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The Cellulase Enzyme Activity of Thermophilic Bacteria from Way Belerang Hot Spring, Lampung

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Abstract

Background: *The demand for cellulase enzymes across various industries continues to rise, while most of the current supply still depends on imports. Therefore, exploring local biological resources, such as thermophilic bacteria capable of producing cellulase, is crucial. This study aims to isolate and evaluate the cellulase enzyme activity of thermophilic bacteria from the Way Belerang Hot Spring in South Lampung, Indonesia.* **Methodology:** *The research involved isolating bacteria from hot spring water samples, conducting qualitative and quantitative assays of cellulase activity, and characterizing the isolates.* **Findings:** *A total of 24 bacterial isolates were obtained, 16 of which demonstrated cellulolytic activity, as indicated by clear zones on 1% CMC agar. The two most promising isolates, S5.1 and S5.24, exhibited the highest cellulolytic indices of 2.728 and 2.395, respectively. These isolates were further tested quantitatively using a glucose standard curve to determine their enzyme activity. The highest cellulase activity for isolate S5.1 was recorded at the 10th hour with 5.1 × 10^{-2} U/mL, while isolate S5.24 showed peak activity at the 14th hour with 3.1 × 10^{-2} U/mL.* **Contribution:** *This study represents the first investigation of thermophilic amylase-producing bacteria from the Way Belerang Hot Spring, highlighting the potential of thermostable amylase enzymes that can withstand high temperatures for future industrial applications*

Keywords: *Cellulase Enzyme; Cellulolytic Index; Enzyme Activity; Thermophilic Bacteria; Way Belerang*

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INTRODUCTION

Thermophilic bacteria are a group of microorganisms capable of growing and surviving in high-temperature conditions, typically ranging from 45°C to 90°C (Kasi, 2020). These microorganisms are able to function and reproduce in such environments. Thermophilic bacteria are commonly found in habitats such as hydrothermal vents, hot springs, and volcanic areas (Mahestri et al., 2021). The exploration of thermophilic bacteria is mainly due to their ability to produce thermostable enzymes, which are enzymes that remain active and stable at high temperatures and can be applied in various industries (Kasi, 2020). One example of a thermostable enzyme produced by thermophilic bacteria is cellulase.

Cellulase is an enzyme capable of degrading cellulose to glucose, cellobiose, and cello-oligosaccharides. The cellulase enzyme system consists of endo-1,4-β-glucanase, exo-1,4-β-glucanase, and β-D-glucosidase. These three enzymes work synergistically to break down cellulose and release reducing sugars as the final products (Kusumaningrum et al., 2019). Cellulase is widely utilized in various industrial sectors that require high temperatures, including biofuel production, the pulp and paper industry, the food industry, animal feed production, and the textile industry (Ajeje et al., 2021). The demand for cellulase continues to increase both globally and nationally; in Indonesia, its usage has grown by approximately 7% annually (Wardani et al., 2023). However, most of the cellulase enzyme required in Indonesia is still met through imports, highlighting the need to explore local biological resources as potential cellulase enzyme producers (Imanisa et al., 2023).

One of the efforts to explore biological resources involves tracing the presence of thermophilic bacteria in various extreme environments in Indonesia, one of which is the Way Belerang Hot Spring. Way Belerang is a natural sulfur-containing hot spring located in a volcanic area in the vicinity of Lampung Province (Firman et al., 2023). The temperature at Way Belerang Hot Spring ranges from 40°C to 65°C, which provides ideal conditions for thermophilic bacteria to survive and grow. Such environmental conditions are likely to support the presence of heat-resistant bacterial strains (Mawati et al., 2021).

The Research on thermophilic bacteria that produce cellulase enzymes has been widely conducted. For example, Majidah et al., (2023) isolated cellulase-producing bacteria from the Ie Suum hot spring in Aceh Besar Regency, obtaining cellulase index values for isolates TS6, TS7, and TS10 of 3.67 cm, 2.33 cm, and 4 cm, respectively, with cellulase activities of 4.7×10^{-3} U/mL, 4.4×10^{-3} U/mL, and 5.5×10^{-3} U/mL. In addition, Sari & Agustien (2012) obtained 28 cellulase-producing bacterial isolates from the Sungai Medang hot spring, with the highest cellulolytic index observed in isolate MII2.2 (5.40 cm) and the highest cellulase activity in isolate MII2.1 (32.14 U/mL). Mukminin (2014) reported four cellulase-producing bacterial isolates from the Pacet hot spring in Mojokerto, with clear zone diameters of 27 mm, 24 mm, 20 mm, and 17 mm, and cellulase activities for isolates PS2, PS3, PS4, and PS8 of 3.9×10^{-3} U/mL, 1.9×10^{-3} U/mL, 7.8×10^{-3} U/mL, and 4.2×10^{-3} U/mL. "Despite extensive research on cellulase-producing bacteria from various Indonesian hot springs (e.g. Sungai Medang, Pacet, Ie Suum), Way Belerang remains uninvestigated for

cellulase activity. Way Belerang is a hot spring located at the foot of Mount Rajabasa in Kalianda, South Lampung. The spring water is naturally rich in sulfur with power pH condition. Exploring this site may uncover novel thermophilic cellulase sources with superior thermostability for industrial applications respectively. This gap serves as the basis for the present study, which aims to isolate and thermophilic cellulase-producing bacteria from Way Belerang Hot Spring and evaluate their cellulase-activity both qualitatively and quantitatively.

METHOD

Hot Spring Sample Collection

Sampling was carried out at the hot spring site by measuring temperature and pH using Termperatur humidity termometer and pH meter (UNI-T^{R}). A total of 200 mL of hot spring water was collected from approximately 10 cm depth at two randomly selected points with GPS coordinates -5.748631, 105.632985. The water samples were placed in thermos bottles, tightly sealed, wrapped in aluminum foil, and labeled accordingly. Sediment samples were collected using a scoop to a depth of approximately 10 cm from three random points. The sediment samples were placed in Falcon tubes (50 ml), tightly sealed, wrapped in aluminum foil, and labeled for identification. The Falcon tubes containing the sediment samples were then placed in a thermos. Both sediment and water samples were transported to the Microbiology Laboratory and processed for bacterial isolation within 24 hours of collection. There are enviromental condition on sampling site:

Table 1. The Enviromental Condition on Sampling Site

Sample	Sampling site	Temperature (^{O}C)	pH
Sediment	1	52	2,2
	2	51	2,4
Water	1	52	2,2
	2	53	2,3

Isolation of Thermophilic Bacteria from Way Belerang Hot Spring

Bacterial isolation was carried out by taking 1 mL of each hot water sample and weighing 1 gram of each sediment sample, which were then added to 9 mL of sterile distilled water. The mixtures were subjected to heat shock at 53°C for 1 minute and homogenized using a vortex mixer. Serial dilutions were performed from 10^{-1} to 10^{-6} using test tubes. From each dilution, 0.1 mL of the sample was inoculated onto petri dishes (120 mm) containing Nutrient Agar (NA) medium and spread evenly using a spreader. The plates were incubated at 53°C for 24–48 hours to allow bacterial colonies to grow. Colonies showing distinct morphological differences were further purified by streaking on NA medium to obtain single colonies. These purified colonies were then incubated again at 53°C for 24 hours (Ifeanyi *et al*. 2014).

Qualitative Assay of Cellulase Enzyme Activity

The qualitative assay of cellulase enzyme activity can be performed using a selective medium containing 1% carboxymethyl cellulose (CMC) (HIMEDIA).

Bacterial isolates for the qualitative test were taken from Nutrient Agar (NA) (HIMEDIA) plates containing pure cultures and then streaked onto several petri dishes containing solid 1% CMC medium. There are no positive and negative controls in qualitative assay. All experiments were conducted in triplicate to ensure the accuracy and reliability of the results. The plates were incubated at 53°C for 5 days. The cellulase enzyme activity was tested using the Congo red method, which involves pouring Congo red solution onto the cultures and allowing it to sit for 15 minutes. The solution was then discarded and the plates were rinsed three times with 1 M NaCl for 15 minutes each time. Bacterial isolates capable of degrading 1% CMC will show clear zones around their colonies. The cellulolytic index (CI) can be determined by measuring the diameter of the colonies. Cellulase activity is categorized as low if the CI < 1, moderate if between 1–2, and high if > 2 (Fauziah & Ibrahim, 2020). The formula for calculating the Cellulolytic Index (CI) according to Nababan et al., (2019) is as follows:

$$CI = \frac{(\text{diameter of clear zone} - \text{diameter of colony})}{\text{diameter of colony}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Bacterial Growth Curve Construction

The bacterial growth phase can be determined by constructing a growth curve, which aims to identify the optimal incubation time for cellulase enzyme production. Two loops of cellulolytic bacterial isolates were inoculated into 15 mL of 1% CMC broth medium and incubated in a shaker at 53°C for 18 hours at 150 rpm. An inoculum of 1.5 mL was taken every 2 hours to measure optical density (OD) or cell density using a spectrophotometer at a wavelength of 600 nm (Baharuddin et al., 2014). Measurements were continued until the OD value began to decline, indicating the onset of the death phase. The growth curve was constructed by plotting OD values against incubation time.

Crude Enzyme Production

The production of crude cellulase enzyme extract was carried out following the method of Marina et al., (2018), A total of 5 mL of bacterial inoculum was inoculated into 250 mL of 1% liquid CMC medium and incubated at 53°C for 48 hours to reach the stationary phase. The crude cellulase extract was obtained by centrifuging the bacterial culture at 10,000 rpm for 10 minutes at 4°C. The centrifugation process aimed to separate the sedimented microbial cells from the supernatant, which contained the crude enzyme extract.

Preparation of Glucose Standard Curve

The glucose standard curve was prepared by first making a glucose stock solution by dissolving 1 gram of glucose in 100 mL of sterile distilled water. To obtain a glucose standard with a concentration of 1 mg/mL, 1 mL of the stock solution was diluted with 9 mL of sterile distilled water. A series of serial dilutions were then prepared with increasing concentrations of 0, 50, 100, 150, 200, 250, and 300 ppm. For each dilution, 0.1 mL of the 1 mg/mL glucose solution was mixed with 1.9 mL of distilled water (Table 2). The glucose solution was then mixed with DNS (3,5-dinitrosalicylic acid) reagent and homogenized using a vortex mixer. The mixture was heated on a hot plate at 100 °C for 5 minutes, then cooled to room temperature. Absorbance was measured

using a spectrophotometer at a wavelength of 550 nm. The absorbance values obtained were statistically processed using Microsoft Excel, with glucose concentrations plotted on the x-axis and absorbance on the y-axis, allowing the regression equation and reaction to be determined (Murtiyaningsih & Hazmi, 2017).

Table 2. The Glucose Concentration

Concentration		Sterile H_2O (mL)	Standard Glucose Stock Solution (mL)
ppm	mg/mL		
0	0,00	2	0,00
50	0,05	1,9	0,10
100	0,10	1,8	0,20
150	0,15	1,70	0,30
200	0,20	1,60	0,40
250	0,25	1,50	0,50
300	0,30	1,40	0,60

The quantitative assay of cellulase activity

The activity of cellulase enzyme can be measured by determining the concentration of reducing sugars using three groups of test tubes: sample, control, and blank. The sample consists of 1 mL of crude enzyme extract mixed with 1 mL of 1 % CMC solution. The control contains 1 mL of 1 % CMC solution and 1 mL of crude enzyme extract, with the reaction stopped beforehand by boiling the mixture in a water bath for 2–3 minutes. The blank contains 1 mL of 1 % CMC solution and 1 mL of distilled water. All three tubes are then incubated at 53 °C for 30 minutes. After incubation, 2 mL of DNS reagent is added, the mixtures are vortexed, and then incubated again in a water bath at 100 °C for 10 minutes. Once cooled to room temperature, absorbance is measured for each tube using a spectrophotometer at a wavelength of 550 nm. The absorbance values are used to analyze enzyme activity with the help of Microsoft Excel to determine the resulting enzyme activity (Murtiyaningsih & Hazmi, 2017). Cellulase activity is determined based on the amount of reducing sugar formed. The glucose concentration produced from the hydrolysis of cellulose by the cellulase enzyme is calculated based on the absorbance value at 550 nm using the following Murtiyaningsih & Hazmi (2017) formula:

$$Absorbance = (As - Ab) - (Ac - Ab) \quad \text{..... (2)}$$

The absorbance value obtained is then inserted into the linear equation derived from the glucose standard curve. The cellulase activity is calculated using the following Mulyasari et al., (2015) formula:

$$Cellulase\ Activity\ (\frac{U}{mL}) = Glucose\ concentration \times \frac{1000}{V \times t \times MW} \quad \text{..... (3)}$$

Description:
As : Absorbance of the sample
Ac : Absorbance of the control
Ab : Absorbance of the blank

V : Volume of enzyme used (mL)
Incubation time (t) : 30 minutes
Molecular weight (MW) of glucose: 180

Characterization of Thermophilic Cellulolytic Bacteria Producing Cellulase Enzyme

The characterization of bacterial isolates refers to the method by Sari & Agustien (2012), which includes both macroscopic and microscopic observations. Macroscopic observation of thermophilic cellulolytic bacterial isolates with the highest cellulolytic index was conducted by examining colony edge, colony color, and colony shape. Microscopic observation was performed through Gram staining. A loopful of pure bacterial isolate was placed on a glass slide, then a drop of distilled water was added and spread to form a thin layer, followed by fixation over a Bunsen flame. Next, one drop of crystal violet was added and left for 30 seconds, then rinsed with distilled water and air-dried. Once dry, iodine solution was added for 1 minute, rinsed with distilled water, and air-dried again. The next step involved adding 70% alcohol for 15 seconds, followed by another rinse with distilled water. After drying, safranin solution was added and left for 1 minute, then rinsed again and dried. Finally, immersion oil was added, and the slide was observed under a microscope at 100x magnification to examine the shape and color of the cells.

RESULT AND DISCUSSION

The Qualitative cellulase-producing bacteria

The result of screening cellulase-producing bacteria showe 16 of 24 isolates exhibited cellulolytic activity, as indicated by the clear zones formed around the bacterial colonies (Figure 1). The formation of clear zones around the isolates indicates cellulolytic activity, which is the result of the cellulase enzyme hydrolyzing the CMC medium. These findings suggest that the bacterial isolates from the Way Belerang Hot Spring have the potential to produce cellulase capable of hydrolyzing the β-1,4-glycosidic bonds in CMC. According to Baharuddin et al., (2022), the distinct clear zones are evidence that the β-1,4-glycosidic bonds linking D-glucose monomers in the cellulose (CMC) chains have been broken down by the cellulase enzyme produced by the cellulolytic bacteria.

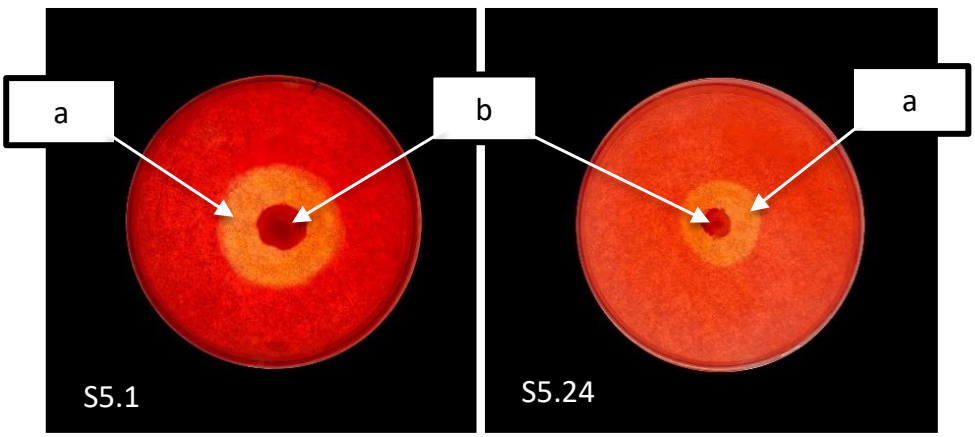

Figure 1. Clear zones indicating cellulase enzyme activity produced by thermophilic bacteria; (a) clear zone (b) bacterial colony (Petri dishes size 120 mm)

The CMC medium hydrolyzed by cellulase enzymes will not retain the Congo red stain. According to Kurniawati (2019), the color change of Congo red indicates cellulose-degrading bacterial activity. Congo red interacts strongly with CMC to produce a reddish color, but if the CMC has been decomposed by cellulolytic microorganisms, the reddish color will turn clear. The clear zones that form can be enhanced by washing with 1 M NaCl. This is because Congo red is a sodium salt of benzidinediazo-bis (1-naphthylamine-4-sulfonic acid) ($C_{3}2H_{2}2N_{6}Na_{2}O6S_{2}$), a soluble dye that can be washed away by sodium salts such as NaCl (Murtiyaningsih & Hazmi, 2017; Yakupova et al., 2019).

Bacterial colonies that exhibited clear zones were then measured for their cellulolytic index, which is the difference between the diameter of the clear zone and the diameter of the bacterial colony. The hydrolysis results of the 16 bacterial isolates are presented in Table 3. The highest cellulase index values were shown by isolate codes S5.1 and S5.24, with indices of 2.728 and 2.395, respectively. According to Fauziah & Ibrahim (2020), a cellulase index value of <1 is categorized as low, between 1–2 as moderate, and >2 as high. A study by Majidah (2023) obtained a higher cellulolytic index than isolates S5.1 and S5.24, reaching 4.00. Similarly, research by Megahati (2017) reported a higher index of 2.90. According to Murtiyaningsih (2017), the variation in cellulolytic activity indices is likely due to differences in the potential of each bacterial isolate to secrete cellulase and degrade the substrate. The higher the cellulase index of an isolate, the greater its cellulolytic activity. The varying levels of cellulase enzyme activity among bacteria are attributed to strain differences, as each bacterium possesses distinct capabilities in degrading cellulose substrates (Kurniawati et al., 2019).

Table 3. Qualitative Cellulolytic Activity of Thermophilic Bacteria

No	Isolat Code	Mean ± Standard Deviation		Index Cellulolytic	Description Index Cellulolytic
		Clear Zone diameter (cm)	Bacterial colony diameter (cm)		
1.	S5.1	4,166 ± 1,314	1,133± 0,406	2,728	High
2.	S5.3	2,575 ± 0,757	1,8 ± 0,31	0,430	Medium
3.	A5.4	2,883 ± 1,338	1,6 ± 0,214	0,786	Medium
4.	S5.8	3,75 ± 0,61	2,35 ± 0,572	0,6234	Medium
5.	S5.11	3,859 ± 0,47	1,583 ± 0,407	1,568	Low
6.	A5.12	3,542 ± 1,09	1,43 ±0,29	1,474	Low
7.	S5.13	3,783 ± 1,07	1,63 ± 0,63	1,480	Low
8.	A5.14	3 ± 0,74	1,45 ± 0,29	1,0615	Low
9.	A5.17	3,61 ± 2,05	1,142 ± 0,364	1,998	Low
10.	A5.18	3,2 ± 0,37	1,366 ± 0,267	1,414	Low
11.	S5.19	2,742 ± 0,27	1,2 ± 0,22	1,327	Low
12.	A5.20	3,43 ± 0,360	1,341 ± 0,113	1,559	Low
13.	S5.21	3,07 ± 0,43	1,266 ± 0,231	1,477	Low

14.	S5.22	3,375 ± 0,475	1,242 ± 0,161	1,716	Low
15.	S5.23	3,53 ± 1,25	1,52 ± 0,426	1,361	Low
16.	S5.24	5,183 ± 0,426	1,533 ± 0,101	2,395	High

Quantitative Activity of Cellulase Enzyme

Measurement of the bacterial growth curve was carried out to obtain information on the optimal incubation time for cellulase production. The glucose standard curve was constructed using six concentration points at 50 ppm intervals, ranging from 50 to 300 ppm. Growth curve analysis of thermophilic bacteria was performed using two bacterial isolates with the highest cellulolytic index values, namely isolate S5.1 and isolate S5.24. Bacterial growth was monitored at 2-hour intervals by measuring the optical density (OD) of bacterial turbidity in CMC broth medium at a wavelength of 600 nm. According to Oktavia (2018), increased turbidity in the inoculum medium indicates an increase in both the number and size of bacterial cells. Additionally, generation time calculation is necessary to predict the population growth of each microorganism over the same time period, in relation to its metabolic activity.

The results of the growth curve (Figure 2) show the bacterial growth phases, including the lag phase, exponential (log) phase, stationary phase, and death phase (Risna et al., 2022). The lag phase was observed from hour 0 to hour 2 for both isolate S5.1 and isolate S5.24, characterized by a very slow growth rate due to the adaptation process to a new environment. The exponential phase occurred between hour 2 and hour 10 for isolate S5.1, and between hour 2 and hour 14 for isolate S5.24. Isolate S5.1 showed a more significant increase compared to S5.24, indicating highly active metabolic processes and cell division under favorable environmental conditions. The stationary phase was reached between hour 10 and hour 12 for isolate S5.1, and between hour 14 and hour 16 for isolate S5.24. During this phase, the bacterial growth rate equals the death rate. Finally, both isolates entered the death phase, marked by a decline in cell numbers, indicating reduced bacterial populations due to nutrient depletion. The decline for isolate S5.24 was observed from hour 16 to hour 72, while for isolate S5.1, the decline began at hour 12 and continued to hour 72.

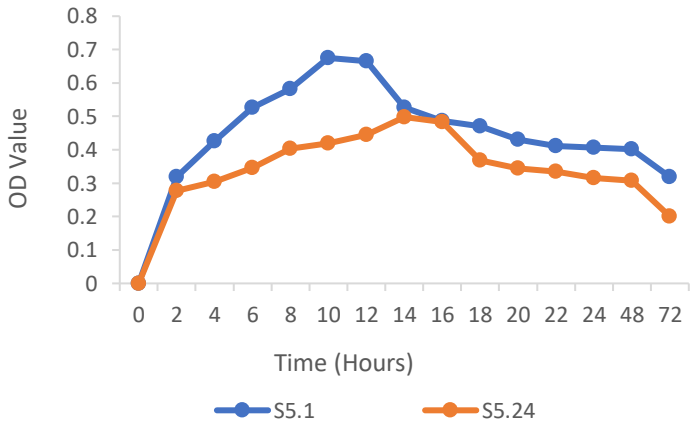

Figure 2. Growth curve of bacterial isolate S5.1 and bacterial isolate S5.24

Based on the growth curve results of both bacterial isolates, it was found that the optimal incubation time to support cellulase enzyme production was at the 10th hour for isolate S5.1 and at the 14th hour for isolate S5.24, which corresponds to the late exponential phase to the early stationary phase. This aligns with the statement by Sonia & Kusnadi (2016) that the production of primary metabolites such as enzymes occurs during the late exponential to stationary phase, making it ideal to be transferred to a new medium with more nutrients. According to Sadhu & Maiti (2013) an increase in the number of bacterial cells leads to enhanced synthesis of cellulase enzymes for cellular metabolic processes. However, once the cellulose in the growth medium is nearly depleted, the bacteria will utilize the glucose released by the cellulase enzyme as an energy source within their cells.

The determination of cellulase enzyme activity is based on the amount of reducing sugar produced, which is indicated by a color change in the DNS solution. Initially yellow, the DNS solution turns reddish-orange upon reaction. According to the study, a deeper color change in the sample corresponds to a higher optical density (OD) value. This is consistent with the statement by Nafiqoh & Suryaningrum (2020), which states that the more intense the color produced by the DNS reagent, the greater the amount of reducing sugar formed. A standard curve was established to determine the glucose concentration from the reducing sugars resulting from the enzymatic degradation of cellulose by cellulase from isolates S5.1 and S5.24, using a glucose standard curve. Glucose was used as the standard because it is a type of reducing sugar produced from the hydrolysis of cellulose by cellulase. The glucose standard curve yielded the linear equation y= 3.236x + 0.001 with a correlation coefficient (R^2) of 0.995 (Figure 3). This equation is used to determine the concentration of reducing sugars produced as the product of cellulase enzyme activity from thermophilic bacteria. The reducing sugar content was calculated by substituting the absorbance values of crude cellulase enzyme extracts into the glucose standard regression equation.

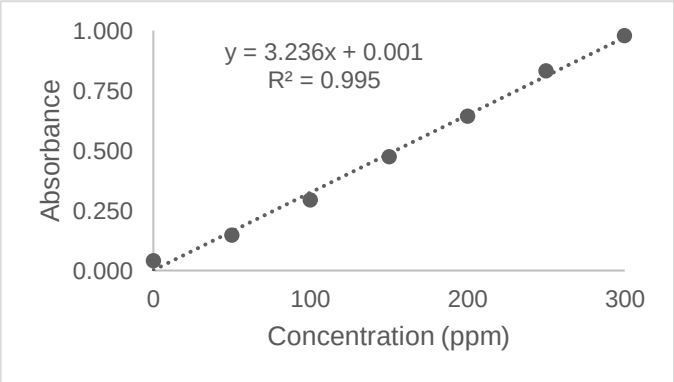

Figure 3. The glucose standard curve

The results of the quantitative cellulase enzyme assay can be seen in (Figure 4), where the blue line represents the growth curve of isolate S5.1, and the gray line represents the enzyme activity of isolate S5.1. Based on the study, it was found that the highest enzyme activity for isolate S5.1 occurred at hour 10, with a value of 5.1×10^{-2} U/mL.

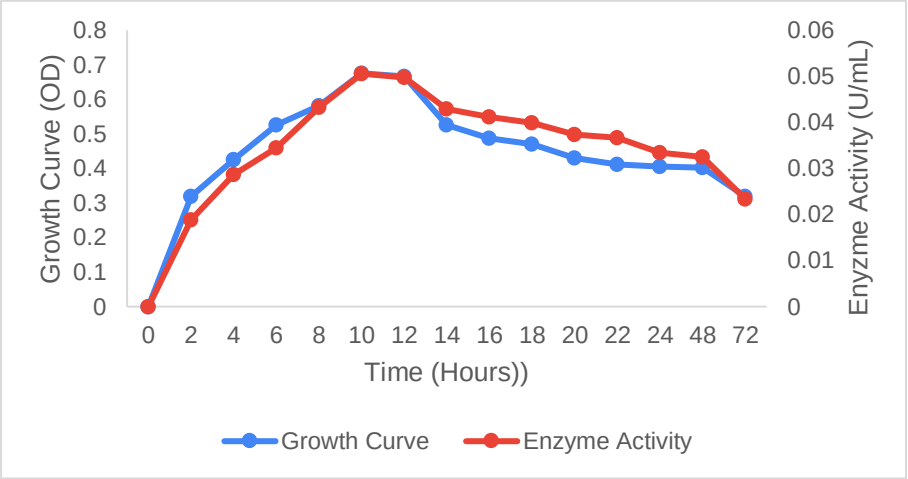

Figure 4. Correlation between growth curve and cellulase enzyme activity of isolate S5.1

The results of the quantitative cellulase enzyme assay for isolate S5.24 are shown in (Figure 5), where the blue line represents the growth curve of isolate S5.24, and the gray line represents the enzyme activity of isolate S5.24. Based on the study, it was found that the highest enzyme activity for isolate S5.24 occurred at hour 14, with a value of 3.1×10^{-2} U/mL.

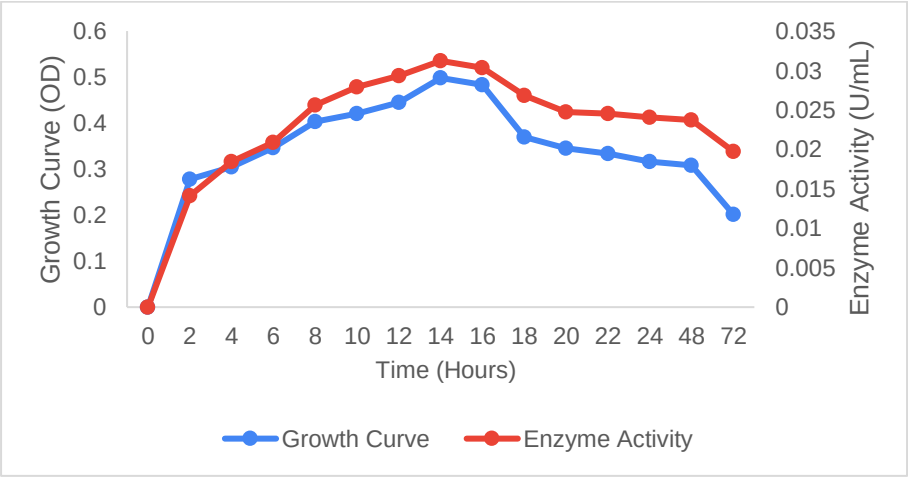

Figure 5. Correlation between growth curve and cellulase enzyme activity of isolate S5.24

The enzyme activity exhibited by bacterial isolates S5.1 and S5.24 is considered high. This aligns with the research conducted by Majidah et al., (2023) on cellulolytic bacteria from the hot spring source Ie Suum in Aceh Besar Regency, which showed cellulase enzyme activities of 4.7×10^{-3} U/mL, 4.4×10^{-3} U/mL, and 5.5×10^{-3} U/mL, respectively. Additionally, Mukminin (2014) isolated four cellulase-producing bacteria from the Pacet Mojokerto hot spring with enzyme activities of 3.9×10^{-3} U/mL, 1.9×10^{-3} U/mL, 7.8×10^{-3} U/mL, and 4.2×10^{-3} U/mL for isolates PS2, PS3, PS4, and PS8, respectively. The results indicate an increase in cellulase enzyme activity accompanied by bacterial cell growth. According to Lestari et al., (2024), the activity of CMC-ase from isolates is influenced by the glucose concentration in the culture. When glucose is limited in the initial cultivation medium, the cells are

stimulated to produce enzymes needed to hydrolyze CMC. At the peak of cellulase enzyme activity, the bacteria release cellulase enzymes maximally into their external environment.

Differences in enzyme activity are caused by each cellulase enzyme isolate originating from different locations. This is in line with Hanifa et al., (2021) statement that variations in cellulase enzyme activity occur because the enzymes come from different growth environments. Daunoras et al., (2024) state that cellulase enzyme activity depend on type of soil ecosystem. n this study, enzyme activity is higher in sediment compared to water, which is due to the sediment environment being more stable and rich in organic materials such as fallen leaves, wood, and other organic matter that contain abundant cellulose—the primary substrate for the growth of cellulolytic bacteria. Irdawati et al., (2018) observation that fallen leaves, twigs, seeds, grasses, pollen, and insect carcasses found in hot springs are organic materials that can be utilized by microorganisms living in those hot spring environments.

Characterization of Thermophilic Cellulolytic Bacteria Producing Cellulase Enzymes

The thermophilic cellulolytic bacterial isolates were further characterized macroscopically, including colony shape, color, edge, and Gram staining. The macroscopic characterization results of isolates S5.1 and S5.24 showed that both had round colony morphologies but differed in colony color and edge. Isolate S5.1 was cream-white with an irregular edge, while S5.24 was cloudy white with a smooth, even edge (Table 4). Gram staining results for both isolates indicated they were Gram-positive rod-shaped bacteria (Figure 6).

Table 4. Macroscopic and Microscopic Characterization of Bacteria S5.1 and S5.24

Isolate	Macroscopic			Microscopic	
	Shape	**Color**	**Edge**	**Gram**	**Cell Shape**
S5.1	Circular	Cream white	Irregular	Positive	Bacil
S5.24	Circular	Cloudy white	Smooth and even	Positive	Bacil

The Bacteria have selective adaptation mechanisms to survive in extreme environments, one of which is by forming endospores. This is what enables Gram-positive bacteria to grow in high-temperature environments. According to Pratiwi & Asri (2022), endospores in some bacteria serve as dormant structures that help bacteria survive under extreme conditions such as high temperatures, drought, UV radiation, extreme pH, nutrient deficiency, and harmful chemicals. Endospores are resistant to extreme temperatures due to several resistance factors, including DNA protection by small acid-soluble proteins (SASPs), accumulation of divalent cations in

the endospore core, dehydration of the endospore core, and the presence of dipicolinic acid (DPA) (Checinska et al., 2015).

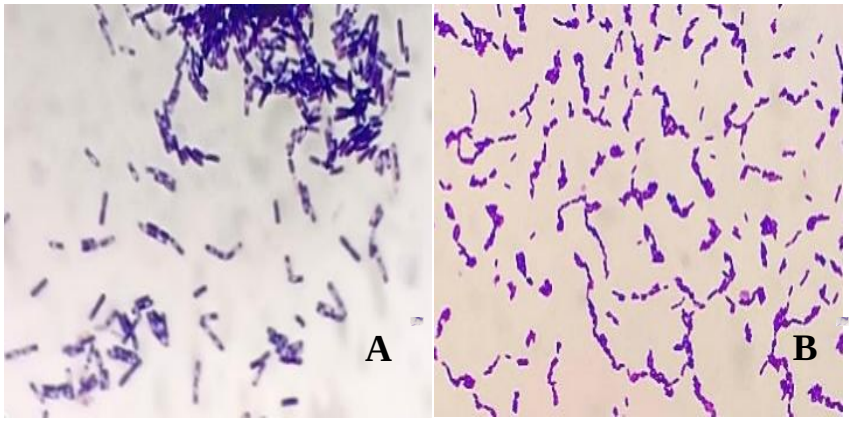

Figure 6. Microscopic character of S5.1 bacterial isolate (A) dan isolat S5.24 bacterial isolate (B)

Endospores are produced by several Gram-positive bacteria, especially those in the *Bacillus* and *Clostridium* groups, which are capable of surviving in extreme environments that cannot be inhabited by other microorganisms. A study by Narayan et al., (2008) characterized thermophilic bacterial isolates from the Savusavu hot springs in Fiji, which showed growth at 90°C and were identified as *Bacillus* sp. through morphological and molecular characterization. Additionally, research by Lv (2023) successfully isolated thermophilic bacteria from hot spring sediments in China, which showed optimal growth at 45°C and were identified as *Clostridium caldaquaticum* sp.

Thermophilic bacteria producing cellulase enzymes have advantages in various industrial applications, mainly due to their ability to produce enzymes that are stable and active at high temperatures (Vavitsas et al., 2022). Cellulase enzymes from thermophilic bacteria exhibit resistance to thermal denaturation, making them highly suitable for use in industrial processes requiring extreme conditions, such as high temperatures and varying pH levels. One of the main industries utilizing these enzymes is the bioenergy sector, particularly in the production of bioethanol from lignocellulosic biomass. In this process, cellulase enzymes are used to hydrolyze cellulose into simple sugars, which are then fermented into ethanol (Ejaz et al., 2021). Additionally, thermophilic cellulase enzymes are also used in the textile industry for fabric biopolishing, in the paper industry for deinking and pulp processing, and in the animal feed industry to improve nutrient availability by breaking down plant fibers (Kuhad *et al.*, 2011).

CONCLUSION

The Termofilik bacteria isolated from Way Belerang were successfully characterized and found to be capable of producing cellulase enzymes. The S5.1 and S5.24 isolates are potential thermophilic bacteria producing cellulase enzymes. Both

isolates demonstrated heat resistance at 53°C. The cellulase enzyme activity test showed that isolate S5.1 had an activity of 5.1×10^{-2} U/mL at hour 10, while S5.24 had an activity of 3.1×10^{-2} U/mL at hour 14. Further characterization is needed to determine whether these isolates can resist even higher temperatures. Additionally, further research is required to determine the optimum cellulase enzyme activity of both isolates under different pH and temperature conditions. This study is the first to explore the potential of thermophilic cellulase-producing bacteria isolated from Way Belerang, highlighting a novel microbial resource for future industrial and biotechnological applications.

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