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Bacillus thuringiensis aizawai HD283 Partial Purified Thermo-Alkaline Metalloprotease Characterization

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ABSTRACT

Objective: The study aimed to classify the alkaline protease produced by *Bacillus thuringiensis aizawai HD283*. **Methods:** The experimental methodology encompassed the following steps: preparation of the inoculum, formulation of production media, enzyme activity assays, protein quantification. **Results:** After ammonium sulfate fractionation, acetone precipitation, and G-100 Sephadex column chromatography purification steps, of *Bacillus thuringiensis aizawai* HD283 partial purified alkaline protease. The purified alkaline protease exhibited a significant activation response to H_2O_2 at concentrations of 1%, 5%, and 10% (v/v), retaining 412%, 342%, and 286% of its activity, respectively. The enzyme activity demonstrated notable increases of 224%, 226%, and 300% in the presence of 10 mM, 5 mM, and 1 mM PMSF, respectively. Alkaline protease activity was inhibited by 10 mM EDTA, $AgNO_3$ and $HgCl_2$ with 44, 60 and 67 % of control respectively, the enzyme's activity increased to approximately 572% in the presence of 10 mM mercaptoethanol. It also increased to 127%, 268%, 292%, and 703% compared to the control group with the addition of calcium chloride, choline chloride, magnesium chloride, and manganese chloride (at 10 mM concentrations, respectively). Acetone at 50% concentration activated the enzyme by 177%. The optimal pH was found to be 8.0 and 10.0, and the enzyme's activity increased with temperature from 30°C to 70°C. The enzyme retained approximately 74% of its original activity at 60°C. At a protein concentration of 0.017 mg/mL, the enzyme's maximum activity was approximately about, 16.640 units/mL/min. The highest maximum velocity (Vmax) of the enzyme was achieved at 30.116 micrograms of tyrosine/ml/min, Km value of casein concentration was 5.448%. **Conclusion:** Based on the chemical classification scheme and the catalytic mechanism observed, the alkaline protease produced in this study was identified as belonging to the class of Thermophilic- Metallo-Alkaline Proteases.

Keywords: Enzyme classification, microbial enzymes, extreme-zymes, biochemical properties, enzymes activators and inhibitors, Km and Vmax.

Introduction

Bacillus thuringiensis is a well-known bacterium in the biopesticide market of past decades, due to its production of crystalline protein toxins effective against many insects. Some strains of this bacterium also produce additional enzymes, such as alkaline protease, which enhance their biological activity against insects. Alkaline protease plays a critical role in catalyzing the conversion of protoxins into their active, low-molecular-weight forms through diverse mechanisms, further contributing to the bacterium's insecticidal efficacy (Li *et al*. 2022).

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Proteases possess a wide range of applications across diverse fields, making them one of the most versatile groups of enzymes. Notably, proteases account for nearly two-thirds of all enzymes utilized in industrial processes, underscoring their significant role in various biotechnological and commercial applications (Sedaghat *et al.,* 2022). Radwan *et al*. (2023) investigated that extracellular proteases from thermo-alkali actinobacteria were characterized as thermophilic alkaline serine proteases (TA-proteases), highlighting their potential as effective, eco-friendly alternatives to chemical and thermal treatments in the dairy industry. El-Sayed *et al*. (2024) used immobilized serine protease effectively to accelerate wound healing. Nour *et al*. (2024) applied successfully alkaline protease produced by *Bacillus safensis* lab418 to control *Meloidogyne incognita*, in addition to the improved plant growth, due to protein interactions between alkaline protease and collagen present in the nematode cuticle, indicating the enzyme's activity in binding and catalysis.

Proteases exhibit diverse applications across multiple industries, including photography, detergents, leather processing, and food production. In the food industry, they play a pivotal role in processes such as protein fermentation, meat tenderization, and dairy product processing, highlighting their versatility and industrial significance (Roshdy *et al*., 2022). Enzymes are distinguished as natural catalysts due to their superior sustainability and efficiency compared to chemical catalysts. However, the role of catalytic enzymes in commercial operations, particularly in industries such as pharmaceuticals, food, and beverages, has been relatively underutilized, despite their potential to enhance process efficiency and environmental compatibility (Chapman, 2018). The development of bio-stimulants and the enhancement of industrial processes aim to maximize productivity and market profitability while simultaneously minimizing environmental impact and resource consumption. This dual focus underscores the importance of sustainable practices in achieving economic and ecological balance (Nourine *et al*., 2023).

Gençkal (2004) classified proteolytic enzymes into four main categories based on their catalytic mechanisms: 1- Serine Proteases: These enzymes are irreversibly inhibited by PMSF (phenylmethylsulfonyl fluoride). They exhibit optimal activity at a pH range of 7 to 11 and an optimal temperature of approximately 70°C. Their molecular weight typically ranges between 18 to 35 kDa. Serine proteases are widely distributed among bacteria, fungi, and yeasts. 2- Aspartic Proteases: Also known as acid proteases, these enzymes function optimally at a pH range of 3 to 4.5. They have a molecular weight ranging from 30 to 45 kDa and are inhibited by pepstatin. Aspartic proteases are further subdivided into three groups: pepsin, retropepsin, and enzymes from pararetroviruses. 3- Cysteine Proteases: Found in both prokaryotes and eukaryotes, these enzymes are active only in the presence of reducing agents. The thiol group of the cysteine residue plays a critical role in their activity, as it is sensitive to oxidation and reacts with various reagents, heavy metals, and iodoacetate. They function optimally at neutral pH and are subdivided into four groups: papain-like, trypsin-like, glutamic-specific, and an additional miscellaneous group. 4- Metalloproteases: These enzymes require divalent metal ions for catalytic activity and are subdivided into four groups: alkaline, neutral, myxobacter I, and myxobacter II proteases.

Classifying enzymes and elucidating their biochemical properties are critical steps in optimizing their efficiency and enhancing their practical applications. This study aims to provide a precise and comprehensive understanding of the optimal conditions for alkaline protease activity, a widely utilized enzyme in various industries.

Additionally, the research investigates the enzyme's biochemical characteristics to expand scientific and industrial insights into its potential applications. By advancing knowledge in this field, the study seeks to promote the development of enzyme-based technologies while reducing reliance on harmful chemical catalysts, thereby contributing to environmental sustainability and the preservation of ecological systems.

This study is the first of its kind to provide a comprehensive biochemical characterization of a thermo-alkaline metallo-protease produced by the Bacillus thuringiensis aizawai HD283 strain. Its scientific novelty lies in the discovery of unique catalytic properties, including: (1) the abrupt activation of the enzyme by the serine protease inhibitor PMSF, which challenges the conventional classification of these inhibitors and suggests an atypical catalytic mechanism; (2) the strong catalytic effect of manganese ions, exceeding 700%, highlighting their crucial role as a cofactor; (3) remarkable thermal stability, retaining 74% of its activity at 60°C, making it a promising candidate for industries requiring harsh conditions; and (4) the presence of two optimal pH values (8 and 10), which may be attributed to

the existence of isoforms of the enzyme. These findings offer new insights into the diversity and mechanism of action of proteases. Bacterial, and opens up prospects for wide-ranging industrial applications.

2. Materials and Methods

Bacterial strain, media used for growth and production of alkaline protease, alkaline protease activity assay and protein determination, it was also explained in detail in our previous article (Roshdy *et. al* 2023 a).

2.1. Assay of alkaline protease

The activity of AP was determined as described by Lowry *et al*. (1951) with some modifications. The standard reaction mixture composition (unless otherwise stated) contained 0.5 ml of enzyme source (culture supernatant or supernatant after purification steps), one ml of glycine-sodium hydroxide buffer 0.1M, pH 9.6, in which 1% casein was dissolved as substrate. The reaction mixture was incubated at 40ºC for 20 minutes after which the enzymatic reaction was stopped by addition of one ml of 20% trichloroacetic acid with thoroughly mixing. The stopped reaction mixture was then centrifuged at 4000 rpm for 10 minutes without disturbing the precipitated proteins.

Series of clean tubes were used 0.5 ml of the clear solution was pipetted in each clean tube and 5 ml of Reagent C were added and mixed well. The mixture was let to stand at room temperature for 10 min. Reagent D was added rapidly to the mixture and mixed immediately. The mixture was allowed to stand at room temperature for 10 min and then the optical density of the developed color was read at 600 nm. The readings of the optical densities of zero time cleared reaction mixtures were subtracted from those of the experimental tubes.

The optical densities were converted to the equivalent grams of tyrosine using standard curve of authentic tyrosine concentrations (10-200 micrograms/ml) that were treated similarly to experimental enzyme-catalyzed reaction mixtures.

The enzyme activity was expressed in term of enzyme units. One AP unit was defined as the amount of enzyme that liberates one-microgram tyrosine per ml enzyme per minute under the specified reaction conditions.

$$\text{Enzyme units} = \frac{\text{Micrograms of tyrosine released X enzyme dilution}}{\text{Reaction time (minutes)}} = \text{Microgram tyrosine/min/ml culture supernatant}$$

Reagents**:**
Reagent A: 2% Na_2CO_3 in 0.1N NaOH
Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% sodium and potassium tartrate
Reagent C: 50 ml of reagent A was mixed with 1 ml of reagent B. Made fresh daily.
Reagent D: 1 part Folin Ciocalteau's phenol reagent + 2 parts water.

2.2. Protein determination

Protein was determined as described by Lowry *et al*. (1951). To determine soluble protein concentrations, series of clean tubes, 0.5 ml of the enzyme solution was pipetted in each clean tube and 5 ml of Reagent C were added and mixed well. The mixture was let to stand at room temperature for 10 min. Reagent D was added, rapidly, to the mixture and mixed immediately. The mixture was allowed to stand at room temperature for 10 min, then the optical density of the developed color was read at 600 nm. The readings of the optical densities of enzyme solutions were noticed.

The optical densities were converted to the equivalent grams of bovine serum albumine using standard curve of authentic bovine serum albumine concentrations (10-200 micrograms/ml) that were treated similarly to experimental enzyme solutions.

2.3. Effect of enzyme inhibitors, surfactants, bleach, and oxidizing agents on the enzyme activity

The effects of Co^{2+}, Ag^{1+}, Mn^{2+}, Hg^{2+}, Ca^{2+} and Mg^{2+} as metalic ions, H_2O_2 as an oxidizing agent, Mercapto-ethanol, PMSF (Phenyl Methyl Sulfonyl Floride), EDTA (Ethelene Di amine Tetra Acetic acid) as inhibitor on alkaline protease activity and DFP (Di- isopropyl Floro Phosphate) as sulfhydryl

agents were investigated to further characterize the enzyme; as shown in details by Roshdy *et al.* (2023 b).

3. Results and Discussion

3.1. Characterization profile of partial purified alkaline protease of *B.t.* aizawai HD283.

In a previous study (Roshdy and Al-Sharshibi, 2024), alkaline protease production was enhanced by *Bacillus thuringiensis aizawai* HD283 using solid-state fermentation (SSF). Wheat bran, an industrial agricultural byproduct, was identified as a super-nutrient substrate, and the addition of fructose as a carbon source nearly doubled enzyme production. Optimal conditions for alkaline protease production included wheat bran particle size less than 1 mm, 200% moisture content, 60% inoculation volume, and a 5-day incubation period. The crude extract peaked at pH 9 and 50°C, exhibiting remarkable stability across a wide temperature range (40–80°C), making it suitable for various biotechnological applications, including detergents, leather tanning, and wastewater treatment.

This motivated us to complete the study on this bacterial strain by testing its effectiveness as an antimicrobial or anti-biofilm agent and purifying the alkaline protease in a research paper that is currently being published. In this research paper, the alkaline protease is characterized biochemically.

3.1. Estimation of activators and inhibitors on the activity of alkaline protease purified by the Sephadex G100 column produced by *B.t. aizawai HD283*.

3.1.1. Effects of metalic ions

Silver nitrate $AgNO_3$ inhibits the enzyme, reducing its activity to 60%. Thiol groups in proteins react with Ag^{+2}, as is known, which may explain the inhibition. Mercury ions also can bind to thiol groups, disrupting enzyme structure and function, Mercury Chloride ($HgCl_2$) inhibits enzyme, reducing activity to 66.94%.

On the other hand, Manganese chloride ($MnCl_2$) has the strongest activating effect, increasing enzyme activity to 703.27%. Manganese ions may act as potent cofactors or stabilize the enzyme's structure. Magnesium chloride ($MgCl_2$) also activates the enzyme, increasing activity to 291.84%. Magnesium ions are common cofactors for many enzymes. Cobalt chloride ($CoCl_2$) strongly activates the enzyme, increasing activity to 267.76%. Cobalt ions may act as cofactors or stabilize the enzyme's active site. Calcium chloride ($CaCl_2$) enhances enzyme activity by 27.35%, suggesting that calcium ions may play a role in stabilizing or activating the enzyme.

Roshdy *et al.* (2023b) illustrated that, $CaCl_2$ and $MgCl_2$ (10 mM) addition to the reaction mixture, increases activity of alkaline proteases by 136% and 147%. The presence of Ca2+ and Mg2+ ions may maintain and/or inhibit the enzyme's activity. Additionally, cobalt, silver, manganese, and mercury ions are inhibitory. Shaikh *et al*. (2018) observed significant protease activation upon the addition of calcium salts, while cobalt and mercury salts inhibited the enzyme. However, Elgammal *et al*. (2021) found that silver salts inhibited proteases. Ullah *et al*. (2022) found that calcium and magnesium salts stimulated protease activity by 125% and 140%, respectively. Lemes *et al*. (2023) reported that alkaline protease produced by *Bacillus gibsonii* 6BS15-4 was slightly activated with a relative activity of 114 upon the addition of magnesium, and its activity decreased to 84% and 97% of the enzyme's activity in the presence of manganese and calcium salts. Mahakhan *et al*. (2023) noted that Mn+2 activates the enzyme; however, CaO, MgSO4, and CaCl2 salts have a lesser effect. Li *et al.* (2023) showed that 5 mM of calcium salts increased enzyme activity by 164.2%, while magnesium and manganese salts inhibited enzyme activity proportionally. Niyonzima *et al.* (2015) observed that alkaline protease was activated by the addition of calcium and magnesium salts and was unaffected by cobalt salts, but mild inhibition occurred with the addition of manganese salts; however, mercury salts inhibited the enzyme by 25%. Harer and Harer (2022) also recorded an increase in enzyme activity with the addition of calcium, magnesium, and manganese salts.

3.1.2. Effects of H_2O_2 as an oxidizing and bleaching agent

Hydrogen peroxide (H_2O_2) activates the enzyme, particularly at lower concentrations, suggesting that mild oxidative conditions may enhance its activity. H_2O_2 (10%, 5%, 1%) activates the enzyme, with the effect increasing as the concentration decreases. At 1% H_2O_2, enzyme activity reaches 412.24%. This could be due to oxidative modifications that enhance enzyme activity.

In a previous study by Roshdy *et al.* (2023b), we found that alkaline protease was not significantly affected by concentrations of 1, 5, and 10% (v/v) H_2O_2, with activity levels of 111, 101, and 66%, respectively. Similarly, Mahakhan *et al*. (2023) found that alkaline protease produced by Bacillus gibsonii 6BS15-4 was unaffected at 0.1% (v/v) H_2O_2.

3.1.3. Inhibitors and chelating agents

Mercaptoethanol, a reducing agent, dramatically increases enzyme activity to 572.24%. This suggests that the enzyme's activity is highly dependent on the reduction of disulfide bonds or the presence of free thiol groups.

Bovine serum albumin (BSA) enhances enzyme activity to 269.39%. BSA is often used as a stabilizing agent, and its effect here suggests it may protect the enzyme from denaturation or aggregation.

Phenylmethylsulfonyl fluoride (PMSF) is a serine protease inhibitor, but in this case, it appears to activate the enzyme.

The activation increases with decreasing PMSF concentration (10mM, 5mM, 1mM), peaking at 1mM (300%). This unexpected result may indicate a secondary mechanism of action.

A notable finding of this study is the activation of the enzyme by PMSF, a known inhibitor of serine proteases (Gençkal, 2004). This atypical activation, which contradicts what is documented in the serine protease literature, suggests that the enzyme under investigation either does not belong to the traditional serine protease family or that PMSF interacts with an allosteric site different from the active site, resulting in an integrative change in the enzyme's structure that enhances its catalytic activity. This hypothesis is supported by studies showing that some metalloproteases may be atypically affected by inhibitors (El-Khonezy *et al*., 2021). Furthermore, this concentration-dependent activation (where activity increased with decreasing PMSF concentration) supports the hypothesis of a complex regulatory mechanism and warrants future studies such as site-directed mutagenesis to identify the PMSF binding site and confirm this mechanism.

EDTA is a chelating agent that binds metal ions, which are often essential for enzyme activity. The significant reduction in activity (43.67%) suggests that the enzyme requires metal ions for its function.

Mercaptoethanol (572.24%) are the most potent activators, highlighting the enzyme's sensitivity to manganese ions and reducing conditions. PMSF, typically an inhibitor of proteases, activates the enzyme in this case. This may indicate a unique mechanism of action or a secondary effect unrelated to protease inhibition.

Roshdy *et al.* (2023b) studied the effect of adding 10, 5 and 1 mM of PMSF on enzyme activity. The activity rates were 82%, 87% and 101% respectively. That is, the inhibitory effect of it did not appear except at very high concentrations. El-Khonezy *et al*. (2015) reported that, PMSF intensely inhibited alkaline protease of *Streptomyces griseus* NCRRT.

In his 2004 master's thesis, which details the classification and nomenclature of protease enzymes, Gençkal (2004) identified serine proteases as being irreversibly inhibited by PMSF. This suggests that the enzyme under study does not belong to the serine protease group. He also noted that metalloproteases are inhibited by chelating agents, including EDTA, but not by sulfhydryl or DFP. However, Shaikh *et al*. (2018) found that the definition of serine protease is achieved by complete inhibition with the addition of PMSF and DFP, because they strongly inhibit the enzyme activity center due to the sulfate of the essential serine residues. Ullah *et al*. (2022) agreed with him, confirming that PMSF is a complete inhibitor of enzyme activity; DFP also inhibited 92% of enzyme activity. This view was also supported by Mahakhan *et al*. (2023), who reported strong inhibition of the serine alkaline enzyme of *Bacillus gibbonii* 6BS15-4 by the addition of PMSF, despite its unaffected activity upon the addition of EDTA. They noted that the enzyme's activity center contains tyrosine residues, which explains why the enzyme's activity was unaffected by the addition of EDTA to the reaction mixture, but was strongly inhibited by the addition of PMSF. (Niyonzima *et al.,* 2015). Harer and Harer (2022) investigated that, enzyme activity was 100% inhibited by PMSF.

3.1.4. Effect of acetone as solvent

Acetone at 50% concentration increases enzyme activity (176.73%). This could be due to acetone's ability to alter the enzyme's conformation or improve substrate accessibility.

By adding 50% acetone to the reaction mixture, Roshdy *et al*. (2023b) recorded an enzyme activity of 39%. This places the enzyme produced by *B.t. dendrolimus* IP 4A/4B, under the category of metalloproteases. This differs from Lemmes *et al*. (2023), where the addition of 50% acetone resulted in a 21.2% inhibition of enzyme activity. They explained this inhibition by the acetone drawing water from and around the center of enzyme activity, thus stopping the enzyme's catalytic effect due to the absence of an aqueous medium.

Figure (1), reveals that, enzyme's activity is highly sensitive, to its chemical environment. Enzyme is sensitive to thiol-reactive agents, as seen with the inhibition by $AgNO_3$ and $HgCl_2$, and the strong activation by mercaptoethanol. The enzyme is highly dependent on metal ions, as evidenced by the strong activation by $CoCl_2$, $MgCl_2$, and $MnCl_2$, and the inhibition by EDTA (which chelates metal ions), mercaptoethanol and H_2O_2 considerably enhance enzyme activity, indicating that metal ions, reducing conditions, and mild oxidative stress positively influence the enzyme. The activation by PMSF is unusual and warrants further investigation to understand the underlying mechanism.

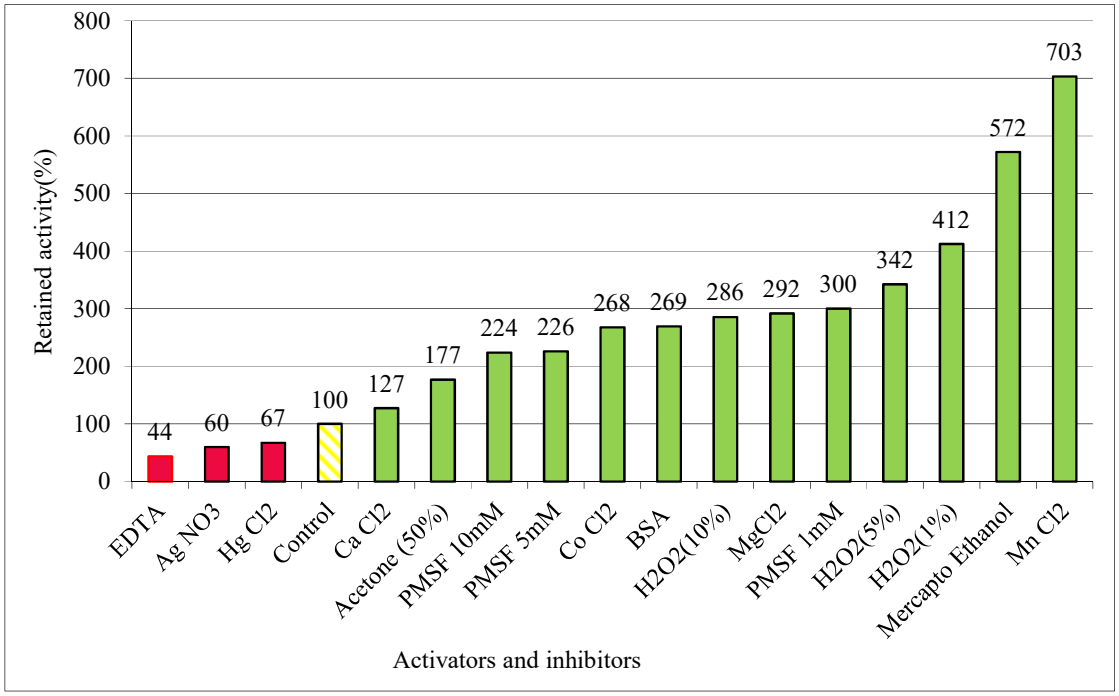

Fig. 1. Activation and inhibition profile of purified alkaline protease produced by *B.t. aizawai HD283*.

The strong activation by $MnCl_2$ and mercaptoethanol suggests that the enzyme may function optimally under reducing conditions with manganese as a cofactor. The manganese ion (Mn^{2+}) exhibited the strongest catalytic effect, at 703.27%, indicating its crucial role as a cofactor for this metal protease. In metal proteases, metal ions play a pivotal role in stabilizing the enzyme's tertiary structure by coordinating with amino acid residues in the active site. They also facilitate electron transfer or maintain the enzyme's active conformation (Shaikh *et al*., 2018). This finding is consistent with previous studies demonstrating significant activation of alkaline proteases by manganese ions (Mahakhan *et al*., 2023; Roshdy *et al*., 2023b), supporting the hypothesis that Mn^{2+} ions are an integral part of the active site or contribute to enzyme stability under alkaline and thermal conditions, making them an ideal candidate for industrial applications requiring metal cofactors.

The unexpected activation by PMSF and H_2O_2 highlights the complexity of the enzyme's regulation and the need for further studies to elucidate its mechanism of action. These findings can guide the optimization of enzyme activity in industrial or research applications.

3.2. Effect of pH on the activity of alkaline protease produced by *B.t. aizawai HD283* after enzyme purification.

The effect of changing pH on the activity of purified alkaline proteases was studied in the reaction medium buffers of glycine-sodium hydroxide and Tris-hydrochloric acid, and illustrated in Figure (2).

The enzyme exhibited two optimal pH values at 8.0 and 10.0, a rare characteristic for proteases. This phenomenon can be explained by several possibilities: (a) the existence of different isoforms of the enzyme, each with an optimal pH value that varies depending on the microenvironment of the active site (Ma *et al*., 2022); (b) the formation of different ionic complexes between the enzyme and the substrate (casein) influenced by the ionization of amino acid side groups at the active site, leading to optimal activity at different pH values (Blandine *et al*., 2016); or (c) a change in the enzyme's overall charge affecting its affinity for the substrate at different pH values. This property enhances the enzyme's versatility in diverse industrial applications, such as detergent manufacturing and X-ray film recycling, where processes require enzymatic activity across a wide pH range (Asar, 2013).

Roshdy *et al.* (2023b) reported that, it has two peak alkaline pH levels, 8.0 and 10.0; not only that, but its activity was 44% at pH 11.0. But Blandine *et al*. (2016) recorded two pH peaks, one acidic at 2.79, which they identified as aspartic protease and the other basic at 9.0, which they described as serine protease. Atrooz and Alomari (2020) also observed two pH peaks at 3 and 9 and attributed this to a molecular enzyme. However, Ma *et al*. (2022) offered a more comprehensive explanation, attributing the presence of more than one pH peak for the enzyme to one of three possibilities: either a difference in the degree of enzyme purification, the formation of two ionized complexes between the enzyme and the substrate, or the presence of different isoforms of the enzyme. El-Shazly *et al*. (2024) reported that, optimum pH for alkaline protease produced by Streptomyces was 10. Harer and Harer (2022) investigated optimum pH 10-11 for alkaline protease produce by *Bacillus thuriengiensis*, it was found that within this pH range, the stability was two, after twenty hours of incubation.

This pH range has several applications, including recycling X-ray films by removing the silver layer from them, and it is also used in the manufacture of detergents (Asar, 2013).

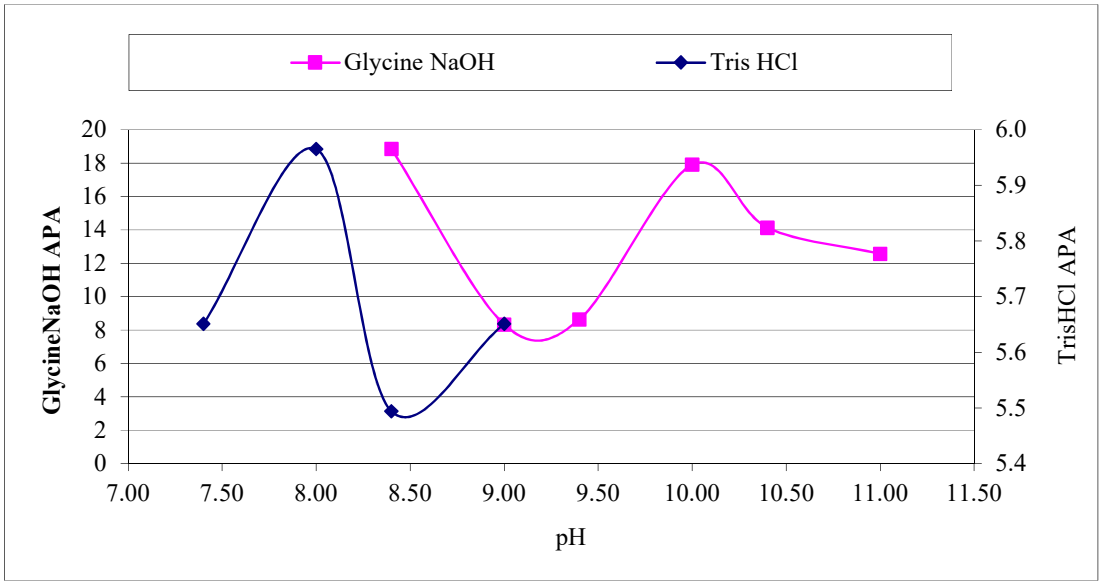

Fig. 2: Effect of pH on the activity of alkaline protease produced by *B.t. aizawai HD283* after enzyme purification.

3.3. Evaluating the effect of temperature changes on the activity of purified alkaline protease produced by *B.t. aizawai HD283*.

Temperature plays a crucial role in enzymatic biocatalysis. Since protein is a key component of enzyme structure, and heat has a strong effect on protein synthesis, particularly in the tertiary and tertiary segments (which can even lead to denaturation at high temperatures), studying the temperature variation in enzyme activity is fundamental to any enzyme study. Figure (3) shows that the optimum

temperature for the enzyme activity under investigation is 70°C. This suggests potential applicability requires further validation for this enzyme, as most industries naturally require high temperatures, especially those involving alkaline proteases, such as detergents and leather manufacturing.

The enzyme's optimum temperature was 70°C, while it retained 74% of its activity at 60°C, demonstrating its thermophilic nature. This high thermal stability is attributed to its protein structure, which features more salt bridges and hydrogen bonds compared to mesophyllic enzymes, as well as a higher proportion of hydrophobic amino acids that enhance its stability at elevated temperatures (Radwan *et al*., 2023). This property makes the enzyme a strong candidate for high-temperature industries, such as detergent and tanning, where it can withstand the demands of hot washing or high-temperature tanning processes.

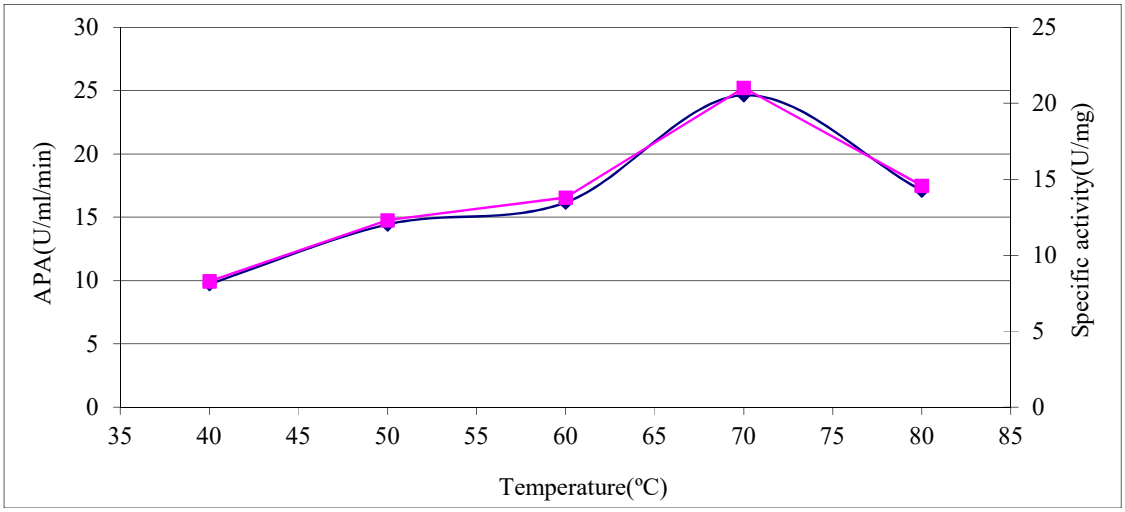

Fig. 3: Evaluating the effect of temperature changes on the activity of purified alkaline protease produced by *B.t. aizawai HD283*.

Roshdy *et al* (2023b) investigated that enzyme activity increased proportionally with increasing temperature until it reached the peak of the curve at 50°C. Activity then decreased to 66% at 60°C and stabilized at this level until 70°C. El-Shazly *et al*. (2024) reported that, optimum temperature for alkaline protease activity was 40 °C. Shaikh *et al*. (2018), Mahakhan *et al*. (2023), (Ullah *et al*. 2022) and Li *et al*. (2023) found that the optimal temperature for enzyme activity was at 55°C, and the enzymatic reaction continued up to 60°C. Whereas (Niyonzima *et al*. 2015 and Elgammal *et al*. 2021) found that the highest value of enzyme activity was at 50°C, only slightly above the temperature range studied, which was from 30 to 60°C.

3.4. Thermal stability assessment of purified alkaline protease produced by *B.t. aizawai HD283*.

The thermal stability of alkaline protease was tested by directly exposing the enzyme to temperatures of 30, 40, 50 and 60 degrees Celsius for 15 minutes, before incubation with the enzyme reaction mixture at 50 degrees Celsius, as reported in Figure (4).

As alkaline protease missed 1.3 % comparative reaction at 50 ºC go on to 19.6% at 80 ºC (Niyonzima *et al.,* 2015). Harer and Harer (2022) reported that optimum temperature for the enzymatic reaction was 45 degrees Celsius, with 100% heat tolerance for an incubation period of 350 minutes. While purified protease retained 46% of control activity at 60 ºC (Ullah *et al.,* 2022). Roshdy *et al.* (2023b) found that, the alkaline protease activity reached about 48% by heating to a temperature of 60 degrees Celsius. Meanwhile, Mahakhan *et al.* (2023) reported that the activity of alkaline protease produced from Bacillus gibsonii 6BS15-4 was unaffected by heating from 30 to 50 °C and a reaction time of 60 minutes. While El-Shazly *et al.* (2024) reported that, alkaline protease retained 5% only of its activity at 70 ºC.

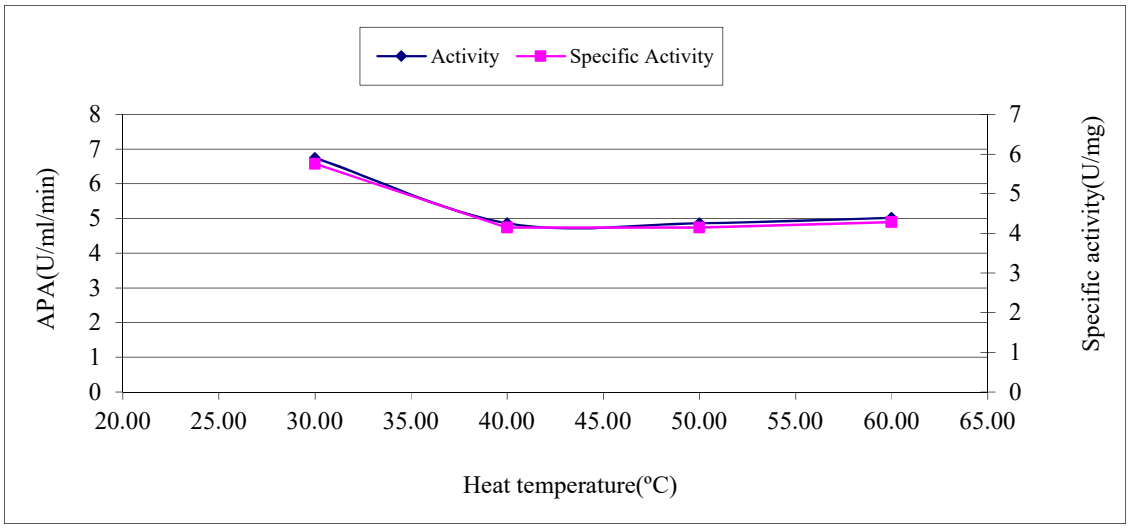

Fig. 4: Thermal stability assessment of purified alkaline protease produced by B.t. aizawai HD283.

3.5. Evaluation of the optimal concentration of purified alkaline protease reaction produced by *B.t. aizawai HD283*.

By comparing graded enzyme concentrations from 0.002 to 0.017 mg protein per ml of reaction solution under standard incubation factors, the optimal concentration for enzyme activity is 0.014 mg protein per ml of reaction solution, where the peak of the curve was at 6.750 U/mL/min, which illustrated in Figure (5).

This value was agreed upon by Roshdy *et al*. (2023b), who found that the optimal concentration of the enzyme protein was 0.014 mg/ml of the reaction solution. Huang *et al*. (2003) found that the optimal concentration of the enzyme protein was 0.34 mg protein/ml of the crude enzyme. However, Aboul-Soud *et al*. (2011) found that the optimal concentration of purified alkaline protease protein produced from *Bacillus sphaericus* was 0.075 mg protein/ml, with an enzyme activity 850 units/min/mg.

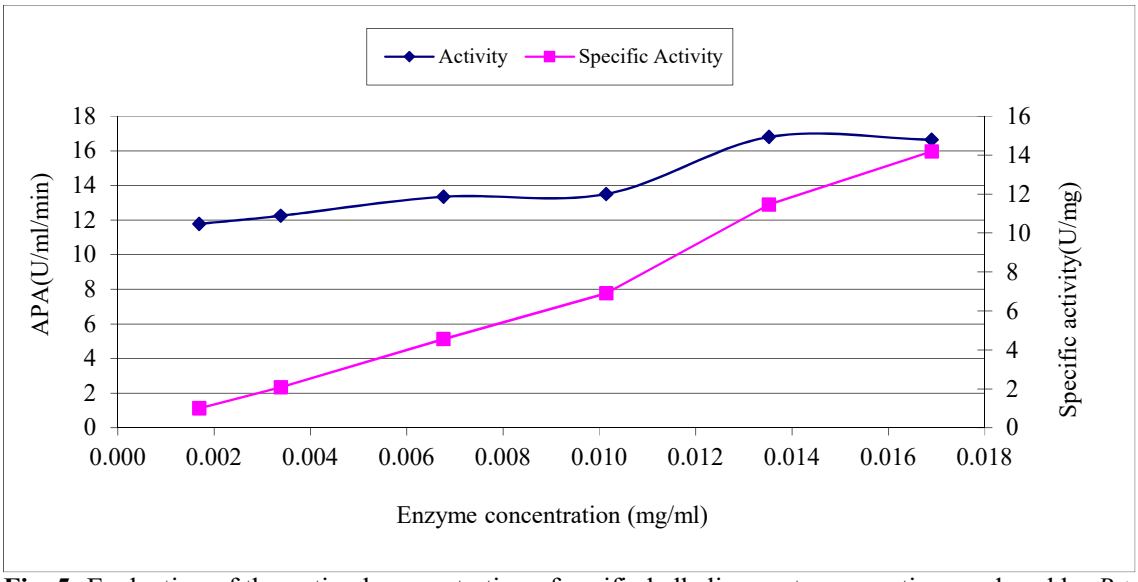

Fig. 5: Evaluation of the optimal concentration of purified alkaline protease reaction produced by *B.t. aizawai HD283*.

3.6. Evaluation of reaction time on the activity rate of purified alkaline protease produced by *B.t. aizawai HD283*.

Studying the optimal reaction time for enzyme activity reveals numerous practical properties of the enzyme and guides it towards optimal industrial application. Figure 6 shows that only 5 minutes was optimal for enzyme activity, indicating potential economic value for the industrial application of the enzyme.

Roshdy *et al* (2023b) also agreed with this finding for the *Bacillus thuringiensis* strain, where five minutes was optimal for enzyme activity. In contrast, alkaline protease activity in Ullah *et al*. (2022) took 35 minutes to peak. Similarly, Aboul-Soud *et al*. (2011) recorded the best alkaline protease activity in *Bacillus sphaericus* at 30 minutes.

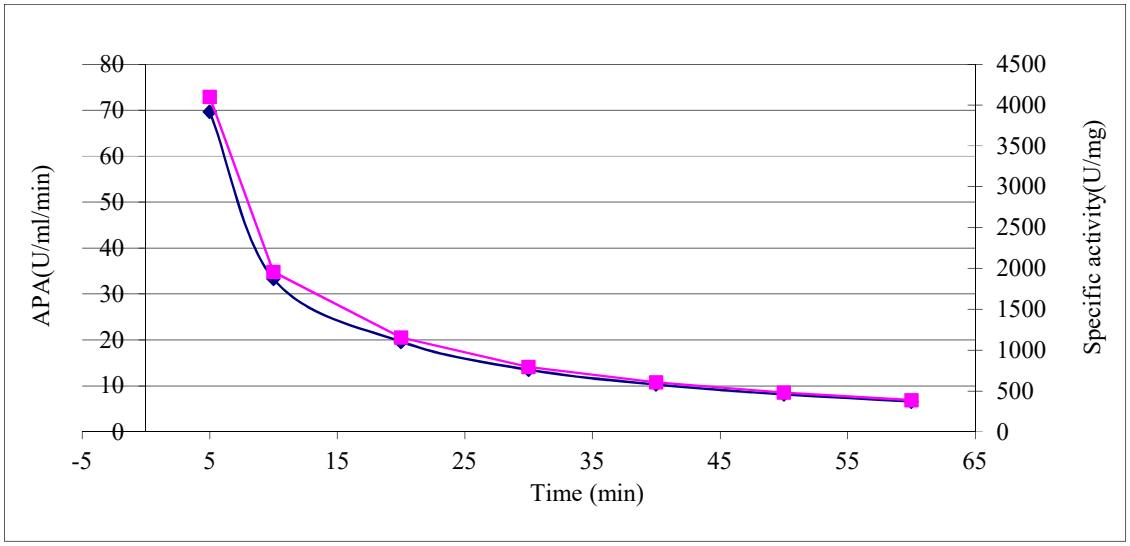

Fig. 6. Evaluation of reaction time on the activity rate of purified alkaline protease produced by *B.t. aizawai HD283*.

3.7. Effect of substrate concentration on purified AP activity produced by B.t. aizawai HD283.

The effect of different substrate concentrations on the rate of the AP catalyzed reactions was studied, in Figure (7), Km and Vmax were illustrated in Figure (8).

Roshdy *et al.* (2023b) illustrated that, the highest maximum velocity (Vmax) was obtained upon using casein as a substrate amounting to 31.821 µg tyrosine/ mL/ min. Determination of Michaelis constant (Km) using the hyperbolic function of Michaelis-Menten Plot of substrate concentration vs. reaction velocity as well as from Lineweaver-Burk reciprocal plot revealed Km values of 0.833%, w/v casein concentration.

Niyonzima *et al.* (2015) reported Km and Vmax of purified alkaline protease were 5.4 mg/ ml and 12.8 U/ml respectively. Km and Vmax of Bacillus thuringiensis alkaline protease were 0.072% (w/v) and 7.952 U/ml respectively (Sugumaran *et al.,* 2012). El-Shazly *et al.* (2024) reported that, Km 19.73 mM and Vmax 56.7 mmol/min/L.

The results of this study have promising probable practical applications in several industrial sectors:

Detergent Industry: The enzyme's activation by H_2O_2 (used as a bleach in detergents) and its thermal stability at 70°C are ideal properties for use in bio-laundry detergents, as they can efficiently remove protein stains under harsh conditions (Mahakhan *et al*., 2023).

Leather Industry: The enzyme's ability to function at an alkaline pH (pH 10) makes it suitable for dehairing in the tanning industry, a process that requires alkaline and thermal conditions (Shaikh *et al*., 2018).

Bioremediation: Inhibiting the enzyme with EDTA (a metal chelator) can be used as a mechanism to control its activity when needed, for example, in the treatment of industrial wastewater containing protein residues, allowing the enzyme reaction to be stopped at the appropriate time. Food industries:

Enzyme activation by calcium and magnesium ions (naturally present in some foods) can be exploited to enhance meat tenderization or protein fermentation in the food industry (Roshdy *et al*., 2022).

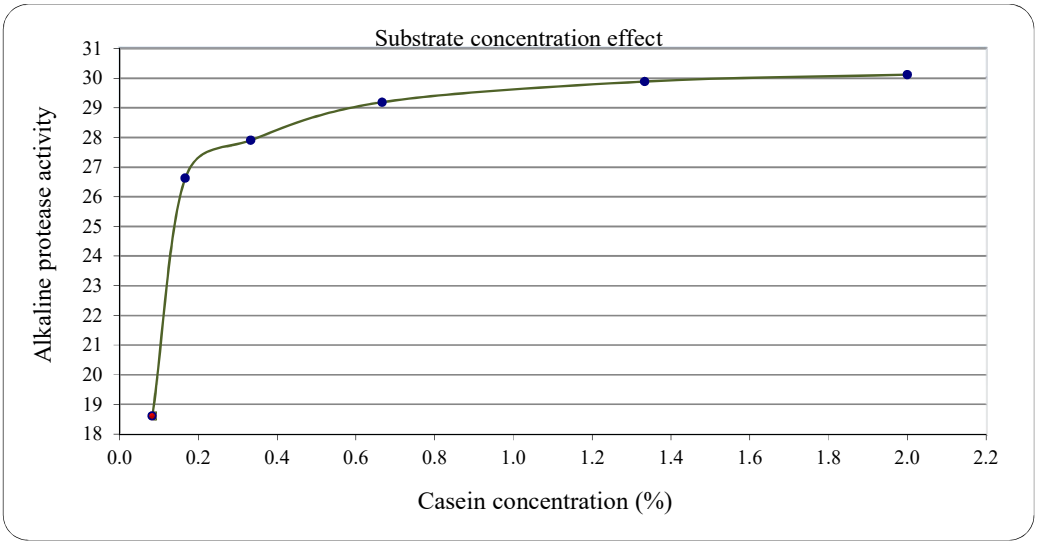

Fig. 7: Evaluation of substrate concentration change on the activity of purified alkaline protease produced by *B.t. aizawai HD283*.

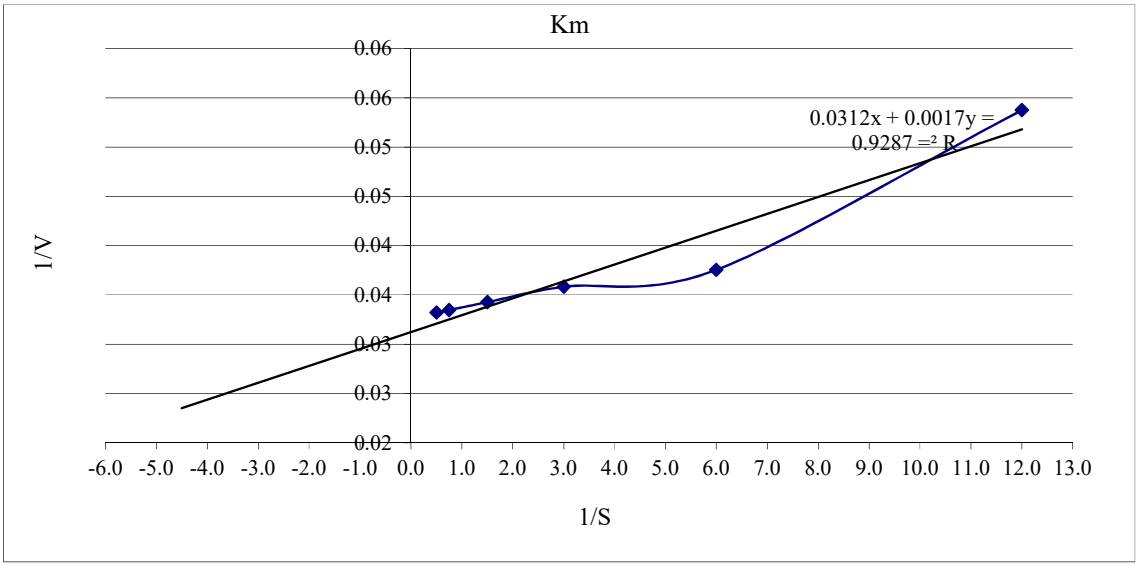

Fig. 8: Lineweaver-Burk Plot for measuring K_m and V_{max} of purified alkaline protease produced by *B.t. aizawai HD283*.

4. Conclusion

Classification and Characterization:

An alkaline protease from *B. thuringiensis aizawai* HD283 was successfully purified and characterized. The study confirmed that it belongs to the thermophilic metallo-alkaline protease family, based on its inhibition by EDTA, activation by metal ions, and thermal stability. The enzyme exhibited promising kinetic properties (Vmax, 30.116 µg/min/mL, Km, 5.448%), making it effective in casein hydrolysis.

Unique Characteristics:

The enzyme was characterized by its unprecedented activation by the serine protease inhibitor PMSF and its strong activation by manganese ions (over 700%), indicating an atypical catalytic mechanism and highlighting the role of metal ions as key cofactors.

Applications:

The enzyme demonstrated exceptional compatibility with harsh conditions, maintaining its high activity in the presence of oxidizing agents (H_2O_2), across a wide pH range (pH 8-10), and at high temperatures (up to 70°C). These properties make it an ideal candidate for use in the biodetergent, leather tanning, and food processing industries, reducing reliance on harmful chemicals and contributing to environmental sustainability.

Future Prospects:

The study recommends further investigation to understand the molecular mechanism of PMSF-induced abrupt activation, by studying the enzyme's crystal structure using techniques such as X-ray crystallography. It also suggests conducting enzyme immobilization studies to enhance its stability and reusability in ongoing industrial applications, and testing its efficiency in breaking down various proteins and environmental pollutants, which could open new avenues for its use in bioremediation.

Conflicts of Interest

No conflict of interest.

Abbreviation List

Alkaline protease (AP)
Alkaline protease Activity (APA)
Bacillus thuringiensis (Bt)
DFP (Di- isopropyl Floro Phosphate)
EDTA (Ethelene Di amine Tetra Acetic acid)
IP (Institute Pasteur)
PMSF (Phenyl Methyl Sulfonyl Floride)
SSF (Solid State Fermentation)

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