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Isolation and Characterization of Protease-Producing Thermophilic Bacteria from Padang Dama Hot Spring, Solok, Indonesia

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Abstract: This study aimed to isolate, screen, and partially characterize protease-producing thermophilic bacteria from the Padang Dama hot spring, Solok, Indonesia. The main issue addressed in this study is the limited information regarding thermophilic bacteria capable of producing protease enzymes from this geothermal environment, despite their potential use in industrial processes requiring stable enzymes. Isolation was carried out using Nutrient Agar followed by incubation at 50 °C for 24 hours. Screening of protease activity was performed using Skim Milk Agar based on clear zone formation, and the proteolytic index (PI) was calculated to evaluate enzyme activity. Selected isolates were further characterized through macroscopic, microscopic, and biochemical analyses. The results showed that three isolates exhibited proteolytic activity, with isolate TUA-109 showing the highest proteolytic index, indicating superior enzymatic potential compared to other isolates. Partial characterization revealed that isolate TUA-109 is a Gram-negative, rod-shaped bacterium with positive catalase activity and motility, which are important traits supporting its adaptability and potential for enzyme production under extreme conditions. These findings highlight the potential of thermophilic bacteria from the Padang Dama hot spring as sources of protease enzymes, with isolate TUA-109 identified as a promising candidate for further investigation, particularly for industrial enzyme production. Future studies are recommended to optimize enzyme production and evaluate its application in industrial processes.

Keywords: Thermophilic bacteria; protease thermostable; hot spring; proteolytic index; characterization

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INTRODUCTION

Thermophilic bacteria are microorganisms capable of growing at high temperatures and are commonly found in geothermal environments such as hot springs and volcanic areas. These microorganisms have attracted significant attention due to their ability to produce thermostable enzymes that remain active and stable under extreme environmental conditions. Thermostable enzymes are particularly valuable for industrial processes because they maintain catalytic activity at high temperatures and under harsh reaction conditions (Burkhardt, 2023). Enzymes function as biological catalysts that accelerate biochemical reactions and play essential roles in various industrial and biotechnological processes (Muqarrahmah et al., 2023). Among the enzymes produced by thermophilic microorganisms, proteases are one of the most important groups due to their ability to hydrolyze peptide bonds in proteins and their wide range of industrial applications, including detergents, pharmaceuticals, food processing, leather processing, and waste treatment (Razzaq et al., 2019). These previous studies highlight the importance of thermophilic bacteria as potential sources of industrially relevant protease enzymes.

The demand for thermostable enzymes continues to increase due to their advantages in industrial applications. Enzymes derived from thermophilic microorganisms exhibit higher stability and catalytic efficiency compared with enzymes from mesophilic organisms, allowing their use under extreme processing conditions (Guta et al., 2024). For example, in the detergent industry, thermostable proteases remain active in high-temperature and alkaline conditions, improving stain removal efficiency, while in food processing, they enhance protein hydrolysis efficiency and help reduce microbial contamination. Geothermal ecosystems are considered important reservoirs of thermophilic microorganisms with diverse metabolic capabilities, and previous studies have reported their potential to produce extracellular enzymes with industrial value (Kumar et al., 2024).

Solok Regency in West Sumatra, Indonesia, possesses several geothermal areas that serve as natural habitats for thermophilic microorganisms. One of these is the Padang Dama hot spring, which has water temperatures ranging from 50–54 °C and a pH of approximately 5. These conditions create a selective environment that favors the growth of thermophilic bacteria, as such microorganisms possess structural and enzymatic adaptations that enable survival and metabolic activity at elevated temperatures (Sarmiento et al., 2022; Guta et al., 2024). In addition, thermophilic enzymes exhibit high thermal stability and catalytic efficiency, supporting microbial activity under extreme environmental conditions (Wang et al., 2021). The surrounding environment is characterized by natural vegetation such as trees, ferns, mosses, and organic litter that may serve as nutrient sources for microbial communities. The availability of organic substrates combined with extreme environmental conditions further enhances the growth and activity of thermophilic bacteria.

Previous studies have shown that geothermal ecosystems harbor diverse microbial communities with significant enzymatic potential, including the production of industrially important enzymes (Colman et al., 2019). Despite the significant industrial importance of protease enzymes, particularly their contribution of approximately 60–65% to the global enzyme market (Mushtaq et al., 2024), information regarding thermophilic bacteria capable of producing protease enzymes from the Padang Dama hot spring remains limited. This knowledge gap is critical, as the discovery of novel thermophilic protease-producing bacteria from this geothermal environment may contribute to the development of efficient and thermostable enzymes for industrial applications.

Therefore, this study aimed to isolate and screen thermophilic protease-producing bacteria from the Padang Dama hot spring, Solok, Indonesia, and to identify isolates with high proteolytic activity. The isolated bacteria were further characterized based on their morphological and biochemical properties. This study is expected to contribute to the understanding of thermophilic protease-producing bacteria from Indonesian geothermal environments and to provide a scientific basis for the development of thermostable protease enzymes for industrial applications, particularly in the detergent, food processing, and biotechnology sectors.

METHOD

This study was conducted using an experimental laboratory approach to isolate, screen, and characterize thermophilic bacteria producing protease enzymes from a geothermal environment. The research was carried out from March to June 2025 at the Biotechnology Laboratory of Biota Sumatera Laboratory, Universitas Andalas. The experimental procedure consisted of several sequential stages, including sample collection, bacterial isolation, screening of proteolytic activity, and characterization of isolates. All experimental processes were performed under controlled laboratory conditions to ensure consistency and reliability of the results.

The materials used in this study included hot spring water samples collected from Padang Dama, Solok Regency, Nutrient Agar (NA) (Merck), Nutrient Broth (NB) (Merck), and Skim Milk Agar (SMA) media (Merck). Additional reagents used for staining and biochemical tests included crystal violet, iodine solution, safranin, alcohol, and hydrogen peroxide (H_2O_2). The equipment used consisted of standard microbiological laboratory instruments, including an autoclave, incubator, laminar airflow cabinet, microscope, micropipettes, and Petri dishes.

Sampling and Sample Preparation

Samples were collected from a geothermal hot spring located in Padang Dama, Solok Regency, West Sumatra, Indonesia, at coordinates 0° 53’ 26.78” S and 100° 39’ 48.64” E. Sampling was conducted at a single point using purposive sampling based on environmental conditions suitable for thermophilic microorganisms. Two bottles of hot spring water were collected aseptically to represent the sampling site. Before sampling, the bottles were sterilized using an autoclave at 121 °C for 15 minutes to prevent contamination. The environmental conditions at the sampling site showed a temperature range of approximately 50–54 °C and a pH of 6. Before bacterial isolation, the samples were incubated at 50 °C for 1 hour to enrich and activate thermophilic bacteria, allowing them to adapt to laboratory conditions and enhance their subsequent growth during isolation.

Isolation of Thermophilic Bacteria

Isolation of thermophilic bacteria was carried out using the pour plate method. This method was selected because it allows the growth of both surface and subsurface colonies, thereby increasing the likelihood of recovering diverse thermophilic bacteria from the sample. After the adaptation process, 1 mL of the sample was aseptically transferred into a sterile Petri dish using a micropipette. Approximately 15 mL of molten Nutrient Agar (NA) medium (Merck) was poured into the Petri dish and gently mixed to ensure even distribution. The medium was allowed to solidify, and the plates were incubated at 50 °C for 24 hours. Colonies that appeared were observed based on their morphological characteristics, including shape, size, color, and margin. Colonies showing distinct morphological characteristics were selected and subjected to purification on Nutrient Agar before further screening of proteolytic activity.

Purification of Thermophilic Bacteria

Purification was performed using the streak plate method. Selected colonies were aseptically transferred using an inoculating loop and streaked onto fresh Nutrient Agar (NA) medium (Merck). The plates were incubated at 50 °C for 24 hours. This process was repeated several times to obtain well-isolated single colonies, ensuring that each colony originated from a single bacterial cell and represented a pure culture. The process was continued until consistent colony morphology was observed across successive streaking. These isolates were then used for subsequent screening and characterization.

Screening of Protease-Producing Bacteria

Screening of protease-producing bacteria was performed using Skim Milk Agar (SMA) medium (Merck). The purified isolates were inoculated onto SMA plates and incubated at 50 °C for 24 hours. Proteolytic activity was indicated by the formation of clear zones surrounding bacterial colonies due to casein hydrolysis. The plates were observed after 24 hours of incubation, and the diameter of the clear zones and colony diameter were measured using a caliper. Measurements were performed in duplicate to ensure accuracy, and the clear zone was measured as the total diameter of the hydrolysis zone surrounding the colony. The proteolytic activity of each isolate was further evaluated by calculating the proteolytic index (PI) using the following formula:

$$\mathrm{IP} = \frac{\text{Clear Zone Diameter} - \text{Colony Diameter}}{\text{Colony Diameter}}$$

Isolates exhibiting higher proteolytic index values were considered to have stronger protease activity and were selected for further characterization (Agustien, 2010)

Characterization of Isolates

Selected isolates showing high proteolytic activity were further characterized based on morphological and biochemical properties. Macroscopic observations were performed to examine colony morphology, including shape, elevation, margin, and color. Microscopic observations were conducted to determine cell shape and arrangement. Gram staining was carried out using standard staining procedures to classify bacterial isolates based on their cell wall characteristics. The staining involved the sequential application of crystal violet, iodine solution, alcohol decolorization, and safranin as a counterstain, followed by observation under a microscope (Paray et al., 2023). Gram-positive bacteria were indicated by purple-colored cells, while Gram-negative bacteria appeared pink or red. Spore staining was performed to identify the presence of endospores using standard staining techniques involving primary staining with heat fixation, followed by microscopic observation (Sunatmo, 2007).

The presence of endospores was indicated by the appearance of distinct, refractile structures within the bacterial cells. The catalase test was conducted by adding hydrogen peroxide (H_2O_2) onto bacterial colonies to detect the presence of catalase enzyme. A positive result was indicated by the immediate formation of oxygen bubbles, while the absence of bubbles indicated a negative result. Motility testing was carried out using semi-solid media to observe bacterial movement based on the pattern of growth from the inoculation line. Diffuse growth radiating outward from the inoculation line indicated motile bacteria, whereas growth confined to the inoculation line indicated non-motile bacteria. All characterization procedures were performed under aseptic conditions to ensure accuracy and reliability of the results (Suryani et al., 2010). Aseptic techniques were maintained by conducting all inoculation and transfer processes in a laminar airflow cabinet, sterilizing inoculating tools using flame or autoclaving, and minimizing exposure to the external environment during handling and microscopic observations.

Data Analysis

Data obtained from this study were analyzed using descriptive qualitative and quantitative approaches. Protease activity was evaluated based on the formation of clear zones around bacterial colonies on Skim Milk Agar. The diameter of the clear zone and colony diameter were measured using a caliper, and the proteolytic index (PI) was calculated using the following formula:

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

The PI values were used to compare the proteolytic activity among isolates and to determine the most potential protease-producing bacteria. All measurements were performed in duplicate, and the average values were used for analysis. Data analysis was conducted using descriptive statistical methods to compare PI values among isolates.

Morphological and biochemical characteristics of the selected isolates were analyzed descriptively to identify their properties and differentiate between isolates. The results of these analyses were interpreted by comparing them with standard microbiological characteristics reported in previous studies. All experimental measurements were conducted in duplicate to ensure data reliability. The results were presented in the form of tables and figures to facilitate interpretation and discussion.

RESULTS AND DISCUSSION

The isolation, screening, and characterization of thermophilic bacteria producing protease enzymes from the Padang Dama hot spring, Solok Regency, resulted in the following findings:

Isolation of Thermophilic Bacteria

Thermophilic bacteria isolated from Padang Dama hot spring, Solok Regency, are presented in Table 1.

Table 1. The number of bacteria and thermophilic bacteria isolate as well as abiotic factors from Padang Dama hot springs, Solok Regency

Abiotic Factor					
Water Temperature (°C)	**pH**	**∑ Colony**	**∑ Bacteria (x10 cfu/mL)**	**∑ Isolate**	**Isolate Code**
54°C	6.3	35	3.5	2	TUA-106 TUA-107
		22	2.2	1	TUA-108
Amount		57	5.7	3	

Details: *TUA (Thermophilic Universitas Andalas)

Figure 1. Colonies and isolates of thermophilic bacteria from Padang Dama hot spring, Solok Regency

The results of thermophilic bacterial isolation obtained from the Padang Dama hot spring are presented in Table 1. A total of three bacterial isolates were successfully obtained using Nutrient Agar (NA) medium and incubation at 50 °C, which supports the growth of thermophilic microorganisms (Sarmiento et al., 2022). The successful growth of these isolates indicates the presence of bacteria adapted to elevated temperature environments (Wang et al., 2021). The number of colonies and isolates obtained in this study is closely associated with the environmental conditions of the sampling site, which exhibited temperatures ranging from 50–54 °C and a pH of approximately 6. These conditions create a selective environment that favors thermophilic bacteria, as high temperatures promote the growth of heat-tolerant microorganisms while inhibiting mesophilic competitors, thereby shaping microbial community structure in geothermal environments (Sarmiento et al., 2022; Kumar et al., 2024).

In addition, slightly acidic conditions may influence microbial diversity and select for specific thermophilic populations capable of adapting to such environments. Therefore, the successful isolation of thermophilic bacteria in this study is strongly influenced by the environmental characteristics of the Padang Dama hot spring, where temperature and pH act as key ecological factors regulating microbial growth and distribution in geothermal systems (Sarmiento et al., 2022; Hedlund et al., 2015; Kumar et al., 2024).

In addition to temperature and pH, other environmental factors such as mineral composition and nutrient availability may also influence the diversity of thermophilic bacteria. Geothermal environments are characterized by complex geochemical compositions, including dissolved minerals and reduced compounds that can support microbial growth (Colman et al., 2019; Inskeep et al., 2013). These minerals can function as essential cofactors in enzymatic reactions and support various metabolic pathways in thermophilic bacteria, thereby enhancing their survival and activity under extreme conditions (Merino et al., 2019). In addition, the availability of organic and inorganic nutrients influences microbial growth by providing energy sources required for cellular processes (Kumar et al., 2024).

These factors create selective pressure that allows thermophilic microorganisms with specific physiological and metabolic adaptations to dominate such environments (Inskeep et al., 2013). Thermophilic bacteria are known to possess thermostable enzymes and specialized cellular adaptations that enable efficient metabolism and structural stability under high-temperature and mineral-rich conditions (Littlechild, 2015). Previous studies have demonstrated that geothermal ecosystems harbor diverse thermophilic microbial communities with significant biotechnological potential (Gallo et al., 2024; Kumar et al., 2024). Differences in environmental conditions, even within similar geothermal systems, can lead to variations in microbial diversity and functional capabilities (Sarmiento et al., 2022). The findings of this study are consistent with these reports, indicating that the Padang Dama hot spring is a promising source of thermophilic bacteria with potential industrial applications.

Screening of Thermophilic Bacteria Producing Protease

Screening of thermophilic bacteria was conducted on thermophilic bacteria isolates obtained from Padang dama Hot Springs, Solok Regency, as presented in Table 2.

Table 2. Average colony diameter and clear zone, as well as the Proteolytic Index (PI) of thermophilic protease-producing bacteria

Isolate Code	$\sum$ Diameter (mm)		$\sum$ Proteolytic Index (PI)
	Colony	Clear Zone	
TUA-108	7.10	28.69	3.04
TUA-109	6.85	28.20	3.12
TUA-110	6.95	27.86	3.01

The results of protease activity screening of thermophilic bacterial isolates obtained from the Padang Dama hot spring are presented in Table 2. All isolates (TUA-108, TUA-109, and TUA-110) demonstrated proteolytic activity, as indicated by the formation of clear zones on Skim Milk Agar (SMA). The proteolytic index (PI) values varied among the isolates, with TUA-109 showing the highest PI value (3.12), followed by TUA-108 (3.04), and TUA-110 (3.01). These results indicate that all isolates have the ability to produce extracellular protease enzymes, although with different levels of activity.

The differences in proteolytic index values among isolates are closely related to variations in metabolic activity and enzyme production capacity. Protease production is influenced by several factors, including bacterial growth phase, nutrient availability, and environmental conditions (Fani et al., 2022). The presence of casein in SMA medium acts as a substrate that induces protease production, where bacteria secrete extracellular enzymes to hydrolyze proteins into simpler compounds for metabolism (Singh et al., 2016). The high proteolytic index value obtained for isolate TUA-109 indicates its superior ability to degrade protein substrates compared to the other isolates.

In the discussion regarding PI and proteolytic activity classification, it would be beneficial to explicitly state how this study's results compare with established standards for proteolytic activity, such as those presented by Ahmad et al. (2013). Isolates with high proteolytic index values are considered to have strong protease-producing capabilities and are potential candidates for further analysis and industrial application. The isolate TUA-109, which exhibited the highest PI value, is therefore selected as the most promising isolate for further characterization.

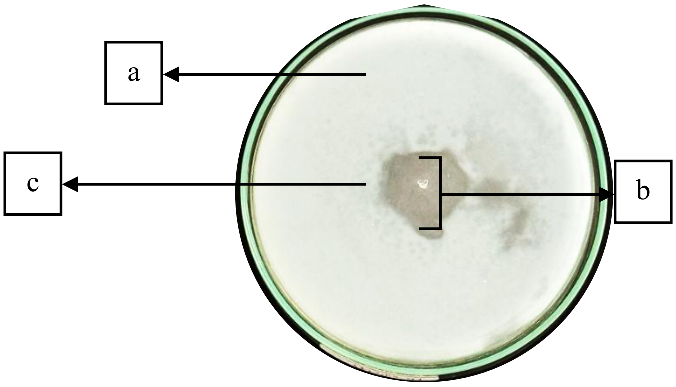

Figure 2. Screening of TUA-109 Isolate: (a) Skim Milk Agar Media (b) Clear Zone (c) Bacteria Colony

The formation of clear zones surrounding bacterial colonies on Skim Milk Agar (SMA), as shown in Figure 2, indicates the ability of the isolates to produce extracellular protease enzymes. These clear zones are formed as a result of casein hydrolysis into soluble peptides and amino acids, reflecting the proteolytic activity of the bacterial isolates (Razzaq et al., 2019; Yuniati et al., 2015). The variation in clear zone diameter among isolates reflects differences in enzyme production capacity. Isolates with larger clear zones tend to exhibit higher proteolytic activity, which is consistent with the proteolytic index (PI) values presented in Table 2. The PI value represents the ratio between the diameter of the hydrolysis zone and the colony diameter, serving as an indicator of the ability of bacteria to degrade protein substrates (Ahmad et al., 2013).

Based on the classification proposed by Ahmad et al. (2013), PI values lower than 2.1 are categorized as low, values between 2.1 and 3.1 as moderate, and values higher than 3.1 as high proteolytic activity. In this study, all isolates fall into the high activity category, indicating strong protease-producing capabilities and suggesting their potential for further study. Similar findings have been reported by Muqarramah et al. (2023), who isolated thermophilic protease-producing bacteria from the Bukik Gadang hot spring in Solok Regency, demonstrating that geothermal environments in this region are promising sources of proteolytic bacteria with significant enzymatic potential. This comparison indicates that thermophilic bacteria from the Padang Dama hot spring exhibit comparable characteristics and potential.

Protease production is influenced by several physiological and environmental factors. Nutrient availability, particularly protein substrates such as casein, plays an important role in inducing enzyme production, while bacterial growth phase also affects enzyme synthesis, with higher activity typically observed during the exponential phase These factors may directly contribute to the variation in PI values observed among the isolates in this study, as differences in metabolic activity, growth rate, and enzyme secretion capacity can result in varying levels of casein hydrolysis (Fani et al., 2022; Kumar et al., 2024).

Characterization of Isolates

Characterization was focused on isolate TUA-109, which exhibited the highest proteolytic index. The analyses included macroscopic, microscopic, and biochemical tests. The results are presented in Table 3.

Table 3. Macroscopic, microscopic, and biochemical characterization of TUA-109

Parameter	Observation
Colony color	White
Colony shape	Rhizoid
Colony elevation	Flat
Colony margin	Entire
Gram stain	Negative
Cell shape	Rod-shaped
Catalase test	Positive (+)
Motility test	Motile

Table 3 presents the results of partial characterization of the thermophilic protease-producing bacterium TUA-109, based on macroscopic, microscopic, and biochemical analyses. Macroscopic observation showed that the isolate formed white colonies with rhizoid-shaped, flat elevation and entire margins, indicating its ability to adapt to high-temperature environments. Microscopic and biochemical characterization revealed that isolate TUA-109 was Gram-negative, rod-shaped, catalase-positive, and motile (Gustiana et al., 2021). These characteristics are consistent with those reported for thermophilic bacteria isolated from geothermal environments, which commonly exhibit rod-shaped morphology, motility, and enzymatic activity that support their survival under high-temperature conditions (Kumar et al., 2024).

The selection of isolate TUA-109 for further study was based on its highest proteolytic index (PI) value among the isolates, indicating superior protease production capability. In addition, its morphological and biochemical characteristics, such as motility and catalase activity, suggest a strong adaptive capacity to environmental stress, which may contribute to enhanced enzyme production and stability (Rampelotto, 2013). These characteristics suggest that the isolate possesses adaptive mechanisms to survive under environmental stress conditions and supports its potential as a thermophilic protease-producing bacterium.

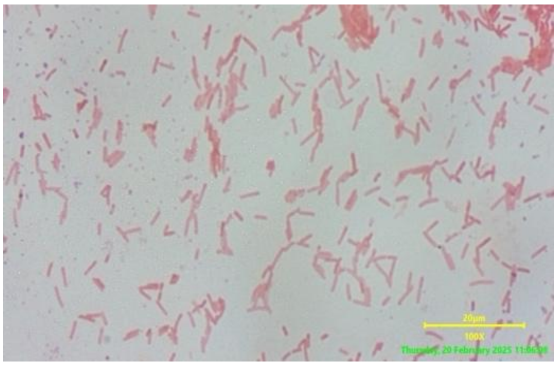

Figure 3. Gram staining of TUA-109

Figure 3 shows the Gram staining result of isolate TUA-109, which was identified as Gram-negative with a rod-shaped (bacillus) cell morphology. This result indicates that the isolate possesses a thin peptidoglycan layer and an outer membrane rich in lipids, which is characteristic of Gram-negative bacteria. The inability of Gram-negative bacteria to retain crystal violet during the staining process is associated with the presence of an outer membrane that is disrupted by alcohol treatment, allowing the primary stain to be removed and replaced by the counterstain, resulting in a red or pink appearance under microscopic observation (Sun et al., 2021; Hummels et al., 2023).

Figure 4. Catalase test of TUA-109

Figure 4 shows the catalase test result of isolate TUA-109, a thermophilic protease-producing bacterium. The formation of oxygen bubbles after the addition of hydrogen peroxide (H_2O_2) indicates that the isolate is catalase-positive. This result confirms the ability of the bacterium to produce catalase enzyme.Catalase plays an important role in protecting bacterial cells from oxidative stress by decomposing hydrogen peroxide into water (H_2O) and oxygen (O_2). This enzymatic activity is commonly found in aerobic bacteria and supports their survival in oxygen-rich environments, including geothermal habitats (Muqarrahmah et al., 2023).

Figure 5. Motility test of TUA-109

Figure 5 shows the motility test result of isolate TUA-109, where bacterial growth spread throughout the medium, indicating motile behavior. This result confirms that the isolate is capable of active movement. Bacterial motility reflects the ability of cells to move independently using structures such as flagella, which play an important role in environmental adaptation. Motile bacteria are generally associated with rod-shaped or spiral cell morphology, while non-motile bacteria are often coccoid in form. This capability allows bacteria to respond to environmental changes, access nutrients, and survive under varying conditions (Muqarrahmah et al., 2023).

The ability of TUA-109 to adapt to environmental conditions may contribute to its efficiency in enzyme production, particularly proteases, which are widely used in industrial applications such as detergents, food processing, and waste treatment (Anwar et al., 2022; Song et al., 2023). Therefore, this isolate has promising potential for further development as a source of thermostable protease enzymes. Future studies are recommended to optimize enzyme production through variations in growth conditions, such as temperature, pH, and substrate concentration, as well as to evaluate enzyme stability and activity under industrial conditions (Wang et al., 2021).

CONCLUSION

This study successfully achieved its objective of isolating and identifying thermophilic protease-producing bacteria from the Padang Dama hot spring, Solok, Indonesia. All isolates exhibited proteolytic activity, with isolate TUA-109 showing the highest potential based on its proteolytic index. The characterization results indicated that this isolate possesses physiological and biochemical traits that support its adaptability to high-temperature environments. These findings highlight the potential of the Padang Dama hot spring as a source of thermophilic bacteria with industrial relevance, particularly for the production of thermostable protease enzymes used in detergent, food, and waste treatment industries. Furthermore, this study contributes to the understanding of thermophilic bacteria from Indonesian geothermal environments. Future research is recommended to optimize enzyme production and evaluate its application under industrial conditions.

RECOMMENDATION

Further studies are recommended to conduct enzyme purification, molecular identification, and optimization of protease production from isolate TUA-109 to enhance its potential for industrial applications.

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