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Isolation and characterization of potential proteolytic and amylolytic bacteria from Bayanan hot spring as bioremediation agents

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ABSTRACT. Hot water temperature can be a place for the development of thermophilic bacteria. Thermophilic bacteria produce thermostable enzymes which are needed in various industrial fields such as agriculture, food, detergent, pharmacology, and bioremediation agents. This study aims to isolate and characterize potential proteolytic and amylolytic bacteria from the Bayanan hot spring. Samples were collected from two distinct locations within the geothermal environment: the hot spring source itself and the associated water storage pools. The collected samples underwent a dilution and isolation process to obtain pure bacterial cultures. Subsequently, these isolates were inoculated onto selective media, including skim milk agar (SMA) and nutrient agar (NA), to assess their proteolytic and amylolytic activities, respectively. The presence and extent of these enzymatic activities were determined by measuring the diameter of the clear zone surrounding each bacterial colony. The analysis revealed the presence of eight proteolytic bacterial isolates and twenty amylolytic isolates. Notably, a hydrolysis index threshold of ≥2.5 was implemented to identify isolates with high potential for proteolytic and amylolytic activity. Based on this criterion, two proteolytic bacterial isolates (codes B2-12-C3 and B1-12-B3) exhibited a proteolytic index exceeding 2.5. Furthermore, eight amylolytic isolates displayed an index above 2.5 (codes B1-10-A1, B1-10-A3, B2-8-A1, B2-8-A2, B2-10-A1, B2-10-A2, B2-12-C1, and B2-12-C2). These isolates, particularly those with high hydrolysis index values, has potential as bioremediation agents due to their demonstrated ability to efficiently hydrolyze proteins and starches.

Keywords: amylase enzymes; bioremediation; Gram staining; protease enzymes; thermophilic bacteria

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INTRODUCTION

Indonesia, a tropical archipelago nation, is characterized by a multitude of volcanic mountain ranges exhibiting high levels of activity (Badan Geologi, 2023). This specific geological context translates to a widespread distribution of geothermal resources across the Indonesian islands, including hot springs (EBTKE, 2020). One of the hot springs in Central Java is the Bayanan Hot Spring, which is located 17 km southeast of Sragen City (Diskominfo, 2023). Right in the hamlet Bayanan, Jambeyan Village, Sambirejo District, Sragen Regency. The Bayanan hot spring have the physical properties of yellow water with a slight smell of sulfur, the pH of the water is close to neutral (pH 6) and the temperature is ± 37°C (Helmi *et al*., 2020).

Hot water temperature can be a place for the development of thermophilic bacteria, which live and grow at temperatures of 20°C to 80°C, usually growing optimally at temperatures of 50°C to 65°C (Canganella & Wiegel, 2014; Pandey *et al*., 2015). Thermophilic bacteria, owing to their production of thermostable enzymes with applications across diverse industries (agriculture, food science, detergents, pharmaceuticals), hold immense promise for bioprospecting (Arora *et al*., 2015; Sharma *et al*., 2019; Finore *et al*., 2023; Hussian *et al*., 2023), while also offering potential for bioremediation and biodegradation of contaminants (Mehetre *et al*., 2019; Mir *et al*., 2021).

A significant research need persists in exploring microbial enzymes capable of degrading polymers like amylase, protease, cellulase, xylanase, and pullulanase (Solanki *et al*., 2021; Xia *et al*., 2021; Šuchová *et al*., 2022). Particularly noteworthy are proteolytic bacteria isolated from hot spring belonging to genera like *Bacillus, Brevibacillus* (Gomri *et al*., 2019; Yasir *et al*., 2019), *Pseudomonas* (Oztas Gulmus *et al*., 2020), *Proteus* (Jardine & Ubomba-Jaswa, 2020), *Thermomonas* (Mohammad

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et al., 2017), and *Staphylococcus* (Banerjee *et al*., 2024). These bacteria are crucial for their ability to secrete proteases into the surrounding environment. These proteolytic enzymes function by hydrolyzing protein polymers into smaller peptide chains and ultimately amino acids (Mótyán *et al*., 2013; Callmann *et al*., 2020). Amylase enzymes, alongside proteases, hold significant importance in various industrial applications, including pharmaceuticals, textiles, food production, and detergents, rely on their functionality. Amylolytic bacteria, particularly those belonging to *Bacillus* (Kiran *et al*., 2018), *Geobacillus* (Soy *et al*., 2019), and *Enterobacter* (Ashkan *et al*., 2020), are prominent producers of these enzymes. Amylases play a crucial role in starch-rich waste management. Examples of such waste include liquid effluents from flour mills, food scraps, and fish pond waste (Champasri *et al*., 2021).

Many studies on thermophilic bacteria from hot springs in Indonesia have been carried out, including bacteria *Pseudomonas* sp. from the Lejja hot spring (Mahmudah *et al*., 2016), *Bacillus cereus* producing amylase enzymes from Way Panas Kalianda hot spring (Mahestri *et al*., 2021). In addition, there were 3 isolates of proteolytic bacteria and 6 amylolytic bacteria that were successfully isolated from the Gedongsongo hot spring (Rukmi *et al*., 2018). The investigation of thermophilic bacteria inhabiting the Bayanan hot spring presents a compelling research avenue. Notably, this exploration would be the first of its kind in this specific geothermal environment, offering the potential to unearth unique enzymatic capabilities. Therefore, this study aims to isolate and identify thermophilic bacteria that produce proteolytic and amylolytic enzymes from the Bayanan hot spring. This research paves the way for the development of thermostable enzymes and bioremediation agents from a previously unexplored geothermal environment.

MATERIALS AND METHODS

Dilution of water samples. The researchers first diluted the water samples in stages up to 10^{-12} dilution. Distilled water was used as the solvent, and each tube received 9 ml. A 1 ml sample was taken and homogenized in the first dilution tube. They then transferred another 1 ml sample from the first dilution tube and homogenized it in the second dilution tube. This process was continued for all subsequent dilutions. The final results used for bacterial isolation were the dilutions at the last three levels: 10^{-8},10^{-10}, and 10^{-12} (Aksani *et al*., 2016).

Bacterial isolation. Bacterial isolation was carried out by pouring a sterile nutrient agar (NA) medium into a sterile petri dish. After 0.1 mL of solid medium, the hot water sample was taken with a micropipette, put into a petri dish, spread with an L stick on the agar surface, and then incubated for 24 h at 37°C. Purification of bacteria is carried out by taking one cycle of bacterial colonies that grew differently on the previous NA medium and inoculating them into each other petri dish.

Identification of morphology and Gram staining. Morphological characteristics of bacteria can be observed using the naked eye (macroscopic). Identification characteristics of bacterial colonies include: color, shape, edges, and elevation of the colony. Microscopic observations were carried out using the Gram staining technique. Bacterial isolate was smeared in a single cycle onto an object glass and then fixed. Crystal violet was dripped onto the bacterial colony and left to sit for 60 s. Then, the preparations are washed using running water and dried. Lugol's solution dripped on the bacterial colony and left to sit for 60 s. The preparations were washed with running water and then air dried. The preparation was then dripped with 2-3 drops of alcohol-acetone solution, then washed again and dried in the air. After that, it was observed under a microscope (Mahestri *et al*., 2021).

Protease and amylase activity test. The protease activity test was conducted by inoculating a pure culture of 24-h-old bacterial isolates on skim milk agar (SMA) media. Isolates were incubated at 37°C for 48 h, and a clear zone was observed in each colony. Colonies that form clear zones are protease-producing bacteria. Indications of bacterial isolates that produce proteases and can properly degrade protein (casein) are characterized by a clear zone around the colony. The amylase activity test was conducted by inoculating a pure culture of 24-h-old bacterial isolates in NA media containing 1% starch. Bacterial isolates were inoculated for 48 h at 37°C on NA media containing starch. The

activity of the amylase enzyme was tested by dripping iodine solution on the surface of the press that was growing bacteria. Isolates that produce amylase are indicated by a clear zone around the bacterial colony. Next, the diameter of the clear zone was measured to determine the proteolytic and amylolytic index of the isolate (Hudzicki, 2009). The proteolytic index is calculated using the formula IP = Clear zone diameter (cm) / Colony diameter (cm). The amylolytic index also uses the formula IA = Clear zone diameter (cm) / Colony diameter (cm).

Data analysis. The data analysis technique was carried out using descriptive analysis techniques. The research data obtained was processed using Microsoft Excel. Data is presented through images, graphs and tables.

RESULTS AND DISCUSSION

An investigation of the Bayanan hot spring revealed a water temperature of 40°C at the source and 37°C in the underlying pool. The hot spring water exhibited a neutral pH of 7. Following isolation procedures, a total of 28 bacterial isolates were obtained from the Bayanan hot spring. Among these isolates, characterization identified 8 exhibiting proteolytic activity and 20 demonstrating amylolytic activity. The isolated proteolytic and amylolytic bacteria displayed diverse colony morphologies based on macroscopic observations. To further characterize these isolates, both macroscopic examination and microscopic Gram staining techniques were employed. Gram staining serves as a diagnostic tool for differentiating bacterial types based on cell wall structure. This difference in cell wall composition leads to variations in dye permeability and response to washing solutions during the staining process. The morphological characteristics of the isolated proteolytic and amylolytic bacteria are presented in Table 1.

Table 1. Characteristics of proteolytic and amylolytic bacteria

No	Isolate code	Morphological characteristics of proteolytic bacteria				Gram staining	
		Colour	Colony shape	Edge	Elevation	Gram	Shape
1	B1-12-B1	Yellowish clear	Irregular	Entire	Flat	+	Basil
2	B1-12-B2	Yellowish clear	Irregular	Entire	Flat	+	Coccus
3	B1-12-B3	Yellowish clear	Irregular	Entire	Flat	+	Coccus
4	B1-12-B4	Yellowish clear	Irregular	Entire	Flat	+	Coccus
5	B2-12-C1	Clear white	Irregular	Undulate	Flat	+	Basil
6	B2-12-C2	Clear white	Irregular	Undulate	Flat	+	Coccus
7	B2-12-C3	Clear white	Irregular	Undulate	Flat	+	Coccus
8	B2-12-C4	Clear white	Irregular	Undulate	Flat	-	Basil
		Morphological characteristics of amylolytic bacteria					
9	B1-10-A1	White	Irregular	Entire	Flat	+	Basil
10	B1-10-A3	White	Irregular	Entire	Flat	+	Basil
11	B2-8-A1	White	Irregular	Undulate	Convex	+	Coccus
12	B2-8-A2	White	Irregular	Undulate	Convex	+	Basil
13	B2-8-A3	White	Irregular	Undulate	Convex	-	Coccus
14	B2-8-A4	White	Irregular	Undulate	Convex	+	Basil
15	B2-10-A1	White	Circular	Entire	Flat	+	Basil
16	B2-10-A2	White	Circular	Entire	Flat	+	Basil
17	B2-10-A3	White	Circular	Entire	Flat	-	Basil
18	B2-10-A4	White	Circular	Entire	Flat	+	Coccus
19	B2-10-B1	White	Irregular	Undulate	Convex	+	Basil
20	B2-10-B2	White	Irregular	Undulate	Convex	+	Coccus
21	B2-10-B3	White	Irregular	Undulate	Convex	-	Basil
22	B2-10-B4	White	Irregular	Undulate	Convex	+	Basil
23	B2-12-B1	Clear	Circular	Entire	Convex	+	Coccus
24	B2-12-B2	Clear	Circular	Entire	Convex	-	Basil
25	B2-12-B3	Clear	Circular	Entire	Convex	-	Basil
26	B2-12-B4	Clear	Circular	Entire	Convex	+	Basil
27	B2-12-C1	Clear white	Irregular	Undulate	Flat	+	Coccus
28	B2-12-C2	Clear white	Irregular	Undulate	Flat	-	Basil

Gram staining of the isolated proteolytic bacteria revealed a predominance of Gram-positive strains, with 7 isolates identified as such and only 1 isolate classified as Gram-negative. Similarly, the Gram staining results for amylolytic isolates demonstrated a majority of Gram-positive bacteria (14 isolates), while 6 isolates were identified as Gram-negative. Fig. 1 visually depicts the distinct microscopic appearance of Gram-negative and Gram-positive bacteria following Gram staining.

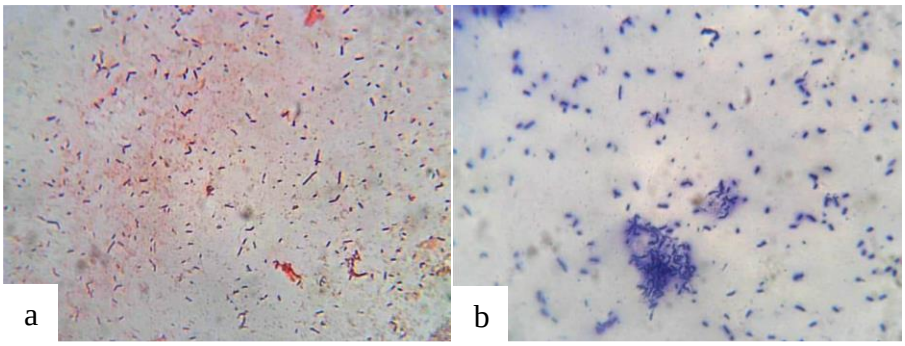

Fig. 1. Results of observations of gram-negative and gram-positive bacteria using a microscope: a. Gram-negative bacteria from isolate B2-10-A3; b. Gram-positive bacteria from isolate B2-12-C3

Proteolytic bacteria are characterized by their ability to produce extracellular proteases. The presence of a clear zone surrounding bacterial colonies grown on SMA media signifies the breakdown of the protein substrate by these proteases (Afrin *et al*., 2024; Kaempe *et al*., 2024). Similarly, amylolytic activity can be assessed by the addition of iodine solution to starch-containing NA media. The formation of a clear zone around the colony indicates the hydrolysis of starch by amylolytic bacterial isolates, thus this enzymatic breakdown converts starch polymers into simpler oligosaccharides or sugar molecules (Sachdev *et al*., 2016; Nimisha *et al*., 2019). Fig. 2 visually depicts the formation of clear zones around colonies exhibiting amylolytic activity.

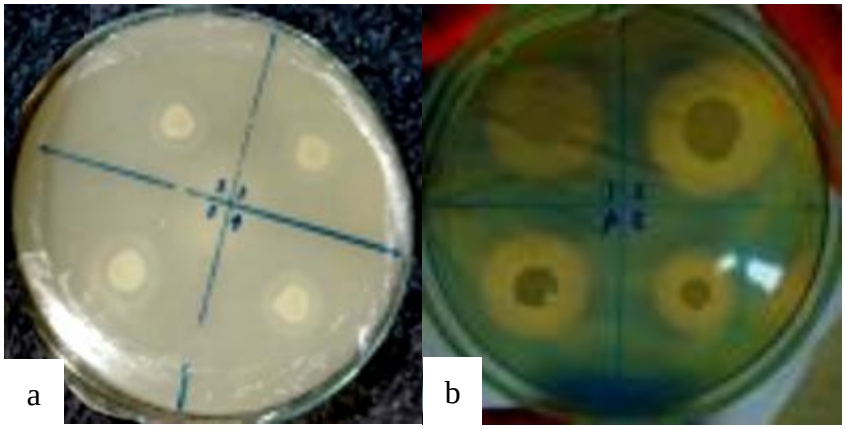

Fig. 2. Isolate bacteria on selection media; a. Proteolytic bacterial isolates and clear zones formed on SMA media, b. Amylolytic bacterial isolates and clear zones formed on NA media supplemented with starch

Following a 48-hour incubation period, both colony diameter and clear zone diameter were measured. A qualitative hydrolysis activity assessment was then performed. The resulting hydrolysis index was categorized as follows: high for a ratio ≥ 3, medium for a range of ≥ 1 to 2.9, and low for a ratio between 0 and 0.9 (Goñi *et al*., 1997). Table 2 presents the results of the proteolytic and amylolytic index measurements.

The isolate with the highest proteolytic index was B2-12-C3, with an index of 2.89, followed by isolate B1-12-B3, with an index of 2.86, and isolate B1-12-B1, with an index of 2.45. The isolate with the lowest proteolytic index is B2-12-C4, with an index of 1.12. Meanwhile, the isolate with the highest amylolytic index was B2-12-C1 with an index of 3.75, followed by isolate B2-8-A2 with an

index of 3.33 and isolate B2-10-A1 with an index of 3.17. The isolate with the lowest amylolytic index was B2-12-B3, with an index of 1.40 (Table 2).

Table 2. Proteolytic and amylolytic index measurement

No	Isolate code	Zone clear diameter (cm)	Colony diameter (cm)	Proteolytic index	Amylolytic index
1	B1-12-B1	1.4	0.6	2.45	-
2	B1-12-B2	1.0	0.6	1.67	-
3	B1-12-B3	2.0	0.7	2.86	-
4	B1-12-B4	1.7	1.1	1.55	-
5	B2-12-C1	1.5	1.3	1.20	-
6	B2-12-C2	1.1	0.6	1.83	-
7	B2-12-C3	1.3	0.5	2.89	-
8	B2-12-C4	1.4	1.3	1.12	-
9	B1-10-A1	1.3	0.5	-	2.60
10	B1-10-A3	1.4	0.5	-	3.11
11	B2-8-A1	1.5	0.5	-	2.90
12	B2-8-A2	2.0	0.6	-	3.33
13	B2-8-A3	1.5	0.8	-	2.00
14	B2-8-A4	1.7	0.9	-	1.83
15	B2-10-A1	1.9	0.6	-	3.17
16	B2-10-A2	1.6	0.6	-	2.58
17	B2-10-A3	1.5	0.7	-	2.31
18	B2-10-A4	1.4	0.7	-	2.15
19	B2-10-B1	1.5	0.7	-	2.14
20	B2-10-B2	1.4	0.6	-	2.33
21	B2-10-B3	1.5	0.7	-	2.23
22	B2-10-B4	1.5	0.8	-	1.81
23	B2-12-B1	1.8	0.9	-	2.12
24	B2-12-B2	1.7	0.7	-	2.43
25	B2-12-B3	1.4	1.0	-	1.40
26	B2-12-B4	2.0	1.0	-	2.00
27	B2-12-C1	0.8	0.2	-	3.75
28	B2-12-C2	2.0	0.8	-	2.67

The potential application of proteolytic and amylolytic bacterial isolates in bioremediation hinges on their hydrolysis capabilities. The evaluation of isolates from this study revealed that all proteolytic bacteria fell within the medium hydrolysis category. Conversely, amylolytic isolates displayed a wider range of activity, with four isolates categorized as high (B1-10-A3, B2-8-A2, B2-10-A1, and B2-12-C1) and the remaining 16 classified as medium. These findings suggest that the amylolytic isolates exhibiting high hydrolysis potential, particularly those with codes B1-10-A3, B2-8-A2, B2-10-A1, and B2-12-C1, warrant further investigation for their suitability in bioremediation processes.

The identification of proteolytic and amylolytic isolates with high hydrolysis potential from a hot spring opens doors for the development of thermostable enzymes applicable in bioremediation processes at elevated temperatures. Conventional bioremediation approaches are often limited by environmental conditions, with temperature being a key factor (Azubuike *et al*., 2016; Jardine, 2022). Many pollutants persist in environments with high temperatures, such as industrial waste sites or post-combustion zones (Gaur *et al*., 2018; Munawer, 2018; Kishor *et al*., 2021). Thermostable enzymes, like those potentially produced by the identified proteolytic and amylolytic amylolytic isolates from local hot spring, can function effectively at these elevated temperatures, accelerating the biodegradation of pollutants. Further characterization of these isolates and their enzymatic machinery could lead to the development of robust bioremediation strategies applicable in challenging environments.

CONCLUSION

Based on the isolation results, all isolates of proteolytic bacteria are in the medium category. Meanwhile, for amylolytic bacterial isolates, there were 4 bacterial isolates in the high category and 16 others in the medium category. 4 isolates of amylolytic bacteria were included in the high hydrolysis category, namely isolates with codes B1-10-A3, B2-8-A2, B2-10-A1, and B2-12-C1. This bacterial isolate has potential as a bioremediation agent because it has high starch hydrolysis ability.

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