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Statistical optimization of alkaline protease production from newly isolated *Pseudomonas* species MTCC 16017

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ABSTRACT

Aims: The present study was investigated to optimize the production parameters using statistical method of the industrially important enzyme alkaline protease from newly isolated strain *Pseudomonas putida* from soil microorganisms.
Methodology and results: Among 50 isolates of extensive screening two highly productive strains were selected. One among after biochemical characterization both laboratory level and by IMTECH Chandigarh was confirmed as *P. putida.* Submerged fermentation was carried out and statistical optimization methods Plackett and Burman and RSM were used to optimize the production parameters.
Conclusion, significance and impact of study: Among the two selected strains out of 50 isolates, *P. putida* was identified as the one of the major protease producer. In this study eleven parameters were selected for the fractional factorial design (Plackett and Burman). Four significant parameters including time, carbon source, nitrogen source and salt showed significant impact on the alkaline protease production. During the study an increase in the alkaline protease activity from 4.659 U/mL to 7.396 U/mL was observed. Based on the above data more complex designs, such as Box Wilson design to study the impact of individual significant variable on the enzyme production as well as interactive effects among these significant variables were carried out. The interactive effect of the most influential parameters resulted in increase in enzyme activity from 7.246 up to 10.818 U/mL in 60 h. Analysis of variance showed the adequacy of the model and verification experiments confirmed its validity.

Keywords: Alkaline protease, Plackett and Burman method, RSM, *Pseudomonas putida*

INTRODUCTION

Proteases termed as Industrial masters occupy the third largest position in enzyme production. Proteases find their applications in various industrial sectors such as in food, detergent, tannery, Photography and other industries (Kumar and Takagi, 1999; De Coninck *et al*., 2000; Gupta *et al*., 2002; Puri *et al*., 2002). Most of the available proteases produced commercially are of microbial origin. It is a renowned fact that environmental and nutritional factors greatly influence extracellular protease production in microorganisms. In our study we included fructose and peptone as carbon, nitrogen sources (Hanlon *et al*., 1982; Kole *et al*., 1988; Banerjee and Bhattacharya, 1993; Kumar and Takagi, 1999; Calik and Ozdamar, 2001; Chi and Zhao, 2003; Rai and Mukherjee, 2009) and environmental factors such as pH, temperature, agitation rate and incubation time (Razak *et al.*, 1994; Gupta *et al.*, 2002; Puri *et al.*, 2002) along with inoculums amount.

The classical method 'one-at-a-time-approach' is the most commonly used to optimize the production parameters to enhance the enzyme yield. This approach consumes lot of time in addition ignores the mutual interactions among various physicochemical parameters. Statistical optimization techniques such as Plackett and Burman (Plackett and Burman, 1946; Moon and Parulekar, 1993; Mekala *et al.*, 2008; Reddy *et al.*, 2008) and RSM (Response Surface Method) 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 (Kunamneni *et al.*, 2005; Reddy *et al.*, 2008). In recent times, numerous statistical experimental designs with response surface method (RSM) have been employed for optimizing enzyme production from microorganisms (Puri *et al.*, 2002; Saran *et al.*, 2007; Rai *et al.*, 2009; Swapna and Sreenivas, 2011). Significant parameters for statistical optimization of protease production using locally isolated *P. putida species* their interaction effects are reported below.

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MATERIALS AND METHODS

Isolation and enzyme production

Soil samples from different regions of Andra Pradesh were collected and protease producing strains were isolated on skim milk agar. After inoculation the plates were incubated at 37 °C for 48 h. After incubation bacterial colonies appearing over skim milk agar medium were identified based on colony characteristics and their identities were confirmed through Gram staining methods and by a series of biochemical tests as prescribed by Bergey's manual. For enzyme production, microbes were cultured in 250 mL of Erlenmeyer flask containing 100 mL culture medium, which consists of 10.0 g of fructose, 3.5 g peptone, 3.5 g yeast extract, 0.5 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$. The inoculated medium was placed in a thermostatic orbital shaker for 48 h at 37 °C and 120 rpm. The culture was centrifuged at 10,000 rpm for 10 min to obtain crude enzyme in the supernatant.

Assay for proteolytic activity

Alkaline protease activity studies were done by applying a modified method given by (Gupta *et al.*, 2002). According to this procedure 0.25 mL of Tris-Hcl (50 mM, pH 10.5) buffer was incubated with 2.5 mL of 0.6% casein dissolved in the same buffer at 30 °C until equilibrium was achieved. An aliquot of 0.25 mL of the enzyme solution was added to this mixture and incubated for 20 min. The reaction was stopped by adding 2.5 mL TCA solution (0.11 M trichloroacetic acid). After 10 min the entire mixture was centrifuged at 5000 *g* for 15 min. Supernatant in the amount of 0.5 mL was mixed with 2 mL of 0.5 M Na_2CO_3 and 1 mL of Folin-Ciocalteu's phenol solution and kept for 30 min at room temperature. The optical densities of the solutions were determined with respect to the sample blanks at 660 nm. For these studies, one alkaline protease unit was defined as the enzyme amount that could produce 1 mg of tyrosine in one minute under the defined assay conditions.

Statistical optimization of protease production using response surface methodology

The alkaline protease production is influenced by various production parameters including nutritional and environmental parameters. Statistical optimization was carried out for higher enzyme production using various nutritional and environmental parameters using 'Design Expert 8.5' software. The Plackett–Burman experimental design was applied to investigate the significance of various medium components on alkaline protease production. Eleven culture variables were tested in two levels: −1 for low level and +1 for high level based on Plackett-Burman matrix design, which is a fraction of a two-level factorial design and allows the investigation of n−1 variables in at least n experiments. The main effect of each variable was calculated simply as the difference between the averages of measurements made at a high setting (+1) and the average of measurements observed at a low setting (−1) of that factor. The levels of these variables were optimized for enhancing the protease yield using a response surface Box–Behnken experiment design. The design matrix with n experimental runs in two blocks with three replicates of the midpoint. The significant variables selected for optimization, were coded as a, b, c … respectively.

$$Y = a_0 + \Sigma\, a_i C_i + \Sigma\, a_{ii} C_i^2 + \Sigma\, a_{ij} C_i C_j$$

where Y is the predicted response (total protease production in U/mL), a_0 is the intercept term, a_i is the linear effect, a_{ii} is the square effect, a_{ij} is the interaction effect, and C_i and C_j are the variables. The above equation was used to optimize the values of independent parameters for the response. Multiple regression analysis, response surface plots and statistical analyses were performed using Design Expert Statistical Software® (Miniapolis USA).

RESULTS AND DISCUSSION

Isolation of bacteria

Bacteria producing alkaline protease were isolated from soil by serial dilution techniques. Superior microbial strain having high productivity is selected from zone of hydrolysis assay. The isolated bacteria having maximum zone of hydrolysis (Figure 1) were identified through a series of biochemical tests (Table 1) as *Pseudomonas putida* for further confirmation the sample was sent to IMTECH Chandigarh and the biochemical characterization results were shown in Table 1.

Table 1: Biochemical characterization results.

Biochemical Test	*Pseudomonas* sp.
Catalase	Negative
Urease test	Negative
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Carbohydrate	Acid
a) Glucose	Positive
b) Sucrose	Positive
c) Maltose	Negative
Gram reaction	Negative
Shape	Rod

Statistical optimization of protease production using Plackett and Burman method

The Plackett-Burman experimental design was applied to investigate the significance of various medium components on alkaline protease production. Eleven

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culture variables were tested in two levels: −1 for low level and +1 for high level based on Plackett-Burman matrix design. In this study the independent variables were screened in 12 combinations according to the matrix. Glucose, peptone, KH_2PO_4 and time were identified as significant parameters affecting alkaline protease production as indicated in (Figure 1).

Response Surface Method

The levels of the four significant variables obtained from Placket and Burman design were optimized for enhanced protease production using response surface Box–Behnken experiment design. The design matrix with 20 experimental runs in two blocks with three replicates of the midpoint is shown in (Table 2). The variables selected for optimization, i.e. glucose, KH_2PO_4, peptone concentration, and incubation time was coded as A, B, C and D respectively. The goodness-of-fit of the model was checked by determining the coefficient of determination (R^2) and adjusted R^2. When R^2 is large, then, the regression has accounted for a large proportion of the total variability in the observed value of Y which favors the

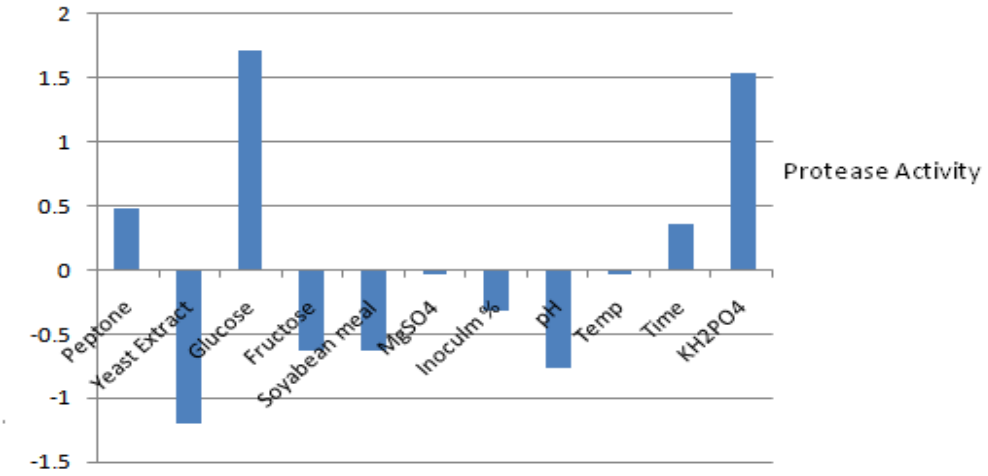

Figure 1: Significant main effects of production parameters from the Plackett-Burman design.

Table 2: Box-Behnken experiment design matrix with observed and predicted responses for different experiments.

Factor A (Glucose) (g/mL)	Factor B (KH_2PO_4) (g/mL)	Factor C (Peptone) (g/mL)	Factor D (Time) (h)
-1	0	0	0
-1	1	1	1
0	0	0	-1
0	0	0	1
0	0	0	0
0	0	0	0
-1	-1	-1	-1
-1	1	-1	1
0	0	1	0
0	0	0	0
0	0	-1	0
0	1	0	0
0	-1	0	0
1	1	1	-1
0	0	0	0
0	0	0	0
1	0	0	0
1	-1	-1	1
-1	-1	1	-1
1	-1	1	1
1	1	-1	-1

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Table 3: Analysis of variance for Response Surface Quadratic model.

Source	Sum of squares	df	Mean square	F-value	P value > F	
Model	8.157E+007	10	8.157E+006	8.29	0.0013	Significant
A-A	0.41	1	0.41	4.20701E-0	0.9995	
B-B	0.21	1	0.21	2.174E-007	0.9996	
C-C	9.600E+006	1	9.600E+006	9.76	0.0108	
D-D	0.25	1	0.25	2.520E-007	0.9996	
AB	2.402E+006	1	2.402E+006	2.44	0.1492	
AC	200E+007	1	1.200E+007	12.2	0.0058	
AD	2.399E+006	1	2.399E+006	2.44	0.1494	
BC	1.199E+007	1	1.199E+007	12.19	0.0058	
BD	2.398E+006	1	2.398E+006	2.44	0.1495	
CD	1.199E+007	1	1.199E+007	12.19	0.0058	
Residual	9.835E+006	10	9.835E+005	8.29		
Lack of fit	9.835E+006	6	1.639E+006	1.535E+006	< 0.0001	Significant
Pure error	4.27	4	1.07			
Cor Total	9.140E+007	20				

regression equation model. The observed values of R^2 (97.88%) explain that the fitted model is 97.88% of the total variation and hence vouches for adequacy of the model and only 2.12% can occur due to chance. P values of each of the parameters and their quadratic and interaction terms are indicated in (Table 3). The significance of individual variables can be evaluated from their P values, the more significant terms having a lower P -value. The values of P>F less than 0.05 indicates that the model terms are significant and this case B, C, AB and B^2 were found to be significant model terms.

Response surface curves were plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal protease yield. Figure 2a-2f represents the response surfaces obtained for the interaction effects of tested variables. The data presented in the response plots indicated that the alkaline protease production increased with an increase in the concentration of fructose up to optimum value and then started decreasing the yield this may be due to catabolic repression effect. Swapna and Sreenivasa Rao (2011) reported fructose as one of the better carbon source than glucose producing higher enzyme yields Calik, Reddy and Razak also reported positive influence of carbon source (Razak *et al*., 1994; Çalık and Özdamar, 2001; Reddy *et al.*, 2008) on enzyme production up to certain extent and further increase resulted in decrease in enzyme production due to catabolic repression Gupta reported organic nitrogen source has positive influence on enzyme yield, which was also observed in our case where peptone prominently has positive influence on enzyme yield (Gupta *et al.*, 2002). Time has profound positive influence on enzyme production and this was also reported by Puri *et al.*, 2002. Increase in time increases the enzyme production and longer timescales decline phase appears may be due to depletion of nutrients or may be auto digestion of the enzyme (Chu *et al.*, 1992). The optimum glucose, peptone concentration is 1.4 g/100mL and time 34 h for maximum

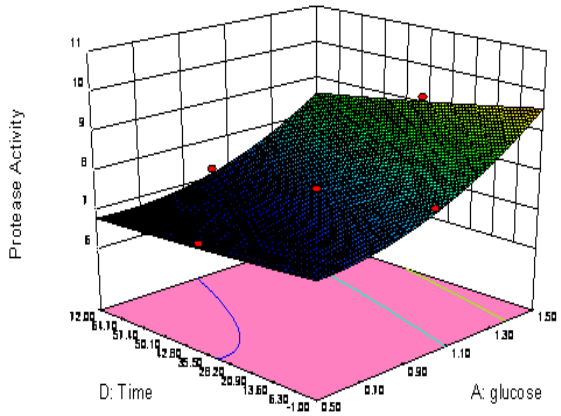

Figure 2(a): Response curve of carbohydrate and time vs protease activity.

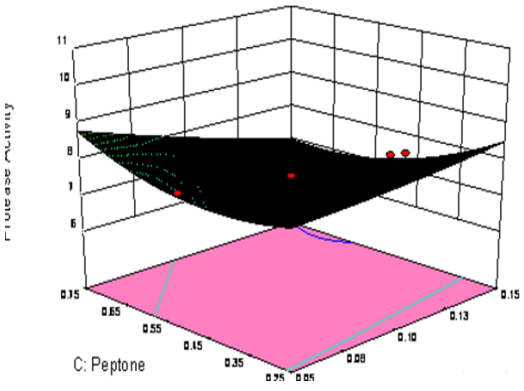

Figure 2(b): Response curve of nitrogen source and KH_2PO_4 vs protease activity.

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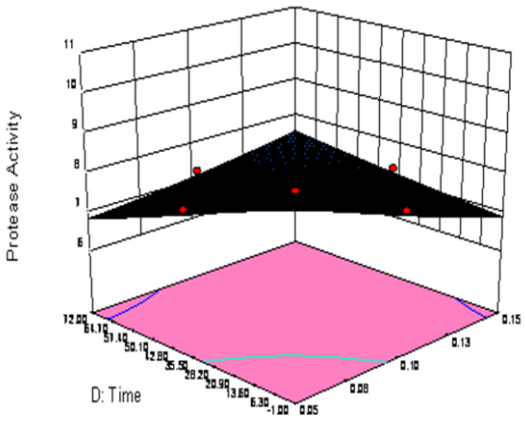

Figure 2(c): Response curve of time and KH_2PO_4 vs protease activity.

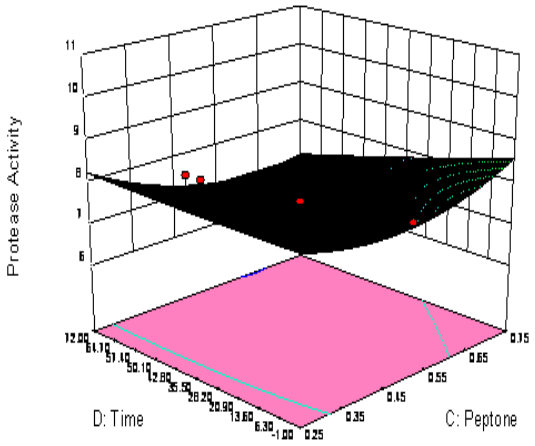

Figure 2(d): Response curve of time and carbon source vs protease activity.

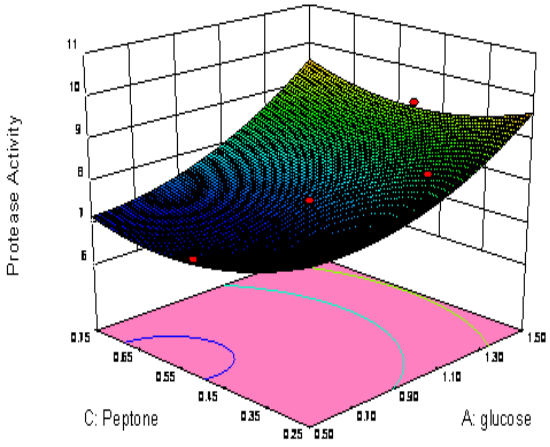

Figure 2(e): Response curve of nitrogen source and carbon source vs protease activity.

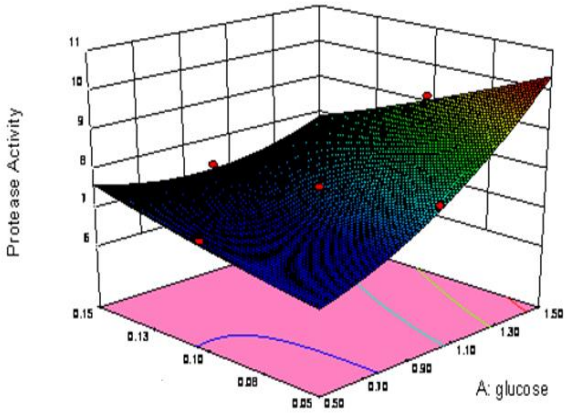

Figure 2(f): Response curve of KH_2PO_4 and carbon source vs protease activity.

yield. KH_2PO_4 concentration has significant influence on protease production and optimum value is 0.06. Validity experiment was conducted with optimum values and similar increase in protease production of 10.898 is observed.

Recent studies were conducted on statistical optimization of different production parameters of different organisms using response surface methodology (Reddy *et al*., 2008; Mekala *et al.*, 2008; Swapna and Sreenivasa Rao, 2010). Every organism is unique in its requirement, no defined media in particular and environmental parameters for maximum enzyme production as it vary for one organism to another. Therefore, each of them has to be considered separately and the requirements have to be optimized accordingly. In this study a 2.3 fold increase from 4.659 U/mL to 10.898 by optimizing production parameters time, Fructose concentration and $MgSO_4$ concentration was observed. Recent study done by Gupta *et al*. (2002) on *Bacillus* sp. RGR-14 for alkaline protease production, reported a 12.85 fold increase by optimizing starch, phosphate ion and inoculums concentrations using the response surface method. Another study performed by Gupta *et al.* (2002) showed that alkaline protease production produced by *Bacillus mojavensis* was improved up to 4.2 fold in a bioreactor of 14 L using RSM.

CONCLUSIONS

Statistical optimization of production parameters considering the interactive effects of the most influential parameters resulted in overall enzyme activity from 4.659 up to 10.889 U/mL in 60 h. A 2.33 fold increase by optimizing time, fructose concentration and $MgSO_4$ concentration is observed. Thus a statistical approach of optimization is very much useful and less time consuming considering the interaction effects provides a basis for conduction short term experiments with better performance.

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