International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Issue 1 | JAN-MAR| 2018 | 308-313
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF α-AMYLASE FROM BACILLIUS SUBTILIS

Rekhani Jyoti Pal[1*] and Zeenat Madan[2] and Gulshan kumar[3]

[1] Department of Biotechnology, S.D. College, Ambala Cantt
[2] Department of Zoology, S.D. College, Ambala Cantt
[3]Department of Biochemistry, Universal Groups of Institute

***Corresponding Author Email: rekhanipal105@gmail.com**

ABSTRACT

The use of α-amylase especially in food, beverage, paper, leather, and textile industries is increasing day by day; so, there is need for other sources of the enzyme to be discovered. These enzymes are among the most important industrial enzymes occupying approximately 25% of the world enzyme market. Various fungi, yeasts and bacteria are used for its derivations. As we know that India being rich in flora and fauna it is also rich source of natural resources, especially the microbes as enzymes producers. This research is aimed at isolation, partial purification and characterization of α-amylase from Bacillus subtillis. To get the partial purified enzymes, raw extract was fragmentised with ammonium sulphate salts in variety of saturated degree. By determining the optimum pH and temperature at which the enzyme activity is maximum, partial characterization of partially purified enzymes was calculated. The result showed that the partially purified enzyme has specific activity of 0.244 ± 0.219 u/mg, there was an increase of 34.5 times than the raw extract. The optimum temperature of the enzyme was 70°C. The optimum pH of the purified enzymes was 6.5, but the enzyme can also work between the pH range of 5.0 to 9.0. Bacillus subtillis can serve as other source of α-amylase especially for industrial purposes.

KEY WORDS

α-Amylase; Bacillus subtillis; Partial Purification; Characterization; Ammonium sulphate; Industrial application

INTRODUCTION

α-Amylases is a protein enzyme that hydrolyses alpha bonds of large[1], alpha-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose.[2] It catalyses internal α-1,4-glycosidic linkages in starch, converting starch into low-molecular-weight products such as glucose, maltose and maltotriose units (Rajagopalan and Krishnan, 2008; Gupta et al., Kandra, 2003). These enzymes are among the most important industrial enzymes occupying approximately 25% of the world enzyme market (Rajagopalan and Krishnan, 2008; Reddy et al., 2003). It is the major form of amylase found in humans and other mammals. [3]

α-Amylases can be derived from several fungi, yeasts, and bacteria. It is also present in seeds containing starch as a food reserve and is secreted by many fungi. However, mostly enzymes from fungi and bacteria are used in industrial sectors (Reddy et al., 2003). Various species of *Aspergillus* had been considered as important organism for commercially production of α-Amylases because of its ease in cultivation and desirable physicochemical properties (Hernandez et al., 2006). The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics. [4]

α-Amylases contain number of distinct protein domains. The catalytic domain has a structure consisting of an 8 standed α/β barrel that contains the active site, interrupted by a ~70-amino acid calcium-binding domain protruding between β stand 3 and α helix 3, and a carboxyl-terminal Greek key β-barrel domain. [5] Several α-amylases contain a β-sheet domain, usually at the C terminus. This domain is organised as a 5-standed antiparallel β-sheet. [6][7] Several α-amylases contain an all-beta domain, usually at the C terminus. [8]

α-amylase called "Termamyl", sourced from Bacillus licheniformis, is also used in some detergents, especially dishwashing and starch-removing detergents. [9]

Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications. [10]

This research work is aimed at isolating, partially purify and characterize α-amylase from *Bacillus subtillis*.

MATERIALS AND METHODS

2.1 Methods

Following are the steps for the reaserch

- Isolation of *Bacillus subtilis*
- Extraction of α-amylase from the *Bacillus subtilis*
- Partial purification of the enzyme and characterization.

2.1.1 Isolation of *Bacillus subtillis*

The bacterial cells used for research were isolated from soil collected from S.D. College Botanical garden in Ambala cantt, Haryana.

Serial dilutions was performed by adding 1g of soil sample to 9 ml sterilized distilled water in a test tube, and 1ml of the mixture in the 1st test tube were transfer to a 2nd test tube containing 9ml of distilled water and same process was done continuously until the last 7th test tube.

Nutrient agar plates were prepared by dissolving 1.2 g peptone, 0.8 g casein hydrolyzate, 0.30 g beef extract, 0.6 g yeast extract and 0.4 g starch in 200 ml distilled water, pH was then adjusted to 7 and then 3 g agar was added in the 500 ml Erlenmeyer flasks, autoclaved at 121°C and 15 lb pressure for 15 min. 0.1ml of the sample from test tubes 4, 5, 6, and 7 were spread onto nutrient agar plates and incubated at 37°C for 48 hours.

Colonies were gram stained followed by gram staining procedure using standard procedure. The gram-positive colonies were subjected to biochemical tests for identification of *Bacillus subtilis*.

2.1.2 Extraction of α-Amylase

The fermentation media containing: NaCl 0.04%, peptone 0.20%, yeast extract 0.10% and starch 1.00%. pH was adjusted to 7 with NaOH were used for the production of α-Amylase. The media was sterilized by autoclaving at 121°C and 15 lb pressure for 15 min, and then cooled at room temperature. After cooling, the media was transferred into test tubes and inoculated with the bacterial isolates and incubated at 37°C for 24 hours.

Cold centrifuge at speed of 6000 rpm for 30 minutes at 4°C was used to separate the raw extract of the α-amylase from bacterial cell present in fermentation media.

2.1.3 Partial Purification of α-Amylase.

2.1.3.1 Fractionation with Ammonium Sulphate

In a variety of saturated degree of ammonium sulphate, a standard procedure was used to get the partially purified enzyme. [11]

The raw extract was brought to 30% saturation of ammonium sulphate, the precipitates were removed by centrifuging at 20,000g for 15 minutes. The resulting supernatant was raised to 50% ammonium sulphate saturation and precipitate was collected by centrifuging at 20,000g for 15 minutes. The resulting supernatant was raised to 70% ammonium sulphate saturation and precipitate was also collected by centrifuging at 20,000g for 15 minutes. The resulting supernatant was raised to 90% ammonium sulphate saturation and precipitate was also collected by centrifuging at 20,000g for 15 minutes. The resulting supernatants were subjected to enzyme assay.

2.1.3.2 Enzyme Assay

Reagents:

1. **Substrate (Starch):** Mixed 1gm of soluble starch in 200ml of 0.1M Phosphate buffer (pH 6.8). Boiled for 3 minutes and cooled at room temperature.
2. **Crude Enzyme.**
3. **1% Sodium chloride:** Necessary for enzyme activity.
4. **DNS (Dinitro Salicyclic acid):** Dissolved 1.6gm of NaOH in 20ml of distilled water. Took 1gm of 3.5 DNS in NaOH Solution. In other beaker took 30gm of Sodium potassium tartrate. Dissolved in 50ml of distilled water. Mixed this DNS solution and finally make the volume upto 100ml with distilled water.

5. **Standard solution of Maltose:** It was prepared by dissolving 200mg Maltose in 100ml of water (2mg / 1ml).

Preparation of Phosphate buffer: Dissolved 0.2M (2.7218 grams) of KH_2PO_4 in 100ml of distilled water to this solution added 0.5M (2.8053 grams) KOH drop by drop till the pH is set to 6.8. Total volume was made up to 200ml with distilled water. So, the final concentration is 0.1M of 200ml Phosphate buffer.

Procedure:

Took 0.5ml of substrate and 0.2ml of 1% NaCl in a test tube and pre-incubated at 37^0C for 10 minutes then added 0.5 ml of crude enzyme and incubated for 15 minutes at 37^0C. Reaction was stopped by addition of 2 ml of DNS (3,5-dinitrosalicylic acid) reagent.[12] Mixed well and kept the test tubes in boiling water bath for 10 minutes. Cooled and diluted with 10ml of distilled water. Read the colour developed at 520 nm. Simultaneously colour development was observed at 520nm. Simultaneously set uped the blank as per the test by adding DNS prior to the addition of enzyme. Standards of different test tubes were set up and repeated the experiment as per the test and measured the colour developed at 520nm absorbance. D-glucose was used to create a standard curve. One unit of enzyme activity was defined as the amount of enzyme releasing reducing sugar equivalent to 1 µmol glucose per minute under the assay condition.

2.1.3.3 Protein Content Estimation

A standard method by [13] was used. Different dilutions of Bovine Serum Albumin (BSA) solutions were prepared by mixing stock BSA solution (1 mg/ ml) and distilled water in test tubes. The final volume in each of the test tubes was 5 ml. The BSA range was 0.2 to 0.8 mg/ ml. From these different dilutions, 0.2 ml protein solutions were pipetted to different test tubes and 2 ml of alkaline copper sulphate reagent was added. The solutions were mixed well and incubated at room temperature for 10 min. 0.2 ml of Folin Ciocalteau solution was then added to each tube and incubated for 30 min. The absorbance of each mixture was then measured using spectrophometer at 600 nm. The absorbance was plotted against protein concentration to get a standard calibration curve. The concentration of the unknown samples was determined from the standard curve plotted.

2.1.4 Partial Characterization of Purified Enzyme

The partial characterization of the purified enzyme was done by determining the optimum pH and optimum temperature at which the enzyme activity is high.

2.1.4.1 Determination of Optimum pH of the Purified Enzyme

2ml of 0.1% starch solution was added in 12 sets of test tubes in triplets, followed by the addition of 4ml of 0.1 M phosphate buffer with pH variations of 4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. 4ml of distilled water was added to the 12^{th} test tube and labelled as blank. All the tubes were incubated at 37°C in a water bath for 5 minute to equilibrate. 0.1ml of the α-amylase was added and mixed in all the test tubes and returned into water bath for further incubation for 5min and 2ml of N\100 iodine solution was added immediately after incubation. The absorbance of each mixture was measured spectrophotometrically at 660 nm using reagent blank.

2.2.4.1.2 Determination of Optimum Temperature of the Purified Enzyme

2ml of 0.1% starch solution was added to 12 sets of test tubes in triplicates, followed by addition of 4ml of 0.1 M phosphate buffer (with pH 7) and 1ml of distilled water. The variations of the temperature used were 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 and 90°C. The test tubes were left at these temperatures for 5 min. 0.1ml of the enzyme preparation was added to each tube. The tubes were incubated for another 5 min then 4ml of N\100 iodine solution was added after the incubation. The absorbance of each mixture was measured spectrophotometrically at 660 nm using reagent blank.

RESULTS

3.1 Extraction and Partial Purification of α-Amylase

Cell growth and release of enzyme after inoculation at 37°C for 24 hours from plate 10^{-5} in broth culture was observed. The culture was more turbid, and bubbles were seen at the top of the liquid as presented in Figure 1. The results for the individual steps during the partial purification procedures are presented in Table 1.

Table 1: Purification table for isolated α-amylase enzyme

Purification Steps	Total Activity (U)	Total Protein (mg)	Specific Activity (u/mg)	Yield (%)	Purification factor (X)
Raw extract	0.046 ± 0.0010	8.238 ± 0.126	0.0043 ± 0	100	1
A.S. 30%	0.0015 ± 0.0020	0.050 ± 0.0001	0.015 ± 0.0021	0.021 ± 0.002	2.431 ± 0.548
A.S. 50%	0.0025 ± 0.00022	0.200 ± 0.0013	0.025 ± 0.0028	0.050 ± 0.005	3.588 ± 0.657
A.S. 70%	0.0295 ± 0	0.900 ± 0.0001	0.034 ± 0.0023	0.642 ± 0	5.681 ± 0
A.S. 90%	0.0068 ± 0.00010	0.0725 ± 0.063	0.244 ± 0.219	0.260 ± 0.003	34.472 ± 28.891

The values are presented as mean ± standard deviation of triplets. A.S. means ammonium sulphate

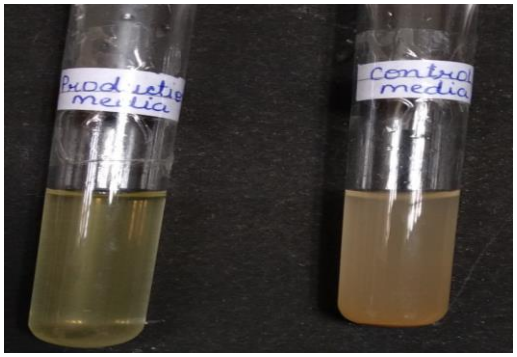

Figure 1: Control and Production broth for α-amylase

3.2 Partial characterization

The enzyme activities (unit) of the partially purified enzyme at the various pH values used are presented in Figure 2, while those of temperatures are shown in Figure 3.

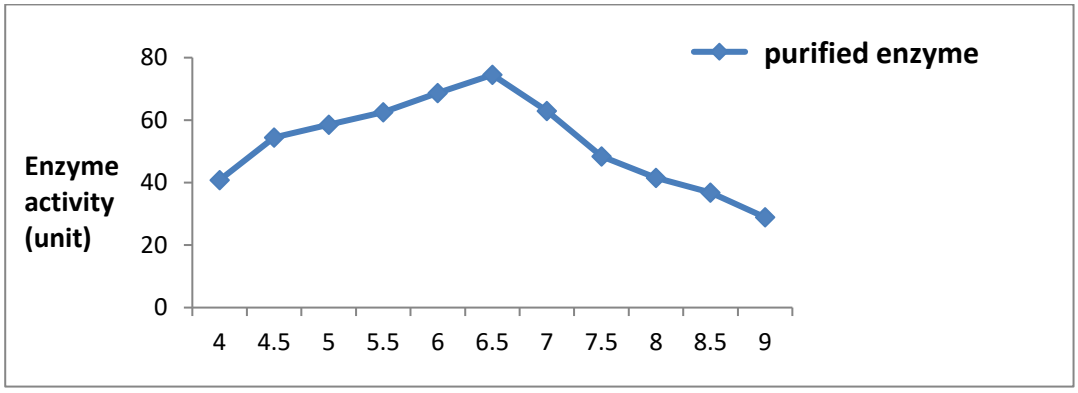

Figure 2: Optimum pH of partially purified α-amylase enzyme

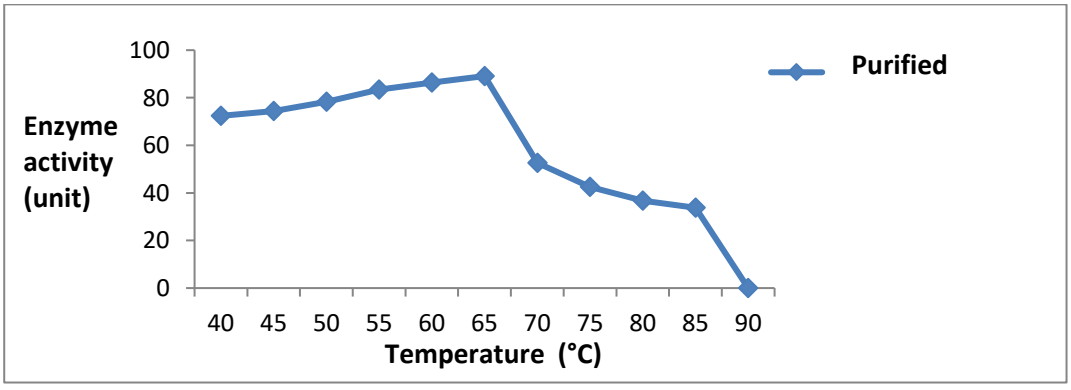

Figure 3: Optimum temperature of partially purified α-amylase enzyme

DISCUSSION

Many micro-organisms especially several species belonging to Bacillus are known to produce variety of extracellular enzymes and they have a wide range of industrial applications. Of these enzymes, amylases are of particular significance to industry.

4.1 Partial Purification

It has been seen that there was high gradual specific activity in each step of purification. The phases of purification of α- amylase from *B. subtilis* can be seen in Table 1. From ammonium sulphate fraction of 90% saturation the highest activity was observed.

The specific activity increases from one purification step to another as follows; 2.4 times for ammonium sulphate fraction of 30%; 3.6 times for ammonium sulphate fraction of 50%; 2.2 times for ammonium sulphate fraction of 70% and 5.4 times for ammonium sulphate fraction of 90%. The increase in specific activity for each purification process indicated that the step of purification used was effective. The results obtained were compared to those obtained by [14] who did purification on α-amylase from *B. subtilis* ITBCCB148. The α-amylase obtained showed increase of specific activity higher than α-amylase from wild *B. subtilis*.

4.2 Partial characterization

4.2.1 Optimal pH

The results obtained indicated that the optimum pH at which the α-amylase activity is high is 6.5. This observation agrees with what was earlier reported by [15, 16]. The α-amylase obtained showed the same optimal pH with α- amylase from wild *B. subtilis* (pH value 6).

4.2.2 Optimal Temperature

Enzyme activity was highest at 65°C. This also agrees with what was reported earlier by [14, 15] and disagree by [16, 17] reported results. The optimum temperatures at which α- amylase activity reaches maximum as reported by many researches were reviewed based on microbial sources by [18].

CONCLUSION

Though some preliminary enzymatic parameters like pH optium and temperature optimum have been determined for alpha amylase from 48 hours of SSF at 37°C, complete purification as well as molecular weight determination is still to be performed. The usefulness of an enzyme from any organism for starch hydrolysis depends upon its potential to degrade native starch to oligosaccharides, glucose and other products at high temperatures and over a wide range of pH. The ability of *Bacillus* sp. to degrade native starch, at a wide range of pH and the thermal stability of α-amylase are the attractive attributes which make this bacterial strain to be a potential source of this enzyme for starch hydrolysis, especially for many applications ranging from bread and baking industry, starch liquefaction and saccharification textile desizing, paper industry, detergent application, analysis in medicinal and clinical chemistry.

REFERANCES

1. Windish W.W and Mhatre N.S. (1965). Microbial amylases, In: Wayne W.U., editor. Advances in applied microbiology, Vol.7. New York: Academic Press 273-304.
2. Maureen Barlow Pugh, ed. (2000). Stedman's Medical Dictionary (27th). Baltimore, Maryland, USA: Lippincott Williams & Wilkins. p. 65.
3. Voet, D., & Voet, J. G. (2005). Biochimie. (2nd). Bruxelles: De Boeck. p. 1583
4. Lonsane B, Ramesh M. (**1990**). Advances in applied microbiology, Vol. 35. San Diego: California Academic Press, 1-56.
5. Abe A, Yoshida H, Tonozuka T, Sakano Y, Kamitori S (December 2005). "Complexes of Thermoactinomyces vulgaris R-47 alpha-amylase 1 and pullulan model oligossacharides provide new insight into the mechanism for recognizing substrates with alpha-(1,6) glycosidic linkages". *FEBS J*. **272** (23): 6145–53.
6. Kadziola A, Sogaard M, Svensson B, Haser R (April 1998). "Molecular structure of a barley alpha-amylase-inhibitor complex: implications for starch binding and catalysis". *J. Mol. Biol*. **278** (1): 205–17.
7. Kadziola A, Abe J, Svensson B, Haser R (May 1994). "Crystal and molecular structure of barley alpha-amylase". *J. Mol. Biol*. **239** (1): 104-21.
8. Machius M, Wiegand G, Huber R (March 1995). "Crystal structures of calcium-depleted Bacillus licheniformis alpha-amylase at 2.2 A resolution". *J. Mol. Biol*. **246**(4): 545-59.
9. *"*The use of enzymes in detergents*".* Faculty of Engineering, Science and the Built Environment, London South Bank University. 20 December 2004. Archived from the original on 20 October 2009. Retrieved 21 November 2009.
10. "Falling Number - Introduction". Perten instruments. 2005. Retrieved 2 November 2009.
11. Yandri, A.S., D. Herasari, T. Suhartati. (**2007**). *Jurnal Sains MIPA*, *Edisi khusus,* 13 (2): 100-106.
12. Yang CH, Cheng KC, Liu WH. (**2003**). *Food Sci. Agric. Chem.,* 5: 35-40.

13. Lowry, O.H., N.J., Rosebrough, A.L., Farr, R.J. Randall (**1951**). *J. Biol. Chem*., 193-265.
14. Yandri A.S., T. Suhartati, S. Hadi. (**2010**). *European Journal of Scientific Research.* 39, 64-74.
15. Hayashida S, Teramoto Y, Inoue T. (**1988**). *Appl*. *Environ. Microbiol*; 54: 1516-22
16. Schwermann B, Pfau K, Liliensiek B, Schleyer M, Fischer T, Bakker EP. (**1994**)
17. *Folia Microbiol* 1985; 30:452
18. Gupta R., Gigras P., Mohapatra H., Goswami V. K., Chauhan B. (**2003**). *Process Biochemistry* 38(11): 1599-1616
19. Burhan, U. Nisa, C. Gokhan, C. Omer, A. Ashabil, and G. Osman, "Enzymatic properties of a novel thermostable, thermophillic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp. Isolated ANT-6" Process Biochemistry, Vol. 38, no.10, pp. 1397-1403, 2003.
20. Das K, Doley R, Mukherjee AK. Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular α-amylase from *Bacillus subtilis* DM-03, a strain isolated from the traditional fermented food of India. Biotechnol Appl Biochem. 2004; 40:291–298.
21. Shaw JE, Lin FP, Chen SC, Chen CC. Purification and properties of an extracellular α-amylase from *Thermus* sp. Bot Bull Acad Sin. 1995; 36:195–200.
22. Carbajal AF, Soto JO. Thermostable α-1,4- and α-1,6-glucosidase enzymes from *Bacillus* sp. isolated from a marine environment. World J Microbiol Biotechnol. 2002; 18:791–795.
23. G. Rajagopalan and C. Krishnan, "α-Amylase production from catabolite derepressed Bacillus subtilisKCC103 utilizing sugarcane bagasse hydrolysate," Bioresource Technology, vol. 99, no. 8, pp. 3044–3050, 2008
24. Z. Baysal, F. Uyar, and Ç. Aytekin, "Solid state fermentation for production of α-amylase by a thermotolerant Bacillus subtilis from hot-spring water," Process Biochemistry, vol. 38, no. 12, pp. 1665–1668, 2003.
25. V. N. Ivanova, E. P. Dobreva, and E. I. Emanuilova, "Purification and characterization of a thermostable alpha-amylase from Bacillus licheniformis," Journal of Biotechnology, vol. 28, no. 2-3, pp. 277–289, 1993.

***Corresponding Author:**
Rekhani Jyoti Pal*
Email: **rekhanipal105@gmail.com**