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Full Length Research Paper

Application of response surface methodology to the optimization of amylase production by *Aspergillus oryzae* MTCC 1847

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This research paper mainly focused on developing a media by optimizing parameters like sweet potato concentration, sodium nitrate concentration, pH, temperature for the maximum production of amylase by *Aspergillus oryzae* MTCC 1847. Optimization of the medium components such as sweet potato (carbon source), sodium nitrate (nitrogen source) and parameters like pH and temperature were studied for the amylase production by using response surface methodology (RSM) based central composite design (CCD). By using the surface plots and response optimizer of MINITAB 14 Software, the maximum enzyme activity of 0.893 IU/ml was predicted when sweet potato concentration was 2.01%, sodium nitrate 0.6%, pH 7.2 and temperature 25°C.

Key words: Sweet potato, *Aspergillus oryzae*, response surface methodology, central composite design.

INTRODUCTION

Amylases are widely distributed and are one of the most studied enzymes.These enzymes have wide scale application ranging from food to effluent treatment. Amylases are a class of enzymes (hydrolases) that are capable of digesting the glycosidic linkages found in starch or glycogen. Under aqueous conditions, amylases act on glycosidic bonds present in starch to liberate glucose, maltose, maltotriose (Delphine et al., 2000; Satish and Aniruddha, 2007), etc.

Amylases can be derived from a variety of living organisms, ranging from microorganisms to plants and humans. Microorganisms are the most important sources for the production of amylases. The *Aspergillus* species produce the extracellular amylase enzyme having significant industrial importance. Fungal amylases are used in food industry, textile and paper industries (Ellaiah et al., 2002; Gigras et al., 2002; Pandey et al., 2000). Agroindustrial waste substrates such as defatted soybean cake, wheat and rice bran can be used for enzyme production (Germano et al., 2003). Dimitrovski and Sapecska reported wheat bran as an appropriate substrate for a-amylase production by *Bacillus* sp. ibi-3 (Dimitrovski and Sapecska, 1995; Iefuji et al., 1996).

The important stage in a biological process is optimization to improve a system and increase the efficiency of the process without increasing the cost (Tanyildizi et al., 2005). The classical single variable optimization method is not only time-consuming and tedious but also does not depict the complete effects of the parameters in the process and ignores the combined interactions between physicochemical parameters. This method can also lead

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Abbreviations: ANOVA, Analysis of variance; **CCD,** central composite design; **RSM,** response surface methodology; **DNS,** dinitrosalicylic acid.

List of symbols: F, Fisher's function; **P,** corresponding level of significance; **T,** student's test; X_1, sweet potato; X_2, sodium nitrate; X_3, pH; X_4, temperature. **Y,** predicted response; **β,** coefficient.

Table 1. Experimental range and levels of the four significant variables used in RSM for amylase production.

Variables	-1	0	+1
Sweet potato (%), X_1	1	1.5	2
Sodium nitrate (%), X_2	0.5	0.625	0.75
pH, X_3	6	6.5	7
Temperature (°C), X_4	30	35	40

to misinterpretation of results. Response surface methodology (RSM) is suited for studying the main and interaction effects of factors on growth or metabolite formation during microbial fermentation. Compared to classical method of optimization, central composite design (CCD) was more effective in bioprocess optimization. A full factorial CCD was applied to study various effects of sweet potato concentration, nitrogen source concentration, pH and temperature to determine the optimal value of these variables on amylase production by *Aspergillus oryzae* MTCC 1847 under shake flask fermentation conditions

MATERIALS AND METHODS

The fungus, *A. oryzae* MTCC 1847 was obtained from Microbial Type Culture Collection and Gene Bank of the Institute of Microbial Technology (IMTECH), Chandigarh, India. It was maintained on Sabouraud dextrose agar (Hi Media, Mumbai) medium. The slants were grown at 30°C for seven days and stored at 4°C. The strain was sub-cultured at four weeks intervals. All chemicals used in this study are of analytical grade from Hi-Media Laboratories Pvt. Ltd., India and Loba Chemie Laboratory, Qualigens Fine Chemicals, India.

Inoculum preparation

Pre-inoculum was prepared by transferring 5 ml of sterilized distilled water to seven days old SDA slant culture and the spore suspension was transfer into 250 ml Erlenmeyer flask containing 100 ml of modified Czapek Dox inoculum medium and incubated at 30°C for seven days. 1% inoculum size is used to inoculate production media.

Substrate preparation

Sweet potato was obtained from the local market in Chennai. It was ground into powder with a blender and passed through a sieve (80/100 mesh size) to remove large size particles and the fine sweet potato powder was used for further studies.

α-Amylase activity assay

α-Amylase was determined as described by Okolo et al. (1995). The reaction mixture consisted of 0.5 ml of 1% soluble starch, 0.25 ml 0.1 M acetate buffer (pH 5.0), 1.5 ml of distilled water and 0.5 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method of Miller (1959). The color developed was read at 575 nm using a spectrophotometer. Glucose was used as the standard. One unit (IU) of α-amylase was defined as the amount of enzyme releasing one µmol glucose equivalent per minute under the assay conditions.

Optimization of amylase production by response surface methodology

Experimental range and level of independent variables such as sweet potato concentration (X_1), sodium nitrate concentration (X_2), pH (X_3) and temperature (X_4) are given in Table 1. Each variable was studied at three different levels (1, 1.5 and 2%) for sweet potato concentration, (0.5, 0.625 and 0.75%) for nitrogen source concentration and (6, 6.5 and 7) for pH and temperature (30, 35 and 40°C). Experimental Design includes 31 runs and fermentation was carried out separately for each with replicates. Upon completion of experiments, amylase activity was taken as a dependant variable or response. A full polynomial model was obtained by a multiple regression technique for four factors using MINITAB 14 to determine the optimum composition of the medium and production conditions.

RESULTS AND DISCUSSION

RSM for optimization of Amylase Production

Interaction between sweet potato concentration, sodium nitrate concentration, pH and temperature was studied for optimization of growth of fungus for amylase production using CCD which has been employed for determination of optimal value for enzyme production.

Experimental design and statistical analysis

Central composite design is one of the response surface methodologies usually utilized to obtain data that fits a full second-order polynomial model. The graphical representation of the model equation results in response surface plots that represent the individual and interactive effects of test variables on the response. A fraction of a coded, 25 CCD with two axial points at a distance $\alpha = 2$ from the design center and six replicates about the center point, making a total of 31 runs, were used to study the screened variables, sweet potato concentration, sodium nitrate, pH and temperature. These parameters were tested at three levels, coded, -1, 0 and +1 for low, middle and high, respectively. The concentration of other medium constituents was kept constant. The concentrations of other components were, KH_2PO_4 0.1%, $MgSO_4$ $7H_2O$ 0.05% and KCl 0.05% (Ho-Soo et al., 2000). The CCD experiment was designed using the MINITAB software package, version 14.0, The Math Works Inc., Natick, MA, USA. The experimental range with the levels of independent variables and experimental plan is shown in Tables 1 and 2, respectively, for medium optimization. The coded and actual (Xi) level of variables is shown in Table 2. All the experiments were carried out in duplicate

Table 2. CCD matrix of independent variables used in RSM with corresponding experimental and predicted values of amylase activity.

Std Order	X1	X2	X3	X4	Amylase activity (IU/ml)	
					Experimental	Predicted
1	-1	-1	-1	-1	0.358	0.395
2	1	-1	-1	-1	0.543	0.518
3	-1	1	-1	-1	0.509	0.512
4	1	1	-1	-1	0.638	0.657
5	-1	-1	1	-1	0.877	0.835
6	1	-1	1	-1	0.858	0.863
7	-1	1	1	-1	0.744	0.750
8	1	1	1	-1	0.897	0.800
9	-1	-1	-1	1	0.556	0.604
10	1	-1	-1	1	0.570	0.632
11	-1	1	-1	1	0.523	0.586
12	1	1	-1	1	0.643	0.636
13	-1	-1	1	1	0.587	0.636
14	1	-1	1	1	0.621	0.570
15	-1	1	1	1	0.439	0.416
16	1	1	1	1	0.341	0.372
17	0	0	0	0	0.639	0.578
18	0	0	0	0	0.614	0.656
19	0	-2	0	0	0.739	0.707
20	0	2	0	0	0.614	0.626
21	0	0	-2	0	0.582	0.492
22	0	0	2	0	0.597	0.667
23	0	0	0	-2	0.636	0.693
24	0	0	0	2	0.550	0.473
25	0	0	0	0	0.809	0.838
26	0	0	0	0	0.812	0.838
27	0	0	0	0	0.819	0.838
28	0	0	0	0	0.828	0.838
29	0	0	0	0	0.841	0.838
30	0	0	0	0	0.887	0.838
31	0	0	0	0	0.867	0.838

and averages of the results were analyzed. Based on the regression analysis a second order polynomial model describes the relationship between the independent variables and amylase activity was developed (Equation 1). When the value of composite desirability is close to 1, the predicted values and model was well fitted with the experimental results.

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+ \beta_4X_4 +\beta_{11}X_{12}+ \beta_{22}X_{22} +\beta_{33}X_{32}+ \beta_{44}X_{42} +\beta_{12}X_1X_2+\beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 \quad \ldots. (1)$$

where Y is the response variable and X_1, X_2, X_3, and X_4 are the coded values of the independent variables: sweet potato concentration, sodium nitrate concentration, pH and temperature respectively.

The response surface plots were used to describe the individual and cumulative effects of the variables as well as the mutual interactions between the variables on the dependent variable. The second degree polynomial equation was maximized by a constraint search procedure using the MINITAB software (Version 14.0, The Math Works, Inc.) to obtain the optimal levels of the independent variables and the predicted maximum amylase Activity. All the experiments were carried out in duplicate and average amylase activity given in Table 2 were subjected to multiple linear regression analysis using MINITAB 14 software. The effect of sweet potato concentration, sodium nitrate concentration, pH and temperature on amylase activity was described in the form of second order polynomial model in coded units (Equation 2).

Table 3. Estimated regression coefficients of second order polynomial model for optimization of amylase production. (R^2 = 0.912).

Coefficient	Estimated coefficient	t- Value	p-Value
β_0	-29.969	-10.041	0.000
β_1	1.590	3.088	0.007
β_2	10.264	4.804	0.000
β_3	5.516	8.092	0.000
β_4	0.480	8.554	0.000
β_{11}	-0.221	-4.657	0.000
β_{22}	-2.735	-3.603	0.002
β_{33}	-0.258	-5.437	0.000
β_{44}	-0.003	-5.363	0.000
β_{12}	0.090	0.355	0.727
β_{13}	-0.094	-1.490	0.156
β_{14}	-0.009	-1.490	0.156
β_{23}	-0.808	-3.184	0.006
β_{24}	-0.054	-2.128	0.049
β_{34}	-0.041	-6.431	0.000

Table 4. Analysis of variance (ANOVA) of second order polynomial model for optimization of amylase production.

Factors	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
Regression	14	0.668	0.047	11.86	<0.001
Linear	4	0.137	0.116	28.99	<0.001
Square	4	0.286	0.071	17.82	<0.001
Interaction	6	0.243	0.040	10.10	<0.001
Residual error	16	0.064	0.004		
Lack-of-fit	10	0.059	0.005	6.79	0.015
Pure error	6	0.005	0.001		
Total	30	0.733			

$$\text{Amylase activity (IU/ml)} = -29.969 + 1.590X_1 + 10.264X_2 + 5.516X_3 + 0.480X_4 - 0.221X_{12} - 2.735X_{22} - 0.258X_{32} - 0.002X_{42} + 0.090\ X_1X_2 - 0.094X_1X_3 - 0.009X_1X_4 - 0.808X_2X_3 - 0.054X_2X_4 - 0.040X_3X_4 \quad \ldots. (2)$$

Response surface plots and contour plots were described by the regression model for CCD which was developed using MINITAB 14 software. The student's t-test was performed to determine the significance of the regression coefficients. The results of statistical analysis including the regression coefficient, t and p values for linear, quadratic and combined effects of the variables are given in the Table 3. The statistical significance of the model was also determined by F-test for analysis of variance (ANOVA) and residuals analysis was performed to validate the model at 95% confidence level. The model fitted well with amylase activity and the optimal values from the model was justified (p = 0.000). The ANOVA given in Table 4 indicates that the linear, quadratic and interaction terms in second order polynomial Model (Equation 2) were highly significant ($p < 0.005$) and adequate to represent the relationship between amylase activity (IU/ml) and sweet potato concentration, sodium nitrate concentration, pH and temperature.

Effect of temperature and pH on amylase activity

Figure 1 shows the effect of temperature and pH on amylase activity, while other variable (sweet potato concentration and sodium nitrate) was fixed at their middle level. It was observed that the amylase activity was less at lower level and increases towards middle, higher levels of pH. Amylase activity was increased at lower level of temperature, whereas it was decreased at middle and higher level of temperature.

Effect of sodium nitrate and pH on amylase

From Figure 2, decreased value of amylase activity at

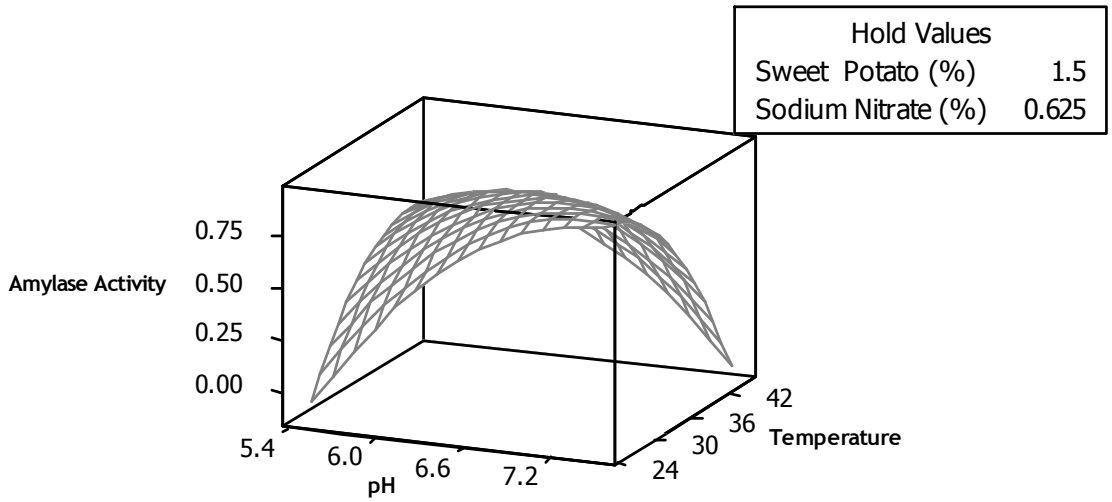

Figure 1. Response surface plot showing the interaction effect of temperature and pH on amylase activity.

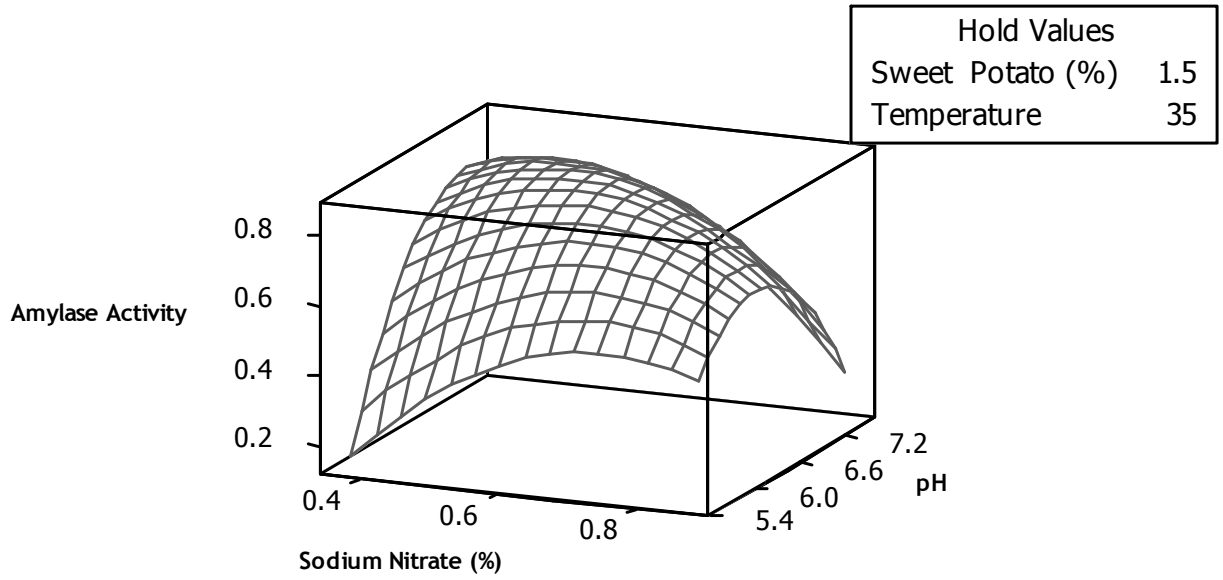

Figure 2. Response surface plot showing the interaction effect of sodium nitrate and pH on amylase activity.

lower and higher level of sodium nitrate and pH, when other variables (sweet potato and temperature) were kept at middle level was observed. Maximum activity was observed at middle level of sodium nitrate and pH.

Effect of sodium nitrate and temperature on amylase activity

Figure 3 shows the effect of sodium nitrate and temperature on amylase activity. It shows that the amylase activity was less at lower and higher level of sodium nitrate concentration and temperature; it was increased at middle level of sodium nitrate concentration and temperature.

Effect of sweet potato and temperature on amylase activity

Figure 4 shows that higher and lower level of sweet potato and temperature has no significant effect on the amylase activity and increased activity was observed at middle level of sweet potato and temperature.

Effect of sweet potato and pH on amylase activity

Figure 5 shows that middle level of sweet potato

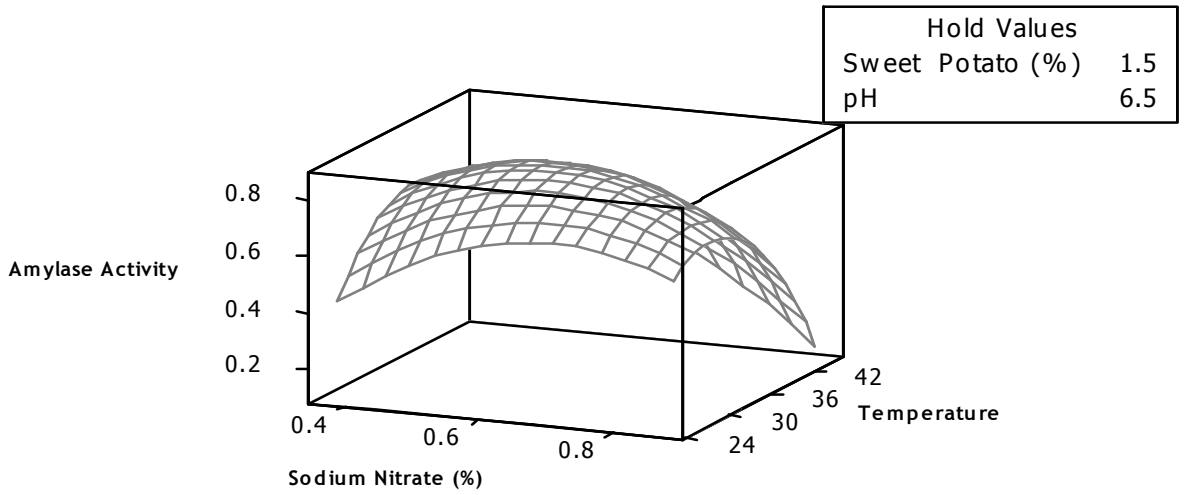

Figure 3. Response surface plot showing the interaction effect of sodium nitrate and temperature on amylase activity.

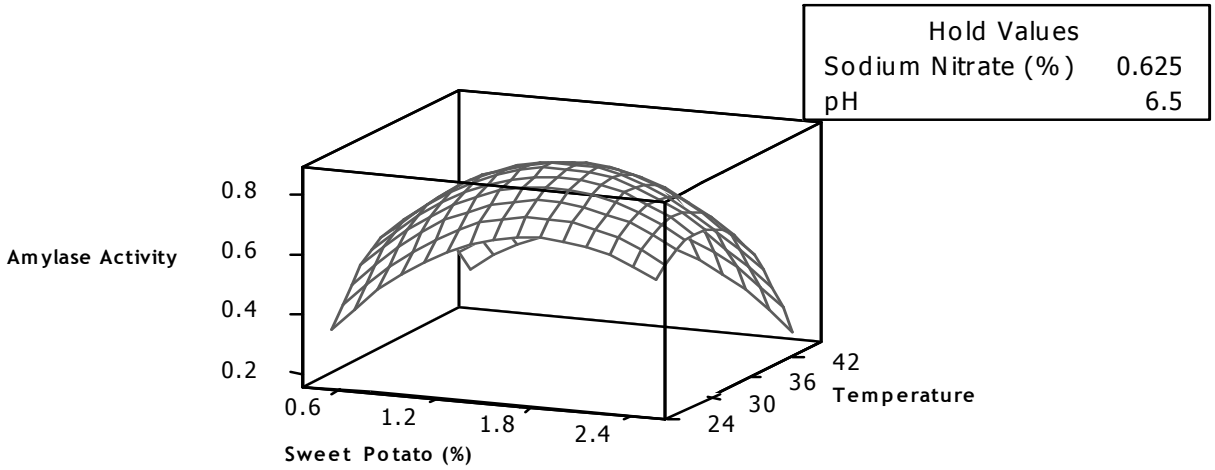

Figure 4. Response surface plot showing the interaction effect of sweet potato and temperature on amylase activity.

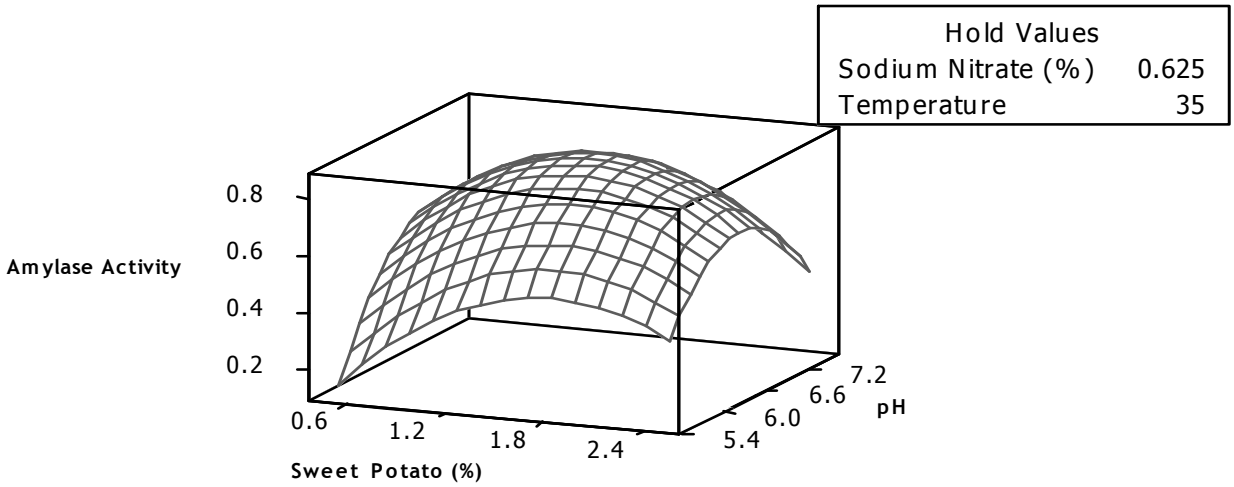

Figure 5. Response surface plot showing the interaction effect of sweet potato and pH on amylase activity.

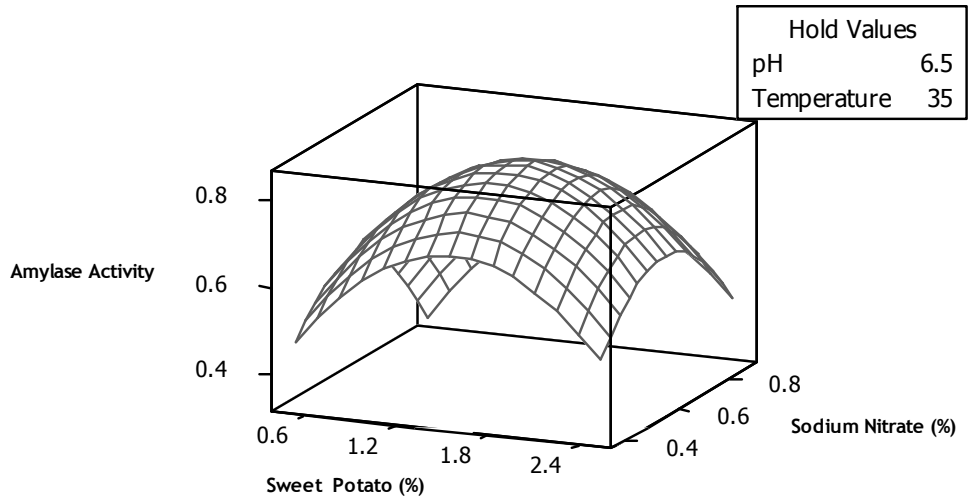

Figure 6. Response surface plot showing the interaction effect of sweet potato and sodium nitrate on amylase activity.

concentration and pH increased the amylase activity and there was no effect at lower and higher levels.

Effect of sweet potato and sodium nitrate on amylase activity

Figure 6 shows the interaction effect of sweet potato and sodium nitrate on amylase activity, while other variable (temperature and pH) were fixed at middle level. It was observed that the amylase activity was increased at middle level of sweet potato and sodium nitrate concentration, and decreased in lower and higher levels.

Conclusion

The amount of carbon source in culture media is important for the growth and production of extra cellular amylase by fungi. It was observed that low level of carbon source is inadequate for the enzyme production and excess carbon is equally detrimental, causing enzyme inhibition. Central composite design, a type of RSM is applied to evaluate and find the conditions leading to the maximum yield of amylase production. It was observed that the linear square and interaction terms were highly significant in the enzyme production. The optimized values obtained for sweet potato, sodium nitrate, pH and temperature were 2.0110%, 0.6%, 7.2 and 25°C, respectively, with the predicted maximized response of amylase activity (0.893 IU/ml).

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