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# Optimization of the fermentation conditions and partial characterization for acido-thermophilic $\alpha$-amylase from *Aspergillus niger* NCIM 548

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**Abstract**−A high performance thermostable $\alpha$-amylase at low pH values has been synthesized. Sugarcane bagasse was hydrolyzed in a dilute acid solution and utilized as carbon source for the growth of *Aspergillus niger* strain NCIM 548. Glucose, xylose and arabinose with the ratio of 1.0 : 0.9 : 0.3 (w/w/w) were detected in the hydrolyzate by HPLC analysis. Optimization of the fermentation conditions for $\alpha$-amylase production was performed by varying four influential parameters such as Sugarcane bagasse hydrolyzate (SBH), $NH_4Cl$, pH and incubation time using a central composite design (CCD) under response surface methodology (RSM). The optimum values of SBH, $NH_4Cl$, pH and incubation time were 20.49, 2.34 g/*l*, 5.65 and 76.67 h, respectively. The acido-thermophilic enzyme showed maximum stability at 70 °C and pH value of 4. The rate constant, $K_m$ and maximum reaction rate, $V_{max}$ were 18.79 g/*l* and 15.85 g/*l*·min, respectively.

Key words: $\alpha$-Amylase, *Aspergillus niger*, Response Surface Methodology, Sugarcane Bagasse Hydrolyzate, Optimization

## INTRODUCTION

Amylolytic enzymes are commercially used to hydrolyze starch molecules to fine diverse products such as dextrins, oligosaccharides and glucose molecules [1,2]. Amylases possess approximately 25-33% of the world marketable enzymes [3]. Today, in most food processing plants, chemical hydrolysis of starch is completely replaced by enzymatic hydrolysis [4]. $\alpha$-Amylases (1,4-$\alpha$-glucan-glucanohydrolases) are extracellular enzymes which catalyze the cleavage of $\alpha$-1,4-glycosidic bonds in starch molecules [5,6]. These enzymes have found extensive applications such as liquefaction and saccharification of starchy substances in food processing, confectionary and beverage industries. There are numerous potential use of enzymes in fine chemicals, pharmaceutical, paper, textile and detergent manufacturing processes [7-11]. Various species of microorganisms such as *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Aspergillus oryzae* are able to produce $\alpha$-amylase [12-14]. $\alpha$-Amylases are derived from various carbon sources. They have distinct properties which make them more suitable for particular applications. For instance, $\alpha$-amylases at low pH values demonstrate high performances which are attractively used in many starch industries [15].

The use of lignocellulosic wastes as a potential carbon source for the living organisms leads to decrease in the cost of bioprocesses. Sugarcane bagasse is a cellulosic agro-industrial waste which is the residue of cane stalks left over after the crushing and extracting the juice from the sugarcane [16]. Daily processing of sugarcane results in thousands of tons of bagasse which are disposed as solid wastes that may be a serious threat to the environment [17]. Bagasse consists of cellulose, hemicellulose, and lignin [16-18]. However, these natural polymers of monomeric sugars need to be hydrolyzed with acids or enzymes in order to liberate fermentable sugars. Several processes have been developed for the conversion of lignocellulosic biomass residues to monomeric sugars; including steam explosion, hot water treatment, pretreatment of the fibers with alkaline solution, acid hydrolysis and wet oxidation process [19]. Once the fermentable sugars are utilized by specific strains of microorganisms, biological products such as enzymes are easily synthesized [2].

Fermentation process variables and media compositions may play a major role in enzyme production. Selection of random variables for fermentation of carbohydrates may not be the method of choice. The method of changing one variable at time is not suitable for optimization of fermentation processes, because the interactions between different variables are overlooked. These limitations can be eliminated by optimizing all the effective parameters collectively using response surface methodology (RSM). RSM is a collection of mathematical and statistical techniques which generates three dimensional plots and displays linear, quadratic and interactive effects of process variables [20].

The purpose of the present study was to determine the optimum fermentation conditions for $\alpha$-amylase production by *A. niger* NCIM 548 in culture medium containing SBH. The effects of four process parameters (concentrations of SBH and $NH_4Cl$, pH and incubation time) on $\alpha$-amylase production were investigated. Also, the partial characterization of the produced enzyme was successfully carried out.

## MATERIALS AND METHODS

### 1. Hydrolysis of Sugarcane Bagasse

Sugarcane bagasse was initially washed with distilled water, dried at 70 °C in an oven (Binder, Germany) and crushed to small pieces. The small particles (40 g) were soaked in 300 ml of dilute sulfuric acid solution (0.25% v/v). The pretreated uniform sugarcane bagasse soaked particles were autoclaved at 121 °C for 60 min. The hydrolyzed bagasse was pressed to separate the unreacted solid fraction.

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The remaining solid particles were removed by Whatman filter paper No. 1 (UK) and the hydrolyzate solution was neutralized to pH 7.0 with 1 M NaOH.

**2. Microorganism and Media**

*Aspergillus niger* NCIM 548 was obtained from National Collection of Industrial Microorganisms (Chandigarh, India). The stock culture was maintained on nutrient agar slants kept at 4 °C. Seed culture was prepared by transferring a full loop of cells from the slant culture into 50 ml of medium (250 ml Erlenmeyer) composed of (g/*l*): glucose, 20; $NH_4Cl$, 2.5; yeast extract, 1; $KH_2PO_4$, 0.3; NaCl, 0.25; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1 g/*l*. The initial pH of the medium was adjusted to 5. The sterilized and inoculated medium was incubated for 72 h at 30 °C in an incubator-shaker (Stuart, UK) with agitation rate of 200 rpm. For the enzyme production, the carbon source of the medium was substituted by SBH solution. The media for enzyme production were sterilized (121 °C/15 min), cooled to room temperature and inoculated with 2.5 v/v% of seed culture and incubated at 30 °C with agitation rate of 200 rpm. Mycelium biomass was removed by centrifugation (7,000 rpm, 10 min) and the supernatant was used for determination of the enzyme activities.

**3. Enzyme Assay**

The amylolytic activity of the enzyme was assayed by incubating 300 µl of enzyme solution with 3 ml of soluble starch (2% w/v) prepared in 0.1 M phosphate buffer (pH=5.0) at 50 °C. The reduced sugar was determined by color-metric method using 3,5-dinitrosalicylic acid (DNS) method [21]. Defined concentration of glucose solution was used as standard. One unit of $\alpha$-amylase activity was defined as the amount of enzyme which liberates 1 µmol of the reducing sugars per minute under the assay conditions.

**4. High-performance Liquid Chromatography (HPLC)**

The solution obtained from sugarcane bagasse was analyzed by HPLC with C-18 reversed phase column (250 mm×4.6 mm; Knauer, Smartline, Germany) thermostatted at 30 °C. The mobile phase (water/acetonitrile (9 : 1, v/v), pH 5.5) was used at a flow rate of 1 ml/min. The products were analyzed with UV detector (190 nm) maintained at 45 °C. Chromatographic grade glucose, galactose, xylose, mannose and arabinose (Merck) were used as standards.

**5. Experimental Design and Optimization of $\alpha$-Amylase Production**

For $\alpha$-amylase production, *A. niger* fermentation conditions were optimized by CCD under response surface methodology. RSM was used to obtain a mathematical model which illustrates the relationships between the independent variables and the response. The effect of four parameters (concentrations of SBH and $NH_4Cl$, pH and incubation time) was investigated. The second-order polynomial coefficients were determined using DESIGN EXPERT 7.0 (Stat-Ease, Inc, Minneapolis, MN, USA) software [22]. Design of 30 experiments was formulated with six replicates at the central points. The regression equation was solved to determine the optimum values of the selected variables. Also, the effects of the influential parameters on $\alpha$-amylase production were investigated by analyzing the response surface plots. Each of the parameters was coded at five levels: $-\alpha$, $-1$, 0, $+1$ and $+\alpha$. In addition, statistical analysis of the model was performed using the analysis of variance (ANOVA).

**6. Enzyme Characterization**

The enzyme activities and stabilities with respect to temperature in the range of 20-90 °C were investigated. Also, the performance of $\alpha$-amylase was evaluated at various pH values of 2-8. To determine the optimum pH and temperature, the mixtures contained 3 ml of soluble starch (2% w/v) and 300 µl of enzyme solution were incubated for 15 min. To compare enzyme activities at various experimental conditions, the amount of reducing sugars liberated in each sample was measured.

Also, initial rates of starch hydrolysis with various substrate concentrations (2-20 g/*l*) in 0.1 M citrate buffer solution (pH=4) at 65 °C were measured. The kinetic constants, $K_m$ and $V_{max}$ were defined by the double reciprocal, Lineweaver-Burk plot.

## RESULTS AND DISCUSSION

**1. Initial Stage of Enzyme Production**

*A. niger* was grown in a medium described in material and method as the carbon source was replaced by SBH solution. In the initial stage of fermentation, the fermentation condition for $\alpha$-amylase production was experimented without any optimization strategy for the defined process variables. The initial experiment had medium composition of (g/*l*): SBH solution, 20; $NH_4Cl$, 1.5; yeast extract, 1; $KH_2PO_4$, 0.3; NaCl, 0.25; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1 and pH value of 5. The culture was incubated for 70 h. The obtained enzyme activity in the initial stage of fermentation without any process optimization was 39.43 U/ml.

**2. Characterization of the SBH**

SBH solution had a sugar concentration of 30 g/*l*. The solution was analyzed by HPLC. The hydrolyzate solution consisted of glucose, xylose and arabinose with proportion of 1.0 : 0.9 : 0.3 (w/w/w), respectively. Glucose is the metabolic fuel of the cell which is responsible for enzyme generation. Adequate amounts of glucose prevent the full expression of the specified genes-proteins involved in the fermentation of numerous carbohydrates even when they are present in high concentration. This phenomenon is known as catabolite repression, which prevents the wasteful duplication of energy for production of enzyme. In the SBH solution, there was limited amount of glucose (10-20 g/*l*) to justify $\alpha$-amylase production. As catabolite repression of $\alpha$-amylase production did not occur in the medium containing SBH solution, utilization of SBH was highly remarkable.

**3. Modeling for Enzyme Production**

The concentrations of SBH solution, $NH_4Cl$, pH and incubation time were selected as influential parameters on $\alpha$-amylase production. CCD was implemented to investigate the interactions between these parameters and also to determine their optimum levels. The actual values and the corresponding coded levels of the independent variables are summarized in Table 1. The values corresponding to center point were chosen from a single-parameter study (data

**Table 1. Experimental range and levels of the independent variables**

| Variables | Symbol | Range and levels | | | | |
|---|---|---|---|---|---|---|
| | | −2 | −1 | 0 | 1 | 2 |
| SBH (g/*l*) | $X_1$ | 10 | 15 | 20 | 25 | 30 |
| $NH_4Cl$ (g/*l*) | $X_2$ | 0.5 | 1 | 1.5 | 2 | 2.5 |
| pH | $X_3$ | 4 | 4.5 | 5 | 5.5 | 6 |
| Incubation time (h) | $X_4$ | 50 | 60 | 70 | 80 | 90 |

**Table 2. Experimental design based on CCD**

| Run no. | Coded values | | | | Activity (U/ml) | |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Actual | Predicted |
| 1 | −1 | −1 | −1 | −1 | 20.53±0.62 | 19.1 |
| 2 | +1 | −1 | −1 | −1 | 10.84±0.15 | 9.8 |
| 3 | −1 | +1 | −1 | −1 | 29.15±0.88 | 28.9 |
| 4 | +1 | +1 | −1 | −1 | 20.66±0.17 | 18.76 |
| 5 | −1 | −1 | +1 | −1 | 21.07±0.19 | 19.02 |
| 6 | +1 | −1 | +1 | −1 | 11.83±0.38 | 10.48 |
| 7 | −1 | +1 | +1 | −1 | 32.21±0.22 | 34.26 |
| 8 | +1 | +1 | +1 | −1 | 22.36±0.41 | 24.88 |
| 9 | −1 | −1 | −1 | +1 | 22.23±0.53 | 18.58 |
| 10 | +1 | −1 | −1 | +1 | 29.26±0.24 | 24.96 |
| 11 | −1 | +1 | −1 | +1 | 33.03±1.02 | 32.14 |
| 12 | +1 | +1 | −1 | +1 | 36.78±0.26 | 37.68 |
| 13 | −1 | −1 | +1 | +1 | 25.44±1.13 | 25.1 |
| 14 | +1 | −1 | +1 | +1 | 33.13±0.46 | 32.24 |
| 15 | −1 | +1 | +1 | +1 | 44.20±0.89 | 44.1 |
| 16 | +1 | +1 | +1 | +1 | 51.22±0.94 | 50.4 |
| 17 | −2 | 0 | 0 | 0 | 13.39±0.08 | 15 |
| 18 | +2 | 0 | 0 | 0 | 10.24±0.38 | 12 |
| 19 | 0 | −2 | 0 | 0 | 17.80±0.34 | 23.64 |
| 20 | 0 | +2 | 0 | 0 | 54.06±0.21 | 51.6 |
| 21 | 0 | 0 | −2 | 0 | 27.83±0.55 | 32.42 |
| 22 | 0 | 0 | +2 | 0 | 46.31±1.13 | 45.06 |
| 23 | 0 | 0 | 0 | −2 | 5.22±0.11 | 5.24 |
| 24 | 0 | 0 | 0 | +2 | 26.87±1.05 | 30.24 |
| 25 | 0 | 0 | 0 | 0 | 48.91 | 48.82 |
| 26 | 0 | 0 | 0 | 0 | 49.26 | 48.82 |
| 27 | 0 | 0 | 0 | 0 | 48.33 | 48.82 |
| 28 | 0 | 0 | 0 | 0 | 48.65 | 48.82 |
| 29 | 0 | 0 | 0 | 0 | 47.76 | 48.82 |
| 30 | 0 | 0 | 0 | 0 | 50.03 | 48.82 |

The actual activities are the mean value of three independent experimental data

not shown). The experimentally obtained activities and statistically predicted data for a total of 30 experiments are presented in Table 2. The relationships between the α-amylase activity and the independent variables in coded units are expressed by the regression equation as follows:

$$Y=48.82-0.75X_1+6.99X_2+3.16X_3+6.25X_4 -8.83X_1^2-2.80X_2^2-2.52X_3^2-7.77X_4^2-0.21X_1X_2 +0.19X_1X_3+3.92X_1X_4+1.36X_2X_3+0.94X_2X_4+1.65X_3X_4 \quad (1)$$

The adequacy of the regression equation and the significance of the coefficients are shown in Table 3. The significance of each coefficient is determined by F and P-values. The F-value presents variation of data about the mean value. A high F-value and a very low probability (P-value) indicate that the model is in good prediction of the experimental results. The adequacy of the model equation was checked by using analysis of variance (ANOVA). The F-value (45.83) with a low probability value (P<0.0001) implies a high significance of the model. The accuracy of the model was also checked

**Table 3. Significance of regression coefficients**

| Model term | Coefficient estimate | Standard error | F-value | P-value |
|---|---|---|---|---|
| Intercept | 48.82 | 1.25 | | |
| $X_1$ | −0.75 | 0.63 | 1.45 | 0.2478 |
| $X_2$ | 6.99 | 0.63 | 124.57 | <0.0001 |
| $X_3$ | 3.16 | 0.63 | 25.51 | 0.0001 |
| $X_4$ | 6.25 | 0.63 | 99.46 | <0.0001 |
| $X_1^2$ | −8.83 | 0.59 | 227.08 | <0.0001 |
| $X_2^2$ | −2.80 | 0.59 | 22.86 | 0.0002 |
| $X_3^2$ | −2.52 | 0.59 | 18.44 | 0.0006 |
| $X_4^2$ | −7.77 | 0.59 | 175.95 | <0.0001 |
| $X_1X_2$ | −0.21 | 0.77 | 0.075 | 0.7880 |
| $X_1X_3$ | 0.19 | 0.77 | 0.061 | 0.8090 |
| $X_1X_4$ | 3.92 | 0.77 | 26.14 | 0.0001 |
| $X_2X_3$ | 1.36 | 0.77 | 3.14 | 0.0966 |
| $X_2X_4$ | 0.94 | 0.77 | 1.51 | 0.2388 |
| $X_3X_4$ | 1.65 | 0.77 | 4.63 | 0.0482 |

ANOVA for the response surface quadratic model

| Source | SS | DF | MS | F-value | P-value |
|---|---|---|---|---|---|
| Model | 6043.15 | 14 | 431.65 | 45.83 | <0.0001 |
| Residual | 141.27 | 15 | 9.42 | | |
| Pure error | 3.06 | 5 | 0.61 | | |
| Total | 6184.42 | 29 | | | |

Adequate precision as signal to noise ratio=21.365; SS, sum of squares; DF, degrees of freedom; MS, mean square

by the multiple correlation coefficients ($R^2$). In this case, the multiple correlation coefficient of 0.9772 indicates that this model is statistically significant and only 2.28% of the total variations is not defined by the regression. The predicted multiple correlation coefficient ($R_P^2$= 0.8706) is in reasonable agreement with the adjusted multiple correlation coefficient ($R_A^2$=0.9558). Moreover, the coefficient of variance (C.V.=9.81%) was low, which implies significant precision and reliability of the experimental data. Low P-values of linear and quadratic terms for $NH_4Cl$, pH and incubation time show high linear and quadratic effects of these parameters on the response. Also, SBH had high quadratic effect on α-amylase production, but the P-value (0.2478) for the linear term of this variable was high indicating insignificant linear effect.

The three-dimensional response surface graphs were drawn to illustrate the main and interactive effects of the independent variables on α-amylase production. In each plot, effects of two variables are depicted, while other variables are held at zero level (Figs. 1-6).

Fig. 1 represents the interaction of nutrients on enzyme activities. The estimated P-value (0.7880) for the interaction of these two factors was high, which indicates low interaction. Lower and higher values of substrate (SBH) did not result in higher enzyme activities. Even at optimum substrate concentration, but with low nitrogen source, the enzyme production rate was also low. With increase in the SBH concentration range of 10-20 g/*l*, the enzyme production was increased. But at higher substrate concentrations, the activity of liberated enzyme was dropped, most probably due to catabolite repression. The maximum enzyme activity was obtained with 20

and 2.2 g/*l* of carbon and nitrogen sources, respectively.

The effect of pH and carbon source on enzyme production was investigated (Fig. 2). An optimum pH of 5.4 was predicted for maximum enzyme activity. As the substrate concentration increased to 20 g/*l*, the enzyme activities gradually increased. At optimum pH and substrate concentration, maximum enzyme activity was 51.56 U/ml. The high P-value (0.809) shows insignificant interaction between the two variables. It was observed that concentration of SBH had more prominent influence on the response. The optimum pH of 5.5, defined by experimental results, corresponds to the predicted pH value of 5.4 obtained by response surface method.

Fig. 3 depicts the interaction of the SBH concentration with incubation time. Maximum enzyme activity was recorded in the middle values of both variables. The P-value (0.0001) shows significant interaction of variables. The production of $\alpha$-amylase increased with an increase of incubation time up to an optimum value. Extended period of incubation might lead to the interaction between $\alpha$-amylase and other components in the media and thus a decrease in the amylolytic enzyme activity [23]. At low incubation time, low substrate concentration may lead to high enzyme activities while at high substrate concentration a longer incubation time is required to have sufficient enzyme activities. In fact, increase of incubation time may result in an increase of optimum SBH concentration. When incu-

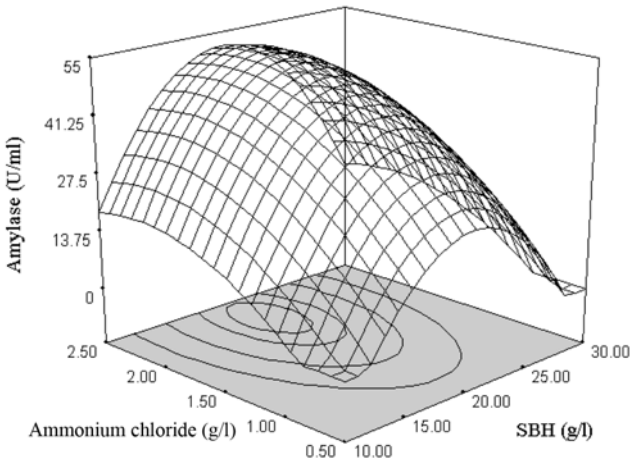


**Fig. 1. Effect of SBH and $NH_4Cl$ on $\alpha$-amylase production by *A. niger*. Other variables held at zero level.**

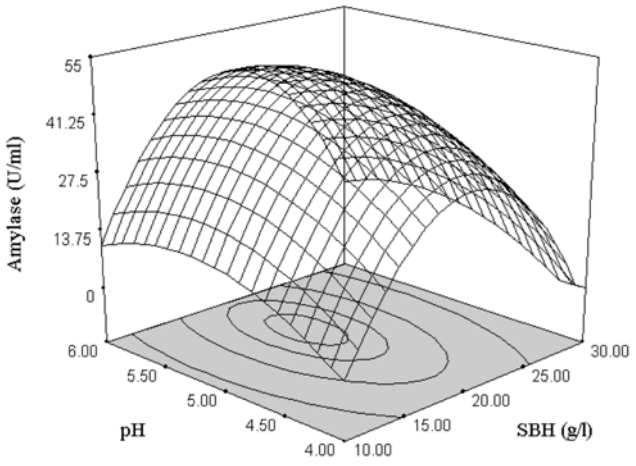


**Fig. 2. Effect of SBH and pH on $\alpha$-amylase production by *A. niger*. Other variables held at zero level.**

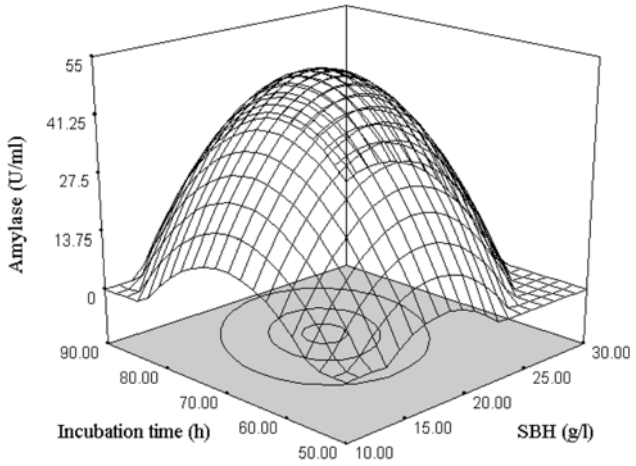


**Fig. 3. Effect of SBH and incubation time on $\alpha$-amylase production by *A. niger*. Other variables held at zero level.**

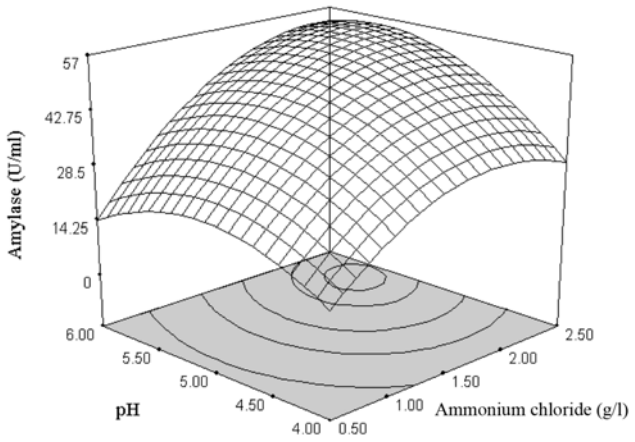


**Fig. 4. Effect of $NH_4Cl$ and pH on $\alpha$-amylase production by *A. niger*. Other variables held at zero level.**

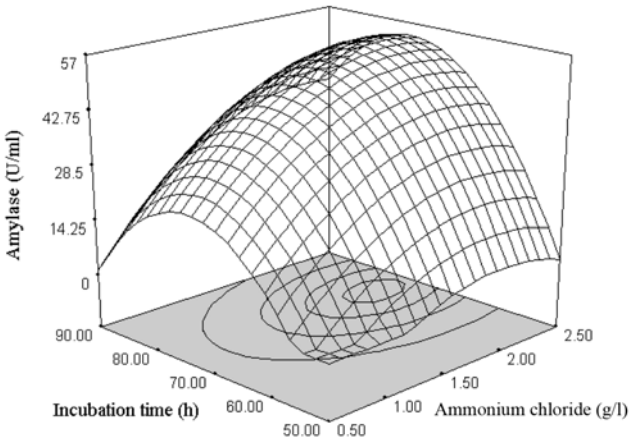


**Fig. 5. Effect of $NH_4Cl$ and incubation time on $\alpha$-amylase production by *A. niger*. Other variables held at zero level.**

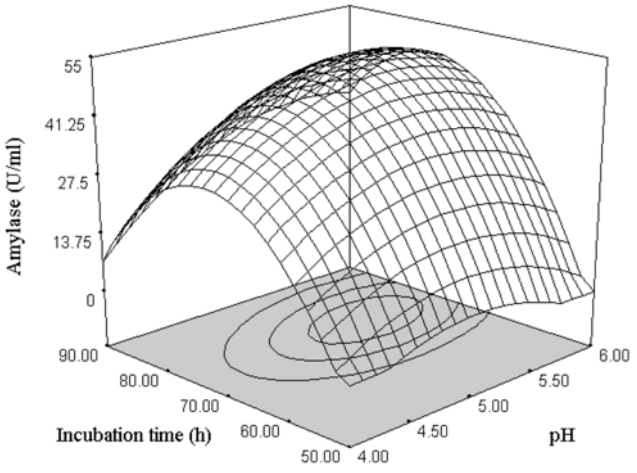


**Fig. 6. Effect of pH and incubation time on α-amylase production by *A. niger*. Other variables held at zero level.**

bation time shifted from 50 to 90 h, the optimum SBH concentration increased from 17.5 to 22 g/*l*.

Fig. 4 shows the interaction of $NH_4Cl$ concentration and pH on enzyme production. The low P-value (0.0966) indicates significant interaction between these variables. When $NH_4Cl$ concentration was varied from 0.5 to 2.5 g/*l*, the optimum pH was shifted from 5 to 5.6. At pH value of 6, the effect of $NH_4Cl$ on α-amylase production was more significant than other pH values.

The response surface curve for the interaction of $NH_4Cl$ concentration and incubation time is represented in Fig. 5. Incubation time had greater influence on enzyme activity. The P-value (0.2388) for the interaction of these two factors shows approximately low interaction. So, incubation time had an insignificant effect on the optimum value for $NH_4Cl$ concentration. The change of incubation time from 50 to 90 h resulted in a slight shift in optimum $NH_4Cl$ concentration (2 to 2.3 g/*l*).

Fig. 6 represents the interaction between pH and incubation time. The low P-value (0.0482) shows significant interaction. For the process with incubation time of 50 h, maximum enzyme production was obtained at pH value of 5 and the optimum pH was slightly shifted to 5.6 as the incubation time increased to 90 h. *A. niger* is able to produce citric acid [24,25]. A long period of incubation leads to higher concentration of citric acid and thus decreases the media pH, which has a negative effect on enzyme synthesis. So, for a process with long incubation time, the initial media pH must be adjusted at higher value to ensure maximum enzyme activity.

## 4. Optimization of α-Amylase Production

The levels of variables for maximum α-amylase production were obtained by mathematical procedure [22]. The stationary point (maximum) of the fitted model was found by taking the first derivatives of Eq. (1) as follows:

$$\begin{aligned} &-0.75-17.66X_1-0.21X_2+0.19X_3+3.92X_4=0\\ &6.99-5.60X_2-0.21X_1+1.36X_3+0.94X_4=0\\ &3.16-5.04X_3+0.19X_1+1.36X_2+1.65X_4=0\\ &6.25-15.54X_4+3.92X_1+0.94X_2+1.65X_3=0 \end{aligned} \quad (2)$$

The system of linear Eqs. (2) was solved and the coded values of the independent variables for maximum α-amylase production were calculated: $X_1$=0.0996, $X_2$=1.6721, $X_3$=1.3001 and $X_4$=0.6665. The optimum conditions for α-amylase production are presented in Table 4. Under optimum conditions, an experiment was conducted and the enzyme activity of 57.24 U/ml was achieved, which was similar to the predicted value (58.76 U/ml) given by the regression model. The activities of liberated enzyme with and without optimum conditions were 57.24 U/ml and 39.43 U/ml, respectively. The activities of enzyme at optimum conditions were enhanced by 45.2%.

**Table 4. Optimum conditions for α-amylase production, predicted by CCD**

| Independent variable | Optimum value |
|---|---|
| SBH (g/*l*) | 20.49 |
| $NH_4Cl$ (g/*l*) | 2.34 |
| pH | 5.65 |
| Incubation time (h) | 76.67 |

## 5. Effect of Temperature and pH on the Performance of α-Amylase

The influence of temperature and pH on stability is shown in Fig. 7. Maximum stability at 70 °C, shows the enzyme thermostability. However, the produced α-amylase has a broad temperature range of activity (Fig. 7(a)). Maximum performance was observed at pH 4 and the enzyme had 90% of its maximum stability for pH in the range of 3-5 (Fig. 7(b)). Activity and stability of the produced α-amylase at low pH make this enzyme highly remarkable for a number of food processing industries such as the starch industry. Starch slurry prepared for liquefaction has a pH of 4.5 and α-amylases pro-

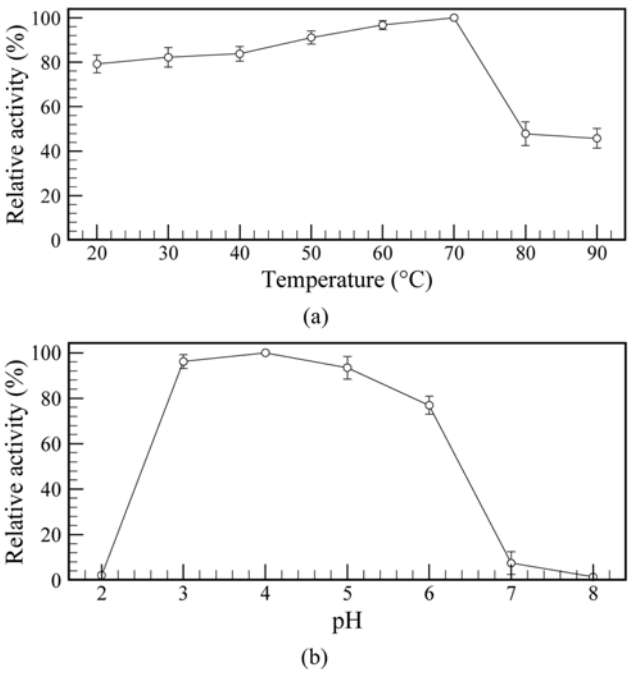


**Fig. 7. Enzyme amylolytic activities after incubation in soluble starch for 15 min. (a) Effect of temperature at pH of 5; (b) Effect of pH at 70 °C; Optimum pH, citrate buffers 0.1 M (pH of 2-4) and phosphate buffers 0.1 M (pH of 5-8). Each value represents the average of two experimental values.**

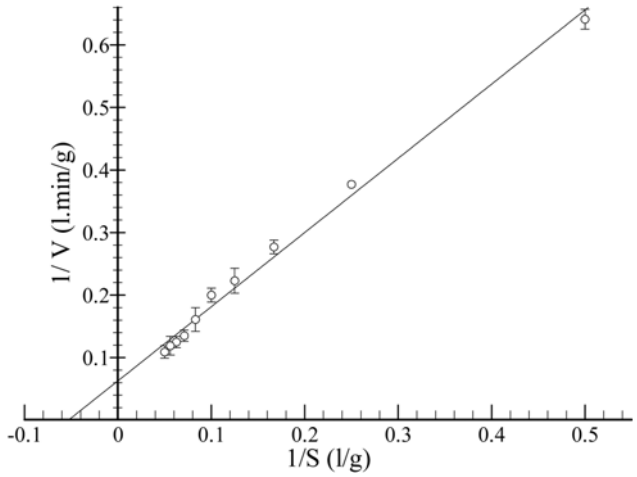


**Fig. 8. Lineweaver-Burk plot for kinetic parameters of *α*-amylase hydrolyzed the soluble starch as substrate. Each value represents the average of two experimental values. $R^2$=0.9925.**

duced from most microorganisms are unstable at low pH. Therefore, the value of pH must be increased to 5.5-6.5, the optimum pH range of commonly used *α*-amylases, with the addition of acid neutralizing chemicals which must be removed by ion-exchange refining. The next process step involves saccharification of the liquefied starch with the optimum operating pH value of about 4-4.5. Therefore, the pH should be decreased to 4-4.5, which requires further chemical addition and refining steps [15]. With the utilization of *α*-amylases which are active and stable at low pH, the costly use of chemicals and refining steps can be omitted. As *α*-amylases have most widely been reported to be unstable at low pH, there exists a great need for engineering of *α*-amylases and also finding new *α*-amylases with low pH performance. High amylolytic activity of the produced enzyme from *A. niger* at low pH values is very important from the economic point of view.

**6. Kinetic Parameters**

The kinetic parameters for enzymatic hydrolysis of *α*-amylase were determined by Lineweaver-Burk plot. The double reciprocal plot was obtained by calculation of the reaction rate with various starch concentrations (Fig. 8). The $K_m$ and $V_{max}$ values were 18.79 g/*l* and 15.85 g/*l*·min, respectively.

## CONCLUSION

The *α*-amylase produced by *A. niger* NCIM 548 was highly active at low pH values. This remarkable property makes the enzyme very important for starch liquefaction in food processing industries. Catabolite repression of *α*-amylase production did not occur in the medium containing the SBH solution (10-20 g/*l*). The optimum values of SBH, $NH_4Cl$, pH and incubation period for *α*-amylase production were 20.49, 2.34 g/*l*, 5.65 and 76.67 h, respectively. Also, the maximum performance of the produced enzyme was obtained at 70 °C and pH value of 4. The rate constant, $K_m$ and maximum reaction rate, $V_{max}$ were 18.79 g/*l* and 15.85 g/*l*·min, respectively.

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