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OPTIMIZATION OF PROTEASE PRODUCTION FROM HUSK OF VIGNA MUNGO BY BACILLUS SUBTILIS NCIM 2724 USING STATISTICAL EXPERIMENTAL DESIGN

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ABSTRACT

Proteases are industrially important enzymes which are best produced employing microbial species. This research was an attempt to study the effect of nutritional ingredients on protease production from *Bacillus subtilis* NCIM 2724 in solid state fermentation using black gram husk, (an agricultural waste) as substrate. Moisture content, Carbon supplement (Maltose) concentration and Nitrogen supplement (Ammonium Chloride) concentration which chiefly effected the production of high protease yields were selected for optimization. Response surface methodology using the Box-Behnken design was used in the design of experiments and in the analysis of results. The maximum productivity of protease under optimum conditions was 712.7792 U/ml. Moisture content 40.88327 %v/w, Maltose concentration 1.61499 %w/w and Ammonium Chloride concentration 2.12112 %w/w was found to be optimum for protease production.

Key words: Protease, *Bacillus subtilis* NCIM 2724, *Vigna mungo* (Black gram) husk, Optimization, Response surface methodology (RSM), Box-Behnken design.

INTRODUCTION

Protease is an enzyme that conducts proteolysis, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases are one of the most important commercial enzymes constituting 60-65% of the global enzyme market [6]. They are used in food processing, detergents, diary industry and leather making [2]. Proteases occur widely in plants and animals, but commercial proteases are produced exclusively from microorganisms like *Aspergillus, Penicillium, Bacillus* and *Rhizopus* genera[7].

Basically proteolytic enzymes are of significant importance in the current biotechnological era in various industries. Proteases have been used in food industry for centuries especially rennet is obtained from fourth stomach of calves in cheese production and papain for tenderizing meats while processing and canning [7].Alkaline proteases are used to remove hair from hides. Proteases can be used in the treatment of diseases and conditions such as cancer and autoimmune diseases and also immunosuppressive agents. Furthermore, proteases may be used as vaccine adjuvants[8].

Most commercial serine proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus* [9]. Microbial proteases account to approximately 40% of the total worldwide enzymes sale. In addition, proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all characteristics desired for their biotechnological applications[7].

Black gram husk is selected as an apt substrate as it is easily available and is of minimal commercial interest being an agricultural waste[11]. Moreover it has a suitable texture for solid state fermentation[12].

The present work aims at a better understanding of the relation between the important independent variables (moisture and C/N concentration) and dependent variable (enzyme yield) to determine optimum conditions for the synthesis of protease enzyme from *Bacillus subtilis* NCIM 2724. The application of RSM and Box-Behnken design which is an efficient statistical technique for optimization of multiple

variables to predict best performance conditions with minimum number of experiments and the results of its application is discussed in the present work.

EXPERIMENTAL

Materials and Methods

The microorganism used, *Bacillus subtilis*(NCIM 2724) was procured from NCIM, Pune. The culture was maintained on nutrient agar medium slant and subcultured for every 21 days. The maintenance medium used for sub culturing is nutrient agar medium with composition as follows: Yeast Extract 1.5 g/l, Beef extract 1.5 g/l, NaCl 5.0 g/l, Peptone 15.0 g/l, Agar 5.0 g/l.

Preparation of Inoculum

Inoculums are prepared by transferring 2ml of suspension from 24 hour old slant culture into 250 ml Erlenmeyer flasks containing production substrate. Substrate used is Black gram husk which is an agricultural waste[10]. Commercial quality of black gram husk was procured from the local market and used as the solid substrate for the production of protease. It is added with minimal moisture content and autoclaved at 121^0C and 12 lb pressure for 20 minutes to sterile it as well as to soften the hard texture so as the microbe digests the husk easily yielding optimal enzyme production.

Production medium and conditions

Production medium contained black gram husk 10gms, maltose 1.5gms, ammonium chloride 2.0gms and moisture content of 4 ml. The pH of the medium was adjusted to 7.0 and autoclaved. The production medium was inoculated with 2 ml of homogenous spore suspension (10^8 CFU/ ml). All fermentations were carried out in 250 ml Erlenmeyer flasks and incubated at 27^0C.The fermented biomass in each case was filtered and centrifuged. The supernatant was ultra-filtered through filter paper and the filtrate was assayed for protease.

Fermentation, Enzyme extraction and Assay

Buffer was prepared by mixing 25ml of glycine solution (0.8gms glycine/100ml distilled water), 22.7ml of NaOH solution (1.5gms NaOH/100 ml distilled water) and made up to 100ml with distilled water. 50ml of the prepared buffer was added to the contents of the flask and kept for half an hour of shaking. Filtration was done using Whatmann No.1 filter paper, the filtrate was centrifuged for 15 minutes. The supernatant was collected and added with Caesin (0.3mg casein/100ml distilled water) in 1:6 ratio and incubated at 37 ºC for 10 minutes. Then 6ml of Trichloroacetic acid solution (24.5gm TCA/100ml distilled water) was added and incubated for 30 minutes, again subjected to centrifugation for 10 minutes. The supernatant was collected and FC reagent (3.3ml FC/10ml distilled water) was added incubated for 30 minutes. The OD Values were taken down at 660 nm. One unit of protease is defined as the quantity of enzyme that liberates one micro mole of tyrosine per minute under the assay conditions [8].

Effect of additional nutrients on protease production

The effects of various additional nutrients (carbon source and nitrogen source) on protease production were studied by adding to Black gram husk. Maltose and ammonium chloride were added as carbon and nitrogen sources[13].

Optimization of selected nutrients using RSM

Box-Behnken design and RSM were used to optimize the concentrations of these factors (Moisture content, Maltose and ammonium chloride) which resulted from the above studies. The lowest and highest concentrations of selected ingredients were Moisture content, 10 and 60 %w/v; Maltose, 0.5 and 3 %w/w; Ammonium chloride, 0.5 and 3.0 %w/w respectively.

Design of RSM experiment

RSM consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria. Prior knowledge and understanding of the process variables under investigation is necessary for achieving a realistic model. The range and levels of experimental variables investigated in this study were presented in Table 1. The central values (zero level) chosen for experimental design were: 40 %v/w- Moisture content (X_1) , 1.5%w/w- Maltose (X_2), 2.0 %w/w- Ammonium chloride (X_3). The production of

protease was optimized using Box-Behnken design[7] when protease production is related to independent variables by a response equation

$$Y = f(x_1, x_2, x_3, \ldots, x_k) \quad (1)$$

The true relationship between Y and x_k may be complicated and, in most cases, it is unknown; however a second-degree quadratic polynomial can be used to represent the function in the range interest.

$$Y = R_0 + \sum_{i=1}^{k} \cdot\cdot R_i X_i + \sum_{i=1}^{k} R_{ii} \cdot X_i^2 + \sum_{i=1, i<j}^{k-1} \cdot \sum_{j=2}^{k} \cdot R_{ij} X_{\cdot i} X_j + € \quad (2)$$

Where $X_1, X_2, X_3 \ldots X_k$ are the independent variables which affect the response Y, R_0, R_i, R_{ii} and R_{ij} (i=1-k, j=1-k) are the known parameters, € is the random error. A second order model is designed such that variance of Y is constant for all points equidistant from the center of the design. The experimental design chosen for the study was a Box-Behnken design that helps in investigating linear, quadratic and cross-product effects of these factors, each varied at these levels and also includes three center points for replication[8].The design is performed because relations for experimental combination of the variables are adequate to estimate potentially complex response functions. The 'STATISTICA' software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface plots[5].

RESULTS AND DISCUSSION

The extra cellular protease enzyme was obtained from the culture filtrate of *Bacillus subtilis* and the yield of enzyme depended on various growth conditions. The production of protease by *Bacillus subtilis* was optimized by response surface methodology with middle range parameters, as it is a powerful technique for testing multiple process variables. Experiments were carried out as per the design and the average protease enzyme activity obtained after 24 hours fermentation with 15 experiments in triplicate from the chosen experimental design are shown in Table 2. The application of RSM[4] yielded the following regression equation, which is an empirical relationship between the enzyme yield and test variables in coded units.

$$Y = 450.3 + 62.95\,X1 + 82.64\,X1X1 + 46.8\,X2 + 51.75\,X2X2 + 48.18\,X3 + 49.85X3X3 - 8X1X2 - 11.2\,X1X3 + 9.12\,X2X3 \quad (3)$$

Where Y= enzyme yield, X_1, X_2, X_3 are the coded values of the moisture content, maltose and ammonium chloride concentrations respectively.

The calculation of regression analysis gives the value of the determination coefficient (R^2 = 0.984) indicates that only 1.6% of the total variations are not explained by the model and the F-value of 159.12 indicates that the protease production by *Bacillus subtilis* has a good model fit due to the high values of R^2 and F. The value of adjusted determination coefficient (Adj. R^2 = 0.956) is also very high which indicate a high significance of the model.The regression coefficients, along with the corresponding p-values for the model were given in Table 3. The p-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. The smaller the p-values,more the significance of the corresponding coefficient. Table 4 shows the various critical values obtained for the selected factors.

Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels (zero for instance) are more helpful in understanding both the main and the interaction effects of these two factors. These plots can be easily obtained by calculating from the model and the values taken by one factor where the second varies (from -1.0 to +1.0, step 0.5 for instance) with constraint of a given Y value. The yield values for different concentration of the variable can also be predicted from the respective response surface plots between moisture content and maltose concentration; maltose concentration and ammonium chloride concentration; moisture content and ammonium chloride

concentration respectively (Figs. 1-3). The results obtained show that the protease activity is increased to 708.39 U/ml from 694.4 U/ml at the same laboratory conditions, due to optimization using Response surface methodology.

Table-1: The range and levels of experimental variables investigated

Variables	Coded levels		
	-1	0	+1
Moisture content (% v/w) X1	35	40	45
Maltose (% w/w) X2	1.0	1.5	2.0
Ammonium Chloride(%w/w) X3	1.5	2.0	2.5

Table-2: The average protease enzyme activity obtained after 24 hours fermentation with 15 experiments in triplicate from the chosen experimental design.

Run No.	X1	X2	X3	Observed protease activity(U/ml)	Predicted protease activity(U/ml)
1	-1	-1	0	302.4	309.4375
2	1	-1	0	448.7	451.3375
3	-1	1	0	421.7	419.0625
4	1	1	0	536.0	528.9625
5	-1	0	-1	311.8	308.6625
6	1	0	-1	455.7	456.9625
7	-1	0	1	428.7	427.4375
8	1	0	1	527.8	530.9375
9	0	-1	-1	410.8	406.9000
10	0	1	-1	476.5	482.2750
11	0	-1	1	490.8	485.0250
12	0	1	1	593.0	596.9000
13	0	0	0	698.2	696.0000
14	0	0	0	694.4	696.0000
15	0	0	0	695.4	696.0000

Table -3: The regression coefficients, along with the corresponding p-values for the model are given

Factor	Effect	p-value	Coefficient
Mean/ Intercept	450.3250	0.000000	450.3250
X1X1	125.900	0.000002	62.3500
X1Y	165.2875	0.000000	82.3437
X2X1	93.6250	0.000007	46.3215
X2Y	103.5125	0.000000	51.7563
X3X1	96.3750	0.000006	48.1875
X3Y	99.7125	0.000000	49.3562
X1X2	-16.0000	0.068258	-8.0000
X1X3	-22.4000	0.023250	-11.2000

X2X3	18.2500	0.046522	9.1250

Table -4: The various critical values obtained for the three selected factors.

Factor	Observed minimum	Critical value	Observed maximum
X1	35.00000	40.88327	45.00000
X2	1.00000	1.61499	2.00000
X3	1.50000	2.12112	2.00000

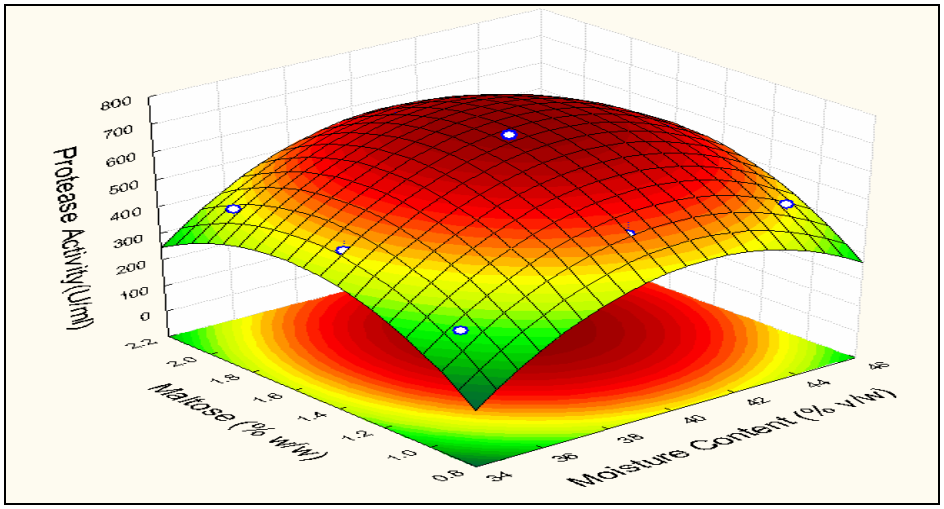

Fig 1: Response surface plots between Moisture content and maltose concentration

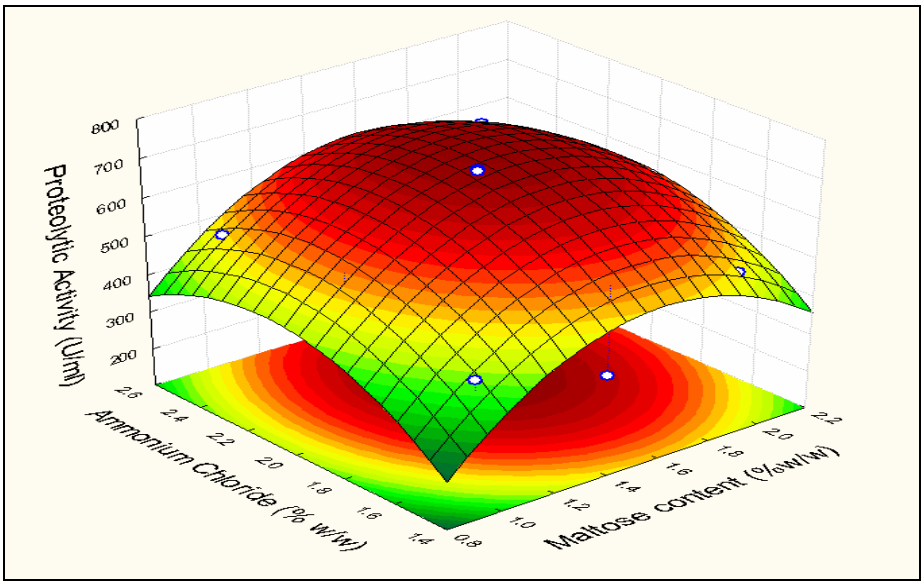

Fig 2: Response surface plots between Maltose concentration and Ammonium chloride concentration

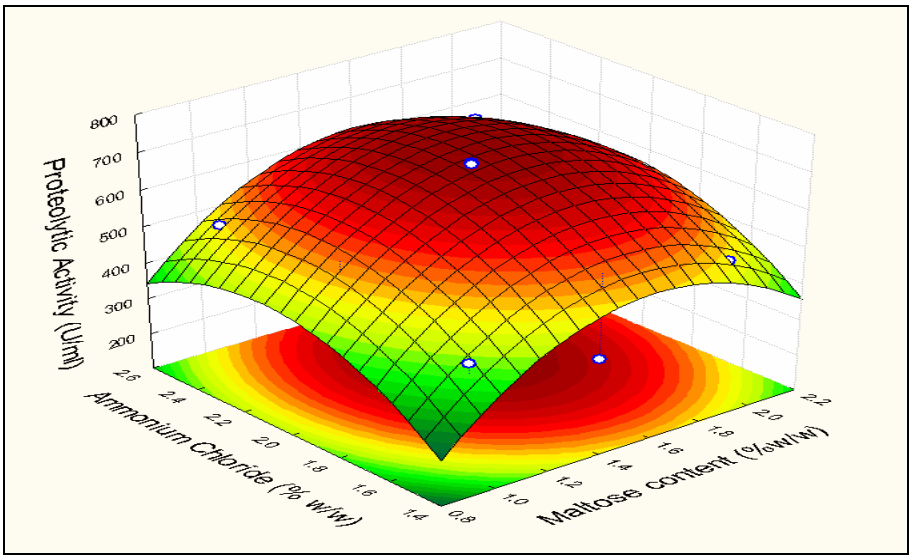

Fig 3: Response surface plots between Moisture content and Ammonium chloride concentration

CONCLUSION

The work has demonstrated the use of a Box-Behnken design by determining the conditions leading to the optimum yield of enzyme production. This methodology could therefore be successfully employed to any process (especially with three levels), where an analysis of the effects and interactions of many experimental factors are referred. Box-Behnken designs maximize the amount of information that can be obtained, while limiting the number of individual experiments required. Response surface plots are very helpful in visualizing the main effects and interaction of its factors. Thus, smaller and less time consuming experimental designs could generally suffice the optimization of many fermentation processes.

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