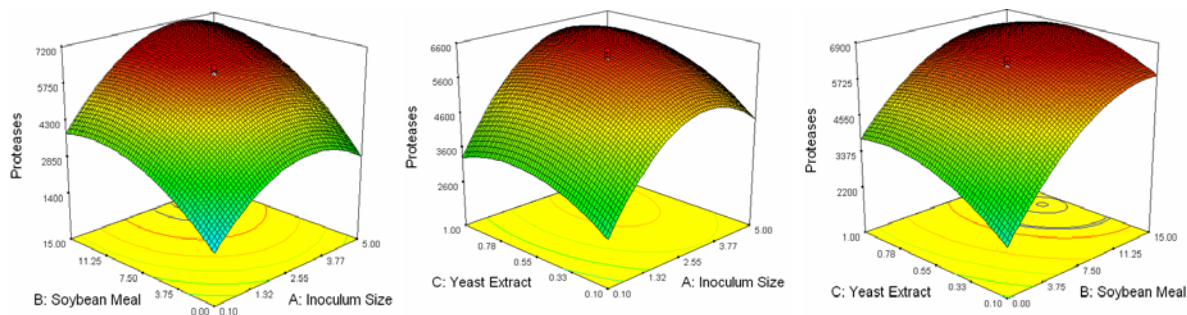


Fig. 3. 3D response surface plot for alkaline protease production (a) interactive effects of Inoculum size and soybean meal keeping yeast extract constant. (b) interactive effects of Inoculum size and yeast extract keeping soybean meal constant (c) interactive effects of soy bean meal and yeast extract keeping soybean meal constant.

In the present study no proteinaceous substrate was required for higher protease production (6981 U/g) which is in agreement with Chellapaan *et al.*, (2005) but not with Venugopal & Saramma, (2006). This may be due to the fact that wheat bran is a protein rich medium and the proteins present in the bran are sufficient for the induction process and no additional protein source is required (Smit *et al.*, 1996). Protease production in absence of wheat bran was 2677 (U/g) and in the absence of soybean meal 2018 (U/g), respectively. The production was increased to 6981 (U/g) in

presence of wheat bran. Increase in protease production in the presence of proteinaceous substrates has also been reported by Phadataré *et al.*, (1993); and Venugopal & Saramma, (2006) (Fig. 4).

The alkaline protease was purified by ammonium sulphate precipitation, ion-exchange chromatography and size exclusion chromatography. The molecular wt. of the purified enzyme was determined by SDS-PAGE under reductive conditions using medium range standard molecular weight markers. It was found to be 45 kDa as shown in Fig. 5.

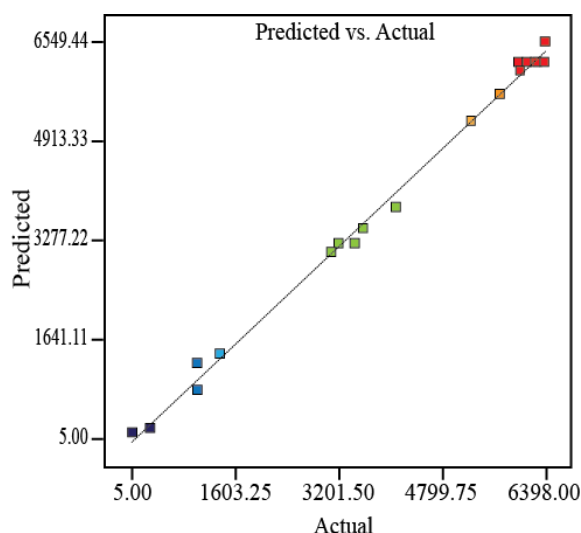


Fig. 4. Plot between predicted and actual productions.

Conclusion

Bacillus subtilis GCU-8 is a potent producer of alkaline protease - a 45 kDa protein, in addition to acid and neutral proteases. Biostatistically optimized solid substrate medium containing equal amounts of soybean meal and wheat bran is capable of producing enhanced amount of alkaline protease. A linear correlation between statistically predicted values and experimental values showed validity of the statistical model.

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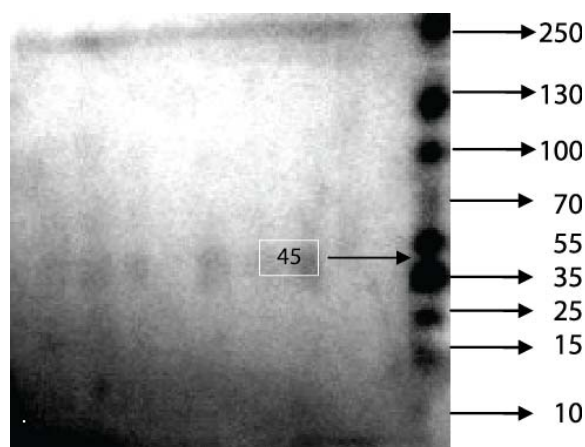


Fig. 5. SDS-PAGE of alkaline protease produced by *Bacillus Subtilis* GCU-8.

Well 1 and 2: Protease sample after purification Well 3: Size marker molecular weight in k Da.

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