Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 10	No. 2	pp. 277-280	2011

Original Research Paper

Isolation and Characterization of a-Amylase Producing *Bacillus subtilis*

S. M. Lokhande
Deptt. of Microbiology, Shri D. M. Burungale College of Science and Arts, Shegaon-444 203, Distt. Buldana, Maharashtra

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 28-9-2010
Accepted: 27-10-2010

Key Words:
α-amylase
Bacillus subtilis
Starch hydrolysis

ABSTRACT

The present study is concerned with production of α-amylase by strains of *Bacillus subtilis* S_5 (3). The fermentation was carried out by continuous shaking containing 25mL of medium in 250 mL Erlenmeyer flask. The maximum production of the enzyme was optimized at pH 7.5, while incubation temperature investigated was 42°C. The production of enzyme was obtained maximum at 48 hrs after incubation.

INTRODUCTION

Bacillus subtilis is one of the most widely used bacteria for production of enzymes (Alexander 1977). Amylases are the most important enzymes used in biotechnology, particularly in process involving starch hydrolysis. α-amylase, an extracellular enzyme degrade α, 1-4 glucosidic linkages of starch and related substrates in an endofasion producing oligosaccharides including maltose, glucose and α limit dextrin (Calik & Ozdanar 2001). Amylases are extensively used in brewing, baking and textile industries. Their broad application make amylases a major product in the enzymes market and has led to the characterization of a large variety of these enzymes (Vieille et al. 2000).

Microorganisms like fungi and bacteria have been extensively screened for α-amylase production (Ivanova et al. 2001). But the *Bacillus* species such as *Bacillus subtilis, Bacillus licheniformis* and *Bacillus sterothermophilus* can be used for better production of α-amylase in shake flask (Mamo & Gessess 1999). The production of amylase is dependent on the strains, compositions of media, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermo stability. The effect of temperature on relative activity of α-amylase from *Bacillus subtilis* was optimized between 60°-70°C for maximum activity (Kim et al. 1995). The production and stability of the enzyme is very sensitive to pH 4.5-10.5 and incubation temperature (Nadia Riaz et al. 2003). Ivanova et al. (1993) observed that α-amylase obtained from *Bacillus subtilis* was stable at pH 6.5-8.0 and incubation temperature of 40°C. The aim of present study is isolation and characterization of α-amylase producing *Bacillus subtilis.*

MATERIALS AND METHODS

Collection of sample: For the isolation of *Bacillus subtilis*, total 24 soil samples were collected from the soil of Shegaon, Maharashtra.

Isolation of *Bacillus subtilis:* Isolation of *Bacillus subtilis* was carried out by using starch agar. Replica plating technique was used for transfer of colonies from starch agar to nutrient agar. Plates of starch agar were treated with iodine. Colonies, which showed the zone of starch solubilization, were selected for further study. Isolates were identified according to various biochemical characterizations. Out of 14 strains, 6 different strains of *Bacillus subtilis* showed highest zone of starch solubilization of which only one strain S_5(3) was selected for further study, which showed zone of starch solubilization (35mm).

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) 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 40°C for 48 h. The fermented broth was centrifuged at 5000 rpm for 15 min.

Enzyme assay: α-amylase estimation was carried out

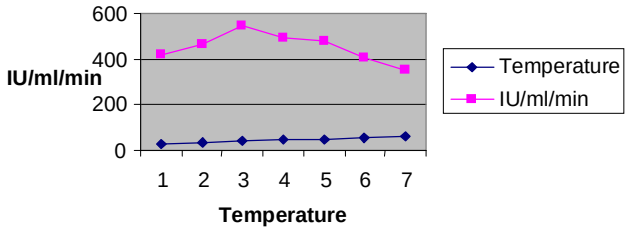

Fig. 1: Effect of different temperatures on production of alpha-amylase by *Bacillus subtilis* S5(3) (pH = 7.5, incubation time period = 48 hr).

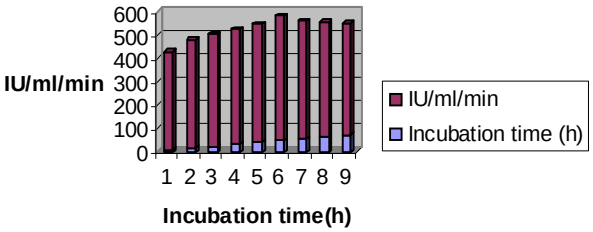

Fig. 2: Rate of alpha-amylase fermentation by *Bacillus subtilis* S5(3) (pH = 7.5, incubation time period = 48 hr, incubation temperature = 40°C).

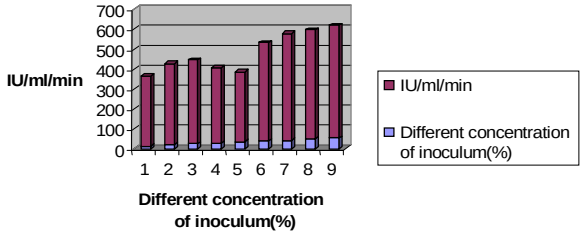

Fig. 3: Effect of different inoculum sizes on production of alpha-amylase by *Bacillus subtilis* S5(3) (pH = 7.5, incubation temperature = 42°C, incubation time period = 48 hr).

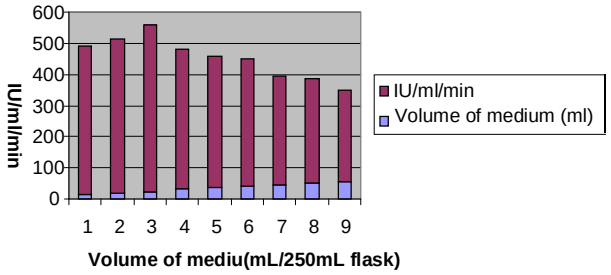

Fig. 4: Effect of different volumes of medium on production of alpha-amylase by *Bacillus subtilis* S5(3).

according to the method of Fisher & Stein (1961). The enzyme solution at pH 7.5 was incubated at 60°C 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 optical density at 546 nm against maltose as standard. The amylase activity was determined in IU/mL/min by applying the following formula (Haq et al. 2002).

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

RESULTS AND DISCUSSION

Effect of incubation temperature: The data given in Fig. 1 show the effect of different incubation temperatures on the production of α-amylase by *B. subtilis* S_5 (3). 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 42°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 42°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* S_5(3) in shake flask. The culture was incubated at 42°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 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* S_5 (3) 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 5% level of inoculum. 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 5.0% (v/v) was found optimum for fermentation.

Effect of different volumes of medium: Table 1 and Fig. 4 shows the effect of different volumes of the basal medium on the production of α-amylase by *B. subtilis* S_5 (3). The level of fermentation medium such as 15, 20, 25, 30, 35, 40, 45, 50 or 55 mL was investigated in 30 mL conical flask. The maximum production of α-amylase (471 IU/mL/min)

was observed in the flask containing 30 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, 30 mL volume of fermentation medium was optimized for the production of α-amylase.

Effect of pH on the activity of enzyme: Fig. 5 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 in decrease of 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 the present study, the fermentation was carried out at different incubation temperatures. The maximum production of the enzyme was observed at 42°C. Biosynthesis of α-amylase was significantly decreased with the increase in the incubation temperature beyond 42°C. 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). In the 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 byproducts in the fermentation medium. The size of inoculum has marked effect on the growth of bacteria and biosynthesis of α-amylase as reported by Allan et al. (1996). In the present study, the effect of different inoculum size was tested for enzyme activity. Among all the inoculum sizes tested, 5% 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

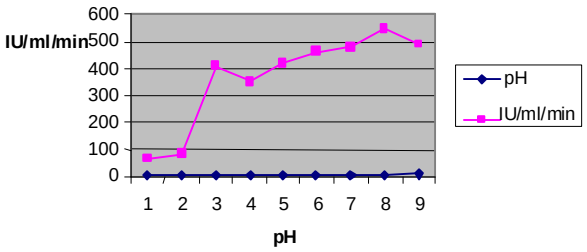

Fig. 5: Effect of different pH values on activity of alpha-amylase (incubation temperature = 42°C, incubation time period = 48 hr).

Table 1: Effect of different volumes of medium on the production of α-amylase by *Bacillus subtilis* S5 (3).

No.	Volume of medium (mL)	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

medium were insufficient to overcome the growth of organism. Hence, the production of the enzyme was also decreased. At low inoculum level, growth of the organism might be reduced and time of organism to enter the stationary phase became increased.

In the present study, different volumes of the fermentation medium (15-55 mL) were evaluated in 250 mL conical flasks. The maximum production of enzyme was obtained at 30 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 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. The different pH values (4-8) of starch solution were tested for the activity of α-amylase. The maximum activity of the enzyme was obtained at slightly alkaline pH of 7.5. At acidic pH, the results were extremely low. It might be due to the enzyme became inactive in the acidic medium (Anyangwa et al. 1993, Castro et al. 1993).

ACKNOWLEDGEMENT

The author is grateful to Dr. Yamini Patil, Head of Microbiology Department, Shri D. M. Burungale Science and Arts College, Shegaon for her continuous inspiration, encouragement, valuable suggestions and moral support during the study.

REFERENCES

Alexander, M. 1997. Introduction of Soil Microbiology. John Wiley and Sons Inc., New York.

Allan, S., Henrik, B.F. and Torbenvedel, B. 1996. A method of designing alpha mutants with predetermined properties alpha amylase variants and detergents containing the variants. Process Biochem., 31: 110-210.

Anyangwa, E.M., Mapsev, C., Musanage, P. and Elemva, M. 1993. The effect and removal of starch in the sugar refining industry. J. Int. Sugar., 95: 210-3.

Calik, P. and Ozdamar, T.H. 2001. Carbon sources affect metabolic capacities of *Bacillus* species for the production of industrial enzymes. Biochem. Engg. J., 8: 61-81.

Castro, G.R., Ferrero, M.A., Mendez, B.S. and Sineriz, F. 993. Screening and selection of bacteria with high amylolytic activity. Acta Biotechnol., 13: 197-201.

Chengyi, W.H., Ming, M. and Jiang, R. 1999. Studies on the properties of alpha-amylase produced by *Bacillus pumilus* 289 (PBX96). Acta Microbial. Sin., 32: 400-404.

Fisher, E. and Stein, I. 1961. α-amylase from human saliva. Biochem. Prep., 8: 27.

Haq, I., Ashraf, H., Ali, S. and Qadeer, M.A. 1997. Submerged fermentation of alpha amylase by *Bacillus licheniformis* GCB–36. Biologia, 39: 45.

Haq, I., Ashraf, H., Zehra, R. and Qadeer, M.A. 1998. Biosynthesis of alpha amylase by *Bacillus subtilis* GCB12 using agricultural byproducts as substrates. Pakistan Biol. Soc., 44: 154.

Ivanova, V., Yankov, D., Kabaivanova, L. and Pashkoulov, D. 2001. Simultaneous biosynthesis and purification of two extracellular *Bacillus* hydrolases in aquous two alpha amylases. Biochem. Engg. J., 8: 61-81.

Vieille, C., Savchenko, A. and Zeikus, J.G. 2000. Amylases microbial. Encyclopidia of Microbiology. 2nd ed., 1: 171-172.

Mamo, G. and Gessesse, A. 1999. Effect of cultivation conditions on growth and alpha amylase production by thermophilic *Bacillus* sp. Lett. App. Microbiol., 29: 61-65.

Kim., T.U., Gum, G.B., Jeong, J.Y. and Young, S.C. 1995. Purification and characterization of maltotetrose forming alkaline alpha amylase from an alkalophilic *Bacillus* strain, GM 8901. Appl. Environ. Microbiol., 61: 3105-3112.

Haq, I., Riaz, N., Ashraf, H., Qadeer, M.A. and Iqbal 2002. Effect of inorganic salts on the biosynthesis of alpha amylase by *Bacillus subtilis*. Ind. J. Plant Sci., 1: 115-121.