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# Purification and characterization of thermostable serine alkaline protease from *Geobacillus* sp. DS3 isolated from Sikidang crater, Dieng plateau, Central Java, Indonesia

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**ABSTRACT** Thermostable proteases that optimally withstand the high-temperature conditions of thermophilic bacteria could be produced and purified, which would be highly beneficial for use in industry. *Geobacillus* sp. is a thermophilic bacterium that can be found in various environmental conditions. The goal of this study was to isolate and characterize thermostable serine protease that had been produced by thermophilic *Geobacillus* sp. strain DS3. The proteolytic index was measured in a solid medium. The expression of protease was optimized by *Geobacillus* sp. DS3 at 50 °C for 18 h. Targeted protease was purified using ammonium sulfate (40-60%) and DEAE Sephadex A-25 resin. Using SDS-PAGE, the molecular weight of the enzyme was predicted to be around 32 kDa. Purified thermostable protease was highly activated at 70 °C, pH 9.6 stable for 1 h, and inhibited by PMSF. Therefore, this enzyme is classified as a thermostable alkaline serine protease. Its kinetic study revealed specific activity of 0.41 U/mg ($V_{max}$) and 0.25 mg/mL ($K_M$). Overall, a thermostable alkaline serine protease from *Geobacillus* sp. DS3 showed high activity at high temperatures and alkaline pH, which is vital for application in industries such as leather processing and detergent formulation.

**KEYWORDS** Thermostable serine alkaline protease; *Geobacillus* sp. DS3; enzyme purification, DEAE Sephadex A-25

## 1. Introduction

Proteases, proteinases, or peptidases are several kinds of enzymes that have their specific function of degrading the protein molecules into short-chain peptides and amino acids (Sharma et al. 2017). Currently, proteases are reported as one of the most widely used for the industry as well as industrial enzymes. The sales of industrial enzymes are around $4.2 billion in value worldwide (Singh et al. 2016; Suberu et al. 2019b), since the proteases play an important role as one of the largest groups of industrial enzymes. Proteases produced by microorganisms have become a massive group of industrial enzymes, about more than 60% of the total global sale of enzymes (Souza et al. 2015). Hence, it has been utilized in several industrial applications and analytical processes, such as the production of leather, pharmaceuticals, protein processing and analysis, foods biotechnology, cosmetic preparations, cleaning processes, diagnostic reagents, and peptide synthesis industries (Souza et al. 2015; Tavano et al. 2018; Zhou et al. 2018).

Thermostable enzymes or thermozymes are commonly synthesized from thermophilic microorganisms at the thermophile condition, which optimal growth temperature of more than 60 ºC and the hyperthermophiles with optimal growth temperatures of more than 80 ºC (Stetter 2006). Thermozymes are utilized for the enzymatic process, which is conducted in high-temperature conditions. Generally, thermostable proteases are defined as proteases that optimally withstand high-temperature conditions and can exist with high catalytic efficiencies and provide resistance from mesophilic microbial contamination (Hussein et al. 2015).

The number of research involved in producing the thermostable enzyme from thermophilic bacteria is still limited in Indonesia. On the other hand, there are potential geothermal sites since Indonesia is located in the ring of fire (volcano line) (Pambudi 2018), including crater Sikidang, Dieng which has the potential biodiversity of thermophilic bacteria. In the previous study, it has been reported that the thermophilic bacterium *Geobacillus* sp. strain DS3 was isolated from Sikidang Crater, Dieng Plateau, Central Java (Witasari et al. 2010). Therefore, the potential protease from this isolated bacteria should be determined and characterised.

The purification of protease has a significant role since the purified enzyme will be utilized to determine the bio-

chemical function, such as the activity and its effect factors, including pH, temperature, activator, and inhibitor, to understand the character of the protease group. Based on the characteristic of active sites and mode of catalytic action, the proteases were grouped into aspartyl or carboxyl protease, cysteine or thiol protease, serine protease, and metalloproteases. Furthermore, regarding pH preferences, proteases could be subdivided into acidic, alkaline, and neutral proteases (Rawlings et al. 2018). Indeed, protease purification methods must be optimized in various strategies based on different purposes. However, the early purification methods could be started from ammonium sulfate precipitation and column chromatography (such as gel filtration and ion exchange).

In this present study, the purification and characterization of protease are conducted to determine thermostable protease produced by *Geobacillus* sp. strain DS3 from Sikidang crater, Dieng plateau, Central Java, Indonesia.

## 2. Materials and Methods

### 2.1. Preliminary protease assay using solid medium

The main purpose of this step was to measure the proteolytic index. Expression of protease in *Geobacillus* sp. DS3 was determined using solid medium of Minimal Synthetic Medium (MSM) (Zilda et al. 2013). The medium contained 0.1% (w/v) NaCl, 0.1% (w/v) $K_2HPO_4$, 0.01% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) yeast extract and 2% (w/v) bacteriological agar was prepared and supplemented with 1% (w/v) skim milk. The incubation was carried out at 30, 40, 50, and 60 ºC for 24 h. The clear zone formed around the colony indicated the ability of the bacterium to produce protease and was designated as the Proteolytic Index (PI). The Proteolytic Index was determined by measuring the diameter of the clear zone around the colony compared to the diameter of the colony.

The MSM medium without agar was used to optimize the expression of protease. Initially, the culture was incubated for 24 h at 50 ºC for seed culture, then 0.5 mL of seed culture was sub-cultured into 5 mL liquid MSM medium. The growth culture was observed at various times of 0, 3, 6, 9, 15, 18, 21, and 24 h. In addition, the temperature optimum was conducted at 30, 40, 50, 60, 70, and 80 ºC.

### 2.2. Protease activity assay

Protease activity was performed using casein as a substrate by the modified Folin and Ciocalteu's method. Two hundred microliter of the crude enzyme was mixed with 500 µL of 0.2 M Glycine-NaOH buffer (pH 10.0) containing 1% (w/v) casein and incubated at 50 ºC for 10 min. The enzyme reaction was stopped by adding 2 mL of 10% (w/v) trichloroacetic acid. The mixture was incubated at room temperature for 15 min, followed by centrifugation at 3046 g at 20 ºC for 15 min. One milliliter of supernatant was mixed with 2.5 mL of 0.5 M $Na_2CO_3$ and then 500 µL of 20% (v/v) 2 N Folin & Ciocalteu's reagent was added. The mixture was incubated at 40 ºC for 10 min then the absorbance of the mixture was measured by GENESYS™ 150 Vis/UV-Vis Spectrophotometer at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the standard assay conditions.

### 2.3. Protein content

The protein concentration was determined using the Bradford methods. A series of BSA standard solutions (0.25 -1.4 mg/mL) was prepared for a standard curve. The assay was performed by pipetting a 10 µl sample and adding 1 mL of Bradford reagent. The mixture was incubated in a dark place for 5 min. The absorbance was read at 595 nm by GENESYS™ 150 Vis/UV-Vis Spectrophotometer.

### 2.4. Purification of protease

The purification of protease was conducted by two steps purification systems; using ammonium sulfate precipitation and DEAE-Sephadex resin. The incubated culture at 50 ºC for 18 h was centrifuged at 3046 g. The supernatant was extracted with ammonium sulfate at various saturation as follows 0-20%, 20-40%, 40-60%, and 60-80%. Each extract was separated by centrifuge at 4 ºC with 3046 g for 20 min. The obtained pellet was suspended in 0.2 M glycine-NaOH buffer pH 10 and dialyzed against the same buffer overnight. After dialysis, the solution was added 85% glycerol with a ratio 1:1 to inhibit protein aggregation and stored at -20 ºC. The protease activity and protein concentration were determined.

The protein fraction of ammonium sulfate (40-60%) was then subjected to further purification using DEAE-Sephadex A-25 prepacked column (15 cm × 10 cm). First, 0.5 mL dialysate was loaded into the prepared column that was equilibrated with 0.5 M Glycine-NaOH buffer pH 10.0 (Buffer A). The unbound proteins were eluted by buffer A. Five milliliters of unbound fractions were collected for 10 fractions. The bound proteins were eluted with 0.5 M NaCl dissolved in the same buffer. The 1.5 mL of bound fractions were collected and detected its protein content at a wavelength of 280 nm. The column was washed by the same buffer until the absorbance of the fraction at 280 nm reached zero.

### 2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) assay

The molecular weights of purified protease were determined using SDS-PAGE method. The gel consisted of 7% (w/v) and 12% (w/v) of stacking and separating gels, respectively. Samples were prepared by mixing purified enzymes in distilled water containing loading dye (Novagen). The prepared sample was incubated at 95 ºC for 5 min and then centrifuged at 1820 g for 5 s. Each sample and the protein marker (Spectra Multicolor Broad Range Protein Ladder, Thermoscientific) were loaded into the wells. Electrophoresis was performed at 120 V for 120 min. Protein bands were visualised by overnight staining with Coomassie dye. Protein bands were observed after

de-staining the gel the following day using the de-staining solution.

### 2.6. The pH optimum, temperature optimum, and stability of enzyme

The pH optimum was determined using ammonium sulfate (40-60%) extract containing 0.716 mg/mL of protein concentration. The optimal pH value of enzyme activity was performed from 5.8 – 10.6 at 50 ºC, 1h. The standard buffers were used, including 0.2 M phosphate buffer (pH 5.8 – 7.2), 50 mM Tris-HCl buffer (pH 7.0 – 9.0), and 50 mM Glycine-NaOH buffer (pH 8.6 – 10.6). Protease activity assay were determined as described earlier.

The 40-60% ammonium sulfate was precipitated in protein solution with a concentration of 0.716 mg/mL for the temperature and stability assays. The effect of temperature on protease activity was conducted at optimum pH obtained. The range of temperatures was performed from 30, 40, 50, 60, 70, 75, and 80 ºC. The enzyme stability was measured by incubating the enzyme at 70 ºC for 3 h. Every hour, 50 µL of the enzyme was mixed with 500 µL of 50 mM Glycine-NaOH buffer pH 9.6 containing 1% casein, and after 10 min the reaction was terminated by adding trichloroacetic acid (Rai and Mukherjee 2009). Protease activity was measured as described earlier.

Inhibitors of 1 mM phenylmethylsulphonyl fluoride (PMSF) for serine protease, 1 mM 2-mercaptoethanol (2-ME) for cysteine protease, and 1 mM ethylenediaminetetraacetic acid (EDTA) for metalloprotease were prepared. The ammonium sulfate extract (40-60%) with a protein concentration of 0.716 mg/mL was pre-mixed with the inhibitors and incubated at RT for 30 min. The residual activity was measured using the protease activity assay. The result was expressed as the percentage of the protease activity measured without an inhibitor (control).

### 2.7. Enzyme kinetic assay

The kinetic assay was conducted using pure enzyme after DEAE-Sephadex A-25 purification system. Various concentrations of casein (0.0025 – 0.02 mg/mL) were used as substrate. Protease kinetic assay was performed at 50 ºC for 3 min. The kinetic rate constants, $K_M$ and $V_{max}$ were determined using the Lineweaver-Burke equation.

### 2.8. Statistical analysis

The proteolytic index, time, and temperature optimum for protease expression were statistically analyzed using SPSS (version 23) IBM one-way ANOVA with a probability level of $p < 0.05$. The other results are mostly represented as mean ±SD of at least three experiments.

## 3. Results and Discussion

### 3.1. Preliminary protease assay using solid medium

To identify the protease expression in *Geobacillus* sp., the preliminary assay using MSM agar containing 1% skim milk at various temperatures was conducted. The clear zone surrounding *Geobacillus* sp. DS3 colonies after 24 h incubation indicated the protease activity was the highest, followed by a higher proteolytic index. Incubation at 50 ºC showed the highest proteolytic index (Table 1). Unfortunately, the colonies and clear zone did not appear in higher temperature incubation at 60 ºC . In a previous study, it was reported that 6 isolates BII-1, BII-2, and BII-6 (*Bacillus licheniformis*); BII-3 and BII-4 (*Bacillus subtilis*); and LII (*Brevibacillus thermoruber*) isolated from Indonesian hot spring formed clear zone on MSM plate agar after incubating at 55 ºC for 30 h (Zilda et al. 2013). Thus, the preliminary result revealed that *Geobacillus* sp. DS3 from Sikidang crater, Central Java has the highest protease activity at 50 ºC in solid MSM medium.

### 3.2. Optimizing the expression of protease

We performed incubation at various times and temperatures to determine optimum conditions for protease expression in *Geobacillus* sp. DS3. The protease activity substantially increased from 0 h until 18 h and then gradually decreased after 18 h incubation (Figure 1a). The highest protease activity was observed at 18 h incubation. Therefore, it was selected for further protease expression conditions in *Geobacillus* sp. DS3 at a different level of temperature conditions. The protease activity was dramatically raised from 40 ºC and suddenly dropped after 50 ºC. Thus, *Geobacillus* sp. DS3 produced protease with the highest enzyme activity at 50 ºC for 18 h of incubation. Protease produced from isolate LII of *Brevibacillus thermoruber*, from Padang Cermin, Lampung, Indonesia, expresses the highest activity at 50 ºC for 22 h incubation (Zilda et al. 2013).

### 3.3. Purification of protease

The highest protease activity at 40-60% saturation in ammonium sulfate purification system. Therefore, this ammonium sulfate extract was utilized for the following purification step using DEAE-Sephadex A-25. The bound fraction of DEAE-Sephadex A-25 purification showed two protein peaks at fractions 19 and 26. Protease activity and protein content of several peaks were measured, but only the highest peak of fraction 19 provided detectable protease activity (Table 2).

The SDS-PAGE assay of ammonium sulfate extract (40-60%) in lane 3 contained several slightly visible bands around 30, 32, and 60 kDa. Lane 5 (ammonium sulfate extract (0-20%) showed a thick band at 30 kDa but did not exhibit any protease activity. Unfortunately, lane 6

**TABLE 1** Proteolytic Index

| Parameters | Temperature (°C) | | | |
|---|---|---|---|---|
| | 30 | 40 | 50 | 60 |
| Proteolytic Index | 1.17 ± 0.07a | 1.63 ± 0.08b | 2.43 ± 0.17c | nm |

Mean with a different superscript in the same indicate significantly different (P<0.05). nm: non-measurable

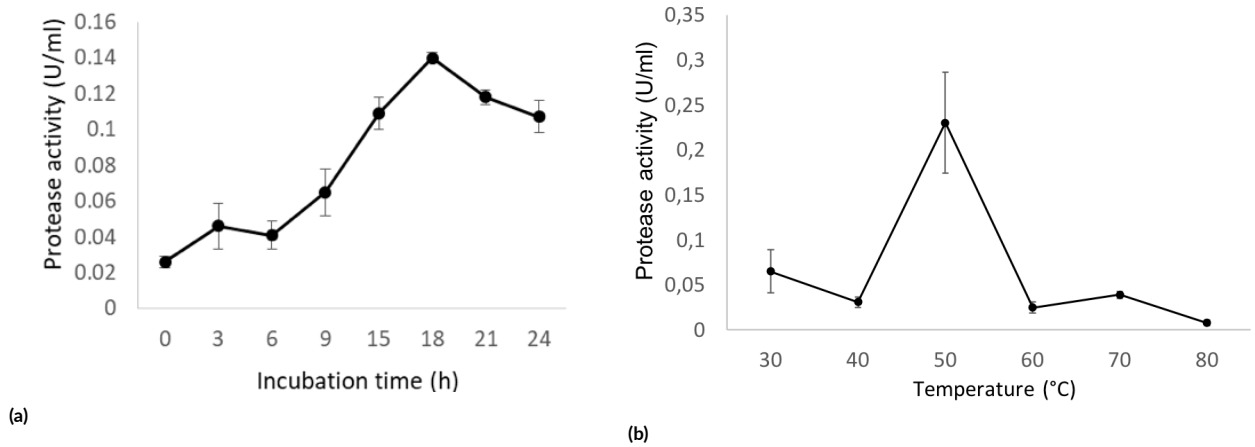


**FIGURE 1** Effect of incubation time and temperature on protease expression from *Geobacillus* sp. DS3 by a liquid MSM medium with 1% skim milk

(fraction 19 of DEAE-Sephadex A-25 purification) was invisible. In general, the molecular weight of alkaline serine proteases from *Bacillus* species has been reported to be 30–45 kDa. The molecular weight of alkaline serine proteases from *B. subtilis* RD7, and *Bacillus lehensis* JO-26 were reported to be 43 kDa (Suberu et al. 2019a), and 34.6 kDa (Bhatt and Singh 2020), respectively. The purified thermostable alkaline serine protease gene of *Geobacillus stearothermophilus* B-1172 has a molecular weight of 39 kDa (Iqbal et al. 2015). The molecular weight of alkaline serine protease from *Bacillus cereus* strain S8 was 71 kDa (non-reducing) and 35 kDa and 22 kDa (reducing), indicating the enzyme existence as a dimer in its native state. Furthermore, after gel filtration (Sephadex G-200) chromatography, its purified enzyme showed a molecular weight of 22 kDa (Lakshmi et al. 2018). Based on this information, we predicted our protease has a molecular weight of around 32 kDa.

### *3.4. pH optimum, temperature optimum, and stability of enzyme*

Different standard buffers (phosphate buffer, tris-HCl buffer and glycine-NaOH buffer) with various pH ranges from 5.8 - 10.6 were applied to determine the optimum pH for protease. The result showed that the crude enzyme has pH optimum in glycine-NaOH buffer at 9.6 for protease activity. At pH below 7, its protease activity gradually decreased from 50% of the maximum activity. The protease activity was maximal at 70 ºC. At a temperature below 40 ºC revealed, protease activity reduction was approximately 60% of the maximum activity. At 80 ºC, it only exhibited 25% of the maximum activity. Furthermore, the result of enzyme stability at 70 ºC indicated that the enzyme was highly stable for 1 h incubation and gradually decreased after 2 h incubation. It still showed protease activity around 63% of its maximal stability at 3 h incubation. These results suggested that the enzyme belongs to the thermostable alkaline protease. An alkaline serine protease from *G. stearothermophilus* (GsProS8) showed optimal activity at pH 8.5 and 50 °C (Chang et al. 2021). Whereas a serine protease from thermophilic *Geobacillus* sp. GS53 optimally worked at 55 °C and pH 8 (Baykara et al. 2021). Iqbal et al. (2015) also reported that a thermostable alkaline serine protease from *G. stearothermophilus* B-1172 was stable at 90 °C and pH 9. The alkaline protease from *B. cereus* strain S8 showed optimum temperature and pH of 70 ºC and 10, respectively (Lakshmi et al. 2018). Besides, Shrinivas and Naik (2011) reported thermostable alkaline protease from *Bacillus* sp. JB 99 showed an optimal temperature at 70 ºC and pH optimum at 11. Thermostable alkaline protease in this study which is stable at 70 °C and pH 9.6 could be applied in the detergent industry. Typically, a detergent protease needs to be active, stable, and compatible with the alkaline environment encountered under harsh washing conditions: pH 9 - 11, the temperature of 20 - 60 °C, as well as high concentrations of salt, bleach, and surfactant.

**TABLE 2** Enzyme activity and total protein of each purification steps

| Purification steps | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (U/mg) | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 200 | 70.2 | 41.6 | 0.59 | 100 |
| Ammonium sulphate extract (40-60%) | 5 | 3.58 | 2.245 | 0.63 | 5.4 |
| DEAE-Sephadex A-25 (Fraction 19) | 1.5 | 0.47 | 0.73 | 1.55 | 1.75 |

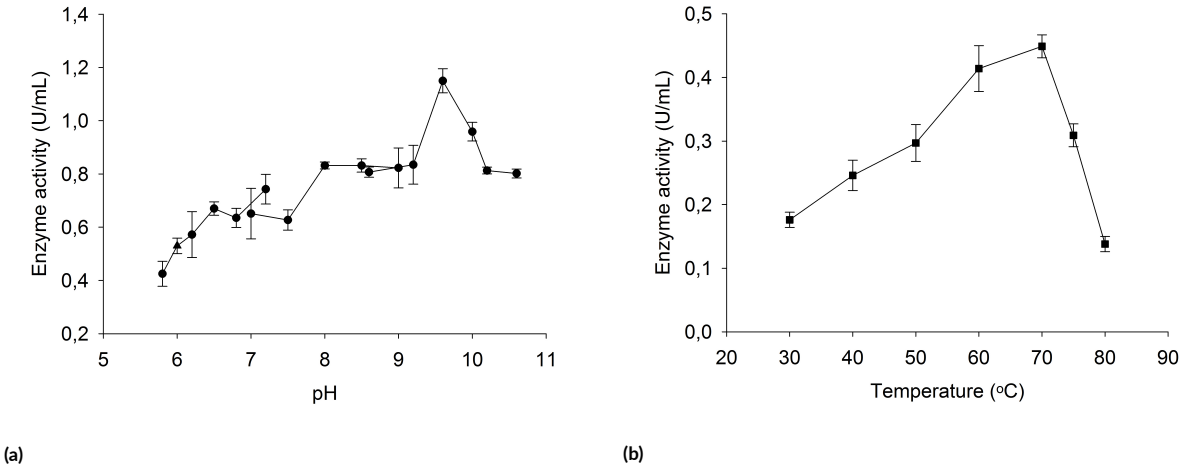


**FIGURE 2** Effect of pH and temperature on protease activity

### *3.5. Effect of protease inhibitors*

Protease inhibitors were subjected to identify groups at the active site of the enzyme. 2-ME (cysteine protease inhibitor) and EDTA (metalloprotease inhibitor) slightly affected the protease activity (Figure 3). The result indicated that SH-group residue did not work in the protease activity of this enzyme. In addition, ions or metals may not be essential for the stability and activity of this enzyme. In contrast, the protease activity is inhibited by PMSF (serine protease inhibitor) due to its lowest residual activity (50%) (Figure 3). Through this finding, the enzyme could be classified as a serine protease. Similarly, some proteases such as GsProS8 from *G. stearothermophilus* (Chang et al. 2021), alkaline protease from *B. cereus* strain S8 (Lakshmi et al. 2018), thermostable alkaline protease from *Bacillus* sp. JB 99 (Shrinivas and Naik 2011) were strongly inhibited by PMSF.

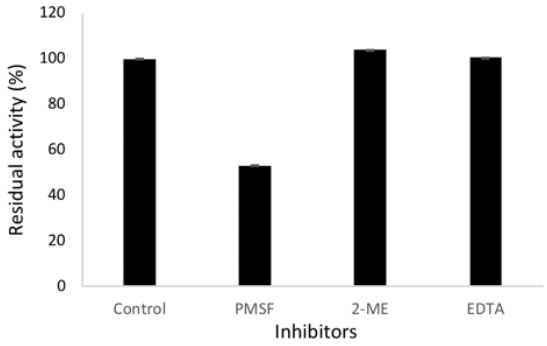


**FIGURE 3** Effect of inhibitors on protease activity. PMSF: Serine protease inhibitor, 2-ME: Cysteine protease inhibitor, and EDTA: Metallo protease inhibitor

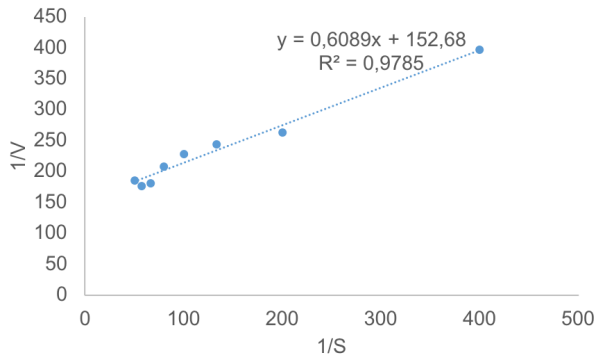


**FIGURE 4** Lineweaver-Burk curve of the purified protease

Keratinases are a group of mostly extracellular serine-proteases that are able to degrade keratins into amino acids. Keratin is a group of fibrous structural proteins of hair, wool, nails, hooves, horns and feather quills, and the epithelial cells in the outermost layers of the skin. Therefore, thermostable serine protease in this study could be potentially used for leather processing applications.

### *3.6. Enzyme kinetic*

The Lineweaver-Burk curve (Figure 4) was obtained by plotting 1/V versus 1/S with a casein substrate concentration of 0.0025 – 0.02 mg/mL. The enzyme has the value $V_{max}$ of 0.41 U/mg, while $K_M$ was 0.25 mg/mL. GsProS8 exhibited Vmax of 231.50 U/mg and KM of 7.37 mg/mL (Chang et al. 2021). A serine protease of thermophilic *Geobacillus* sp. GS53 showed 137.8 U/mg of specific activity (Baykara et al. 2021). Lakshmi et al. (2018) reported that alkaline protease from *B. cereus* strain S8 mentioned $K_M$ and $V_{max}$ of 3.3 mg/mL and 15 U/mg, respectively. Lower $K_M$ value of protease reflects a stronger binding affinity of this enzyme to substrate. Thus, the thermostable alkaline protease from *Geobacillus* sp. DS3 in this study exhibited a higher affinity than the other alkaline serine proteases.

## 4. Conclusions

In conclusion, thermostable serine alkaline protease from *Geobacillus* sp. DS3 isolated from Sikidang crater, Indonesia, had an optimal condition for enzyme activity at

70 ºC with pH 9.6. However, further studies related to the effect of surfactant, metal ion, organic solvents, and bleaching agents that are most crucial for industries are required to be performed.

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## Authors' contributions

This work was conceived and designed by LDW and SP. Experimental work was carried out and interpreted by SP. LDW, SP and AN contributed to data analysis and manuscript preparation. All authors have read and approved the manuscript.

## Competing interests

The authors declare no conflicts of interest.

## References

Baykara SG, Sürmeli Y, Şanlı Mohamed G. 2021. Purification and Biochemical Characterization of a Novel Thermostable Serine Protease from *Geobacillus* sp. GS53. Appl. Biochem. Biotechnol. 193(5):1574–1584. doi:10.1007/s12010-021-03512-0.

Bhatt HB, Singh SP. 2020. Cloning, expression, and structural elucidation of a biotechnologically potential alkaline serine protease from a newly isolated haloalkaliphilic *Bacillus lehensis* JO-26. Front. Microbiol. 11:1–16. doi:10.3389/fmicb.2020.00941.

Chang C, Gong S, Liu Z, Yan Q, Jiang Z. 2021. High level expression and biochemical characterization of an alkaline serine protease from *Geobacillus stearothermophilus* to prepare antihypertensive whey protein hydrolysate. BMC Biotechnol. 21(1):1–13. doi:10.1186/s12896-021-00678-7.

Hussein AH, Lisowska BK, Leak DJ. 2015. The genus geobacillus and their biotechnological potential. Adv. Appl. Microbiol. 92:1–48. doi:10.1016/bs.aambs.2015.03.001.

Iqbal I, Aftab MN, Afzal M, Ur-Rehman A, Aftab S, Zafar A, Ud-Din Z, Khuharo AR, Iqbal J, Ul-Haq I. 2015. Purification and characterization of cloned alkaline protease gene of *Geobacillus stearothermophilus*. J. Basic Microbiol. 55(2):160–171. doi:10.1002/jobm.201400190.

Lakshmi B, Muni Kumar D, Hemalatha K. 2018. Purification and characterization of alkaline protease with novel properties from *Bacillus cereus* strain S8. J. Genet. Eng. Biotechnol. 16(2):295–304. doi:10.1016/j.jgeb.2018.05.009.

Pambudi NA. 2018. Geothermal Power Generation in Indonesia, a country within the Ring of Fire: Current Status, future development and policy. Renewable Sustainable Energy Rev. 81:2893–2901. doi:10.1016/j.rser.2017.06.096.

Rai SK, Mukherjee AK. 2009. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04. Bioresour. Technol. 100(9):2642–2645. doi:10.1016/j.biortech.2008.11.042.

Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. 2018. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the panther database. Nucleic Acids Res. 46(D1):D624–D632. doi:10.1093/nar/gkx1134.

Sharma KM, Kumar R, Panwar S, Kumar A. 2017. Microbial alkaline proteases: Optimization of production parameters and their properties. J. Genet. Eng. Biotechnol. 15(1):115–126. doi:10.1016/j.jgeb.2017.02.001.

Shrinivas D, Naik G. 2011. Characterization of alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity. Int. Biodeterior. Biodegradation 65(1):29–35. doi:10.1016/j.ibiod.2010.04.013.

Singh R, Kumar M, Mittal A, Mehta PK. 2016. Microbial Enzymes: Industrial progress in 21st Century. 3 Biotech 6(2):1–15. doi:10.1007/s13205-016-0485-8.

Souza PM, Bittencourt ML, Caprara CC, Freitas Md, Almeida RP, Silveira D, Fonseca YM, Ferreira Filho EX, Pessoa Junior A, Magalhães PO, et al. 2015. A biotechnology perspective of fungal proteases. Braz. J. Microbiol. 46(2):337–346. doi:10.1590/s1517-838246220140359.

Stetter KO. 2006. Hyperthermophiles in the history of life. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 361(1474):1837–1843. doi:10.1098/rstb.2006.1907.

Suberu Y, Akande I, Samuel T, Lawal A, Olaniran A. 2019a. Cloning, expression, purification and characterisation of serine alkaline protease from Bacillus subtilis RD7. Biocatal. Agric. Biotechnol. 20:101264. doi:10.1016/j.bcab.2019.101264.

Suberu Y, I A, Samuel T, Lawal A, Olaniran A. 2019b. Optimization of protease production in indigenous *Bacillus species* isolated from soil samples in Lagos, Nigeria using response surface methodology. Biocatal. Agric. Biotechnol. 18:101011. doi:10.1016/j.bcab.2019.01.049.

Tavano OL, Berenguer-Murcia A, Secundo F, Fernandez-Lafuente R. 2018. Biotechnological applications of proteases in Food Technology. Compr. Rev. Food Sci. Food Saf. 17(2):412–436. doi:10.1111/1541-4337.12326.

Witasari LD, Prijambada ID, Widada J, Andang D,

Wibawa A. 2010. Cloning of thermostable DNA polymerase gene from a thermophilic *Brevibacillus* sp. isolated from Sikidang Crater, Dieng Plateu, Central Java. Indones. J. Biotechnol. 15(2):72–78. doi:10.22146/ijbiotech.7825.

Zhou C, Qin H, Chen X, Zhang Y, Xue Y, Ma Y. 2018. A novel alkaline protease from alkaliphilic Idiomarina sp.. C9-1 with potential application for eco-friendly enzymatic dehairing in the leather industry. Scientific Rep. 8(1):1–18. doi:10.1038/s41598-018-34416-5.

Zilda DS, Harmayani E, Widada J, Asmara W, Irianto HE, Patantis G, Fawzya YN. 2013. Screening of thermostable protease producing microorganisms isolated from Indonesian hotspring. Squalen Bull. Mar. Fish. Postharvest Biotechnol. 7(3):105. doi:10.15578/squalen.v7i3.5.