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Isolation, Screening and Partial Characterization of Thermophilic Bacteria Producing Protease from Bukik Gadang Hot Springs, Solok Regency

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Abstract – Research about the isolation, screening and partial characterization of thermophilic bacteria that produce protease from Bukik Gadang hot springs, Solok Regency, was carried out at the Basic Biology Laboratory, UPT Basic and Central Laboratory, Andalas University in January - April 2022. This study aims to obtain isolates of thermophilic bacteria that indicated protease production and to determine the partial characterization of thermophilic bacteria that indicated protease production from Bukik Gadang hot springs, Solok Regency. This study used a survey method and purposive sampling technique. The results of this study obtained 18 isolates from 20 isolates of thermophilic bacteria which indicated protease production, with one isolate of TPBG-03 having the potential to produce protease. Partial characterizationby 10 isolates of thermophilic bacteria indicated protease production was generally Gram positive, bacillus cell shape, spore-forming, catalase positive and motile.

Keywords – Hot Springs, Isolation, Protease, Screening, Thermophilic Bacteria.

I. INTRODUCTION

Enzymes are catalysts that can increase the speed of specific chemical reactions. Without enzymes, a chemical reaction will take place very slowly. Enzymes become biocatalysts that can support various industrial processes. This is because the enzyme has high efficiency and effectiveness, and the reaction does not cause by- products [1].

Protease enzymes are one class of enzyme that are crucial to the expansion of industry. In the industrial enzyme market, hydrolytic enzymes account for around 75% of sales, of which 60% are proteolytic enzymes. The economic value of the commercial enzyme protease is substantial, reaching up to 200 million US dollars annually. The development of the field of biotechnology can provide progress in increasing the production of protease enzymes and their applications, so that protease enzymes become a supporting factor in increasing the value of human life [2].

The application of protease enzymes in industry is very broad, including food and non-food industries such as detergents [3], meat tenderization and leather tanning. In industrial applications, enzymes are used by requiring enzymes that are resistant to heat or resistant to extreme environments. Because the main factor that can damage enzymes is temperature, an enzyme that is thermostable is needed. A protease is able to hydrolyze a protein into simpler compounds such as peptide bonds and amino acids. Protease enzymes can be found in various organisms, both prokaryotes and eukaryotes [4]. Thermophilic bacteria are one of these organisms and can create enzymes that can withstand high temperatures.

Thermophilic bacteria are able to survive and thrive in high temperature conditions due to their content of enzymes and

proteins that are more stable and heat-resistant. The lipid membrane of thermophilic cells contains many saturated fatty acids, which form very strong hydrophobic interactions and protein-synthesizing molecules (ribosomes and other components) are stable to heat. Thermophilic bacteria are one of the producers of thermostable enzymes that can be isolated from geothermal environments such as thermophilic hot springs because of their ability to adapt to extreme temperatures, with optimal temperatures ranging from 45°C - 80°C. Geothermal springs are good habitats for thermophilic bacteria [5].

Hot springs in West Sumatra can be found in several areas, one of which is in Solok Regency, namely the Bukik Gadang hot spring located in Bukik Gadang, Lembang Jaya District. This hot spring has the potential to act as a habitat for the growth of thermophilic bacteria that produce several enzymes, such as proteases. Field surveys that have been carried out at this location indicate that there are several hot spring points with temperatures ranging from 45°C - 60°C with pH intervals of 7 - 8. Around the hot springs there are rocks overgrown with moss and various types of vegetation, such as ferns, grasses, trees and litter. The remains of organisms that have died in hot spring locations, such as dry leaves, branches, and wood, can be used as a carbon source for bacteria. The Bukik Gadang hot spring has an alkaline pH and has the potential to have a greater diversity of bacteria compared to other hot springs. Therefore, it is necessary to conduct research on the isolation, screening and partial characterization of thermophilic bacteria producing protease from Bukik Gadang hot springs, Solok Regency.

II. RESEARCH METHODOLOGY

A. Sample Collection

Water samples were taken using the purposive sampling technique at 5 points in the Bukik Gadang hot spring, Solok Regency. Observed abiotic factors such as water temperature, air temperature, water pH and bioticfactors around hot water.

B. Isolation of Thermophilic Bacteria

Isolation is done by incubating the water in the sample bottle according to the temperature at the time of sampling for 1 hour. Then 1 mL of water in the sample bottle was taken, then pipetted into a petri dish and then 15 mL of Nutrient Agar (NA) medium was poured, then incubated at 50°C for 24 - 48 hours [6]. The method used is pour plate by pouring water sample first and followed by Nutrient Agar (NA) medium. Observed bacterial colonies that have grown such as color, shape, elevation, size and colony margins.

C. Purification of Thermophilic Bacteria

Bacteria were inoculated using a needle loop on each bacterial colony that grew differently in Nutrient Agar (NA) medium previously in another 20 petri dishes containing Nutrient Agar (NA) medium and scraping using the quadrant method, then the bacteria were incubated in an incubator for 24 hours at 50°C [6]. This is done to obtain pure cultures of thermophilic bacteria that live in hot springs.

D. Screening for Protease-Producing Thermophilic Bacteria

The thermophilic bacteria isolates were inoculated on a petri dish using an ose needle on 1% Skim Milk Agar (SMA) medium, then incubated in an incubator at 50°C for 24 - 48 hours. The diameter of the bacterial colonies and the clear zone formed around the bacterial colonies were measured with a caliper and then the Proteolytic Index (PI) was determined with the formula:

$$PI = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diameter}}$$

The bacterial isolates with the highest Proteolytic Index (PI) were recorded [7].

E. Partial Characterization of Protease-Producing Thermophilic Bacteria

Partial characterization was carried out by observing macroscopic, microscopic and biochemical tests. Parameters of macroscopic observations were carried out based on observations of the morphology of thermophilic bacteria including the shape of the colony, the periphery of the colony, the elevation of the colony and the color of the colony. Parameters of microscopic observation were carried out by Gram staining and sporesto determine the nature of Gram, cell shape and location of spores. Observations of biochemical tests were carriedout by means of a catalase test and also a motility test.

III. Result And Discussion

A. Isolation of Thermophilic Bacteria

Isolation of thermophilic bacteria from 5 hot spring points in Bukik Gadang, Solok Regency, obtained the number of thermophilic bacteria that grew, namely 23.3 x 10 cfu/mL with 20 isolates of thermophilic bacteria (Table 1). Abiotic factors in the Bukik Gadang hot spring have water temperatures with intervals of 45°C - 60°Cand water pH with intervals of 7 - 8.

Table 1. Isolates of thermophilic bacteria and abiotic factors from the Bukik Gadang hot spring, Solok Regency

Point	Abiotic Factors		∑ Bacteria (x 10 cfu/mL)	∑ Isolate	Isolate Code
	Water Temperature (°C)	Water pH			
I	46	7	2,9	3	TPBG-01 TPBG-02 TPBG-03
II	45	7	3,8	4	TPBG-04 TPBG-05 TPBG-06 TPBG-07
III	45	7	9,6	6	TPBG-08 TPBG-09 TPBG-10 TPBG-11 TPBG-12 TPBG-13
IV	60	8	4,6	4	TPBG-14 TPBG-15 TPBG-16 TPBG-17
V	45	7	2,4	3	TPBG-18 TPBG-19 TPBG-20
Total Number			23,3	20	

Description: *TPBG = Bukik Gadang Thermophilic Protease

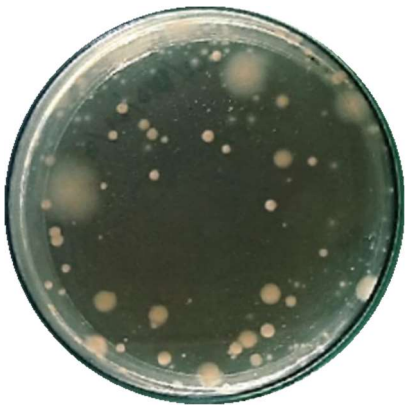

Figure 1. Colonies of thermophilic bacteria at point II

Thermophilic bacteria obtained from the purification have different morphological characteristics in terms of shape, color, elevation and margins on each isolate of thermophilic bacteria. Abiotic factors such as temperature are one of the factors that

affect the diversity and physiological characteristics of thermophilic bacteria. Hot prings with an alkaline pH have a high mineral content, allowing thermophilic bacteria to survive which affects the growth and diversity of thermophilic bacteria [8]. Differences in the growth of the number of colonies of thermophilic bacteria can be influenced by environmental conditions that support bacterial life, both biotic and abiotic factors, such as the presence of dry leaves, grass and moss, as well as temperature and pH [9].

B. Screening for Protease-Producing Thermophilic Bacteria

Screening of thermophilic bacteria which was carried out on 20 bacterial isolates showed that as many as 18 isolates of thermophilic bacteria were indicated to produce protease enzymes (Table 2). The proteolytic index (PI) of the isolates of thermophilic bacteria producing protease from Bukik Gadang hot springs ranged from 0.31 -

2.51 with a potential isolate that was TPBG-03 with an average value of 2.51. The isolates of thermophilic bacteria which indicated the production of protease enzymes were characterized by the formation of a clear zone around the isolates of thermophilic bacteria in the media (Figure 2).

Table 2. Average Proteolytic Index (PI) of thermophilic protease-producing bacteria from Bukik Gadang hotsprings

Point	Isolate Code	Σ Diameter (mm)		Σ Proteolytic Index (PI)
		Colony	Clear Zone	
I	TPBG-01	21,79	35,83	0,64
	TPBG-02	6,61	18,57	1,80
	TPBG-03	6,70	23,58	2,51
II	TPBG-04	19,02	40,78	1,14
	TPBG-05	18,03	37,95	1,10
	TPBG-06	24,30	34,15	0,40
	TPBG-07	30,48	43,19	0,41
III	TPBG-08	19,61	31,32	0,59
	TPBG-09	24,76	41,28	0,66
	TPBG-10	30,42	39,94	0,31
	TPBG-11	5,50	13,30	1,40
	TPBG-12	5,42	7,65	0,41
	TPBG-13	3,31	-	-
IV	TPBG-14	26,96	36,52	0,35
	TPBG-15	5,01	10,16	1,02
	TPBG-16	5,44	7,52	0,39
	TPBG-17	6,21	13,34	1,14
V	TPBG-18	24,58	39,29	0,59
	TPBG-19	4,73	-	-
	TPBG-20	6,91	11,82	0,71

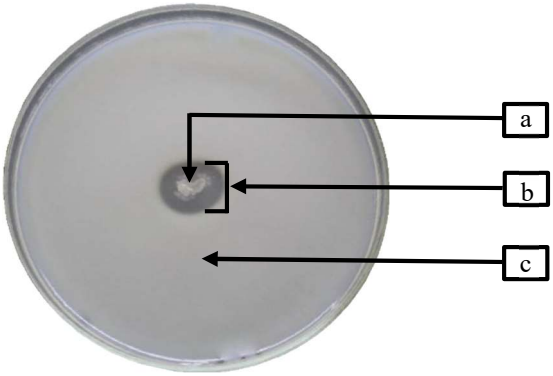

Figure 2. Screening of isolate bacteria TPBG-11

a) Bacterial colonies b) Clear zone c) Skim Milk Agar (SMA)

Casein contained in Skim Milk Agar (SMA) media serves as a substrate for bacteria to produce protease enzymes which will be hydrolyzed into peptides and amino acids marked by a clear zone around the bacterial colonies. Skim milk media is good for selection of bacteria that can secrete extracellular proteolytics to produce protease enzymes qualitatively [10].

The proteolytic activity of bacteria was influenced by the source of nutrition, the different types of bacteria and the speed of bacterial growth in the media of each isolate. If the nutrients in the media are lacking, the bacteria will not grow optimally [11]. The difference in the clear zone formed was caused by the enzyme activity of each isolate that was secreted into the medium. The low activity of the enzyme is influenced by changes in the structure of the enzyme which causes a decrease in the rate of the catalyst because the active site of the enzyme cannot be used to bind the substrate properly [12].

C. Partial Characterization of Protease-Producing Thermophilic Bacteria

Partial characterization of thermophilic bacteria was carried out on 10 bacterial isolates indicated to produce proteases by observing macroscopic, microscopic and biochemical tests (Table 3). According to Padder *et al* (2017) [13], bacteria have diversity in terms of color, shape, elevation and margins. The morphological characteristics of the colonies of thermophilic bacteria obtained were cream, yellowish and white; shape in the form of circular and irregular; elevation in the form of flat, raised and convex; and margins in the form of entire,undulate and serrate.

Table 3. Characterization of macroscopic, microscopic and biochemical tests of isolates of thermophilic bacteriaproducing protease

Observation	Isolate Code									
	TPBG-01	TPBG-02	TPBG-03	TPBG-04	TPBG-05	TPBG-09	TPBG-11	TPBG-15	TPBG-17	TPBG-20
Macroscopis										
Color	White	Yellowish	Crem	Yellowish	Crem	Yellowish	Crem	White	White	Yellowish
Form	Circular	Circular	Irregular	Circular	Circular	Circular	Circular	Irregular	Circular	Irregular
Elevation	Flat	Raised	Flat	Convex	Convex	Raised	Raised	Raised	Flat	Raised
Margin	Undulate	Serrate	Undulate	Entire	Entire	Entire	Entire	Undulate	Entire	Undulate
Microscopis										
Gram stain	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Cell shape	Diplo bacil	Strepto bacil	Diplo bacil	Strepto bacil	Diplo bacil	Strepto bacil	Diplo bacil	Diplo bacil	Diplo bacil	Strepto Bacil
Spore stain	Sub terminal	Sub terminal	Sub terminal	Sub terminal	Sub terminal	Sub terminal	Sub terminal	Sub terminal	Sub terminal	Sub terminal
Motility	Motil	Motil	Motil	Motil	Motil	Motil	Motil	Motil	Motil	Motil
Biochemical Test										
Catalase	+	+	+	+	+	+	+	+	+	+

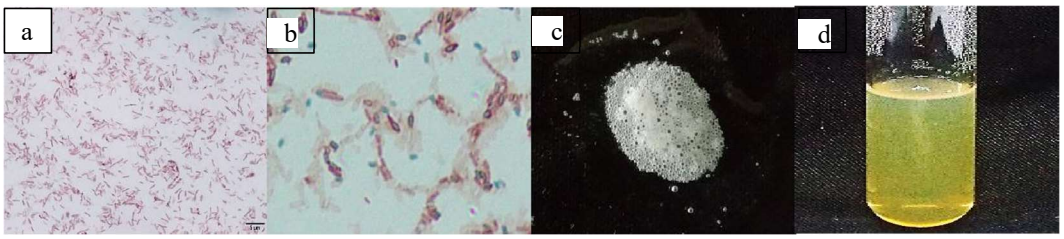

Figure 3. Partial characterization of protease-producing thermophilic bacteria

(a) Gram stain (b) Spore stain (c) Catalase test (d) Motility test

On macroscopic observations with Gram staining, it was found that all isolates of thermophilic protease- producing bacteria were Gram positive and were in the form of bacilli. The TPBG-11 isolate was Gram positive with the shape of the cell was bacil (Figure 3a). Gram-positive bacteria have a thick layer of peptidoglycan on the

bacterial cell wall so that they can maintain the given crystal violet [14]. Spore staining was performed on isolates of thermophilic bacteria which indicated that they were Gram-positive protease-producing bacteria in the form of bacilli and were found to be positive for producing spores. TPBG-04 isolate had spores in vegetative cells (endospores) which were marked by a green color formed and located in the sub-terminal part (Figure 3b). The location of the spores formed in the cell consists of three types central, terminal and sub-terminal. Spores are formed when subjected to stress which serves to protect bacteria from unfavorable external factors by surviving extreme environments such as heating, drought due to high temperatures, radiation, lack of nutrients, acid conditions, freezing and chemicals. Bacteria form spores when environmental conditions are no longer optimal for growth and development, for example the medium dries up, the nutrient content shrinks and so on [15].

The biochemical test was carried out by testing catalase to determine the activity of catalase on thermophilic bacteria. The TPBG-03 isolate was catalase positive so it was classified as an aerobic bacterium (Figure 3c). This is indicated by the presence of oxygen produced on the surface of the isolate when a solution of hydrogen peroxide (H_2O_2) is dripped. So that aerobic bacteria can survive in that environment, oxygen can still be dissolved well in hot springs. Hydrogen peroxide (H_2O_2) is formed during aerobic metabolism so that microorganisms growing in aerobic circles must decompose the material. Bacteria are able to produce catalase or peroxidase enzymes by breaking down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) [16].

A motility test was carried out to determine the ability of bacterial isolates to move and move as in the motile TPBG-11 isolate (Figure 3d). The shape of bacterial cells that are classified as motile is generally spiral and bacilli, while bacteria that are classified as immotile are cocci. Motile bacteria indicate that the organism has the ability to move on its own. This property is caused by the presence of whip motors (flagellates) or cilia so that bacterial cells can move in the media. The movement of these bacteria is carried out for adaptation and survival [17].

IV. Conclusion

Thermophilic bacteria which indicated protease producing 18 isolates from 20 isolates with one isolate TPBG-03 which was potential to produce protease. Partial characterization was carried out on 10 isolates of thermophilic bacteria with indications of protease production, which are generally Gram positive, bacillus cell shape, have spores, are catalase positive and motile.

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