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Isolation and characterization of thermo-proteolytic bacteria from hot springs in the Mariarotu Tourism Area (MTA), Yapen Islands, Papua

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ABSTRACT

Thermophilic bacteria are able to survive in extreme environmental conditions with high temperatures, generally ranging from 45–80°C. Thermophilic bacteria are also capable of producing protease enzymes. This study aimed to isolate and identify thermophilic bacteria from the Mariarotu hot spring, Yapen Islands Regency, Papua. The method used in this study was a survey followed by laboratory identification. Water samples were collected from two hot spring points with temperatures of 39–49°C. A total of six isolates were obtained and characterized based on macroscopic, microscopic, and biochemical tests. Based on this characterization, all isolates belonged to the genus Bacillus. These results indicate that the Mariarotu hot spring has potential as a habitat for thermophilic bacteria producing thermostable enzymes with possible applications in biotechnology. Further molecular-based studies are required for more accurate species identification.

Key words: bacteria; characterization; enzyme; isolate; thermophilic.

INTRODUCTION

Indonesia is one of the countries with extremely high biodiversity and is estimated to naturally harbor at least 10,000 species of microorganisms (Cappucino & Sherman, 1992; Kurnia, 2008). Hot springs are one of the extreme ecosystems characterized by high temperatures, varying pH, and distinctive mineral content. Such environments provide favorable habitats for thermophilic microorganisms capable of producing thermo-stable enzymes that remain active at high temperatures, giving them considerable potential for industrial applications in food, pharma-ceuticals, and biotechnology (Hastuti *et al.*, 2011; Lee *et al.*, 2022; Ortega-Villar *et al.*, 2024).

A number of studies have successfully isolated thermo-proteolytic bacteria from various hot springs in the world and in Indonesia, showing the high diversity of microorganisms in these ecosystems (Nafia *et al.*, 2022; Santosa & Setyaningrum, 2024). Most studies are still focused on western Indonesia, such as Java and Sumatra. In contrast, eastern Indonesia, including Papua, which has abundant hot spring potential, remains very limited in terms of microbiological exploration.

Thermophilic microorganisms are microorganisms that can live at temperatures of 45–88°C. They contain heat-resistant proteins that resist denaturation, enabling them to adapt to extreme-temperature environments (Kumar & Nussinov, 2001). Several thermostable enzymes

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have slightly different amino acid compositions from the same enzymes in mesophilic bacteria, including a higher content of hydrophobic amino acids; one example is protease (Madigan *et al*., 2000; Domingguez *et al*., 2005).

Various thermostable enzymes have been identified from thermophilic bacteria, including amylase, DNA polymerase, xylanase, chitinase, lipase, and protease (Domingguez *et al*., 2005; Bozoglu *et al*., 2015; Scully & Johan, 2015; Ladeira et al., 2015). Bacillus strains are among the main protease producers used commercially. Proteases have been widely applied in the pharmaceutical, leather, laundry, food, and waste-processing industries. Various potential local isolates of thermophilic bacteria in Indonesia have been identified from several hot springs, especially on the islands of Java and Sumatra (Linawati, 2005; Agustini, 2006; Muharni, 2009; Agustien, 2010).

Isolation of thermophilic bacteria from hot springs in Papua was first reported by Runtuboi *et al*. (2014) from hot springs in Merauke Regency. A subsequent study by Ima *et al*. (2015) reported four thermophilic *Bacillus* sp. isolates from hot springs in Merauke Regency that showed proteolytic activity. The potential of thermophilic bacteria as enzyme producers widely distributed in Papua is one of the main reasons for this study. One of the areas selected for this study was the hot spring in Mariarotu Village, part of the Mariarotu Tourism Area (MTA), Yapen Islands Regency. The purpose of this study was to isolate and identify the characteristics of thermophilic bacteria from the hot spring source in the MTA area of Yapen Islands Regency.

MATERIALS AND METHODS

Time and place of research

This study was conducted over a period of 3 months in the Microbiology Laboratory, Department of Biology, FMIPA, Cenderawasih University. The bacterial samples examined originated from the hot spring in Mariarotu Village, Kosiwo District, Yapen Islands Regency, Papua. The method used in this study was an experiment conducted in the laboratory.

Materials and tools

The equipment used in this study included an incubator, vortex mixer, refrigerator, autoclave, analytical balance, Laminar Air Flow (LAF), laboratory glassware, and other supporting equipment. The materials used in this study included nutrient broth (NB), Plate Count Agar (PCA), Triple Sugar Iron Agar (TSIA), 70% alcohol, and Gram-staining reagents.

Research procedure

This study consisted of several stages, namely isolation, macroscopic observation (colony characteristics), microscopic observation (cell shape, Gram reaction, and spores), and biochemical tests.

Bacterial isolation

Bacterial isolation was carried out by collecting water samples using sample bottles that had been sterilized beforehand. A total of 100 mL of water sample was taken from the water surface at one point of the Mariarotu hot spring source at temperatures between 45–65°C, with two replications. Water pH was measured using universal pH paper, and physical water parameters such as odor, taste, and sample color were also recorded.

The water samples were stored in a sample box container and then taken to the laboratory for analysis within 24 hours after collection. The samples were then inoculated onto PCA medium and incubated at 50°C for 24 hours (Krieg *et al*., 2010; Patasik *et al*., 2015; Bozoglu *et al*., 2015). Colonies that grew were re-isolated on fresh medium to obtain pure isolates. Further identification was carried out based on macroscopic characteristics, microscopic characteristics, and biochemical tests, including colony character, Gram reaction, spores, carbohydrate fermentation (glucose, lactose, fructose, maltose), indole production, catalase production, methyl red test, Voges–Proskauer test, TSI test, and Simon’s test (Capucino & Sherma, 1992).

Data analysis

Data analysis in this study was conducted using a descriptive-analytical approach, which involved integrating data from macroscopic and microscopic observations as well as biochemical tests to obtain comprehensive bacterial identification results. This data analysis method aims to present the data objectively and provide an in-depth analysis to understand the relationships between parameters and the validity of the resulting identifications.

RESULTS AND DISCUSSION

Characteristics of water samples

Water sampling was carried out at two points, namely at the spring source and in the pool. Based on observations of the physical parameters, the temperatures at the two sampling points did not differ significantly, ranging from 39–40°C. The water felt warm, was clear, and had a fresh taste. According to Lee *et al.* (2022), temperature is one of the factors that influence microbial growth. Thermophilic microbes grow best at high temperatures, such as in hot springs.

The pH values of the two samples were also very similar, around pH 7–7.3. This indicates that the water was neutral and safe for consumption, and that such a pH is also very favorable for aquatic life and plants around the site. Sharma *et al.* (2024) found that the pH or acidity level, determines enzyme stability and microbial metabolism; most bacteria generally grow at a neutral pH (6.5–7.5).

The dissolved oxygen (DO) levels measured at the sampling locations indicate conditions that support the survival of surrounding organisms. Measurement of dissolved oxygen (DO) is an important parameter in determining water quality because it plays a role in the respiration of aquatic organisms and in the decomposition of organic matter (Effendi, 2003).

Table 1. Characteristics of water samples from the hot spring source.

Sampling point	Physical characteristics			
	Temperature (°C)	pH	Taste	DO
1	40	7.3	Fresh	5
2	39	7.1	Fresh	5

The environmental conditions described above indicate that this environment is a suitable habitat for thermophilic bacteria. Thermophilic bacteria can grow and develop at temperatures around 45–80°C, and some are even able to grow at temperatures up to 100°C. According to Lestari (2000), the ability of thermophilic bacteria to live under high-temperature environmental conditions is due to the fact that they possess enzymes and proteins that are almost entirely heat-stable compared with those of mesophilic bacteria. Water characteristics serve as ecological selection factors that determine the composition of microbial communities. Any change in these parameters will alter the structure and function of microbial communities, including their adaptive capacity and metabolite production.

Isolation and characterization of bacteria

Isolation of bacteria from the two hot spring points produced six isolates, with three isolates obtained from point I and coded A (1A, 2A, 3A), whereas three isolates from point II were coded B (1B, 2B, 3B).

Sampling hot water at different points can affect the number of isolates obtained. Table 2 shows the bacterial isolates identified macroscopically based on several characteristics, including colony shape, cell shape, margin, and size, whereas microscopic characterization evaluated several traits such as cell shape, color, size, the presence or absence of endospores, and catalase activity. Biotic and abiotic factors are factors that influence environmental conditions and thereby support the growth of thermophilic bacteria in hot springs. Biotic factors at the sampling sites included fallen leaves, grasses, mosses, and other organic sources that serve as energy sources for microorganisms (Rahmadani *et al.*, 2015).

Observations of the six isolates showed relatively similar macroscopic and microscopic

Table 2. Results of macroscopic, microscopic, and catalase characterization.

Isolate	Macroscopic Test Results									Identification (Bergey's Manual of Determinative Bacteriology)
	Morphology					Staining			Catalase	
	Colony shape	Color	Margin	Size	Surface	Cell shape	Gram	Endospore		
1A	Circular	White	Entire	Small	Smooth and shiny	Rod	+	+	+	*Bacillus* sp.
2A	Circular	Yellow	Indented	Small	Smooth and shiny	Rod	+	+	+	
3A	Circular	White	Entire	Small	Smooth and shiny	Rod	+	+	+	
1B	Irregular	White	Entire	Small	Smooth and shiny	Rod	+	+	+	
2B	Irregular	White	Serrate	Medium	Rough	Rod	+	+	+	
3B	Circular	White	Indented	Medium	Wrinkled	Rod	+	+	+	

Table 3. Physiological characteristics with biochemical tests.

No	Sample code	Indol	SC	TSIA	SIM	MR-VP	Man	Mal	Lak	Suk	Glu	Katalase
1.	1A	+	+	–	+	–	+	+	+	+	+	+
2.	2A	+	+	–	+	+	+	+	+	+	+	+
3.	3A	–	+	–	+	–	+	+	–	+	+	+
4.	1B	–	+	–	+	+	+	–	–	+	+	+
5.	2B	+	+	–	+	–	+	+	+	–	+	+
6.	3B	–	+	–	+	+	–	+	–	+	+	+

Note: - = negative, + = positive, SC = Simmon Citrate, TSIA = Triple Sugar Iron Agar, SIM = Sulfur Indole Motility, MR = Methyl Red, VP = Voges Proskouer.

characteristics. Macroscopically, most isolates showed circular to irregular colony shapes with entire, indented, and serrate margins. Colony surfaces were generally smooth and shiny, although some isolates had rough and wrinkled textures. Colony color was dominated by white, except for isolate 2A, which was yellow (Table 2). This variation indicates phenotypic differences among isolates, even though they belong to the same bacterial group (Cappuccino & Sherman, 2014).

Based on microscopic observations, all isolates were bacilli and exhibited a Gram-positive reaction, indicating the presence of a cell wall with a high peptidoglycan content. All isolates were able to form endospores, which are characteristic of certain bacteria in coping with extreme environmental conditions (Madigan *et al*., 2018).

Catalase test results showed that all isolates were positive, as indicated by the formation of oxygen bubbles (Figure 1d) after the addition of H_2O_2. Bacterial isolates are capable of producing the enzyme catalase, which breaks down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). Hydrogen peroxide itself is a reactive compound that is toxic to cells because it can damage important components such as proteins, lipids, and nucleic acids. Therefore, the presence of the enzyme catalase serves as an indicator of the bacterium's physiological adaptation to aerobic conditions.

This indicates the ability of the bacteria to produce the catalase enzyme to break down peroxide compounds, a characteristic commonly found in aerobic bacteria (Leboffe & Pierce, 2012).

Based on the identification results in this study (Table 2), all isolates showed characteristics that point to the genus Bacillus, with phenotypic variations visible in the biochemical tests (Table 3). These results are consistent with various previous studies that reported the dominance of bacteria from the genus Bacillus in extreme environments such as hot springs.

Several recent studies support these results, one of which is Corneles *et al.* (2023) on the Lahendong hot spring, North Sulawesi, which reported that the thermophilic bacteria found were dominated by the genera *Bacillus* and *Geobacillus*, both of which are capable of producing thermostable enzymes such as lipase.

The isolation and phenotypic testing of the six bacterial isolates showed positive reactions in the catalase test, citrate utilization (SCA), and metabolic activity in the SIM test, indicating that the isolates are aerobic bacteria with good metabolic adaptation capacity. However, the biochemical test results showed significant phenotypic heterogeneity, especially in the indole test, MR-VP, and carbohydrate fermentation patterns. This variation suggests the presence of several species or strains within the genus Bacillus. This reflects high metabolic flexibility, which is an important strategy for microorganisms to survive in extreme environments such as hot springs.

The broad carbohydrate fermentation pattern shown by most isolates, especially toward glucose, sucrose, and mannitol, indicates that the isolates possess a relatively complete enzymatic system for degrading various organic substrates. Not all isolates were able to ferment lactose or maltose, indicating limitations in specific enzymes such as β-galactosidase or certain amylolytic enzymes. This variation is an important indicator of metabolic differences among isolates, further strengthening the evidence for species diversity within the same habitat.

This study shows the same pattern as many previous studies reporting that hot springs are ecosystems with high microbial diversity, although they are often dominated by bacterial groups such as *Bacillus*. Sharma *et al.* (2024) showed that this bacterial group is capable of producing secondary metabolites such as hydrolitic enzymes, antimicrobial compounds, and other bioactive molecules that play a role in adaptation to extreme environments. The production of these metabolites not only serves to ensure survival, but also provides a competitive advantage within complex microbial communities.

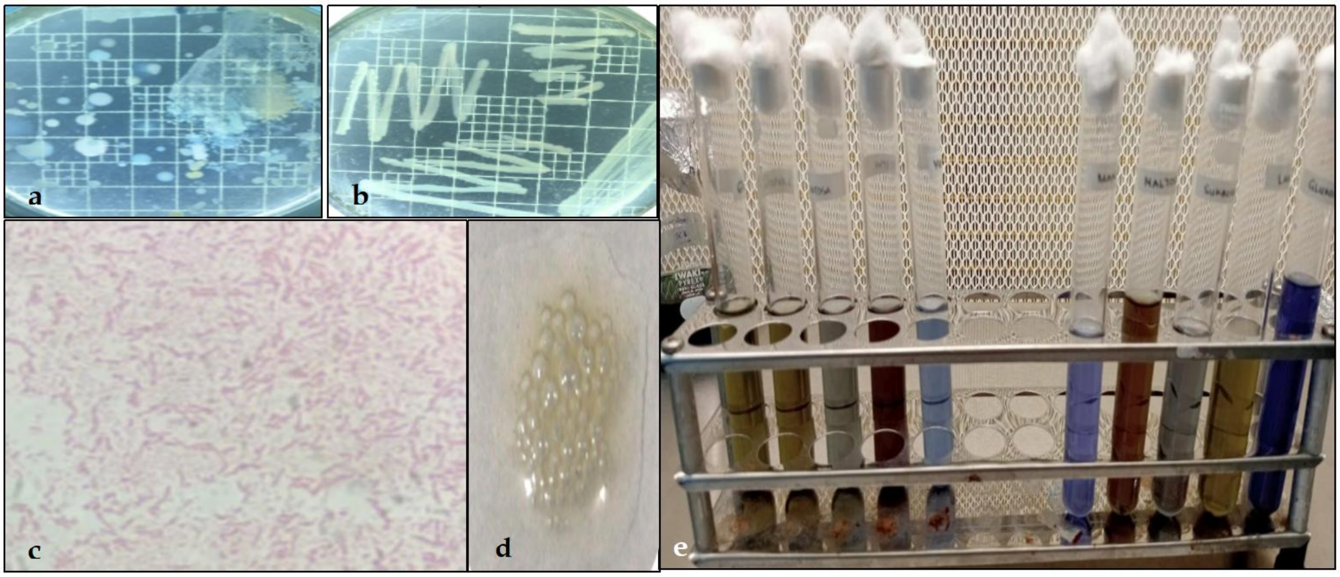

Figure 1. Characteristics of the bacterial isolates. (a) and (b) bacterial colony morphology, (c) staining and cell morphology (rod shape) and Gram-positive reaction in the isolates, (d) positive catalase reaction in the isolates, (e) biochemical test results of the isolates.

This similarity indicates that the isolates obtained in this study are relevant not only taxonomically but also functionally.

The phenotypic identification approach used in this study has limitations in distinguishing species specifically. Metagenomic studies show that microbial communities in hot springs are much more complex than those detected through conventional culture-based methods (Zhang *et al*., 2025). The limitations of the phenotypic approach lie not only in its low taxonomic resolution but also in its inability to capture the intraspecific genetic variation that plays a crucial role in ecological function. Microorganisms that are phenotypically similar may exhibit significant differences at the genomic level, particularly in genes involved in secondary metabolic pathways. In extreme environments such as hot springs, high selective pressure drives molecular adaptation.

Metagenomic analysis can reveal the existence of the “rare biosphere”—groups of microbes with low abundance but important ecological roles—which often go undetected by conventional methods. The presence of these microbes holds the potential to serve as a source of high-value enzymes or new bioactive compounds. This study also has important implications for biotechnology, as isolates with broad metabolic capabilities have potential as sources of thermostable enzymes that can be utilized in the food, pharmaceutical, and environmental industries.

CONCLUSION

In this study, identification was based on phenotypic characteristics, including macroscopic and microscopic features as well as biochemical tests. The results of the characterization indicate that all six isolates belong to the genus Bacillus.

This study not only confirmed the dominance of the genus Bacillus but also the presence of phenotypic diversity; therefore, further research using a molecular approach, such as 16S rRNA gene analysis, is needed to obtain a more accurate identification.

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