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Production and characterization of extracellular protease from horse fecal–originated *Bacillus amyloliquefaciens* SLBD

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ABSTRACT

Proteases are valuable industrial enzymes, yet most commercial preparations in Indonesia remain imported, underscoring the need to identify local, efficient producers. This study reports, for the first time, the production and characterization of an extracellular protease from *Bacillus amyloliquefaciens* SLBD, isolated from horse feces. The crude extracellular enzyme was collected at different growth phases and evaluated for proteolytic index, specific activity, and catalytic properties using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate. The crude enzyme exhibited a specific activity of 8.82 U mg^{-1}, Km of 0.519 mM, Vmax of 192.31 μmol min^{-1}, and kcat/Km of 1.23×10^5 M^{-1} s^{-1}. Optimal activity was observed at pH 7–8 and 50 °C, with more than 80% stability retained between pH 6–10 and temperatures up to 60 °C. Activity was moderately affected by organic solvents but remained above 70% in methanol and ethanol, and was largely unaffected by nonionic detergents. Metal ions such as Zn^{2+} slightly enhanced activity, whereas Hg^{2+} and EDTA caused strong inhibition, implying partial metal dependence. Collectively, the enzyme demonstrates broad operational stability and tolerance to diverse physicochemical conditions. The findings highlight *B. amyloliquefaciens* SLBD as a promising feces-derived strain producing a robust, solvent- and detergent-tolerant extracellular protease suitable for biotechnological and industrial applications.

Keywords: Bacillus amyloliquefaciens SLBD; Extracellular protease; Enzyme characterization; Kinetic parameters; Faecal bacteria

INTRODUCTION

Proteases are among the most important industrial enzymes, accounting for nearly 60% of the global enzyme market (Kumari *et al.*, 2015). They catalyze the hydrolysis of peptide bonds in proteins, thereby generating peptides and free amino acids that serve diverse applications in the detergent, leather, food, pharmaceutical, and livestock feed industries (Razali *et al*., 2021a; Aruna *et al*., 2023; Chan *et al*., 2022). In the livestock sector, proteases are particularly valued for improving protein digestibility, nutrient absorption, and feed conversion efficiency in poultry and ruminants (Khairunisa *et al.*, 2016; Hou *et al.*, 2017; Philipps-Wiemann, 2018; Rodríguez -Soriano *et al.*, 2025). Owing to their broad substrate specificity and catalytic versatility, proteases have become indispensable in modern bioprocessing and sustainable bioindustry (Aruna *et*

al., 2023).

Globally, the demand for industrial proteases continues to grow, driven by the expansion of the detergent and animal feed sectors, which are projected to reach multi-billion-dollar markets by 2030 (Chen *et al.*, 2024). In Indonesia, as reported by Sanjaya *et al.*, (2024) the majority of enzymes used in industry—including proteases—are still imported, leading to high dependency on foreign suppliers and increased production costs. This situation emphasizes the urgent need to explore and develop indigenous enzyme sources that are both cost-effective and adapted to local industrial conditions. Consequently, there has been growing scientific attention toward isolating novel protease-producing microorganisms that exhibit unique catalytic traits suitable for diverse process environments such as high temperature, alkaline pH, or high salt conditions (Uttatree and Charoenpanich, 2018).

Proteases can be derived from plants, animals, and microorganisms; however, bacterial proteases are generally preferred due to their high productivity, ease of genetic manipulation, and broad catalytic adaptability (Song *et al.*, 2023). Within bacteria, *Bacillus* and *Pseudomonas* groups are particularly recognized as prolific producers of extracellular proteases . Members such as *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus cereus* have long been exploited for large-scale production of alkaline and neutral proteases owing to their robust secretion systems and stability under harsh process conditions (Contesini *et al.*, 2018; Harwood and Kikuchi, 2022).

In the search for novel or unique proteases, bacteria isolated from extreme or unconventional environments are often promising sources, as their enzymatic systems have evolved to function under specialized ecological pressures (Littlechild, 2015). Shiddiq *et al.* (2025) have isolated a cellulolytic bacterium, *Bacillus amyloliquefaciens* SLBD, from an unconventional niche—fresh animal feces. The fecal environment provides a unique selective pressure characterized by high organic load, fluctuating pH, and variable temperature, fostering microbial communities with versatile enzymatic capabilities for degrading macromolecules (Ndlela and Schmidt, 2016). Although earlier enzymatic screening by Shiddiq *et al.* (2025) demonstrated remarkable cellulolytic activity in this isolate, whole-genome sequencing subsequently revealed the presence of multiple open reading frames annotated as protease-encoding genes. This finding suggested that *B. amyloliquefaciens* SLBD might also produce extracellular proteases, a hypothesis that had not been experimentally confirmed.

Previous studies have documented several *Bacillus* species as efficient protease producers (Contesini *et al.*, 2018; Harwood and Kikuchi, 2022); however, reports on proteases from *B. amyloliquefaciens* remain limited and fragmentary, particularly from isolates originating from animal feces. The potential of this species to produce extracellular proteases with distinct catalytic and stability profiles remains underexplored. Addressing this knowledge gap could contribute valuable information on microbial diversity and expand the repertoire of industrially relevant proteases. Therefore, the present study aimed to confirm and characterize the production of extracellular protease by *Bacillus amyloliquefaciens* SLBD. For the first time, the proteolytic activity of an *amyloliquefaciens* strain originating from animal feces is demonstrated, including its production pattern across different growth stages. Furthermore, the effects of pH, temperature, and metal ions on enzyme activity and stability were systematically investigated. The findings provide new insight into the catalytic potential of *B. amyloliquefaciens* SLBD and highlight its promise as a novel source of industrial protease.

MATERIALS AND METHODS

Preparation of Extracellular Crude Enzyme at Different Growth Phases

The extracellular crude enzyme (CE) of *Bacillus amyloliquefaciens* SLBD was prepared following a modified protocol adapted from Lindang *et al.*, (2021), Razali *et al.* (2020) and Yahya *et al.*, (2025). The bacterial strain was cultivated in 500 mL of Luria–Bertani (LB) broth (Merck, Darmstadt, Germany) and incubated at 37 °C under continuous shaking. To monitor bacterial growth, aliquots of the culture were collected at regular intervals, and the optical density (OD) was measured at 600 nm using a spectrophotometer to construct a growth curve. At each sampling point, a portion of the culture

was also taken for enzyme extraction. Cultivation was terminated when the culture has reached the end of stationary phase of early death phase. The collected samples were centrifuged at 8000 × g for 15 minutes at room temperature to obtain the cell-free supernatant (CFM). Each supernatant was filtered through a 0.22 µm membrane to remove residual cells, yielding the extracellular crude enzyme fraction corresponding to that specific growth phase. These CE samples were subsequently used for protease activity assays to determine the enzyme production profile throughout bacterial growth.

Proteolytic Index

The proteolytic activity of the extracellular fraction was determined using the disc diffusion method on casein agar plates according to Yahya *et al.* (2025) with adjustment for volume of proteases and the use of casein as substrate. Briefly, casein agar was prepared as the protein substrate, allowing visualization of proteolysis through the formation of a clear halo zone. Sterile paper discs (6 mm in diameter) were placed on the agar surface and each disc was spotted with an equal volume (50 µL) of the cell-free medium (CFM) collected from different growth phases of *Bacillus amyloliquefaciens* SLBD. A known protease solution and sterile LB broth were used as the positive and negative controls, respectively. The plates were incubated at 37 °C for 24 hours, followed by measurement of the diameters of the clear zones, including the discs, in two perpendicular directions using a digital caliper, and the average values were recorded. The Proteolytic Index (PI) was expressed according to the following formula:

$$\text{Proteolitix Index (PI)} = \frac{\text{Halo zone diameter (mm)}}{\text{paper disc diameter (mm)}}$$

Protease Activity Assay Using Suc-AAPF-pNA Substrate

The specific proteolytic activity of the crude enzyme was determined using the chromogenic substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA) as described previously by Falkenberg *et al.*, (2022) with slight modifications on the amount of enzyme used in the assay and the use of UV-Vis spectrophotometer, instead of microplate reader. The assay was carried out at 30°C in 100 mM Tris–HCl buffer (pH 8.0). A stock solution of Suc-AAPF-pNA (110 mM) was prepared in dimethyl sulfoxide (DMSO) and diluted 1:100 in the reaction mixture immediately before use. The enzymatic reaction was initiated by adding 0 to 2 mg/mL of crude enzyme to the substrate solution and monitored spectrophotometrically at 410 nm to detect the release of p-nitroaniline. Absorbance was recorded every 30 seconds for 5 minutes using a UV–Vis spectrophotometer with a 1 mL cuvette. The molar extinction coefficient of p-nitroaniline (ε_{410} = 8.48 $mM^{-1} \bullet cm^{-1}$) was used to calculate product formation. One unit (U) of protease activity was defined as the amount of enzyme required to liberate 1 µmol of p-nitroaniline per minute under the specified assay conditions. The enzyme's specific activity was expressed as units per milligram of protein (U mg^{-1}). All measurements were performed in triplicate, and the mean values ± standard deviations were reported.

Determination of Kinetic Parameters

The kinetic parameters of the protease were evaluated using the synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) under the same reaction conditions described in the protease activity assay. Enzyme reactions were carried out at 37 °C in 100 mM Tris–HCl buffer (pH 8.6), with varying substrate concentrations up to 2.5 mM. The substrate was freshly diluted from a 110 mM stock solution prepared in DMSO prior to each assay. The reactions were initiated by the addition of the crude enzyme, and the increase in absorbance due to the release of *p*-nitroaniline was continuously recorded at 410 nm for 5 minutes. The initial reaction rates (Vo) were calculated from the linear portion of the absorbance–time curve using the molar extinction coefficient of *p*-nitroaniline (ε_{410} = 8.48 $mM^{-1} \cdot cm^{-1}$). The Michaelis–Menten kinetic parameters, Km (Michaelis constant) and Vmax (maximum velocity), were determined according to Razali *et al.* (2022). The kcat value was calculated based on the enzyme concentration used in the assay, and the catalytic efficiency (kcat/Km) was derived accordingly.

pH and Temperature-dependence and Stability

The effects of pH and temperature on protease activity were determined using the synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA), following the procedure described Falkenberg *et al.* (2022) for the specific activity assay with minor modifications. For the determination of optimum pH, the enzyme activity assay was performed at 37 °C using 100 mM buffer systems covering different pH ranges: citrate–phosphate (pH 3.0–6.0), phosphate (pH 7.0–8.0), Tris–HCl (pH 8.5–9.0), and glycine–NaOH (pH 9.0–11.0). The reactions were conducted as described in the specific activity assay, and the absorbance at 410 nm was measured to determine the amount of *p*-nitroaniline released.

To determine the optimum temperature, the enzyme assay was carried out at various incubation temperatures ranging from 20 to 80 °C under the optimal pH previously established. The relative activity was calculated by comparing the activity at each pH or temperature to the highest activity obtained in the respective series, which was defined as 100%. For pH stability, aliquots of the crude enzyme were pre-incubated in the same buffer systems as described above (without substrate) at the respective pH values for 30 minutes at 30 °C. Similarly, thermal stability was evaluated by pre-incubating enzyme samples at different temperatures (ranging from 30 to 80 °C) for 30 minutes in Tris–HCl buffer (pH 8.6). After the pre-incubation period, the residual protease activity was immediately measured under the standard assay conditions using Suc-AAPF-pNA as substrate. The enzyme activity at each pH or temperature condition was expressed as relative activity, in which the highest observed activity within the respective set of conditions was defined as 100%. All other activity values were then normalized proportionally against this maximum value, allowing comparison of enzyme performance across different pH or temperature conditions. The following formula was used to calculate the relative activity (%) :

$$\text{Relative Activity (\%)} = \frac{\text{Ai}}{\text{Amax}} \times 100\%$$

Where:

Ai = enzyme activity at a specific pH or temperature ($U.mg^{-1}$)

Amax = highest observed enzyme activity among all tested conditions ($U.mg^{-1}$), defined as 100%

Effect of Metal Ions and EDTA on Protease Activity

The influence of selected metal ions and the chelating agent ethylenediaminetetraacetic acid (EDTA) on protease activity was evaluated under standard assay conditions modified from Majeed *et al.*, (2024) and Falkenberg *et al.* (2022). Metal ions including Hg^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+} and EDTA were tested using their respective analytical-grade salts ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, $MnSO_4$, $MgCl_2$, $NiCl_2$, and $ZnCl_2$). Each metal ion or EDTA was added to the enzyme solution at a final concentration of 5 mM and pre -incubated with the crude enzyme for 30 minutes. Following incubation, the residual protease activity was assayed using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) as the substrate, under the same conditions as described for the specific activity determination. A reaction mixture without any additives served as the control, and its activity was defined as 100%. The relative enzyme activity in the presence of each metal ion or EDTA was expressed as a percentage of the control activity (without metal ion or EDTA), according to the following formula:

$$\text{Relative Activity (\%)} = \frac{\text{Ai}}{\text{Acon}} \times 100\%$$

Where:

Ai = Observed activity of the enzyme under metal ion or EDTA treatment ($U.mg^{-1}$)

Acon = Observed activity of the enzyme without treatment ($U.mg^{-1}$), defined as 100%

Effect of Organic Solvents, Surfactants, and Oxidant on Protease Activity

The stability of the protease toward various organic solvents, surfactants, and oxidizing agents was evaluated under standard assay conditions modified from Majeed *et al.* (2024) and Falkenberg *et al.* (2022) with minor modifications. Organic solvents including ethanol, dimethyl sulfoxide (DMSO), hexane, and methanol, as well as the surfactants sodium dodecyl sulfate (SDS), Triton X-100, Tween 20, and Tween 80, and the oxidizing agent hydrogen peroxide (H_2O_2), were tested for their effects on enzyme activity. Each compound was added to the crude enzyme solution at a final concentration of 10%

(v/v) in a total reaction volume of 1 mL. Mixtures containing surfactants and H_2O_2 were incubated for 30 minutes, while those containing organic solvents were incubated for 10 minutes, all at 50 °C and pH 8.0. Following incubation, 1 mL of substrate solution containing *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) was added to initiate the reaction, and enzyme activity was measured under the standard assay conditions described previously. The relative enzyme activity in the presence of organic solvent or surfactant or oxidant was expressed as a percentage of the control activity (without organic solvent or surfactant or oxidant), according to the following formula:

$$\text{Relative Activity } (\%) = \frac{\text{Ai}}{\text{Acon}} \times 100\%$$

Where:

Ai = Observed activity of the enzyme under organic solvent or surfactant or oxidant ($U.mg^{-1}$)

Acon = Observed activity of the enzyme without treatment ($U.mg^{-1}$), defined as 100%.

RESULTS AND DISCUSSION

Growth Profile and Proteolytic Activity of *Bacillus amyloliquefaciens* SLBD

The growth pattern and proteolytic activity of *Bacillus amyloliquefaciens* SLBD are shown in Figure 1. The bacterial growth, represented by the optical density at 600 nm (Abs_{600}), exhibited a typical sigmoidal curve with a lag phase during the first 4–6 hours, followed by a rapid exponential increase between 6 and 14 hours, and reaching the stationary phase after approximately 16 hours of incubation. In parallel, the proteolytic index (PI) began to increase after 6 hours, peaked at 12 hours, and subsequently declined, even though cell density remained stable throughout the later incubation period. As shown in Figure 1, no significant difference ($P > 0.05$) was observed in the PI values of the extracts obtained up to 12 hours of growth, indicating that the maximum proteolytic activity was reached around this time, followed by a significant decline after 20 hours of incubation.

The observed pattern reflects a growth-associated enzyme production profile, in which extracellular protease synthesis occurs primarily during the late exponential and early stationary phases. At this stage, cell proliferation begins to slow due to nutrient limitation, especially of carbon and nitrogen sources, which often triggers the secretion of extracellular hydrolytic enzymes such as proteases, cellulases, and amylases to degrade macromolecules in the surrounding environment (Gupta *et al.*, 2002). This strategy enables the bacterium to recycle nutrients and sustain metabolic activity under nutrient-depleted conditions. In *Bacillus* species, protease production is tightly regulated by a complex network of global regulatory systems, including DegS–DegU, Spo0A, and AbrB, which respond to nutrient signals and population density (Shiwa *et al.*, 2015; (Velho *et al.*, 2013; Farhangi *et al.*, 2024). During the transition to stationary phase, the accumulation of intracellular signalling molecules such as cyclic-di-GMP and quorum-sensing peptides induce the expression of genes responsible to extracellular enzyme synthesis. Therefore, the peak in protease production at 12 hours coincides with the period when cells sense nutrient exhaustion and initiate secondary metabolite production, including extracellular proteases, to enhance survival and competitiveness in the environment (Rao *et al.*, 1998). The subsequent decline in proteolytic activity observed after 14–16 hours may result from several factors. First, prolonged incubation can lead to au-

Table 1. Kinetic Parameters of the Unpurified Protease Extract from *Bacillus amyloliquefaciens* SLBD using the AAPF Synthetic Substrate.

Kinetic Parameter	Value
Vmax (µmol·min⁻ ¹)	192.31
Km (mM)	0.519
kcat (s^{-1})	64.1
kcat/Km ($M^{-1}·s^{-1}$)	1.23×10^5

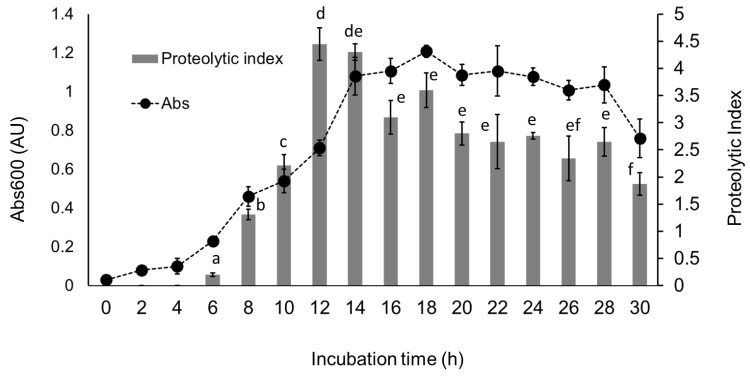

Figure 1. Growth curve and proteolytic index of *Bacillus amyloliquefaciens* SLBD during cultivation. Bacterial growth and proteolytic index were monitored over 36 h in basal medium containing casein substrate. The proteolytic index was determined by clear-zone diameter on casein agar plates.

tolysis or degradation of secreted proteases by other proteolytic enzymes present in the culture supernatant. Second, changes in pH due to metabolic by-product accumulation (e.g., ammonia or organic acids) may alter enzyme stability and reduce catalytic efficiency. Third, feedback repression mechanisms, in which high concentrations of free amino acids or small peptides inhibit further enzyme synthesis, are known to downregulate protease gene expression during the stationary phase (Antelmann *et al.*, 2003; Chang and Lee, 2020; Razali *et al.*, 2021).

Although some reports indicated that *Bacillus* species produced proteases optimally during the stationary phase (Harwood and Kikuchi, 2022; Majeed *et al.*, 2024; Zhang *et al.*, 2022), and Sharma *et al.* (2017) reported that the variations in the timing of enzyme production are also commonly observed among species. These differences are likely attributed to distinct molecular regulatory mechanisms. In general, however, bacteria have been reported to produce maximum level of protease during the late exponential or, more commonly, the stationary phase. Nevertheless, protease synthesis may also occur at earlier stages, as these enzymes play essential roles in various metabolic functions. In certain bacteria, early secretion of protease facilitates autolytic turnover and biofilm detachment, thereby enhancing nutrient availability and ecological adaptability. Consistent with these observations, protease activity in *Bacillus amyloliquefaciens* SLBD was detectable throughout almost all growth phases, except during the lag phase, where its absence is likely due to the limited protein synthesis at that stage. Collectively, these results indicate that *B. amyloliquefaciens* SLBD exhibits a growth-associated and nutrient-responsive protease production profile, where enzyme synthesis is triggered by nutrient depletion and regulated through quorum-sensing and global transcriptional control. The 12-hour incubation point is thus the most suitable period for harvesting the crude enzyme to obtain the highest proteolytic yield before degradation and feedback inhibition reduce the enzyme concentration in the culture medium.

Specific Activity of Crude Protease from *Bacillus amyloliquefaciens* SLBD

The specific activity of the extracellular protease from *Bacillus amyloliquefaciens* SLBD was determined based on the linear correlation between enzyme concentration and product formation rate using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF) as substrate. As illustrated in Figure 2, the crude enzyme exhibited a specific activity of approximately 8.82 U mg^{-1}. This specific activity represents the catalytic efficiency of the unpurified extracellular fraction and reflects the total activity derived from multiple proteolytic isoforms secreted by *B. amyloliquefaciens* SLBD. In general, the specific activity of *Bacillus proteases* can vary widely among species and strains. For instance, *Bacillus* sp. DA 5.2.3 exhibited a specific activity of 40.9 $U.mg^{-1}$ (Jamilah *et al.*, 2009), *Bacillus subtilis* K-5 showed 111.56–124.72 $U.mg^{-1}$ (Shad *et al.*, 2024), a *B. cereus* strain isolated from cooked

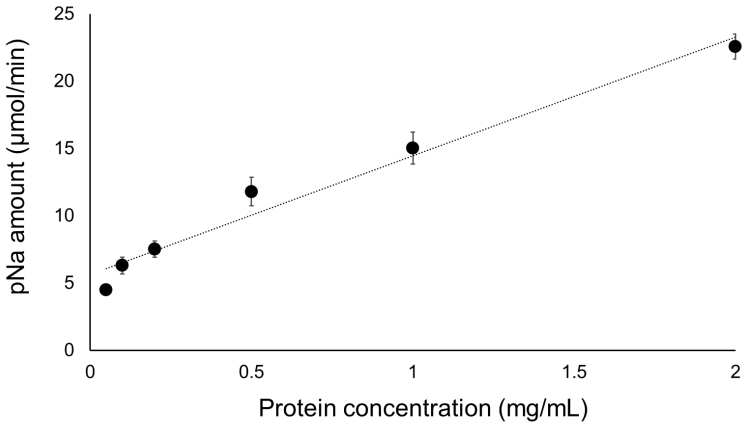

Figure 2. Effect of protein concentration of the unpurified extract from *B. amyloliquefaciens* SLBD on its catalytic activity toward the AAPF synthetic substrate. Enzyme activity was quantified based on the rate of p-nitroaniline (pNA) release per minute during substrate hydrolysis.

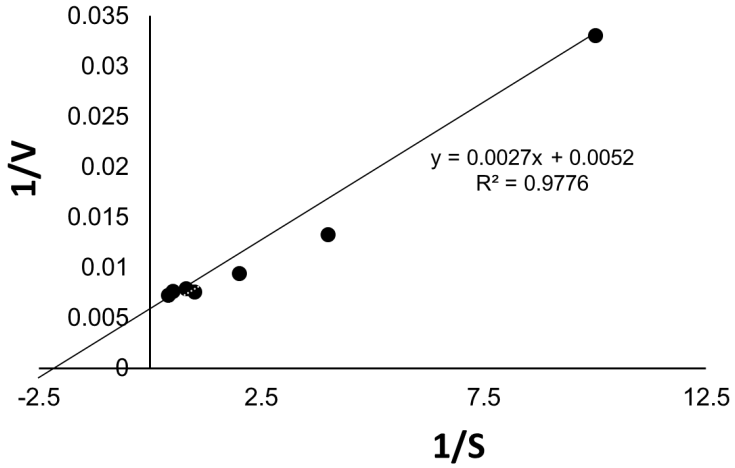

Figure 3. Effect of pH on protease activity (A) and stability (B) of the unpurified extract from *B. amyloliquefaciens* SLBD. The range of pH used in the measurement was 3.0 – 11.0

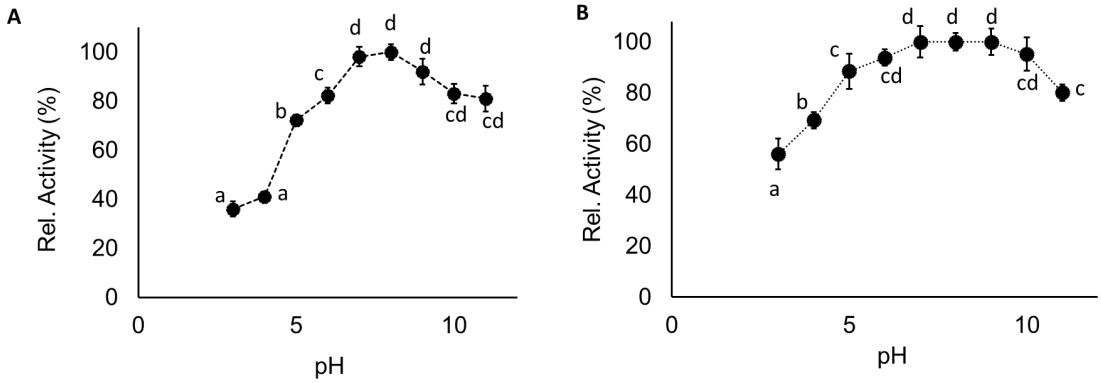

Figure 4. Lineweaver–Burk plot of the unpurified protease extract from *B. amyloliquefaciens* SLBD using the AAPF synthetic substrate. The different letters adjacent to the means indicate significant differences at $P < 0.05$.

food recorded 1.20 U.mg^{-1} (Salam Khattab and Al-Nazzal, 2024), while *Bacillus sp*. DEM05 reached as high as 1075 U.mg^{-1} (Mohamadi *et al.*, 2021). Such variations are strongly influenced by factors including the degree of enzyme purification, the assay conditions, and the inherent physiological characteristics of each *Bacillus* strain. Patil and Jadhav (2017) further demonstrated that among 28 *Bacillus* isolates obtained from dairy industry soil, the specific activities differed markedly—confirming that each species possesses distinct protease profiles in both type and quantity, resulting in diverse activity magnitudes.

However, the intercept value (5.62 μmol mL^{-1} min^{-1}) indicates the presence of background or non-specific hydrolytic activity, which is typical for crude enzyme preparations. This residual activity was likely attributed to co-extracted host proteins, co-purified non-specific esterases or lipases, and/or minor spontaneous substrate hydrolysis under the assay conditions, as also implied by Razali *et al.* (2021). Contamination during experimental procedures was minimized through the implementation of aseptic techniques and appropriate blank controls, and was therefore considered unlikely to be a major contributing factor. Accordingly, all measured activities were corrected by subtracting the intercept value. To improve assay specificity in future studies, additional purification steps (e.g., affinity or size-exclusion chromatography), as well as the inclusion of heat-inactivated controls or specific inhibitors, are recommended to better discriminate between true enzymatic activity and non-specific hydrolysis (Obeng *et al.*, 2017; Goh *et al.*, 2018). Moreover, these steps are expected to enhance the specific activity by removing non-proteolytic proteins and concentrating the active component. Previous reports on *Bacillus* protease purification have shown that specific activity often increases dramatically—ranging from several-fold to over a hundred-fold—after just a single chromatographic step (Lakshmi *et al.*, 2018; Ullah *et al.*, 2022). This substantial enhancement reflects the effectiveness of chromatographic separation in enriching the active enzyme fraction and eliminating non-enzymatic proteins. The observed activity also demonstrates the enzyme's suitability for catalytic applications such as protein hydrolysis and feed processing, given its efficient turnover rate. When expressed relative to reaction duration (5 min), the enzyme produced approximately 44.1 μmol p-nitroaniline mL^{-1} in total, confirming its high potential for further bioprocess optimization and partial purification for industrial use.

Kinetic Characterization of *Bacillus amyloliquefaciens* SLBD Protease

The kinetic behavior of the extracellular protease from *Bacillus amyloliquefaciens* SLBD was analyzed using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) as substrate, and the kinetic constants were derived from the Lineweaver–Burk plot (Figure 4, Table 1). The enzyme exhibited a maximum velocity (Vmax) of 192.31 μmol·min^{-1}, an apparent Km of 0.519 mM, a turnover number (kcat) of 64.1 s^{-1}, and an overall catalytic efficiency (kcat/Km) of 1.23 × 10^{5} M^{-1}·s^{-1}. It is important to note that these parameters were obtained using the crude extracellular enzyme (unpurified extract), which represents the collective catalytic contribution of secreted enzymes in the culture supernatant. In line with the observed intercept value (5.62 μmol mL^{-1} min^{-1}), indicating background or non-specific hydrolytic activity, the derived kinetic parameters may also be influenced by such contributions. This background activity likely arises from co-extracted proteins, non-specific proteases, or minor spontaneous substrate hydrolysis under assay conditions. Consequently, the reported kinetic constants should be interpreted as apparent values, reflecting the combined effects of target and non-specific catalytic activities. Further purification and the inclusion of appropriate controls are therefore recommended to obtain more accurate intrinsic kinetic parameters of the target enzyme. As such, the kinetic values reflect the apparent activity of the protease mixture rather than a single purified isoenzyme. The presence of other extracellular proteins or minor contaminating enzymes could influence the observed kinetics by altering substrate accessibility or local microenvironmental conditions (e.g., ionic strength, pH buffering, or protein–protein interactions). Consequently, the measured Km and Vmax likely represent aggregate kinetic behavior, potentially underestimating the true catalytic efficiency of the target protease.

Despite these limitations, the obtained Km

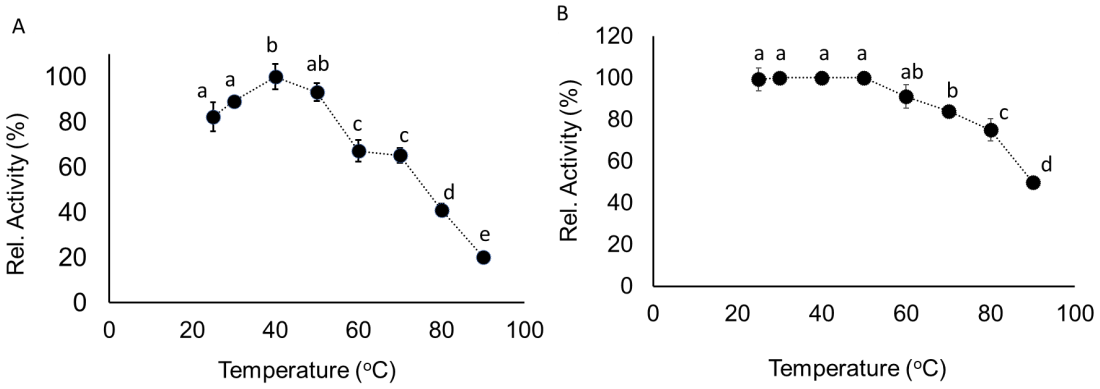

Figure 5. Effect of temperature on protease activity (A) and stability (B) of the unpurified extract from B. amyloliquefaciens SLBD. The range of temperature used in the measurement was 25 – 90 °C. The different letters adjacent to the means indicate significant differences at $P < 0.05$.

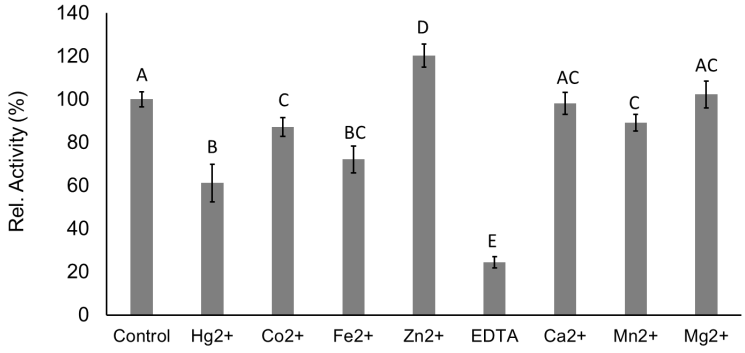

Figure 6. Effect of metal ions and chelating agent on protease activity of the unpurified extract from B. amyloliquefaciens SLBD. The different letters above the bars indicate significant differences at $P < 0.01$.

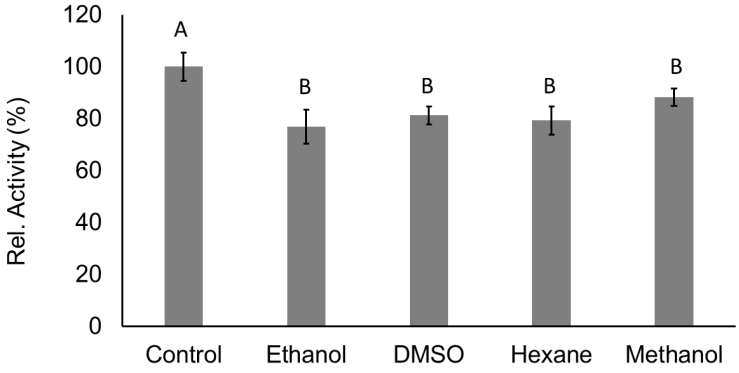

Figure 7. Effect of organic solvent and oxidizing agent on protease activity of the unpurified extract from *B. amyloliquefaciens* SLBD. The different letters above the bars indicate significant differences at $P < 0.01$.

(0.519 mM) indicates a strong substrate affinity toward the Suc-AAPF-pNA peptide, consistent with those reported for extracellular serine proteases from *Bacillus* species, which typically range from 0.2 to 1.0 mM for synthetic chromogenic substrates (Rao *et al.*, 1998; Gupta *et al.*, 2002). The moderate kcat (64.1 s^{-1}) further suggests that the enzyme exhibits efficient substrate turnover, comparable to unpurified alkaline proteases from other *Bacillus* isolates. The relatively high catalytic efficiency (1.23×10^5 $M^{-1} \cdot s^{-1}$) implies that the enzyme performs rapid and specific hydrolysis of peptide bonds even without purification, reflecting substantial catalytic potential in its native extracellular form.

The ability of the crude enzyme to achieve such kinetic performance suggests that *B. amyloliquefaciens* SLBD secretes proteases in functional, properly folded conformations, possibly stabilized by extracellular cofactors or metal ions naturally present in the culture medium. Furthermore, the enzyme's effective turnover under mild assay conditions (pH 8.6, 30 °C) and preference for a synthetic peptide substrate implies that it may share functional characteristics with serine-type proteases, which often exhibit strong activity toward AAPF-type substrates. However, confirmation of this classification would require further validation through inhibitor assays (e.g., PMSF sensitivity) or genomic annotation of protease-coding genes. Overall, the kinetic parameters derived from the crude enzyme preparation indicate that *B. amyloliquefaciens* SLBD produces a highly active extracellular protease system with notable affinity and turnover for peptide substrates. While purification is expected to refine these values and provide clearer mechanistic insight, the results already demonstrate strong biocatalytic potential, underscoring the enzyme's applicability for industrial processes where crude or semi-purified enzyme preparations are preferred for cost efficiency and robustness.

Effect of pH on Activity and Stability of *Bacillus amyloliquefaciens* SLBD Protease

The influence of pH on the catalytic performance and stability of the extracellular protease from *Bacillus amyloliquefaciens* SLBD is shown in Figure 4. Enzyme activity increased progressively from acidic to neutral conditions, reaching its maximum at pH 7–8, while a gradual decline was observed beyond this range. Statistical analysis indicated that activities at pH 7 and 8 were not significantly different ($P > 0.05$), suggesting that the enzyme maintains a broad optimum within the neutral to mildly alkaline range. Even at pH 9–10, the protease retained more than 80% of its relative activity, whereas strong acidity (pH 3–4) markedly reduced catalytic performance. The enzyme also demonstrated remarkable stability between pH 6 and 10, maintaining 90–100% of its residual activity after 30 minutes of pre-incubation, with a noticeable decline only under highly acidic or strongly alkaline conditions.

The neutral-to-alkaline optimum and wide operational stability window are typical features of many *Bacillus*-derived extracellular proteases (Saleem *et al.*, 2020; Majeed *et al.*, 2024). This pH behavior suggests that the enzyme's catalytic residues remain properly ionized for peptide bond cleavage in this range, and its tertiary structure is sufficiently rigid to resist conformational changes caused by pH fluctuations. Although the catalytic mechanism of SLBD protease has not yet been confirmed, its optimal performance in near-neutral to alkaline conditions hints at a possible serine-type protease, as these enzymes generally exhibit maximum activity within similar pH intervals due to the need for a deprotonated nucleophilic residue at the catalytic center (Rao *et al.*, 1998; Gupta *et al.*, 2002). The active site of serine proteases relies on a histidine-mediated charge relay system that functions efficiently under slightly basic environments; however, other protease types (e.g., metalloproteases) can display overlapping profiles, and therefore, further inhibitor or genomic analyses would be required to verify this assumption.

The enzyme's ability to remain active and stable across a wide pH spectrum demonstrates structural robustness and environmental adaptability. Such resilience is often associated with surface-exposed acidic residues that maintain electrostatic balance and preserve protein folding under varying proton concentrations (Saeki *et al.*, 2007). The gradual decline in activity observed at higher pH (≥10) could result from perturbation of ionic interactions within the catalytic pocket or partial unfolding of surface loops critical for substrate binding. Conversely, reduced activity under acidic conditions likely stems from protonation of critical residues involved in substrate anchoring

or catalysis, leading to suboptimal charge distribution and decreased substrate accessibility. Collectively, these results indicate that the *B. amyloliquefaciens* SLBD protease is a pH-tolerant enzyme with a neutral–alkaline optimum (pH 7–8) and broad operational stability (pH 6–10). These characteristics suggest its potential suitability for biotechnological processes that require stable catalytic activity under mildly alkaline environments, such as detergent formulations, peptide hydrolysis, or animal feed digestion enhancement.

Effect of Temperature on Activity and Stability

The effect of temperature on enzyme activity and stability of the extracellular protease from *Bacillus amyloliquefaciens* SLBD is illustrated in Figure 5A. Enzymatic activity increased progressively from 25 °C to 40 °C, reaching a maximum at 50 °C, and then gradually decreased at higher temperatures. Statistical analysis revealed that activities between 40 °C and 50 °C were not significantly different ($P > 0.05$), indicating that the protease maintains near-optimal catalytic efficiency in this range. Beyond 50 °C, enzyme activity dropped sharply, retaining approximately 65% at 60 °C and less than 30% at 80 °C, with a substantial loss at 90 °C. The temperature stability profile (Figure 5B) demonstrated that the enzyme retained >95% of its initial activity after 30 min incubation at 25–50 °C, and approximately 80% activity at 60 °C. However, prolonged exposure to 70 °C and above led to a marked decline in residual activity, suggesting thermal denaturation of the protein structure. The enzyme was highly stable up to 50 °C ($P > 0.05$) and exhibited significant activity loss only beyond 60 °C.

This temperature-dependent pattern is consistent with many *Bacillus*-derived proteases, which often show moderate thermophilicity and maintain functionality between 40 °C and 60 °C (Septianingrum *et al.*, 2024; Amin *et al.*, 2025; Gupta *et al.*, 2002; Majeed *et al.*, 2024). The optimum at 50 °C reflects a balance between catalytic turnover and thermal stability of the enzyme's tertiary conformation. Increased temperature generally enhances enzyme–substrate collision frequency and reduces substrate viscosity, promoting faster catalysis. However, at temperatures above the stability threshold, disruption of non-covalent interactions—such as hydrogen bonds and hydrophobic packing—leads to partial unfolding and loss of active-site geometry, thereby reducing activity (Rao *et al.*, 1998). Interestingly, comparative analyses of alkaline protease-producing *Bacillus* species have generally shown that most isolates are mesophilic, displaying optimal enzyme activity within a temperature range of 30–37 °C (Rai and Mukherjee, 2010; Mukherjee *et al.*, 2008; Abidi *et al.*, 2008). In contrast, the protease from *Bacillus amyloliquefaciens* SLBD demonstrated maximum activity at 50 °C, which is noticeably higher than the typical mesophilic range.

The broad stability observed up to 50–60 °C implies that the SLBD protease possesses a relatively rigid structure, possibly supported by stabilizing salt bridges, disulfide bonds, or bound divalent cations such as Ca^{2+}, which are known to enhance thermostability in *Bacillus* proteases

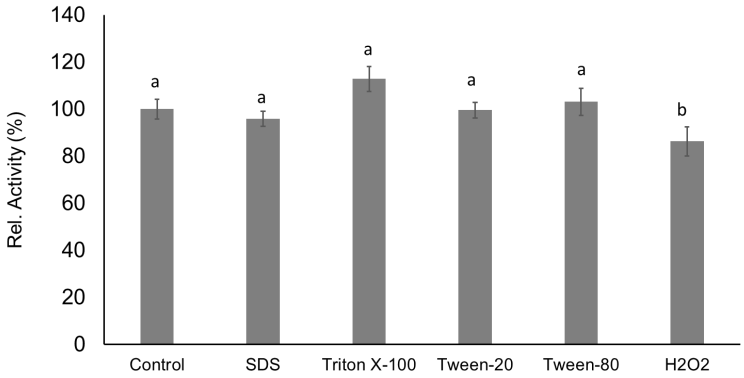

Figure 8. Effect of detergent and surfactants on protease activity of the unpurified extract from B. amyloliquefaciens SLBD. The different letters above the bars indicate significant differences at $P < 0.01$.

(Saeki *et al.*, 2007). This thermal resilience is advantageous for industrial and biotechnological applications that involve elevated processing temperatures, as higher operating temperatures improve substrate solubility, minimize microbial contamination, and increase reaction rates.

It should be emphasized that these results were obtained from the crude extracellular fraction, which contains a complex mixture of secreted proteins, cofactors, and potentially stabilizing components from the growth medium. Such components may contribute to the observed apparent thermostability, as interactions among proteins or with medium constituents (e.g., salts, calcium ions, or peptides) can protect the catalytic protein from heat-induced denaturation. Conversely, the presence of minor contaminant enzymes could influence the overall activity profile, causing deviations from the true intrinsic behavior of the purified protease. Thus, while the current data provide a reliable overview of the enzyme's performance in its native extracellular environment, further purification would be necessary to precisely delineate the intrinsic thermal characteristics of the protease itself. Nonetheless, the broad activity and stability range observed up to 60 °C, possibly supported by stabilizing salt bridges, disulfide bonds, or bound divalent cations such as Ca^{2+}, which are known to enhance thermostability in *Bacillus* proteases (Saeki *et al.*, 2007), implies that *B. amyloliquefaciens* SLBD secretes a moderately thermostable protease system with practical resilience under elevated temperatures. The ability to retain activity in the crude form also highlights its potential suitability for direct application without extensive purification, thereby lowering production costs for industrial enzyme formulations.

Effect of Metal Ions and Chelating Agent (EDTA) on Protease Activity of Crude Extract

The influence of selected metal ions and the chelating agent EDTA on the activity of the crude extracellular protease from *Bacillus amyloliquefaciens* SLBD is shown in Figure 6. Distinct statistical groupings (different letters above bars, $P < 0.01$) indicate significant differences among treatments. The addition of Zn^{2+} markedly enhanced protease activity, reaching approximately 120% of the control, while Hg^{2+} and EDTA produced the most pronounced inhibitory effects, reducing activity to ~60% and ~20%, respectively. The presence of Co^{2+}, Fe^{2+}, and Mn^{2+} caused moderate decreases in catalytic efficiency (70–85% of control), whereas Ca^{2+} and Mg^{2+} maintained activities comparable to the untreated enzyme ($P > 0.01$), suggesting a stabilizing rather than activating effect.

Because these assays were conducted using the unpurified (crude) enzyme extract, the results reflect the net effect of metal ions on the entire extracellular protein milieu rather than on a single purified protease. Interactions among multiple secreted enzymes or native cofactors in the crude fraction may modulate the apparent influence of each ion. For instance, trace divalent cations naturally present in the culture supernatant could already stabilize the enzyme structure, thereby minimizing additional effects from supplementation. Conversely, chelation by EDTA would remove such native stabilizing ions, explaining the drastic decline in activity, a pattern commonly observed in metallo-dependent or metal-stabilized proteases (Rao *et al.*, 1998; Gupta *et al.*, 2002). In numerous reports on *Bacillus* proteases, EDTA has been shown to cause a marked reduction in enzymatic activity, primarily due to its metal-ion chelating effect (Septianingrum *et al.*, 2024; Yang *et al.*, 2000).

The strong inhibition observed in the presence of Hg^{2+} is characteristic of catalytic environments containing cysteine or histidine residues, where mercuric ions can form covalent or coordinate bonds with essential thiol or imidazole groups, leading to irreversible enzyme inactivation. In contrast, the slight activation caused by Zn^{2+} suggests that this ion may contribute to the enzyme's structural rigidity or catalytic stability, possibly by interacting with surface residues or reinforcing folding within the active site region. A similar phenomenon was previously reported by Yang *et al.* (2000) for proteases from *Bacillus cereus*. Meanwhile, in addition to Hg^{2+}, Zn^{2+} was also reported to inhibit the protease from *Bacillus subtilis* D9 (Mahmoud *et al.*, 2021). These contrasting responses indicate that the behavior of metal ions toward *Bacillus* proteases is species-dependent, reflecting variations in active-site architecture and metal-binding motifs. Notably, the neutral effects of Ca^{2+} and Mg^{2+} observed in this study are consistent with their

well-established roles as nonspecific stabilizers that preserve enzyme conformation by shielding charged residues and preventing thermal unfolding (Saeki *et al.*, 2007).

Collectively, these results indicate that the crude protease system of *B. amyloliquefaciens* SLBD is moderately tolerant to most divalent cations, while being sensitive to heavy metals and chelating agents. The strong inhibition by EDTA implies that the enzyme may require divalent metal ions—either as structural cofactors or stabilizing agents—to maintain its active conformation. Such characteristics are commonly reported among *Bacillus metalloproteases* or metal-assisted serine proteases, though definitive classification would require inhibitor and metal reconstitution assays using purified enzyme preparations. Nonetheless, the persistence of activity across several ionic environments reinforces the enzyme's potential for industrial applications where metal ion fluctuations are common, such as in feed, detergent, or waste-treatment formulations.

Effect of Organic Solvents on Protease Activity of Crude Extract

The influence of various organic solvents on the activity of the crude extracellular protease from *Bacillus amyloliquefaciens* SLBD is shown in Figure 7. Exposure to ethanol, methanol, DMSO, and hexane at a final concentration of 10% (v/v) resulted in a moderate reduction in enzyme activity compared with the solvent-free control. Statistical analysis revealed that all solvent-treated samples differed significantly from the control at $P < 0.01$, indicating that the presence of these organic solvents adversely affected protease activity to some extent. Among the tested solvents, methanol-treated samples exhibited the highest residual activity (~85%), whereas hexane and ethanol caused slightly greater reductions, with residual activities of approximately 75–80%.

Because these assays were conducted using the unpurified (crude) enzyme extract, the observed responses likely reflect the combined behavior of the target protease and other extracellular proteins or stabilizing components in the supernatant. The crude extract may contain protective substances such as peptides, polysaccharides, or ions that partly buffer solvent-induced denaturation. Nevertheless, the consistent reduction in activity across all solvents suggests that hydrophobic and polar solvent molecules interfere with enzyme–water interactions, thereby perturbing tertiary structure or displacing essential bound water molecules from the active site (Castillo *et al.*, 2006; Klibanov, 2001). Organic solvents can also alter the dielectric environment around catalytic residues, decreasing conformational flexibility and substrate binding efficiency (Bansal *et al.*, 2010).

Although a slight reduction in catalytic activity was observed, the SLBD protease retained more than 70% of its initial activity in the presence of common organic solvents, indicating moderate solvent tolerance. This property is valuable for industrial biocatalysis, where enzymes often function in solvent-rich or biphasic systems. Several organic solvent–resistant *Bacillus* proteases have been reported previously, including those from *Bacillus sp.* JER02 (Badoei-Dalfard and Karami, 2013), *Bacillus subtilis* strain Rand (Abusham *et al.*, 2009), *Bacillus pat-agoniensis* DB-5 (Li *et al.*, 2025), *Bacillus sp.* DEM05 (Mohamadi *et al.*, 2021), and *Bacillus circulans* M34 (Sari *et al.*, 2015). Similar resilience has been reported for other *Bacillus* proteases used in peptide synthesis and detergent formulations, which remain partially active in the presence of short-chain alcohols or aprotic solvents (Gupta *et al.*, 2002; Saeki *et al.*, 2007). These findings suggest that solvent tolerance is not unique among *Bacillus* proteases; however, to our knowledge, such a characteristic has not been previously reported for feces-derived *Bacillus amyloliquefaciens*, nor for any isolate originating from Indonesia. This highlights the novelty of the SLBD strain as a potential source of a solvent -tolerant protease adapted to unconventional environments. The stability observed in crude form also implies potential suitability for direct application without extensive purification, particularly in processes such as enzymatic hydrolysis of complex substrates or waste bioconversion, where residual medium components may enhance enzyme robustness.

Effect of Detergents or Surfactants on Protease Activity of Crude Extract

The effect of various detergents on the activity of the crude extracellular protease from

Bacillus amyloliquefaciens SLBD is shown in Figure 8. The enzyme exhibited high tolerance toward all tested surfactants, maintaining 80–100% of its initial activity after incubation. Statistical analysis indicated that the differences among treatments were not significant ($P > 0.05$), as represented by the identical letters above the bars, suggesting that detergent exposure did not markedly affect enzyme performance. Slight variation was observed among treatments: the enzyme retained slightly higher relative activity in the presence of Triton X-100 and Tween 80, while marginal decreases were observed with SDS and Tween 20. Because this assay utilized the unpurified (crude) extracellular fraction, the observed results reflect the net activity of the enzyme system in its native extracellular matrix, where natural cofactors, peptides, and other secreted proteins may help preserve structural stability. Such protective interactions likely mitigate the denaturing effects typically caused by surfactants. Nonionic surfactants such as Triton X-100 and Tween 80 are known to be less disruptive to protein folding, often enhancing the solubility of hydrophobic regions and preventing aggregation, whereas anionic surfactants like SDS can partially interfere with hydrophobic interactions, leading to minor losses of activity (Rao *et al.*, 1998; Gupta *et al.*, 2002).

The detergent tolerance of the SLBD protease closely resembles that reported for other *Bacillus* proteases used in industrial applications. For example, proteases from *Bacillus licheniformis* F11 (Ilmiah *et al.*, 2018), *Bacillus cereus* (Doddapaneni *et al.*, 2009), *Bacillus sp*. APCMST-RS3 (Maruthiah *et al.*, 2015), and *Bacillus sp*. APR-4 (Kumari *et al.*, 2015), *Bacillus subtilis* PF10 (Bhange *et al.*, 2016) commonly retain 70–100% of their activity in the presence of detergents such as Triton X-100 or SDS. Accordingly, the ability of *B. amyloliquefaciens* SLBD protease to maintain high activity when exposed to various detergents and surfactants—even in its crude, unpurified form—not only reflects the general resilience typical of *Bacillus* proteases but also highlights its intrinsic conformational stability and suitability for direct industrial application without extensive purification. Such detergent compatibility is particularly advantageous for proteases intended for detergent formulations, cleaning agents, leather processing, and protein stain removal—applications in which both catalytic persistence and surfactant tolerance are critical for operational efficiency.

CONCLUSION

Bacillus amyloliquefaciens SLBD produces a growth-associated extracellular protease with peak activity at 12 hours. The crude enzyme shows good catalytic efficiency (Km = 0.519 mM; kcat/Km = 1.23×10^5 $M^{-1} \cdot s^{-1}$), optimum activity at pH 7–8 and 50 °C, and stability across pH 6–10 and up to 60 °C. Zn^{2+} enhances activity, while Hg^{2+} and EDTA inhibit it, indicating metal dependence. The enzyme is also tolerant to organic solvents and detergents. Overall, this protease is stable, efficient, and show potential for biotechnological and industrial applications.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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