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# *SCREENING AND CHARACTERIZATION OF THERMOPHILIC BACTERIA FROM THE SAPAN HOT SPRING, SOUTH SOLOK REGENCY, AS PRODUCERS OF THERMOSTABLE PROTEASE ENZYMES*

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**Abstract**

Thermostable protease enzymes can be produced by thermophilic bacteria that act as biocatalysts in protein hydrolysis and have extensive industrial applications. The supply of stable and high-performance protease enzymes is still insufficient, encouraging exploration of thermophilic bacteria from extreme environments as potential producers. The aim of this study was to identify and characterize the macroscopic, microscopic, physiological, and biochemical features of thermophilic bacterial isolates that indicated protease production. This study was conducted using descriptive methods through rejuvenation, screening, and characterization of thermophilic bacteria isolated from the Sapan Hot Spring, South Solok Regency, as potential thermostable protease producers. Based on the results, six protease-producing thermophilic bacterial isolates, namely TUA-01, TUA-02, TUA-11, TUA-21, TUA-26, and TUA-31, with the highest Proteolytic Index (PI) value being isolate TUA-26. Characterization of the six protease-producing thermophilic bacterial isolates revealed differences in colony morphology characteristics, Gram-positive, rod-shaped cells, subterminal spores, motility, and catalase-positive. The thermophilic bacterial isolate TUA-26 has the potential to produce thermostable protease enzymes at high temperatures, making it highly promising for use in various biotechnology and industrial applications.

**Keywords**: *Characterization; Protease; Screening; Thermophilic Bacteria; Thermostable Enzyme*

**Abstrak**

Bakteri termofilik mampu menghasilkan enzim protease termostabil yang berperan sebagai biokatalisator dalam menghidrolisis protein dan memiliki aplikasi yang luas di berbagai bidang industri. Ketersediaan enzim protease yang stabil dan berkinerja tinggi masih terbatas, sehingga mendorong eksplorasi bakteri termofilik dari lingkungan ekstrem sebagai produsen potensial. Tujuan penelitian ini untuk mengetahui kemampuan dan karakter isolat bakteri termofilik yang berindikasi penghasil protease secara makroskopis, mikroskopis, uji fisiologi, dan biokimia. Penelitian ini dilakukan dengan menggunakan metode deskriptif melalui tahapan peremajaan, skrining, dan karakterisasi bakteri termofilik yang diisolasi dari sumber air panas Sapan, Kabupaten Solok Selatan, yang berpotensi sebagai penghasil protease termostabil. Penelitian ini memperoleh 6 isolat bakteri termofilik penghasil protease yaitu TUA-01, TUA-02, TUA-11, TUA-21, TUA-26, dan TUA-31 dengan nilai Indeks Proteolitik (IP) tertinggi yaitu isolat TUA-26. Karakterisasi yang dilakukan pada keenam isolat bakteri termofilik yang berindikasi penghasil protease memiliki perbedaan karakteristik morfologi koloni, bersifat Gram-positif, sel *rod-shaped*, memiliki subterminal spora, positif motil dan katalase. Isolat bakteri termofilik TUA-26 memiliki potensi menghasilkan enzim protease yang bersifat termostabil pada suhu tinggi sehingga berpeluang besar dimanfaatkan dalam berbagai aplikasi bioteknologi dan industri.

**Kata Kunci**: *Bakteri Termofilik; Enzim Termostabil; Karakterisasi; Penapisan; Protease*

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## INTRODUCTION

Enzymes are catalysts with exceptional catalytic strength that can speed up reactions, they are essential for biological processes in particular macromolecular compounds (Warshel & Bora, 2016). Enzymes bind to substrates on their active sites, forming enzyme-substrate complexes, and undergo conformational changes that facilitate the formation of reaction products. Many industrial processes are supported by enzymes, which are very efficient biocatalysts. The advantages of enzymes include high specificity to substrates, optimal efficiency and catalytic activity, the ability to operate over a wide temperature range, and the production of reactions that do not generate by-products. The global enzyme market is estimated to reach USD 7.0 billion, with Europe and North America being the largest consumers at 30% and 40% respectively (Kocabas & Grumet, 2019).

Thermophilic bacteria are capable of producing thermostable enzymes that have the unique ability to remain stable and active at high temperatures even when the ambient temperature increases, whereas enzymes generally undergo denaturation when exposed to high temperatures. These enzymes have a maximum activity temperature above the growth temperature of the microorganisms that produce them, making them heat-resistant and highly valuable commercially. The stable conformation of the active site allows the enzyme's catalytic activity to function optimally when conditioned at the ideal temperature. The use of thermostable thermophilic bacteria has several advantages, including reducing contamination from unwanted microorganisms, reducing fermentor cooling costs by reducing water and energy consumption for sterilization, being able to synthesize enzymes with high catalytic activity, growing easily on simple substrates, and reproducing rapidly in a short time, thereby lowering production costs (Abdel-Naby *et al*., 2017; Mumecha *et al*., 2021).

Protease is one of the enzymes produced by thermophilic bacteria. This enzyme type may lead to new industrial uses. This enzyme belongs to an essential group that can catalyze the hydrolysis of peptide bonds in polypeptides or proteins by releasing amino acids and peptides (Wu *et al*., 2018). Protease enzymes have the general properties of proteins, such as being easily denatured at high temperatures or other extreme conditions. Enzymes used in industry are those that are heat-resistant or resistant to extreme environments. Therefore, an enzyme that is thermostable or resistant to high temperatures is needed, because the main factor that can damage enzymes is temperature (Nanda *et al.,* 2017).

Proteases have a wide range of commercial applications in fields like agriculture, chemistry, industry, and medicine. Potential protease-producing bacteria come from the genera Bacillus, Pseudomonas, Clostridium, Proteus, and Serratia, while fungi include Aspergillus and Rhizopus. Future Market Insight (FMI) reported that the protease enzyme market was worth USD 2.1 billion in 2023 and is expected to reach USD 3 billion globally (Srivastava *et al.,* 2024). Proteases are widely used in the detergent, pharmaceutical, food, dairy and cheese, beer clarification, paper and pulp, leather tanning, textile, and waste treatment industries (Murugappan *et al.,* 2020; Abdella *et al.,* 2023; Ashwini *et al.,* 2023; Song *et al.,* 2023).

Thermophilic bacteria can grow in high-temperature environments, optimally at temperatures above 45°C – 80°C (Aanniz *et al.,* 2015; Kumar *et al*., 2019). Local microbial resources need to be explored in order to increase local species diversity. Thermophilic bacteria have a widespread habitat on earth, one of which is hot springs (Ejaz *et al.,* 2020). Hot springs are a good example of a habitat that thermophilic bacteria may adapt to. Research on thermophilic bacteria from the Sapan Hot Spring remains very limited, particularly regarding their potential as protease-producing microorganisms. A study by Irdawati *et al.* (2023) reported the isolation of several thermophilic bacterial consortia, namely MSS18 & MS15, MSS15 & MS16, and MS18 & MS16, which demonstrated potential for enhancing xylanase enzyme production. This research is necessary to obtain thermophilic bacterial isolates capable of producing thermostable proteases with potential applications in various food and non-food industries.

Thermophilic bacteria have stable and heat-resistant enzymes and proteins, and their thermophilic cell lipid membranes contain many saturated fatty acids that can form very strong hydrophobic bonds. Furthermore, thermophilic bacteria have stronger hydrogen bonds in their DNA, which keeps it stable even at high temperatures. This ability enables thermophilic bacteria to maintain optimal metabolic activity in extreme environments such as hot springs. Various biotic and abiotic factors also influence

the ability of thermophilic bacteria to adapt to extreme environments, such as fallen leaves, grass, moss, or other organic sources, as well as high mineral content that produces alkaline pH as an energy source that affects the growth of thermophilic bacteria (Nnolim & Nwodo, 2020; Dasdemir *et al.,* 2020).

This study was conducted using a collection of thermophilic bacterial isolates from the Biotechnology Laboratory, which were isolated from the Sapan Hot Spring in South Solok Regency, Indonesia, in 2021. The collection of thermophilic bacterial isolates consisted of 31 isolates from three locations, such as Sapan Aia Angek, Maluluang, and Balun, with 15, 10, and 6 isolates, respectively. The environmental conditions at the hot springs have a temperature range of 50°C – 83°C and a pH of 7.8 – 8.3, which can support the growth of thermophilic bacteria. Additionally, both abiotic and biotic factors influence this growth because various vegetation and dead organic matter serve as carbon sources for the bacteria. The Sapan Hot Spring is classified as alkaliphilic, making it potentially more diverse in bacteria compared to other hot springs and capable of producing enzymes. Therefore, research on the screening and characterization of thermophilic bacteria from the Sapan Hot Spring, South Solok regency as producers of thermostable protease enzyme is needed.

## METHODS

### Rejuvenation of Thermophilic Bacteria

The thermophilic bacterial isolates used in this research were collected from the Biotechnology Laboratory, Andalas University, which were isolated from the Sapan Hot Spring, South Solok Regency. Rejuvenation of thermophilic bacterial isolates is carried out by streaking the isolates on Nutrient Agar medium in test tubes using an ose needle. The thermophilic bacteria were incubated at 50°C for 24 hours.

### Screening of Thermophilic Bacteria

Thermophilic bacterial isolates were inoculated on 1% Skim Milk Agar medium in Petri dishes using an ose needle, then incubated at 50°C for 24 – 48 hours. The ability of bacteria to produce protease is indicated by the formation of a clear zone around the colony due to protein hydrolysis by protease from the bacterial isolate. The diameter of the colony and the clear zone around the colony were measured using a caliper, then the Proteolytic Index (PI) was calculated using the formula:

$$\text{Proteolytic Index (PI)} = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diameter}}$$

Isolates with the highest Proteolytic Index (PI) value were recorded as isolates with the best proteolytic activity (Agustien, 2010).

### Characterization of Protease-Producing Thermophilic Bacteria

Characterization was carried out by observing the macroscopic, microscopic, physiological, and biochemical features of thermophilic bacterial isolates that indicated protease production.

#### *Macroscopic Observation*

Macroscopic observations were conducted directly based on the morphology of each thermophilic bacterial isolate growing on Nutrient Agar medium. The morphological characteristics of the colonies included color, form, margin, elevation, and texture. These observations were conducted to determine the purity of the culture and identify the bacteria.

#### *Microscopic Observation*

Microscopic observation parameters were performed using Gram staining and spore staining to determine Gram properties, cell shape, and spore location. Gram staining was performed using crystal violet, safranin, lugol or iodin, and 96% alcohol, while spore staining was performed using malachite green. Gram-positive bacteria are characterized by purple-colored bacterial cells, while Gram-negative bacteria are characterized by red-colored bacterial cells. Furthermore, colored bacteria were observed under a microscope at an enlargement of 1000x with the addition of immersion oil. The shape of bacterial cells and their color were then observed (Prescott *et al.,* 1990).

***Physiological Observations***

Physiological observations were conducted to determine the presence or absence of movement (motility) in bacterial isolates using semi-solid media in vertical test tubes. Afterward, the medium was incubated at 50°C for 24 – 48 hours. The result of motility in bacteria is characterized by traces of bacterial movement (Baehaki *et al*., 2019).

***Biochemical Observations***

Biochemical observations were performed with a catalase test using 2 – 3 drops of 3% hydrogen peroxide ($H_2O_2$) solution on a glass slide. A positive catalase test result was indicated by the presence or absence of air bubbles. This test was conducted to determine the ability of bacteria to produce the enzyme catalase, as well as to distinguish between aerobic and anaerobic bacteria.

## RESULT AND DISCUSSION

### Result

A total of 31 thermophilic bacterial isolates were isolated from the Sapan Hot Spring, South Solok Regency from three locations, such as Sapan Aia Angek, Maluluang, and Balun with 15, 10, and 6 isolates, respectively. The abiotic factors of the Sapan Hot Spring have a water temperature ranging from 50°C to 83°C and alkaline water pH ranging from 7.8 to 8.3. Screening of thermophilic bacteria conducted on 31 isolates showed that 6 thermophilic bacterial isolates indicated the production of protease enzymes (Table 1).

**Table 1.** Average Proteolytic Index (PI) of Thermophilic Bacteria from the Sapan Hot Spring as Producers of Thermostable Protease Enzyme

| Isolate Code | Σ Diameter (mm) | | Σ Proteolytic Index (PI) |
|---|---|---|---|
| | Colony | Clear Zone | |
| **TUA-01** | 30.99 | 46.42 | 0.49 |
| **TUA-02** | 14.39 | 26.95 | 0.87 |
| **TUA-11** | 14.28 | 28.20 | 0.97 |
| **TUA-21** | 37.10 | 54.77 | 0.47 |
| **TUA-26** | 8.860 | 25.82 | 1.91 |
| **TUA-31** | 24.24 | 37.95 | 0.56 |

The Proteolytic Index (PI) values of protease-producing thermophilic bacterial isolates from the Sapan Hot Spring ranged from 0.47 to 1.91, with a potential isolate that was TUA-26 being the most promising thermophilic bacteria as a producer of the thermostable protease enzyme. Thermophilic bacterial isolates that indicated protease enzyme production were characterized by the formation of a clear zone around the thermophilic bacterial isolates on Skim Milk Agar medium by hydrolyzing proteins into simpler compounds such as peptides and amino acids (Figure 1).

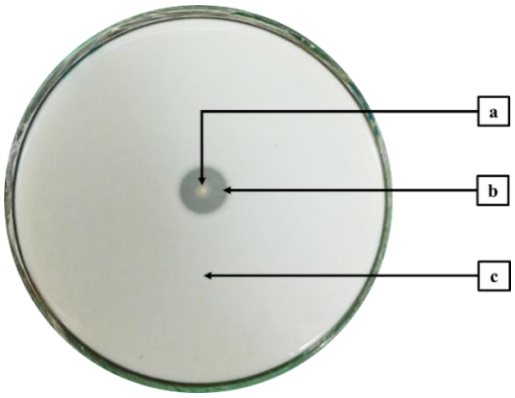


**Figure 1.** Screening of Thermophilic Bacterial Isolate TUA-26
(a) Bacterial Colony; (b) Clear Zone; (c) Medium Skim Milk Agar

Characterization of the six thermophilic bacterial isolates as producers of thermostable protease enzyme was performed through macroscopic, microscopic, physiological, and biochemical observation (Table 2).

**Table 2.** Characterization of Macroscopic, Microscopic, Physiological, and Biochemical Tests of Protease-Producing Thermophilic Bacteria

| Observation | Isolate Code | | | | | |
|---|---|---|---|---|---|---|
| | **TUA-01** | **TUA-02** | **TUA-11** | **TUA-21** | **TUA-26** | **TUA-31** |
| **Macroscopic** | | | | | | |
| Color | Cream | Yellow cream | Cream | Milk cream | Cream | Cream |
| Form | Circular | Circular | Irregular | Irregular | Circular | Circular |
| Margin | Entire | Undulate | Undulate | Lobate | Entire | Entire |
| Elevation | Flat | Raised | Raised | Flat | Raised | Raised |
| Texture | Moist | Moist | Mucoid | Dry | Moist | Moist |
| **Microscopic** | | | | | | |
| Gram stain | + | + | + | + | + | + |
| Cell shape | Short rods | Short rods | Short rods | Rods | Short rods | Rods |
| Cell arrangement | Single bacillus | Diplobacilli | Single bacillus | Single bacillus | Diplobacilli | Single bacillus |
| Spore stain | + | + | + | + | + | + |
| Spore position | Subterminal | Subterminal | Subterminal | Subterminal | Subterminal | Subterminal |
| **Physiological** | | | | | | |
| Motility test | + | + | + | + | + | + |
| **Biochemical** | | | | | | |
| Catalase test | + | + | + | + | + | + |

The colony morphology characteristics of protease-producing thermophilic bacteria are cream, yellow cream, and milk cream in color; circular and irregular in shape; entire, lobate, and undulate in margin; flat and raised in elevation; dry, mucoid, and moist in texture. The six protease-producing thermophilic bacterial isolates belonged to the Gram-positive, rod-shaped cells, subterminal spores, motility, and catalase-positive, such as isolate TUA-26 (Figure 2).

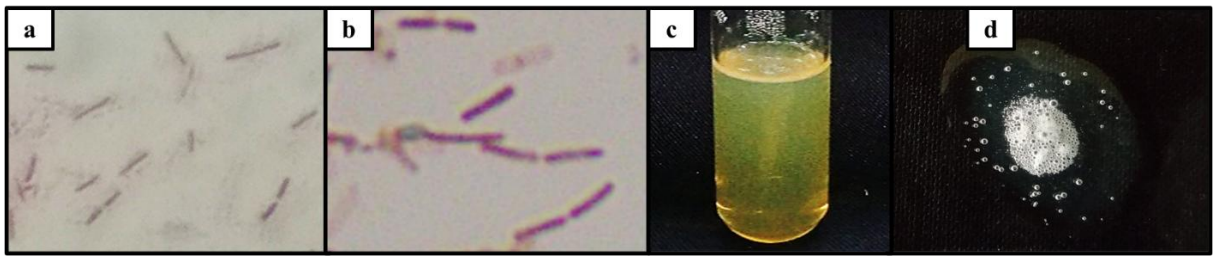


**Figure 2.** Characterization of Thermophilic Bacterial Isolate TUA-26
(a) Gram Stain; (b) Spore Stain; (c) Motility Test; (d) Catalase Test

**Discussion**

The Sapan Hot Spring has favorable environmental conditions such as temperature and alkaline pH with higher mineral content, thus potentially supporting greater diversity of thermophilic bacteria compared to acidophilic hot springs (Pandey *et al*., 2014). Thermophilic bacteria can produce protease due to proteolytic activity characterized by the formation of a clear zone on Skim Milk Agar medium. The proteolytic index indicates the relative ability to produce protease (Ginting, 2020). Bacterial proteolytic activity can be influenced by the nutritional sources employed in the medium. Among the minerals present in skim milk are calcium, casein, magnesium, and phosphorus. Casein is a milk protein consisting of phosphoproteins that bind with calcium to form calcium salts called calceneate. The different microorganism types, the growth rates of each isolate in the medium, and the enzyme activity of each isolate that secretes enzymes into the medium are all linked to the variations in the clear zones that are seen. Structural alterations in the enzyme can result in a decrease in its activity, which lowers

the catalytic rate since the active site can no longer bind the substrate as well (Suleiman *et al.,* 2020). Based on research by Suryanti *et al.* (2025), eleven thermophilic bacterial isolates producing protease from the Way Belerang hot spring in Lampung were found to have proteolytic index values ranging from 0.3 to 3.59, with isolate A9.5 having the highest protease activity value. Nafia *et al.* (2021), obtained proteolytic index values ranging from 0.16 to 1.9 for 20 thermophilic bacteria isolates producing protease from the Nglimut Gonoharjo Kendal hot spring in Central Java, with isolate T5 having the highest protease activity, identified as *Bacillus subtilis* subsp. *spizizenii* strain NBRC 101239. Simonapendi *et al.* (2019), obtained seven thermophilic bacterial isolates from the Mosso hot spring in Jayapura City, three of which had protease activity with proteolytic index (PI) values ranging from 1.51 to 2.55.

The thermophilic bacterial isolates that indicated protease production were characterized by observing the macroscopic, microscopic, physiological, and biochemical features. Bacterial colonies can be identified by their macroscopic morphological features, which include their color (cream, yellow cream, and milk cream); form (circular, irregular, filamentous, spindle, and rhizoid); margin or edge (entire, lobate, undulate, serrate, and filamentous); elevation or surface (flat, raised, convex, and umbonate); and texture (dry, moist, and mucoid). Macroscopic observations of this study reveal different colony morphology characteristics, such as color, form, margin, elevation, and texture. Mohammad *et al.* (2017), also reported that the morphological characteristics of ten thermophilic bacterial isolates from the Jordanian hot spring showed some variation in colony color, edge, shape, and texture that were gray, cream, and white; cloudy or transparent; rough or smooth; with regular or irregular edges.

Microscopic observation using Gram staining and spore staining was performed as an initial step in identifying bacterial genus. Thermophilic bacteria can be classified as Gram-positive or Gram-negative, aerobic or anaerobic, and some of them can form spores (Tarik *et al.,* 2015). Gram-positive bacteria are characterized by their purple or violet color after safranin counterstaining because they can retain the crystal violet dye even after treatment with a decolorizing agent, as in isolate TUA-26 (Figure 2a). Gram-negative bacteria, on the other hand, are red because they are unable to hold onto the dye. Both kinds of bacteria initially absorb the crystal violet during the staining procedure, but because Gram-negative bacteria have a high lipid content in their cell walls, their color fades when exposed to alcohol. Gram-positive bacteria have a thicker peptidoglycan layer in their cell walls than Gram-negative bacteria, which is the reason for this discrepancy (Baehaki *et al*., 2019). Sporulation staining was performed on thermophilic bacterial isolates that indicated Gram-positive rod-shaped cells that produce protease. The location of the spores formed within the cell consisted of central, terminal, and subterminal. Isolate TUA-26 had spores within the vegetative cell (endospores), which were characterized by a green color located in the subterminal part (Figure 2b). Spores are formed when bacteria experience stress, serving to protect them from unfavorable external factors by surviving in extreme environments such as heat, drought due to high temperatures, radiation, nutrient deficiency, acidic conditions, freezing, and chemicals. Mong *et al*. (2022), six moderate thermophilic bacterial strains, namely BM7, BS5, NS1, NS3, NS4, and NW6, were recorded as rod-shaped, Gram-positive, and endospore-forming from Ba Ria-Vung Tau and Khanh Hoa hot springs in Vietnam.

Through motility test, physiological observations were made to ascertain the protease-producing thermophilic bacterial isolates capacity for movement or dispersal. Bacteria are classified as motile when they move, grow, and spread throughout the medium, whereas they are non-motile if they only grow in a straight line at the inoculation site. All six protease-producing thermophilic bacterial isolates are motile such as isolate TUA-26 (Figure 2c), as demonstrated by their movement on the surface of the medium. Motile bacteria are capable of movement or displacement because they have motility organs in the form of flagella or cilia, while non-motile bacteria do not have motility organs that support movement. This bacterial movement is carried out for adaptation in order to survive (Pollit & Diggle, 2017). In the study by Sabaria *et al.* (2024), based on motility tests, there were four protease-producing thermophilic bacterial isolates that were positively motile, namely BT1, BT2, BT3, and BT4 from the Ie Seum hot springs, Aceh Besar.

Catalase tests of protease-producing thermophilic bacterial isolates were used to make biochemical observations. All six protease-producing thermophilic bacterial isolates tested positive in the catalase test such as isolate TUA-26 (Figure 2d), indicating that the isolates belong to the aerobic bacteria group.

Aerobic bacteria are able to thrive in hot springs because oxygen can still dissolve there. A positive reaction was indicated by the formation of oxygen bubbles on the surface of the colony when a drop of hydrogen peroxide ($H_2O_2$) solution was added, indicating that protease-producing thermophilic bacterial isolates are capable of producing the enzyme catalase. The catalase or peroxidase enzyme is produced by bacterial cells by breaking down $H_2O_2$ into water ($H_2O$) and oxygen ($O_2$). The catalase enzyme plays a role in the mechanism of cell integrity defense and enzymatic stability in extreme environmental conditions against oxidative stress, thereby protecting cells from damage caused by the accumulation of free radicals and Reactive Oxygen Species (ROS) (Yuan *et al*., 2021). Based on macroscopic, microscopic, physiological, and biochemical characterization, the six protease-producing thermophilic bacterial isolates exhibited Gram-positive, rod-shaped cells, subterminal spores, motility, and catalase-positive characteristics, which are consistent with the characteristics of the genus Bacillus. Similar results have been previously reported by Guta *et al*. (2024), the morphological and biochemical test results revealed that all 45 bacterial isolates from hot springs in Ethiopia were rod-shaped cells, Gram-positive, catalase-positive, endospore formers, motile, as well as identified from three genera (Bacillus, Paenibacillus, and Brevibacillus) and five species (*B. licheniformis, B. cereus, P. thiaminolyticus, P. dendritiformis,* and *B. borstelensis*). This study has certain limitations, as bacterial identification was based only on morphological, physiological, and biochemical characteristics, which may not provide accurate species-level identification. Therefore, future studies should include molecular identification using 16S rRNA gene sequencing and comprehensive thermal stability analysis of the protease enzyme to better assess its industrial potential.

## CONCLUSION

The six isolates of thermophilic bacteria indicated to be protease producers were obtained from the Sapan Hot Spring, South Solok Regency namely TUA-01, TUA-02, TUA-11, TUA-21, TUA-26, and TUA-31, with the highest Proteolytic Index (PI) value being isolate TUA-26. The characterization of macroscopic, microscopic, physiological, and biochemical features indicated that the six protease-producing thermophilic bacterial isolates had different colony morphology characteristics, Gram-positive, rod-shaped cells, subterminal spores, motility, and catalase-positive. One of the thermophilic bacterial TUA-26 isolates has the potential to produce a thermostable protease enzyme at high temperatures, making it highly promising for use in various biotechnology and industrial applications. Future research should focus on molecular identification through 16S rRNA gene sequencing, detailed characterization of protease thermal stability and activity, and optimization of production conditions to support its application.

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