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Optimization of Pectinase Production from *Manihot utilissima* by *Aspergillus niger* NCIM 548 Using Statistical Experimental Design

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Abstract: The effect of nutritional ingredients on pectinase production was studied using *Aspergillus niger* NCIM 548 in submerged fermentation. Substrate concentration (Tapioca starch), C/N ratio (Glucose/Ammonium sulphate), salt concentration (Potassium dihydrogen ortho phosphate) produced high pectinase yields and were selected for optimization. A 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 pectinase under optimum conditions was 22.87 U mL^{-1}. Tapioca starch concentration 3.71% w/v, C/N ratio 5.94 and salt concentration 0.256% w/v were found to be optimum for pectinase production. This method was efficient because only 15 experiments are necessary to assess these conditions and the model accuracy was very satisfactory, as the coefficient of determination was 0.984.

Key words: Pectinase, *Aspergillus niger* NCIM 548, *Manihot utilissima* (tapioca starch), Optimization, Response Surface Methodology (RSM), Box-Behnken design

INTRODUCTION

Pectinases are a group of enzymes that break the glycosidic bonds of the long chain galacturonic acid residues of pectic substances. These enzymes are classified according to the criteria whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, whether pectinase act by trans-elimination or hydrolysis, or whether the cleavage is random. They are Pectin Esterases (PE), depolymerizing enzymes and protopectinases. Pectinolytic enzymes are of significant importance in the current biotechnological era with their all embracing applications in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives and in the alcoholic beverages and food industries (Ranveer *et al*., 2005). They have a share of 25% in the global sales of food enzymes (Ranveer *et al*., 2005).

Among the commercial pectinolytic enzymes, preparations obtained by the industrial cultivations of *Aspergillus niger* are some of the most popular (Godfrey and West, 1996). Since Pectinases are widely used enzyme for different industrial applications, it is necessary to use inexpensive and readily available raw materials for its production. Carbon sources especially of agrarian source are more suitable because they are cost effective, renewable and available in larger quantities.

The present research aim at a better understanding of the relation between the important independent variables (nutrients and substrate concentration) and dependent variable (enzyme yield) to determine optimum conditions for the synthesis of pectinase enzyme from *Aspergillus niger* NCIM 548. The application of RSM and Box-Behnken design which is an efficient statistical technique for

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optimization of multiple variables to predict best performance conditions with minimum number of experiments and the results of which are discussed in the present study. The research was done at the Center for Biotechnology, Department of Chemical Engineering, Andhra University, India in the year 2006.

MATERIALS AND METHODS

Microorganism

Aspergillus niger NCIM 548 obtained from NCL, Pune, India was used in the present investigation. The culture was maintained on potato dextrose agar slants at 4°C and subcultured every 4 weeks.

Inoculum Preparation

Ten milliliter of sterile water was transferred to *Aspergillus niger* culture (6 days old) and the culture was dislodged using sterile inoculating needle.

Production Medium and Conditions

Production medium contained (g L^{-1}) Tapioca starch 30, Glucose 30, Ammonium sulphate 10 and Potassium dihydrogen ortho phosphate 2. The pH of the medium adjusted to 4.5 and autoclaved. The production medium was inoculated with 1 mL of homogenous spore suspension (10^7-10^8 spores mL^{-1}). All fermentations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of production medium at 30°C on a rotary shaker (120 rpm). The fermented biomass in each case was filtered and centrifuged. The supernatant was ultra-filtered through filter paper and the filtrate was assayed for pectinase.

Effect of Additional Nutrients on Pectinase Production

The effects of various additional nutrients (carbon source, nitrogen source and mineral salts) on pectinase production were studied by adding to Tapioca starch. Glucose, ammonium sulphate, potassium dihydrogen ortho phosphates were added as carbon, nitrogen sources and mineral salts, respectively.

Optimization of Selected Nutrients Using RSM

Box-Behnken design and RSM were used to optimize the concentrations of these factors (Tapioca starch, glucose/ammonium sulphate, potassium dihydrogen ortho phosphate) which resulted from the above studies. The lowest and highest concentrations of selected ingredients were Tapioca starch, 1 and 5% w/v; ratio of glucose/ammonium sulphate, 2 and 8; potassium dihydrogen ortho phosphate, 0.1 and 0.3% w/v, respectively.

C/N Ratio

To rationalize the carbon source (Glucose) with respect to nitrogen source (ammonium sulphate), a term called C/N Ratio was used.

$$\text{C/N Ratio} = \frac{\text{Glucose concentration (mg mL}^{-1})}{\text{Ammonium sulphate concentration (mg mL}^{-1})}$$

Assay for Pectinase Activity

Liquid samples obtained from submerged fermentation were filtered using muslin cloth. The reaction mixture containing 0.3 mL diluted sample was added to a solution containing 1 mL of 0.9% of substrate and 0.7 mL of 0.1 M acetate buffer with pH 4.5. Samples were incubated at 45°C for

Table 1: Independent variables in the experimental plan

	Coded levels		
Variables	-1	0	+1
Tapioca starch concentration (% w/v) X_1	1.0	3.0	5.0
C/N ratio X_2	2.0	5.0	8.0
Potassium dihydrogen ortho phosphate (% w/v) X_3	0.1	0.2	0.3

30 min. Reducing sugars were determined by Dinitro Salicylic Acid (DNS) method using galacturonic acid as reference (Miller, 1959). One unit of pectinase was defined as the quantity of enzyme that liberates one micro mole of galacturonic acid per minute under the assay conditions

Experimental Design and Optimization by RSM

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 central values (zero level) chosen for experimental design were: 3% w/v- Tapioca Starch (X_1), C/N Ratio (X_2) -5, 0.2% w/v-Potassium dihydrogen ortho phosphate (X_3) (Table 1). The production of pectinase was optimized using Box-Behnken design (Box and Behnken, 1960; Gopinath *et al*., 2003a, b; Anbu *et al*., 2005), when pectinase production (4) is related to independent variables by a response equation

$$Y = f(X_1, X_2, X_3, \ldots, X_K) \quad (1)$$

The true relation ship 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 (Annadurai and Sheeja, 1998),

$$Y = R_0 + \sum_{i=1}^{k} R_i X_i + \sum_{i=1}^{k} R_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} R_{ij} X_i X_j + \varepsilon$$

Where, X_1, X_2, X_3… 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, ϵ 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. 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.

RESULTS AND DISCUSSION

The extra cellular pectinase enzyme was obtained from the culture filtrate of *Aspergillus niger* and the yield of enzyme depended on various growth conditions. The production of pectinase by *Aspergillus niger* 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

Table 2: The Box-Behnken design matrix employed for three independent variables in coded units along with observed values

Run No.	X_1	X_2	X_3	Observed values
1	-1	-1	0	7.120
2	1	-1	0	11.080
3	-1	1	0	13.565
4	1	1	0	19.850
5	-1	0	-1	7.980
6	1	0	-1	17.050
7	-1	0	1	15.980
8	1	0	1	20.250
9	0	-1	-1	7.460
10	0	1	-1	13.890
11	0	-1	1	15.950
12	0	1	1	19.350
13	0	0	0	20.980
14	0	0	0	20.950
15	0	0	0	20.960

Table 3: Analysis of variance (ANOVA) for the quadratic model

Sources of variation	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
$X_1+X_1X_1$	113.57	2	56.78	49.22	0.0005
$X_2+X_2X_2$	156.74	2	78.37	67.93	0.0002
$X_3+X_3X_3$	96.85	2	48.42	41.97	0.0007
X_1X_2	1.35	1	1.35	1.17	0.3285
X_1X_3	5.76	1	5.76	4.99	0.0757
X_2X_3	2.29	1	2.29	1.98	0.2174
Error	5.76	5	1.15		
Total SS	365.97	14			

$R^2 = 0.984$; Adjusted $R^2 = 0.956$; MS residual = 1.1536

per the design and the average pectinase enzyme activity obtained after 2 days fermentation with 15 experiments in triplicate from the chosen experimental design are shown in Table 2. The application of RSM (Box *et al*., 1978; Khuri and Cornell, 1987) yielded the following regression equation, which is an empirical relation ship between the enzyme yield and test variables in coded units

$$Y = 12.66 + 2.94\ X_1 + 3.13\ X_2 + 5.34\ X_3 + 0.58\ X_1X_2 - 1.2\ X_1X_3 - 0.75\ X_2X_3 + 1.73\ X_1X_1 + 2.3\ X_2X_2 + 2.19X_3X_3 \quad (2)$$

Where, Y is the enzyme yield, X_1, X_2, X_3 are the coded values of the substrate concentration, C/N ratio, salt concentration, 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 pectinase production by *A. niger* has a good model fit due to the high values of R^2 and F (Table 3). The value of adjusted determination coefficient (Adj. $R^2 = 0.956$) is also very high which indicate a high significance of the model (Akhnazarova and Kafarov, 1982). The parity plot showed a satisfactory correlation between the experimental and predicted values (Fig. 1), where in the points cluster around the diagonal line which indicates the good fit of the model, since the deviation between the predicted and experimental values was less.

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 (Table 4). The smaller the p-values, the bigger the significant of the corresponding coefficient (Cui *et al*., 2006). Table 4 showed that the regression coefficients of the linear terms (X_1, X_2, X_3) and the quadratic coefficients (X_1X_1, X_2X_2) were significant at 1% level and a quadratic term (X_3X_3) was significant at 5% level. The Pareto chart (Fig. 2) also accord with the above mentioned.

Table 4: Model coefficients estimated by linear regression

Factors	Coefficient	Computed t-value	p-value
Intercept	-28.980	-7.23	0.000791
X_1	7.370	6.65	0.001158
X_2	6.376	8.21	0.000435
X_3	149.850	5.87	0.002089
X_1X_1	-0.863	-6.17	0.001618
X_2X_2	-0.512	-8.24	0.000429
X_3X_3	-219.480	-3.92	0.011080
X_1X_2	0.097	1.08	0.328510
X_1X_3	-6.000	-2.23	0.075730
X_2X_3	-2.525	-1.41	0.217450

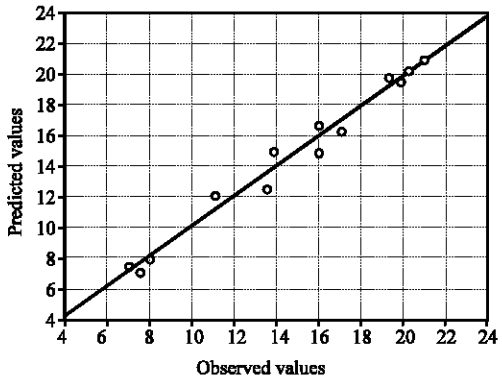

Fig. 1: Parity plot showing the distribution of experimental vs. predicted values of pectinase activity

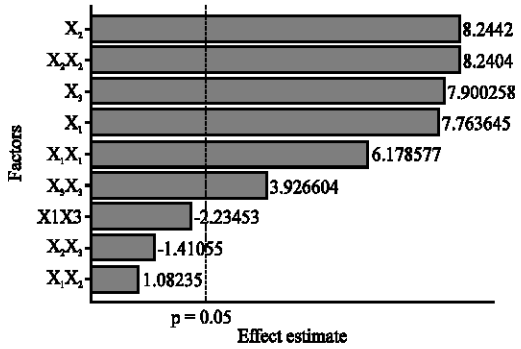

Fig. 2: Representative graphic to show the influence of variables studied of the model

The model Eq. 2 indicated that salt concentration (X_3) had a significant effect ($p<0.01$) on Y and it had the largest coefficient followed by C/N ratio (X_2) and Substrate concentration (X_1). Positive coefficients of X_1, X_2 and X_3 indicated a linear effect to increase yield Y, however, interactive term (X_1X_3, X_2X_3) had negative effect that decrease yield Y. The statistical analysis of the design shows a high precision of the polynomial model that reflects high degree of fitting between the predicted and the experimental data. This great similarity between the predicted and the observed results reflects the accuracy and applicability of the Box- Behnken model in the optimization processes (Kimmel *et al*., 1998; Lofty, 2007).

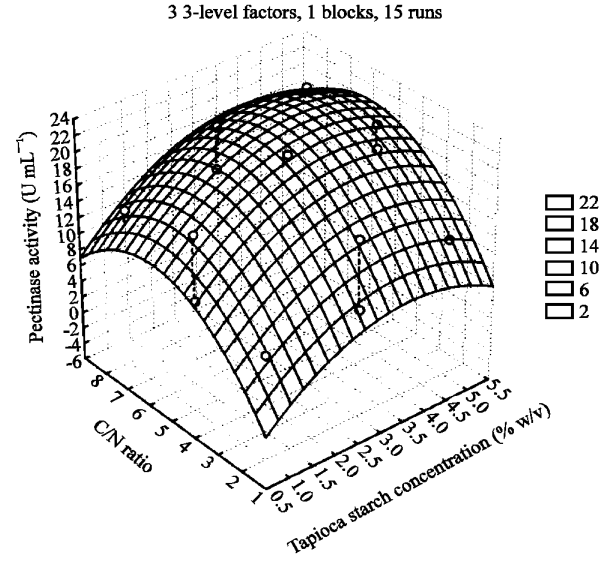

Fig. 3: Response surface plot showing the effect on tapioca starch concentration, C/N ratio on the production of pectinase. Other variables are held at zero level

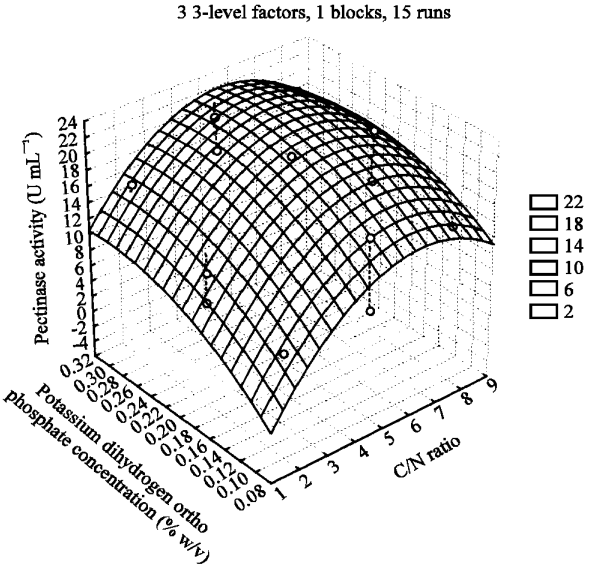

Fig. 4: Response surface plot showing the effect on C/N ratio, Potassium dihydrogen ortho phosphate concentration on the production of pectinase. Other variables are held at zero level

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 (Fig. 3-5). The maximum predicted value is indicated by

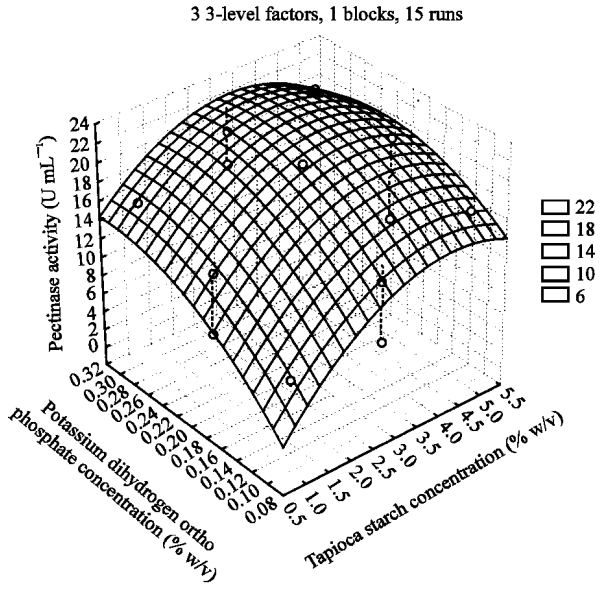

Fig. 5: Response surface plot showing the effect on Tapioca starch concentration, Potassium dihydrogen ortho phosphate concentration on the production of Pectinase. Other variables are held at zero level

Table 5: Critical values of the quadratic model

Factors	Observed minimum	Critical value	Observed maximum
X_1(% w/v)	1.0	3.710	5.0
X_2	2.0	5.940	8.0
X_3(% w/v)	0.1	0.256	0.3

Predicted value at solution: 22.87

the surface confined in the response surface diagram. An increase in pectinase yield with increase in Substrate concentration versus C/N ratio was observed (Fig. 3). An increase in pectinase yield with increase in Substrate concentration versus salt concentration was observed (Fig. 4). An increase in pectinase yield with increase in C/N ratio versus salt concentration was observed (Fig. 5).

The analysis revealed a maximum pectinase activity of 22.87 U mL^{-1} (Table 5) which was 9.0085% more than that of the run number (13) could be achieved at the concentration points where Tapioca starch concentration, C/N ratio and Potassium dihydrogen ortho phosphate concentration were 3.71% w/v, 5.94 and 0.256% w/v, respectively.

CONCLUSIONS

The study 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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