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ISOLATION AND CHARACTERIZATION OF THERMOPHILIC BACTERIA AS AMYLASE ENZYME PRODUCED BY HOT SPRING IN RIANIATE SAMOSIR, INDONESIA

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ABSTRACT

Thermostable enzymes (such as amylase) are enzymes with great potential to overcome industrial technical problems associated with high-temperature processes. This type of enzyme can be isolated from thermophilic bacteria obtained from hot springs, such as hot springs Rianiate, Samosir. The objectives of this study were to obtain isolates, morphological and biochemical characterization, followed by determining the potential of selected isolates qualitatively. Hot water samples were determined by purposive sampling from two location points, with temperatures 40 °C, 43 °C and 45 °C with a pH of 4. Isolation of thermophilic bacteria using Nutrient Agar (NA) media. There were 12 thermophilic bacterial isolates from two sample locations isolated with the distribution of 2 isolates at 40 °C and 10 isolates at 43 °C, while at 45 °C there were no isolates. The twelve isolates were able to produce an amylase enzyme. The amylase enzyme production was determined by the presence of a clear zone around the colony on the starch agar medium after being dripped with iodine solution. The three isolates that produced the largest clear zone were sp1(2), sp3(1) and sp6, namely 83.1 mm, 79.9 mm and 80.2 mm, respectively. A biochemical test was carried out on isolates with the largest clear zone diameter value. The characterization results showed that the isolate was predicted to belong to the genus *Bacillus* sp. These results indicate that the thermophilic bacteria from the hot springs of Rianiate, Samosir have the potential to produce amylase enzymes.

Keywords: Amylase Enzyme, Hot Springs, Rianiate, Thermophilic Bacteria.

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INTRODUCTION

Enzymes play an important role in the industrial world such as the textile industry, detergents, food and beverage ingredients, chemicals, medicines and the leather industry.^{1,2,3,4,5,6} One type of enzyme that is produced by many microorganisms is the amylase enzyme. Amylase enzymes have a very wide distribution and are one of the most studied types of enzymes. This enzyme has applications on a very wide scale ranging from the textile industry to a very wide range of tests.⁷ Amylase demand in the world is very high, in 2004 alone, sales reached the US \$ 2 billion, while amylase used for the food and beverage industry in 2004 was valued at around the US \$ 11.⁸

To obtain enzymes that are resistant to high temperatures, it is necessary to screen microorganisms that can produce thermostable enzymes from various natural sources, such as hot springs.⁹ The use of enzymes derived from microorganisms is generally in great demand because it has stability at high temperatures and can be produced in large quantities¹⁰. Biological processes when operated at high temperatures will reduce the risk of contamination by other organisms.¹¹⁻¹³ Thermophile microbes can produce thermophile enzymes so that enzymatic reactions can run faster, accelerate diffusion, increase the solubility of materials, reduce the viscosity and surface tension of the media.¹⁴ This study aims to obtain thermophilic bacterial isolates that have the potential to produce amylase enzymes that have activity at high temperatures.

EXPERIMENTAL

Material

The samples used were hot water from Rianiate hot springs, nutrient agar (NA), yeast extract, NaCl 0.9% (Wida NS), agar flour, lugol (Merck), starch, Bacto Peptone (Merck), iodine solution (Merck). Malachite

Green 5% (Sigma Aldrich), Crystal Violet (Merck), Safranin (Sigma Aldrich), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 3% H_2O_2 solution (Merck).

Hot Water Sampling

Hot water samples were taken using the Sianturi method.¹⁵ Water samples suspected to contain thermophilic bacteria were taken from two different point groups, namely temperatures of 40°C, 43°C and 45°C. A total of 100 mL of sample was taken using a sterile dipper from the surface and then put into a hot water flask to maintain temperature stability. The sample was taken to the Microbiology Laboratory of the State University of Medan for the isolation process.

Sample Isolation of Hot Water

The sample in a thermos was shaken to make it homogeneous, then 1 ml was taken and poured into a petri dish containing agar nutrients which were still liquid. Next, shake the petri dish so that the suspension is even in the medium. After freezing, the petri dish was incubated at a growing temperature of 45°C for 24 hours. The growing bacterial colonies were inoculated back into a sterile petri dish containing the same medium using the quadrant method, then incubated at 45°C for 24 hours until pure single colonies grew.

The Screening Thermophilic Bacteria of Amilas Enzyme-Producing

The pure thermophilic bacterial isolates were grown on amylolytic selective bacterial media for 24 hours. Then the growing isolates were tested in the form of a suspension. The suspension is made by taking 1-2 ose of bacterial culture isolates that are 1-day old into a sterile test tube containing 0.85% physiological NaCl solution. The mixture was homogenized with vortex, the turbidity of the mixture was compared to the turbidity of Mac Farland Solution scale 1 which was equivalent to $3.108 \text{ CFU} / \text{ml}$. A total of 0.1 ml of bacterial suspension was pipette on paper discs on starch agar medium. The cultures were incubated for 24 hours at 45°C. The growing bacterial isolates were dripped with iodine solution to select amylase-producing bacteria. Isolates that produce amylase are indicated by the presence of a clear zone around the bacterial colony.¹⁶

The Morphological Characterization of Macroscopic Thermophilic Bacterial Isolates

Bacterial isolates that have the potential to produce amylase enzymes are then grown back in Nutrient Agar (NA) media for 24 hours at 45°C using the free scouring method. Each isolate occupies a different petri dish to facilitate macroscopic observation. Furthermore, the bacterial colony morphology was observed including the shape, color, edge and elevation of the bacteria.

Microscopic Observation with Gram Stain

Pure and 24-hour old isolate cultures were taken using a loop needle, then smeared preparations (on a glass object) were made using distilled water and then dried. Then it is fixed on top of the bunsen. The preparation is then dripped with a solution of gram A (Crystal violet) and left for 3 minutes, then rinsed with running water. Then the preparations are dripped again with alcohol until the remaining dye is gone (about 30 seconds), rinsed again with running water. At the final stage, the preparation is dripped with salt solution C (safranin) and allowed to dry. Before the observation is carried out, the preparation is first dripped with immersion oil, then the observation is carried out under a microscope with a magnification of 100x.

Catalase Test

The catalase test was carried out using a 3% H_2O_2 solution which was dropped on a glass slide containing pure bacterial isolates. The test was positive if air bubbles are seen.

Endospore Staining

Pure cultures of amylolytic bacteria were taken slightly aseptically using a loop needle and suspended with sterile distilled water in a glass slide, then the preparations were fixed over a bunsen fire. The preparation was dripped with Malachit green. The preparation was placed on a wire that has been heated over boiling water for 10 minutes. The preparation was washed carefully under running water. The preparations were

dripped with safranin, stand for 30 seconds, then washed using running water and dried carefully. The preparations were observed under a microscope. The test was positive if the vegetative cells are red and the spores are green.

RESULTS AND DISCUSSION

Thermophilic Bacteria Isolate from Rianiate Hot Spring, Samosir

From the isolation results, 12 bacterial isolates from different temperatures were obtained consisting of two isolates at 40°C, ten isolates at 43°C, while at 45°C there were no isolates (Table-1). The isolated bacterial obtained were transferred to the new Nutrient Agar (NA) medium in a quadrant until a single colony was obtained (Fig.-1) for further macroscopic and microscopic observations. Besides of the culture stock was also made on slant media with the same media and incubated at the same temperature. After the isolates have grown, the cultures were stored in a refrigerator for further testing.

Table-1: Distribution Isolates of Bacterial Thermophilic the Results Isolation from Three Sample Points

Sample Points	pH	Temperature	Number of Isolates	Observation of Bacterial Growth
I	4	40 °C	2	++
II		43 °C	10	++++
III		45 °C	0	-

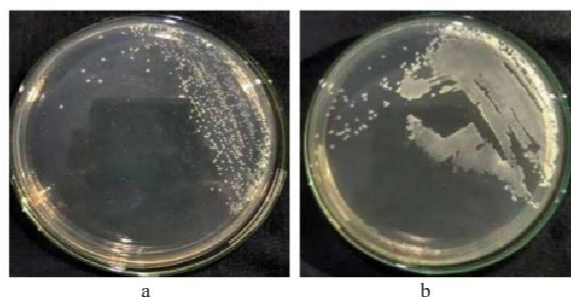


Fig.-1: Single Isolate Obtained by Quadrant Method. (a) Isolate sp4, and (b) Isolate sp6.

The difference in bacterial growth was caused by the ability of bacteria to adapt to the environment where they grow. At sample point, I was a shower that flows directly from a hot spring. Sand and rocks are the basis for the samples made to surround this fountain. Sample point II was the flow of hot springs that are not collected and water directly from the ground to form a puddle. This second sample point is not used for bathing at all. Meanwhile, sample point III was a pond made with a certain size and coated with ceramics. The presence of biotic components can support the growth of thermophilic microorganisms. Dimawan et al. reported that fallen leaves, twigs, grass seeds, pollen, and insect carcasses around hot springs are organic materials that can be utilized by microorganisms that live in hot springs.¹⁷

The Screening Thermophilic Bacteria of Amilas Enzyme-Producing

This stage aims to determine the activity of the amylase enzyme produced by thermophilic bacterial isolates. This activity was shown by the ability of bacteria to hydrolyze starch. Colonies that are capable of hydrolyzing starch will form a clear zone around the colony after being dripped with iodine solution (Fig.-2). The clear zone of 11 isolates was measured using a caliper. The results of measuring the clear zone diameter were shown in Table-2.

Table-2: Diameter of the Clear Zone of Amylase-Producing Thermophilic Bacteria from Rianiate Hot Springs

Isolate Code	Clear of Zone Diameter (mm)
Sp1	0.1
Sp2(2)	83.1
Sp2(1)	20.5
Sp3(1)	79.9
Sp3(2)	57.3

Sp4	4.3
Sp5	3.1
Sp6	80.2
Sp8	27.6
Sp10(1)	31.7
Sp10(2)	0.7

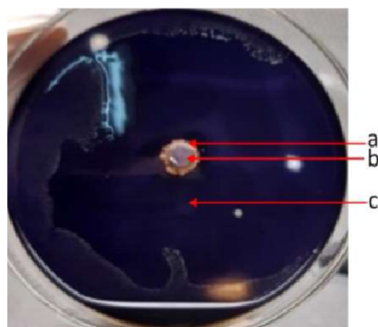


Fig.-2: Screening of Amylase-producing Thermophilic Bacteria Isolate sp6 from Hot Springs Rianiate, Samosir. (a) Clear Zone; (b) Thermophilic Bacterial Colonies; and (c) Starch Agar Medium dripped with Iodine Solution

There were 3 isolates with the largest diameter, namely isolates sp1 (2), sp3 (1) and sp6. The clear zone formed from each isolate was different. This was because the ability of each isolate to hydrolyze starch also varies. The environment and the genes possessed by bacteria were factors that affect the ability of the isolates to hydrolyze starch. According to Raharjo et al. the clear zone occurs because the amylase enzyme secreted by bacterial cells hydrolyzes the starch molecules around them so that a starch-iod complex is formed with a blue-black color.¹⁸

Results of Macroscopic Morphological Characterization of Isolates Bacterial Thermophilic

The pure cultures were grown on Nutrient Agar media with free streaks where each isolate was grown on different petridishes to facilitate macroscopic observation. Colony characterization showed that the shape varied from round, round with raised edges, irregular, diffuse, and round with shell edges. The colony colors were yellow and white. The edges of the colonies are smooth, grooved, and irregular, while the elevation types were raised, flat, convex, and grow into the medium (Table-3). Ochman et al. reported that the phenotypic characteristics of bacteria were not static, so it was possible that the same bacteria can show different morphological characteristics.¹⁹

Microscopic Characterization of Thymophilic Bactrery Isolates by Gram Staining

The results of the microscopic characterization of thermophilic bacterial isolates with gram staining indicated that most isolates had the form of bacilli cells (Table-4), were red (Fig.-3) and all isolates were gram-negative bacteria (Table-4).

Table-3: Results of Macroscopic Morphological Characterization of Thermophilic Bacterial Isolates from Hot Springs of Rianiate, Samosir

Isolate Code	Colony of Color	Colony Forms	Colony Elevation	Colony Edge
Sp1	Yellow	Round with embossed edges	Arises	Slippery
Sp1(2)	White	Round	Arises	Slippery
Sp2(1)	Yellow	Irregular and diffuse	Flat	Notched
Sp3(1)	Yellow	Round	Convex	Slippery
Sp3(2)	White	Round	Flat	Slippery
Sp4	Yellow	Round with embossed edges	Flat	Notched
Sp5	White	Round	Convex	Irregular

Sp6	White	Round with embossed edges	Flat	Notched
Sp8	White	Round	Flat	Irregular
Sp10(1)	Yellow	Round	Arises	Slippery
Sp10(2)	White	Round with clam edges	Grow into medium	Notched
Sp11(1)	Yellow	Round with embossed edges	Arises	Slippery
Sp11(2)	White	Round	Arises	Notched

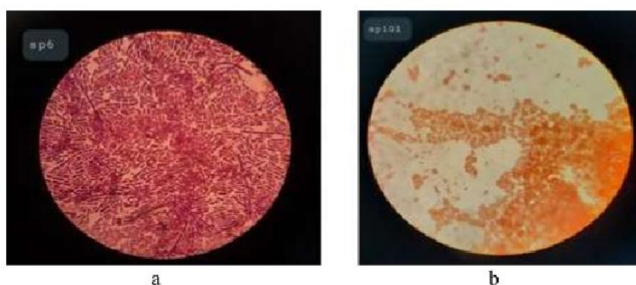


Fig.-3: Gram Staining Test Results. (a) Isolate sp10 (1) and (b) Isolate sp6.

Table-4: Microscopic Observation of Thermophilic Bacterial Isolates by Gram Staining

Isolate Code	Isolate Form	Gram
Sp1	Basil	Negative
Sp1(2)	Basil	Negative
Sp2(1)	Basil	Negative
Sp3(1)	Basil	Negative
Sp3(2)	Basil	Negative
Sp4	Basil	Negative
Sp5	Cocci	Negative
Sp6	Basil	Negative
Sp8	Basil	Negative
Sp10(1)	Cocci	Negative
Sp10(2)	Cocci	Negative
Sp11(1)	Basil	Negative
Sp11(2)	Basil	Negative

Biochemical Test Results By Using Catalase

The biochemical test by using catalase aims to determine the catalase enzyme produced by thermophilic bacteria. Thermophilic bacterial isolate catalase test results showed positive results. It was characterized by the formation of bubbles which were oxygen gas when introduced into a tube containing 3% Hydrogen Peroxide (Fig.-4). The same thing was reported by Silaban et al. where after the addition of H_2O_2 solution gas bubbles form, this indicates that the bacteria are positive for the amylase enzyme.²⁰

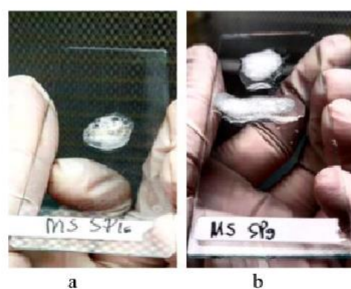


Fig.-4. Catalase Test Results (a) Isolate sp6 and (b) Isolate sp9.

Endospore Staining Results

The pure culture was tested by endospore staining. This staining principle was used to differentiate spores from vegetative cells. The dye most often used to color spores was malachite green (Schaeffer and Fulton) which remains bound to the spores after washing with water, and safranin is used as a "counterstain". In this way, the endospores that were still present in vegetative cells and free spores will be green-blue, while vegetative cells will be red to pink. From the results of endospore staining, it was found that the thirteen thermophilic bacterial isolates that had been isolated positively formed endospores (Table-5). A positive result was shown if the vegetative cells are red and the endospores are green (Fig.-5).

The endospores produced by *Bacillus* aim was to protect thermophilic bacteria from unfavorable environmental conditions such as drought, nutrient deficiency, freezing, and chemicals. This type of bacterial endospores is resistant to environmental changes, is resistant to heat, and certain chemical disinfectants for a long time. If the environment is good, the endospores will experience sporogenesis and from vegetative cells.

Table-5: Observation of Endospore Staining of Thermophilic Bacteria

Isolate Code	Isolate Form
Sp1	+
Sp1(2)	+
Sp2(1)	+
Sp3(1)	+
Sp3(2)	+
Sp4	+
Sp5	+
Sp6	+
Sp8	+
Sp10(1)	+
Sp10(2)	+
Sp11(1)	+
Sp11(2)	+

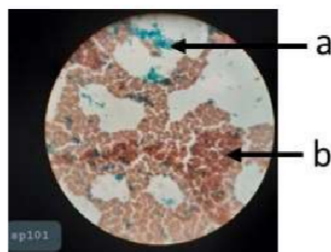


Fig.-5: Endospore Staining Test Results. (a) Endospores and (b) Vegetative Cells.

CONCLUSION

There were 13 isolates of thermophilic bacterial culture which were amylo thermophilic with the largest clear zone diameter, namely isolates sp1 (2), sp3 (1) and sp6 with diameters of 83.1 mm, 79.9 mm and 80.2 mm, respectively. Thermophilic bacteria from Rianiate hot springs, Samosir have the potential to produce amylase enzymes.

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