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Production of α-Amylase from Starch Using *Aspergillus niger* NCIM 548

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Abstract: Synthesis of extracellular α-amylase by *Aspergillus niger* strain NCIM 548 was investigated in a submerged culture using starch as carbon source. The highest amylolytic activities obtained in the media with initial starch concentrations of 10, 20, 30, 40 g/l were 20.80, 29.78, 35.6, 37.45 U/ml, respectively. The data were fitted in Michaelis-Menten equation. The rate constant, K_s and maximum activity, A_m were 19.64 g/l and 57.80 U/ml, respectively. The produced enzyme displayed maximum stability at 74°C and pH value of 3. Trace metal ions such as Mn^{2+}, Ca^{2+}, Na^{+}, Co^{2+} and Ni^{2+} enhanced enzymatic hydrolysis of starch.. In contrary, the presence of Cu^{2+}, Zn^{2+} and Mg^{2+} may hinder the amylolytic activities.

Key words: α-Amylase · *Aspergillus niger* · Enzyme activities · Enzyme kinetics · Enzyme stability · Enzyme hydrolysis

INTRODUCTION

α-Amylase as amylolytic extracellular enzyme is one of the most significant biological based products, has wide range of industrial applications in food, beverage, textile, paper and detergent industries [1-5]. Amylase has the ability to hydrolyze polysaccharides such as starch into simple monomeric sugar constituents [6]. Although these hydrolytic enzymes can be produced from plants and animals, but microorganisms are the major enzyme producers for industrial scale production of amylase [1]. Amylases are originated and produced by number of species of microorganisms such as *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Thermomyces lanuginosus* and *Aspergillus oryzae* [7-10].

The enzymatic reaction is affected by several process variables. The optimal reaction conditions were investigated using response surface methodology (RSM). RSM is a statistical technique applied for modeling and optimizing the influential process parameters [11]. Optimization by changing one independent variable and keeping other parameters constant is time-consuming and does not account for the interactions between the variables. These limitations can be eliminated by the use of RSM [12].

The purpose of present study was to investigate the production of amylase by *A. niger* using starch as carbon source. Also, the activities and characteristics of the produced enzyme were determined.

MATERIALS AND METHODS

Microorganism and Amylase Production: *Aspergillus niger* NCIM 548 was obtained from National Collection of Industrial Microorganisms (Chandigarh, India). The stock culture was maintained on nutrient agar slants at 4°C. Seed culture was prepared by transferring a full loop of the cells from the slant culture into the medium contained: glucose, yeast extract, NH_4Cl, KH_2PO_4, NaCl, $MgCl_2.6H_2O$ and $CaCl_2.2H_2O$ with concentration of 20, 1, 2.5, 0.3, 0.25, 0.2 and 0.1 g/l, respectively. The repaired medium had initial pH values in the range of 5-6. The culture was agitated (200 rpm) and incubated at 30°C for 48 h. The soluble starch was used as carbon source for the propagation of the microorganism and production of enzyme. The enzyme production media with initial pH of 6 were sterilized at 121°C for 15 min. The sterilized medium was inoculated with a 2.5% inoculum level and incubated at 30°C and agitated at 200 rpm. Mycelial mass was

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removed by centrifugation (4°C, 7000 rpm for 10 min). The amylolytic activities of the produced enzyme in the supernatant were measured.

Enzyme Activity: The reaction mixture contained 3 ml of soluble starch (20 g/l) solution made in 0.1 M phosphate buffer (pH = 5) and 300 μl of appropriately diluted cell-free culture-supernatant. The reaction was conducted at 50°C. The concentrations of the liberated reducing sugar and the remaining starch were measured by 3, 5-dinitrosalicylic acid (DNS) and iodine solution, respectively [13, 14]. The activity of one unit enzyme was defined as the amount of enzyme releasing 1 μmol of glucose equivalents per minute.

Effect of Substrate Concentration: The variations of starch and glucose concentrations and amylase production in four media with the initial starch concentrations of 10, 20, 30 and 40 g/l were investigated. Also, the amylolytic activities of the enzyme obtained from the above media were evaluated with the Michaelis-Menten equation as stated below:

$$A=\frac{A_m.S}{K_S+S} \qquad (1)$$

where A, A_m, K_s and S imply activity, maximum activity, Michaelis-Menten rate constant and starch concentration, respectively. A_m and K_s were determined by double reciprocal Lineweaver-Burk plot.

Effect of pH and Temperature on Enzyme Stability: In enzymatic reaction, several parameters affect the enzyme stability. The optimum pH and temperature for amylase performance were determined by central composite design (CCD) under response surface methodology. The RSM is a mathematical and statistical method which is used to model the relationships between the independent variables and the response. The independent variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad (2)$$

where x_i is the dimensionless coded value of the ith independent variable; X_i is the actual value of the ith independent variable; X_o is the actual value of the ith independent variable at the center point and ΔX_i is the step change value. The second-order polynomial model is explained in the literatures [11].

The DESIGN EXPERT 7.0 (Stat-Ease, Inc, Minneapolis, MN, USA) software was used to study the interactive effect of pH and temperature on amylolytic activity. Each of the variables was coded at five levels: α, -1, 0, +1 and +α. The model was statistically analyzed using the analysis of variance (ANOVA).

A total of 13 experiments were conducted with five replicates at the central points. The samples of soluble starch with pH values of 3 to 8 were prepared by phosphate buffer solution. The reaction mixtures contained 3 ml of each sample and 300 μl of enzyme solution which was originated from the medium with the initial starch concentration of 40 g/l. The enzyme reaction was conducted for 10 min at the corresponding temperature according to the experimental plan designed by CCD. The amount of reducing sugars liberated in each experiment was determined.

Effect of Metal Ions on Enzyme Activity: Impact of trace metal ions (8 mM) on enzyme activities and amylase performance was investigated. The use of trace metal ions as cofactor and center metal ions for holding protein molecules were discussed [15]. These trace metal ions are Na^+, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} and Ca^{2+}. The amylase was incubated for 15 min in the presence of various metal ions at optimum values of pH and temperature obtained by CCD. The amylolytic activity in each case was compared with the activity in control sample without presence of metal ions.

RESULTS AND DISCUSSION

The concentration profile for starch consumption and glucose liberation are shown in Fig. 1. The amylase production occurred while starch was consumed and glucose was liberated in the media. As fermentation started, the starch was hydrolyzed to glucose which was consumed by the microorganism to produce amylase. For starch concentrations of 10 and 20 g/l, all of the soluble starch in the media was hydrolyzed in 24 and 30 h, respectively. In the media with the initial starch concentrations of 30 and 40 g/l, the soluble starch was completely degraded after 36 h of fermentation. In the beginning, glucose concentration progressively increased to 6.1, 12.1, 16.2 and 22.89 g/l, then it decreased to nearly zero g/l at 54, 60, 72 and 78 h in the media with the initial starch concentrations of 10, 20, 30 and 40 g/l, respectively.

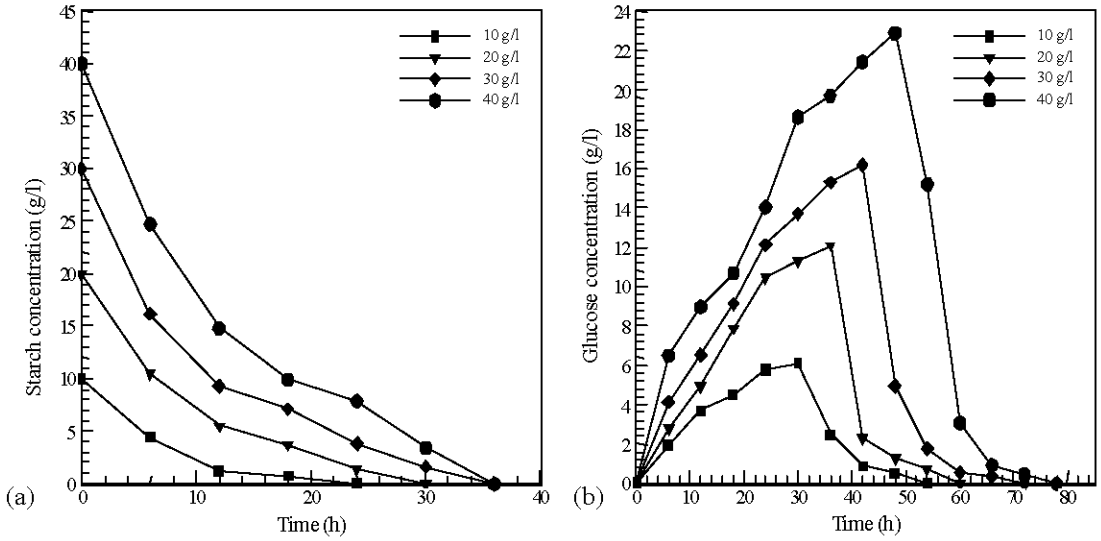

Fig. 1a,b: Concentration profiles in media with initial starch concentrations of 10 to 40 g/l (a) Starch, (b) Glucose

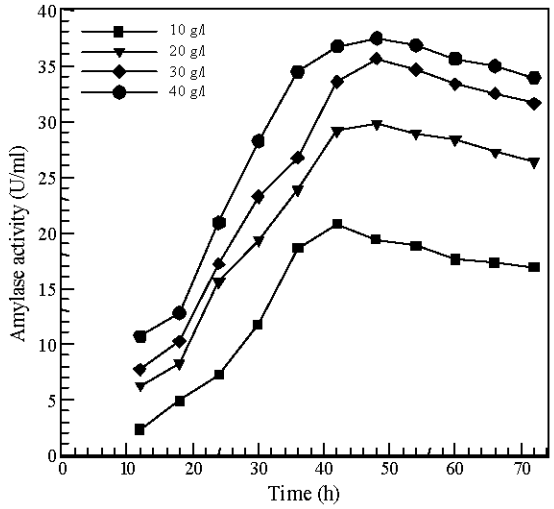

Fig. 2: Liberated amylase activity in the media with initial starch concentrations of 10 to 40 g/l

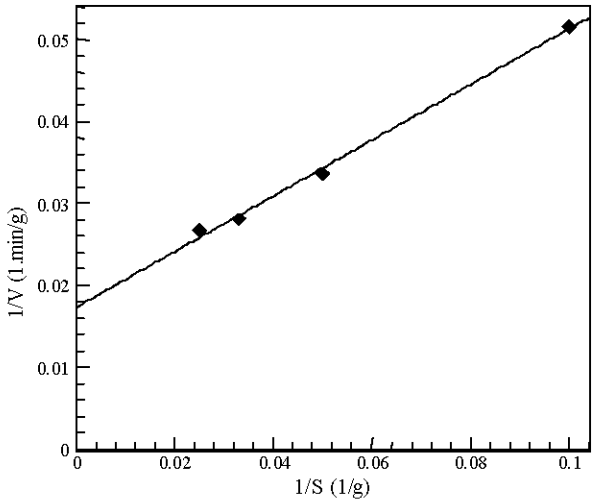

Fig. 3: Lineweaver-Burk plot, the relationship between the initial starch concentration and the amylolytic activity of enzyme

The enzyme activity was gradually developed while the population of biomass and mycelial mass in the media was increased. The maximum amylase activities of 20.8, 29.78, 35.6 and 37.45 U/ml were obtained from the media with the initial starch concentrations of 10, 20, 30 and 40 g/l, respectively (Fig. 2).

The A_m and K_s values were determined using double reciprocal Lineweaver-Burk plot. The values of A_m and K_s were 57.80 U/ml and 19.64 g/l, respectively. As the information drawn from this model, for the highest starch concentration, there was no substrate inhibition for amylase production. The Lineweaver-Burk plot is shown in Fig. 3.

The interactive effect of pH and temperature on the stability of amylase was studied by CCD. The range and levels of the variables are given in Table 1. The amounts of reducing sugars liberated in each experiment along with the predicted responses are represented in Table 2. The relationships between reducing sugar production and the independent variables in coded units are expressed by the regression equation as follows:

$$Y = 12.04 - 0.75X_1 - 2.22X_2 - 1.01X_1X_2 - 0.46X_1^2 - 0.77X_2^2 \quad (4)$$

Table 1: Experimental range and levels of the independent variables

		Range and levels				
Variables	Symbol	-2.5	-1	0	1	2.5
Temperature (°C)	X_1	30	45	55	65	80
pH	X_2	3.0	4.5	5.5	6.5	8.0

Table 2: Experimental design based on central composite design

	Coded values		Reducing sugar (g/l)	
Run no.	X_1	X_2	Actual	Predicted
1	-1	-1	12.14	12.77
2	+1	-1	13.08	13.29
3	-1	+1	10.37	10.35
4	+1	+1	7.26	6.83
5	-2.5	0	11.19	11.04
6	+2.5	0	7.14	7.29
7	0	-2.5	13.05	12.78
8	0	+2.5	1.45	1.68
9	0	0	12.43	12.04
10	0	0	12.02	12.04
11	0	0	11.94	12.04
12	0	0	12.15	12.04
13	0	0	11.89	12.04

Table 3: Significance of regression coefficients

Model term	Coefficient estimate	Standard error	F-value	P-value
Intercept	12.04	0.150		
X_1	-0.75	0.093	64.66	<0.0001
X_2	-2.22	0.093	572.63	<0.0001
X_1X_2	-1.01	0.190	28.94	0.001
X_1^2	-0.46	0.050	85.88	<0.0001
X_2^2	-0.77	0.050	236.70	<0.0001

ANOVA for the response surface quadratic model

Source	SS	DF	MS	F-value	P-value
Model	131.79	5	26.360	186.02	<0.0001
Residual	0.99	7	014.000		
Pure error	0.19	4	0.047		
Total	132.78	12			

Adequate precision as signal to noise ratio = 45.399; SS: sum of squares; DF: degrees of freedom; MS: mean square.

Table 4: Effect of metal ions on amylase performance

Metal ion	Relative activity (%)
None (control)	100.0
$MnCl_2$	117.4
$CaCl_2$	112.1
NaCl	108.6
$CoCl_2$	105.3
$NiCl_2$	103.4
$MgCl_2$	89.7
$ZnCl_2$	23.8
$CuCl_2$	11.5

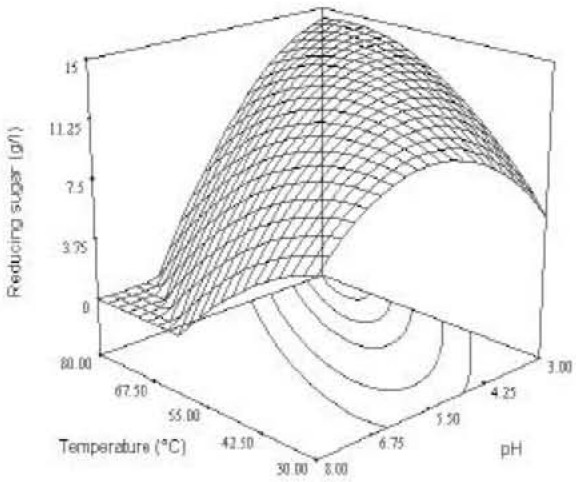

Fig. 4: Response surface plot, effect of pH and temperature on enzyme stability

The adequacy of the regression equation and the significance of coefficients are shown in Table 3. The F-value (186.02), probability value (P<0.0001) and the coefficient of variance (C.V. = 3.6%) confirm the high significance of the model. The multiple correlation coefficients (R^2) determine the accuracy of the model. In this case, the multiple correlation coefficient of 0.9925 implies that this model is statistically accurate. The predicted multiple correlation coefficient (R^2_p=0.9094) is in reasonable agreement with the adjusted multiple correlation coefficient (R^2_A=0.9872).

As shown in Fig. 4, an increase in the reaction mixture temperature, reduces the optimum pH for the stability of amylase. When temperature increased from 30 to 80°C, the optimum pH decreased from 5.7 to 3. The high interaction between these two parameters is also confirmed by P-value (0.001). Maximum stability was observed at 74°C and pH value of 3.

Metal ions catalyze enzyme reactions as they promote the reactions also serve as proton donor to neutralize negative charges. In addition, metal ion's charge creates more acidic molecules to bond with water molecule [15]. Table 4 shows the effect of various metal ions on amylolytic enzyme activities. The metal ions of Mn^{2+} and Ca^{2+} resulted in an increase of enzyme activities. Amylase activity was also slightly enhanced by use of metal ions such as Na^{+}, Co^{2+} and Ni^{2+}. In contrary, presence of some metal ions may have negative impact as some of the ions such as Cu^{2+}, Zn^{2+} and Mg^{2+} caused the enzymatic activity slightly decrease.

CONCLUSION

Starch was hydrolyzed into monomeric sugar constituents which supported the growth of *A. niger* and amylase production. Amylolytic activities of 20.80, 29.78, 35.6 and 37.45 U/ml were obtained in the media with the initial starch concentrations of 10, 20, 30, 40 g/l, respectively. The relationship between the amylase production and the initial starch concentration was fitted in Michaelis-Menten model with rate constant and maximum activity of 19.64 g/l and 57.80 U/ml, respectively. Maximum stability of the produced enzyme was observed at 74°C and pH value of 3. Some trace metal ions were used as promoters for the enzymatic activities. The trace metal ions such as Mn^{2+}, Ca^{2+}, Na^{+}, Co^{2+} and Ni^{2+} showed positive impact on the amylase performance. In contrary, the presence of Cu^{2+}, Zn^{2+} and Mg^{2+} declined or inhibited the amylolytic activities.

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