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OPTIMIZATION OF PECTINASE PRODUCTION BY *ASPERGILLUS NIGER* USING CENTRAL COMPOSITE DESIGN

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ABSTRACT

Pectinases are a group of enzymes that catalyze the breakdown of pectin. Pectinase producing *Aspergillus niger* was obtained from a five-day old Eba (Cassava flakes). Response surface methodology was used for optimizing the process of the pectinase produced. Four independent variables which are, temperature, pH, substrate concentration and time of Heating at 70^{o}C were used to optimize the significant correlation between the effects of the variables on pectinase production. A second-order polynomial was fitted to data and validated by ANOVA. The results revealed maximum pectinase production at pH 6.0, 50^{o}C Temperature, 0.02% substrate concentration and the enzyme lost all its activity within 7 min of heating at 70^{o}C. The study revealed that optimization of pectinase through RSM could improve the enzymatic characteristics and yield of the enzyme. The models used were highly significant with a correlation coefficient (R^2) of 0.901

Keywords: Pectinase; *Aspergillus niger*; Response Surface Methodology; Central Composite Design

OPTIMISATION DE LA PRODUCTION DE LA PECTINASE PAR ASPERGILLUS NIGER AIDE PLAN COMPOSITE CENTRAL

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Résumé

Pectinases sont un groupe d'enzymes qui catalysent la décomposition de la pectine. La production de la pectinase Aspergillus niger a été obtenu à partir d'un vieux de cinq jours (EBA) flocons de manioc. La méthodologie de surface de réponse a été utilisée pour optimiser le processus de la pectinase produite. Quatre variables indépendantes qui sont, de la température, du pH, de la concentration du substrat et le temps de chauffage à 70o C ont été utilisées pour optimiser la corrélation significative entre les effets des variables sur la production de la pectinase. Un polynôme de deuxième degré a été monté aux données et validées par ANOVA. Les résultats ont révélé la production maximale de la pectinase à pH 6,0, 50 °C Température ambiante et à un 0,02 % de la concentration du substrat et l'enzyme a perdu toute son activité dans les 7 min de chauffage à 70oC. L'étude a révélé que l'optimisation de la pectinase par RSM pourrait améliorer le rendement et les caractéristiques enzymatiques de l'enzyme. Les modèles utilisés étaient très significatives avec un coefficient de corrélation (R2) de 0,901

Mots-clés: la pectinase, Aspergillus niger ; la méthodologie de surface de réponse ; plan composite centra

INTRODUCTION

Pectinases are industrially important enzymes (1). They are a group of enzymes that catalyze the breakdown of pectin (2). The two major sources of pectinase are from plants and microorganisms but microbial source of the enzyme is becoming increasingly important (3).The role of pectinase in the clarification of green, yellow and red apple juice had been reported (4). *Rhizopus* sp. isolated from deteriorated grapes produce pectinase (5). A total of 44 fungal strains isolated from different agricultural and non agricultural soils in Chittoor district, Andhra Pradesh in South India revealed only four trains identified as *Aspergillus niger, Aspergillus flavus, Aspergillus japonica*s and *Chaetomium globosum* as pectinase producing microorganisms under submerged fermentation (6). Pectinases have the ability to break down a variety of polysaccharides found within fruit juice extract to release soluble sugars which can clarify the juice producing a clearer, sweeter product (7). The immense potential of pectinase from microbial source especially from *Aspergillus niger*, a fungi which has been given the Generally Regarded as Safe (GRAS) status for clarification of fruit juice has necessitated the optimization of the production process of pectinase to achieve maximum yields. Response surface Methodology had been identified and widely used as an efficient tool for the optimization of different physiochemical parameters with the advantage of determining the influence of variables on enzyme units and to optimize the variables in order to achieve maximum yields under the best possible economic conditions (8). This investigation therefore reports the optimization of the production process of pectinase by *A.niger* by submerged fermentation.

MATERIALS AND METHODS

Isolation of *Aspergillus niger*

Aspergillus niger was obtained from a five-day-old Eba (Cassava flakes) and cultured on potato dextrose agar (PDA) for 4 days at room temperature. Pure cultures were obtained by sub-culturing and further subculturing on potato dextrose agar (PDA). *A. niger* was identified by morphological characterization and the results compared with the "Atlas (9). Pure cultures were maintained on potato dextrose agar (PDA) slants and sub cultured periodically throughout the duration of this research work.

Enzyme Production

This was carried out by submerged fermentation (SMF) according to the method described (10). The fermentation medium was made up of 0.1% NH_4NO_3, 0.1% $MgSO_4.7H_2O$, 0.1% $NH_4H_2PO_4$ and 1% pectin autoclaved at 121^{o}C for 20min. One disc of actively growing *A. niger* from a 96-h-old culture medium was inoculated each into seven different flask containing 50ml fermentation medium using a cork borer of 10mm diameter and separated from a single flask by filtration using muslin cloth and the filtrate was analyzed daily for pectinase activity.

Pectinase Assay

Pectinase assay was carried out according to the method of (11). 0.5g of the pineapple pectin (substrate) will be weighed and dissolved in 100ml of sodium acetate buffer at a pH of 5.0. The protein content will be determined according to (12).

Partial purification of Pectinase

This was carried out using activated charcoal according to the method (13). Three percent (w/v) of activated charcoal was added to the crude pectinase (pH 4.5) and incubated at 30^{o}C for 30min with occasional stirring. The mixture was centrifuged at 2500rpm in a bench centrifuge for 10min. Pectinase activity and the protein content were determined according to the methods described earlier.

Pectinase Production Optimization

Optimization of the physical parameters for maximum pectinase production was carried out using the response Surface Methodology which is a powerful and efficient mathematical approach widely applied in the optimization of enzymes for industrial processes. Central Composite Factorial Design was used in the optimization of the conditions for pectinase production. A total of 30 experiments were used in the study. The independent variables studied were pH (X_1), temperature (X_2, ^{o}C), Substrate Concentration (X_3, mg/ml) and Time of heating (X_4, min). The response (dependent variable) was pectinase activity (Units/ml). Each independent variable was studied at three coded levels (-1, 0, +1). The minimum and maximum levels of each independent variable and the experimental design with respect to their coded and uncoded levels (Table 1). The minimum and maximum ranges of variables and full experimental plan with respect to their values in actual and coded forms (Table 2). The relationship between the coded values and actual values are as described (Eq.1)

TABLE 1: LEVELS OF THE FOUR INDEPENDENT VARIABLES (FACTORS) USED IN RSM

Variables	Unit	Low (- 1)	Actual (0)	High (+ 1)
pH (x_1)	-	3.0	4.5	6.0
Temp. (x_2)	^0C	30	45	60
Substrate Conc. (x_3)	*mg/ml*	0.2	1.6	3.0
Time of Heating (x_4)	*min.*	0	15	30

First Degree Polynomial (First-Order Model)

$$y=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+e\equiv\beta_0+\sum_{i=1}^{p}\beta_ix_i+e \quad (1)$$

Second Degree Polynomial (Second-Order Model)

$$y=\beta_0+\sum_{i=1}^{p}\beta_{ii}x_i^2+\sum_{i}\sum_{j>1}\beta_{ij}x_ix_j+e \quad (2)$$

Where i, j are linear, quadratic coefficients, respectively, while β is regression coefficient, y is response variable (pectinase activity), *k* is the number of factors studied and optimized in the experiment, *e* is the random effect and β_0 is the intercept. The second order model used to fit the response to the independent variables is as described (Eq. 2). A second-order regression analysis of the data was carried out to get empirical model that defines response in terms of the independent variables. Analysis of variance (ANOVA) was performed in coded levels of variables to study the effects of the independent variables. The 2D graphs were generated to understand the effect of selected variables individually and in combination to determine their optimum level (Fig. 1a – f))

Coding Method

$$X_j=\frac{ActualLevel-(HighLevel+LowLevel/2)}{(HighLevel-LowLevel/2)}$$

RESULTS

The results of this study revealed that the Response Surface Methodology (RSM) used to optimize the parameters (pH, temperature, substrate concentration, and time of heating) and the Central Composite Design with a total of thirty experiments produced experimental and predicted values of yields of pectinase are in good agreement with the observed values (Table 2). The competence of the model and fitness evaluated by using ANOVA (Analysis of variance) and regression coefficients for the experimental design used (Tables 3, 4 & 5).

TABLE 2: CENTRAL COMPOSITE ROTATABLE DESIGN OF THE VARIABLES WITH ENZYME ACTIVITY AS RESPONSE

Run	pH (x_1)	Temp. (x_2)	Substrate Conc. (x_3)	Time of Heating (x_4)	Actual Value	Predicted Value
1	- 1	+ 1	- 1	- 1	0.238	0.227
2	0	0	0	0	0.300	0.308
3	+ 1	+ 1	- 1	- 1	0.315	0.329
4	0	0	0	0	0.491	0.451
5	- 1	+ 1	+ 1	+ 1	0.128	0.134
6	+ 1	- 1	+ 1	- 1	0.100	0.109
7	0	0	0	0	0.089	0.067
8	0	0	0	0	0.001	0.002
9	0	0	0	0	0.015	0.019
10	0	0	0	0	0.051	0.061
11	- 1	- 1	+ 1	+ 1	0.003	0.005
12	- 1	- 1	- 1	+ 1	0.001	0.002
13	+ 1	+ 1	+ 1	- 1	0.001	0.007
14	0	0	0	0	0.001	0.003

TABLE 2 (CTD)

Run	pH (x_1)	Temp. (x_2)	Substrate Conc. (x_3)	Time of Heating (x_4)	Actual Value	Predicted Value
15	0	0	0	0	0.002	0.005
16	- 1	- 1	+ 1	+ 1	0.506	0.511
17	+ 1	- 1	+ 1	+ 1	0.023	0.039
18	+ 1	+ 1	- 1	+ 1	0.009	0.011
19	0	0	0	0	0.006	0.008
20	- 1	+ 1	+ 1	- 1	0.003	0.002
21	+ 1	- 1	- 1	+ 1	0.001	0.004
22	0	0	0	0	0.070	0.086
23	+ 1	+ 1	+ 1	+ 1	0.015	0.018
24	- 1	+ 1	- 1	+ 1	0.009	0.008
25	0	0	0	0	0.043	0.048
26	+ 1	- 1	- 1	- 1	0.214	0.300
27	0	0	0	0	0.161	0.182
28	- 1	- 1	- 1	- 1	0.101	0.126
29	0	0	0	0	0.005	0.008
30	0	0	0	0	0.031	0.044

TABLE 3: ESTIMATED REGRESSION COEFFICIENTS FOR THE SECOND-ORDER MODEL

Term	Coefficient	Standard Error of Coefficients	T_{-value}	P_{-value}
Constant	.102787	.086512	1.1881	.0022
x_1 (pH)	.235401	.342109	.6881	.0001
x_2 (Temperature)	.541989	.210006	2.5808	.0003
x_3 (Substrate Conc.)	1.000452	.419412	2.3854	.0012
x_4 (Time of Heating)	.000231	.994188	.00002	.05904
$x_1 x_2$	2.985412	1.004518	2.9719	.0004
$x_1 x_3$	2.000341	3.783198	.5287	.0701
$x_1 x_4$	1.620954	3.033128	.5344	.06713
$x_2 x_3$	.011299	.457899	.0247	.0804
$x_2 x_4$	1.446698	2.459163	.5883	.0576
$x_3 x_4$	2.094219	2.859674	.7323	.0159
x_1^2	.851953	1.009412	.8440	.0009
x_2^2	-.528971	.124976	-4.2326	.0901
x_3^2	1.095518	1.459731	2.8996	.0000
x_4^2	1.951987	.179346	16.1674	.0002

TABLE 4: ANALYSIS OF VARIANCE (ANOVA) FOR RESPONSE SURFACE SECOND-ORDER MODEL

Source of Variation	*Df*	Sum of Squares	Mean Square	F_{-value}	P_{-value}	Remarks
FO	4	.043345	.010836	.1802	.1198	Not Significant
TWI	6	.137678	.022946	.3815	.0009	At least one i s significant
PQ	4	.094199	.023549	.3915	.0104	At least one is significant
Residuals	15	.902111	.060140			
Lack of Fit	12	.356319	.029693	.4937	.0211	Not Significant

TABLE 5: STATISTICAL ANALYSIS OF PECTINASE PRODUCTION

Model Term	Values
Multiple *R*-squared	0.9008
Adjusted *R*-squared	0.8992
Std. Dev.	0.0190
PRESS	0.0309

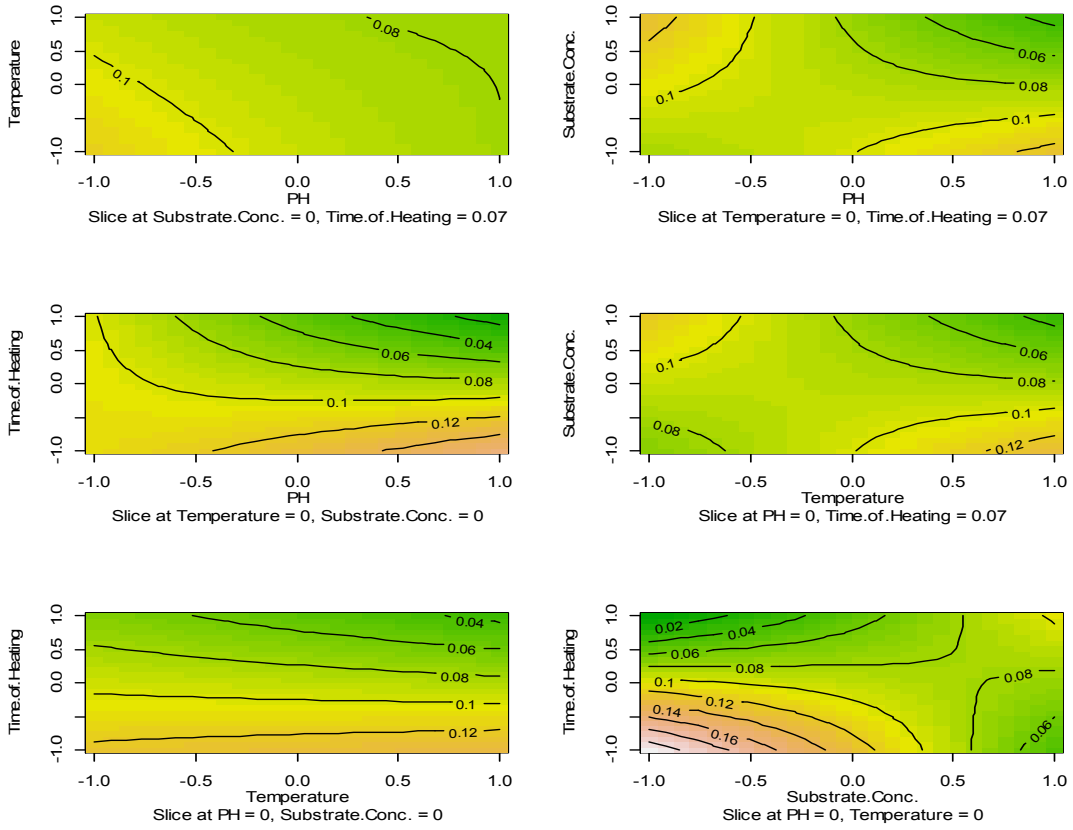

Fig.1. (a) Effect of temperature and pH on the production of pectinase keeping substrate concentration and time of heating at zero level (coded)
(b) Effect of substrate concentration and pH on the production of pectinase. Temperature and time of heating were held at zero level (coded)
(c) Effect of Time of heating and pH on the production of pectinase. Substrate concentration and temperature were kept at zero level (coded)
(d) Effect of substrate concentration and temperature on the production of pectinase with pH and time of heating kept at zero level (coded)
(e) Effect of time of heating and temperature on the production of pectinase. Other variables pH and substrate concentration were held at zero level (coded)
(f) Effect of time of heating and substrate concentration on the production of pectinase. Other variables, pH and temperature were held at zero level (coded).

DISCUSSION

The results of this study revealed that the Response Surface Methodology (RSM) used to optimize the parameters (pH, temperature, substrate concentration and time of heating) and the Central Composite Design with a total of thirty experiments was successful for the production process of pectinase produced by *A.niger*. Ibrahim and Elkhidir (14) reported that Response Surface Methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions. It has successfully been used in the optimisation of bioprocesses. They reported their data using RSM for maximization of enzyme production and minimization of the cost for cellulase, xylanase and phytase by different researchers**.** The Production optimization of a heat-tolerant alkaline pectinase from *Bacillus subtilis* ZGL14 with optimal temperature and pH of 50°C and 8.6 respectively was reported (15). Handa *et al*. (16) reported a novel strain *Rhizopus* sp. C4 isolated from compost for the production of pectinase and Response surface methodology (RSM) was employed to optimize the various environmental parameters (temperature, moisture and incubation days) were studied statistically for a total of 20 runs using central composite design for pectinase production. They obtained the highest yield of the enzyme, i.e., 11.63 IU/mL from 1:3.5 moisture ratios in 7 days at 30°C.

Conclusion

The different parameters of temperature, pH, substrate concentration and time of heating ha d a great effect on the production of pectinase by *A. niger*. These relationships can be explained by the second-order polynomial. The two dimensional contour plots were used to set the optimum values of the variables and significant improvement in the production of pectinase by *A. niger* was observed.

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