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Screening, Characterization and 16S rRNA Sequencing of Thermophilic Bacteria Producing Amylase and Protease from Pekonina Hot Springs, South Solok

Hadela Yuri Putri, Anthoni Agustien* and Feskaharny Alamsjah

Department of Biology, Faculty of Mathematics and Natural Sciences Andalas University

Padang 25163, West Sumatra

***Abstract*— Amylase and protease enzymes are play a major role in food industries, textile, medicine, paper and detergent industries so that the level of need for amilase and protease is very high, especially those with thermophilic properties. This study aims to obtain amilase and protease-producing thermophilic bacteria, determine the growth and activity of amilase and protease enzymes and analyze the molecular-based characterization of amilase and protease-producing thermophilic bacteria from Pekonina hot springs, Solok Selatan. This research uses survey method. The results obtained 5 bacteria isolats from pond I and 5 bacteria isolats from pond II which can produce amilase and protease enzymes. The macroscopic observation of thermophilic bacteria showed that the colonies were circular, irregular, and rhizoid. Margins on bacterial isolates are entire, undulate, lobate, rhizoid. The elevation of the bacterial isolates were flat and convex. Colony colors obtained are white, cream and yellowish. Microscopic observation showed that all bacterial isolates were Gram positive. The shape of the cells in the isolates were bacilli, and diplobasil and coccus. BTPAP-04 is the isolate that had the highest amylolytic index and proteolytic index value are 2.36 mm and 2.96 mm. Molecular identification of 16S rRNA was performed on BTPAP-04 isolate was identified as *Geobacillus stearothermophilus* strain IFO 12550 and *Geobacillus stearothermophilus* strain R-35646.**

Keywords*— Thermophilic Bacteria, Amylase, Protease, *Geobacillus stearothermophilus

I. INTRODUCTION

The demand of the enzyme in Indonesia tends to increase from year to year, which is enzyme is one of the natural products that plays an important role in various industrial applications, ranging from food processing to high value chemical products. Currently, enzymes have been widely used for various commercial purposes in industry, and enzymes can be obtained from various sources at a lower cost. Thermophilic bacteria are able to produce enzymes that are stable at high temperatures.

Thermophilic microorganisms whose optimal growth temperature is 50°C and above have attracted great attention among extremophiles because they are a source of thermostable enzymes (such as amylase, cellulase, chitinase, pectinase, xylanase, protease, lipase, and DNA polymerase). This enzyme exhibits unique features suitable for carrying out biotechnological processes at high temperatures (Singh et al., 2011). Their cellular components are also thermostable, including their enzymes, sometimes referred to as extremozymes, which are known to withstand high temperatures and highly acidic and alkaline conditions, and generally exhibit increased resistance to denaturation and proteolysis (Vielle and Zeikus, 2001).

Corresponding Author: Anthoni Agustien

Enzymes that are widely used in industry are Amylase and Protease. Amylase (EC3.2.1.1; 1,4-α-D-glucan-glucanohydrolase) is one of the most important industrial enzymes that can be used in a number of industrial processes including brewing, textiles and detergents (Pandey et al., 2000; Gupta et al., 2003). Amylase applications are also widespread in the paper, food and fermentation industries, for example in bread making, glucose and fructose syrups, sweeteners and alcoholic beverages (Haq et al., 2010). Proteases are enzymes that hydrolyze peptide bonds on protein or polypeptide substrates (Gupta and Khare, 2007). Protease enzymes are one of the most important groups of enzymes, which are widely used in the food, pharmaceutical, detergent industries (Seiffzadeh et al., 2008; Dias et al., 2008; Synowiecki, 2010). The use of enzymes in detergent formulations increases the ability of detergents to remove stains and makes detergents safe for the environment (Linet et al., 2009).

DNA sequencing provides insight into the regulatory elements in the genome of each cell, and the variation in their activity in different cell types and individuals. Differences in these DNA regulatory regions can be demonstrated through sequencing and can provide insight into the phenotypic basis (Clark, 2016). In prokaryotic organisms, there are three kinds of rRNA, namely 23S rRNA (S=Svedberg units; 2900 nucleotides), 16S rRNA (1550 nucleotides), and 5S rRNA (120 nucleotides). Among the three rRNA molecules, 16S rRNA is the most commonly used. The 16S rRNA molecule has a lot of genetic information and is easier to analyze.

Hot springs are a natural environment for thermophilic microorganisms. Microbes from hot springs generally produce enzyme thermophiles which are of interest to many enzyme-based industries. One of the hot springs in West Sumatra that has not been reported for its potential to produce both amylase and protease is the Pekonina Hot Spring, South Solok.

II. RESEARCH METHODOLOGY

The research was conducted from January-June 2021 at the Sumatra Biota Laboratory, Andalas University and Microbiology Research Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang. This study uses a survey method. Furthermore, the sampling method is purposive sampling technique. The research stage includes the isolation of bacteria from two points in pond I and two points for pond II in Pekonina hot springs, South Solok, West Sumatra (Figure 1). We need to make a medium, namely nutrient agar medium to isolate bacteria, starch agar medium and skim milk agar for the screening process for thermophilic bacteria. The hot water sample was homogenized and then heated using a hotplate according to the temperature at the time of sampling. In a 1 ml pipette, put into a Petridish and then 15 ml of NA medium was poured and then incubated at 50^0C for 24-48 hours with two repetitions (Leboffe and Pierce, 2010). Slanted cultures of these thermophilic bacteria were inoculated using a ose needle on starch medium and skim milk medium. Then incubated at 50^0C for 24-48 hours. After that, the diameter of the bacterial colony and the clear zone formed around the bacterial colony were measured, after which the Amylolytic and Proteolytic Index were determined.

$$\text{IA/IP} = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{Colony diameter}}$$

The isolates that had the highest IA/IP were recorded (Agustien, 2010). After determining the IA/IP values of several isolates obtained, then one isolate with the highest index was taken and the isolate characterization was observed. Bacterial characterization was carried out by macroscopic, microscopic and chemical tests on thermophilic bacteria. The isolates of thermophilic bacteria that have a highest amylolitic and proteolitic index were identified using the 16S rRNA method. The results of the study were described descriptively and to determine the types of thermophilic bacteria in Pekonina hot springs, South Solok which produce amylase and protease enzymes.

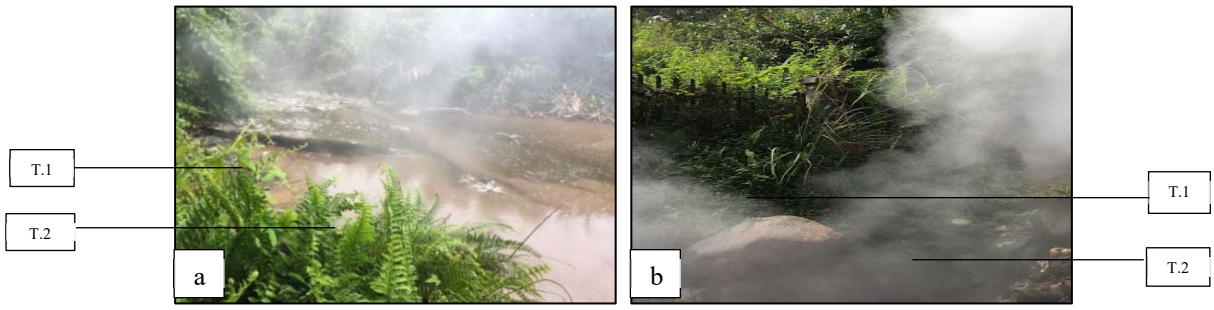


Figure 1. Location of Pekonina Hot Springs, South Solok

Description :

T.1 : first point

T.2 : second point

a : pond I

b : pond II

III. RESULT AND DISCUSSION

Based on research that has been carried out isolation of amylase and protease-producing thermophilic bacteria from Pekonina hot springs, South Solok, West Sumatra, 16 isolates were obtained (Table 1), then screening was carried out and only 10 isolates were proven to produce amylase and protease with the appearance of a clear zone around the bacteria (Table 2)

Table 1. Isolates of thermophilic bacteria from Pekonina Hot Springs

	Pond	Tempature	pH	Colonies	Total Isolate	Isolate Code
1.	Pond I Point 1	87^{0}C	7,6	57	5	BTPAP-01 BTPAP-02 BTPAP-03 BTPAP-04 BTPAP-05
2.	Pond I Point 2	82^{0}C	7,6	31	3	BTPAP-06 BTPAP-07 BTPAP-08
Total				88	8	
3.	Pond II Point 1	79^{0}C	7,7	49	4	BTPAP-09 BTPAP-10 BTPAP-11 BTPAP-12
4.	Pond II Point 1=2	77^{0}C	7,7	42	4	BTPAP-13 BTPAP-14 BTPAP-15

						BTPAP-16
Total				91	8	
Total Number				179	16	

Table 1 shows that the results of the isolation of thermophilic bacteria in Pekonina hot springs obtained 16 bacterial isolates, namely five isolates in pool I point 1, three isolates in pool I point 2, four isolates in pool II point 1 and four isolates in pool II point 2. In pond I point 1, the number of colonies and isolates of thermophilic bacteria was higher than in other ponds. This is caused by the temperature of the pond and the presence of organic waste around the pond which can be seen in Figure 1. Biotic conditions in the hot springs environment where there are grasses, mosses and other organic sources found around the hot springs so that they can be used as an energy source. Abiotic factors also affect the presence of thermophilic bacteria such as alkaline pH caused by high mineral content, causing a high diversity of microorganisms (Agustien, 2010). Temperature is the most important factor in determining microbial diversity and physiological characteristics (Zheng et al., 2011). According to Panda et al., (2013) the higher the temperature, the more diverse the bacteria obtained.

Tabel 2. Average Amylolytic Index (AI) and Proteolytic Index (PI) of thermophilic bacteria isolates Pekonina Hot Springs

No.	Isolate Code	Amylolytic Indekx (AI)	Proteolytic Index (PI)
1.	BTPAP-01	0.97	0.39
2.	BTPAP-02	0,70	0.97
3.	BTPAP-04	2.36	2.96
4.	BTPAP-05	0.47	0.91
5.	BTPAP-06	1.13	2.17
6.	BTPAP-11	0.72	0.53
7.	BTPAP-13	0.74	1.71
8.	BTPAP-14	0.39	1.00
9.	BTPAP-15	1.91	0.22
10.	BTPAP-16	1.52	0.09

Table 2 shows the highest amylolytic and proteolytic index is BTPAP-04 from pond I with amylolitic and proteolitic index value 2.36 mm and 2.96 mm. Thermophilic bacteria producing amylase and protease was characterized by the formation of a clear zone on the substrate media, it was proven that the isolate had the ability to decompose amylase and protease substrates. According to Pisano et al., (2019) stated that Skim Milk Agar is an effective medium for the growth and selection of proteolytic bacteria characterized by the emergence of a clear zone around the media. The formation of the clear zone is caused by amylase and protease enzymes that can hydrolyze starch and skim milk in solid media into simpler compounds (Winarno, 1986). The difference in clear zones in each isolate was caused by the amount and enzyme activity of each isolate secreted in different media. The activity of the bacterial isolates was determined by the concentration of the enzyme, the sequence of the amino acid-forming enzymes and the type of amino acids that make up the enzyme (Agustien, 2010).

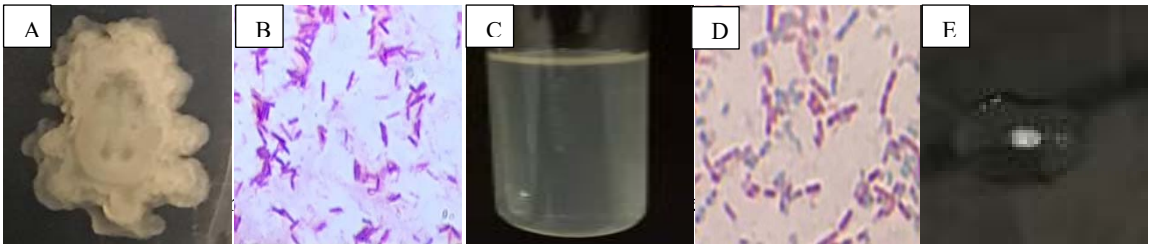
The size of the clear zone depends on the amount of glucose monomer produced from the starch hydrolysis process. The greater the amount of glucose monomer produced, the greater the clear zone formed around the colony (Black and Jacquelyn, 2012).

The formation of a clear zone around the bacterial colonies because the amylase and proteases produced by bacteria are secreted in a solid medium containing a substrate. Furthermore, the process of hydrolysis of the substrate contained in the medium occurs, therefore the merdium in the petri dish looks clear. The size of the clear zone formed depends on the ability of the bacteria to hydrolyze the growth medium substrate (Irfan et al., 2012; Ebrahimpour and Kariminik, 2015).

Table 3. Characterization of Macroscopic, Microscopic, and Biochemical Tests of Amylolytic and Proteolytic Bacteria Isolates

Observation	Bacterial Isolate from Pond I					Bacterial Isolate from Pond II				
	BTPAP-01	BTPAP-02	BTPAP-04	BTPAP-05	BTPAP-06	BTPAP-11	BTPAP-13	BTPAP-14	BTPAP-15	BTPAP-16
Macroscopic										
Form	Circular	Circular	Irregular	Circular	Rhizoid	Irregular	Circular	Circular	Circular	Circular
Margin	Entire	Undulate	Lobate	Entire	Rhizoid	Lobate	Entire	Entire	Entire	Entire
Elevation	Flat	Convex	Convex	Flat	Flat	Convex	Flat	Convex	Flat	Flat
Color	White	Crem	Crem	White	Crem	White	Yellowish	White	Crem	White
Microscopic										
Gram Stain	+	+	+	+	+	+	+	+	+	+
Cell Shape	Bacil	Coccus	Bacil	Bacil	Diplobacil	Bacil	Bacil	Bacil	Bacil	Bacil
Spore Stain	+	-	+	+	+	+	+	+	+	+
Motility	Motil	Non-motil	Motil	Motil	Motil	Motil	Motil	Motil	Motil	Motil
Biochemical Test										
Catalase	+	+	+	+	+	+	+	+	+	+

Initial conventional identification was in the form of morphological observations, from 10 isolates identified circular bacterial were seven isolates, two isolates with irregular shape and one isolate in rhizoid shape. According to Dar et al., (2015) the types of isolates can be distinguished based on differences in size, color and morphological character of the colony. In Figure 2 BTPAP-04 isolate has an irregular shape and lobate-shaped edges. Colony morphology on isolates from Pekonina hot springs, South Solok. According to Cappucino and Sherman (1987) that in general, bacterial colonies are circular, irregular and rhizoid in shape and the edges are entire, lobate and undulate.



Description :

A : Bacterial Forms

B : Bacilli Gram Positive Stain

C : Motility Test

D : Endospore staining

E : Catalase test

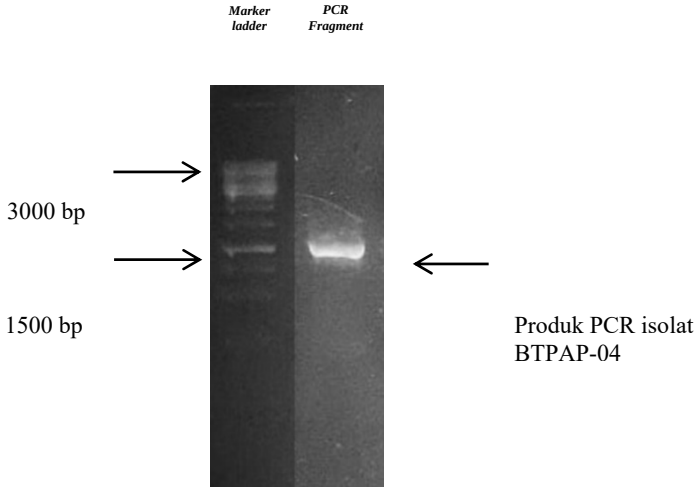


Figure 3. 16S rRNA gene amplification of isolate BTPAP-04

Figure 3 can be seen the results of the amplification of BTPAP-04 bacterial isolates using universal primers with the 16S rRNA method with a PCR product of 1450 bp. This indicates that the amplification using universal primers in the 16S rRNA method is close to 1500 bp. The 16S rRNA gene can be used as a molecular marker because this molecule is present in every organism with identical functions in all organisms, so that a universal primer can be designed for all groups (Pangastuti, 2006).

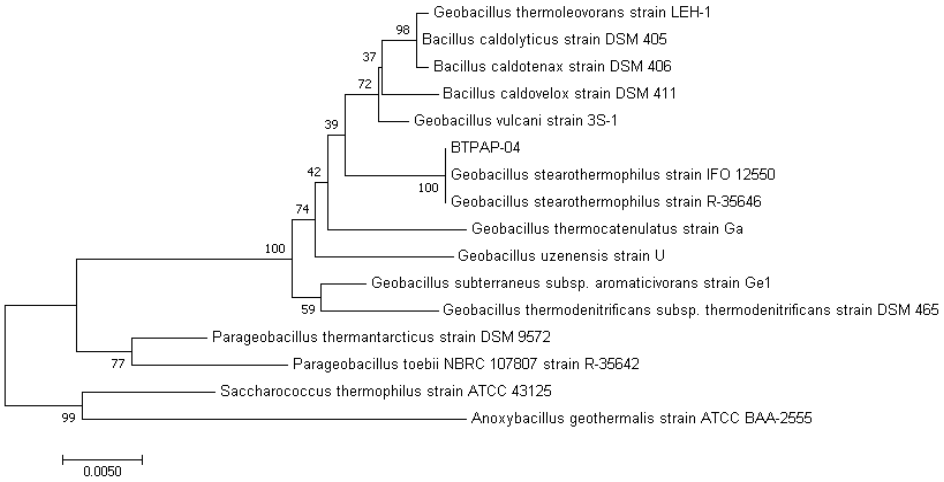


Figure 4. Bacterial phylogenetic tree of BTPAP-04 isolate based on 16S rRNA gene by neighbor joining (NJ) analysis. Note: the 0.005 scale indicates the evolutionary distance in the branch length, the number on the branch indicates the bootstap value of 1000 (ML/NJ/MP)

BTPAP-04 isolate is an isolate of thermophilic bacteria where this isolate is close similarity to *Geobacillus stearothermophilus* strain IFO 12550 and strain R-35646 where these bacteria are also bacteria from different locations with similarity values of 99.20% and 99.13% with the bootstrap percentage value of the NJ tree with a value of 100%, the isolate BTPAP-04 is strongly suspected to be identified as the bacterium *Geobacillus stearothermophilus*. This is also supported by the p-distance data between BTPAP-04 and the bacteria Geobacillus stearothermophilus strain IFO 12550 and strain R-35646 in the gene bank 0.000 which means that there is no evolutionary distance between the two.

Geobacillus stearothermophilus was first identified in 1920 and given the name Bacillus stearothermophilus, then reclassified as a member of the genus Geobacillus in 2001. This bacterium is thermophilic which can be obtained from hot air sources, marine sediments. *G. stearothermophilus* is a Gram-positive rod-shaped bacterium, a member of the genus Firmicutes and has an optimal growth temperature of 55-130 °C (Wickham Laboratories, 2021). *G. stearothermophilus* produces proteases which are commonly used for the production of artificial aspartame (Lemieux et al., 2006). In the study of Khemakhem et al., (2009) the species obtained were *G. stearothermophilus* which produces protease which has been used for industrial applications. Geobacillus species, particularly *G. stearothermophilus*, have been isolated from various geothermal areas including hot springs (McMullan et al., 2004). *G. stearothermophilus* can also produce amylase. Vihinent and Mantsala (1989) found that *G. stearothermophilus* was proven to produce amylase and tested its thermostability and characterized its extra and intracellular enzymes. Research by Damardjati et al. (1997) obtained G. Stearothermophilus from TII-12 isolates producing-amylase isolated from the crater of the Dieng Mountains.

IV. CONCLUSION

The results obtained 5 bacteria isolats from pond I and 5 bacteria isolats from pond II which can produce amilase and protease enzymes. The macroscopic observation of thermophilic bacteria showed that the colonies were circular, irregular, and rhizoid. Margins on bacterial isolates are entire, undulate, lobate, rhizoid. The elevation of the bacterial isolates were flat and convex. Colony colors obtained are white, cream and yellowish. Microscopic observation showed that all bacterial isolates were Gram positive. The shape of the cells in the isolates were bacilli, and diplobasil and coccus. BTPAP-04 is the isolate that had the highest amylolytic index and proteolytic index value are 2.36 mm and 2.96 mm. Molecular identification of 16S rRNA was performed on BTPAP-04 isolate was identified as *Geobacillus stearothermophilus* strain IFO 12550 and *Geobacillus stearothermophilus* strain R-35646.

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