



# Characteristics of Cellulase Producing Bacteria Isolated from Gold Mine Soil

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## ABSTRACT

Land degradation has created an urgent need for innovative restoration strategies based on microbial applications. The microbiological approach using cellulase enzymes produced by cellulolytic bacteria is a prospective step to address problems related to land restoration and biodegradation of complex organic matter in the environment. This study aimed to isolate and characterize cellulolytic bacteria from post-mining soil collected at the Martabe gold mine site in South Sumatra, Indonesia. Isolation and selection of cellulolytic bacteria was performed on 1% carboxymethyl cellulose (CMC) medium, resulting of two non-pathogenic Gram-positive isolates, TSUS3.4.1 and TSUS3.4.2, with cellulolytic index of 2.51 and 2.26, respectively. Hemolysis tests on blood agar confirmed the semi- and non-pathogenic activity of both isolates. The growth curve and enzyme-specific activity was tested and measured in minimal nutrition broth with addition of 1% CMC with the highest activity values of 0.37 U/mg for TSUS3.4.1 and 0.17 U/mg for TSUS3.4.2 at the 27th hour after incubation. TSUS3.4.1 isolate has optimum cellulase activity at pH 6 and 30°C. TSUS3.4.2 isolate has optimum cellulase activity at pH 7 and temperature 30°C. These findings highlight the isolates potential positioning them as promising candidates for biotechnological applications in post-mining land rehabilitation and organic waste degradation.

**Keywords:** biodegradation, cellulolytic bacteria, cellulose, organic matter, post-mining

## INTRODUCTION

Mining activities leave the soil with low fertility, high heavy metal content, and pH that tends to be extreme (Soares *et al.* 2012). These extreme conditions create selective pressures that push only certain microorganisms with high adaptive capabilities to survive. Microbes that are able to thrive in such an environment are thought to have unique metabolic potential including the ability to produce stable and active enzymes in hostile environmental conditions. One of the enzymes that is important in the process of recycling plant biomass is cellulase which plays a role in degrading cellulose into simple sugar. Certain microorganisms, including cellulolytic bacteria, were found to be able to adapt in marginal environments (Rastogi *et al.* 2009). Previous research also shown that cellulolytic bacteria can be found in a variety of habitats, such as soils with low fertility rates (Dewiyanti *et al.* 2022), organic waste (Fu *et al.* 2024), and post-industrial soils (Murugesan *et al.* 2019).

Cellulose is the main component of organic waste and is one of the forms of biomass that has a great abundance on earth. Cellulose has a rigid complex structure and is most abundant in plant cell walls to

provide structural strength (Habibi *et al.* 2010). The consequences of cellulose complex structure are that it is difficult to be degraded naturally in the environment. Inadequate organic waste management through waste burning procedure cause methane emissions and environmental pollution so there is crucial to find alternative for more efficient organic waste management (Rahayu *et al.* 20204). Cellulase enzyme is an important catalyst to hydrolyze cellulose into glucose, its simpler form of monomer, so that it can be an alternative in biodegradation efforts to make organic waste more environmentally friendly (Pratiwi *et al.* 2022). The application of cellulolytic bacteria from mining land may support both economic value and ecological restoration via organic matter decomposition.

## METHODS

### Isolation and Selection of Cellulolytic Bacteria

Soil sample were collected from the Henny Dump 2015 area of Martabe Gold Mining site, North Sumatra, Indonesia where shown in Figure 1. The sample was then placed in labeled zip lock plastic bag.

Isolation of cellulolytic bacteria was carried out through serial dilution method. One gram of soil was diluted with 9 mL of 0.85% NaCl then spreaded on nutrient agar (NA) medium then incubated at 27°C for 24 hours. Each colony that grew then purified on

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another NA plate until single, isolated colony was confirmed. The identification of the macroscopic and microscopic characteristics of each isolate refers to Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994). Each purified colony was inoculated to selective basal media with 1% CMC and incubated at room temperature for 48 hours. Visualization of clear zones was carried out using 0.1 % Congo red then rinsed with 1 M NaCl (Theater & Wood 1982). The measurement of the cellulolytic index was carried out by dividing the value of the clear zone diameter minus the diameter of the colony by the diameter of the colony formed

### Hemolytic Assay

Hemolytic activity assay performed on isolates that were positive for a cellulolytic index to determine their pathogenicity properties (Meryandini *et al.* 2009). A total of one loop full of selected isolates was inoculated in a blood agar medium then incubated at room temperature for 48 hours. The positive control  $\beta$ -hemolysis used was *Pseudomonas aeruginosa*.

### Growth Curve and Specific Enzyme Activity Assay

The initial inoculum is made by inoculating 1-2 lops of isolate into 30 mL nutrient broth (NB). The inoculum was incubated at room temperature using a shaker at 80 rpm for 10 hours. A total of 1500  $\mu$ L from the inoculum into 150 mL of production media, i.e. selective basal media with a substrate of 1% CMC (Bui 2014) without the addition of glucose then incubated at room temperature with a shaker at 80 rpm. Sampling for the growth curve is carried out every 3 hours from the 0th to the 39th hour. Each sampling was measured for turbidity using a spectrophotometer at a wavelength of 600 nm.

The production of enzyme crude extracts was carried out in each sampling using a separation technique based on molecular weight by centrifugation at a speed of 10,000 rpm for 10 minutes at a

temperature of 4°C. The extracellular cellulase enzyme will be on the supernatant part so that it is separated immediately after centrifugation. Each sample was stored in a refrigerator at a temperature of 4°C (Miller 1959). Measurement of the cellulase activity of the crude extract enzyme was carried out by the DNS method (Bradford 1976). The substrate used is 1% CMC dissolved in a 0.1 M phosphate buffer pH 7 at room temperature. Each 1 mL of substrate was reacted with 1 mL of enzyme sample and incubated for 20 minutes. A total of 1 mL of dinitro salicylic acid (DNS) was added to each sample and then homogenized with a vortex and heated with boiling water for 15 minutes. The absorbance value is measured at a wavelength of 540 nm. One unit of cellulase activity is defined as the number of enzymes that produce 1  $\mu$ mol of glucose per minute.

Protein levels were tested for each sample using the Bradford method (Bradford 1976). The specific activity of each sample is known using the calculation:

$$\text{Specific enzyme activity (U/mg)} = \frac{\text{Cellulase activity (U/mL)}}{\text{Protein concentration (mg/mL)}}$$

### Cellulase Enzyme Characterization

The characterization of the pH and optimum temperature of the enzyme is carried out by the DNS method (Miller 1959). The crude extract enzymes were reacted with a 1% CMC substrate dissolved in 0.1 M of citrate buffer (pH 4–6), 0.1 M of phosphate buffer (pH 7 and 8), and 0.1 M of glycine buffer (pH 9 and 10) and then incubated at room temperature for 20 minutes. A total of 1 mL of DNS was added to each sample and then homogenized with a vortex and heated with boiling water for 15 minutes. The absorbance value is measured at a wavelength of 540 nm. Buffers with optimum pH values are used as substrate solvents for optimum temperature testing. Incubation was carried out at temperatures of 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C (Mubarik *et al.* 2003).

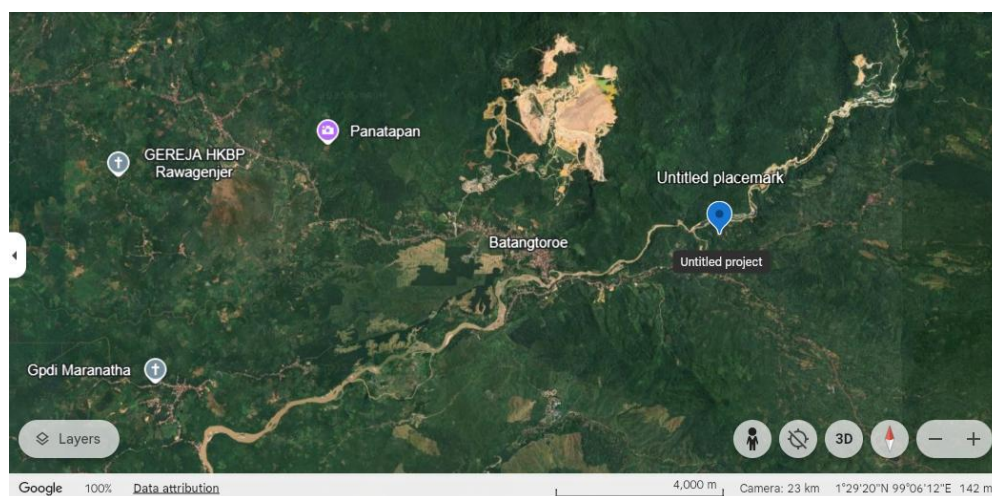


Figure 1 Sampling map in Martabe Mining Site, North Sumatra.

## RESULTS AND DISCUSSION

### Colony and Cells Morphology of the Bacterial Isolates

A total of fifteen isolates with different colony morphologies were successfully isolated from the mine soil by the serial dilution method. The fifteen purified isolates were inoculated on a selective basal media with a 1% CMC substrate then incubated at room temperature for 48 hours. Five of the fifteen positive isolates had clear zones around the colony. Two isolates were chosen, TSUS. 3. 4. 1 and TSUS. 3. 4. 2, to further characterize the morphology of colonies and cells (Table 1). The two isolates tend to have uniform morphological appearance, namely irregular shapes, raised elevations, undulate margins, and white colony color with opaque transparency. The microscopic character of each colony is rod and belongs to Gram-positive bacteria (Figure 2).

Positive cellulolytic activity is indicated by visualization of clear zones around the colony (Figure 3). The isolates of TSUS3.4.1 and TSUS3.4.2 respectively had cellulolytic index of 2.51 and 2.26 (Table 2).

### Hemolytic Activity

The two isolates differ in the type of hemolytic activity (Figure 4). TSUS3.4.1 isolates are seen to have  $\alpha$ -hemolysis or partial hemolysis activity that indicated by the appearance of greenish-yellow zones around the colony. Meanwhile, TSUS3.4.1 isolates were seen to have no hemolytic activity. The positive control  $\beta$ -hemolysis used was *Pseudomonas aeruginosa*.

### Growth and Cellulase Production of the Isolates TSUS3.4.1 and TSUS3.4.2

TSUS3.4.1 showed a sharply increased growth rate from the beginning of incubation to reaching an

exponential phase between the 3rd to the 18th hour (Figure 5A). The isolate experienced a decrease in growth rate at the 18th hour and gradually moved into the stationary phase until the 39th hour. The growth curve of TSUS3.4.1 showed the first peak at the 12th hour with a value of 0.17 U/mg and reached the highest activity value at the 27th hour since the beginning of incubation with a value of 0.37 U/mg. Furthermore, enzyme production gradually decreased. TSUS3.4.2 showed a sharply increased growth rate from the beginning of incubation until reached the exponential phase between the 3rd and 18th hours. The isolates experienced decrease in growth rate at the 18th hour and gradually move into the stationary phase until the 39th hour. Enzyme activity peaked at the 27th hour from the beginning of incubation with a value of 0.17 U/mg and then decreased production (Figure 5B).

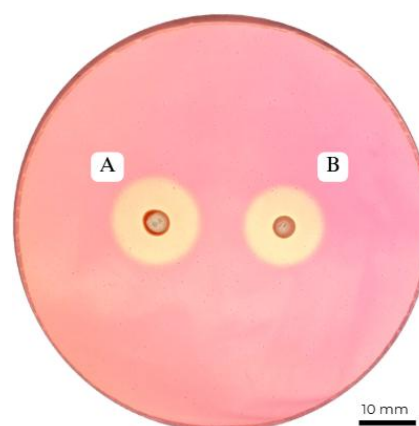


Figure 3 Clear zone formation (indicate cellulose enzyme activity) after 48 hours of incubation of selected isolates A) TSUS3.4.2 and B) TSUS3.4.1, respectively.

Table 1 Bacterial colony and cells characteristics of selected isolates

Code	Macroscopic characteristics				Microscopic characteristics	
	Shape	Margin	Elevation	Color	Gram	Shape
TSUS3.4.1	Irregular	Undulate	Raised	White	+	Rod
TSUS3.4.2	Irregular	Undulate	Raised	White	+	Rod

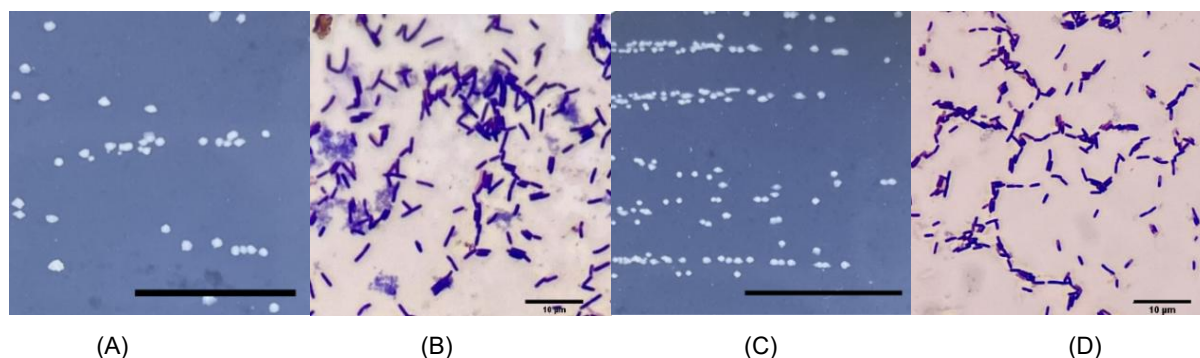


Figure 2 Morphology of colonies (A and C) and Gram staining results (B and D) of isolates TSUS3.4.1 and TSUS3.4.2, respectively. Scale bar: 10µm.

Table 2 Cellulolytic index value of the isolates

Isolate	Colony diameter (mm)	Clear zone diameter (mm)	Cellulolytic index
TSUS3.4.1	4.06±0.05	14.30±0.18	2.51
TSUS3.4.2	4.81±0.21	15.68±0.19	2.26

Note: ± = Standard deviation (SD)

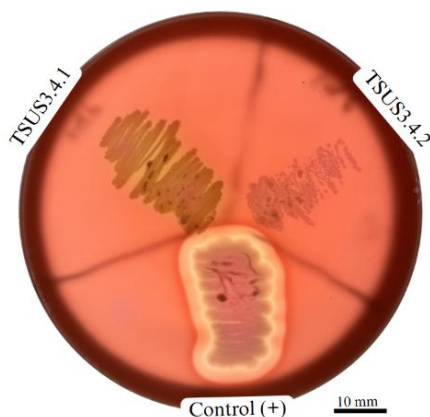


Figure 4 Hemolytic activity of bacterial isolates, TSUS3.4.1 and TSUS3.4.2 with positive control.

#### Cellulase Activity at Various pH and Temperatures

The optimum substrate pH condition for the enzyme production of the TSUS3.4.1 isolate enzyme is at pH 6 with a value of 0.03 U/mL and decreases at neutral pH. Meanwhile, TSUS3.4.2 isolate produces the most optimum enzyme at pH 7 with a value of 0.02 U/mL (Figure 6).

Both isolates had the most optimum cellulase activity at 30°C with TSUS3.4.1 and TSUS3.4.2 isolates having values of 0.024 U/mL and 0.018 U/mL, respectively (Figure 7). The activity of the two isolates were decreasing as the incubation temperature increases. Both isolates showed that rising temperatures did not stop cellulase activity.

## DISCUSSIONS

Soils affected by mining activities present an extreme environment due to its low pH and nutrient content. These conditions create selective conditions for rhizosphere microorganisms to adapt. This adaptation includes the ability to regulate hydrolytic enzymes to hydrolyze a variety of complex compounds in the environment, including cellulolytic enzymes. The result of this research shows that bacteria with the ability to degrade cellulose can be isolated from the mine soil. Cellulolytic bacterial selection using a 1% CMC substrate as an inducer successfully obtained two potential isolates, namely TSUS3.4.1 and TSUS3.4.2. Both have similar macroscopic and microscopic characteristics. Cellulolytic bacteria are able to express cellulase due to substrate-induced regulation of enzymes. Each bacterial strain can produce different

cellulase enzyme complexes depending on their genes and the type of carbon source utilized.

The value of the hydrolytic index is categorized into low activity if the value at  $\leq 1$ , moderate activity for the value between 1 – 2, and high activity if the value at  $\geq 2$  (Choi *et al.* 2005). TSUS3.4.1 and TSUS3.4.2 respectively have index of 2.51 and 2.26 which are quite potential and indicate that the two selected isolates are capable of producing extracellular cellulase enzymes in high activity. This value is higher than the LA2 isolate index identified as *Pseudomonas* sp. with value of 0.78 (Anggriani *et al.* 2023). The cellulase enzyme is synthesized intracellularly then subsequently secreted into the environment where it hydrolyzes cellulose into simpler molecules, such as cellobiose which can be further broken down into glucose. The results of this hydrolysis are again used by bacteria in their own metabolic processes (Datta 2024). Congo red appears its reddish color due to the presence of an extended electron conjugation system within its molecular structure. This azo dye binds to cellulose through hydrogen bonding between the hydroxyl groups of cellulose and the amino groups of its own structure (Gupta *et al.* 2012). The hydrolysis of cellulose causes the loss of these bonds thus creating a visualization of the clear zone.

TSUS3.4.1 and TSUS3.4.2 were placed on a blood agar medium then incubated for 48 hours. The two isolates appear to have differences in the type of hemolytic activity (Figure 3). TSUS3.4.1 isolates are seen to have  $\alpha$ -hemolysis or partial hemolysis activity indicated by the appearance of greenish-yellow zones around the colony. Partial hemolysis occurs due to the reduction of hemoglobin to methemoglobin by enzymes that oxidize hemoglobin. This phenomenon is often associated with low levels of microbial virulence and is more of an opportunistic pathogen such as *Escherichia coli* (Verma *et al.* 2020). Meanwhile, TSUS3.4.1 isolates appear to have no hemolytic activity. This indicates that the isolates do not secrete hemolysin compounds which can lead to increased virulence.

The selection of production media with basalt medium consisting only essential minerals, nitrogen, and yeast extract to support the initial phase of growth aims to simulate the nutrient-poor conditions of the gold mining soil where the isolate originated. The addition of a substrate in the form of 1% CMC as the main carbon source aims to induce the production of cellulase enzymes specifically. Previous research reported that *Bacillus* sp. isolated from soil is capable of having

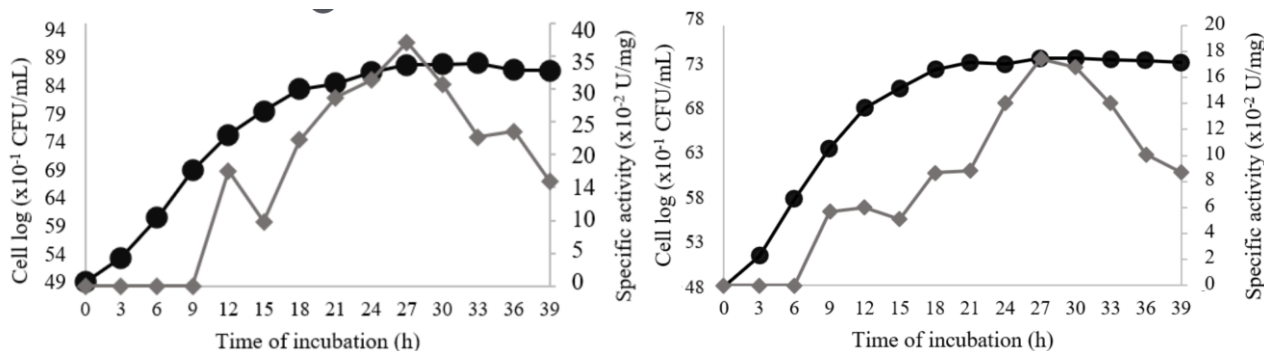


Figure 5 Growth curve with enzyme specific activity assay of the isolate TSUS3.4.1(A) and TSUS3.4.2 (B) in basal broth media + 1% CMC measured at 27 °C, pH 7.0.

cellulase activity in the range of 0.8–1 U/mL in media with 5 % glucose enrichment (Sethi *et al.* 2013).

The growth curve of TSUS3.4.1 isolate showed the highest activity value at the 27th hour since the beginning of incubation with value of 0.37 U/mg. Furthermore, enzyme production gradually decreases. These values indicate that cellulase is expressed from the early logarithmic phase and continues to reach its optimum activity in the early stationary phase. This result is consistent with the previous research which reported that the highest enzyme activity emerged from the exponential phase to the early stationary phase in cellulolytic bacteria isolated from mangrove soil (Dewiyanti *et al.* 2022). This pattern of activity is very commonly found in the production of extracellular enzymes that function in the degradation of complex substrates such as cellulose by bacteria. The production activity of the TSUS3.4.1 isolate enzyme also had the same pattern and reached its peak at the 27th hour from the beginning of incubation with value of 0.17 U/mg then the production gradually decreased. Both isolates produced greater specific activity compared to the results reported in previous studies with similar culture media at 54 hours of incubation with value of 0.097 U/mg of protein (Dewiyanti *et al.* 2022).

Both isolates exhibit optimum cellulase activity values at different pH. TSUS3.4.1 isolate exhibited optimum enzymatic activity at pH 6, whereas TSUS3.4.2 isolate showed its optimum activity at pH 7. The activity of the two isolates decreased in the alkaline pH range from 8 to 10 with the TSUS3.4.2 isolate showing the least activity. This phenomenon is consistent with previous findings which reported that certain cellulolytic bacterial isolates tend to produce cellulase optimally at pH values at 6 (Theater & Wood 1982). Enzyme activity is strongly influenced by pH conditions due to the ionic properties of carboxyl (-COOH) and amino (-NH<sub>2</sub>) groups where pH levels outside the optimum range can alter the enzyme's conformation and lead to denaturation (Chetan *et al.* 2011).

Both isolates had the most optimum cellulase activity at 30°C with TSUS3.4.1 and TSUS3.4.2 isolates having values of 0.024 U/mL and 0.018 U/mL,

respectively (Figure 7). The enzymatic activity of both isolates declines progressively with increasing incubation temperature. These findings are consistent with previous research which reported that cellulolytic bacteria isolated from extreme environments exhibit optimum productivity at 30°C with values of 0.38 U/mL and 1.4 U/mL, indicating that both isolates produce mesozyme-type enzymes (Balla *et al.* 2022). This decrease in activity results from elevated temperatures leading to the denaturation of enzymes structure and thereby affecting its productivity (Kabir & Ju 2023). Both isolates showed that rising temperatures did not stop cellulase activity.

Temperature is a factor that also affects the work productivity of enzymes. Rising temperature toward the enzyme's optimum increases kinetic energy, thereby accelerating substrate conversion (Andreas *et al.* 1999). Incubation temperature within the enzyme's optimum range may lead to denaturation due to alterations in its conformation. Temperature induced changes in substrate structure can also interfere with the enzyme's ability to form enzyme-substrate complexes (Meryandini *et al.* 2009). Enzyme activity remains present between 40°C and 90°C, indicating that the enzymes produced by both isolates have good thermal resistance. Enzymes that remain active at high temperature enable more rapid and efficient fermentation process by enhancing the reaction kinetics involved in substrate conversion (Borthakur *et al.* 2024). TSUS 3.4.1 and TSUS3.4.2 isolates have the potential to be used as biological agents in the biodegradation process of organic waste. The two isolates demonstrate potential as bio-activators for the revegetation of post-mining land also as candidates for industrial-scale cellulase production through appropriate optimization strategies.

## CONCLUSION

Two cellulolytic bacterial isolates, TSUS3.4.1 and TSUS3.4.2, were successfully isolated from soil samples collected from a gold mining site in Martabe,

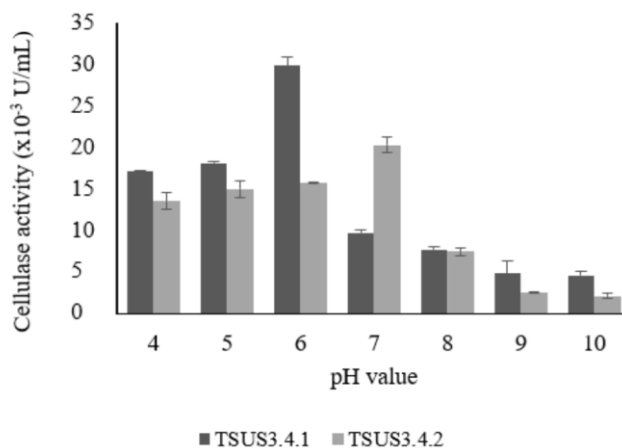


Figure 6 Cellulase activity of TSUS3.4.1 and TSUS3.4.2 bacterial isolates at various pH.

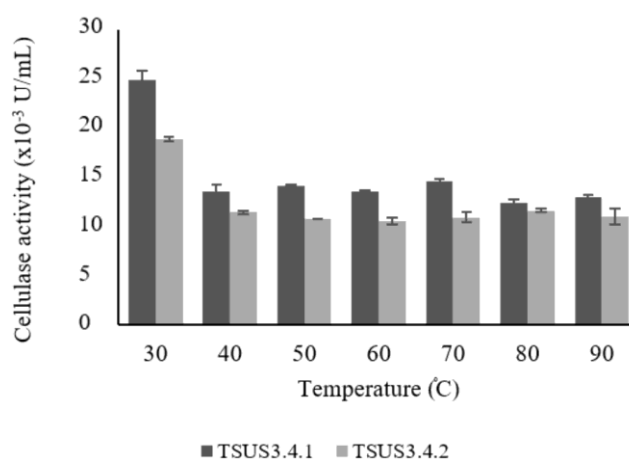


Figure 7 Cellulase activity of TSUS3.4.1 and TSUS3.4.2 isolate at various temperature.

North Sumatra, Indonesia. The cellulolytic index values of isolates TSUS3.4.1 and TSUS3.4.2 were 2.51 and 2.26, respectively. Both isolates demonstrated measurable cellulase activity with distinct optimal conditions, reflecting physiological diversity among local cellulolytic bacteria. These findings suggest that such isolates hold potential as sources of enzymes for future biotechnological applications in biomass conversion.

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