Middle East Journal of Agriculture Research
Volume: 15 | Issue: 02| April – June| 2026

EISSN: 2706-7955 ISSN: 2077-4605
DOI: 10.36632/mejar/2026.15.2.6
Journal homepage: www.curresweb.com
Pages: 96-105

Purification and Evaluation of Antimicrobial Activity and Biofilm Formation of *Bacillus thuringiensis aizawai* Strain HD283

Amira M. Roshdy[1], May A Elhefni[2] and Sahar E. Hamed[3]

[1]Department of Microbial Chemistry, Biotechnology Research Institute, National Research Centre, 33 El Buhouth St., Dokki, Cairo 12622, Egypt.
E-mail: - amiraroshdy82@yahoo.com, https://orcid.org/0000-0001-6809-9949
[2]Agricultural Chemistry Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt; Email: mayali@mans.edu.eg. https://orcid.org/0000-0002-5582-9591
[3]Agricultural Biotechnology Department, Faculty of Agriculture, Damietta University, Damietta, Egypt; Email: s.hamed@du.edu.eg .

Received: 15 Mar. 2026 **Accepted:** 10 May 2026 **Published:** 30 May 2026

ABSTRACT

Objective: The study aimed to evaluate antimicrobial and antibiofilm activities, and purify the alkaline protease enzyme produced by *Bacillus thuringiensis aizawai HD283*. **Methods:** The experimental methodology encompassed the following steps: preparation of the inoculum, evaluate antimicrobial and antibiofilm activities, formulation of production media, enzyme activity assays, protein quantification, ammonium sulfate precipitation, acetone fractionation, and final purification using Sephadex Gel filtration chromatography. **Results:** After cultivating *Bacillus thuringiensis aizawai HD283* under optimal environmental conditions and extracting the crude enzyme, as described in our previous research, the enzyme was subjected to the following purification steps: The purification pattern was as follow, the most active ammonium sulfate fractionation portion was at 60-75 % ammonium sulfate saturation, with 2.16 up to 2.41 % yield recovery, while the most active fraction by acetone precipitation, was wide range from 60-90 % acetone with yield recovery ranged between 13.4 to 12.0 %. Whereas, G-100 Sephadex column chromatography (final purification step) has 1.44 purification factor and 4.5 % final yield recovery of extracted enzyme.

Keywords: Microbial enzymes, biochemical properties, alkaline protease purification, ammonium sulfate saturation, acetone precipitation, G-100 Sephadex column chromatography.

1. 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).

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 evaluated for their anti-biofilm activity against a mixed *Bacillus* biofilm model. Proteases from *Streptomyces sp.* ACD/G413 and *Streptomyces exfoliates* 15/G710 demonstrated significant biofilm removal efficiencies of 55% and 58.69%, respectively, and were characterized as thermophilic alkaline serine proteases (TA-proteases), highlighting their potential as effective, eco-friendly alternatives to chemical and thermal treatments in

Corresponding Author: May A Elhefni, Agricultural Chemistry Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt.

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 *et al.,* 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).

2. Materials and Methods

2.1. Antimicrobial and biofilm Activities Evaluation

Nutrient Agar, Mueller Hinton Agar and Mueller Hinton broth media were purchased from Condalab, Spain**.** Microbial pathogens were kindly donated from the culture collection of Immunology and Microbiology Dep., Faculty of Medicine (boys), Al-Azhar University.

2.2. Antimicrobial Activity Evaluation

The ability of the cell-free extract (CFE) of the targeted strain to prevent the microbial growth was investigated against the standard Pathogen strains, Gram Positive bacteria (*Staphylococcus aureus, Bacillus subtilis*), and Unicellular fungi (*Candida albicans*). Pre-activation of pathogens were carried out by inoculating in the Nutrient broth medium for 24 h. at 37ºC for bacterial strains, while fungal strain was inoculating in Potato Dextrose Broth (PDB) medium for 48 h. at 28ºC under shaking condition. Screening of the tested CFE was preliminary take place at a fixed concentration (i.e. 50 µg/ mL) using Mueller Hinton Agar (MHA) medium based on the inhibition zone diameter (mm) according to agar-well diffusion procedure.

2.3. Biofilm Activity Evaluation

Otherwise, evaluation of the biofilm production by the targeting strain was also assessed qualitatively by Congo red Agar (CRA) plate method. In this method, test organisms were inoculated onto CRA plates, which were then incubated aerobically at 37°C for 48 hours. In contrast to red colonies, which were interpreted as being produced by strains that did not produce biofilm, black colonies with a dry crystalline consistency were indicated as strains that produce biofilm. In addition, biofilm activity of the targeted strain was also applied quantitatively by Crystal violet method (Radwan *et al*., 2022).

2.4. 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* ., 2023a).

2.5. Purification Steps of AP

2.5.1. Ammonium sulfate precipitant

Ammonium sulfate precipitation was carried out by using automatic equation program, cited from, https://www.encorbio.com/protocols/AM-SO4.htm

2.5.2. Acetone Fractionation

This step of purification was conducted as mentioned by Chaykin (1966). The resulting precipitate was separated by cooling centrifugation. The precipitate was dissolved in 10 mL distilled water and

mixed well. The enzyme supernatant was taken to be re-precipitated by the subsequent addition of suitable milliliters of Acetone to bring the enzyme solution to the next saturation level.

2.5.3. Sephadex Gel Filtration Chromatography

Purification by Sephadex G-100 was carried out as described by Aboul-Soud *et al.* (2011).

3. Results and Discussion

In previous study (Roshdy and El-Shershaby 2024), the production of alkaline protease by *Bacillus thuringiensis aizawai HD283* using solid-state fermentation (SSF) was optimized. Wheat bran, an agro-industrial byproduct, which identified as the superior nutritive substrate, and supplementation with fructose as a carbon source enhanced enzyme production by nearly two-fold. Optimal conditions for alkaline protease production included wheat bran particle size <1 mm, 200% moisture content, 60% inoculum size, and a 5-day incubation period. The crude extract had peak at pH 9 and 50°C, with notable stability through a broad temperature range (40–80°C), making it suitable for various biotechnological applications, including detergents, leather processing, and wastewater treatment.

Which prompted us to complete the study on this bacterial strain by testing its effectiveness as an antimicrobial or anti-biofilm on the one hand, and on the other hand, the purification and biochemical characterization of the alkaline protease.

3.1. Antimicrobial and Antifungl Activities Evaluation

The antimicrobial potential of the free cell extract against *Staphylococcus aureus, Bacillus subtilis*, and *Candida albicans* was evaluated using the agar-diffusion method. The results presented in Table 1 indicate that a weak antifungal effect was observed against the white extracts, with the diameter of the inhibition zone being 3 mm. When compared with standard antimicrobial agents, the antifungal activity of the free cell extract was significantly lower than that of amphotericin B (5 mM) but similar to fluconazole (3 mM). Ciprofloxacin, a broad-spectrum antibiotic, showed the highest antibacterial activity, with the diameter of the inhibition zones being 5 mm against *Staphylococcus aureus* and 6 mm against *Bacillus subtilis*. Cephradine, a beta-lactam antibiotic, showed moderate activity (3 mm inhibition zone) against *Bacillus subtilis*, while fluconazole had no effect on the bacterial strains.

Table 1: Antimicrobial activity of the CFE (Inhibition zone mm)

Sample code	Inhibition Zone (mm)		
	Staphylococcus aureus	***Bacillus subtilis***	***Candida albicans***
Image of growth			
CFE	ND	ND	3
Fluconazole [a]	-		3
Amphotericin B	-		5
Cephradine [b]	ND	3	-
Ciprofloxacin	5	6	-

[a, b] Fluconazole, Amphotericin B and Cephradine, Ciprofloxacin were used as standard antifungal and antibacterial agents at 25 µg/mL, respectively. ND: not determined.

However, the free cell extract (FCE) did not show any detectable antibacterial activity against *Staphylococcus aureus* or *Bacillus subtilis*, as no zones of inhibition (ND) was observed.

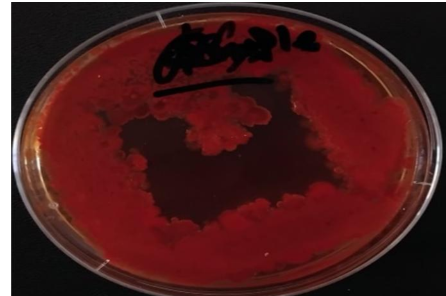
These results therefore suggest that CFE lacks antibacterial activity, and is largely against Gram-positive bacteria. The observed weak antifungal effect may be attributed to the presence of low concentrations of antifungal metabolites, such as chitinase or secondary metabolites with limited bioactivity against *Candida albicans*.

These results highlight a significant discrepancy in antimicrobial activity between CFE and ciprofloxacin (for bacteria) or amphotericin B (for fungi). The weak antifungal effect (3 mm inhibition) suggests that CFE may contain low-potency antifungal compounds, warranting further chemical characterization. The results of this study suggest that the CFE of *Bacillus thuringiensis aizawai HD283* lacks antibacterial properties but exhibits weak antifungal activity against *C. albicans*.

3.2. Assessing Biofilm Formation

Biofilm formation ability of the target *Bacillus thuringiensis aizawai HD283* strain was assessed using both qualitative (Congo red agar, CRA) and quantitative (crystal violet) methods. As shown in Table 2, the target strain failed to form biofilms in either assay. The absence of biofilm formation indicates that this *Bacillus thuringiensis aizawai HD283* strain lacks the genetic and physiological capacity to form biofilms under the conditions tested. Since biofilm formation is a major virulence factor for many pathogenic and environmental bacteria, the inability of this strain to form biofilms may limit its ability to persist in hostile environments or adhere to surfaces. The failure of the strain to develop biofilms also suggests the possible absence or down regulation of key biofilm-associated genes. Many *Bacillus* species rely on structural proteins, such as TasA and BslA, to form the biofilm matrix. The absence of these proteins may explain the observed phenotype. Alternatively, environmental factors such as carbon source availability, quorum sensing molecules, or iron concentration may influence biofilm formation. Comparative genomic analysis may provide further insight into whether this strain possesses the genetic components required for biofilm development. In addition, profiling the transcription under different environmental conditions may help clarify whether biofilm-associated pathways are suppressed under the conditions tested.

Table 2: Biofilm activity of the targeted strain

Biofilm formation by CRA		Biofilm production by crystal violate
Targeted strain		Not detected

Furthermore, the strain does not appear to form biofilms under tested laboratory conditions. Song *et al*. (2023) reviewed detailed studies on microbial proteases, highlighting their numerous advantages, including their diversity in optimal pH ranges for enzymatic activity (acidic, neutral, and alkaline proteases); their tolerance to industrial conditions such as high temperatures; and their broad industrial market, encompassing food and beverage, detergent, leather, animal feed, waste recycling, microbial fermentation, and other industries. Furthermore, the major bioinformatics databases for microbial enzymes are vast, containing several million protease genes whose functions have yet to be fully elucidated. The Agriculture Institute (2025) website explained that the production of fermented dairy products is mainly affected by the performance of bacterial starters - which are carefully selected; and to ensure the optimal performance of these starters, it is necessary to understand and precisely control the environmental and biological factors during the fermentation process.

As mentioned, alkaline protease is the most widely used enzyme in industrial applications, many of which rely on microbial starters as one of the fermentation steps. Therefore, having a microbial enzyme with suitable performance for industry and for preparing the fermentation environment for the starter without competing with the starter for the components of the growth medium is an ideal choice.

3.3. Purification scheme of crude alkaline protease produced by *B.t. aizawai HD283*.

The purification process involved ammonium sulfate precipitation, acetone fractionation, and Sephadex Gel filtration chromatography, then, studying catalytic mechanism and biochemical properties of the enzyme.

3.3.1. Fractionation of crude AP produced by *B.t. aizawai HD283* by Ammonium Sulfate

The ammonium sulfate fractionation process reported in (Table 3), demonstrated weighty variations in alkaline protease activity (APA), purification efficiency, and yield recovery across different saturation levels. The crude enzyme exhibited an initial activity of 174 U/mL/min, with a total enzyme activity of 8715 units, a total soluble protein content of 44.5 mg/mL, and a specific activity of 196 U/mg. As the ammonium sulfate saturation increased, distinct trends in enzyme purification and recovery were observed.

At lower saturation levels (15–45%), the enzyme activity and yield recovery were relatively low, with specific activities ranging from 163 to 321 U/mg, the 30% saturation level achieved a specific activity of 321 U/mg and a purification factor of 1.64, indicating moderate purification efficiency. However, the yield recovery at this stage was only 13%, suggesting notable enzyme loss during precipitation.

The most effective purification was achieved at 60% ammonium sulfate saturation, where the enzyme activity increased to 64 U/mL/min, with a total enzyme activity of 3215 units and a specific activity of 424 U/mg. This step yielded a purification factor of 2.16 and a yield recovery of 37%, highlighting its efficiency in concentrating the enzyme while retaining a substantial portion of its activity. Similarly, the 75% saturation level showed a high specific activity of 472 U/mg and a purification factor of 2.41, although the yield recovery decreased to 26%, indicating partial enzyme loss at higher saturation levels.

At 90% saturation, both enzyme activity and yield recovery declined significantly, with a specific activity of 183 U/mg and a yield recovery of 18%. This suggests that excessive ammonium sulfate concentrations may lead to protein denaturation or co-precipitation of impurities, reducing the overall purification efficiency.

In summary, the 60–75% ammonium sulfate saturation range proved optimal for alkaline protease purification, balancing high specific activity, purification factor, and yield recovery. The results underscore the importance of optimizing saturation levels to maximize enzyme recovery and purity while minimizing losses during the purification process.

These findings align with previous studies, such as those by Roshdy *et al*. (2023b) and Ullah *et al*. (2022), who reported similar trends in ammonium sulfate fractionation for protease purification. The purification of alkaline protease using ammonium sulfate fractionation has been widely reported in the literature, with varying degrees of efficiency. El-Sayed *et al*. (2024) found that the most active ammonium sulfate fractionation portion was at 60-75% ammonium sulfate saturation, with 2.16 up to 2.41% yield recovery. As Roshdy *et al*. (2023b) observed that the most active fraction was achieved at 60–75% ammonium sulfate saturation, yielding a purification factor of 1.93, with a total activity of 645 units and a specific activity of 508 U/mg. Similarly, Ullah *et al*. (2022) found that 70% saturation with ammonium sulfate achieved a total enzymatic activity of 890,368 units and a high-efficiency purification fold of 6.5. In contrast, Li *et al*. (2023) demonstrated that a saturation range of 40–60% ammonium sulfate achieved a 2.3-fold purification with a total activity of 1732.8 units, highlighting the variability in optimal saturation levels depending on the enzyme source and experimental conditions. These findings collectively underscore the importance of optimizing ammonium sulfate saturation levels to maximize enzyme yield and purification efficiency, as demonstrated in the current study. As El-Shazly *et al.* (2024) reported that, 20–70% ammonium sulfate fractionation was optimum for producing enzyme and partially purified to fivefold.

Table 3: Purification of crude alkaline protease (AP) from *Bacillus thuringiensis aizawai HD283* using ammonium sulfate fractionation

Ammonium sulfate content (%)	Enzyme activity (units/ml/min)	Total enzyme activity units	Total soluble protein (mg/ml)	Specific activity (units/mg)	Purification factor (X)	Recovery percentage (%)
Extracted enzyme	174	8715	44.5	196	1.00	100
15	33	1645	7.1	231	1.18	19
30	23	1163	3.6	321	1.64	13
45	24	1215	7.5	163	0.83	14
60	**64**	**3215**	**7.6**	**424**	**2.16**	**37**
75	45	2233	4.7	**472**	**2.41**	26
90	32	1581	8.7	183	0.93	18

3.3.2. Purification of ammonium sulfate fractions of alkaline protease produced by *B.t. aizawai HD283*, by acetone precipitation.

Acetone is commonly used in protein and enzyme purification due to its ability to selectively precipitate proteins. Table (4) analysis the effect of acetone concentration (15% to 90%) on enzyme activity, enzyme activity varies with acetone concentration. The highest enzyme activity was observed at 60% acetone (117 U/ml/min), while the lowest activity was recorded at 30% acetone (39 U/ml/min). This suggests that the optimal acetone concentration for enzyme activation is around 60%. Total enzyme units follow the same trend as enzyme activity. The highest total enzyme units were observed at 60% acetone (1170 U), while the lowest was at 30% acetone (387 U). This reflects the direct relationship between enzyme activity and total enzyme units. The amount of soluble protein also varies with acetone concentration. The highest soluble protein concentration was observed at 60% acetone (4.5 mg/ml), while the lowest was at 75% acetone (1.9 mg/ml). This indicates that acetone concentration affects the amount of soluble protein. Specific activity (enzyme activity per milligram of protein) increases with higher acetone concentrations, peaking at 75% acetone (339 U/mg). This suggests that higher acetone concentrations (75%) lead to more efficient enzyme purification. The purification factor increases with higher acetone concentrations, reaching its maximum at 75% acetone (1.73). This indicates that higher acetone concentrations contribute to increased enzyme purity. Yield recovery also varies with acetone concentration. The highest yield recovery was observed at 60% acetone (13.4%), while the lowest was at 30% acetone (4.4%). This suggests that the optimal acetone concentration for enzyme recovery is around 60%. Higher acetone concentrations (75% and 90%) result in lower amounts of soluble protein, which may indicate selective precipitation of unwanted proteins. Recommendations, 60% acetone concentration can be adopted as the optimal concentration to achieve a balance between enzyme activity, yield recovery, and purification efficiency. However, if the goal is to achieve the highest specific activity and purity, 75% acetone may be the better choice. These results highlight the importance of optimizing purification conditions to achieve the highest efficiency in enzyme extraction. Acetone, as a precipitating agent, can be effective in enzyme purification, but the optimal concentration must be determined based on the specific goals of the study (high enzyme activity, high purity, or high recovery).

El-Sayed *et al*. (2024) found that the most active fraction by acetone precipitation, was wide range from 60-90% acetone with yield recovery ranged between 13.4 to 12.0%. Roshdy *et al*. (2023b) found that, the best purification was 3.92, with a total specific activity of 1032 units/mg, at acetone saturation levels of 30-45%. Shaikh *et al*. (2018) specific activity was 4975.942 U/mg; Niyonzima *et al*. (2015) achieved 13.6 units/mg specific activity and purification fold about 2.6.

Table 4: Purification of ammonium sulfate fractions of alkaline protease produced by *B.t. aizawai HD283*, by acetone precipitation.

Acetone (%)	Enzyme activity (units/ml/min)	Total enzyme activity units	Total soluble protein (mg/ml)	Specific activity (units/mg)	Purification factor (X)	Recovery percentage (%)
15	51	512	3.3	153	0.78	5.9
30	39	387	2.8	139	0.71	4.4
45	43	427	2.4	179	0.92	4.9
60	**117**	**1170**	**4.5**	261	1.33	**13.4**
75	65	650	1.9	**339**	**1.73**	7.5
90	**105**	**1048**	**3.9**	267	1.36	**12.0**

3.3.3. Purification of alkaline protease produced by *B.t. aizawai HD283* culture, fractions which precipitated by acetone, through a G-100 Sephadex column.

Table (5) presents column fractionation, column fractions range from 25 mL to 155 mL, representing different elution volumes during the purification process, each fraction corresponds to a specific stage of protein elution from the column. Enzyme activity (APA) varies across the fractions, with the lowest activity observed at 55 mL (6 U/ml/min) and the highest at 115 mL (16 U/ml/min). This indicates that the enzyme elutes most effectively around the 115 mL fraction. Total enzyme units follow a similar trend to enzyme activity, with the lowest value at 55 mL (157 U) and the highest at 115 mL (391 U). This confirms that the 115 mL fraction contains the highest amount of active enzyme. Total soluble protein concentration varies across fractions, with the lowest concentration at 25 mL and 35 mL (0.7 mg/ml) and the highest at 125 mL (2.8 mg/ml). The 125 mL fraction has a significantly higher protein concentration, but this does not correlate with high enzyme activity, suggesting the presence of non-enzymatic proteins. Specific activity (enzyme activity per milligram of protein) is a key indicator of enzyme purity. The lowest specific activity is observed at 25 mL (0 U/mg) and 125 mL (77 U/mg), while the highest is at 65 mL (327 U/mg). This suggests that the 65 mL fraction contains the purest form of the enzyme. The purification factor reflects the degree of enzyme purity relative to the initial sample. The lowest purification factor is at 25 mL (0.00) and 125 mL (0.39), while the highest is at 65 mL (1.67). This indicates that the 65 mL fraction is the most purified. Yield recovery represents the percentage of enzyme activity recovered in each fraction relative to the total activity. The lowest recovery is at 55 mL (1.8%), while the highest is at 115 mL (4.5%). This suggests that the 115 mL fraction contains the highest amount of recovered enzyme.

The 115 mL fraction shows the highest enzyme activity (16 U/ml/min), total enzyme units (391 U), and yield recovery (4.5%). This makes it the most effective fraction for enzyme recovery. The 65 mL fraction has the main specific activity (327 U/mg) and purification factor (1.67), indicating that it contains the purest form of the enzyme. The 125 mL fraction has the highest total soluble protein concentration (2.8 mg/ml) but low specific activity (77 U/mg) and purification factor (0.39). This suggests that this fraction contains a significant amount of non-enzymatic proteins. The 25 mL fraction shows no enzyme activity (0 U/ml/min) and a purification factor of 0.00, indicating that this fraction does not contain the target enzyme. The 115 mL fraction is the best choice, as it provides the highest enzyme activity, total enzyme units, and yield recovery. For maximum enzyme purity, the 65 mL fraction is the most suitable, as it has the highest specific activity and purification factor. The 125 mL fraction contains a high concentration of total soluble protein but low enzyme purity, suggesting the presence of contaminants.

The data demonstrate the importance of fractionation in enzyme purification. Different fractions contain varying levels of enzyme activity, purity, and recovery, highlighting the need to optimize the purification process to achieve the desired balance between yield and purity. The 115 mL fraction is ideal for maximizing enzyme recovery, while the 65 mL fraction is optimal for achieving high enzyme

purity. These results can guide further optimization of the purification protocol for industrial or research applications.

In agreement with Harer and Harer (2022) who partial characterized a thermally stable serine alkaline protease, also produced from *Bacillus thuringinsis*. The extracted enzyme was 17.04-fold purified, with 8.47% of the enzyme recovered by ammonium sulfate precipitation, followed by dialysis and then Sephadex G-200 columns. Roshdy *et al.* (2023b) reported that, Sepahdex G-100 columns achieved the best enzyme activity, with 234 total activity units and 1408 units/mg of specific activity, accompanied by a purification of approximately 5.36. Shaikh *et al*. (2018) achieved a total enzyme activity of 31907.27, a specific activity of 8741.72 units/mg, and a purification of 3.12-fold. Ullah *et al*. (2022) reported a total alkaline protease activity of 208.591, a specific activity of 8902 units/mg and a purification coefficient of 35.91.

Table 5: Purification of alkaline protease produced by *B.t. aizawai HD283* culture, fractions which precipitated by acetone, through a G-100 Sephadex column.

Sephadex G-100 fractionions (mL)	Enzyme activity (units/ml/min)	Total enzyme activity units	Total soluble protein (mg/ml)	Specific activity (units/mg)	Purification factor (X)	Recovery percentage (%)
25	8	206	0.7	0	0.00	2.4
35	8	205	0.7	291	1.49	2.4
45	8	212	1.0	203	1.04	2.4
55	6	157	0.9	171	0.87	1.8
65	11	265	0.8	**327**	1.67	3.0
75	8	192	0.8	243	1.24	2.2
85	12	299	1.0	293	1.49	3.4
95	11	265	1.0	270	1.38	3.0
105	12	302	1.1	273	1.39	3.5
115	16	**391**	1.4	282	1.44	**4.5**
125	9	215	2.8	77	0.39	2.5
135	9	237	1.3	182	0.93	2.7
145	9	217	1.1	195	1.00	2.5
155	7	186	1.0	182	0.93	2.1

4. Conclusion

The results show that CFE of *Bacillus thuringiensis aizawai* HD283 exhibits weak antifungal activity against *C. albicans* but lacks antibacterial activity against S. aureus and B. subtilis. In addition, the target strain does not form biofilms under standard laboratory conditions, which may affect its ability to persist in natural environments. So, further chemical and genetic studies are needed.

This study partially purified the alkaline protease enzyme produced by *Bacillus thuringiensis aizawai HD283*. Through a systematic purification process involving ammonium sulfate precipitation, acetone fractionation, and Sephadex Gel filtration chromatography, the enzyme was isolated with a final yield recovery of 4.5% and a purification factor of 1.44.

5. Recommendations

These findings have several implications:

Advanced techniques such as HPLC-MS and GC-MS should be used to identify the active compounds present in the CFE. Fractionation of the extract may reveal minor bioactive components that were not detectable in the comprehensive assay. The antimicrobial spectrum should also be expanded, with future studies investigating the efficacy of CFE against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and additional fungal pathogens (*Aspergillus* spp.). Time-kill assays and minimum inhibitory concentration (MIC) determinations could provide a more accurate measure of antimicrobial activity.

Whole-genome sequencing of a *Bacillus thuringiensis aizawai HD283* strain could identify genes encoding antimicrobial peptides or biofilm-associated proteins. RNA-seq analysis could be used to assess differential gene expression in response to different environmental stresses.

If *Bacillus thuringiensis aizawai HD283* strain is confirmed to be nonpathogenic plus non-biofilm-forming, it could have potential applications in agriculture as a biocontrol agent with low risk of environmental persistence.

Conflicts of Interest
No conflict of interest.

Abbreviation List

Alkaline protease (AP)
Alkaline protease Activity (APA)
Bacillus thuringiensis (Bt)

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