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Characterization of a Halostable Metalloprotease from the Halophilic Bacterium *Bacillus clausii* J1G-0%B

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Abstract

Protein plays a crucial role as a biocatalyst in various industries, particularly in breaking down proteins into amino acids. The demand for proteases capable of functioning under extreme conditions, such as high salinity, temperature, and pH, is increasing. To address this, the exploration of bacteria that produce stable proteases in such environments is essential. *Bacillus clausii* J1G-0%B, a halophilic bacterium isolated from Madura salt ponds, thrives in salinity levels of 0-20% NaCl. This study aims to obtain and characterize the protease produced by *Bacillus clausii* J1G-0%B, focusing on its activity and stability under extreme conditions. The research involved screening, production, and purification of the protease using ammonium sulfate fractionation and dialysis. Protease activity was measured using the Kunitz method, and protein content was determined using the Lowry method. Characterization included optimizing enzymatic conditions (pH, temperature, NaCl concentration), identifying metalloprotease types, and analyzing enzyme kinetics and thermodynamics. The study successfully produced protease using a halophilic medium with casein and 5% NaCl. After 96 hours of incubation, the protease exhibited a specific activity of 654.737 U/mg. Optimal activity was observed at pH 7, 50°C, and 10% NaCl, with stability between 2.5% and 15% NaCl concentration. Enzyme kinetics revealed a high affinity for casein, with a K_M value of 0.164 mg/mL and V_{max} of 13.182 µmol/mL·min. Thermodynamic analysis indicated high stability, as shown by a positive ΔG_i value (+105.84 kJ/mol), a low inactivation constant (k_i = 0.0031 min^{-1}), and a long half-life ($t_{½}$ = 223.548 minutes). EDTA chelation tests confirmed that the protease is a metalloprotease. The halostable protease from *Bacillus clausii* J1G-0%B shows significant potential for industrial applications and bioremediation in high-salinity environments.

1. Introduction

Enzymes, serving as biocatalysts, are extensively utilized across various industrial sectors due to their selective and specific functionality. Proteases, in particular, represent approximately 60% of the global enzyme demand. However, a significant challenge in industrial applications is reduced enzymatic activity and stability when environmental conditions—such as pH, temperature, and salt concentration—fluctuate [1, 2].

To address this, ongoing efforts are focused on discovering enzymes that maintain stability in high-salt environments, known as halostable enzymes. Halophilic bacteria, capable of thriving in high-salt or hypersaline conditions, play a crucial role in this search [3]. These bacteria adapt to such environments by accumulating salts and dissolved organic matter (osmolytes) within their cytoplasm. This accumulation helps prevent cytoplasmic fluid loss by binding fluids with salts and osmolytes like ectoine and glycine betaine [4].

Budiharjo *et al.* [5] reported that halophilic bacteria isolated from Madura salt ponds are capable of producing halostable protease enzymes. These enzymes exhibit resistance to hypersaline environments, primarily due to

the shielding effect. This effect arises from the binding of salt cations to negatively charged amino acid residues, such as glutamic acid and aspartic acid, on the surface of halostable enzymes [3]. *Bacillus clausii* J1G-0%B, an orange-pigmented halophilic bacterium also isolated from Madura salt ponds [6], shares a similar habitat with the halophilic bacteria studied by Budiharjo *et al.* [5]. Given this habitat similarity, *Bacillus clausii* J1G-0%B is predicted to have a high potential for producing halostable protease.

Preliminary tests have demonstrated that *Bacillus clausii* J1G-0%B can produce halostable protease. This study aims to determine the characteristics of halostable protease produced by *Bacillus clausii* J1G-0%B in a halophilic medium containing 5% NaCl. The characterization focuses on determining the optimal conditions for enzymatic reactions, including pH, temperature, and NaCl concentration, as well as analyzing enzyme kinetics, thermodynamics, and conducting metalloprotease tests.

2. Experimental

2.1. Tools and Materials

The tools utilized in this study included standard research glassware, inoculation loop, micropipettes (Corning 4075 Lambda Plus Single-Channel Pipettor), spirit burners, analytical balances (Ohaus Pioneer PA214 Analytical Balance), autoclaves, laminar airflow (Humanlab Class II Model CB-150-H), centrifuges (Corning LSE Compact Centrifuge, Speed: 200-6000 rpm), incubators (Memmert IN-30), incubator shakers (Memmert Water Bath Shaking Model WBN14), and a Thermo Scientific™ GENESYS 10S UV-VIS spectrophotometer.

The materials used in this study include skim milk (technical grade), crystal violet, safranin, ethanol, KCl, trisodium citrate, tryptone, $MgSO_4.7H_2O$, NaCl, $FeCl_3.6H_2O$, TCA (trichloroacetic acid), NaH_2PO_4, Na_2HPO_4, BSA (bovine serum albumin) solution, Na_2CO_3, Na-EDTA, NaOH, $CuSO_4.5H_2O$, Folin-Ciocalteu reagent, Na/K tartrate, tyrosine, casein, and ammonium sulfate (all from Merck), as well as yeast extract (Oxoid), agar (technical grade), aluminum foil, cotton, gauze, and filter paper (all technical grade). The bacterial sample used was the halophilic *Bacillus clausii* J1G-0%B.

2.2. Rejuvenation *Bacillus clausii* J1G-0%B

Halophilic bacterial isolates, *Bacillus clausii* J1G-0%B, were inoculated into liquid halophilic media containing 0.1% (w/v) tryptone, 0.05% (w/v) yeast extract, 0.1% (w/v) KCl, 0.3% (w/v) trisodium citrate, 2% (w/v) $MgSO_4.7H_2O$, 5% (w/v) NaCl, and 0.032% (w/v) $FeCl_3.6H_2O$. The bacterial culture was incubated in a shaker incubator at 150 rpm and 37°C for 72 hours.

2.3. Confirmation *Bacillus clausii* J1G-0%B

Rejuvenated halophilic bacterial cultures of *Bacillus clausii* J1G-0%B were subjected to Gram staining for bacterial confirmation. A single drop of the bacterial culture was placed on a glass slide, followed by the addition of two drops of crystal violet. The slide was allowed to stand for one minute, then tilted, rinsed with distilled water, and dried. Iodine solution was then applied, and the slide was left for one minute before being tilted, rinsed with distilled water, and dried. Absolute ethanol was used as a decolorizing agent for 30 seconds, followed by rinsing with distilled water and drying. Two to three drops of safranin were added and allowed to stand for 30 seconds, and then the slide was washed with distilled water and dried. Finally, oil immersion was applied to the preparation, and the slide was observed under a microscope at 400× magnification.

2.4. Screening Protease Potential

The solid halophilic medium was prepared with the following components: 0.3% KCl, 0.3% trisodium citrate, 0.05% yeast extract, 2% $MgSO_4.7H_2O$, 5% NaCl, 0.032% $FeCl_3.6H_2O$, 0.168% casein, and 1.2% agar, all dissolved in 50 mL of distilled water. After preparing the medium, 100 µL of bacterial culture was added, spread evenly, and incubated at 37°C for 24 hours. Following incubation, the bacterial culture was transferred to a new halophilic solid medium, where the casein substrate was replaced with 1% skim milk. Using an inoculation needle, the bacterial culture was spotted onto this fresh halophilic solid medium, which was then incubated at 37°C for an additional 24 hours.

2.5. Determination of Optimal Conditions in Halophilic Media

The optimal conditions for protease production were determined by gradually replacing tryptone with casein, optimizing pH, and identifying the ideal growth time. The process began with incrementally substituting tryptone with casein until optimal growth was achieved with 100% casein. Bacteria were cultured in media with varying tryptone ratios (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100) to establish the best casein composition for protease production. Following this, the pH of the medium was varied to identify the optimal pH range, using 0.1 M phosphate buffer for pH values of 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8, and 0.1 M ammonia buffer for pH values of 9, 9.5, 10, 10.5, and 11. Protease production was monitored for 168 hours. Every 12 hours, samples were tested for protease activity and turbidity (optical density) at 600 nm to assess the effectiveness of the conditions.

2.6. Protease Production

Bacillus clausii J1G-0%B was inoculated into halophilic media containing the optimized casein substrate and incubated in a shaker incubator under the previously determined optimal pH, temperature, and time conditions.

2.7. Isolation and Purification of Protease

The bacterial culture was centrifuged at 6000 rpm for 20 minutes under cold conditions to separate the filtrate (crude enzyme extract) from the bacterial cell mass. The crude extract was then subjected to ammonium sulfate fractionation with various saturation ranges: 0-40%, 40- 60%, and 60-100%, all conducted under cold conditions and washed with 0.05 M phosphate buffer at

pH 6. After obtaining the protease precipitate, dialysis was performed to remove the salt from the protease. This process was carried out for 24 hours in cold conditions using 0.01 M phosphate buffer, with buffer changes every 4-6 hours. Each purification step was evaluated for enzyme activity and protein content, and the specific activity was calculated.

2.8. Determination of Activity, Protein Content, and Specific Activity of Protease

Protease activity was assessed using the Kunitz method [7]. To perform the assay, 0.5 mL of enzyme solution was mixed with 0.625 mL distilled water, 2.5 mL of phosphate buffer at pH 7, and 0.125 mL of 2% casein solution. The mixture was incubated at 37°C for 30 minutes. After incubation, 1.5 mL of 10% TCA solution was added, and the mixture was gently stirred and allowed to stand at room temperature for 30 minutes. The absorbance was then measured at 285 nm using a UV-Vis spectrophotometer. For the control, 10% TCA solution was added before incubation, and 2% casein solution was added after incubation. One unit of protease activity is defined as the amount of enzyme required to release 1 µmol of tyrosine from casein per milliliter of reaction volume per minute [8].

Protein content was determined using the Lowry method [9]. To perform the assay, 1 mL of the enzyme sample solution was mixed with 5 mL of base solution and left to stand while stirring for 10 minutes. Following this, 0.5 mL of Folin-Ciocalteu reagent was added, and the mixture was allowed to react for 30 minutes. The absorbance of the Folin-Ciocalteu-protein complex was then measured at 760 nm using a UV-Vis spectrophotometer. Distilled water served as the control, replacing the enzyme solution. Protein levels were quantified using a linear equation derived from a BSA protein standard curve. Specific activity was calculated as the protease activity units per milligram of protein [10].

2.9. Characterization of Halostable Proteases

Enzyme characterization, including assessments of pH, temperature, salinity, kinetics, and stability, aims to identify the optimal conditions for enzyme activity, evaluate the enzyme's tolerance to various environmental factors, and understand its reaction rates and stability. This characterization is essential for optimizing enzyme performance in industrial and research applications.

2.9.1. Determination of Optimum pH, Temperature, and NaCl Levels

To determine the effect of pH on protease activity, unit activity testing was performed at pH 5, 6, 7, and 8 using 0.01 M phosphate buffer. Enzyme incubation temperatures were varied at 30, 37, 40, 50, and 60°C. Additionally, NaCl concentrations were tested at 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20%, all dissolved in phosphate buffer. Following these tests, protease activity and protein levels were measured to calculate the specific protease activity.

2.9.2. Kinetics and Thermodynamics Enzyme

The substrate concentration was varied at 0.5, 1, 1.5, 2, and 2.5% to determine the K_M and V_{max} values. Thermal stability was assessed by incubating the enzyme at optimal pH and temperature for different durations (0, 10, 20, 30, 40, 50, and 60 minutes) before measuring its unit activity. The inactivation rate constant (k_i), half-life ($t_{½}$), and the energy change due to denaturation (ΔG_i) were calculated based on these measurements [10].

2.9.3. Determination of Halostable Metalloprotease Type

The type of metalloprotease was identified by adding a metal chelating agent, Na-EDTA, to the enzyme sample solution at concentrations of 0, 0.25, 0.5, 0.75, and 1 mM. The specific activity of the remaining protease was then measured to determine the metalloprotease type.

3. Results and Discussion

3.1. Rejuvenation and Confirmation *Bacillus clausii* J1G-0%B

Rejuvenation of *Bacillus clausii* J1G-0%B involved regrowing the bacteria from stock to obtain a fresh, uncontaminated culture. The rejuvenated bacterial culture was then subjected to a bacterial confirmation stage to ensure the sample was pure and phenotypically consistent with *Bacillus clausii* J1G-0%B. Visual observations confirmed that the sample bacteria were indeed *Bacillus clausii* J1G-0%B. As illustrated in Figure 1, the bacteria produced an orange pigment (1a), exhibited rod/bacillus morphology (1b), and were identified as Gram-positive (1c).

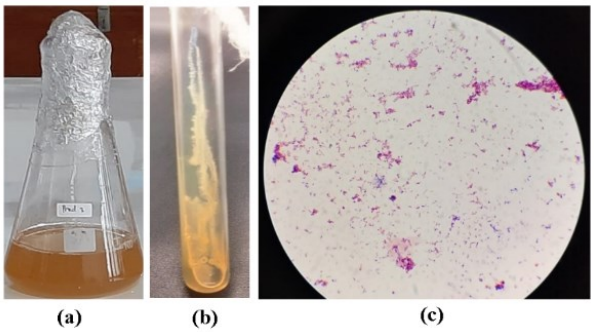

Figure 1. Confirmation of bacterial samples: (a) liquid halophilic media, (b) solid halophilic media, and (c) positive Gram staining results for *Bacillus clausii* J1G- 0%B

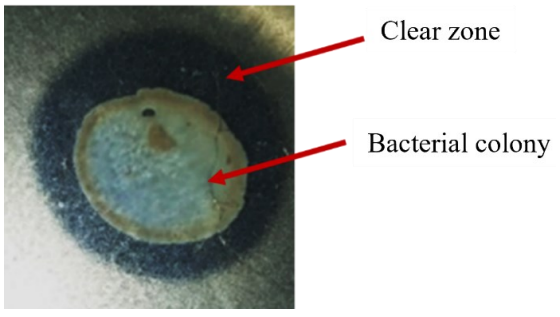

Figure 2. Screening results for protease potential of *Bacillus clausii* J1G-0%B

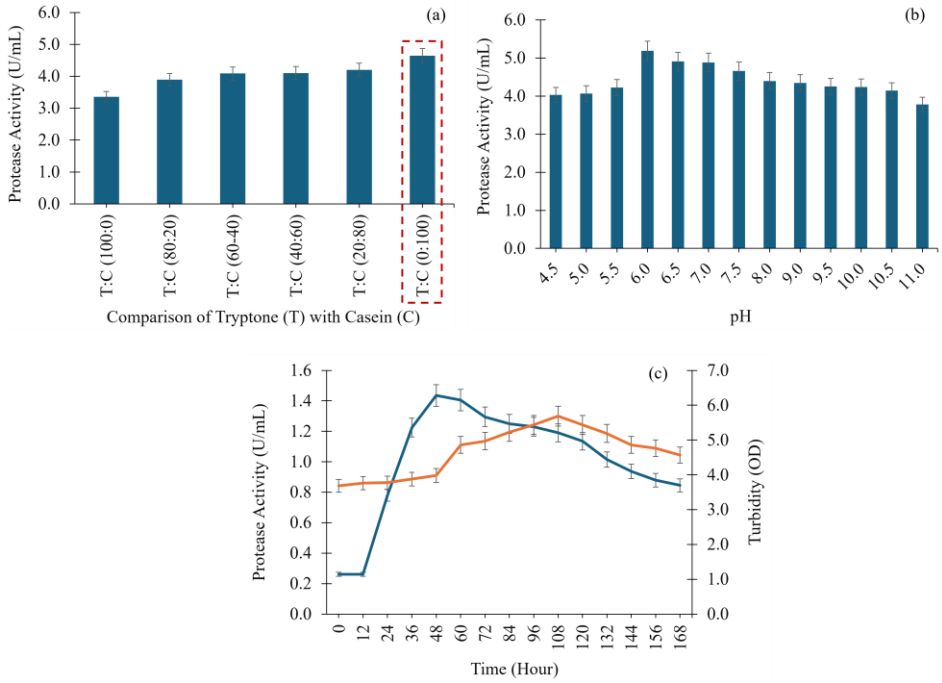

Figure 3. Optimal conditions for halophilic media (a) Tryptone: Casein concentration (T:C), (b) pH, (c) growth curve of *Bacillus clausii* J1G-0%B (blue line) and protease activity (orange line)

3.2. Screening for Protease Potential

The protease potential of *Bacillus clausii* J1G-0%B was assessed based on the formation of a clear zone around the bacterial colony, indicative of casein protein hydrolysis in the screening media by the protease produced [11]. The results, as shown in Figure 2, reveal a clear zone surrounding the bacterial colony, demonstrating that *Bacillus clausii* J1G-0%B possesses protease production potential.

3.3. Optimal Growth Media for *Bacillus clausii* J1G-0%B in Producing Halostable Proteases

Determining optimal conditions is crucial for achieving optimal protease production. In this study, optimization involved modifying the substrate from tryptone to casein, resulting in an increase in protease activity from 3.4 U/mL (T:C = 100:0) to 4.64 U/mL (T:C = 0:100), as illustrated in Figure 3a. The pH variation results indicated that the optimal pH for protease production was 6, with an activity of 5.19 U/mL (Figure 3b). Additionally, protease activity was assessed at different incubation times, with the highest activities recorded at 96 hours (5.44 U/mL) and 108 hours (5.6 U/mL). The optimal production time was determined to be 96 hours, representing the peak activity before a decline occurred, as shown in Figure 3c. The results from determining the optimal conditions for casein concentration, pH, and incubation time were then applied to produce protease from *Bacillus clausii* J1G-0%B.

3.4. Production, Isolation, and Purification of Halostable Proteases

Protease production by *Bacillus clausii* J1G-0%B was conducted under optimal conditions using casein-modified halophilic media, at pH 6, 37°C, and incubated for 96 hours. The resulting crude protease extract exhibited a specific activity of 35.246 U/mg. This extract was subsequently fractionated with ammonium sulfate, with the highest specific activity observed in the (60- 100)% fraction, reaching 428.987 U/mg. Further dialysis of this fraction to remove salt resulted in a specific activity of 654.737 U/mg. As shown in Table 1, the combined ammonium sulfate fractionation and dialysis steps enhanced protease purity by 18.6 times.

Table 1. Purification results of halostable protease

Enzyme	Volume of enzyme (mL)	Activity** (Unit/mL)	Protein content (mg/mL)	Activity specific (U/mg)	Purity (time)
Crude extract	2.6	4.511	0.128	35.246	1
Fraction (0-60%)	27	5.792	0.025	231.695	6.574
Fraction (60-100%)*	33.0	14.157	0.033	428.987	12.2
Dialysis	43.0	11.785	0.018	654.737	18.6

Information :

* = Fractionation ammonium sulfate with the highest protease (fraction 60-100%).

** = One unit of protease activity is defined as the amount of protease required to decompose 1 µmol tyrosine from casein in 1 milliliter of reaction volume per minute.

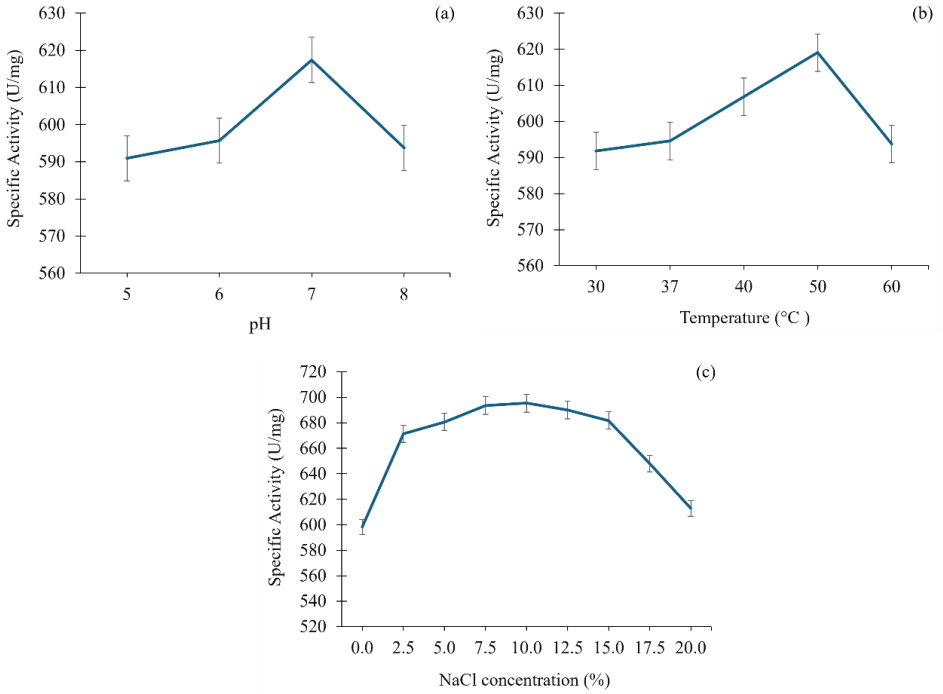

Figure 4. Optimal characteristics of halostable proteases: (a) Effect of pH variation, (b) Effect of temperature variation, (c) Effect of NaCl concentration variation

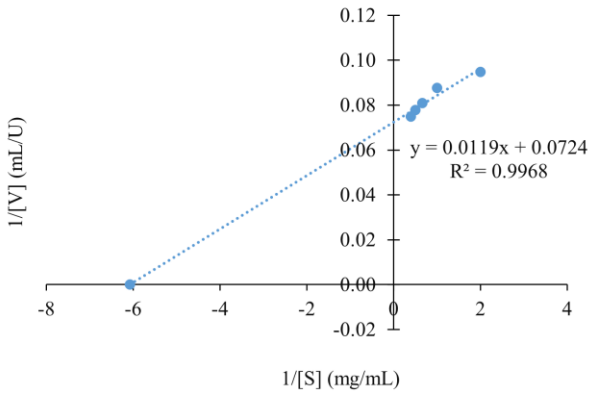

Figure 5. Lineweaver-Burk Plot of halostable proteases

3.5. Characterization of Halostable Proteases

3.5.1. Determination of Optimum pH, Temperature, and NaCl Levels

Protease characterization involved assessing the effects of pH, temperature, and NaCl concentration on enzyme activity. As environmental conditions significantly impact protease activity [12], determining the optimal conditions is crucial for maximizing the hydrolytic efficiency of halostable protease on casein substrates. The characterization results indicated that the optimal conditions for halostable protease activity were achieved at a pH of 7, with a specific activity of 617.416 U/mg (Figure 4(a)); at a temperature of 50°C, with a specific activity of 619.087 U/mg (Figure 4(b)); and at a NaCl concentration of 10%, with a specific activity of 695.401 U/mg (Figure 4(c)).

The pH condition significantly influences the electrostatic interactions between charged amino acids and disrupts the hydrogen bonds of proteins with water, leading to changes in the enzyme's conformation and activity [13]. As illustrated in Figure 4(c), the protease produced by *Bacillus clausii* J1G-0%B exhibits halostable properties, as indicated by its high and stable specific activity within a NaCl concentration range of 2.5% to 15%, with an optimal level at 10%. The stability of protease activity across a broad range of NaCl concentrations demonstrates the ability of the halostable protease to adapt to varying saline conditions, making it suitable for applications in soy sauce production, leather tanning, and bioremediation in high-salinity environments.

3.5.2. Kinetics of Protease Enzymes

The binding of the enzyme-substrate [ES] complex and the conversion of the substrate into the product can be assessed by determining the V_{max} and K_M values, which are derived from enzyme kinetics calculations. The Michaelis-Menten equation is a widely utilized model for enzyme kinetics [14]. This model is applicable to most simple enzymatic reactions and is commonly employed due to its relevance. To determine the V_{max} and K_M values, experiments were conducted varying the casein substrate concentrations at 0.5, 1, 1.5, 2, and 2.5% to measure the enzymatic reaction rates. The resulting data were analyzed using a Lineweaver-Burk plot (Figure 5), which provides a linear representation of the Michaelis-Menten equation (1/V versus 1/[S]).

The data presented in Figure 5 demonstrate that an increase in substrate concentration leads to a higher rate of enzymatic reaction, which aligns with the findings reported by Nelson *et al.* [15]. The Lineweaver-Burk plot provided a K_M value of 0.164 mg/mL and a V_{max} value of 13.182 µmol/mL·min. A lower K_M value, coupled with a higher V_{max}, indicates a strong affinity of the protease for the casein substrate [16].

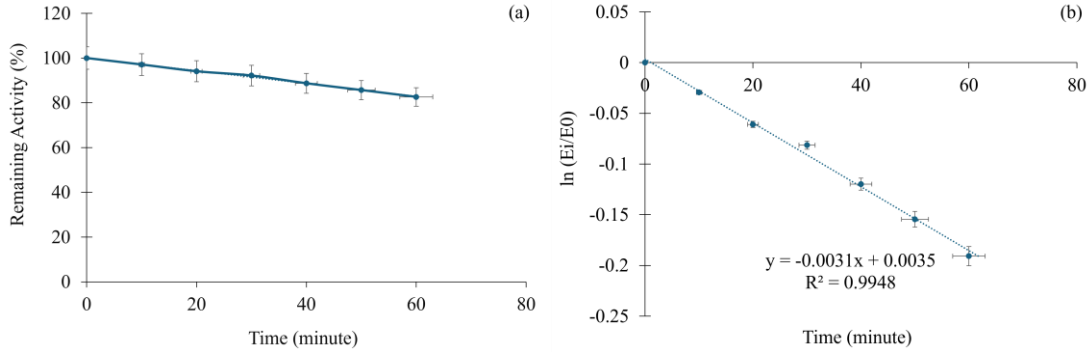

Figure 6. Results of thermodynamic tests of halostable protease: (a) Thermal stability and pH, (b) ln(E_i/E_o) vs. time graph

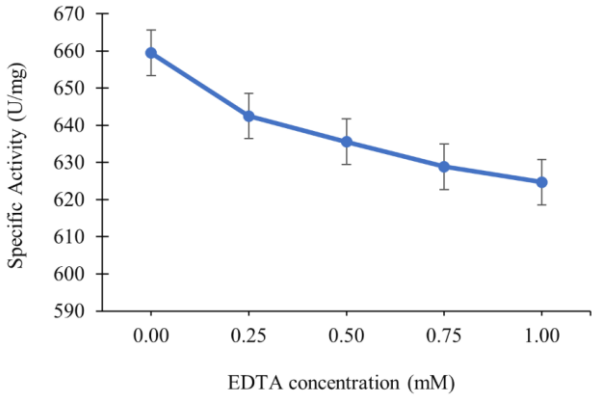

Figure 7. Effect of EDTA on the specific activity of halostable proteases

Thermodynamic analysis was performed by correlating the effect of time on the residual activity of halostable protease and correlating E_i (enzyme activity at time t) and E_o (initial enzyme activity) through the ln (E_i/E_o) vs. t graph, which can determine the value of k_i, $t_{½}$, and ΔG_i. The relationship between the values of k_i, $t_{½}$, and ΔG_i was explained using the first-order reaction rate equation and the Gibbs free energy equation.

The first-order reaction rate equation illustrates that enzyme activity decreases log-linearly with time, as described by the equation ln (E_i/E_o) = -kt, where k is the first-order inactivation rate constant [17]. Using this equation, $t_{½}$, which is the time required for the enzyme activity to reach half of its initial concentration, can be calculated. This is a key measure of enzyme stability and is given by $t_{½} = \ln 2/k_i$ [18]. The first-order of k_i is then used to calculate ΔG_i value at various temperatures using the equation $\Delta G_i = -RT \ln (k_i h / K_b T)$ [17], where h is Planck's constant (6.63×10^{-34} J s), and K_b is Boltzmann's constant (1.381×10^{-23} JK^{-1}). The results from the thermodynamic analysis of the halostable protease enzyme indicate its stability concerning pH and thermal conditions, as shown in Figure 6.

Based on the data analysis in Figure 6b, the values determined are ΔG_i is 105.84 kJ/mol, the $t_{½}$ value is 223.548 minutes, and k_i is 0.0031 min^{-1}. The relatively low k_i value indicates that the halostable protease is resistant to inactivation, suggesting a stable enzyme structure [19]. The $t_{½}$ value, which reflects the time required for the enzyme to lose half of its activity, is 223.548 minutes, indicating a long duration before the protease becomes 50% inactive [20]. The positive free energy change of inactivation (ΔG_i = +105.84 kJ/mol) indicates that a significant amount of energy (105.84 kJ/mol) is required to denature the halostable protease. This suggests that the conformation of the protease from *Bacillus clausii* J1G-0%B is highly stable and resistant to inactivation [16]. Compared to the research conducted by Suwarso *et al.* [10], the values of ΔG_i, $t_{½}$, and k_i for the halostable protease derived from *Bacillus clausii* J1G-0%B demonstrate notably high thermal and pH stability.

3.5.3. Determination of Metalloprotease

Metalloproteases are a subclass of protease enzymes [21]. To determine if an enzyme is a metalloprotease, a chelating agent such as EDTA (Ethylene diamine tetraacetic acid) is added to the enzyme solution [22]. EDTA acts as a chelating agent by binding metal ions, such as Zn^{2+}, Ca^{2+}, or Mg^{2+}, which serve as cofactors for metalloprotease enzymes [23].

Based on Figure 7, there is a noticeable decrease in protease activity with the addition of EDTA, indicating that the halostable protease produced by *Bacillus clausii* J1G-0%B is a metalloprotease. Metalloproteases are protease enzymes that contain metal ions in their active sites, which are essential for catalytic reactions and support the active site [23]. The addition of EDTA to the metalloprotease enzyme solution chelates the metal ions from the enzyme's active site, resulting in reduced enzyme activity [24]. Additionally, Figure 7 demonstrates that low concentrations of EDTA are more effective in reducing enzyme activity than higher concentrations. At low concentrations, EDTA effectively chelates the essential metal ions required for enzymatic catalysis, leading to a noticeable decrease in activity. Once all available metal ions are bound and the enzyme's active sites are saturated, further addition of EDTA does not significantly affect enzyme activity [25]. Research by Lopata *et al.* [25] corroborates this finding, showing that at low EDTA concentrations (30 µM), the K_M value increased, while at higher concentrations (100 µM and 300 µM), enzyme activity decreased to the point of saturation.

4. Conclusion

In this study, protease was successfully produced by *Bacillus clausii* J1G-0%B using a modified halophilic medium with casein, 5% NaCl, and an optimal production

time of 96 hours, resulting in a specific activity of 654.737 U/mg. Characterization revealed optimal protease activity occurred at pH 7, 50°C, and 10% NaCl. The protease demonstrated stability within the NaCl concentration range of 2.5% to 15%, indicating its halostability and suitability for application in both low and high salinity conditions. Enzyme kinetics showed a K_M value of 0.164 mg/mL and a V_{max} of 13.182 µmol/mL·min, indicating a high affinity of the protease for the casein substrate. The thermodynamic analysis provided a k_i value of 0.0031 min^{-1}, $t_{½}$ value of 223.548 minutes, and ΔG_i value of +105.84 kJ/mol. These values suggest that the halostable protease has a high level of stability, with a positive ΔG_i indicating substantial energy required for denaturation and a long half-life demonstrating resistance to inactivation. Furthermore, the protease was classified as a metalloprotease due to the observed decrease in activity upon EDTA chelation of metal cofactors. The results indicate that the halostable protease from *Bacillus clausii* J1G-0%B holds significant potential for applications in various fields, including the food industry and bioremediation of high-salinity environments.

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