

Characterization of thermophilic bacteria from Ie Seum Hot Springs, Aceh Besar, Indonesia as producers of protease enzyme

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Abstract. Sabaria E, Yasmin Y, Ismail YS, Bessania MA, Putri I, Fitri L. 2024. Characterization of thermophilic bacteria from Ie Seum Hot Springs, Aceh Besar, Indonesia as producers of protease enzyme. *Biodiversitas* 25: 1867-1874. Thermophilic bacteria are microorganisms that can survive in high-temperature environments exceeding 75°C. The Ie Seum Hot Springs in Aceh Besar, Indonesia are known to be a place where these thermophilic bacteria inhabit. In such extreme conditions, proteins and enzymes are often denatured, making these bacteria of interest due to their ability to adapt and produce enzymes such as proteases. Proteases are called proteolytic enzymes that can be applied both in the economic and medical fields. This study aimed to isolate and identify thermophilic bacteria species that have the highest potential for protease production in Ie Seum Hot Spring, Aceh Besar by analyzing the 16S rRNA gene and characterizing them using biochemical tests. Based on the result, seven thermophilic bacterial isolates, namely BT1, BT2, BT3, BT4, BT5, BT6, and BT7 were obtained, each showing different colony characteristics. The biochemical tests also revealed differences metabolic activity of each isolate. Of these isolates, BT4 exhibited the highest proteolytic index (4.65). Additionally, the 16S rRNA gene sequence analysis showed that the BT4 strain belongs to the genus *Bacillus* and has high similarities to *Bacillus* sp., *B. licheniformis* and *B. sonorensis*. These findings suggest that BT4 has significant potential as a source of thermophilic protease enzymes.

Keywords: *Bacillus*, hot springs, Ie Seum, protease, thermophilic bacteria

INTRODUCTION

Enzymes have significant industrial importance, owing to their extensive utilization in the production of high-quality consumer goods. As biocatalysts, enzymes facilitate all anabolic and catabolic processes, by reducing the activation energy of biological reactions. The wide range of enzymatic activities has resulted in their widespread application in industrial processes, including food processing, pharmaceutical production, and biofuel production. The continued discovery of novel enzymes and the development of efficient methods for their production and utilization is expected to further expand their industrial importance (Sandoval and Hyster 2020). Although the catalytic potential of enzymes has been recognized for decades, recent progress in biotechnology has led to their expanded utilization as biocatalysts. Hydrolytic enzymes, in particular, have garnered significant attention and are widely employed in various industrial applications (Do et al. 2020). Proteases are the most powerful and efficient enzymes as they degrade complex protein molecules into amino acids and peptides. Proteases are divided into alkaline protease, neutral protease and acid protease. Meanwhile, alkaline proteases are the industrial sector's most exploited or utilized enzymes. Protease enzymes can be produced by animals, plants and microorganisms. The production of enzymes from animal and plant sources is still limited to

exploration due to the low efficiency of the production process, ethical issues, and environmental reasons (Song et al. 2023). Proteases produced by microorganisms have advantages compared to enzymes produced by plants and animals because they are easy, cost-effective, and their production is consistent (Raveendran et al. 2018). Proteases produced from microorganisms are applied in detergent, leather, food, bread dough, tenderizing and beverage industries (Song et al. 2023). Their significant producers include *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., and *Aeromonas* sp. Given its frequent use, protease has the greatest potential for research and commercialization, making it a promising area of research (Masi et al. 2021). Ginting et al. (2019) explained that proteases have garnered significant attention due to their immense economic value. These enzymes are particularly well-suited for industrial processes that require high temperatures (Ardhi et al. 2020). Thermophilic bacteria are known as one of the most promising producers of protease enzymes. Enzymes from thermophilic bacteria are highly effective and advantageous in certain applications, such as enhancing the reaction rate, the solubility of reactants and non-volatile products, and decreasing contamination from mesophilic microorganisms (Sharma et al. 2019; Sang et al. 2020). Thermophilic bacteria are a group of microorganisms that can live in temperatures exceeding 75°C (Pandey et al. 2015). Many of these bacteria are capable of producing thermostable

proteins and enzymes, which remain functional even at high temperatures that typically denature other proteins and enzymes (Lischer et al. 2020). While living organisms, including thermophilic bacteria, are natural sources of enzymes, their use is often constrained by seasonal fluctuations, low concentrations, high processing costs, and limited availability (Alrumman et al. 2018). Thermophilic bacteria have been found to produce a protease enzyme, which is synthesized by the Gram-positive bacteria *Geobacillus thermoglucosidarius*. This bacterium is distinct from the *Bacillus* genus and can thrive between temperatures ranging from 40 to 70°C (Suleiman et al. 2020). In another study, thermophilic bacterial isolates were found to have proteolytic activity at a temperature of 55°C (Ginting 2020). Thermophilic bacteria are often found in hot springs, volcanic craters, and composting regions (Ejaz et al. 2020). Ie Seum is one of the hot springs in Indonesia which is located in Aceh Besar. This area is known to be inhabited by thermophilic bacteria. Previous research has succeeded in isolating thermophilic bacteria from Ie Seum hot springs as producers of amylase (Zuraidah et al. 2020) and cellulase enzymes (Majidah et al. 2023). However, as far as we have searched the literature, thermophilic bacterial isolates that can produce protease have not been found. Thus, this study aimed to investigate the potential of thermophilic bacteria in producing protease enzymes, characterizing them so that bacterial species isolated from the Ie Seum Hot Springs, Aceh Besar, Indonesia can be identified. In this study, the isolation of thermophilic bacteria is carried out at different temperatures to obtain the diversity of microorganisms that exist in the thermal environment. Meanwhile, the characterization of the thermophilic bacteria observed was morphological, physiological and molecular using 16s rRNA.

MATERIALS AND METHODS

Procedures

Water sampling

The water samples were collected from the Ie Seum hot springs located in the Masjid Raya Sub-District of Aceh Besar District, Aceh, Indonesia using a random sampling technique, as depicted in Figure 1. Before sample collection, the physical and chemical properties of the water were determined using a digital pH meter and thermometer. The temperature at points I, II, and III was found 85°C, 75°C, and 65°C, respectively, as depicted in Figure 1. The water samples were then collected as much as 100 mL at a depth of 20-50 cm from the surface and put into sterile heat-resistant flasks. The flasks were stored in an incubator at a temperature appropriate to the original environment in the Microbiology Laboratory at the Faculty of Mathematics and Natural Sciences of Universitas Syiah Kuala for further analysis.

Isolation of thermophilic bacteria

Isolation of thermophilic bacteria in Ie Seum Hot Spring is carried out using the pour plate technique. A total of 1 mL of sample was taken and then transferred to a sterile Petri dish, then transferred to NA (Nutrient Agar) medium then homogenized and allowed to solidify. Then incubated at 50°C for 48 hours. Each colony that grew with different morphology was purified by streaking in quadrants on new NA medium. After that, it was incubated again at 50°C for 48 hours until a single colony grew. Each steps were replicate twice (duplo) (Bahri et al. 2021). The work is carried out using sterile tools and materials and in laminar airflow to prevent contamination.

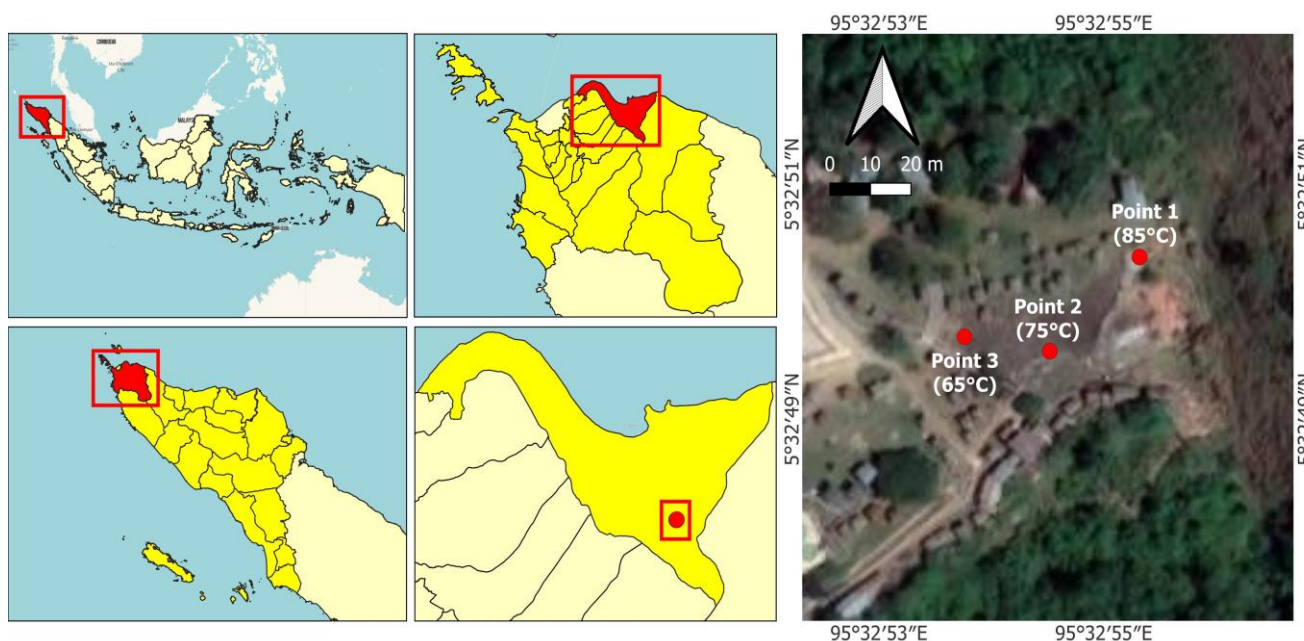


Figure 1. Hot spring sampling points: Point 1 (5.5473228 N, 95.5485450 E), Point 2 (5.5471089 N, 95.5483468 E), and Point 3 (5.5471683N, 95.5481856E)

Morphological and physiological characterization of thermophilic bacteria

The thermophilic bacteria were subjected to morphological and physiological characterization. Macroscopic and microscopic observations were used for the morphological characterization. Macroscopic characterization included the observation of colony form, color, margin, surface, and elevation. Microscopic characterization was performed by Gram staining the bacteria cells and observing them under 1000x magnification. Physiological characterization was conducted using several tests, including:

- i. Catalase test is used to test whether bacteria has the catalase enzyme or not. This enzyme can break down hydrogen peroxide into water and oxygen. If oxygen gas bubbles form when hydrogen peroxide is added to the bacterial culture, it indicates the presence of the catalase enzyme.
- ii. The Sulfide Indole Motility (SIM) test is used to differentiate bacteria based on their ability to produce indole and their motility. This test was carried out by culturing bacteria into SIM media and incubated for 24 hours at 50°C. After incubation, 10 drips of Kovac's reagent were added to the media for the indole test. If indole is produced, a red color forms at the top layer of the medium and if the bacteria are motile, there will be encroachment around the media puncture.
- iii. MR-VP test is used to differentiate between bacteria based on their ability to ferment glucose and produce specific metabolic end products. This test was carried out by culturing bacteria into MRVP media and incubated for 24 hours at 50°C. After incubation, 5 drips of methyl red were added to the media for MR test. Meanwhile, for VP test, 5 drips of alpha naftol and 5 drips of KOH were added to media.
- iv. Triple Sugar Iron Agar (TSIA) test used to differentiate bacteria based on their ability to ferment sugars, produce hydrogen sulfide (H₂S), and reduce sulfur. This test was carried out by culturing bacteria into TSIA media by stabbing at the bottom of the media (butt) and streaking at the top of the inclined media surface (slant). Then medium incubated for 24 hours at 50°C. The results are interpreted based on the color changes and observations in both the butt and the slant of the tube.

These tests helped to determine the presence or absence of specific enzymes and metabolic pathways, which provided insight into the physiological characteristics of the thermophilic bacteria.

Quantitative protease activity test

The test was conducted by culturing a single bacterial colony for 24 hours at 50°C. A clear zone on the surface of the Skim Milk Agar (SMA) medium indicated a positive test. The isolates were then photographed and their diameter was measured using a digital caliper. Furthermore, the Proteolytic Index (IP) was calculated, and the isolates with the highest proteolytic index were identified (Hamdani et al. 2019). The proteolytic index provides a more accurate

measurement of the ability of protease enzymes to break down protein substrates. The following formula was used to calculate the proteolytic index:

$$IP = \frac{(CZD - CD)}{CD}$$

Where:

- IP : Index proteolitik
 CZD : Clear zone diameter
 CD : Colony diameter

Molecular identification of thermophilic bacteria based on 16S rRNA

The bacteria tested for analysis using 16s rRNA is refreshed into new media and it must be ensured that it is pure from other bacterial contamination. This aims to ensure that the identification results obtained are accurate. Molecular identification was performed at the Brackish Water Aquaculture Fisheries Center in Ujong Batee, Aceh Besar, Indonesia (BPBAP-UB). The bacterial genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA). Subsequently, the concentration of the extracted genomic DNA was quantified using NanoDrop™ method. The DNA amplification was carried out using a PCR Thermal Cycler T100™. Universal primers, Bact 27F (5' AGAGTTGATCCTGGCTCAG 3') and UniB 1492R (5' GGTTACCTTGTTACGACTT 3'), were used in the PCR procedure. The amplified DNA was analyzed using a UV-transilluminator such as the ChemiDoc Bio-Rad™ after electrophoresis, and the 16S rRNA gene was sequenced using the Sanger sequencing technique (also known as Sanger dideoxy sequencing) (Ardhi et al. 2020).

RESULTS AND DISCUSSION

Isolation and characterization of thermophilic bacteria

This study involved the isolation of seven thermophilic bacteria at three different temperatures, namely 85°C, 75°C, and 65°C, at a neutral pH of 7 (Table 1). Previous research by Khalila et al. (2020) isolated 16 thermophilic bacteria, with 4, 5, and 7 isolates at 70°C, 60°C, and 50°C. The microbial population in hot springs can be influenced by several factors. As reported by Runtuboi et al. (2018), the location of the sample can affect the number of bacteria collected due to the varying environmental conditions that promote microbial growth. Additionally, biotic variables like organic sources such as fallen leaves, grass, and moss serve as the energy source for microbes, as noted by Tuysuz et al. (2020). On the other hand, abiotic factors such as high mineral content causing an alkaline pH have been found by Nnolim and Nwodo (2020) to influence the occurrence of thermophilic bacteria, leading to the growth of various microorganisms.

Table 1. Morphological characteristics of thermophilic bacteria

The temperature of the sample collected	Isolate code	Morphological characteristics						
		Shape	Color	Margin	Elevation	Surface	Cell form	Gram staining
85°C	BT1	Irregular	Cream	Undulate	Flat	Smooth	Bacilli	Positive
	BT2	Circular	White	Entire	Flat	Smooth	Bacilli	Positive
75°C	BT3	Irregular	Cream	Entire	Convex	Smooth	Bacilli	Positive
	BT4	Irregular	White	Undulate	Flat	Smooth	Bacilli	Positive
	BT5	Circular	Cream	Entire	Convex	Smooth	Bacilli	Positive
65°C	BT6	Circular	Yellow	Entire	Flat	Smooth	Bacilli	Positive
	BT7	Irregular	White	Undulate	Flat	Smooth	Bacilli	Positive

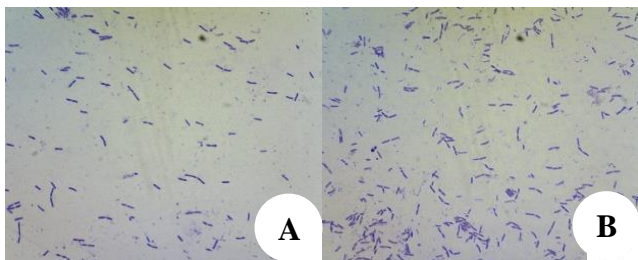
**Figure 2.** Gram Staining of thermophilic bacteria. A: isolate BT4; B: Isolate BT7

Table 1 shows the distinct morphological forms of the collected thermophilic bacteria isolates. The shape of the margins was undulating (wavy) and complete, with circular and irregular colonies. The elevation characteristics were convex and flat, while the resulting colors were varied, ranging from light brown to white and yellow, with a smooth surface. Previous research by Kasi et al. (2021), isolated thermophilic bacteria from Pincara hot springs and also observed morphological characteristics, such as round and irregular forms, complete and undulating edges, as well as convex and flat elevations. Similar observations were seen for newly thermophilic bacteria isolates in the Ie Seum tourism region hot springs in Aceh Besar, Indonesia as reported by Khalila et al. (2020).

In this study, the morphology of bacterial cells was observed, revealing a bacilli form that was characterized as Gram-positive (Figure 2). However, unlike the findings of Fachrial et al. (2021), who isolated thermophilic protease-producing bacteria from the Dolok Tinggi Raja Hot Springs in North Sumatra, the Gram staining results varied among the isolates, with some showing positive results and others negative. According to Marathe et al. (2018), the Gram stain is a useful tool for characterizing bacterial types based on their cell wall composition, with Gram-positive bacteria possessing a thick peptidoglycan layer and Gram-negative bacteria having a thick lipid layer.

Table 2 shows the results of physiological tests performed on seven different isolates of thermophilic bacteria. The tests included TSIA, SC, Sucrose, Lactose, SIM, Indole, Catalase, and MRVP. Overall, the isolates showed variable results in the physiological tests, indicating differences in their biochemical characteristics. According to Table 2, thermophilic bacteria isolates exhibited distinct physiological characteristics. On the TSIA media, only BT1, BT5, and BT7 do not produce gas, while other isolates produce it. BT1 produces a yellow color change throughout the media,

which means this bacteria can ferment glucose, lactose or sucrose. BT5 and BT7 produce a red color change throughout the media, which means none of the three sugars are fermented by bacteria. Meanwhile, BT2, BT3, BT4 and BT6 showed a red slant and yellow butt which means these bacteria can only ferment glucose. In the citrate test, BT1 and BT4 showed positive results, while the remaining were negative. According to the report, bacteria can use citrate as a source of carbon and energy (Azizah et al. 2020). BT1 isolates were the only ones with lactose-fermenting colonies, while others produced negative results. The lactose test was conducted by Jitpakdee et al. (2021) to determine whether bacteria can ferment disaccharide sugar into acid and gas. BT1, BT2, BT3, BT4, BT6, and BT7 produced positive results in the indole test, while BT5 recorded negative results. This test was conducted to determine the presence of the tryptophanase enzyme in bacteria capable of hydrolyzing the amino acid tryptophan into indole and pyruvic acid, as described by Das et al. (2019). Only BT1 isolates had positive results in the Sucrose test, while others were negative. According to Harlé et al. (2020), the positive result for sucrose was due to the bacteria's ability to decrease the sugar concentration in the sample solution.

In the motility test, three isolates, namely BT5, BT6, and BT7, yielded negative results, while others were positive. According to Sanam et al. (2022), thermophilic microorganisms possess motility. Furthermore, BT1, BT2, BT3, BT5, and BT6 produced the catalase enzyme, while the others, such as BT4 and BT7, were negative. According to Vanniyasingam et al. (2019), the favorable results were attributed to the ability of microorganisms to decompose hydrogen peroxide. All thermophilic bacteria isolates failed the Voges Proskauer (VP) test, or the VP and the Methyl Red tests were negative. Khosravi et al. (2021) stated that the MR-VP test is positive when the microorganism can oxidize glucose to produce a highly concentrated acid.

Bacillus-classified thermophilic bacteria isolates were evaluated based on their morphology and physiology. According to Logan and Vos (2015), *Bacillus* colonies have a variety of shapes, ranging from round to irregular and white to yellow, except for those that are black, brown, orange, pink, or yellow. The edges varied, ranging from flat and wavy to crenate with emerging convex elevations. The form and size of the colonies differed by species. This research reported that 4 thermophilic bacteria were isolated from Bora hot springs in the District of South Sulawesi (Ifandi et al. 2018).

Table 2. Physiological characteristics of thermophilic bacteria

Isolate code	Physiological test							
	TSIA	SC	Sucrose	Lactose	motility	Indole	Catalase	MRVP
BT1	y/y, g(-), H ₂ S(-)	+	+	+	+	+	+	-
BT2	r/y, g(+), H ₂ S(-)	-	-	-	+	+	+	-
BT3	r/y, g(+), H ₂ S(-)	-	-	-	+	+	+	-
BT4	r/y, g(+), H ₂ S(-)	+	-	-	+	+	-	-
BT5	r/r, g(-), H ₂ S(-)	-	-	-	-	-	+	-
BT6	r/y, g(+), H ₂ S(-)	-	-	-	-	+	+	-
BT7	r/r, g(-), H ₂ S(-)	-	-	-	-	+	-	-

Note: -: negative, +: positive, y/y: yellow slant/ yellow butt, r/r: red slant/red butt, r/y: red slant/red butt, r/y: red slant/yellow butt: red, g: gas, TSIA: Triple Sugar Iron Agar, SC: Simmon Citrate, MR: Methyl Red, VP: Voges Proskauer

Table 3. Measurement of the diameter of the clear zone and the proteolytic index formed from bacteria

Isolate code	Clear zone diameter (cm)	Colony diameter (cm)	Proteolytic index
BT1	1.68	1.50	0.12
BT2	8.57	7.45	0.15
BT3	4.51	4.00	0.13
BT4	5.65	1.01	4.65
BT5	0.97	0.42	1.30
BT6	0.95	0.20	3.75
BT7	2.08	0.50	3.16

Proteolytic activity of thermophilic bacteria

Proteolytic activity of thermophilic bacteria was demonstrated by the clear zone that showed on the surface of the SMA medium (Table 3). Table 3 demonstrates the protease enzyme production capabilities of the isolates, as evidenced by the clear zone formation on the SMA medium. The largest and smallest clear zones were produced by the BT2 and BT6 isolates, with diameters of 8.57 and 0.95 cm, respectively.

According to Hamdani et al. (2019), a clear zone greater than 2 centimeters around the colony is an indicator of a good protease-producing strain. Artha et al. (2019) suggest that thermophilic bacteria isolates form clear zones due to their ability to secrete protease into the environment, leading to the hydrolysis of protein in milk and the subsequent clearing of the surrounding colonies. The proteolytic index provides a relative measure of protease production capacity. BT4 and BT1 isolates generated the highest and lowest proteolytic indices at 4.65 and 0.12, respectively. The proteolytic index ranges from 1.60 to 3.00, with the differences possibly influenced by bacterial species, as suggested by Dhayalan et al. (2022). Therefore BT4, was further analysed by 16S rRNA gene sequencing.

Molecular identification protease-producing bacteria

DNA was extracted from the genome of a thermophilic bacteria isolate (BT4) for PCR analysis of the 16S rRNA. PCR amplification of the 16S rRNA gene of BT4 showed band at 1500 bp (Figure 2). The DNA had a concentration of 193.8 ng/L and a purity of 1.86 at A260/A280. According to Marasco et al. (2022), the purity of nucleic acid was determined by the ratio A260/A280. A value close to 1.8 in A260/A280 indicated DNA purity, while below

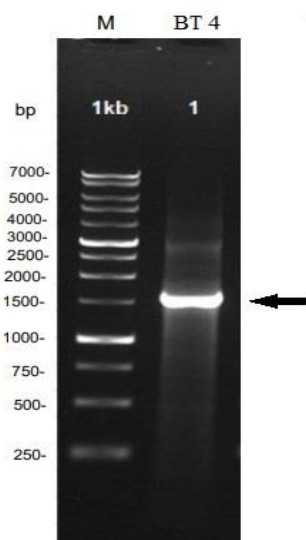
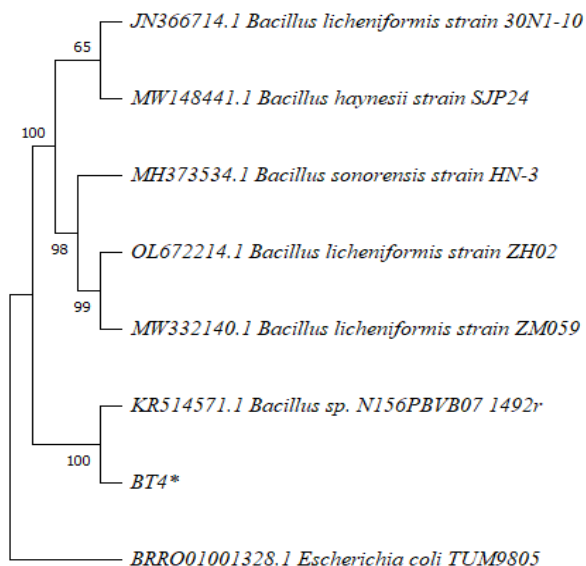
1.8 showed the presence of phenol, protein, or other contaminants. The sample was amplified using two oligonucleotide primers (Alameri et al. 2022). Using these primers, Bact 27F and UniB 1492R an amplicon of the fragment measuring 1500 bp was generated (Octarya et al. 2022).

The PCR sequencing results were analyzed using BLASTn through the GenBank database at NCBI, and the alignment of the BT4 isolate sequences and the five most closely related bacterial sequences are shown in Table 4. BLAST, as described by Samal et al. (2021), identifies regions of sequence similarity and compares nucleotide or protein sequences in a database, calculating the statistical match. Based on the results in Table 4, the BT4 isolate exhibited the highest degree of similarity with *Bacillus licheniformis* strain 30N1-10. As noted by previous studies (Kadaikunnan et al. 2015), the percentage of identity is a critical factor in identification, with values >97%, >99%, and 97% indicating genus level, species level, and low levels of homology between the sequencing results and the sequences in the database.

The closer the e-value is to 1, the less similarity between the sequencing results and the GenBank data. According to Saryono et al. (2022), the expectation value (E-value) determines the similarity between sequencing results and GenBank data. Phylogenetic tree analysis using the Neighbor-Joining (NJ) method with a bootstrap value of 1000 repetitions (1000 replicates) showed that BT4 isolates formed a monophyletic group with *Bacillus* sp. strain N156PBVB07. The Neighbour-Joining (NJ) method is a commonly used algorithm in the field of phylogenetics that is based on the estimation of evolutionary distances between pairs of operational taxonomy units (Figure 3). This method groups taxa according to their evolutionary distances, where each branch in the phylogenetic tree evolves at a different rate (Calogero et al. 2022). The bootstrap value is a measure of the reliability of the reconstructed tree topology and reflects the accuracy of the data model used in the analysis, as pointed out by Alghamdi (2022). Strain BT4 is closely related to *Bacillus* sp. The closer the e-value is to 1, the less similarity between the sequencing results and the GenBank data. According to Saryono et al. (2022), the Expectation value (E-value) determines the similarity between sequencing results and GenBank data.

Table 4. Alignment data of several BLAST organisms with thermophilic bacteria

Description	Max score	Total score	Query cover	E value	Identity	Accession
<i>Bacillus licheniformis</i> strain 30N1-10	1718	1718	99%	0.0	97.89%	JN366714.1
<i>Bacillus sonorensis</i> strain HN-3	1716	1716	99%	0.0	97.89%	MH373534.1
<i>Bacillus</i> sp. strain N156PBVB07	1716	1716	99%	0.0	97.89%	KR514571.1
<i>Bacillus licheniformis</i> strain ZH02	1716	1716	99%	0.0	97.89%	OL672214.1
<i>Bacillus licheniformis</i> strain ZM059	1716	1716	99%	0.0	97.89%	MW332140.1

**Figure 3.** Amplification of BT4 DNA using Bact 27F- UniB 1492R primers**Figure 4.** Results of phylogenetic tree reconstruction of BT4 isolates with several organisms from BLAST

Phylogenetic tree analysis using the Neighbor-Joining (NJ) method with a bootstrap value of 1000 repetitions (1000 replicates) showed that BT4 isolates formed a

monophyletic group with *Bacillus* sp. strain N156PBVB07 (Figure 4). The Neighbour-Joining (NJ) method is a commonly used algorithm in the field of phylogenetics that is based on the estimation of evolutionary distances between pairs of operational taxonomy units. This method groups taxa according to their evolutionary distances, where each branch in the phylogenetic tree evolves at a different rate (Calogero et al. 2022). The bootstrap value is a measure of the reliability of the reconstructed tree topology and reflects the accuracy of the data model used in the analysis, as pointed out by Alghamdi (2022).

Strain BT4 is closely related to *Bacillus* sp. *Bacillus* are a group of Gram-positive bacteria that are widely distributed in nature. They are known to be facultative anaerobes, meaning they can grow in the absence of oxygen but can also switch to an oxygen-dependent lifestyle. Feizabadi et al. (2021) reported on this physiological trait of *Bacillus* sp. The genus *Bacillus* includes both pathogenic and non-pathogenic species, with some species capable of producing endospores under specific environmental conditions. These endospores have an oval shape and allow the bacterium to remain dormant for long periods. While the formation of endospores was the original criterion for defining the genus *Bacillus*, some closely related species may be placed in a different genus within the Firmicutes phylum, as reported by Wang et al. (2022).

Based on the research results, it was found that 7 isolates of thermophilic bacteria could produce protease enzymes, namely BT1, BT2, BT3, BT4, BT5, BT6, and BT7. The ability of thermophilic bacteria to produce protease enzymes was seen from the largest clear zone value in the BT4 isolate of 85.8 mm and the smallest in BT6 of 9.7 mm. Proteolytic index measurements produced the largest value in BT3, namely 4.5, and the smallest in BT1 isolate namely 0.02. This BT4 strain belongs to the genus *Bacillus* and has high similarities to *Bacillus* sp., *B. licheniformis* and *B. sonorensis*. Many species of *Bacillus* are known to produce protease enzymes, including *Bacillus licheniformis* (Contesini et al. 2017) and *Bacillus sonorensis* (Arzita et al. 2019). Likewise, this BT4 isolate as *Bacillus* has great potential for further research into industrial applications such as food, pharmaceuticals and others regarding the protease enzyme it produces.

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