Process optimization by Placket-Burman Designs for the Production of Protease Enzyme from Bacillus Subtilis under Submerged Fermentation

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Abstract - Protease constitutes a large and complex group of enzyme which plays an important nutritional and regulatory role in nature. A variety of microorganisms such as bacteria, fungi, yeasts and actinomycetes are known to produce this enzyme. Commercially proteases are the most important industrial enzymes accounting for about 60% of the total enzyme market. The objective of the present study was to evaluate protease production *Bacillus subtilis* under submerged fermentation. Initially a Plackett – Burman experimental design was used to find out the significant parameters and these were further optimized employing a Response Surface Methodology. Software Minitab 15 (Minitab Inc, USA) was used for experimental design, data analysis and quadratic model building. The detailed results are discussed.

Key Words: *Bacillus subtilis*, Protease, Response surface methodology, Fermentation

I. INTRODUCTION

Most commercial proteases are mainly neutral and alkaline. Alkaline proteases are of considerable interest in view of their activity and stability at alkaline pH they find several application in wine and beer production, the paper industry, starch industry, leather industry, in bioremediation, baking industry, detergent formulations as well as in the pharmaceutical & diagnostic industry. The alkaline protease generally produced by submerged fermentation. The culture conditions that promote protease production were found to be significantly different from the culture conditions promoting cell growth. It is essential to optimise the culture conditions to develop an economically feasible technology for the enzyme.

Placket and Burman designs (Plackett & Burman 1946) are fractional factorial designs used when one needs to screen a large number of factors to identify those that may be important (i.e., those that are related to the dependent variable of interest), In such situations a design that allows one to test the largest number of factor main effects with the least number of observations is desired.

II. METHODOLOGY

2.1 Statistical experimental designs

Process optimizations are essential in improving the productivity and to understand the effect of parameters on the fermentation. In the conventional method for the optimization of enzyme production, the “one variable at a time” approach is used, which involves changing one parameter at a time while keeping the other entire parameters constant (Chen, 1994). The optimized concentration of the previous experiment is then incorporated in the next experiment. The same procedure is followed for all the parameters to complete the optimization (Young *et al*, 1985). But this process is cost, labour and time intensive, and also does not consider the interaction between variables. An alternative and more efficient approach is the use of statistical methods. Several statistical methods ranging from two factorial to multi-factorial designs are available (Monaghan & Koupal, 1989). Placket and Burman design are most widely used. The Placket and Burman design has the interaction effects of variables confounded with new main effects. Because the added factors are created by equating (aliasing), the "new" factors with the interactions of a full factorial design, these designs always will have 2^k runs e.g., 4, 8, 16, 32, and so on. Full factorial design is fractionalized in a different manner, to yield saturated designs where the number of runs is a multiple of 4, rather than a power of 2.

In an experimental procedure for studying the effects of process parameters (independent variables) under question, the selection of high (1) and low (-1) values of the variable is very critical (Greasham & Inamine, 1986). The difference between the levels of each variable must be large enough to ensure that the optimum response will be included. After performing the experiments, the responses obtained are analyzed statistically to determine the effect of that variable on the response, experimental errors and the significance of the influence of each variable on the response (Nelson, 1982). The effect of a variable is the difference between the average response of that variable at higher and lower levels. Probability tests are run to determine the level of significance of the effects of each variable. The design of experiments and analyses of responses are now routinely done using software made for the purpose eg – Statistical (Statsoft Inc, USA), Design Expert (Stat-Ease, USA) etc. The objective of present study was to determine the effect of process variables in production of protease by *Bacillus subtilis* under SmF, using a fractional factorial (Plackett & Burman) experiment design.

III. RESULTS AND DISCUSSION

3.1 Optimization of the SmF production of protease by Bacillus subtilis

Submerged Fermentation production of protease was done. A Plackett and Burman (Plackett & Burman, 1946) design was employed to determine the effect of individual parameters affecting protease production by the bacteria under SmF. The composition of mineral salt solution and the important physical parameters affecting enzyme production were screened in a design with 6 variables at two levels in a total of 20 experimental runs (Table1).

Table1: Plackett & Burman design matrix for the optimization of variables influencing protease production

Run	Glucose	Peptone	pH	Inoculum size	Inoculum age	Incubation time	Activity
1	5	5	4	0.5	36	72	1.06
2	0.5	5	10	0.5	36	72	0.96
3	0.5	0.5	10	5	12	72	0
4	5	5	4	0.5	12	12	2.123
5	0.5	0.5	4	5	12	72	0
6	5	0.5	4	5	36	12	7.5
7	5	5	10	5	12	12	9.65
8	5	0.5	4	0.5	12	72	0
9	0.5	5	4	5	12	72	0
10	0.5	5	10	0.5	12	12	6.56
11	5	0.5	10	0.5	36	72	1.008
12	0.5	5	10	5	36	12	13
13	0.5	5	4	5	36	72	0.912
14	5	0.5	10	5	12	12	6.948
15	5	5	4	5	36	12	15
16	5	5	10	0.5	12	72	0
17	0.5	0.5	4	0.5	12	12	1.351
18	0.5	0.5	4	0.5	36	12	0
19	0.5	0.5	10	0.5	36	12	2
20	5	0.5	10	5	36	72	1.152

The parameters tested were: Incubation time, inoculums size, inoculums age, concentrations of glucose, peptone and medium pH. The variables were tested at two levels: a higher level designated as +1 and a lower level designated as -1. The actual and coded values tested for each parameter are given in Table 2.

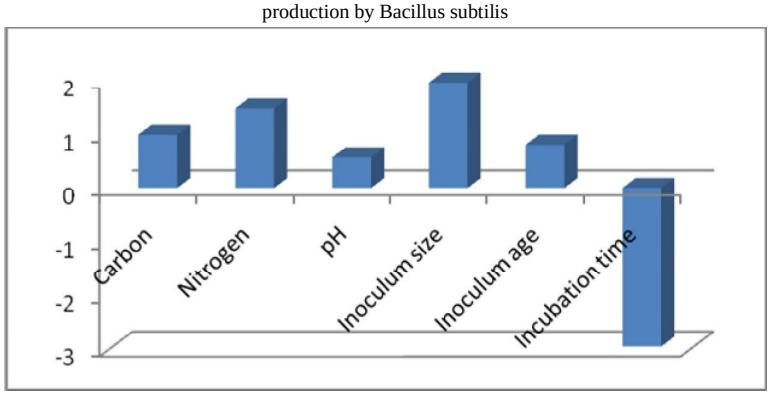
Table 2: Actual levels of variables tested with the factorial design and their effects on protease production

Code	Parameter name	Low level (-1)	High Level (+1)
X_1	Glucose (g/L)	0.5	5
X_2	Peptone (g/L)	0.5	5
X_3	pH	4	10
X_4	Inoculum size	0.5	5
X_5	Inoculum age (Hours)	12	36
X_6	Incubation time (Hours)	12	72

Experimental runs were performed according to the design and the response (Enzyme activity) was recorded. A factorial model was fitted for the main effects using Design Expert software (Statease Corp, USA).

Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on protease production. The most significant parameters affecting protease production and the best combination and levels of parameters for improved production were identified.

Figure 1: Estimated effect of process parameters on protease production by Bacillus subtilis



The parameters with largest effects were incubation time, inoculums size and nitrogen source. Incubation time influenced protease production negatively while the other two has a positive effect.

3.2Optimization of enzymatic saccharification by Box–Benhken design

A Box–Behnken design (Box and Behnken, 1960) was employed to determine the effects of independent variables on the response and factor interactions with different combination of variables. This included three level four factorial designs to investigate and validate the process parameters affecting protease production by *Bacillus subtilis.* Three independent variables – Inoculum size, incubation time and nitrogen source concentration were studied at three levels -1, 0 and +1, which corresponded to the low, medium and high values, respectively. The variable input parameters were inoculums size (5-8%, w/w), incubation time (6-18 hours) and nitrogen source concentration (5-8%) and the protease yield was the output factor. The results were analyzed by software Minitab 15 (Minitab Inc., USA). Both the linear and quadratic effects and the possible interactions of the four variables were calculated.

Table 3: Box–Behnken design

RunOrder	Nitrogen	Inoculum size	Incubation time	Activity
1	5	6.5	18	6.64
2	8	6.5	18	8.334
3	5	5	12	9.471
4	5	6.5	6	0
5	8	5	12	10.1
6	5	8	12	8.47
7	6.5	6.5	12	11.20
8	8	6.5	6	0.15
9	6.5	8	18	8.42
10	6.5	6.5	12	11.2
11	6.5	8	6	0.15
12	6.5	6.5	12	11.2
13	6.5	5	6	0.06
14	8	8	12	12.16
15	6.5	5	18	9.65

3.3 Optimization of protease production from Bacillus subtilis by Box–Behnken design

The objective of the experimental design was to optimize the protease production by *Bacillus subtilis* under submerged fermentation. Since step wise optimization by single parameter at a time cannot examine all the possible combinations of independent variables, statistical experimental design tools for optimization are important. Experimental design and experimental protease yields are presented in Table. Polynomial equation for the model used was as below:

Protease yield = $0.7704\ X_1 - 0.0101\ X_2 + 4.0855\ X_3 - 0.9694\ X_1^2 - 0.1804\ X_2^2 - 6.4496\ X_3^2 + 0.7652\ X_1X_2 + 0.3860\ X_1X_3 - 0.3300\ X_2X_3$

where X_1, X_2 and X_3 are nitrogen source concentration, inoculums size and incubation time respectively.

Table 4 - The analysis was done using coded units.
Estimated Regression Coefficients for Activity

Term	Coef	SE Coef	T	P
Constant	11.2000	0.4101	27.313	0.000
Nitrogen	0.7704	0.2511	3.068	0.028
Inoculum size	-0.0101	0.2511	-0.040	0.969
Incubation time	4.0855	0.2511	16.270	0.000
Nitrogen*Nitrogen	-0.9694	0.3696	-2.623	0.047
Inoculum size*Inoculum size	-0.1804	0.3696	-0.488	0.646
Incubation time*Incubation time	-6.4496	0.3696	-17.449	0.000
Nitrogen*Inoculum size	0.7652	0.3551	2.155	0.084
Nitrogen*Incubation time	0.3860	0.3551	1.087	0.327
Inoculum size*Incubation time	-0.3300	0.3551	-0.929	0.395

S = 0.710256 PRESS = 40.3570
R-Sq = 99.16% R-Sq(pred) = 86.51% R-Sq(adj) = 97.64%

P - Value was used as a tool to check the significance of each of the coefficients. Smaller the p-value, more significant is the correlation with the corresponding coefficient. The regression coefficient for protease production was found to be best with nitrogen source (p-value 0.028) and incubation time (p-value 0.000). The details are shown in Table.

Table 5: Analysis of variance (ANOVA) of the response surface model
Analysis of Variance for Activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	296.533	296.533	32.9481	65.31	0.000
Linear	3	138.279	138.279	46.0930	91.37	0.000
Square	3	154.879	154.879	51.6265	102.34	0.000
Interaction	3	3.374	3.374	1.1247	2.23	0.203
Residual Error	5	2.522	2.522	0.5045		
Lack-of-Fit	3	2.522	2.522	0.8408	*	*
Pure Error	2	0.000	0.000	0.0000		
Total	14	299.055				

Response surface curves were plotted to find out the interaction of variables and to determine the optimum level of each variable for maximum response. Surface plot showing the interaction between a pair of factors is shown in Figures .

The effects of incubation time and nitrogen source on protease production are shown in Fig. At low levels of incubation time and nitrogen source the protease yield was low. Significant improvement in protease production could be obtained by increasing incubation time and nitrogen source. When the incubation time was set at high level (14 hours) and nitrogen source concentration (7.0 -7.5%), the protease production reached a maximum value (12 U/ml).

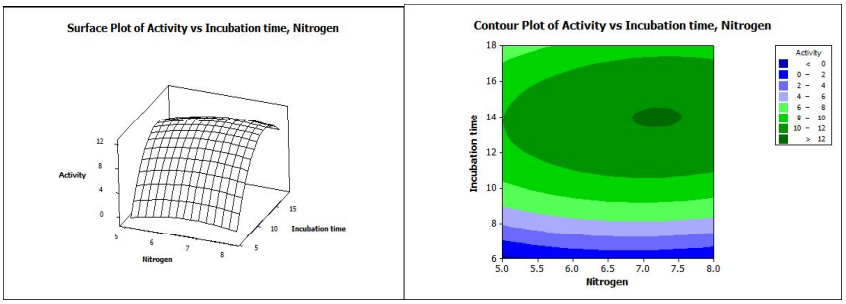


Fig 2: Surface and contour plots showing interactions of incubation time and nitrogen source on protease production

The effects of inoculums size and nitrogen source on protease production are shown in Fig. At low levels of inoculums size and nitrogen source the protease yield was low. Significant improvement in protease production could be obtained by increasing inoculums size and nitrogen source. When the inoculums size was set at high level (7.0 – 8.0%) and nitrogen source concentration (7.0 -8.0%), the protease production reached a maximum value (11.4 U/ml).

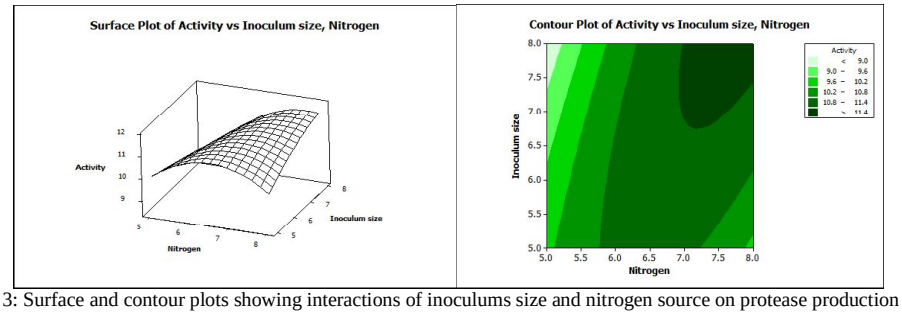


Fig 3: Surface and contour plots showing interactions of inoculums size and nitrogen source on protease production

Fig. shows enzyme production as a function of incubation time and inoculums size. At low levels of incubation time, protease production is low. Maximum production was observed in the incubation time 12-17 hours. Inoculum size 5.0- 8.05 gave same level of enzyme production.

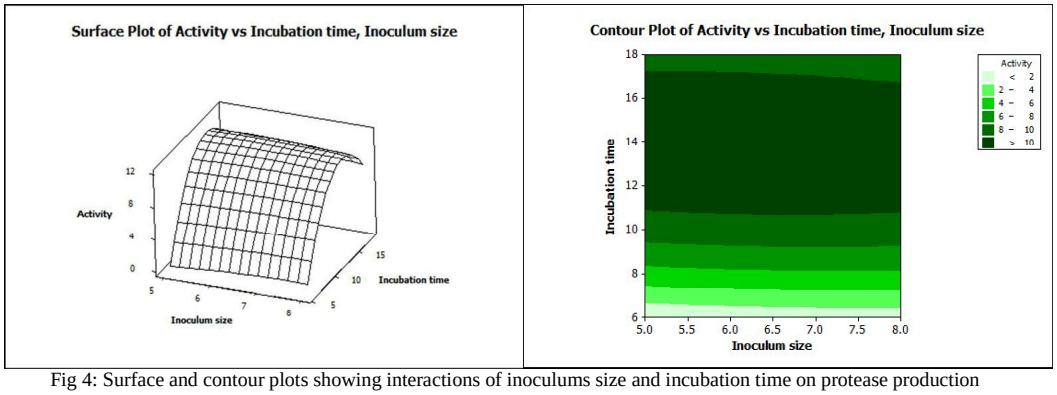


Fig 4: Surface and contour plots showing interactions of inoculums size and incubation time on protease production

The most favorable condition found in the considered range for protease production were nitrogen source concentration (8%), inoculums size (8%) and incubation time (12 h) with protease production of 12.16 U/ml.

IV. VALIDATION OF THE EXPERIMENTAL MODEL

For the validation of the model, three confirmation experiments were performed within the range of levels defined previously. Correlation analysis was performed on the actual responses and predicted values for each solution and the correlation coefficient was found, hence the empirical models developed were reasonably accurate.

V. CONCLUSION

Optimisation of the parameters for the enzyme production was done by using Response surface methodology. Plackett – Burman experimental design gave Nitrogen source, inoculum size, incubation time as significant parameters. The Model was significant since the R^2 value was 81.49%. Evaluation of the interactions between different physiological and nutritional parameters were done with response surface methodology and the optimum conditions obtained were Nitrogen source7-7.5g/l, Inoculum size 5-8.5% v/v, Incubation period 12-17 hrs .

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