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Isolation and Identification of Thermophilic Bacteria from Lahendong Hot Spring, North Sulawesi

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ABSTRACT

Thermophilic bacteria are a group of procaryotic organisms that can grow in high temperature around 45^0C until 90^0C. Thermophilic bacteria are able to produce lipase thermostable enzyme. Lipase thermostable enzymes produced from thermophilic bacteria are able to catalyze the hydrolysis of triglycerides into glycerol and free fatty acids and the synthesis of esters in organic solvents. Lipase thermostable enzymes are potential to be used in industrial production. Lipase enzymes have been used for the production of laundry detergents as additives or to replace chemical detergents because of their environmental friendliness. This research was aimed to isolates and identified themophilic bacteria from Lahendong hot springs, North Sulawesi and to conduct the morphological characterization, biochemical test, and molecular identification with 16S rRNA. Results showed that isolates obtained from the- Lahendong hot spring have high similarities with Geobacillus kaustophilus, Bacillus cereus, and Geobacillus lituanicus. The three isolates are able to produce lipase thermostbale enzyme.

***Keywords**: Geobacillus kaustophilus; Bacillus cereus; Geobacillus lituanicus; Lipase Thermostable enzyme; Lahendong Hot Spring*

ABSTRAK

Bakteri termofilik merupakan kelompok bakteri yang beradaptasi dengan kondisi lingkungan yang bersuhu tinggi, yaitu dengan suhu berkisar 45^0C sampai 90ºC. Bakteri termofilik mampu menghasilkan enzim termostabil. Salah satunya enzim lipase termostabil yang merupakan kelas hidrolase dan dapat mengkatalis hidrolisis trigliserida menjadi gliserol dan asam lemak bebas serta sintesis ester dalam pelarut organik enzim ini telah digunakan dalam bidang industri. Enzim lipase telah digunakan untuk produksi deterjen cucian sebagai aditif atau untuk menggantikan deterjen kimia karena keunggulannya yang ramah lingkungan. Tujuan penelitian ini adalah untuk mengisolasi dan mengidentifikasi bakteri termofilik yang berhasil di isolasi dari sumber air panas lahendong sulawesi utara dan untuk mengetahui karakteristik morfologi, uji biokimia, identifikasi secara molekular menggunakan metode 16S rRNA dan untuk mengetahui keberadaan enzim lipase pada isolat yang teridentifikasi. Hasil penelitian menunjukkan bahwa isolat yang diperoleh dari pemandian air panas Lahendong memiliki kemiripan yang tinggi dengan Geobacillus kaustophilus, Bacillus cereus, dan Geobacillus lituanicus. Ketiga isolat tersebut mampu menghasilkan enzim termostbal lipase.

***Kata kunci**: Geobacillus kaustophilus; Bacillus cereus; Geobacillus lituanicus; Enzim Lipase Termostabil; Sumber Air Panas Lahendong*

BACKGROUND

Lahendong is the one of the geothermal areas in North Sulawesi, this place located 30 km south of Manado the capital city of North Sulawesi province, at an altitude of about 750 m above sea level. The utilization of Lahendong area as a geothermal one of them is geothermal power generation (Jefferson, 2020). The hot spring in this place are water springs geothermal that rises from the earth's crust to the ground surface. Hot springs vary in size and produce water which is in the

warm to very hot temperature range. Hot springs undergo a boiling process which makes the water evaporate and flow away from the source and experience cooling (Sasa, 2020). Lahendong is the geothermal area that can serve as a habitat for thermophilic bacteria (Huwae, 2020).

Thermophilic bacteria are group prokaryotic organisms that can grow in high temperature conditions that are 45^{o}C until 90^{o}C. They are found naturally widespread in the surface area of hot springs, volcanic craters or volcanic areas (Labeda, 1990). Thermophilic bacteria are able to produce enzymes that are resistant to extreme temperatures. Enzymes produced by thermophilic bacteria have the selectivity and stability of the substrate in abnormal conditions, especially high temperatures and extreme pH (Sasa, 2020). Thermophilic bacteria have protein that are resistant to hot conditions and denaturation so that thermophilic bacteria can adapt on extreme temperature. Thermophilic bacteria can provide thermostable enzyme and be used in various industries (Kumar and Nussinov, 2001).

Thermostable enzymes can be isolated from thermophilic microorganisms that live in hot springs (Dessy *et al.*, 2019). Thermostable enzymes have a slightly different amino acid composition because they contain many hydrophobic amino acids and are very important because of their intrinsic thermostability and resistance (Marthariana, 2010). One of the thermostable enzymes is lipase which is the second largest commercially produced enzyme used in various industries for the production of fine chemicals, cosmetics, pharmaceuticals and biodiesel including detergents (Colla *et al*., 2010). Lipase enzymes have been used for the production of laundry detergents as additives or to replace chemical detergents because of their environmental friendliness and better ability to remove oil stains without damaging the texture of fabrics (Sanchez, 2011). Lipase (triacylglycerol hydrolase) is a class of hydrolases that can catalyze the hydrolysis of triglycerides into glycerol and free fatty acids and the synthesis of esters in organic solvents (Susanti, 2017).

Research reported to the identify of lipase produce by thermophilic bacteria has been carried out previously by Septiani (2017) who isolated Al96 isolate a thermostable lipase enzyme producer with optimum pH of 10. Dessy *et al*. (2019) which reported 14 isolates of thermophilic bacteria with 5 isolates of positive thermophilic bacteria produced lipase enzymes with the highest lipase activity of 2.20 units/mL. Asnawi *et al.* (2014) who reported the existence of the genus Bacillus sp. Lipase activity is indicated by a color change during titration with Phenolphatalein indicator from colorless to pink, with lipase activity occurring at 48 hours with a power of hydrogen (pH) of 7 and a temperature of 45^{o}C. However, this far research of isolation and identification of thermophilic bacteria from Lahendong hot spring North Sulawesi is lacking. Therefore, the proposed research will focus on that topic.

METHODS

Hot water and mud samples for this study were obtained from the Lahendong hot spring with one location sampling (**Figure 1**). The research process was carried out at the Microbiology Laboratory of the Pharmacy Study Program, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University. This research was conducted from 10 March 2022 until 28 May 2022.

Figure 1. Sampling location

Bacterial isolation is the process of taking bacteria from the media or environment of origin, and growing them on artificial media so that pure cultures are obtained from the isolation (Singleton and Sainsbury, 2006). Macroscopic identification of colony morphology can be viewed from various aspects, namely shape, edge, height, size, surface, viscosity or density, odor, transparency, and colony pigmentation (Alcamo, 2001). Microscopic identification of cell morphology is determined by looking at the smear of the culture that has been stained under a microscope and seeing how the shape of the cell, the nature of the gram, and the ability to form spores of the bacteria are. Biochemical tests were carried out to determine the characteristics and specificity of bacteria by looking at their enzymatic activity, as well as to strengthen the data obtained so that they are easily identified, several biochemical tests were applied, including the indole production test, carbohydrate fermentation test, citrat usage test, methyl red test, Voges proskauer test, urease test, catalase test, and H_2S test (Cappuccino and Sherman, 1987). Bacterial identification using the 16S rRNA gene for molecular detection methods. The 16S rRNA gene is a gene that is used to determine the phylogenetic and taxonomy of bacteria that is carried out molecularly (Janda and Abbott, 2007) and in its utilization, the 16S rRNA gene is often used in various fields because of its advantages, especially in the process of identifying bacteria as marker genes (Akihary and Kolondam, 2020).

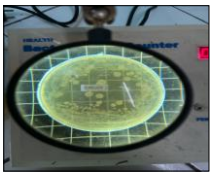
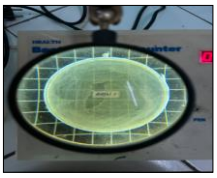
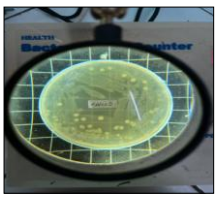
The lipase enzyme test was conducted by taking 2-3 oozes of bacterial inoculum and inoculation into an Erlenmeyer flask containing Nutrient Broth Media by mixing 99 mL of ddH_2O plus 0.792 gr NB and adding 10% Olive Oil. Then the Erlenmeyer was covered with aluminum foil and wrapping and then incubated at 60^0C for 72 hours. Furthermore, the crude lipase enzyme formed was harvested from the culture by taking 10 mL of crude lipase enzyme and placed in a centrifugation tube and then centrifuged at 3200 rpm for 30 minutes. After that the supernatant formed was taken and transferred to another Erlenmeyer to be tested for enzyme activity. The lipase enzyme activity test was carried out by making a phosphate buffer solution (pH 5,7,9) by adding KH_2PO4 and NaOH and the pH was measured using litmus paper. After that, 2 mL of olive oil was added, 1 mL of crude enzyme solution (supernatant) was added and it was cultivated at 60^oC for 30 minutes. Furthermore, the mixture of crude enzyme substrate was inactivated by adding 10 mL of acetone: alcohol (1:1) solution. Then it was titrated with 0.05 M NaOH by adding 3 drops of phenolphthalein. The titration is stopped when a pink color change occurs.

DATA ANALYSIS

Analysis for this research use Macroscopics identification, Microscopics identification, Lipase enzyme test, PCR and Sequencing of the 16s rRNA Gene, and Biochemical test.

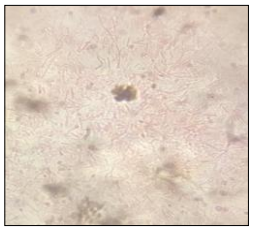
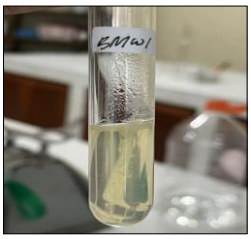
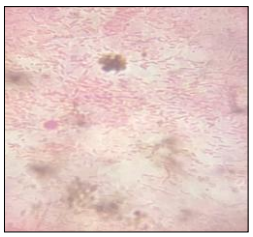
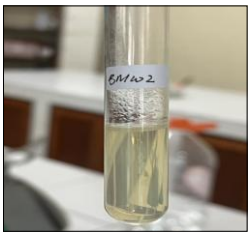
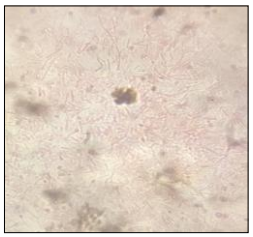
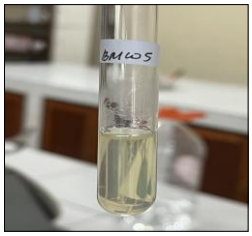
RESULTS AND DISCUSSION

Macroscopics identification

Isolation Code	Macroscopic Observation	Isolate figure
BMW1	Colony Shape Irregular, Colony Edge Lobate, Colony Elevation Convex, Colony Size Moozerate and Colony Color Yellowish White	
BMW2	Colony Shape Circular, Colony Edge Entire, Colony Elevation Flat, Colony size Pinpoint and Colony Color Yellowish White	
BMW5	Colony Shape Circular, Colony Edge Lobate, Colony Elevation Flat, colony Size Moozerate and Colony color Yellowish White.	

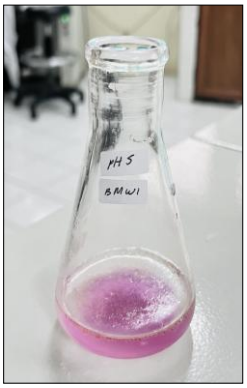
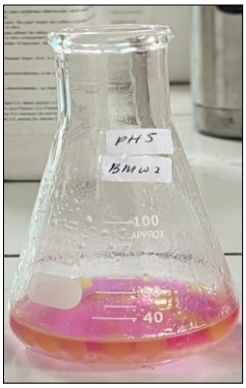
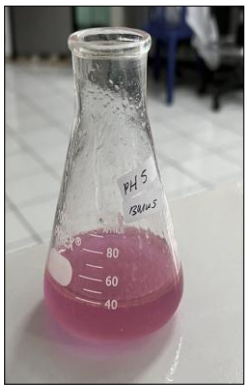
Results of macroscopic observations of bacterial colony morphology of BMW1, BMW2, and BMW5 are presented in Table. The three isolates of thermophilic bacteria had the same colony color, namely Yellowish White. However, the colony shape differed amongst the isolates, namely irregular and circular. The isolates obtained also showed variations in terms of edge and colony size. The edges of the colony are lobate and entire. For the colony size, the 3 isolates obtained showed Pinpoint and Moozerate sizes. Furthermore, the BMW2 and BMW5 isolates have the same elevation, which is flat.

Microscopic identification

Isolate Code	Cell shape	Gram stainning	Motilation
BMW1	Basil long	Gram Negative 	Positive 
BMW2	Basil short	Gram Positive 	Negative 
BMW5	Basil long	Gram Negative 	Negative 

Results from Gram staining showed that isolates BMW1 and BMW5 are Gram negative whilst isolate BMW is a Gram positive. All isolates appeared to have basil (rod) shape. Gram negative bacteria are bacteria that are unable to maintain the crystal violet color on their cell walls when staining is done (Radji, 2010). The bacterial cell wall is in the form of lipoprotein, with the addition of acid alcohol, the lipid will dissolve so that the protein part is still intact and the lipid part is perforated or formed pores, so that the painting with safranin will fill the pores and a purple-red color arrangement occurs purple-red will blend and form a pink color, so the color on Gram Negative is pink. Gram stain is very important for bacterial classification and identification of bacterial species. Gram-Positive bacteria are bacteria that maintain a violet crystal dye during the gram staining process so that it will be blue or purple under a microscope. Gram-Negative bacteria are bacteria that do not retain violet crystal dyestuffs during the gram staining process so that they will be red when observed with a microscope (Radji, 2010).

Lipase enzyme test

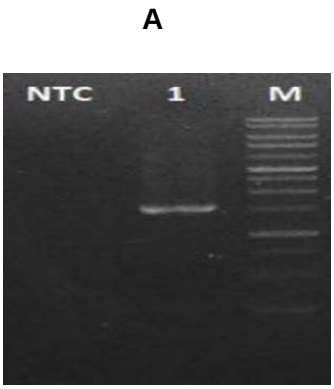
Isolate Code	Buffer solution		
	pH 5	pH 7	pH 9
BMW1			
BMW2			
BMW5			

Based on the results obtained, the three thermophilic bacterial isolates namely BMW1, BMW2, and BMW5 were able to produce lipase enzymes. The activity of the lipase enzyme is indicated by a color change when titrated with Phenolphatalein indicator from colorless to pink due to changes in the pH of the solution. A pink color appears when NaOH can no longer bind to fatty acids, thus giving the solution an alkaline nature and causing a pink color as an indication of changes in the pH of the solution (Paskevicius, 2001). According to Putranto (2006) environmental conditions where enzymes can catalyze substrates at optimum pH. A pH that is far from optimum conditions causes enzyme inactivation because the enzyme is damaged in protein structure so that its activity is reduced and even becomes inactive. Based on what was reported by Asnawi (2017) lipase activity reached the optimum condition at pH 7.0. After reaching the optimum condition, the activity decreased.

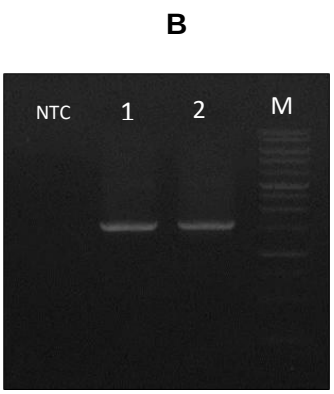
Lipases show pH dependent activity, generally at neutral pH 7.0 or up to pH 4.0 and lipases at pH 8.0 are stable. Based on research conducted by Septiani (2017) reported that the AL 96 isolate tested at a fraction of 30-50% showed the optimum pH, namely pH 10.0. Similar results were shown by lipase in the 50-70% fraction, there was an optimum pH, namely pH 10.0. This is because at pH 10.0 the important proton donating and accepting groups on the catalytic side of the enzyme are in the desired state so that the catalytic activity is high.

Microbial lipases are more valuable than those of plant or animal origin due to the wide range of available catalytic activities, high yield production, and simplicity of genetic manipulation, absence of seasonal fluctuations, regular supply, safer and more convenient stability and very high growth rates of microorganisms on economical media (Reetz, 2013).

Moleculer identification by PCR and Sequencing 16S rRNA gene



A. Visualization of Amplification DNA BMW1



B. Visualization of Amplification DNA BMW2 and BMW5

Based on the results of electrophoresis of the amplified bacterial DNA fragment it can be observed that the DNA fragment are in the range of 1423-1430 bp. The results obtained were then edited using software Geneious 10.1.3 and analyzed using the BLAST method to look for similar sequences available in the Gen Bank. From the results of the analysis sent by PT. Genetics Science Indonesia

using the 16S rRNA method and the BLAST search method BMW2 and BMW5 are 100% similar to *Bacillus cereus* and *Geobacillus lituanicus*. Based on the results obtained for isolate BMW1 and analyzed using the BLAST method to look for similar sequences available in the GenBank based on the results, isolate BMW1 has a 100% similarity level with the species *Geobacillus kaustophillus.*

According to Vasie *et al.* (2016) reported the presence of *Bacillus cereus* bacteria isolated from rice flour using the Rhodamin B agar plate method and obtained lipase enzyme activity of 343 U/ml. Based on research conducted by Duta and Ray (2009) reported the lipase enzyme activity of *Bacillus cereus* isolate with an incubation temperature of 60^0C and pH 8. at a depth of 2000 m with a temperature of 60^0C and pH 6,5 then incubated on agar plates with an incubation temperature of 60^0C for 48 hours. *Geobacillus lictuanicus* Strain N-3 is reported to have a clooze relationship with *Geobacillus thermoleovorans* DSM 5366T, having a sequence similarity of 99.4% Kuisiene (2004). According to research reported by Zhu *et al.*, (2014) who succeeded in finding isolates of the bacterium *Geobacillus* sp. EPT9 which has a thermostable lipase enzyme that was successfully isolated from deep sea thermophiles at an optimum temperature of 55^0C with a pH range of 5,0 until 10,0. The lipase enzyme that was successfully isolated showed 50% of its maximum activity in the pH range of 7,5 until 9,0. At higher pH > 9,0, there was a decrease in lipase activity, and at pH 9,5 there was no lipase enzyme activity. According to Ozdemir *et.al.*, (2021) reported the use of lipase gene (gklip) from isolates of *Gebacillus kaustophillus* cloned into pET28a (+) vector with N-Terminal 6x His-tag. The gklip gene is expressed heterologously in host cells. In this study, gklip showed optimal activity at pH 8 and a temperature of 50^0C. Furthermore, the clip shows 53% activity at pH 7.

Biochemical Test

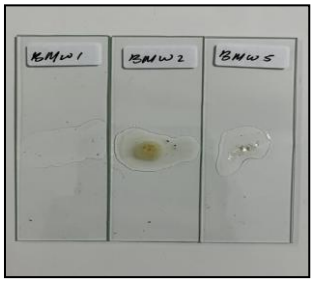
Isolation Code	**Citrate Test**	**Sugar Fermentation Test**	**Catalase Test**
BMW1	Negative (-)	Negative (-)	Negative (-)
BMW2	Negative (-)	Negative (-)	Negative (-)
BMW5	Positive (+)	Negative (-)	Positive (+)

Based on the previous results, BMW1 and BMW2 isolates were used for Citrate Test, Sugar Fermentation Test, with an incubation temperature of 37^0C for 48 hours. The results obtained were negative because there was no color change in TSIA media and CSA media. For Catalase Test negative results were also obtained because after dripping with H_2O_2 there were no bubbles. The BMW5 isolate for the Citrate Test with an incubation temperature of 37^0C for 48 hours obtained positive results because the blue color changed on the CSA medium and for the Sugar Fermentation Test there was a black precipitate, which means negative results. For the Catalase Test obtained positive results because there were bubbles formed after being dripped with H_2O_2. All results of biochemical test

confirmed the results of Gram staining which showed that the three isolates are basil.

Sugar Fermentation Test

Citrate Test



Catalase Test

CONCLUSIONS

Three isolates of thermophilic bacteria that produce lipase enzymes were successfully isolated from Lahendong Hot Spring, North Sulawesi. The three isolates of thermophilic bacteria exhibited different morphological and biochemical characteristics. By using PCR and Sequencing of rRNA gene, it was shown that the DNA sequences shared high similarities with *Geobacillus kaustophilus, Bacillus cereus, and Geobacillus lituanicus.*

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