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Screening of Metalloprotease-Producing Thermophilic Bacteria and Optimization of Trace Elements for Enzyme Activity

Mellanie Alia Putri[1], Anthoni Agustien(*)[1], Yetty Marlida[2], Ayumi Rizci Puspita[1]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences,
Universitas Andalas, Padang, West Sumatera 25163, Indonesia;
[2] Department of Animal Husbandry, Faculty of Animal Husbandry,
Universitas Andalas, Padang, West Sumatera 25163, Indonesia

***Corresponding Author:** anthonieagustien@sci.unand.ac.id/aagustien@gmail.com

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Abstract

Background: *Thermophilic bacteria are potential sources of thermostable enzymes with important applications in biotechnology and industry. Among these enzymes, metalloproteases are of particular interest because their catalytic activity depends on metal ions. This study aimed to screen thermophilic bacterial isolates from hot springs in Solok Regency, West Sumatra, Indonesia, for metalloprotease production and to evaluate the effects of trace elements on metalloprotease activity.* **Methodology:** *Four thermophilic bacterial isolates (TUA-104, TUA-106, TUA-109, and TUA-113) were cultivated in protease production medium (pH 8.0) at 50°C for 24 hours, and crude enzymes were obtained by centrifugation. Metalloprotease screening was conducted using EDTA inhibition assays through well diffusion and specific protease activity analyses. The isolate showing the strongest metalloprotease characteristics was further evaluated using 5 ppm Ca^{2+}, Mn^{2+}, and Zn^{2+} supplementation.* **Findings:** *The results showed that isolates TUA-106, TUA-109, and TUA-113 exhibited characteristics of metalloprotease-producing bacteria. Among them, TUA-106 showed the most consistent inhibition pattern, with specific activity decreasing from 0.780 U/mg in the control to 0.522 U/mg after treatment with 10 mM EDTA. Zn^{2+} produced the highest specific activity (0.284 U/mg), followed by Mn^{2+} (0.263 U/mg), whereas Ca^{2+} resulted in the lowest activity (0.036 U/mg).* **Contribution:** *These findings indicate that TUA-106 is a potential Zn-dependent metalloprotease-producing thermophilic bacterium and provide new insight into the metalloprotease-producing potential of thermophilic bacteria from Solok geothermal environments for future biotechnological applications.*

Keywords: *EDTA; Enzyme Activity; Metalloprotease; Thermophilic Bacteria; Trace Element*

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INTRODUCTION

The growing demand for industrial enzymes has driven the search for biocatalysts capable of maintaining high activity and stability under harsh processing conditions. Enzymes used in the food, detergent, pharmaceutical, and biotechnology industries are frequently exposed to elevated temperatures, extreme pH, and various chemical agents that may reduce their catalytic performance. Consequently, thermostable enzymes have attracted considerable attention because they can retain their structural integrity and catalytic efficiency under such conditions, thereby improving process efficiency and reducing operational costs. In this context, thermophilic microorganisms have emerged as promising sources of thermostable enzymes due to their natural adaptation to high-temperature environments (Gallo et al., 2024; Hussain & Leong, 2023; Wang et al., 2025).

Thermophilic bacteria are microorganisms that thrive at elevated temperatures and are widely distributed in geothermal environments such as hot springs, hydrothermal vents, and volcanic regions. These microorganisms have attracted considerable attention because they produce thermostable enzymes that remain active and stable under extreme environmental conditions. Due to their thermal stability and resistance to denaturation, thermostable enzymes have been extensively explored for applications in biotechnology, food processing, pharmaceuticals, detergents, and waste management industries (Hussain & Leong, 2023; Shomali & Danish-Daniel, 2024; Guta et al., 2024; Gallo et al., 2024; Razzaq et al.,2019).

Among industrially important enzymes, proteases are one of the most widely utilized enzyme groups because of their ability to hydrolyze proteins into peptides and amino acids. Microbial proteases are particularly attractive due to their high catalytic efficiency, ease of production, and adaptability to various environmental conditions (Afrin et al., 2024; Wang et al., 2025; Solanki et al., 2021; Song et al., 2023; Adetunji et al., 2023). Based on their catalytic mechanisms, proteases are classified into several groups, including serine proteases, cysteine proteases, aspartic proteases, and metalloproteases. Metalloproteases constitute an important class of proteolytic enzymes that require metal ions, particularly Zn^{2+}, for catalytic activity and structural stability (Mushtaq et al., 2024). These metal ions participate directly in peptide bond hydrolysis and contribute to maintaining the active conformation of the enzyme.

The identification of metalloproteases is commonly performed using ethylenediaminetetraacetic acid (EDTA), a metal-chelating agent capable of binding essential metal ions from the enzyme active site. The removal of these metal ions may reduce or inhibit enzymatic activity, making EDTA inhibition assays a simple and effective approach for screening potential metalloprotease-producing microorganisms (Punnarath et al., 2025). Several studies have reported thermophilic bacteria producing metalloproteases from various extreme environments, highlighting their potential for industrial and biotechnological applications (Mushtaq et al., 2024; Sodagar et al., 2024).

In addition to their role in catalysis, metal ions contribute significantly to enzyme stability, folding, and activity. Several trace elements, including Zn^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}, have been reported to influence protease production and enzymatic performance. Zn^{2+} commonly functions as a catalytic cofactor, whereas Ca^{2+} contributes to enzyme stabilization and resistance to thermal denaturation. Therefore, optimization of trace elements represents an important strategy for improving metalloprotease activity and determining suitable conditions for enzyme production (Mushtaq et al., 2024; Punnarath et al., 2025).

Indonesia possesses abundant geothermal resources that support diverse thermophilic microbial communities. Previous studies have reported the occurrence of protease-producing thermophilic bacteria from hot springs in Solok Regency, West Sumatra, highlighting the potential of this geothermal area as a source of industrially important microorganisms (Putri et al., 2026). Four thermophilic bacterial isolates (TUA-104, TUA-106, TUA-109, dan TUA 113) from the culture collection of the Biotechnology Laboratory, Sumatra Biota Laboratory, Universitas Andalas, were selected for this study based on their proteolytic potential. However, information regarding their metalloprotease-producing potential and the influence of trace elements on enzyme activity remains limited. To date, no study has specifically evaluated thermophilic bacterial isolates from hot springs in Solok, Indonesia, using EDTA inhibition assays, specific protease activity analyses, and trace element optimization. Therefore, this study aimed to screen thermophilic bacterial isolates for metalloprotease production and evaluate the effects of different trace elements on metalloprotease activity. The findings are expected to contribute to the exploration of geothermal microbial resources as potential sources of thermostable metalloproteases for industrial and biotechnological applications.

METHOD

This study was conducted using a laboratory experimental method with a descriptive approach. The research was carried out at the Biotechnology Laboratory, Sumatra Biota Laboratory, Universitas Andalas, Padang, Indonesia, from December 2025. Four thermophilic bacterial isolates (TUA-104, TUA-106, TUA-109, and TUA-113) previously isolated from hot springs in Solok Regency, West Sumatra, Indonesia, were used in this study. Based on previous characterization, TUA-104 was identified as a Gram-positive *Bacillus*, whereas TUA-106, TUA-109, and TUA-113 were Gram-negative rod-shaped bacteria. The isolates were screened for metalloprotease production using EDTA inhibition assays through well diffusion and specific protease activity analyses. The isolate exhibiting the strongest metalloprotease characteristics was subsequently used for trace element optimization.

Preparation of Protease Production Medium

The protease production medium was prepared by dissolving 3g KH_2PO_4, 3 g K_2HPO_4, 3 g $MgSO_4$, 5 g NaCl, and 20 g casein in sterile distilled water and adjusting the final volume to 1000 mL. The medium pH was adjusted to 8.0 prior to sterilization. The medium was heated until completely dissolved and then dispensed into Erlenmeyer flasks. Sterilization was performed using an autoclave at 121 °C and 15 psi for 15 min before use (Agustien, 2010).

Preparation of Bacterial Culture

Four thermophilic bacterial isolates (TUA-104, TUA-106, TUA-109, and TUA-113) previously isolated from hot springs in Solok Regency, West Sumatera, Indonesia, were used in this study. Each isolate was inoculated into 100 mL of protease production medium in a 250 mL erlenmeyer flask and incubated at 50 °C with agitation at 150 rpm for 24 h. The cultures were subsequently centrifuged at 6000 rpm for 20 min, and the supernatants were collected as crude enzyme extracts for metalloprotease screening.

Metalloprotease Screening by Well Diffusion Assay

Metalloprotease screening was performed using an EDTA inhibition assay following a modified method of Nageswara et al., (2019). Crude enzyme extracts were incubated with EDTA at concentrations of 1.0, 2.5, and 5.0 mM for 10 min at 50 °C. A total of 100 µL of each enzyme mixture was loaded into 6 mm diameter wells prepared on skim milk agar (SMA) plates. After incubation at 50 °C for 24 h, metalloprotease activity was evaluated based on the formation of clear zones surrounding the wells. Clear zone formation was evaluated qualitatively based on the presence or absence of hydrolysis zones surrounding the wells. The reduction or absence of clear zones after EDTA treatment was considered indicative of metalloprotease activity.

Metalloprotease Screening Based on Specific Protease Activity

Metalloprotease-producing isolates were further screened based on specific protease activity using EDTA as an inhibitor following the method of Punnarath et al., (2025). Crude enzyme extracts were incubated with EDTA at concentrations of 1, 5, and 10 mM for 2 h at 50 °C. Crude enzyme extracts without EDTA treatment (0 mM EDTA) were used as the control. Protease activity was determined using the casein hydrolysis method according to Takami et al., (1989), and absorbance was measured at 578 nm, while protein concentration was measured using the Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) as the standard protein, and absorbance was measured at 750 nm. Specific protease activity was calculated as the ratio of protease activity to protein concentration and expressed as units per milligram of protein (U mg^{-1} protein) using the following equation,

$$\text{Specific Activity}\left(\frac{\text{Unit}}{\text{mg}}\right)=\frac{\text{Protease Activity (Unit/ml)}}{\text{Protein Content (mg/ml)}}$$

Effect of Trace Elements on Metalloprotease Activity

The isolate exhibiting the strongest metalloprotease characteristics during the screening stage was selected for trace element optimization. The crude enzyme extract was treated separately with 5 ppm Ca^{2+}, Mn^{2+}, and Zn^{2+} solutions and incubated for 24 hours prior to protease activity determination. An enzyme extract without trace element supplementation was used as the control treatment. The effects of these trace elements on metalloprotease production were evaluated based on specific protease activity. Protease activity and protein concentration were determined, and specific protease activity was calculated as the ratio of enzyme activity to protein concentration. The trace element resulting in the highest specific protease activity was considered the most favorable factor for metalloprotease activity. The crude enzyme extract was treated separately with 5 ppm Ca^{2+}, Mn^{2+}, and Zn^{2+} solutions.

Data analysis

Data obtained from metalloprotease screening and trace element optimization were analyzed descriptively and presented in tables and figures. All experiments were performed in duplicate (n= 2), and the results were expressed as the average of two independent measurements. Potential metalloprotease-producing isolates were identified based on their responses to EDTA treatment in well diffusion assays and specific protease activity analyses. The effects of Ca^{2+}, Mn^{2+}, and Zn^{2+} on metalloprotease activity were evaluated by comparing the specific protease activities obtained under each treatment. The trace element producing the highest specific protease activity was considered the optimum trace element for metalloprotease activity.

RESULTS AND DISCUSSION

Metalloprotease Screening by Well Diffusion Assay

The results of metalloprotease screening using the well diffusion assay are presented in Figure 1. The assay was performed by treating crude enzyme extracts with EDTA and observing the formation of clear zones on skim milk agar. The results showed that isolate TUA-104 consistently formed a clear zone around the well after EDTA treatment, whereas isolates TUA-106, TUA-109, and TUA-113 exhibited reduced or no clear zone formation. The persistence of a clear zone indicates that proteolytic activity remained active despite EDTA treatment, while the reduction or absence of a clear zone suggests inhibition of enzymatic activity by the chelating agent.

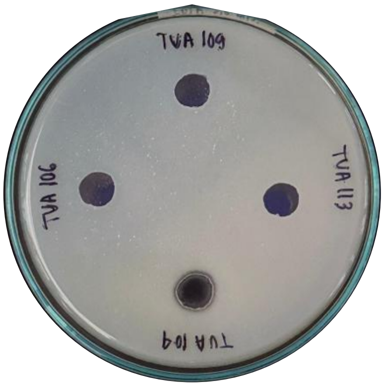

Figure 1. EDTA inhibition assay of thermophilic bacteria isolates

EDTA is widely used as a metalloprotease inhibitor because it can chelate divalent metal ions that are required for enzymatic catalysis (Mushtaq et al., 2024). Metalloproteases depend on metal cofactors, particularly Zn^{2+}, which participate directly in peptide bond hydrolysis and contribute to enzyme stability and catalytic efficiency (Mushtaq et al., 2024). Therefore, the removal of these metal ions by EDTA may reduce or completely inhibit proteolytic activity, making EDTA inhibition assays an effective approach for screening metalloprotease-producing microorganisms (Punnarath et al., 2025). Similar screening approaches have been successfully applied for identifying metalloproteases from thermophilic and environmental bacterial isolates (Sodagar et al., 2024; Afrin et al., 2024).

The persistence of a clear zone in isolate TUA-104 after EDTA treatment suggests that the protease produced by this isolate is less dependent on metal ions for catalytic activity. This response may indicate the presence of non-metalloprotease enzymes or proteases with lower sensitivity to metal chelation (Afrin et al., 2024). Similar observations have been reported in bacterial isolates producing proteases that retained catalytic activity despite EDTA treatment because their catalytic mechanisms were not primarily dependent on metal cofactors (Sodagar et al., 2024).

In contrast, isolates TUA-106, TUA-109, and TUA-113 exhibited inhibition of proteolytic activity after EDTA treatment, as indicated by the reduction or absence of clear zone formation. This result suggests that the proteases produced by these isolates depend on metal ions for catalytic activity and therefore may belong to the metalloprotease group. The inhibitory effect of EDTA on microbial proteases has been widely reported as an important characteristic of metalloproteases because these enzymes require metal ions to maintain catalytic activity and structural stability (Mushtaq et al., 2024; Punnarath et al., 2025).

Similar observations have been reported in several metalloprotease-producing bacteria where EDTA treatment significantly reduced proteolytic activity due to metal ion chelation (Soussou et al., 2023). Han et al., (2023) also reported that EDTA reduced metalloprotease activity by more than 90% through the removal of catalytic metal ions from the enzyme active site. Therefore, based on the well diffusion assay, isolates TUA-106, TUA-109, and TUA-113 were considered potential metalloprotease-producing bacteria and were subsequently evaluated through specific protease activity analysis to further confirm their metalloprotease characteristics.

Metalloprotease Screening Based on Specific Protease Activity

The specific protease activities of thermophilic bacterial isolates following EDTA treatment are presented in Table 1. The results showed that EDTA affected the specific protease activity of all tested isolates, although the degree of inhibition varied among isolates. In the absence of EDTA, isolates TUA-106, TUA-109, and TUA-113 exhibited specific activities of 0.780, 0.830, and 0.820 U mg^{-1} protein, respectively. Following EDTA treatment, isolate TUA-106 showed a gradual decrease in specific activity as EDTA concentration increased, whereas TUA-109 and TUA-113 exhibited fluctuating responses. The trend of specific protease activity in response to increasing EDTA concentrations is presented in Figure 2.

Table 1. Specific protease activity of thermophilic bacterial isolates following treatment with different concentrations of EDTA

EDTA (mM)	Isolate	Specific Activity (U/mg)
0	TUA-106	0.780
	TUA-109	0.830
	TUA-113	0.820
1	TUA-106	0.741
	TUA-109	0.545
	TUA-113	0.684
5	TUA-106	0.632
	TUA-109	0.754
	TUA-113	0.730
10	TUA-106	0.522
	TUA-109	0.588
	TUA-113	0.770

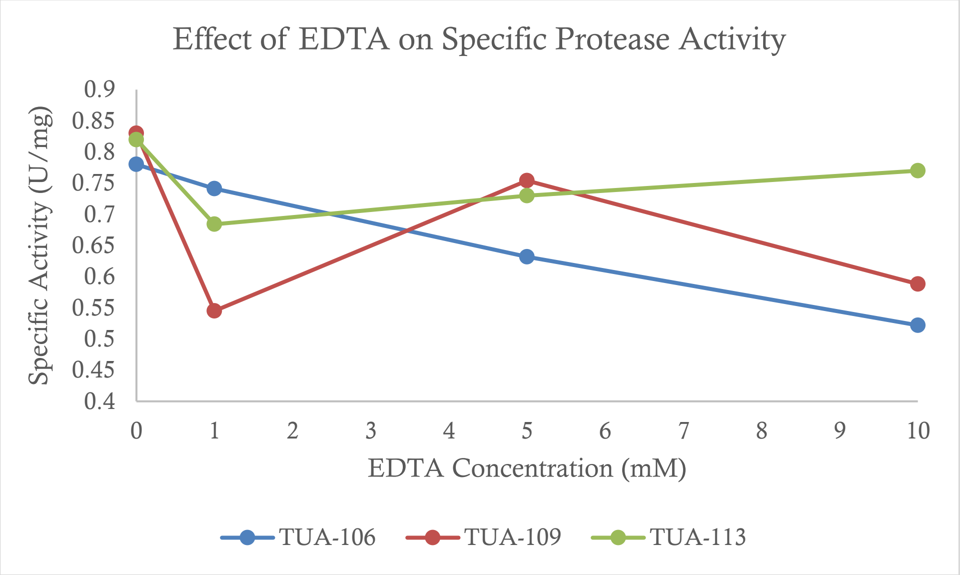

Figure 2. Effect of EDTA concentration on specific protease activity of thermophilic bacterial isolates

The reduction in specific protease activity following EDTA treatment indicates the involvement of metal ions in enzymatic catalysis. EDTA acts as a metal-chelating agent that binds divalent metal ions and removes them from the active site of metalloproteases, thereby reducing enzymatic activity (Mushtaq et al., 2024). Similar inhibitory effects have been reported in thermophilic and environmental bacterial proteases, where EDTA treatment resulted in significant reductions in enzymatic activity due to the sequestration of catalytic metal cofactors (Noskova et al., 2023; Punnarath et al., 2025).

Among the tested isolates, TUA-106 exhibited the most consistent inhibition pattern in response to increasing EDTA concentrations. Its specific protease activity decreased progressively from 0.780 U mg^{-1} protein in the control treatment to 0.741, 0.632, and 0.522 U mg^{-1} protein following treatment with 1, 5, and 10 mM EDTA, respectively. A gradual decline in enzyme activity with increasing concentrations of EDTA is commonly regarded as a characteristic feature of metalloproteases because their catalytic activity depends on the availability of metal ions, particularly Zn^{2+} (Mushtaq et al., 2024). Similar observations were reported by Vinothini et al., (2025), who demonstrated that increasing concentrations of EDTA progressively inhibited bacterial metalloprotease activity through the removal of catalytic metal ions.

In contrast, isolates TUA-109 and TUA-113 exhibited less consistent responses to EDTA treatment. Although both isolates showed reductions in specific activity at certain EDTA concentrations, fluctuations were observed at higher concentrations. Since the enzyme preparations used in this study were not purified, the measured proteolytic activity likely reflected the combined contribution of several protease forms. Consequently, inhibition of one protease component by EDTA may have been partially compensated by the activity of other proteases, resulting in non-uniform responses across EDTA concentrations. Similar variability has been reported in crude protease systems containing multiple proteolytic enzymes with distinct catalytic properties and inhibitor sensitivities (Naveed et al., 2020). Such variations may indicate the presence of multiple protease systems with different sensitivities to EDTA or the coexistence of metalloproteases and non-metalloproteases within the crude enzyme extract (Afrin et al., 2024). Similar differences in inhibitor sensitivity among bacterial proteases have been attributed to variations in enzyme structure, catalytic mechanisms, and cofactor requirements (Kati & Balci, 2023).

Metal ions play important roles not only in enzymatic catalysis but also in maintaining enzyme conformation and structural stability. The removal of these ions by chelating agents such as EDTA may disrupt substrate binding and catalytic efficiency, resulting in reduced proteolytic activity (Han et al., 2023). Based on the inhibition patterns observed in this study, isolate TUA-106 demonstrated the strongest evidence of metalloprotease production among the tested isolates. Therefore, TUA-106 was selected for subsequent trace element optimization to evaluate the effects of different metal ions on metalloprotease activity.

Effect of Trace Elements on Metalloprotease Activity

The effects of different trace elements on the specific metalloprotease activity of isolate TUA-106 are presented in Table 2. The results showed that trace element supplementation affected enzyme activity to different extents. Zn^{2+} produced the highest specific activity (0.284 U/mg), followed by Mn^{2+} (0.263 U/mg), both of which were higher than the control treatment (0.250 U/mg). In contrast, Ca^{2+} supplementation resulted in a substantial decrease in specific activity to 0.036 U/mg. This reduction may indicate that Ca^{2+} was not required as a catalytic cofactor by the protease produced by isolate TUA-106. Excess Ca^{2+} may interfere with enzyme conformation or substrate binding, thereby reducing catalytic efficiency. In addition, metalloproteases exhibit different metal-ion preferences, and the presence of non-essential metal ions may alter the catalytic environment and negatively affect enzyme activity. Similar inhibitory effects have been reported in microbial proteases, where unsuitable metal ions reduced enzymatic activity through conformational changes or disruption of catalytic processes (Han et al., 2023; Mushtaq et al., 2024).

Table 2. Effect of Trace Elements on Specific Metalloprotease Activity

Trace Elements	Specific Activity (U/mg)
Control	0.250
Ca^{2+}	0.036
Mn^{2+}	0.263
Zn^{2+}	0.284

The increased specific activity observed following Zn^{2+} and Mn^{2+} supplementation suggests that these metal ions contribute to maintaining enzyme stability and catalytic activity. Metal ions are known to function as essential cofactors that support protein conformation and enzymatic performance in various microbial proteases (Chandrangsu et al., 2017). In particular, Zn^{2+} plays a crucial role in metalloproteases by participating directly in peptide bond hydrolysis and maintaining the structural integrity of the enzyme active site (Mushtaq et al., 2024; Varghese et al., 2023; Wang et al., 2020). Metalloproteases are generally classified as zinc-dependent proteolytic enzymes because zinc ions function directly in catalytic activity and contribute to enzyme stability (Ojo-Omoniyi et al., 2024). Furthermore, Zn-dependent enzymes utilize zinc ions to stabilize the catalytic center and facilitate substrate hydrolysis (Di Leo et al., 2023). The highest activity observed in the presence of Zn^{2+} therefore indicates that the protease produced by isolate TUA-106 may be a zinc-dependent metalloprotease.

The addition of Mn^{2+} also increased specific activity compared with the control treatment. Manganese has been reported to act as an enzyme activator by improving protein conformation and enhancing catalytic efficiency in several microbial proteases (Martin et al., 2022; Chandrangsu et al., 2017). Similar stimulatory effects have been observed in thermophilic microorganisms, where Mn^{2+} supplementation improved enzyme activity and stability (Sodagar et al., 2024; Saeed et al.,2023). These findings suggest that manganese may support the catalytic performance of the metalloprotease produced by isolate TUA-106.

In contrast, Ca^{2+} supplementation caused a marked reduction in specific activity. Although calcium ions are often associated with enzyme stabilization, their effects depend on enzyme structure and cofactor requirements. Certain metal ions may compete with essential catalytic cofactors or alter enzyme conformation, resulting in reduced enzymatic activity (Sattar et al., 2017; Mushtaq et al., 2024). Therefore, the substantial decrease in activity observed in this study suggests that Ca^{2+} is not a favorable cofactor for the metalloprotease produced by isolate TUA-106.

The reduction in protease activity following EDTA treatment observed in the previous screening assay, together with the increase in activity after Zn^{2+} supplementation, provides strong evidence that the enzyme produced by TUA-106 belongs to the Zn-dependent metalloprotease group. EDTA inhibits metalloproteases by chelating metal ions from the enzyme active site, whereas Zn^{2+} supplementation helps maintain catalytic activity and structural stability (Mushtaq et al., 2024; Di Leo et al., 2023). Therefore, the response pattern observed in this study supports the classification of TUA-106 as a potential Zn-dependent metalloprotease-producing thermophilic bacterium.

CONCLUSION

Thermophilic bacterial isolates TUA-106, TUA-109, and TUA-113 showed potential as metalloprotease producers based on EDTA inhibition and specific protease activity analyses. Among the tested isolates, TUA-106 exhibited the strongest metalloprotease characteristics. Trace element optimization showed that Zn^{2+} produced the highest specific activity (0.284 U/mg), followed by Mn^{2+} (0.263 U/mg), whereas Ca^{2+} decreased enzyme activity. These results indicate that TUA-106 is a potential Zn-dependent metalloprotease-producing thermophilic bacterium from Solok hot springs. This study expands current knowledge of thermophilic metalloprotease-producing bacteria from geothermal environments in West Sumatra and provides a basis for future enzyme purification and molecular characterization. Furthermore, the results highlight the potential of geothermal microbial resources as sources of thermostable enzymes for industrial and agricultural applications.

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