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OPTIMISATION OF BROMELAIN PURIFICATION FROM PINEAPPLE PEEL (*Ananas comosus* L.) USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Bromelain, a cysteine protease with broad industrial uses, can be efficiently extracted from pineapple processing residues, offering a sustainable method for waste valorisation. This study aimed to optimise the purification of bromelain from pineapple (*Ananas comosus* L.) peel using dialysis combined with Response Surface Methodology (RSM) based on a Box-Behnken design. A crude enzyme extract was obtained through different extraction media, followed by partial purification through ammonium sulfate precipitation and subsequent dialysis. The effects of pH (6 - 8), dialysis duration (20 - 30 h), and buffer-to-sample ratio (100:1 - 200:1, v/v) on specific activity were assessed. A quadratic regression model was applied to the bromelain purification process with statistically significant results ($p < 0.01$, $R^2 = 0.9971$). The optimal conditions were determined as pH 6.93, a dialysis time of 25.16 h, and a buffer-to-sample ratio of 134.50 (v/v), yielding a predicted specific activity of 235.86 U/mg protein, which closely matched the experimental value of 235.48 U/mg protein. The purified bromelain demonstrated high stability within pH 6 - 8 and at temperatures of 40 - 50 °C. SDS-PAGE analysis confirmed a predominant protein band at 25 - 30 kDa. These findings show that dialysis combined with statistical optimisation offers an effective and scalable method for bromelain purification, supporting the sustainable use of pineapple processing waste. This study provides one of the first systematic optimisations of dialysis parameters for bromelain purification from pineapple peel, highlighting its potential as a low-cost alternative to conventional chromatographic techniques. Compared with previously reported purification approaches, the proposed method demonstrates competitive specific activity while significantly reducing operational complexity and cost.

Keywords: Bromelain, pineapple peel, dialysis, response surface methodology, Box-Behnken design, enzyme purification

1. INTRODUCTION

Pineapple (*Ananas comosus* L.) is one of the most important tropical fruits widely utilised in the food processing industry to produce juices, concentrates, jams, and other value products. Global pineapple production has shown a steady increase, reaching approximately 29.6 million tons in 2023, driven by rising consumer demand. However, industrial processing generates a substantial amount of waste, accounting for approximately 30–50% of the total fruit mass, mainly consisting of peels, cores, and crowns. These residues pose environmental challenges if they are not efficiently managed [1],[2].

In recent years, the valorisation of agro-industrial by-products has become a key focus within the framework of the circular economy and sustainable bioprocessing. Pineapple peel, in particular, has been identified as a valuable biomass rich in sugars, organic acids, minerals, and structural polysaccharides such as cellulose, hemicellulose, and pectin [3],[4]. More importantly, it represents a valuable source of bromelain, a proteolytic enzyme complex with considerable food and cosmetic industrial relevance [5],[6].

Bromelain belongs to the cysteine protease family and exhibits broad substrate specificity and high catalytic efficiency. It has been extensively studied due to its diverse biological activities,

including anti-inflammatory, antimicrobial, antioxidant, and fibrinolytic effects [6],[7]. These properties enable its application in various sectors such as food processing, pharmaceuticals, nutraceuticals, and cosmetics [8],[9]. In dermatological applications, bromelain has demonstrated promising potential in wound healing and acne treatment through protein degradation and inhibition of pathogenic microorganisms [7],[10].

While bromelain is distributed throughout various parts of the pineapple plant, several studies have confirmed that the peel and other processing residues retain significant enzyme activity, making them viable, low-cost raw materials for enzyme recovery [3],[11]. The utilisation of such waste streams not only enhances economic value but also contributes to environmental sustainability by reducing agro-industrial waste [12]. Despite its promising applications, the industrial use of bromelain is limited by challenges related to enzyme purity, stability, and recovery efficiency. Crude extracts often contain a mixture of proteins and non-protein impurities, which reduce specific activity and hinder downstream applications [13]. Therefore, developing efficient and economically viable purification strategies is crucial.

Various purification techniques have been investigated, including ammonium sulfate precipitation, ultrafiltration, aqueous two-phase systems, and chromatographic methods such as ion-exchange and affinity chromatography [13], [14]. While chromatographic techniques provide high purity, they are generally expensive and difficult to scale up. Alternatively, dialysis offers a simple and cost-effective method for desalting and removing low-molecular-weight impurities. Although various purification techniques, such as chromatography and aqueous two-phase systems, have been extensively studied, these methods are often limited by high operational costs, complexity, and scalability issues. In contrast, dialysis remains underexplored as a tunable purification step, particularly when integrated with statistical optimisation tools such as RSM. To date, there are no systematic studies investigating the combined effects of dialysis parameters (pH, duration, and buffer-to-sample ratio) on bromelain purification efficiency, especially pineapple peel biomass.

Response surface methodology (RSM), especially when combined with Box-Behnken design (BBD), has been widely applied as a powerful statistical tool for optimising complex bioprocesses. It enables the evaluation of multiple variables and their interactions with fewer experimental runs, thereby improving efficiency and reliability [15],[16]. Although RSM has been successfully applied in bromelain extraction and other enzyme systems, its application in optimising purification processes, particularly dialysis, remains limited [4],[17].

In addition, accurate evaluation of enzyme purity is critical for validating purification efficiency. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is a standard analytical technique for determining molecular weight and assessing protein purity. Combined with enzyme activity assays and specific activity measurements, it provides a comprehensive evaluation of enzyme quality and functionality [18].

Therefore, this study aims to optimise the purification of bromelain from pineapple peel using dialysis combined with RMS. The purification efficiency is evaluated through stability, specific activity and SDS-PAGE analysis. The objective of this study is expected to provide a scientific basis for developing an efficient and sustainable purification strategy, contributing to the valorisation of pineapple processing waste for industrial applications.

2. MATERIALS AND METHODS

2.1. Materials

Fresh pineapple peels (*Ananas comosus* L.) were collected from a local fruit processing facility and thoroughly washed with distilled water to remove adhering impurities. The cleaned peels were cut into small pieces and either used immediately for extraction or stored at −20 °C until further use.

All chemicals and reagents used in this study were of analytical grade. Casein, trichloroacetic acid (TCA), and ammonium sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate buffer components (Na_2HPO_4 and NaH_2PO_2), sodium hydroxide (NaOH), and hydrochloric acid (HCl), disodium ethylenediaminetetraacetate (EDTA) were obtained from Xilong Scientific Co., Ltd. (Shantou, China). Dialysis membranes (molecular weight cut-off 10-12 kDa) were procured from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA).

2.2. Preparation of crude bromelain extract

Crude bromelain extract was prepared based on commonly reported extraction procedures for pineapple residues with minor modifications [2],[13]. Fresh pineapple peels were homogenised with extraction media at a solid-to-liquid ratio of 1:0.5 (w/v). Three extraction systems were investigated, namely phosphate buffer (0.05 M), Tris-HCl buffer (0.05 M), and distilled water, all adjusted to pH 7.0. The homogenate was filtered through muslin cloth to remove insoluble residues. The filtrate was collected as a crude enzyme extract and stored at 4 °C until further analysis.

2.3. Ammonium sulfate precipitation

Partial purification of bromelain was carried out by ammonium sulfate fractionation according to Gul et al., with slight modifications [19]. The crude bromelain extract was subjected to stepwise precipitation using ammonium sulfate at different saturation levels (30 - 60%) under continuous stirring at 4 °C to ensure uniform distribution of the salt. The mixture was then incubated at 4 °C for 1 h to allow protein precipitation. The precipitated proteins were recovered by centrifugation at 4000 rpm for 20 min. The resulting pellet was collected and resuspended in 0.05 M phosphate buffer (pH 7.0) for further purification.

2.4. Dialysis and experimental design

The resuspended protein fraction was subjected to dialysis using a membrane with a molecular weight cut-off of 10 -12 kDa against phosphate buffer (0.05 M, pH 7.0) at 4 °C to remove residual salts and low-molecular-weight impurities. Process optimisation was performed using Response Surface Methodology (RSM) based on a Box-Behnken design. Three independent variables were selected: pH (X_1, 6 - 8), dialysis time (X_2, 20-30 h), and buffer-to-sample ratio (X_3, 100 - 200, v/v). Each independent variable was coded at three levels (−1, 0, +1), corresponding to low, central, and high values, respectively, as presented in Table 1.

Table 1. Coded and actual levels of independent variables used in the Box-Behnken design

No.	Variable	Symbol	-1	0	1
1	pH	X_1	6	7	8
2	Dialysis time (h)	X_2	20	25	30
3	Buffer-to-sample ratio (v/v)	X_3	100	150	200

The experimental design and statistical analysis were carried out using JMP® 18 Pro software (SAS Institute Inc., Cary, NC, USA). Specific activity (U/mg protein) was used as the response variable. A second-order polynomial model was fitted to the experimental data, and analysis of variance (ANOVA) was applied to evaluate the significance of the model and interaction effects. All experiments were conducted in triplicate, and data are presented as mean ± standard deviation. Statistical significance was evaluated at $p < 0.05$.

2.5. Determination of proteolytic activity

Proteolytic activity of bromelain was determined using the casein digestion unit method as described by Gul et al., with slight modifications [19]. Casein (0.5%, w/v) was prepared in phosphate buffer (0.05 M, pH 7.0). The reaction mixture consisted of 0.8 mL phosphate buffer, 0.2 mL activation solution (0.006 M EDTA and 0.03 M L-cysteine), and 1 mL casein solution, followed by preincubation at 37 °C for 10 min. The reaction was initiated by adding 1 mL of the enzyme solution and incubating at 37 °C for 15 min. The reaction was terminated by adding 3 mL of 5% (w/v) TCA. After centrifugation at 3000 rpm for 20 min at 4 °C, the absorbance of the supernatant was measured at 275 nm. A standard curve was constructed using L-tyrosine. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine per minute under the assay conditions.

The amount of tyrosine released was determined from the standard curve, and proteolytic activity (A) was calculated as follows:

$$A\ (U/mL) = \frac{C \times f}{t \times V_e} \qquad \text{(Eq.1)}$$

where C is the amount of tyrosine released (µg), f is the dilution factor, ttt is the reaction time (min), and V_e is the volume of enzyme used (mL).

Total activity (TA) and specific activity (SA) were calculated using the following equations:

$$TA\ (U) = A \times V \quad \text{(Eq.2)}$$

$$SA\ (U/mg) = \frac{A}{P_t} \quad \text{(Eq.3)}$$

where V is the total volume of enzyme extract (mL) and P_t is the protein concentration (mg/mL).

Activity recovery (AR) was calculated as:

$$AR\ (\%) = \frac{TA_{after}}{TA_{before}} \quad \text{(Eq.4)}$$

Protein concentration was determined using the Bradford method [20], with bovine serum albumin (BSA) as the standard. Absorbance was measured at 595 nm.

2.6. Stability studies of bromelain

pH stability: The pH stability of the purified bromelain was assessed by maintaining the enzyme within various buffer systems (pH 4.0-9.0) at 37 °C for 30 min. After incubation, the residual activity was determined under standard assay conditions and expressed as a percentage of the initial activity [6].

Thermal stability: Thermal stability was determined by subjecting the enzyme solution to a temperature gradient (30-70 °C) for a 30 mim incubation period. After incubation, the samples were cooled to room temperature, and residual activity was measured under standard assay conditions and expressed as relative activity (%) [6].

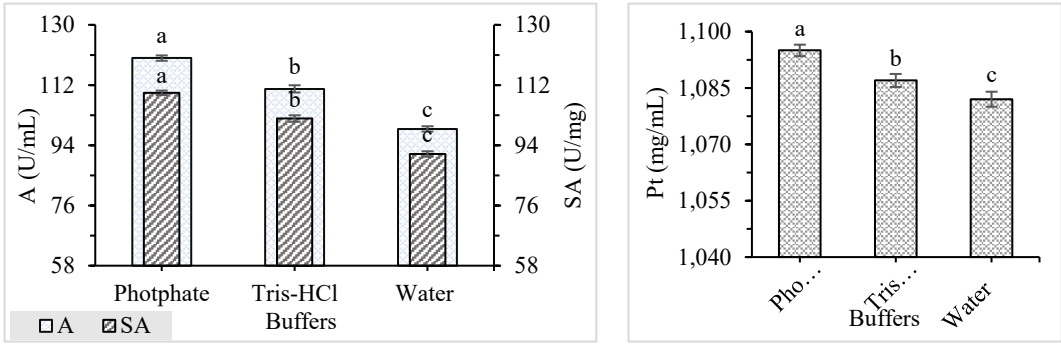
2.7. SDS-PAGE analysis

Protein samples were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a SureCast™ Handcast System (Thermo Fisher Scientific, USA). The gel consisted of a 4% (w/v) stacking gel and an 8% (w/v) resolving gel prepared according to the manufacturer's instructions. Electrophoresis was carried out in Tris–glycine running buffer (1×), prepared from a 10× Novex™ Tris–Glycine SDS buffer (Thermo Fisher Scientific, USA). The gels were run at a constant voltage of 125 V for 100 min. Following electrophoresis, protein bands were visualised using Coomassie Brilliant Blue staining.

3. RESULTS AND DISCUSSION

3.1. Effect of extraction buffer on crude bromelain activity

The extraction medium significantly influenced bromelain recovery from pineapple peel. As shown in *Fig. 1*, the extraction buffer had a noticeable impact on bromelain recovery from pineapple peel. Phosphate buffer resulted in the highest enzyme activity and specific activity, followed by Tris–HCl buffer, whereas distilled water showed the lowest values. A similar pattern was observed for total protein content, suggesting that the choice of extraction medium directly affects both protein solubilisation and enzyme performance. The better performance of the phosphate buffer is likely related to its ability to maintain a stable near-neutral pH, which is favourable for preserving the structural integrity and catalytic function of bromelain. In addition, the ionic environment provided by phosphate ions may help stabilise protein molecules during extraction, thereby reducing aggregation or activity loss. In contrast, the use of distilled water may lead to pH fluctuations and partial enzyme destabilisation due to the absence of buffering capacity [21], [22]. This observation agrees with previous studies reporting that bromelain exhibits higher stability and activity under near-neutral conditions in suitable buffer systems [23]. Based on these results, phosphate buffer was selected for subsequent experiments.

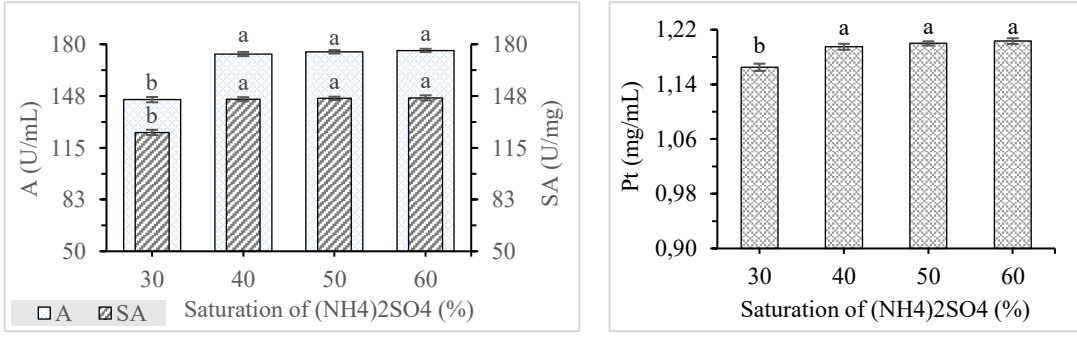


Different letters (a–c) indicate statistically significant differences at $p < 0.05$, as determined by one-way ANOVA.

Fig. 1. Effect of extraction buffer on enzyme activity (A), specific activity (SA), and total protein content (P_t) of crude bromelain

3.2. Effect of ammonium sulfate concentration on bromelain activity

As shown in *Fig. 2*, ammonium sulfate concentration had a pronounced effect on the precipitation efficiency of bromelain. Both enzyme activity (A) and specific activity (SA) increased from 30% to 40% saturation, indicating improved recovery of active enzyme within this range. However, further increases in salt concentration (50–60%) led to a decline in both A and SA, despite a slight increase in total protein content (P_t). This behaviour can be attributed to the salting-out effect, where increasing ionic strength reduces protein solubility and promotes precipitation. At moderate saturation (~40%), bromelain is preferentially precipitated, resulting in higher enzymatic activity and purity. In contrast, higher salt concentrations may promote co-precipitation of non-target proteins and potentially induce partial structural alterations, leading to reduced catalytic efficiency [19], [22]. This trend is consistent with previous studies reporting that bromelain and similar plant proteases exhibit optimal recovery at intermediate ammonium sulfate saturation levels, typically around 40% [19], [24].



Different letters (a–c) indicate statistically significant differences at $p < 0.05$, as determined by one-way ANOVA.

Fig. 2. Effect of ammonium sulfate saturation on enzyme activity (A), specific activity (SA), and total protein content (P_t) of bromelain

3.3. Optimisation of bromelain purification

The optimisation of bromelain purification was performed using RSM based on a Box-Behnken design. The experimental design matrix and corresponding responses are presented in *Table 2*, where the specific activity ranged from 124.33 to 238.17 U/mg protein, indicating a strong dependence of purification efficiency on the investigated variables.

A second-order polynomial model was developed to describe the relationship between the independent variables and the response (Y), expressed in coded form as follows:

$$Y = 232.75 - 10.46X_1 + 7.51X_2 - 16.98X_3 - 11.41X_1X_3 + 19.54X_2X_3 - 48.01X_1^2 - 16.93X_2^2 - 25.13X_3^2$$

Table 2. Box-Behnken design matrix and experimental results for bromelain purification optimization

Run	Coded variables (X_1, X_2, X_3)	Actual variables			Specific activity (U/mg protein)	
		X_1 (pH)	X_2 (Dialysis time, h)	X_3 (Buffer-to-sample ratio, v/v)	Experimental	Predicted
1	(+1,0,+1)	8	25	200	124.331 ± 3.574	120.756
2	(0,−1,+1)	7	20	200	143.478 ± 3.180	146.658
3	(+1,−1,0)	8	20	150	143.740 ± 0.394	144.134
4	(−1,0,+1)	6	25	200	166.823 ± 2.328	164.495
5	(+1,+1,0)	8	30	150	169.708 ± 0.853	170.561
6	(−1,0,−1)	6	25	100	172.069 ± 3.574	175.643
7	(+1,0,−1)	8	25	100	175.216 ± 2.328	177.544
8	(−1,−1,0)	6	20	150	177.315 ± 0.853	176.462
9	(−1,+1,0)	6	30	150	180.462 ± 0.392	180.07
10	(0,+1,+1)	7	30	200	198.037 ± 2.721	200.758
11	(0,+1,−1)	7	30	100	198.823 ± 3.180	195.643
12	(0,−1,−1)	7	20	100	222.432 ± 2.721	219.709
13	(0, 0, 0)	7	25	150	231.611 ± 1.137	232.748
14	(0, 0, 0)	7	25	150	228.463 ± 4.285	232.748
15	(0, 0, 0)	7	25	150	238.168 ± 5.420	232.748

The presence of significant quadratic terms (X_1^2, X_2^2, X_3^2) confirms the non-linear behaviour of the system, suggesting an optimal region rather than a linear trend. Among the linear effects, dialysis time (X_2) showed a positive contribution, whereas pH (X_1) and buffer-to-sample ratio (X_3) exhibited negative effects within the studied range. This indicates that excessive pH or dilution conditions may negatively impact enzyme stability, while appropriate dialysis duration enhances purification efficiency. The improvement in specific activity after dialysis can be attributed to the selective removal of low-molecular-weight impurities, salts, and inhibitory compounds, which may otherwise interfere with enzyme activity measurements.

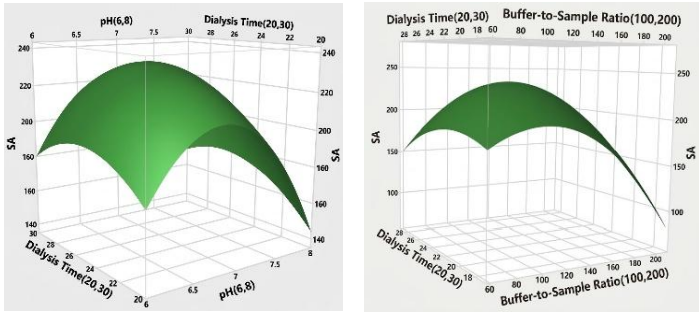
Table 3. Analysis of variance (ANOVA) and predicted optimum conditions for bromelain specific activity optimisation using the Box-Behnken design.

Source	DF	Sum of squares	Mean square	Fvalue	Pvalue	Predicted optimum conditions
1. ANOVA for the model						
Model	9	16541.205	1837.91	75.188	0.0001*	+ pH: 6.93 + Dialysis time: 25.16 h + Buffer-to-sample ratio: 134.50 (v/v) + **Predicted SA:** 235.863 (U/mg protein) **+ Experimental SA at optimum:** 235.482 (U/mg protein)
Error	5	122.220	24.44			
Total	14	16663.425				
2. Lack-of-fit test						
Lack of fit	3	73.18737	24.3958	0.9951	0.5366	
Pure error	2	49.03239	24.5162			
Total error	5	122.21976		**RSq:** 0.9971		

The adequacy of the model was evaluated by analysis of variance (ANOVA), as shown in *Table 3*. The model was statistically significant (F = 75.188, p = 0.0001), while the Lack-of-fit was not significant (p = 0.5366), confirming that the model adequately describes the experimental data.

The interaction effects of the variables are illustrated by the response surface plots in *Fig.3*. A pronounced curvature was observed for the interaction between pH and dialysis time, indicating their

combined influence on enzyme purification (*Fig.3a*). Similarly, the interaction between dialysis time and buffer-to-sample ratio showed a synergistic effect, suggesting improved mass transfer during dialysis at appropriate conditions (*Fig.3b)*. In contrast, the negative interaction between pH and buffer ratio implies that simultaneous increases in these variables may destabilise the enzyme system. The negative effect of buffer-to-sample ratio suggests that excessive dilution may reduce effective enzyme concentration and promote structural instability due to prolonged exposure to aqueous environments.



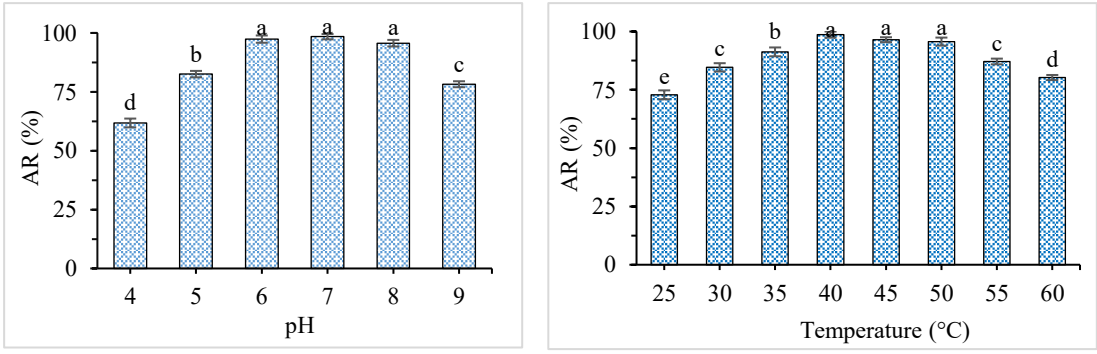
a. pH × Dialysis time *b. Dialysis time × Buffer-to-sample ratio*

Fig 3. Response surface plot for the interaction effects of independent variables on specific activity.

From the developed model, the optimal conditions were determined as pH 6.93, dialysis time of 25.16h, and a buffer-to-sample ratio of 134.50 (v/v). Under these conditions, the predicted specific activity was 235.863 U/mg protein. Experimental validation yielded a comparable value (235.482 U/mg protein), with a deviation of less than 1%, demonstrating the high predictive accuracy of the model. These results confirm the effectiveness of RSM in optimising bromelain purification and are consistent with previous studies reporting improved enzyme recovery using statistical optimisation approaches [4], [25].

3.4. Effect of pH and temperature on bromelain activity recovery

As shown in *Fig. 4,* bromelain exhibited high stability within pH 6.0–8.0, with maximum activity near neutral conditions, while a significant decline was observed under more acidic and alkaline environments. This behaviour is associated with changes in the ionisation state of active-site residues, particularly the catalytic cysteine, which directly influences enzyme conformation and catalytic efficiency. Similar pH-dependent stability profiles have been reported for bromelain derived from pineapple by-products, confirming that near-neutral conditions are optimal for maintaining enzymatic functionality [26], [27].



Different letters (a–c) indicate statistically significant differences at $p < 0.05$, as determined by one-way ANOVA.

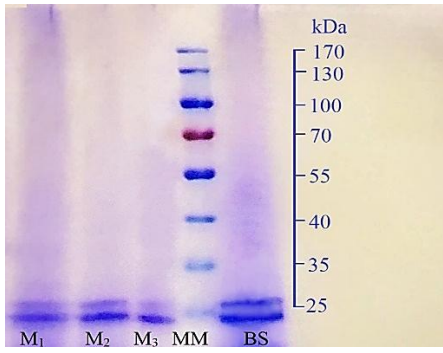
Fig. 4. Effect of pH and temperature on the activity recovery of purified bromelain

Temperature also played a critical role in enzyme stability, with bromelain retaining high activity in the range of 40–50 °C, followed by a gradual decline at higher temperatures due to thermal denaturation. This trend is consistent with recent studies indicating that bromelain from peel, core, and stem generally exhibits moderate thermostability within 40–60 °C, beyond which irreversible structural changes occur, leading to rapid loss of activity [28], [29]. Although stem bromelain has been reported

to possess slightly higher thermal stability, bromelain extracted from peel shows comparable activity but may be more sensitive to processing conditions and purification strategies. Overall, the present results are in good agreement with recent literature, confirming that bromelain from pineapple peel exhibits moderate thermostability typical of plant cysteine proteases. This characteristic makes it suitable for applications under mild processing conditions, while highlighting the importance of temperature control to minimise enzymatic inactivation during downstream processing.

3.5. SDS-PAGE analysis

As shown in *Fig. 5*, the protein profiles of the extracted samples (M_1 - M_3), commercial bromelain (BS), and the molecular weight marker (MM) were clearly resolved by SDS-PAGE. A distinct protein band was consistently observed in all extracted samples within the molecular weight range of approximately 25-30 kDa, corresponding closely to the band detected in the commercial bromelain standard. The presence of a dominant band at this position suggests that bromelain was successfully extracted as the major protein component. The alignment with the commercial standard supports the identification of the enzyme and demonstrates the effectiveness of the purification process. Although a dominant band at 25-30 kDa was observed, the presence of faint additional bands indicates that the enzyme preparation is partially purified rather than homogeneous. Further purification steps, such as ion-exchange chromatography, may be required to achieve higher purity levels. A progressive reduction in band intensity was noted from M1 to M3, correlating with the sample dilution levels, while the band migration remained consistent. This confirms that the structural integrity of the enzyme was maintained throughout the extraction and purification stages, with no detectable fragmentation or degradation. The observed molecular weight range is consistent with previous reports, which indicate that bromelain derived from pineapple residues typically falls within 24 to 33 kDa [2].



MM: molecular weight marker.

BS: commercial bromelain.

M_1 - M_3: extracted samples at different dilution levels.

Fig.5. SDS-PAGE profile of bromelain extracted from pineapple peel.

4. CONCLUSIONS

This study successfully developed an optimised purification process for bromelain from pineapple peel using dialysis integrated with RMS. The results demonstrated that both extraction and purification parameters significantly influence enzyme recovery and specific activity. Phosphate buffer and 40% ammonium sulfate saturation were identified as optimal pre-treatment conditions. The Box-Behnken model exhibited high statistical significance and predictive accuracy, enabling the determination of optimal dialysis conditions (pH 6.93, 25.16 h, and buffer-to-sample ratio of 134.50) that yielded high specific activity with minimal deviation between predicted and experimental values. The purified bromelain showed good stability under near-neutral pH and moderate temperature conditions, and SDS-PAGE confirmed its molecular integrity. This result highlights the potential of combining dialysis with statistical optimisation as a simple, scalable, and cost-effective approach for enzyme purification. The findings contribute to the sustainable utilisation of pineapple processing waste and support the development of value-added bioproducts for industrial applications.

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