

Screening and Isolation of Thermophilic Amylase-Producing Bacteria from Ulubelu Hot Springs in Tanggamus Lampung

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Abstract

Thermophilic bacteria generally thrive in hot springs and produce thermostable enzymes. These enzymes retain catalytic activity at high temperatures and play an important role in various industrial applications. This study aims to conduct screening, isolation, and characterization of thermophilic amylase-producing bacteria from the Ulubelu hot spring located in Tanggamus Regency, Lampung. Hot spring water samples from a location with a temperature of 60 °C and pH 5.0 yielded eight bacterial isolates with amylolytic activity, by the formation of clear zones at the bacterial growth sites on selective LB medium containing starch. Among the isolates obtained, Ulb A3.7 exhibited the largest clear zone diameter and was therefore selected for further characterization. Morphological and physiological analyses revealed that Ulb A3.7 is a Gram-negative rod-shaped bacterium. The Ulb A3.7 isolate tested positive for oxidative-fermentative metabolism, oxidase activity, and glucose utilization. The crude α -amylase from Ulb A3.7 exhibited optimum activity at 80 °C, with a value of 254.75 U/mL, and demonstrated a broad activity range from 50 °C to 100 °C. The enzyme was able to retain its amylolytic activity for up to 300 minutes of incubation, indicating its potential for application in high-temperature industrial processes.

Keywords: α -amylase, hot spring, thermostable

1 Introduction

Enzymes are biological catalysts with high specificity that catalyze the conversion of substrates into products. Due to their environmentally favorable properties, enzymes have increasingly replaced chemical catalysts in industrial processes, resulting in decreased greenhouse gas emissions and reduced energy consumption [1]. Approximately 5–6% of the 3,000 known enzymes are currently utilized in industrial applications [2]. These enzymes are derived from various sources, including animals and plants; however, the majority are obtained from microbes due to their rapid growth, high enzyme production, and ease of genetic modification to enhance functional properties.

Among industrial applications, the starch industry represents a major field of enzyme utilization, accounting for approximately 15–20% of global enzyme usage, with α -amylase as the primary enzyme [3, 4]. α -Amylase (EC 3.2.1.1) is an endoamylase that catalyzes the hydrolysis of α -1,4 glycosidic bonds in starch and related polysaccharides, producing shorter oligosaccharides [5]. Thermostable variants of this enzyme have attracted considerable attention because of their ability to retain catalytic activity and stability at elevated temperatures, making them highly suitable for high-temperature industrial processes [6, 7]. The thermostable α -amylase remain active at high temperatures, above

50 °C and can function efficiently even at 80–90 °C or higher [8].

Recent developments in enzyme research have highlighted the potential of thermostable enzymes from thermophilic microorganisms to address the limitations of conventional enzymes under harsh industrial conditions [3, 9]. These thermophilic microorganisms possess unique metabolic and physiological adaptations that enable them to produce thermostable enzymes capable of functioning efficiently under extreme environmental conditions [10, 11]. This thermostable nature is also commonly found in enzymes from thermophilic bacteria, which have adapted to natural hot environments such as volcanic soils and hot springs [12, 13].

Exploration of thermophilic bacteria from hot springs as the potential sources of thermostable α -amylase has become a key focus in enzyme screening, where isolates are evaluated for their ability to produce heat-stable amylase. Numerous geothermal sites across Indonesia—such as those in Padang, Riau, Minahasa, Lampung, West Java, Malang, and Central Sulawesi—have been identified as promising reservoirs of thermophilic microbial communities capable of producing thermostable enzymes [14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24]. Lampung hosts several hot springs, including those in Natar, Ulubelu, and Kalianda. The Ulubelu hot springs, with temperatures ranging from 60 °C to 90 °C, provide a suitable environment for thermophilic bacteria. This study aims to screen and isolate thermophilic α -amylase-producing bacteria from the Ulubelu hot springs in Tanggamus Regency, Lampung.

2 Method

The research was conducted in two main stages. The first stage involved sampling from hot springs, isolation of thermophilic bacteria, and screening for amylase-producing bacteria. The second stage focused on α -amylase production and biochemical characterization, including determination of the optimum temperature and pH, as well as thermostability. All reagents used in this study were of analytical grade and purchased from reputable commercial suppliers.

Samples were collected from the three distinct sites within the Ulubelu hot springs, Tanggamus, Indonesia. On-site measurements of temperature and pH were recorded, and samples were immediately stored in sealed thermos flasks for transport to the Laboratory of Biochemistry Research Group at Institut Teknologi Sumatera. Upon arrival, samples were incubated at 50 °C and

agitated at 150 rpm for 24 hours. Bacterial isolation was performed by culturing the samples in Luria Bertani (LB) broth containing 1% tryptone (HiMedia, India), 1% NaCl (Merck, Germany), and 0.5% yeast extract (Criterion, USA), followed by plating onto LB agar supplemented with 2% bacto agar (HiMedia, India) to obtain single colonies through successive subculturing. α -Amylase-producing isolates were screened by inoculating the strains onto LB agar plates supplemented with 1% soluble starch (Merck, Germany), incubated at 50 °C for 48 hours, and subsequently overlaid with 0.3% iodine solution to visualize hydrolysis zones indicative of amylolytic activity. Selected isolates were further characterized based on morphological and physiological properties through biochemical assays, including the methyl red test, oxidative-fermentative test, protease assay, and oxidase assay. These analyses were conducted at the Balai Veteriner, Bandar Lampung.

The selected isolate, Ulb A3.7, was cultivated in 5 mL starter LB-medium, and the actively growing culture was transferred into 50 mL production medium, followed by incubation at 50 °C and 150 rpm for 24 hours. After incubation, the culture was centrifuged at 3000 rpm for 20 minutes to separate the extracellular enzyme (supernatant) and intracellular enzyme (pellet). To evaluate amylase activity, a glucose standard curve was constructed using glucose solutions of 0.5–2.5 mM, each mixed with 1 mL dinitrosalicylic acid (DNS) reagent (HiMedia, India), incubated at 50 °C for 10 minutes, and absorbance measured at 500 nm. The DNS assay quantifies reducing sugars released from starch hydrolysis. Under alkaline and high-temperature conditions, reducing sugars such as glucose reduce 3,5-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid, producing a reddish-brown complex measurable at 500 nm. The intensity of the color is directly proportional to the concentration of reducing sugars produced by α -amylase activity. One unit of α -amylase activity is defined as the amount of enzyme capable of producing 1 mmol of reducing sugars (as glucose) per minute under the specified assay conditions. Amylolytic activity was tested under various temperatures (50–100 °C) using 200 μ L extracellular enzyme and 200 μ L of 1% soluble starch, with each component preincubated for 1 minute at the test temperature. After 10-minute incubation, 400 μ L DNS was added to stop the reaction, followed by heating at 90–100 °C for 10 minutes, dilution with 1.2 mL distilled water,

centrifugation (3000 rpm, 10 min), and absorbance reading at 500 nm. The optimum temperature was determined by comparing enzyme activity across the temperature treatments. A similar approach was used for pH optimization at pH 4.0, 7.0, and 10.0, using buffer-dissolved starch. The reactions were performed in 50 mM acetate buffer (pH 4.0), 50 mM phosphate buffer (pH 7.0), and 50 mM Tris-Cl buffer (pH 10.0). Enzyme thermostability was assessed at the determined optimum temperature over incubation times ranging from 60 to 300 minutes, with residual activity measured using the DNS method as described. All experiments were performed in triplicate sets. The values were determined and are presented as the mean $\pm$ standard deviation. Statistical significance between groups was evaluated using a two-sample t-test, with $p < 0.05$ considered statistically significant.

3 Result and Discussion

Samples were collected from the Ulubelu hot spring in Tanggamus, Lampung, Indonesia, located at coordinates 5°21'34.2"S 104°35'59.9"E. Sampling was carried out at three different points, where the water temperature and pH were measured on-site: Point 1 (A1) had a temperature of 90 °C and pH 3.0, Point 2 (A2) had a temperature of 60 °C and pH 6.0, and Point 3 (A3) had a temperature of 60 °C and pH 5.0.

Based on the isolation results from sample point 3 (A3), a total of eight distinct bacterial isolates were obtained, designated as Ulb A3.1 through Ulb A3.8. Bacteria that are able to grow at temperatures ranging from 50 °C to 90 °C are classified as thermophilic. All isolates demonstrated growth at 50 °C, indicating their thermophilic nature. Growth at this temperature suggests these bacteria are adapted to moderately high-temperature environments [25]. Morphological observations revealed that the colonies were circular, slightly convex, white in appearance, and well-separated from each other.

Screening of thermophilic α -amylase-producing bacteria was indicated by the formation of clear zones at the bacterial growth sites on selective LB medium containing starch. These clear zones, which appeared after the application of KI/I₂ solution, suggested starch hydrolysis by thermophilic bacterial isolates. As shown in Figure 1, isolate Ulb A3.7 exhibited the largest clear zone among all tested isolates, indicating its highest starch-degrading ability and therefore it was selected for further analysis of its crude α -amylase activity.



Figure 1. Screening of α -amylase-producing bacteria

Morphological characterization of the α -amylase-producing bacterial isolate was performed using Gram staining. The isolate was identified as Gram-negative with rod-shaped colony morphology (Figure 2). Isolate Ulb A3.7 tested positive for both oxidative and fermentative metabolism as well as oxidase activity.



Figure 2. Gram staining of α -amylase-producing bacteria Ulb A3.7

Based on the production results shown in Figure 3, the medium inoculated with Ulb A3.7 appeared turbid, indicating active bacterial growth. The optical density (OD) measurement yielded a value of 0.778, suggesting a high cell density that may be associated with enhanced amylase production depending on the growth phase and culture conditions.



Figure 3. α -Amylase production by Ulb A3.7 isolate

As shown in Figure 4, Ulb A3.7 exhibited increasing amyolytic activity from 50 °C, reaching an optimum at 80 °C with a maximum activity of 254.75 U/mL. Although a slight decrease was observed at 90 °C and 100 °C (Figure 4A), the enzyme remained substantially active across the entire temperature range of 50–100 °C. The optimum pH for its activity was found to be pH 4.0, at which the enzyme achieved 254.38 U/mL of activity (Figure 4B). While the activity gradually declined at higher pH values, the enzyme retained considerable activity across a broad pH range of 4.0–10.0. These findings indicate that Ulb A3.7 amylase functions most effectively under acidic conditions and exhibits remarkable thermal tolerance, likely reflecting the physicochemical characteristics of its native habitat, which is estimated to have a temperature of around 60 °C and a pH of approximately 5.0.

Compared to previously reported α -amylases, Ulb A3.7 demonstrates a notably higher temperature optimum. For instance, the crude α -amylase from *Bacillus licheniformis* BT5.9 exhibited an optimum temperature of 50 °C and pH 5.0 [20], while the crude enzyme from BR002 and BR015 isolates showed activity optimum at around 50 °C and 70 °C, with pH optimum of 8.0 [21]. Similarly, *Geobacillus* sp. DS3, although purified, showed optimum activity at 50 °C and pH 7.0 [19]. Another thermophilic isolate, *Anoxybacillus thermarum* FRM-RBK0, showed optimum activity at 80 °C and pH 7.0 using a crude enzyme [22] closer to Ulb A3.7 in terms of temperature but still differing in pH. Partially purified α -amylase from isolate HAT1 had an optimum temperature of 70 °C and pH 6.5 [23], and another reported crude α -amylase had an optimum temperature of 60 °C [18]. These comparisons highlight that Ulb A3.7 not only

functions effectively at a higher temperature but also prefers more acidic conditions than most previously studied isolates, indicating its potential for applications in acidic starch hydrolysis processes such as glucose or fructose syrup production, bakery fermentation, and other food processing operations requiring low-pH conditions [8].

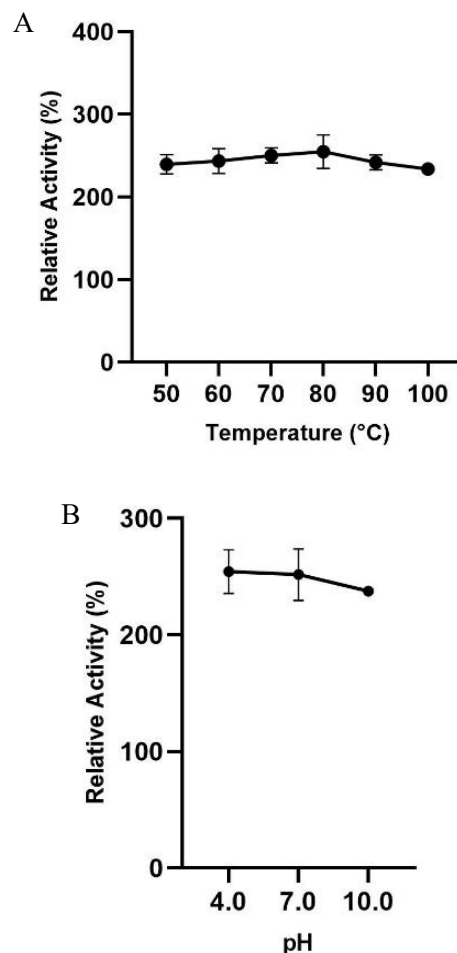
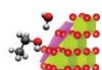


Figure 4. The hydrolytic activity profiles of Ulb A3.7 toward soluble starch are depicted in different temperatures (A) and pH levels (B)

The optimum stability of amyolytic activity from the Ulb A3.7 isolate was observed at 120 minutes of incubation at 80 °C, with the highest recorded activity of 261.08 U/mL (Figure 6). This indicates that the enzyme maintains its maximum stability and functionality at this incubation period under elevated temperature conditions. The enzyme exhibited relatively stable amyolytic activity up to 300 minutes of incubation at 80 °C. Statistical analysis using a two-sample t-test indicated no significant difference in enzyme activity across the tested incubation times,



suggesting that the observed variations were not statistically significant. Although the structural features of Ulb A3.7 remain uncharacterized and the enzyme has not yet been produced recombinantly, its ability to retain activity at 80 °C for up to 300 minutes suggests the presence of inherent thermostabilizing characteristics. These may include a high proportion of hydrophobic or charged residues, salt bridges, or other stabilizing interactions commonly found in thermophilic enzymes [26].

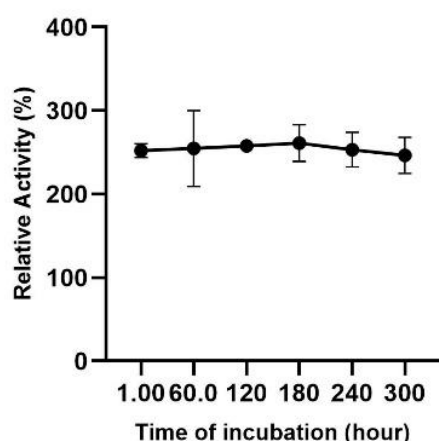


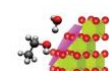
Figure 5. Thermal stability of amylase activity from the Ulb A3.7 isolate at 80°C at various incubation times

4 Conclusion

Thermophilic amylase-producing bacteria were successfully isolated from Ulubelu hot spring, Lampung, with eight distinct colonies obtained. The Ulb A3.7 isolate is a Gram-negative rod-shaped bacterium. The crude enzyme of Ulb A3.7 showed stable amylolytic activity across 50–100 °C, and remained active across a wide incubation time and pH range. Further studies using 16S rRNA sequencing are recommended to confirm its genetic identity.

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