Vol. 13, No.1: 1 — 5
May 2024
DOI:
https://dx.doi.org/10.22487/25411969.2024.v13.i1.17057

Natural Science
Journal of Science and Technology

Original article

Stability Study of Thermophilic Crude Amylase PL-16 against pH and Temperature Alterations

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Keywords: *Amylase, Thermophile, pH, Temperature, Stability*

Article history:
Received 25 March 2024
Accepted 05 May 2024
Published 31 May 2024

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Abstract

A stability study of previously isolated and selected crude amylase PL-16 has been performed. The purpose of this study was to determine the effect of pH and temperature alterations on the stability of crude amylase enzymes PL-16 produced by thermophilic bacteria found in Pulu hot springs in Central Sulawesi. Stability characterization was carried out by storing and incubating crude amylase for 24 hours and at elevated heat prior to activity assay at varied pH and temperatures. Enzyme activity following characterization was determined by the DNS method and UV-VIS spectrophotometry at a wavelength of 540 nm, using maltose as the standard. The results showed that at storage for 24 hours with pH varied from 5-12, the crude amylase enzyme activity was able to maintain its 85-95% stability in the pH range of 7-9, then decreased in stability at pH of 10-12. While at 2 hours storage in elevated temperature, it maintained 84-85% stability against heat at 70-80°C, then decreased at higher temperatures. Our results suggested that crude amylase PL-16 is a promising candidate for industrial enzyme application due to its capability to maintain stability in high temperatures and neutral pH.

INTRODUCTION

The utilization of microbes to produce enzymes offers several benefits. These include the ability to grow them in large quantities, potentially producing them infinitely, all at a lower cost and with increased efficiency (Sundarram and Murthy, 2014). Furthermore, microbes that live in extreme environments produce enzymes that have distinct characteristics in activity against alterations of alkalinity, pH, pressure, and temperature (Elleuche *et al*., 2014). These characteristics are essential for industrial applications, especially those that involve enzymes in the production process (Littlechild, 2015).

Amylase is one of the enzymes that is used in industrial applications for the production of starch, detergent, fuel alcohol, textiles, and paper. Amylase enzymes produce glucose, dextrin, and maltose as an end product by hydrolyzing starch at specific α-glycosidic bonds. For example, in the starch conversion industry, hydrolyze starch in a liquefaction process to produce fructose and glucose syrup (De Souza and de Oliveira, 2010). It consists of alpha-, beta-, and gamma-amylases, but among them, alpha-amylase is the most widely used (Kumar and Chakravarty, 2018).

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How to Cite: Satrimafitrah, *et al.* “Stability Study of Thermophilic Crude Amylase PL-16 against pH and Temperature Alterations” Natural Science: Journal of Science and Technology. Vol. 13, No. 1: 1–5, May 2024.

Microbial amylase production necessitates two optimal temperatures. The first temperature supports microbial growth, while the second optimizes enzyme biosynthesis. Thermophilic amylases, known for their high-temperature tolerance and activity, notably have optimal activity at 70-100°C where the structure of normal enzymes is usually disrupted (Farooq, *et al.*, 2021). In terms of pH, it has a significant role in the production and secretion of amylase. It also determines the stability of protein structure, particularly alpha helices and beta sheets with electrostatic interactions. Amylases have a wide range of optimum pH, i.e. 4-8 (Kumari, *et al.*, 2019).

Previously, we screened and isolated crude thermophilic amylase from Pulu hotspring and studied its optimum pH and temperature working condition (Satrimafitrah, *et al.*, 2020). We also studied its best incubation time and agitation speed to measure the enzyme activity in producing the highest maltose (Satrimafitrah, *et al.*, 2021). In this paper, we report the effect of storage in various temperatures and pH has altered the activity of crude amylase PL-16 to understand better its stabilities.

MATERIALS AND METHODS

Bacillus sp. isolate PL-16 from Pulu hot spring, NA (Nutrient Agar) media, MSM (Minimum Synthetic Medium), Starch, Maltose, Phosphate buffer (pH 6.0 - 8.0), Sodium acetate buffer (pH 5.0), Glycine-NaOH buffer (pH 9.0-12.0), Distilled water, Universal pH paper, DNS (3,5-dinitro salicylic acid) reagent, and 70% ethanol

Production of Crude Amylase

Following the previously described method (Satrimafitrah, *et al.*, 2020), crude amylase enzymes were produced using a minimal synthetic medium (MSM) containing 1% starch as the carbon source. The MSM also included specific concentrations of potassium phosphate (KH_2PO_4), sodium chloride (NaCl), ammonium sulfate ($(NH_4)_2SO_4$), and yeast extract, all dissolved in 100 mL of distilled water. Initially, a starter culture was incubated at 60°C with constant stirring (180 rpm) for 24 hours. This culture was then transferred to fresh MSM containing 1% starch to promote further enzyme production for another 24 hours. Finally, the culture was centrifuged at 8000 rpm at 4°C to separate the cells from the crude amylase enzyme supernatant.

Activity Assay of Crude Amylase

Amylase activity was measured and calculated with the previously described method (Satrimafitrah, *et al.*, 2020). The assay involved a reaction mixture containing 1 mL of 1% starch solution (substrate) prepared in a 0.05 M phosphate buffer (pH 7.0), and 1 mL of the crude enzyme solution. This mixture was incubated for 15 minutes at 60°C to allow the enzyme to break down the starch. To stop the reaction, 2 mL of a 3,5-dinitro salicylic acid (DNS) reagent was added to each tube. Control samples were prepared by adding the DNS reagent before introducing the crude enzyme, while blank samples received the DNS reagent before adding both the enzyme and the substrate. All tubes were then boiled for 15 minutes to develop color and cooled to room temperature. The amount of reducing sugars (products of starch breakdown) was measured using a spectrophotometer at a wavelength of 540 nm. In this assay, one unit of amylase activity is defined as the amount of enzyme required to produce 1 micromole of reducing sugars, specifically in the form of maltose, per minute under the specified reaction conditions.

Effect of pH Alteration against Enzyme Stability

To find out the stability of crude amylase in varied pH, the enzyme solution was stored for 24 h at 4°C with the following pH buffers; sodium acetate buffer pH 5.0, phosphate buffer pH 6-8, and glycine NaOH buffer pH 9-12. After that, the activity of crude amylase was measured after incubation at 60°C at pH 7 for 15 minutes, as previously described (Satrimafitrah, *et al.*, 2020). The enzyme stability is determined by calculating the difference in enzyme activity before and after 24 h storage.

Effect of Increased Temperature against Enzyme Stability

The effect of temperature on the stability of the crude amylase enzyme was tested by incubating the crude extract at temperatures of 50, 60, 70, 80, 90, and 100°C for 2 hours and then immediately storing it in ice cubes. After that, the activity of crude amylase was measured after incubation at 60°C at pH 7, for 15 minutes as previously described (Satrimafitrah, *et al.*, 2020). The enzyme stability is determined by calculating the difference in enzyme activity before and after 2 h of elevated heating.

RESULTS AND DISCUSSION

In the previous study, we screened 30 potential isolates that have promising amylolytic properties and among them, the *Bacillus* sp., PL-16 had the highest activity (Satrimafitrah, *et. al*,. 2020). Furthermore, we optimized the incubation condition to 48 h and agitation speed to 180 rpm which produced the highest amylase activity (Satrimafitrah, *et. al.*, 2021). However, to

understand further the characteristics of our crude enzyme for future utilization in industrial applications, it is important to find out its capability to maintain stability during certain conditions, such as pH and temperature alterations. Our fresh crude amylase enzyme worked optimally at pH 8 and a temperature of 80°C, nevertheless, how it maintained optimal activity after a certain storage time and elevated temperature remains unclear. To reveal that, we stored fresh crude PL-16 amylase enzymes for one day at 4°C, in varied pH buffers, and concurrently, at elevated temperatures for 2 h, then we calculated enzyme activities before and after storage.

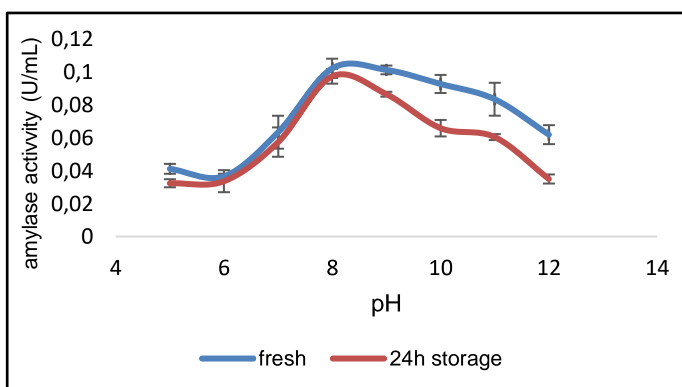

Figure 1. Activity of crude amylase PL-16 in varied pH before (blue line) and after (red line) 24 h storage.

Enzyme activity before and after 24 h storage at various pH is shown in Figure 1. Both enzyme activities are optimum at pH 8 and slightly decrease after reaching pH 9 to 12. Twenty-four-hour storage crude enzyme loss 5 % of activity at pH 8, and decreased to 14-28% at pH 9-11. One day incubation in specific pH exhibits a moderate reduction of enzyme activity indicating the capability of crude enzyme to maintain structure stability.

Another *Bacillus* sp. from Hotspring at Jordan that produced amylase with an optimum pH of 8.5 was able to maintain 64% of original activity after 2 h stored at pH 6 (Carvalho, *et. al.*, 2008), α-amylase from thermophilic *Bacillus* sp. Ferdowsicous has maximum activity at pH 4.5, was stable above 75% at pH 4-7.5, and decreased dramatically at higher pH (Asoodeh, *et. al.*, 2010), and purified α-amylase from *Geobacillus* sp. TF14 preserved 70% and 50% stability at its optimum pH 5 and 9 following 48 h incubation (Keskin and Ertunga, 2017). Meanwhile, despite of decreasing of decreasing stability to 70% at pH 10-11, our crude amylase retained 95% stability at optimum pH 8 after 24 h incubation, indicating promising stability properties. Furthermore, it is also more stable at acidic pH, suggesting that our enzyme is acidophilic amylase (Figure 2).

Enzymatic reactions rely on the pH of surrounding solutions. Enzyme active sites, composed of amino acids with charged groups (carboxyl, amino, thiol, imidazole, phenolic hydroxyl, etc.), are highly sensitive to pH changes. These changes influence the ionization state of the amino acid residues, consequently affecting the structure of the active site, its binding affinity for the substrate, and, ultimately, catalytic activity (Miyanaga and Uno, 2011).

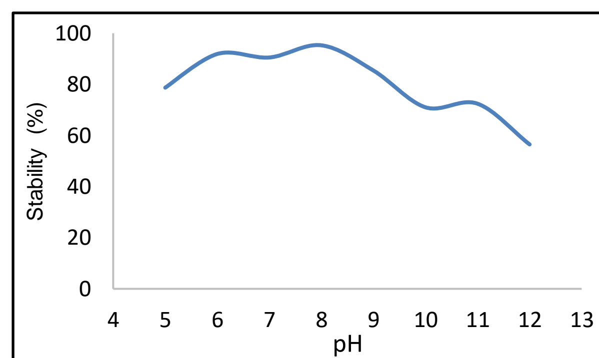

Figure 2. Stability of crude amylase PL-33 at varied pH

The reveal of how our crude enzyme retains activity at certain temperature alterations, we heated it at 50-100°C for 2 h prior to activity measurement, and freshly extracted crude enzyme was used as control. The results showed that the crude enzyme was quite stable in maintaining activity with declining activity of 16% ,15%, and 13% at temperatures of 50-80°C, while it lost 50% activity at 100°C (Figure 3). The crude enzyme retains 83-86% of stability at 60-80°C and declines to 50% at 100°C, indicating that our crude amylase is quite stable in retaining activity even after 2 hours storage at increasing temperatures (Figure 4.).

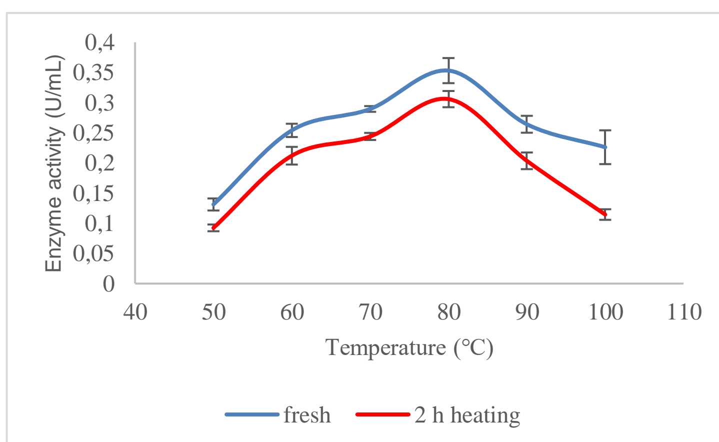

Figure 3. Activity of crude amylase PL-16 in varied temperatures before (blue line) and after (red line) 2 h heating.

Numerous stability studies of thermophilic amylases from *Bacillus* sp have been reported. Free amylase enzyme from *Geobacillus* sp. TF14 retain nearly all activity after 48 h incubation, and almost all activity was lost at 72 h and 90°C (Keskin and Ertunga, 2017); amylase from a *Bacillus* sp. isolate A3-15 maintained 95% stability after 100°C for 30 minutes (Arikan, 2008), purified amylase from *Bacillus licheniformis* So-B3 retained 66.7% stability after 2 h preincubation at 70°C (Fincan, *et al*., 2021), amylases from *B. licheniformis* and *Bacillus cereus* respectively drop stability to 70% and 100% following 75°C (Rakaz, *et al*., 2021), and alpha-amylase from Extremophile *Bacillus* sp. FW2 retains 83% of stability at 45°C and 32 % at 75°C after 12 h preincubation (Pham, *et al*., 2021). Even though our crude amylase enzyme PL-16 exhibited higher stability compared to some enzymes aforementioned above, it is an impurified enzyme that certainly contains several amylases, which probably act together in maintaining enzyme activities. Furthermore, due to different experimental procedure, it is also burdensome to compare them. Usually, concentrated enzymes are more stable than diluted ones, as a result of reactions of non-covalent intermolecules, which leads to sensitivity to heat (Hameed and Ul-Haq, 2020).

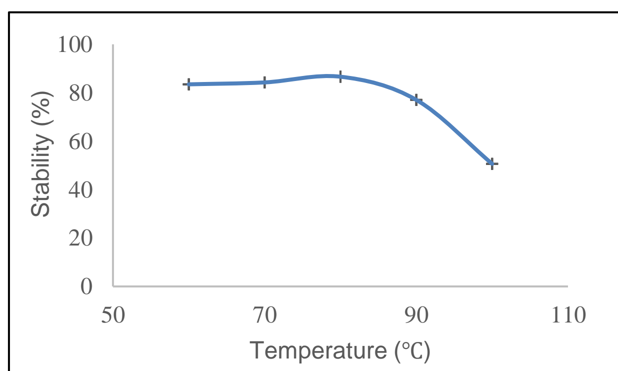

Figure 3. Activity of crude amylase PL-16 in varied temperatures before (blue line) and after (red line) 2 h heating.

Enzymes are functional protein, biological catalysts comprised of polypeptide chains folded into intricate three-dimensional conformations known as tertiary structures. This conformation is critical for their catalytic function, as it orchestrates the formation of a specialized microenvironment termed the active site. The active site exhibits a complementary geometry to the substrate molecule, facilitating specific interactions. However, exposure to elevated temperatures disrupts the non-covalent interactions that stabilize the protein's tertiary structure. This disruption unfolds or denatures the protein, resulting in a loss of its characteristic three-dimensional shape and rendering the active site non-functional (Lewis and Stone. 2023). The specific 3D- structure of amylase enzyme depends on multiple chemical interactions (ionic bonds, hydrogen bonds, and hydrophobic interactions) of certain amino acid side chains inside enzyme. Heat alteration disturbs interaction and leads to unfolding protein structure, and in the end, disrupts stability. (Judge and Dodd, 2020).

CONCLUSION

Crude amylase PL-16 isolated from thermophilic Bacillus sp. is a promising candidate for industrial application, since it has good stability in the acidic to neutral pH range and at higher temperatures. However, this crude enzyme still needs further investigation to reveal, and most importantly, undergoing a purification process to understand its properties better.

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