Thermal stability of partially purified α-amylase produced by Brevibacillus borstelensis R1 from the coastal waters of Bay of Bengal, Visakhapatnam

K. Suribabu1, T. Lalitha Govardhan1 & K. P. J. Hemalatha2

1 PG Department of Microbiology and Research Centre, Dr. Lankapalli Bullayya Post-graduate College, Visakhapatnam-530 013, A.P, India.
2 Department Microbiology, Andhra University, Visakhapatnam-530 003, A.P, India.

[E-mail: ksuribabu_sda@yahoo.com]

Received 11 September 2015; revised 07 September 2016

Highest α-amylase activity of Brevibacillus borstelensis R1 was observed at 37°C (6133±58U/ml). The α-amylase retained its activity after 10 hrs exposure to 40°C with negligible variations in its activity. Alpha-amylase have several applications in starch processing, desizing of textiles, paper sizing, detergent additive, bread improvement, ethanol production, sewage treatment, effluent treatment and other fermentation processes.

Highest α-amylase activity was observed at 37°C (6133±58U/ml), while the lowest at 70°C (2360±40U/ml). Only 38.47% activity remained at 70°C. Optimum temperature of partially purified enzyme was similar to the crude enzyme. Partially purified enzyme was tested for its stability (1-10 hours) at temperatures ranging from 40°C to 70°C. A at 20°C it retained 34.2% activity, at 25°C 44.1%, at 37°C 34.7%, at 50°C 56.2%, at 60°C 38.2% and at 70°C it retained 30.8% activity upon exposure for about 10 hrs.

[Keywords: α-amylase, Thermal stability, Brevibacillus borstelensis R1]

Introduction

Several reports are available on α-amylase production by thermophilic bacteria1-9. Extreme thermophiles like species of Pyrococcus10, Thermococcus spp.11, Rhodothermus marinus12, Thermobifida fusca NTU2213 and Geobacillus thermodenitrificans HRO1014 also produced α-amylase.

The rate of an enzyme catalyzed reaction increases with rise in temperature till it reaches the maximum. Effect of temperature is due to thermal stability of enzyme, alteration of pKₐ’s by the heat of ionization, affinity of an enzyme for the activators or inhibitors, different temperature coefficients and change in rate limiting functions15. The α-amylase activity with optimum temperature range 30°C-50°C was reported in Bacillus spp.16 and 17. Optimum temperature range, 55°C-70°C for alpha-amylase activity was reported in Bacillus spp.18. Extreme optimum temperature for amylase activity range 75°C-100°C was reported in Bacillus spp.19.

All the enzymes have a narrow temperature range for their efficient functioning. The enzyme activity declines at temperature beyond optimum temperature. It is important to understand the therm stability of the enzyme. It was reported that the amylase was optimally active with a half-life of 3hrs at 100°C in Bacillus thermooleovorans NP518. Enzyme was stable retaining more than 90% of its original activity after 60min exposure at 90°C and 20min exposure at 95°C in Bacillus licheniformis mutant 790220. The enzyme was reported to be stable at 90°C for 10min in Bacillus sp.21. The optimum temperature for α-amylase activity was 40°C and 71% of the activity was still maintained until 30 min after heating at 80°C in Bacillus sp.22. Thermal stability of amylase in bacteria was studied by El-Safey & Ammar (2002), Pimpa (2004) and Alva et al. (2007).
Materials and Methods

Marine water samples were collected with sterile BOD bottles from coastal area of Rushikonda, Visakhapatnam, Andhra Pradesh, India. By using protocols from Bergey’s Manual Determinative Bacteriology, the colony, morphological and biochemical characterization of the high yielding α-amylase bacteria were identified as *Brevibacillus borstelensis* R1. Ideal purification should optimize both the purity and the concentration of the metabolite. Alpha-amylase is an extracellular enzyme. Precipitate collected from 70% (NH₄)₂SO₄ salting out was dissolved in required amount of 0.1M phosphate buffer (pH 6.8). Dialysis was conducted to get rid of the ions in the protein. After dialysis in the buffer for 24hrs, the sample was subjected to gel filtration. The fraction number 38° had shown highest α-amylase activity (3793±12U/ml) with protein concentration (4.8mg/ml). Fold purification obtained (3.9) when the sample was subjected to sephadex G-100 gel filtration. Specific activity increased from 202.6U/mg to 790.21U/mg protein. The optimized α-amylase production in PK medium by submerged fermentation (SmF) was subjected to varying physical parameters such as incubation period, inoculum size, optimum temperature, optimum pH and salinity.

Maltose produced by the hydrolytic activity of α-amylase on α-1, 4 linkages present in polysaccharides, reduce 3, 5 dinitro salicylate to an orange red colored 5-nitro 3-amino salicylate which can be measured at 520nm. Starch substrate [0.5ml of 0.5% in 0.1M phosphate buffer (pH 6.8)] was mixed with 1% (0.2ml) NaCl in a test tube and pre incubated at 37°C for 10 minutes. Supernatant collected from the centrifugation of the production media was used as enzyme source, 0.5ml of this was added to the reaction mixture. The reaction was terminated by the addition of 1.0 ml of 3, 5-dinitrosalicylic acid reagent [1.0 gm DNS in 0.8% NaOH, 60% Na K tartrate]. After incubation at 37°C for 15 minutes, the contents were mixed well and kept in boiling water bath for 10 minutes. Then they were cooled and diluted with 10 ml of distilled H₂O. The color developed was read at 520nm. One unit of enzyme activity is defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions.

The effect of temperature on α-amylase activity was checked by adding 0.5 ml of starch (0.5% in phosphate buffer at pH 6.8) to 0.2ml of NaCl (1%) and pre-incubated for 10minutes at 37°C. To this mixture partially purified enzyme (0.5ml) was added and incubated at different temperatures 4°C, 20°C, 25°C, 37°C, 50°C, 60°C and 70°C for 15 minutes. One milliliter of 3, 5- dinitrosalicylic acid reagent (1%) was added to stop the reaction. Estimation of α-amylase activity was carried out according to DNS method. The effect of temperature stability of α-amylase activity was tested by adding 0.5 ml of starch (0.5% in phosphate buffer at pH 6.8) to 0.2ml of NaCl (1%) and pre-incubated for 10minutes at 37°C. Partially purified enzyme (0.5ml) was added and incubated at different temperatures 4°C, 20°C, 25°C, 37°C, 50°C, 60°C and 70°C for different hours (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10). For control the partially purified amylase was incubated at 37°C for 15 minutes. One milliliter of DNS reagent (1%) was added to stop the reaction. All the experiments were conducted in triplicate. The results were given as mean value ± standard deviation. The conditions were analyzed to determine the significant difference between the variables by one way ANOVA, two way ANOVA and correlation analysis by using the scientific graph pad (Prism 6.1version software). Analysis of variance (ANOVA) refers to the examination of differences among the sample means. It is used to examine the significance of the difference amongst more than two sample means at the same time. Correlation analysis is concerned with measuring the strength or degree of relationship between variables. The measure of correlation is called the correlation co-efficient. The correlation measures the closeness of the relationship between variables.

Results and Discussion

There was 3.9 fold purification of α-amylase when the crude enzyme was subjected to (NH₄)₂SO₄ precipitation and gel filtration. The α-amylase was found to be useful in bakery, food, fodder for poultry, automation dishwashing and laundry industries. These are only pilot-scale experiments and there is a need to carry out experiments which are safe to health. The α-amylase was also useful in solving the problem of water pollution of industrial effluents and sewage water by hydrolyzing the substrates.

The highest amylase activity was observed at 37°C (6133±58U/ml), lowest at 70°C (2360±40U/ml) (Fig. 1). Only 38.47% activity remained at 70°C. The optimum temperature of partially purified enzyme was similar to the crude enzyme.
The stability of amylase was tested for its stability for 1-10 hours at temperatures ranging from 4°C-70°C. At 4°C the activity of amylase (3100±20U/ml) remained nearly constant for 1hr to 10hrs (Fig. 2) with negligible variations in activity. At 20°C the activity of amylase (4693±64U/ml) remained nearly stable for 5hours and later showed decrease in activity and remained constant (2627±12U/ml) for 7-9hrs and reached the lowest (1627±12U/ml) after 10hrs (Fig. 3). There was 34.2% activity retained after 10hrs. At 25°C the activity of amylase (5367±58U/ml) remained stable for 8hrs and reached the lowest (2367±58U/ml) after 10hrs (Fig. 4). There was 44.1% activity retained after 10hrs.

At 37°C the activity of amylase (6133±58U/ml) remained nearly stable for 6hrs and later decreased to the lowest activity (2133±58U/ml) after 10hrs (Fig. 5). There was 34.7% activity retained after 10hrs.

At 50°C the activity of amylase was 3047±50U/ml. The activity of the amylase showed stepwise constant decrease in activity for 1-3hrs, 4-7hrs and 8-10hrs (2513±23U/ml, 2013±23U/ml and 1713±23U/ml respectively) (Fig. 6). There was 56.2% activity retained after 10hrs. After 60°C the activity of amylase was 2807±46U/ml. The activity of the amylase was constant for 1-2hrs (2573±12U/ml), 3-6hrs (2073±12U/ml), 7-9hrs (1873±12U/ml) and later decreased (1073±12U/ml) to the lowest after 10hrs (Fig. 7). There was 38.2% activity retained after 10hrs. At 70°C the activity of amylase was 2360±40U/ml. The activity of the amylase was decreased and remained constant for 1-3hrs (2027±31U/ml) and 4-6hrs (1527±31U/ml) and later decreased (727±31U/ml) to the lowest after 10hrs (Fig. 8). There was 30.8% activity retained after 10hrs. The highest stability of amylase was found at 4°C (3000±12 U/ml) for 10hours.
The optimum temperature of partially purified α-amylase of *B. borstelensis* R1 was studied. Highest amylase activity was observed at 37°C (6133±58U/ml). Only 38.47% activity remained at 70°C. The optimum temperature of partially purified enzyme was similar to the crude enzyme.

The partially purified enzyme was tested for its stability for 1-10hours at temperatures ranging from 4°C-70°C. The α-amylase retained its activity after 10hrs exposure to 4°C with negligible variations in activity, at 20°C it retained 34.2% activity, at 25°C it retained 44.1% activity, at 37°C it retained 34.7% activity, at 50°C it retained 56.2% activity, at 60°C it retained 38.2% activity and at 70°C it retained 30.8% activity upon exposure to about 10hrs. Similar reports on thermal stability were reported in *Bacillus* sps. Estimation of amylase was done by using DNS method. Enzyme was optimally active with a half-life of 3hrs at 100°C in *Bacillus thermooleovorans* NP5. Enzyme activity increased as the temperature rose gradually from 75°C to 100°C. The enzyme was reported to be fairly stable retaining more than 90% of its original activity after 60min exposure at 90°C and 20min exposure at 95°C of *Bacillus licheniformis* mutant 7902. In *Bacillus* spp. the enzyme was reported to be not very stable at 60°C and was completely inactivated after 10minutes at 80°C. The enzyme was stable at 90°C for 10min and at 100°C it still retained 26% of its initial activity. Amylase from *Bacillus licheniformis* CUMC305 showed many interesting features. Purified enzyme showed maximal activity at 90°C, pH 9.0 and 91% of it’s activity remained at 100°C. Stability of the amylase from *Bacillus stearothermophilus* could be enhanced if liquefied thick starch slurries (at 80°C and pH 6.9) were provided as feed/substrate in the presence of Ca²⁺. Suribabu K et al., worked over *Brevibacillus borstelensis* R1 with relation to isolation, optimization of physical parameters, media, carbon, nitrogen, mineral source under submerged fermentation along with that partial purification and kinetics of partially purified alpha-amylase was also studied. The highest α-amylase yielding isolate was identified as *Brevibacillus borstelensis* R1 through 16S rRNA gene sequence analysis and Mega BLAST score. BLASTN score was employed to construct the phylogenetic tree by Suribabu K and Hemalatha KPJ (2016).  

**Conclusion**

The highest α-amylase activity was observed at 37°C (6133±58U/ml), while the lowest at 70°C (2360±40U/ml). Only 38.47% activity remained at 70°C. Optimum temperature of partially purified enzyme was similar to the crude enzyme. Partially purified enzyme was tested for its stability (1-10hours) at temperatures ranging from 4°C to 70°C. The α-amylase retained its activity after 10hrs exposure to 4°C with negligible variations in its activity. At 20°C it retained 34.2% activity, at 25°C 44.1%, at 37°C 34.7%, at 50°C 56.2%, at 60°C 38.2% and at
70°C it retained 30.8% activity upon exposure for about 10 hrs.

Acknowledgements
Authors thank Management of Dr.Lankapalli Bullayya College, Visakhapatnam for the financial support and facilities provided to make this work possible.

References


33. Suribabu K, Lalitha Govardhan T and Hemalatha KPJ, Influence of carbon sources on α-amylase production by Brevibacillus sp. under submerged Fermentation. Inter. jr. of research in eng. and tech., 03 (02) (2014) 292-299.


