

Original Research Paper

# Optimization and Molecular Identification of Protease-Producing Thermophilic Bacterial Isolate TUA-26

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**Abstract:** Protease enzymes are used as biocatalysts that can accelerate a reaction. Thermostable enzymes produced by thermophilic bacteria are needed in the industrial field because of their ability to withstand high temperatures. The thermophilic bacterial isolate TUA-26 that produces protease was to analyze the optimum conditions of temperature, pH, agitation, trace elements, carbon sources, and nitrogen sources; the stability of protease enzymes; and identify the species based on analysis of 16S rRNA sequences. The research method was conducted experimentally using the Response Surface Methodology (RSM) type of Central Composite Design (CCD) in software statistic design expert 13. The optimum conditions for the thermophilic bacterial isolate TUA-26 to produce protease at 70°C, 8 pH, 150 rpm, Zn trace element, glucose carbon source, and NaNO<sub>3</sub> nitrogen source. Protease activity produced by the thermophilic bacterial isolate TUA-26 increased before and after optimization. The protease enzyme stability test still lasts up to 80% for 7 h from the initial activity. Molecular identification using 16S rRNA of isolate TUA-26 that has similarities with *Brevibacillus borstelensis* strain UTM105. The thermophilic bacterial isolate TUA-26 that produces protease can be improved through optimization by engineering the extrinsic environment and producing thermostable enzymes, which is of great interest for commercial and industrial use in both food and non-food fields.

**Keywords:** Optimization, Protease, Response Surface Methodology, Thermophilic Bacteria, Thermostable Enzyme

## Introduction

Enzymes are catalysts that can accelerate a reaction with extraordinary catalytic power as well as be key to biological processes in a specific macromolecular compound. The most widely used enzyme in biotechnological processes is the protease, which has a specific function in the hydrolysis of proteins (Ibrahim *et al.*, 2021). Protease is one of the largest enzymes with very broad hydrolytic activity and accounts for about 60% of the total enzyme sales in the world, of which 52% are alkaline proteases (Naveed *et al.*, 2021).

The application of protease enzymes is widely used in the fields of food and non-food industries, such as dairy products, pharmaceuticals or medicines, bakery, cosmetics, brewing, detergent industry, paper, pulp, silk, photography, leather processing, skin sampling, textiles, household waste management and industry (Dutta *et al.*, 2016; Si *et al.*, 2018; Adelere and Lateef, 2019; Emran *et al.*, 2020). In

energy, operating costs, facilitate the separation of volatile compounds and be more stable at longer storage times. Thermostable enzymes produced by thermophilic bacteria are needed in the industrial field because they have properties that are stable to high temperatures, so they are perfectly suited for processes in various industrial fields. The use of thermophilic bacteria can reduce the occurrence of contamination from undesirable microorganisms and reduce the cost of cooling the fermentor by reducing water and energy consumption for sterilization (Chen and Jiang, 2018).

The thermophilic bacteria live in extreme areas, optimally growing at temperatures above 45°C and hyperthermophiles grow above 80°C (Aanniz *et al.*, 2015; Kumar *et al.*, 2019). Each has specific properties and different optimal environmental conditions for producing enzymes. The growth of thermophilic bacteria can be enhanced through optimization by engineering its extrinsic environments, such as medium composition,

medium pH, temperature, age and amount of inoculum, production time, agitation, sources of carbon and nitrogen so that the production of enzymes produced is more optimal (Shajahan *et al.*, 2017).

The thermophilic bacterial from the collection of Biotechnology Laboratory that was isolated from Sapan hot spring, South Solok, Indonesia in 2021, namely with the TUA-26 isolate code that has the highest proteolytic index (IP) value of the other five isolates. The collection of thermophilic bacteria isolated from Sapan hot spring has a total of 31 isolates that come from three points, including Sapan Aia Angek, Maluluang, and Balun with each isolate having a total of 15, 10, and 6 isolates. The conditions of Sapan hot spring are that has a temperature range of 50-83<sup>o</sup>C and 7.8-8.3 pH. Sapan hot springs are classified as alkaliphilic, so that has the potential to have more diverse bacteria than other hot springs and the ability to produce enzymes. Optimization used Response Surface Methodology (RSM) and molecular identification with 16S rRNA sequence analysis to introduce the potential and diversity of thermophilic bacteria from hot springs in the region of Indonesia that can be exploited in the field of industry. The response surface method could be employed in modeling the factors and response to predict and optimize the parameters affecting the response. Recently, RSM has been often applied to the optimization parameters of enzyme purification (Alhelli *et al.*, 2016). This research can obtain protease-producing thermophilic bacterial isolate as an alternative in overcoming the problem of the number of protease stocks that can be used in various fields of industrial applications. Therefore, research was conducted on the optimization and molecular identification of thermophilic bacterial isolates TUA-26 that produce proteases.

## Materials and Methods

### *Bacterial Rejuvenation*

The rejuvenation of thermophilic bacterial isolate TUA-26 collector protease producer from the Biotechnology Laboratory, Universitas Andalas is carried out by scratching the bacteria isolate on the reaction tube containing the medium Nutrient Agar (NA).

### *Growth Profile of Thermophilic Bacterial Isolate*

Inoculated isolates with loop needle oblique cultures on a 100 mL production medium. Then incubated at 50<sup>o</sup>C, and agitated at 150 rpm for 24 h. After that, 5 mL of inoculum is piped into 95 mL of production medium. 3 mL of bacterial culture was sampled (1.5 mL for turbidity measurement and 1.5 mL for enzyme activity testing), with turbidity measurement using a spectrophotometer at a wavelength of 600 nm ( $t_0$ ). Bacterial culture footage was carried out at intervals of 1 h and stopped after a decrease in the growth of bacterial isolates (Agustien *et al.*, 2015).

### *Protease Assay*

Protease activity was determined by a modification of the method of Takami *et al.* (1989), starting with making a 1% casein substrate, the substrate solution is piped into 0.5 mL, then combined with up to 0.5 mL of the enzyme solution, 0.25 Tris-HCl buffer at 50 mm pH 8.0 and incubated at 60<sup>o</sup>C for 15 min. After that, 0.5 mL of TCA is added and incubated at room temperature for 20 min. Centrifuged at 6000 rpm for 20 min, the supernatant was taken to 0.375 mL and transferred into a test tube, adding 1.25 mL Na<sub>2</sub>CO<sub>3</sub> and 0.25 mL 1N Folin-Ciocalteu's. Optical Density (OD) readings are performed at a wavelength of 578 nm. The determination of the value of blanks is carried out in the same way and 0.5 mL of enzyme is replaced with 0.5 mL of distilled water. The determination of standard values is also carried out using 0.5 mL protease samples replaced with 0.5 mL of 5 mm tyrosine. One Unit (U) of enzyme activity is the amount of enzyme required to produce 1 mole of tyrosine per minute under test conditions.

### *Determination of Optimal Conditions for Protease Production*

Determination of optimum conditions including temperature, pH, agitation, trace element, carbon sources, and nitrogen sources. The optimization of temperature, pH, and agitation for protease production was carried out by Response Surface Methodology (RSM) using a Central Composite Design (CCD) type experimental design at statistical software Design Expert 13, State-Ease, Inc., Minneapolis, USA. Variations in treatment factors at temperature (A) are 60, 70, and 80<sup>o</sup>C; pH (B) is 7, 8, and 9; and agitation (C) is 100 rpm, 150 rpm, and 200 rpm was determined to approach One-Variable-at-a-Time (OVAT) to find out the center point to be optimized. Analysis of Variance (ANOVA) was used to examine the model's significance and suitability. The response results are presented in the form of a three-dimensional graph and statistical analysis. Further, the determination of trace elements is Ca, Zn, and Mn; variations of carbon sources are citric acid, glucose, lactose, and maltose; nitrogen sources are KNO<sub>3</sub>, NaNO<sub>3</sub>, NH<sub>4</sub>Cl, and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> with a concentration of 1% each. Each 5 mL of inoculum is piped into the protease production medium based on variations and then the production medium is extracted over a certain period (idiophase). The production medium is centrifuged at 6000 rpm for 20 min so that an enzyme solution is obtained and enzyme activity is determined.

### *Stability Enzyme Assay*

The enzymes obtained at optimum conditions are transferred to Erlenmeyer 100 mL, then heated in a beaker filled with water to a temperature of 60<sup>o</sup>C, and the Erlenmeyer-containing enzymes into the beaker. A

sampling of enzyme solution at 1 h intervals until protease activity drops, then a test of protease enzyme activity (Agustien *et al.*, 2015).

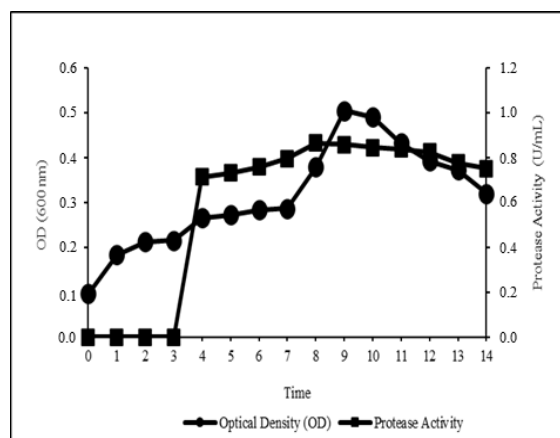
### Molecular Identification

Identification was carried out on isolates of protease-producing TUA-26 thermophilic bacteria by the 16S rRNA method using universal primers. DNA isolation was performed using Kit GeneJET Genomic DNA Purification Kit-ThermoFisher, and DNA amplification was performed using a primer 16S rRNA-27F (*forward*) ((5' AGA GTT TGA TCM TGG CTC AG 3') and primer 16S rRNA-1525R (*reverse*) (5' AAG GAG GTG WTC CAR CC 3'). PCR fragments were analyzed using an automated DNA sequencer. The PCR was then programmed to run at pre-denatured conditions 98°C for 5 min, denatured 98°C for 30 sec, annealing 50 °C for 30 sec, primer elongation or extension 72°C for 90 sec, carried out for 30 cycles, and post-PCR 72 °C for 5 min. The PCR product was sequenced commercially at 1<sup>st</sup> Base Sequencing Services Malaysia to determine the DNA base order. The results of the sequence were confirmed on GenBank. DNA sequences were aligned using the "Clustal X" and "NJ tree" programs using bootstrap with 1000x for phylogenetic trees (Tamura *et al.*, 2013).

## Results

### Growth Profile and Protease Activity

The growth profile and protease activity of the TUA-26 thermophilic bacterial isolate were incubated at 50 °C and agitated at 150 rpm (Fig. 1). Bacterial growth undergoes several phases, namely the lag phase (adaptation), exponential phase (log), stationary phase, and death phase. The growth profile of isolate TUA-26 occurred for 14 h with a very short lag (adaptation) phase, namely at 0-1 h. The exponential phase (log) occurs from the 1<sup>st</sup> h until the 9<sup>th</sup> h.



**Fig. 1:** Growth profile and protease activity of TUA-26 thermophilic bacterial isolate

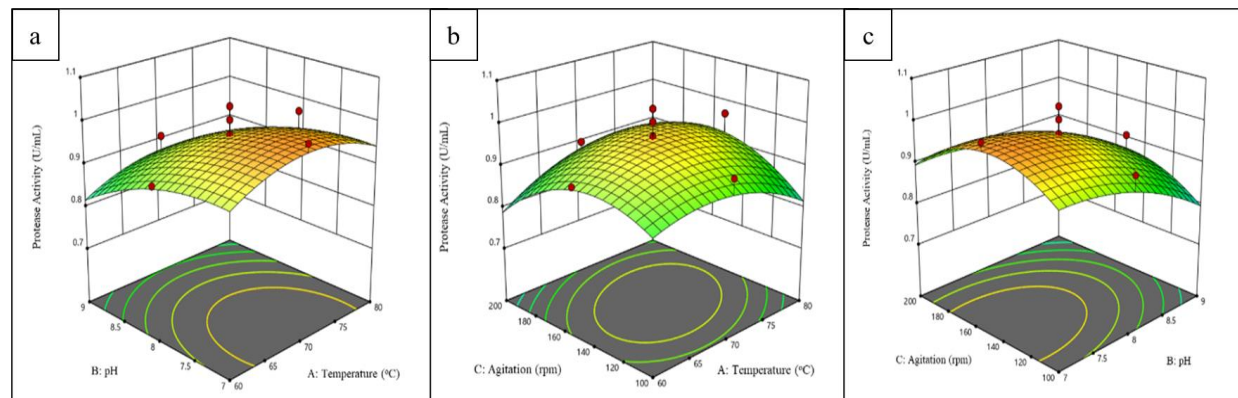
### Optimum Conditions for Protease Production

The determination of optimum conditions for protease production includes temperature, pH, agitation, trace elements, carbon sources, and nitrogen sources. Optimization of temperature, pH, and agitation was carried out with Response Surface Methodology (RSM), a Central Composite Design (CCD) type experimental design on statistical design expert 13 Software. A combination of temperature (A), pH (B), and agitation (C) obtained 20 treatments with 6 repeat center points (70°C, 8 pH, 150 rpm) (Table 1).

Response surface plots in Fig. 2. show three-dimensional shapes characterized by the presence of color differences. The optimum point on the response surface plots of three-dimensional shapes is at the highest point marked with the red area. The highest protease activity was at 70°C, 8 pH, and agitation of 150 rpm with an activity value of 1.035 U/mL. The determination coefficient (R<sup>2</sup>) and the adjusted determination coefficient (Adj-R<sup>2</sup>) were 0.4836 and 0.6811. The F-value and p-value of the model were obtained as 5.51 and 0.0067 (Table 2). The ANOVA F-value and p-values for the selected parameters demonstrate the efficacy and precision of the model.

**Table 1:** The results of temperature, pH, and agitation optimization for protease production using Central Composite Design (CCD)

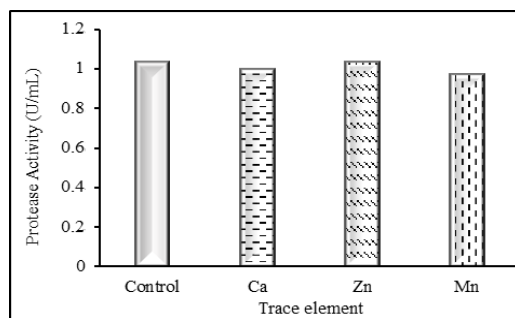
Run	Temperature (°C)	pH	Agitation (rpm)	Protease Activity (U/mL)
1	80	9	200	0.765
2	70	8	100	0.936
3	80	7	200	0.890
4	60	7	100	0.917
5	70	9	150	0.908
6	70	8	150	0.888
7	70	8	150	0.935
8	80	7	100	0.821
9	80	9	100	0.709
10	70	8	200	0.898
11	60	9	100	0.756
12	70	8	150	1.035
13	70	7	150	1.010
14	70	8	150	0.970
15	60	7	200	0.792
16	60	9	200	0.714
17	70	8	150	0.871
18	80	8	150	0.969
19	70	8	150	1.004
20	60	8	150	0.917



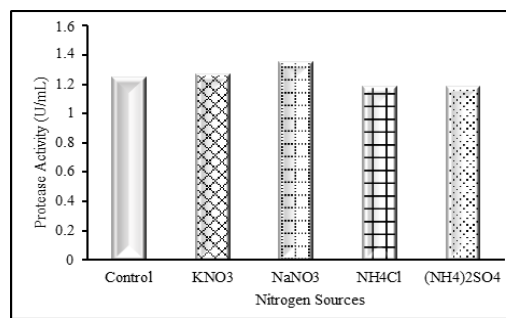
**Fig. 2:** Response surface plots three-dimensional shape (3D) protease activity (U/mL) of TUA-26 thermophilic bacterial isolate to optimization of the variable factors; (a) temperature (°C) and pH; (b) temperature (°C) and agitation (rpm); (c) pH and agitation (rpm)

**Table 2:** ANOVA for the CCD of the selected production parameters

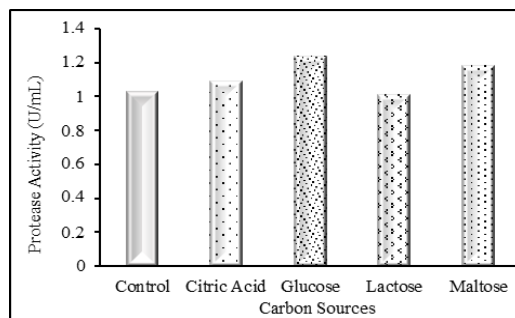
Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1487	9	0.0165	5.5100	0.0067	Significant
A-temperature	0.0003	1	0.0003	0.1122	0.7446	
B-pH	0.0334	1	0.0334	11.1400	0.0075	
C-agitation	0.0006	1	0.0006	0.2134	0.6540	
AB	5.000E07	1	5.000E07	0.0002	0.9900	
AC	0.0107	1	0.0107	3.5500	0.0888	
BC	0.0006	1	0.0006	0.2042	0.6610	
A <sup>2</sup>	0.0074	1	0.0074	2.4800	0.1461	
B <sup>2</sup>	0.0036	1	0.0036	1.1900	0.3006	
C <sup>2</sup>	0.0168	1	0.0168	5.5900	0.0397	
Residual	0.0300	10	0.0030			
Lack of fit	0.0091	5	0.0018	0.4383	0.8068	not significant
Pure error	0.0208	5	0.0042			
Cor total	0.1786	19				



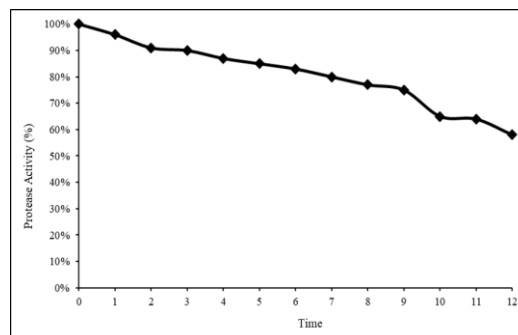
**Fig. 3:** Protease activity of TUA-26 thermophilic bacterial isolate against trace elements optimization



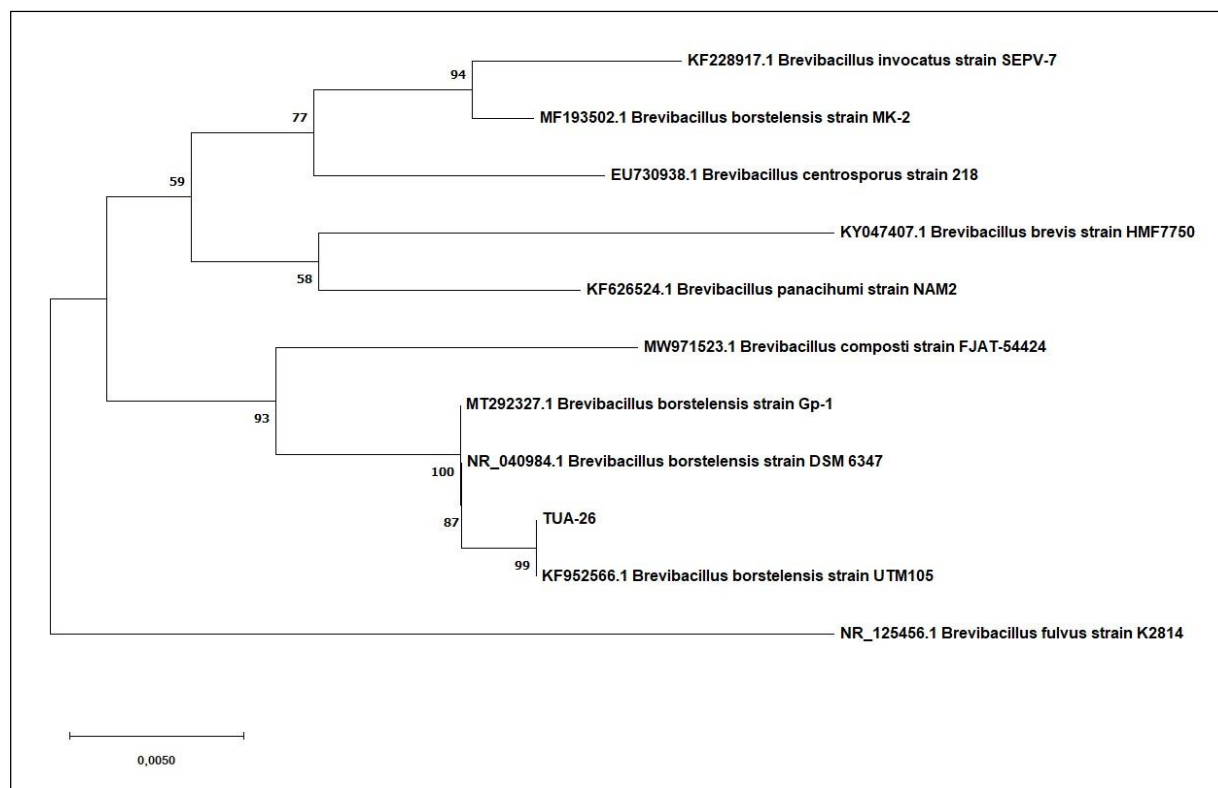
**Fig. 5:** Protease activity of TUA-26 thermophilic bacterial isolate against nitrogen sources optimization



**Fig. 4:** Protease activity of TUA-26 thermophilic bacterial isolate against carbon sources optimization



**Fig. 6:** Protease stability test of TUA-26 thermophilic bacterial isolate



**Fig. 7:** Phylogenetic tree of TUA-26 isolate based on 16S rRNA with Neighbor Joining (NJ) analysis. The phylogram using bootstrap percentages of 1000 replicates and nucleotide positions are substituted by 0.005 in the bar

The effect of giving trace elements for protease production has the highest protease activity, namely Zn with a value of 1.033 U / mL (Fig. 3). The carbon source applied to isolates of TUA-26 thermophilic bacteria resulted in varying values of protease activity. The carbon source that has the highest protease activity value is glucose with an activity value of 1.239 U/mL (Fig. 4). The nitrogen source that has the highest activity value is NaNO<sub>3</sub> (sodium nitrate) with an activity value of 1.347 U/mL (Fig. 5).

#### Protease Stability Test

Proteases produced by isolates of TUA-26 thermophilic bacteria have stable enzyme catalytic power even in hot environmental conditions. The enzyme stability test of isolate TUA-26 showed that protease stability at the 7<sup>th</sup> h began to decrease to 80% from the initial activity to the 12<sup>th</sup> h, which is 58% of the initial activity (Fig. 6).

#### Identification Based on 16S rRNA Gene Sequence Analysis

Amplification of isolates of protease-producing TUA-26 thermophilic bacteria was carried out using a universal primer based on 16S rRNA sequence analysis. From the

results of PCR products of 1500 bp, the same results were obtained as the size of the PCR product according to the primary size used. The BLAST nucleotide sequence of TUA-26 thermophilic bacteria isolates with 15 isolates contained in the gene bank has a similarity value of 96.68% - 100% with an E-value of 0.0 which shows more significant alignment with BLAST results. The base composition of the 16S rRNA gene isolates of protease-producing TUA-26 thermophilic bacteria with BLAST bacteria is the average percentage of T(U) = 19.3%; C = 24.7%; A = 24.3% and G = 31.7%. Phylogenetic analysis was performed with Neighbor-Joining (NJ) on MEGA (Molecular Evolutionary Genetic Analysis) software version 10.2.2. Isolates of thermophilic bacteria TUA-26 have a very close kinship with isolates of *Brevibacillus borstelensis* strain UTM105 with a similarity value of 99.93% (Fig. 7).

#### Discussion

Bacterial cells are very active in dividing and cell metabolism takes place quickly because the nutrients in the medium are well fulfilled so that bacteria can grow optimally. The stationary phase of the TUA-26 isolate was not detected because it proceeded very quickly or had a very short time. The death phase starts in the 10<sup>th</sup> h,

because new cells form more slowly than dead cells. The highest protease activity was at the 8<sup>th</sup> h with a value of 0.865 U/mL, that produced at harvest time showing that the bacteria can produce stable enzymes (Irdawati *et al.*, 2018). Shaikh *et al.* (2023), revealed the highest protease activity value of 0.92 U/mL from the isolate of *Bacillus nakamurai* strain PL4.

Optimal conditions can be determined if it produces high enzyme activity at harvest time. After reaching optimal conditions, enzyme activity will decrease or decrease the value of protease enzyme activity. The thermophilic bacteria evolved outstanding genetic and physiological mechanisms that allowed them to adjust their systems, particularly their enzymatic activity, to survive in harsh environments (Mohammad *et al.*, 2017). Enzymes produced at optimum conditions (pH, temperature, and agitation) are resistant, stable, and avoid denaturation (Atalah *et al.*, 2019).

Response Surface Methodology (RSM) is described visually through response surface plots and plot contours to determine the shape of the relationship between responses and their independent variables. This method is frequently used to optimize the production of proteins (Behravan and Hashemi, 2021). In this experiment (Fig. 2), the protease activity variable (y) was influenced by three independent variables, namely temperature ( $x_1$ ), pH ( $x_2$ ), and agitation ( $x_3$ ). The values of independent variables ( $x_1$ ,  $x_2$ , and  $x_3$ ) that have been obtained using the right model formulation cause protease activity values to be optimal. Model selection analysis is carried out based on the sum of squares of the model sequence (Sequential Model Sum of Squares), testing model inaccuracy (Lack of Fit/ LOF), and statistical model summary (Model Summary Statistics). The analysis of variance in the p-value model of TUA-26 isolates is less than 0.0500, so it is significant for response (protease activity) by the CCD (Alshammari *et al.*, 2015). Lack of Fit (LOF) is insignificant, it indicates that its terms and degree of accuracy in predicting the data were suitable (Prajapati *et al.*, 2021). Experimental design types used by Central Composite Design (CCD), this design has also been considered economic because of the minimal experimental runs and estimates of the efficiency of the independent parameters and their interactions (Ahmad *et al.*, 2015). Sharif *et al.* (2023), also optimized thermophilic bacterial isolates to produce enzymes using Response Surface Methodology (RSM) type CCD from Tatta Pani hot spring, Pakistan.

Trace elements can be activators and inhibitors of enzyme activity. An enzyme requires an activator in a catalytic reaction to increase the speed of the enzymatic reaction, but in this study by giving trace elements in the form of Ca, Zn, and Mn in isolates of TUA-26 thermophilic bacteria for protease production tends to reduce the value of protease activity produced. Structural changes in the

enzyme can impair its ability to connect with the substrate, so enzyme activity decreases (Azar *et al.*, 2020). Similar findings were revealed by Baykara *et al.* (2021), that trace elements Ca and Zn can increase protease activity from isolates of *Geobacillus* sp. strain GS53.

The carbon and nitrogen source that has the highest protease activity value is glucose and NaNO<sub>3</sub>. Glucose can produce enough energy to trigger the expression of genes involved in the processes of biosynthesis and enzyme secretion in increasing protease activity. Glucose and maltose are relatively good sources of carbon to increase protease activity (Jingying and Yan, 2023). Sodium nitrate (NaNO<sub>3</sub>) provides a suitable source of nitrogen for bacterial growth and protein synthesis. Nitrogen is an important component in protein structure, so it is indispensable in increasing protease production. The source of nitrogen is necessary for cell growth, while the element carbon is used to increase biosynthetic energy. The source of carbon and nitrogen becomes organic compounds, which are very important for the development of thermophilic bacteria in producing metabolites (Talhi *et al.*, 2022). Sudha *et al.* (2018), reported the carbon source that has the highest activity value is glucose, while ammonium chloride has the lowest protease activity value compared to other types of nitrogen sources from *Exiguobacterium profundum* sp. MM1.

Thermostable enzymes have a molecular structure composed of thermostable protein molecules in cells and the association of enzyme protein compounds with other molecules such as lipids, polysaccharides and other proteins causes the formation of a compound that has a mechanism that allows it to remain stable when facing conditions that can activate it. Factors that cause enzymes to be thermostable are increased hydrogen bonds and salt bridges in proteins from enzymes, the amino acid composition of enzyme proteins has decreased the amount of cysteine and serine while the amount of arginine and tyrosine increased markedly and the amount of amino acid proline is less found in the  $\alpha$ -helical structure of proteins. Heat treatment makes it simple to purify thermostable enzymes (Sana, 2015). The advantages of thermostable enzymes include their durability, enhanced resistance to organic solvents, decreased danger of microbial contamination, reduced activity losses during processing even at high temperatures, and a potential advantage in pre-treatments of ionizable material (Yadav *et al.*, 2018).

The increase in activity indicates that the production of enzymes by bacteria is influenced by several factors such as temperature, pH, agitation, trace elements, carbon sources, and nitrogen sources. Thermostable enzymes can maintain most of the activity produced by bacteria even under high-temperature conditions. In general, enzymes with an activity percentage of 50% or more after heating to elevated temperatures can be

considered thermostable. Temperature has a significant impact on both the concentration of the intended end product and the growth that an organism experiences (Farinas, 2015). Protease activity in a wide temperature range is characteristic in detergent formulation applications (Hammami *et al.*, 2017).

Isolate TUA-26 were identified as *Brevibacillus borstelensis*. This is also supported by p-distance data between TUA-26 isolates and *Brevibacillus borstelensis*. *P-distance* in the gene bank is 0.000 which means there is no evolutionary distance between the two. Neighbor-Joining (NJ) analysis is the basis for making a phylogenetic tree based on the difference between two sequences where a phylogenetic tree with a high bootstrap value of at least 70% is a good phylogenetic tree. Similar, sequencing analysis of PCR fragments from thermophilic bacterial isolates from the Kharga Oasis hot springs in New Velly, Egypt, showed that ten isolates had similarities with *Brevibacillus borstelensis* MA-49 (Kortam *et al.*, 2023), *B. marinus* strain SCSIO 07484T (Wang *et al.*, 2021), *B. composti* sp. nov. strain FJAT-54423T (Tang *et al.*, 2021). Thermophile bacteria *B. borstelensis* 707 have degradation of polyethylene (Hadad *et al.*, 2005).

## Conclusion

Optimization of enzyme production against isolates of TUA-26 thermophilic bacteria has an effect on increasing protease enzyme activity. The optimum conditions for protease enzyme production are temperature 70 °C, 8 pH, 150 rpm by Response Surface Methodology, Zn trace element, glucose carbon source, and NaNO<sub>3</sub> nitrogen source. The stability of the enzyme isolate of protease-producing TUA-26 thermophilic bacteria is thermostable and still lasts 80% for 7 h. Molecular-based analysis of isolate TUA-26 has similarities with *Brevibacillus borstelensis* strain UTM105. The thermostability of protease enzymes derived from thermophilic bacteria makes them potential and valuable for various industrial applications. Accordingly, it is necessary to carry out further research on genetic engineering for the large-scale production of protease enzymes and their applications.

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## Author's Contributions

**Anthoni Agustien:** Conceived the original idea, wrote the manuscript, designed the study, reviewed, and approved the manuscript.

**Mufidhatul Muqarramah:** Materials and equipment engagement, literature search.

**Feskaharny Alamsjah:** Designed research methodology and analyzed.

## Ethics

This article is completely original and has never been published previously viewed material. The correspondent author certifies that all other authors have read and accepted such and such work there is no ethical contradiction.

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