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Full Length Research Paper

Statistical optimization of substrate, carbon and nitrogen source by response surface methodology for pectinase production using *Aspergillus fumigatus* MTCC 870 in submerged fermentation

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Optimization procedure using response surface methodology (RSM) based on face centered central composite design (FCCCD) with three factors (concentrations of wheat flour, glucose and ammonium nitrate) was used in order to investigate the effect of these parameters on the production of pectinase from *Aspergillus fumigatus* MTCC 870. According to the results of RSM, concentrations of wheat flour, glucose and ammonium nitrate were significant ($p<0.05$) on enzyme production. As a result of this optimization, maximum pectinase activity (15.46 IU/mL) was achievable at wheat flour (0.25 g/100 mL), glucose concentration (0.25 g/100 mL) and ammonium nitrate concentration (0.25 g/100 mL).

Key words: Optimisation, enzyme activity, submerged culture, modelling, FCCCD, pectinase.

INTRODUCTION

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. RSM defines the effect of the independent variables, alone or in combination, on the process. In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process. It has been successfully applied to optimizing conditions in food, chemical and biological processes (Ismail, 2005). With this perspective, a study was initiated with the goal of designing a low cost media through RSM using wheat flour as substrate and glucose and ammonium nitrate as carbon and nitrogen sources respectively, which would promote maximum pectinase production of a strain.

Enzymes that hydrolyze pectic substances, which contribute to the firmness and structure of plant cells, are known as pectolytic enzymes or pectinases. Based on their mode of action, these include polygalacturonase (PG), pectin esterase, pectin lyase and pectate lyase. Pectinases are extensively used in the industrial clarification of wine and fruit juice, in tomato pulp and oil extraction, in chocolate and tea fermentation and in vegetable waste treatment. In the fruit juice extraction and clarification process, these are used together with amylases whereby a reduction of 50% in the filtration time is observed. Furthermore, in combination with other enzymes like cellulases, arabinases and xylanases they have shown to increase the pressing efficiency enormously. Recent applications have emerged in the treatment and degumming of natural fibers used in paper and textile industry. For example pectinases in conjunction with amylases, lipases, cellulases and hemicellulases have been used to remove the sizing agents from cotton, in a safe and eco-friendly manner by replacing toxic soda. Moreover, pectinases are used in animal feed production reducing the feed viscosity and increasing the

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Table 1. Central composite design in coded units of variables for pectinase production by *Aspergillus fumigatus.*

Std. Run	Wheat flour (X_1)	Glucose (X_2)	Ammonium nitrate (X_3)	Activity (IU/mL)	
				Experimental	Predicted
1	-1	-1	-1	4.147	3.276
2	-1	-1	+1	4.147	4.633
3	-1	+1	-1	12.252	12.973
4	-1	+1	+1	16.588	15.462
5	+1	-1	-1	6.220	5.279
6	+1	-1	+1	9.425	8.250
7	+1	+1	-1	6.220	5.279
8	+1	+1	+1	7.352	7.768
9	-1	0	0	9.237	10.027
10	+1	0	0	6.960	7.988
11	0	-1	0	1.697	2.585
12	0	+1	0	6.262	7.192
13	0	0	-1	4.713	5.132
14	0	0	+1	5.655	7.055
15	0	0	0	6.499	5.961
16	0	0	0	6.777	5.961
17	0	0	0	6.515	5.961
18	0	0	0	6.623	5.961
19	0	0	0	6.917	5.961
20	0	0	0	6.074	5.961

absorption of nutrients. In the industrial market they contribute to almost 25% of the global enzyme sales, where this contribution is estimated to increase further by the year 2009. Therefore in order to meet this high demand, it is highly important to produce pectinase enzyme in a cost effective and productive way (Akhnazarova and Kafarov, 1982; Naidu and Panda, 1998; Kashyap et al., 2001; Tari et al., 2007).

Factors like carbon and nitrogen sources and their concentrations have always been of great interest to the researchers in the industry for the low cost media design. It is also known that 30-40% of the production cost of industrial enzymes is estimated to be the cost of growth medium. These sources together with factors like agitation speed and inoculation ratio, besides their effect on the product formation, have been determined to play significant role in the determination of the final morphology of the culture. Therefore, it is of great significance to optimize the conditions for cost-efficient enzyme production (Panda and Naidu, 2000; Sathyanarayana and Panda, 2003).

MATERIALS AND METHODS

Microorganism and spore production

A. fumigatus MTCC 870 was purchased in the lyophilized form, from IMTECH, Chandigarh. The propagation of this culture was done on PDA agar slant medium incubated at 30°C until well sporulation (72 h). The spore suspensions were used as inoculum containing 2 x 10^7 spores/mL.

Production medium

Total of 20 shake flasks media (50 mL in 250 mL Erlenmeyer), including the repetitions performed at center points, were prepared according to the CCD (Table 1) for optimization step and experiments were conducted at 30°C for 72 h with an initial pH of 5.0 and agitation speed was maintained at 160 rpm. The media for production are contained in g/L: $(NH_4)_2SO_4$ -1, $MgSO_4$ -5, KH_2PO_4 -5 and $FeSO_4$ – 0.05. The substrate used for the production was wheat flour, the concentrations of which were varied between 2.5 and 12.5 g/L. The incubation temperature and time for each of the steps were 30°C and 72 h, respectively (Octavio et al., 1999; Sadhana et al., 2006; Jose et al., 2007).

In addition to this medium, glucose and ammonium nitrate were added according to the initial concentrations given in the design to study their effect (as carbon and nitrogen source) on the production of pectinase. After 72 h of incubation, the samples from each flask were assayed for enzyme activity.

Enzyme assay

Pectinase activity was assayed according to the procedure given by Minjares-Cassanco et al. (1997) by using pectin as substrate. The reaction mixture (1 mL) containing equal amounts of substrate (1%) prepared in citrate buffer (0.05 M pH 4.4) and suitably diluted enzy-

Table 2. Experimental variables in coded and actual units.

Independent variable	Coded unit		
	-1	0	1
Wheat flour (X_1) (g/100mL)	0.25	0.75	1.25
Glucose (X_2) (g/100mL)	0.05	0.15	0.25
Ammonium nitrate (X_3) (g/100mL)	0.05	0.15	0.25

Table 3. Estimated regression coefficients of second order polynomial model for optimization of pectinase production by *Aspergillus fumigatus*.

Factor	Estimated coefficient	Standard deviation	t- value	p-value
Constant	5.961	0.4034	14.779	0.000
X_1	-1.019	0.3711	-2.747	0.021
X_2	2.303	0.3711	6.209	0.000
X_3	0.961	0.3711	2.591	0.027
X_1^2	3.046	0.7076	4.305	0.002
X_2^2	-1.072	0.7076	-1.516	0.160
X_3^2	0.131	0.7076	0.186	0.856
X_1X_2	-2.827	0.4149	6.815	0.000
X_1X_3	0.001	0.4149	0.000	1.000
X_2X_3	0.283	0.4149	0.682	0.511

me was incubated at 40°C for 30 min. After incubation 3 mL DNS solution was added to stop the reaction and tubes were kept in boiling water for 10 min. On cooling, the developed colour was read at 575 nm using spectrophotometer. The amount of released reducing sugar was quantified using galacturonic acid as standard. The enzyme activity was calculated as the amount of enzyme required to release one micromole equivalent of galacturonic acid per minute under assay condition.

Experimental design and statistical analysis

In this study, the effects of independent variables, initial concentrations of wheat flour (X_1), glucose (X_2) and ammonium nitrate (X_3) were investigated on the response of pectinase activity using RSM. A face centered CCD design with three-factors and three-coded level was used to determine the production of pectinase.

Analysis of data and generation of response surface graphics was done using MINITAB 15 trial version. After running the experiments and measuring the pectinase activity levels, a second order model including interactions was fitted to the response data:

$$y = \beta_0 + \sum_{1}^{k}\beta_i x_i + \sum_{1}^{k}\beta_{ii} x_i^2 + \sum_{i<j}\sum\beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y is the predicted response, k the number of factor variables, β_0 the model constant, βi the linear coefficient, Xi the factor variable in its coded form, βii the quadratic coefficient, and β_{ij} is the interaction coefficient (Nair and Panda, 1997; Zong-ming et al., 2008).

In dimensionless coordinate system, the upper and lower levels of factors are at +1 and -1, respectively. The coordinates of the centre point of the design are Zero. These coded values of x_j are then used to build a regression model to fit the experimental data. The factors and levels were followed according to Table 2. The equation for transforming casual factors to coded form (Torbjorn et al., 1998; Montgomery, 2007):

$$x_j = \frac{Z_j - Z_j^c}{\Delta Z_j} \quad (2)$$

The analysis of variance (ANOVA) tables was generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined.

RESULTS AND DISCUSSION

Results obtained after running 20 trials according to the statistical design are shown in Table 1. The three factors and three-levels used in experiment are given in Table 2. The predictive model equation for pectinase production is given below:

$$Y = 5.961 - 1.0193x_1 + 2.303x_2 + 0.961x_3 + 3.046x_1^2 - 2.827x_1x_2 \quad (3)$$

Estimates values of factor coefficients, interactive terms, quadratic terms and probability values (p-values) are shown in Table 3. The effects with p-values higher than 0.05 are not significant at the 95% confidence level and were discarded. The sign of the effect marks the perfor-

Table 4. Analysis of variance (ANOVA) for second order polynomial model for optimization of pectinase production by *Aspergillus fumigatus.*

Factor	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	p-value
Model	9	171.495	19.055	13.84	0.000
Linear	3	72.707	24.237	17.60	0.000
Square	3	34.192	11.397	8.28	0.005
Interaction	3	64.596	21.531	15.64	0.000
Residual error	10	13.768	1.377		
Lack-of-fit	5	13.348	2.670	31.77	0.001
Pure error	5	0.420	0.084		
Total sum of squares	19	185.263			

Table 5. Fit statistics for enzyme activity.

Parameter	Master model	Predicted model
Mean	7.014	7.014
R-square	92.57%	90.39%
Adj. R-square	85.88%	86.95%
RMSE	1.1734	1.127
CV	16.729	16.079

mance of the response. In this way, when a factor has a positive effect, the response is higher at the high level and when a factor has a negative effect the response is lower at high level. In the analysis of variance (ANOVA) through quadratic model are given in Table 4. The contribution of factors X_1: wheat flour, X_2: glucose, X_3: ammonium nitrate, X_1^2: (wheat flour)^2, X_1*X_2: (wheat flour * glucose) were found to be significant in explaining the production of pectinase at 5% level of significance. The significance of X_1^2 indicated that there was a curvature in the response surface of enzyme activity. Glucose and ammonium nitrate were found to be influencing individually. The interaction between wheat flour and glucose concentration was also found significant. The lack of fit was found to be insignificant ($p<0.05$) which indicated the absence of block effect in the experiment. The fit statistics for enzyme activity model are shown in Table 5. The analyses were carried out using coded units. The R^2 value for the master model and predicted model was found to be 92.57 and 90.39%, respectively. The adjusted R^2 value for the predicted model was 86.95%.

The study clearly indicates that this strain with the optimized conditions can be considered as a potential pectinase producer for different industrial applications. The contour plots described by the regression model were given in Figures 1, 2 and 3 by keeping third factor at their middle level to illustrate the effects of the independent variables, and combined effects of each independent variable upon the response variable.

A three-dimensional response surface plot (Figure 4) shows the interaction effect of varying concentration of carbon source (glucose concentration) and nitrogen source (ammonium nitrate) with fixed concentration of substrate (wheat flour) at its centre point (0.75 g/100 mL). It is observed that the high pectinase activity is achieved at higher concentrations of glucose and ammo-nium nitrate. In addition to these conditions maximum activity required lower concentration of wheat flour concentration (Figures 5-6). The critical values (opti-mum), the wheat flour, glucose and ammonium nitrate concentrations were determined by setting the derivatives of Eq.(3) of $\partial Y/\partial X_1$, $\partial Y/\partial X_2$, and $\partial Y/\partial X_3$ to zero yielding opti-mal values 0.25, 0.25, 0.25 g/100 mL, respectively with maximum enzyme activity of 15.46 IU/mL.

The normal probability plot (Figure 7) of the residuals is an important diagnostic tool to detect and explain the systematic departures from the assumption that errors are normally distributed and are independent of each other and that the error variance are homogeneous. The residual plot (Figure 8) which shows equal scatter of the residual data above and below the x-axis indicates that the variance was independent of the value of pectinase production, thus supporting the adequacy of the least square fit.

Conclusions

To date, few reports are available in literature regarding the production of pectinase from *A. fumigatus.* Therefore, this study will serve as a base line of the initial studies in this field. The study does not only provide novel information on the growth requirement of this organism, but also serves as an example for the application of the statistical techniques to the fungal systems for choosing the optimized conditions. Through these optimization experiments, the optimal conditions for maximum conditions for

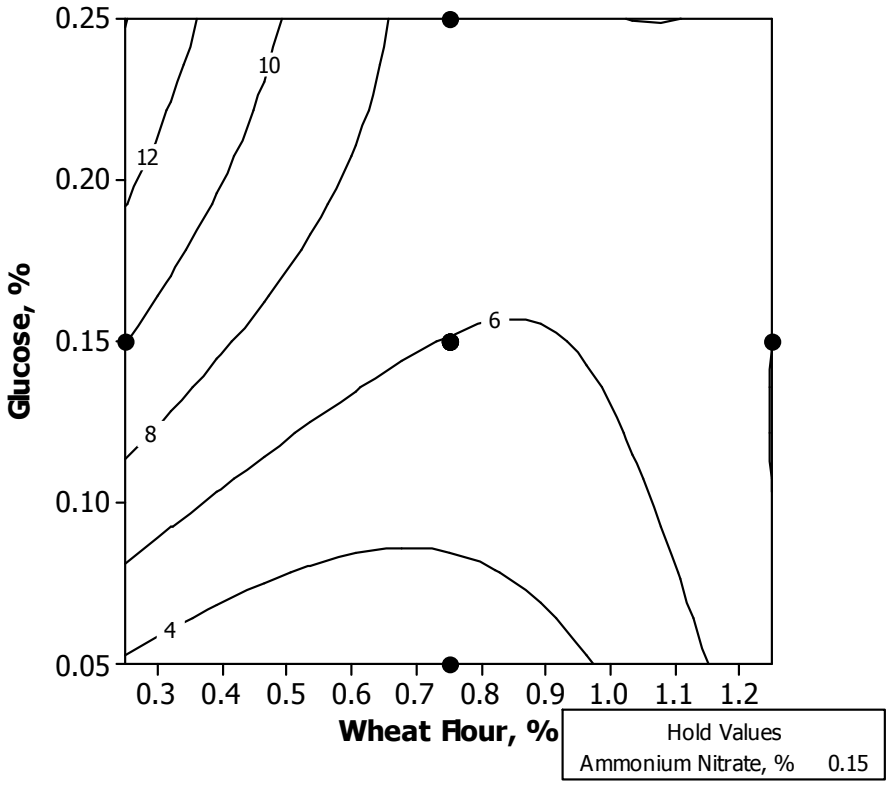

Figure 1. Contour plot for the effect of glucose and wheat flour on enzyme activity.

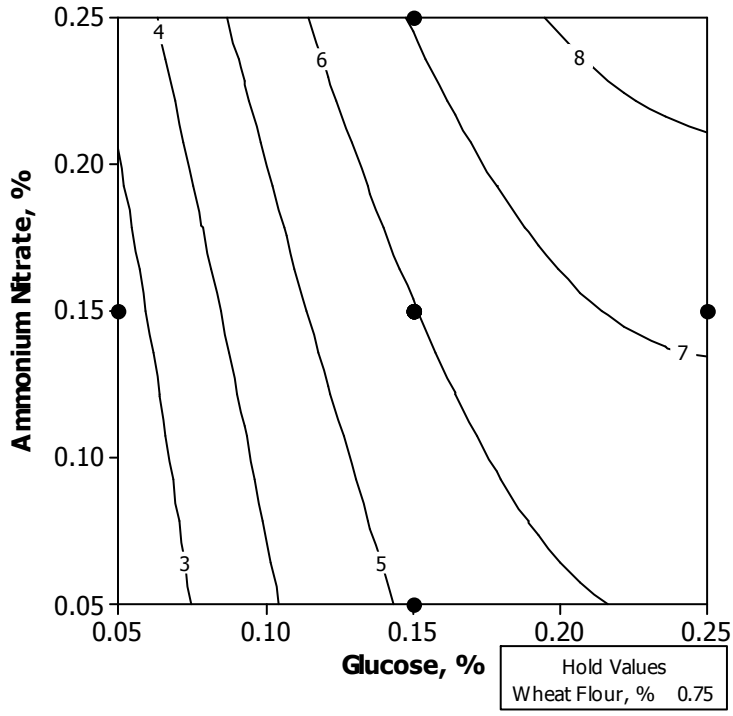

Figure 2. Contour plot for the effect of ammonium nitrate and glucose on enzyme activity.

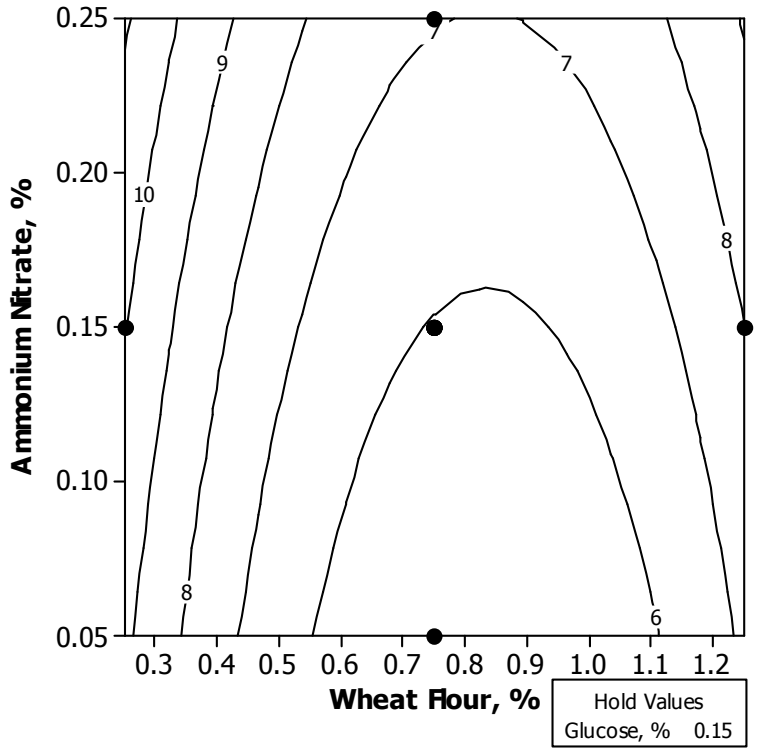

Figure 3. Contour plot for the effect of ammonium nitrate and wheat flour on enzyme activity.

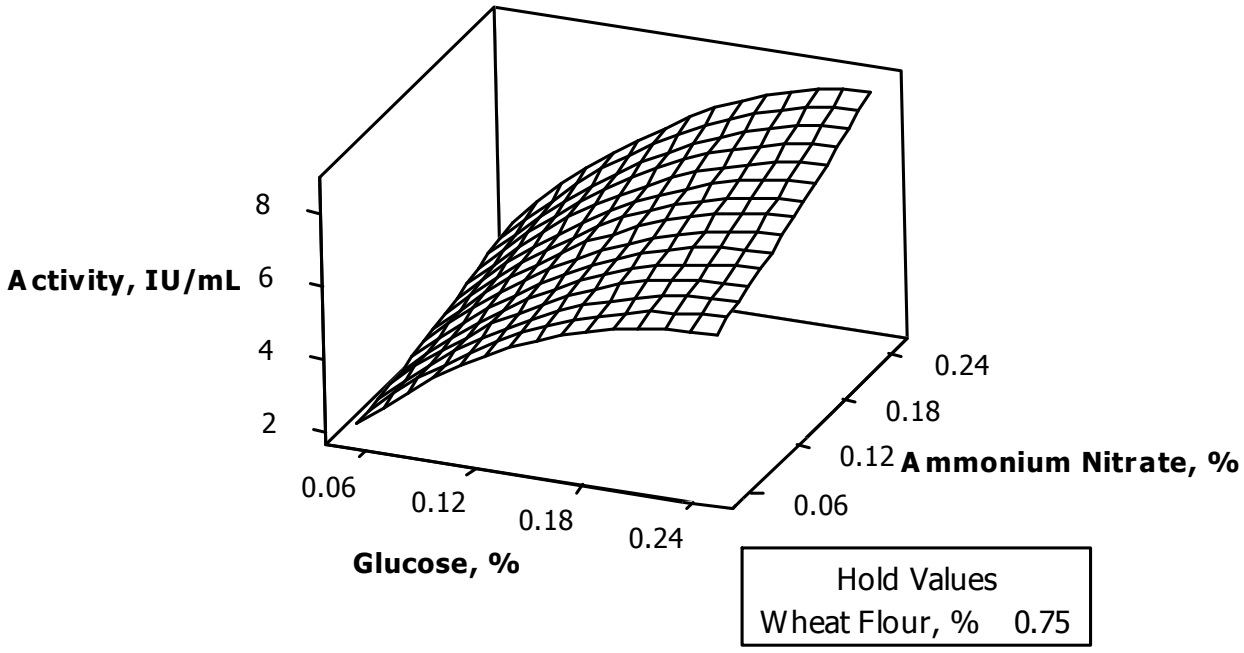

Figure 4. Surface plot for the effect of carbon and nitrogen on enzyme activity.

pectinase production (15.46 IU/mL) were to use wheat flour at 0.250 g/100 mL, high level of carbon source (glucose-0.250g/100mL) and nitrogen source (ammonium nitrate - 0.250 g/100 mL).

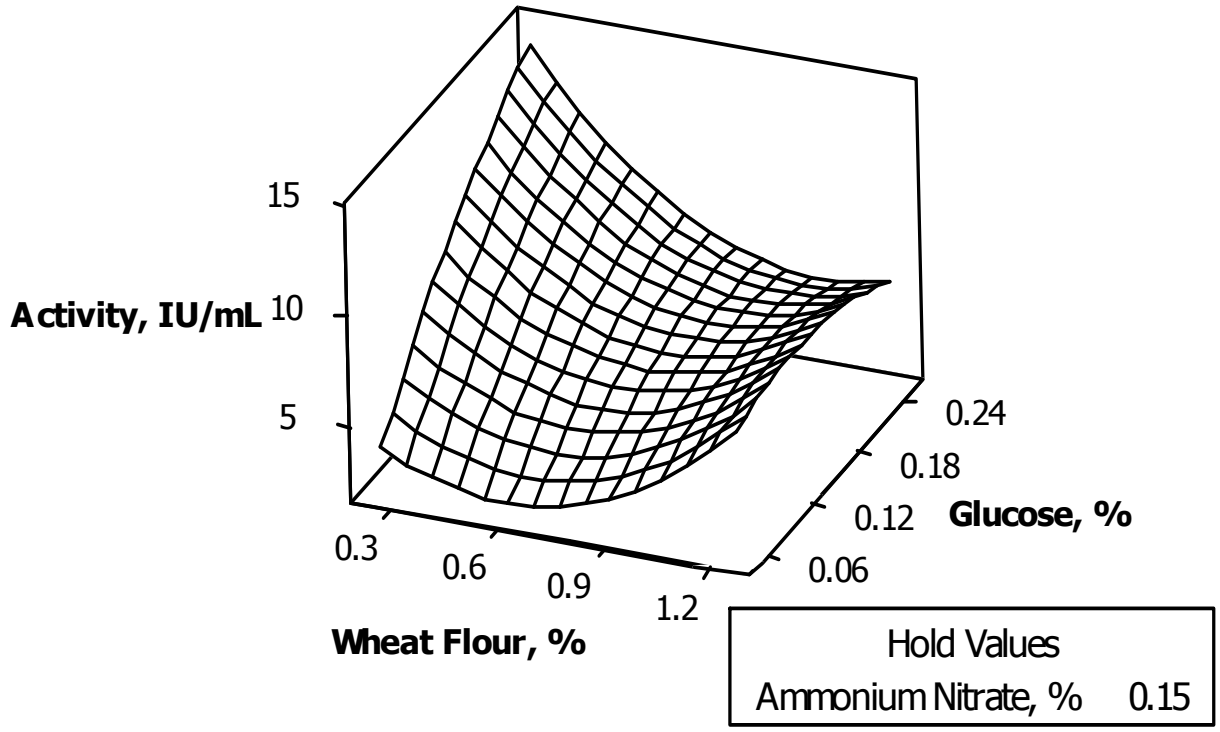

Figure 5. Surface plot for the effect of carbon and wheat flour on enzyme activity.

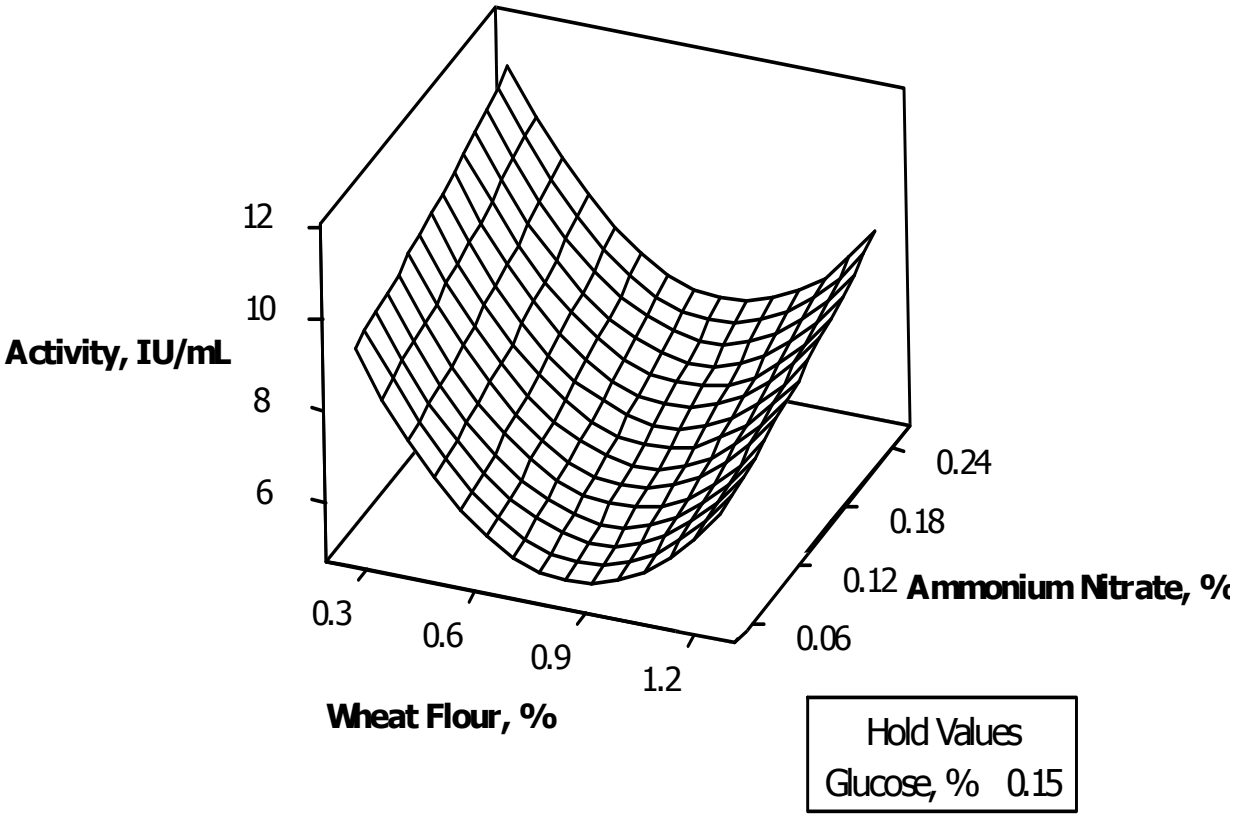

Figure 6. Surface plot for the effect of ammonium nitrate and wheat flour on enzyme activity.

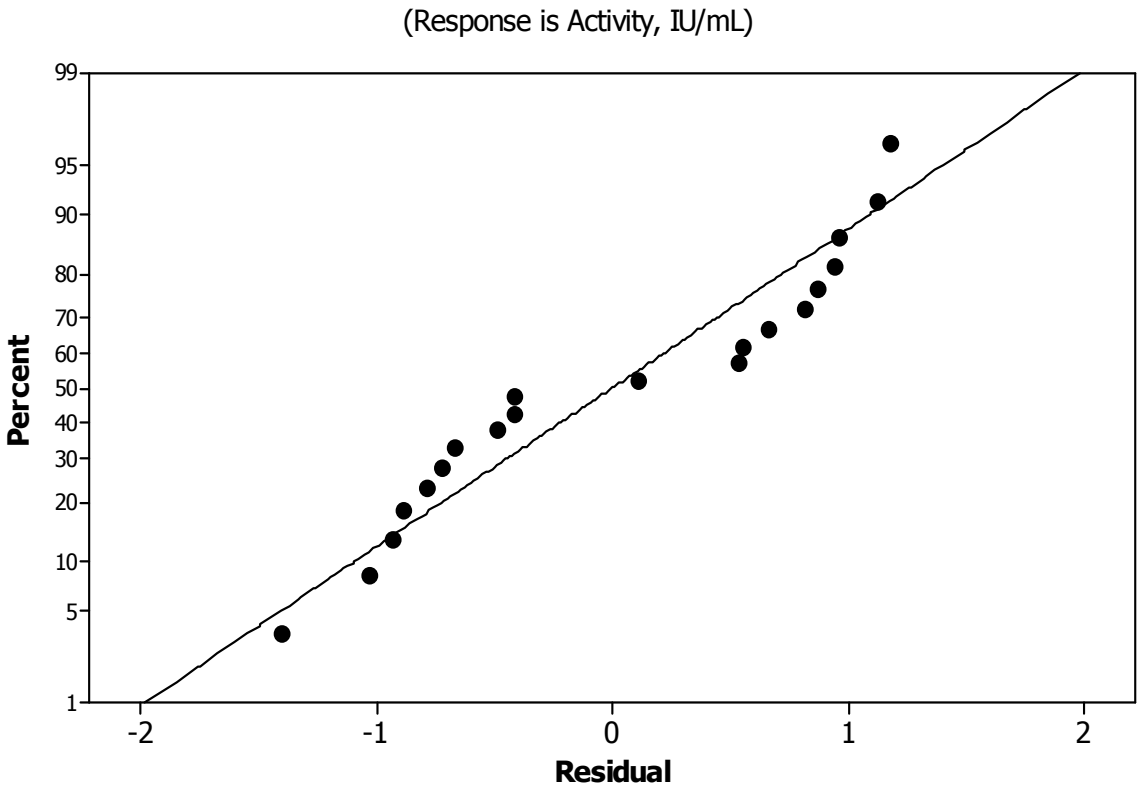

Figure 7. The Normal probability plot for pectinase production model.

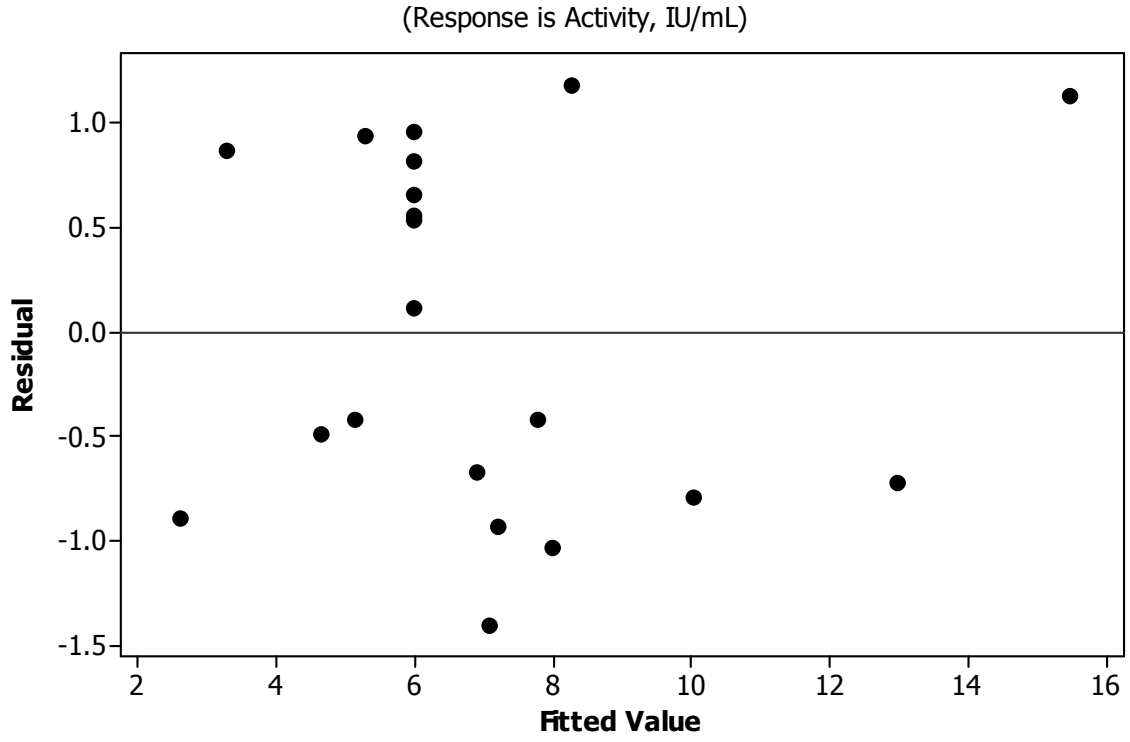

Figure 8. The Residual plot for pectinase production model.

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