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Characterization of α-Amylase by *Bacillus subtilis*

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ABSTRACT

The present study is concerned with the production of α-amylase by mutant strain of *Bacillus subtilis* GCBUCM-25. The fermentation was carried out in by continuous shaking containing 25 mL of medium in 250 mL Erlenmeyer flask. The maximum production of enzyme was optimized at the pH 7.5, while the incubation temperature investigated was 40ºC, the volume of basal medium at 25 mL and inoculum size at 4% were also optimized. The production of the enzyme was obtained maximum at 48 hours after incubation (535 IU/mL/min).

Key Words: α-amylase; *Bacillus subtilis*

INTRODUCTION

Alpha (α)-amylase, an extracellular enzyme degrades α, 1-4 glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrin (Calik & Ozdamar, 2001). This enzyme is extensively used in many industries including starch liquification, brewing, food, paper, textile and pharmaceuticals (Ohdan *et al*., 2000). These uses have placed greater stress on increasing α-amylase production and search for more efficient processes.

Microorganisms like fungi and bacteria have been extensively screened for α-amylase production (Ivanova *et al*., 2001). But the *Bacillus* species such as *B. subtilis, B. licheniformis* and *B. sterothermophilis* can be used for the better production of α-amylase in shake flask (Mamo & Gessesse, 1999). However, the mutant strain of *B. subtilis* was found to be the best for biosynthesis of α-amylase. The production of amylase is dependent on the strains, composition of media, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermostability. The effect of temperature on the relative activity of α-amylase from *B. subtilis* was detected and temperature was optimized between 60ºC-70ºC, for maximum activity (Kim *et al.,* 1995). The production of α-amylase by *B. subtilis* during both the exponential and stationary phases of growth. The rate was slightly slower during the stationary phase than the exponential phase. The production and stability of the enzyme is very sensitive to pH and incubation temperature. Organism grew at pH between 4.5-10.5. Ivanova *et al.* (1993) observed that α-amylase obtained from *B. subtilis* was stable at pH 6.5 –8.0 and incubation temperature 40ºC. The 25 mL volume of basal medium along with the 1mL of inoculum size was also optimized. In our present study the cultural conditions were optimized for the best production of α-amylase.

MATERIALS AND METHODS

The mutant strain of *B. subtilis* GCBUCM-25 was obtained from Biotechnology Research Laboratory, Department of Botany Government College Lahore. The strains were stored in paraffin oil containing nutrient starch agar medium.

Inoculum preparation. Vegetative inoculum was used in present studies which was prepared according to the method of Haq *et al.* (1998).

Fermentation technique. The fermentation was carried out in 250 mL Erlenmeyer flask. Fifty mL of the fermentation medium containing (g/L) *Pearl millet* starch 20.0, lactose 10.0, nutrient broth 15.0, $(NH_4)_2SO_4$ 5.0, $CaCl_2$ 2.0, NaCl 2.0 in 1000 mL of Phosphate buffer (pH 7.5) was transferred to 250 mL cotton plugged Erlenmeyer flask. The flasks were sterilized in the autoclave at 121°C and 15 lbs pressure and then cooled at room temperature. One ml of vegetative bacterial inoculum (24 h old) was transferred to each flask. The flasks were then placed in the rotary incubator shaker (200 rpm) at 40ºC for 48 h. Then, the fermented broth was centrifuged at 5000 rpm for 15 min. The solid free supernatant was used for the estimation of α-amylase and biomass. However, residuals left in the centrifuge tube were used for substrate consumption. All the experiments were carried out in triplicates.

Enzyme assay. α-amylase estimation was carried out according to the method of Fisher and Stein (1961). The enzyme solution at pH 7.5 was incubated at 60ºC using 1% soluble starch solution. The reducing sugars were measured by adding 3,5-dinitro salicylic acid reagent, boiling for 5 min, cooling and measuring the O.D (Optical Density) at 546 nm in the spectrophotometer against maltose as standard. The amylase activity was determined in IU/mL/min by applying the following formula (Haq *et al.,* 2002).

$$\text{IU/ml/min} = \frac{\text{Activity of enzyme} \times 1000}{\text{Molecular wt. of maltose} \times \text{time of incubation}}$$

Biomass. The biomass was determined tubermeterically at 650 nm. The biomass was converted into g/L according to the method of Hariuchi *et al*. (1993). The substrate free culture samples were centrifuged (15,000 rpm) at 4°C, cell free supernatant was discarded and the centrifuged tubes were oven dried at 105°C. Biomass was estimated in comparison with the standards.

Treatment effects were compared by the method of Snedecor and Cochrane (1980). Significance has been presented as Duncan's multiple range tests in the form of probability (*P*) values.

RESULTS AND DISCUSSION

Effect of incubation temperature. The data of Fig.1 shows the effect of different incubation temperatures on the production of α-amylase by *B. subtilis* GCUCM-25. The fermentation was carried out at 30, 35, 40, 45, 50, 55 or 60°C in rotary incubator shaker. The maximum production of α-amylase was obtained at 40°C (545 IU/mL/min). As the incubation temperature was increased, the production of the enzyme was decreased. The production of the enzyme was greatly inhibited at 30°C (291 IU/ml/min). Thus the incubation temperature 40°C was selected for maximum production of enzyme.

Rate of α-amylase fermentation. Fig. 2 shows the time course of α-amylase fermentation by *Bacillus subtilis* GCUCM-25 in shake flask. The culture was incubated at 40°C for different intervals of time (0-72 h). The production of enzyme was reached maximum (535 IU/mL/min) at 48 h after inoculation. Further increase in incubation period however, did not show any significant increase in enzyme production rather it was decreased. Thus optimum time of enzyme synthesis was found to be 48 h after inoculation.

Effect of different inoculum sizes. Fig. 3 shows the effect of different size of inoculum on the production of α-amylase by *B. subtilis* GCUCM-25 in shake flask. The vegetative inoculum at the level of 1-8% was studied for the production of enzyme. The maximum production of enzyme was obtained at 4% level of inoculum (542 IU/mL/min). As the level of inoculum was increased, the production of the enzyme was decreased. At low level of inoculum the production of enzyme was insignificant. Thus the inoculum level of 4.0% (v/v) was found optimum for fermentation studies.

Table I. Effect of different volumes of medium on the production of α-amylase by *Bacillus subtilis* GCUCM-25

No	Volume of medium (mL/250mL flask)	IU/ml/min
1	15	321
2	20	432
3	25	538
4	30	471
5	35	399
6	40	314
7	45	288
8	50	275
9	55	264

Initial temperature = 40°C; pH = 7.5; Incubation time period = 48 h

Effect of different volumes of medium. Table I shows the effect of different volume of the basal medium on the production of α-amylase by *B. subtilis* GCUCM-25. The level of fermentation medium such as 15, 20, 25, 30, 35, 40, 45, 50 or 55 mL was investigated in 25 mL conical flask. The maximum production of α-amylase (538 IU/mL/min) was observed in the flask containing 25 mL of the fermentation medium. As the volume of fermented medium was increased, the production of the enzyme was decreased gradually. At low level of the volume of fermentation medium the production of the enzyme was insignificant. However, 25 mL volume of fermentation medium was optimized for the production of α-amylase.

Effect of pH on the activity of enzyme. Fig. 4 shows the effect of initial pH of reaction mixture (enzyme substrate complex) for the activity of α-amylase. The enzyme activity was extremely low at pH 4.0 (68 IU/mL/min). The activity of enzyme was gradually increased and found maximum at pH 7.5 (547 IU/mL/min). Further increase in the initial pH resulted decrease in the activity of α-amylase. However, the pH of reaction mixture for the hydrolysis of starch was found to be optimum at 7.5.

The production and stability of α-amylase depends upon temperature. In present study, the fermentation was carried out at different incubation temperatures. The maximum production of enzyme was observed at 40ºC. Biosynthesis of α-amylase was significantly decreased with the increase in the incubation temperature beyond 40ºC. It might be due to that at high temperature, the growth of the bacteria was greatly inhibited and hence, enzyme formation was also prohibited (Haq *et al.,* 1997; Chengyi *et al*., 1999) Optimization of the volume of fermentation medium is very necessary for air supply, nutrient supply, growth of microorganisms and production, of enzyme (Mimura & Shinichi, 1999; Ivanova *et al*., 2001). In present study, the rate of enzyme was increased with the increase in the fermentation period and reached maximum 48 h after inoculation. It might be due to the organism entered in the incubation period resulted in the decreased production of α-amylase. It may be due to the accumulation of other by products in the fermentation medium. The size of inoculum has marked effect on the growth of the bacteria and biosynthesis of α-amylase as reported by Allan *et al*. (1996). In present study, the effect of different inoculum size was tested for enzyme activity. Among all the inoculum sizes tested, 4% inoculum was found sufficient for the production of enzyme. As the inoculum size was increased, the production of enzyme was decreased. It might be due to increase in the inoculum size; the growth of the organism was significantly increased. The nutrients present in the medium were insufficient to overcome the growth of organism. Hence, the production of the enzyme was also

Fig. 1. Effect of different temperatures on the production of alpha amylase by *Bacillus subtilis* GCUCM-25 (pH =7.5, Incubation time period =48 h)

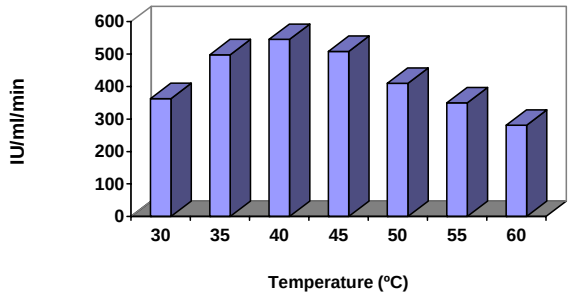


Fig. 2. Rate of α-amylase fermentation by *Bacillus subtilis* GCBUCM-25 (pH =7.5, Incubation time period = 48 h, Incubation temperature =40ºC)

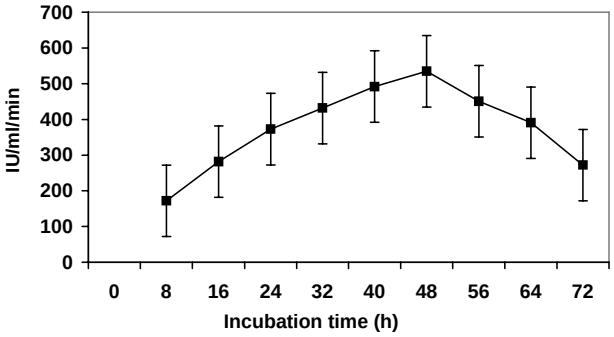


Fig. 3. Effect of different inoculum sizes on the production of alpha amylase by *Bacillus subtilis* GCUCM-25 (pH = 7.5, Incubation temperature = 40ºC, Incubation time period = 48 h)

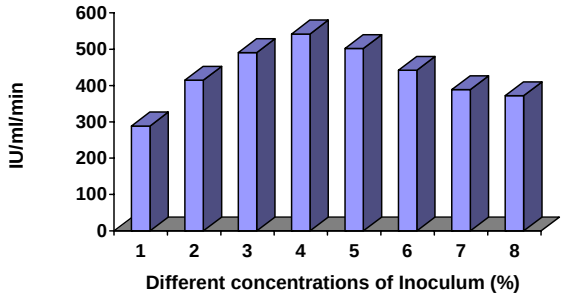
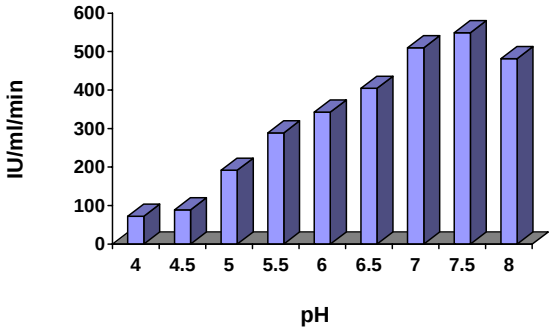


Fig. 4. Effect of different pH on the activity of α-amylase (Incubation temperature =40ºC, Incubation time period = 48 h)



decreased. At low inoculum level, growth of the organism might be reduced and time of organism to enter in the stationary phase became increased.

In present study, different volumes of the fermentation medium (15-55 mL) were evaluated in 250 mL conical flask. The maximum production of enzyme was obtained at 25 mL of the fermentation medium. As the volume of the medium was increased, the production of enzyme was decreased. It might be due to the reduction in the agitation rate of medium, decrease in air supply and subsequently enzyme production. At low concentration of fermentation medium, the production of enzyme was also decreased. It might be due to the nutrients present in the fermentation medium were not sufficient for the growth of bacteria.

The hydrolytic action of α-amylase is greatly effected by pH. In present study, the different pH (4-8) of starch solution was tested for the activity of α-amylase. The maximum activity of the enzyme was obtained at slightly alkaline pH 7.5. At acidic pH the results were extremely low. It might be due to the enzyme was inactive in the acidic medium (Anyangwa *et al*., 1993; Castro *et al.,* 1993).

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