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Optimization Of Alkaline Protease Production From Locally Isolated Bacillus Sp. Bacillus Firmus From Soil Microorganisms In Batch Culture Using Statistical Design

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Abstract
Microbial strains were isolated and characterized from various soil sources for higher alkaline protease production. As the next step statistical optimization was carried out for higher enzyme production using various nutritional and environmental parameters using 'Design Expert 8.5' software. In this study eleven parameters were selected for the fractional factorial design (Plackett Burman) where as three significant parameters including time, carbon source and $MgSO_4$ showing significant impact on the alkaline protease production. During the study an increase in the alkaline protease yield from 0.491 U/ml to 5.497U/ml was observed. Based on the above data more complex designs, such as Box Wilson design (RSM) to study the impact of individual significant variations 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 a yield improvement from 5.49 up to 7.989 U/ml in 32 h . Analysis of variance showed the adequacy of the model and verification experiments confirmed its validity.

Keywords: Alkaline protease, Plackett Burman method, RSM, Bacillus sp.

I. 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 [6- 8, 12, and 22]. Most of the available proteases produced commercially are of microbial origin. The alkaline proteases produced by *Bacillus* species are by far the most important group of enzymes produced commercially [21]. 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 [1-4, 10, 11, 15] and environmental factors such as pH, temperature, agitation rate and incubation time [18, 20-21] 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 Placket & Burman [9, 14, 17, 20] and RSM (Response Surface Method) which includes factorial design and regression analysis, helps in evaluating the effective factors and building models to study the interaction and select optimum conditions of variables for a desirable response [13-14]. In recent times, numerous statistical experimental designs with response surface methodology (RSM) have been employed for optimizing enzyme production from microorganisms [21-23, 25]. Here we report significant parameters for statistical optimization of protease production using locally isolated *Bacillus species* and we have also reported their interaction effects.

Ii. Material And Methods
2.1 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 37^0C for 48 hours. 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 [24] as prescribed by Bergey's manual. The identification was confirmed by sending sample for confirming identification by IMTECH Chandigarh. For enzyme production, microbes were cultured in 250 ml of the Erlenmeyer flask containing 100 ml culture medium, which consists of 10.0 g of fructose, 3.5 peptone g, 3.5 g yeast extract, 0.5 g K2HPO4, 0.1 g MgSO4.7H2O.The inoculated medium was placed in a thermostatic orbital shaker for 48 hrs at 37°C and 120 RPM (revolutions per second). The culture was centrifuged at 10,000 RPM for 10 min to obtain a crude enzyme in the supernatant.

2.2 16s RNA Sequencing

Molecular identification of the superior isolate was achieved by 16Sr DNA sequencing. Sequencing was done using forward primer (16SF Universal AGA GTT TGA TCC TGG CTC AG) and reverse primer (16SR Universal ACG GCT ACC TTG TTA CGA CTT). PCR reactions were performed with the following program forthe16SrRNAgene:30cycles consisting of 95ºC for1min, 55 ºC for1min and 72 ºC for 1.5min, followed by a final extension step of 5 mi n at 72 ºC. The16SrRNAsequence was analyzed by an automated DNA sequencer (Applied Bio systems). The sequence was analyzed for homology using (CLUSTALW).

2.3 Assay For Proteolytic Activity

Alkaline protease activity studies were done by applying a modified method given by Takami et al. [7]. According to this procedure 0.250 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 ^{0}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 *m*g of tyrosine in one minute under the defined assay conditions.

2.4 RSM Methodology

The Alkaline protease production is influenced by various production parameters including nutritional and environmental parameters. The Plackett–Burman experimental design [20] 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 in 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 of the response. Multiple regression analysis, response surface plots and statistical analyses were performed using Design Expert Statistical Software® (Minneapolis USA).

Iii. Results And Discussion

Among 50 isolates one superior strain showing high activity was selected. The strain was biochemically characterized at laboratory level as *Bacillus sp. And* further confirmed by IMTECH Chandigarh, The results of biochemical characterization of the superior strain producing alkaline protease are shown in (Table 1) . From the 16S RNA sequencing analysis the strain is 99% similar to *Bacillus Firmus.* The Phylogenetic tree constructed is shown below (Fig. 1).

3.1 Optimum RPM And Inoculms %

Influence of RPM (100, 120, 140, 160, 180, and 200) and inoculums percentage (2, 4, 6, and 8) on alkaline protease production was studied and activity of protease v's varying RPM rates and inoculums percentages is represented schematically in following graphs (Fig. 2a, b). In this study higher protease production is observed at 160 RPM and 6% inoculums %.

3.2 Statistical Optimization Of Protease Production Using Plackett & Burman Method

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 .In this study the independent variables were screened in 12 combinations according to the matrix. Fructose, MgSO4 and time were identified as significant parameters affecting alkaline protease production as indicated in (Fig .3) And the significance of the model is given by the ANOVA regression analysis (Table 2).

3.2 Response Surface Method

The levels of the three significant variables obtained from Placket & Burman design were optimized for enhanced protease production using response surface Box–Behnken experiment design .The design matrix with 17 experimental runs in two blocks with three replicates of the midpoint is shown in (Table 3). The variables selected for optimization, i.e., Fructose concentration, MgSO4 concentration and incubation time were coded as A, B, and C 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 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. (Fig. 4a-4c) represents the response surfaces obtained for the interaction effects of testing 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. Sen reported fructose as one of the best carbon sources than glucose producing higher enzyme yields. Calik , Reddy and Razak also reported a positive influence of carbon source on enzyme production up to a certain extent and further increase resulted in a decrease in enzyme production due to catabolic repression. Time has a profound positive influence on enzyme production and this was also reported by Puri and Beg. 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 by the enzyme [5]. The optimum fructose concentration is 1.4 g/100ml and time 34h for maximum yield. MgSO4 concentration has no any significant influence on protease production and optimum value is 0.06 [10].

Iv. Conclusion

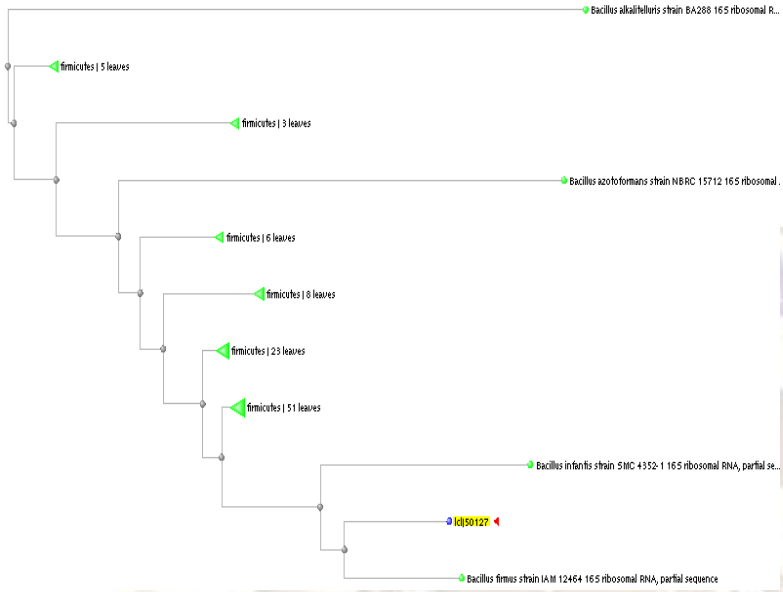
Statistical optimization of production parameters considering the interactive effects of the most influential parameters resulted in overall yield improvement from 0.49 up to 7.989 U/ml in 32 h. A 16 fold increase by optimizing time, Fructose concentration and MgSO4 concentration are observed. Thus a 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.

in the observed value of Y which favors the 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 4). 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.

Validity experiment was conducted with optimum values and similar increase in protease production of 7.898 is observed.

Drastic studies were conducted on optimization of different production parameters of different organisms using response surface methodology [9, 13, and 14]. Every organism has unique in its requirement, no defined media in particular and environmental parameters for maximum enzyme production as it varies 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 16 fold increase from 0.45 U/ml to 7.989 by optimizing production parameters time, Fructose concentration and MgSO4 concentration was observed. Recent studies done by Chauhan and Gupta [8] on Bacillus sp. RGR-14 for alkaline protease production, Reported a 12.85 fold increase by optimizing starch, casamino acid, phosphate ion and inoculums concentrations using the response surface method. Another study performed by Beg et al. [21] Showed that alkaline protease production produced by Bacillus mojavensis was improved up to 4.2 fold in a bioreactor of 14 l usingRSM.

Fig.1 Phylogenitic Tree



Results

Table 1 Biochemical Characterization

S.No	*Test*	*Result*
1	*Gram stain*	*Positive*
2	*Color*	*White*
3	*Indole Test*	*Negative*
4	*Methyl Red Test*	*Negative*
5	*Voges Proskauer Test*	*Negative*
6	*Citrate Utilization*	*Negative*
7	*Casein Hydrolysis*	*Positive*
8	*Starch Hydrolysis*	*Positive*
9	*Urea Hydrolysis*	*Negative*
10	*Cytochrome Oxidase*	*Negative*
11	*Catalase Test*	*Positive*
12	*Nitrate Reduction*	*Negative*
13	*H_2S Production*	*Positive*

Fig.2a Agitation vs. Protease Activity, Growth

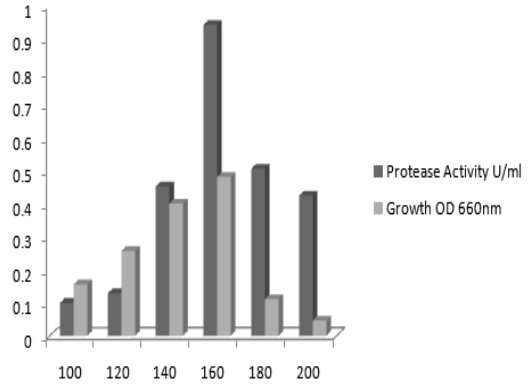
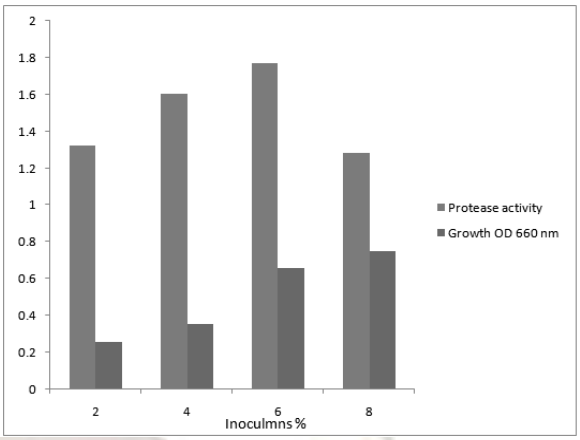


Fig.2b Inoculums' % vs. Protease Activity, Growth



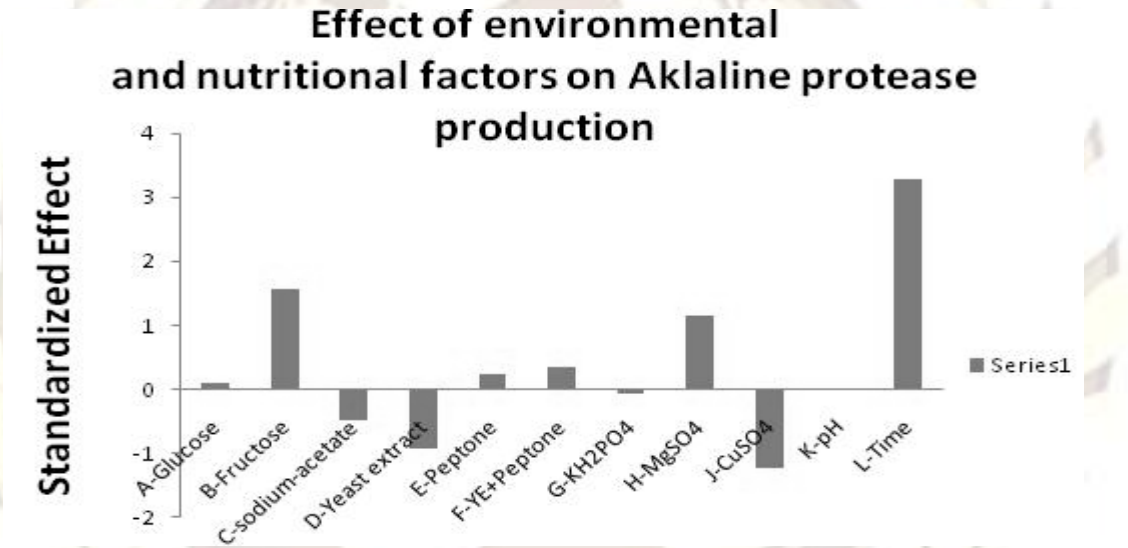


Fig.3 Significant main effects of production parameters from the Plackett Burman design.

Table 2 ANOVA regression analysis for placket & Burman design

Source	Sum of Squares	DF	Mean Square	F-Value	P-valueProb>F	
Model	48.21	4	12.05	21.94	0.0005	Significant
B-Fructose	*7.28*	*1*	*7.28*	*13.26*	*0.0083*	
H-MgSO4	*3.9*	*1*	*3.9*	*7.1*	*0.0322*	
J-CuSO4	*4.7*	*1*	*4.7*	*8.55*	*0.0222*	
L-Time	*32.33*	*1*	32.33	*58.86*	*0.0001*	

Table 3: Box-Behnken experiment design matrix with observed and predicted responses for different experiments.

Std	Fructose Conc. mg/ml A	MgSO4 Conc. mg/ml B	Time H C	Alkaline Protease Activity Observed	Alkaline Protease Activity predicted
1	0.5	0.06	32	7.899	7.859
2	1.5	0.06	32	7.2	7.381
3	0.5	0.1	32	7.17733	6.979
4	1.5	0.1	32	7.47767	7.501
5	0.5	0.08	30	6.49933	7.179
6	1.5	0.08	30	6.37833	7.201
7	0.5	0.08	34	6.888	6.979
8	1.5	0.08	34	7.49333	7.501
9	1	0.06	30	7.50167	7.76
10	1	0.1	30	6.745	7.38
11	1	0.06	34	7.44333	7.5
12	1	0.1	34	7.09633	7.32
13	1	0.08	32	7.265	7.33
14	1	0.08	32	7.214	7.33
15	1	0.08	32	7.476	7.33
16	1	0.08	32	7.27333	7.33
17	1	0.08	32	7.42533	7.33

Table 4: Analysis of varriance for Response Surfcae Quadratic model

S.no	Source	Sum of Squares	dF	Mean Square	F-value	Prob>F
1	Model	2.03	9	0.23	4.09	0.0382(significant)
2	*A-A*	9.17E-04	*1*	9.17E-04	*0.017*	*0.9011*
3	*B-B*	0.3	*1*	*0.3*	*5.42*	*0.0527*
4	*C-C*	*0.4*	*1*	*0.4*	*7.31*	*0.0305*
5	*AB*	0.25	*1*	*0.25*	*4.52*	*0.071*
6	*AC*	0.13	*1*	*0.13*	*2.39*	*0.1661*
7	*BC*	0.042	*1*	*0.042*	*0.76*	*0.4122*
8	A^2	0.079	*1*	*0.079*	*1.43*	*0.2703*
9	B^2	*0.25*	*1*	*0.25*	*4.57*	*0.0698*
10	C^2	*0.6*	*1*	*0.6*	*10.95*	*0.0129*
11	Residual	0.39	7	0.055		
12	*Lack of fit*	*0.34*	*3*	*0.11*	*8.71*	*0.0315(significant)*
13	*Pure Error*	*0.051*	*4*	*0.013*		
14	Cor Total	2.42	*16*			

$R^2 = 0.9788$ and $R^2adj = 0.9152$

Response Curves

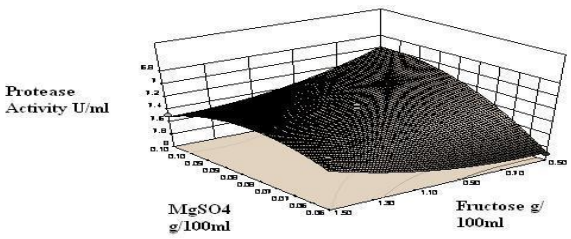


Fig. 4a Response curve of carbohydrate and metal ion vs. Protease activity

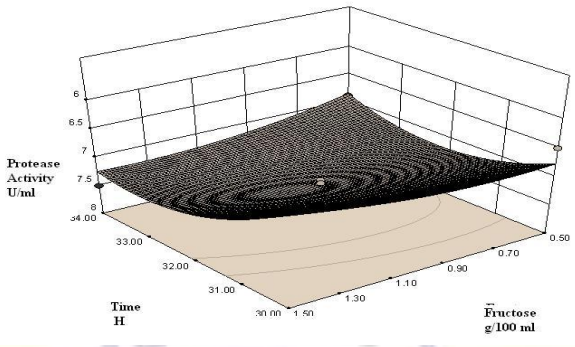


Fig. 4b Response curve of carbohydrate and time vs. Protease activity

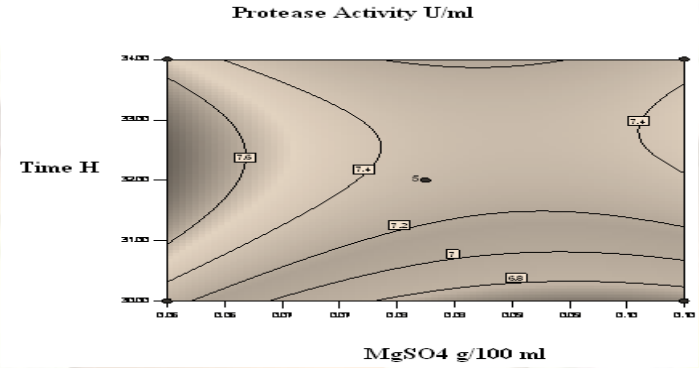


Fig. 4c Response curve of time and metal ion vs. Protease activity

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