RESEARCH ARTICLE

Effects of Precipitation Methods on Protease Yield and Proteolytic Activities of Protease Enzyme from Different Maturity Stages of Starfruit (Averrhoa Carambola)

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Abstract

This study investigates the impact of three precipitation methods, ammonium sulfate (40%), acetone, and acetone with trichloroacetic acid (TCA) on the yield and activity of protease extracted from *Averrhoa carambola* (starfruit) at various maturity stages (unripe, semi-ripe, and ripe). The study employed response surface methodology (RSM) to optimize hydrolysis conditions, specifically temperature, and pH, for maximum proteolytic activity. The proteases were incubated in buffers at pH values ranging from 3.5 to 8.5 for 12 hours, and the proteolytic activity was assessed. Additionally, temperature effects were evaluated by incubating proteases at temperatures between 40$^{\circ}C$ and 90$^{\circ}C$. The storage stability of the proteases was monitored over a 7-day period at temperatures $<4^{\circ}C$. The results revealed that ammonium sulfate precipitation (40%) yielded the highest proteolytic activity (PA), particularly at the unripe stage, where it reached 0.34 units. Conversely, acetone with TCA precipitation exhibited the highest specific activity, with values of up to 2.38 U/mg at the ripe stage, indicating that although the total protease yield was lower, the enzymes extracted were more efficient per unit of protein. Acetone precipitation showed intermediate values for both PA and specific activity. Numerical optimization identified the optimal conditions for proteolytic activity as 65$^{\circ}C$ and pH 6.5, achieving a maximum activity of 0.862 units/mL. Despite the promising proteolytic activity under optimized conditions, a significant reduction in enzyme activity was observed during a 7-day storage period at $<4^{\circ}C$. This finding underscores the importance of considering both total proteolytic activity and specific activity when selecting the optimal protease extraction method. Overall, this study demonstrates that ammonium sulfate precipitation is the most effective method for extracting protease from starfruit, particularly at the unripe stage for higher yield, while acetone with TCA precipitation offers advantages in producing more active proteases, suitable for applications requiring enzymatic efficiency.

Keywords: starfruit, maturity stages, precipitation methods, proteolytic activity, specific activity

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1 Introduction

Proteases are examples of plant enzymes that are extensively utilized in both the food and medicine sectors [1]. According to [2], proteases function as protein-digesting enzymes, also known as proteolytic enzymes, which break down proteins. It can be categorized as per its characteristics [3], such as microbial, animal, or plant origins, catalytic site, and catalytic action [4]. Proteases aid in the hydrolysis of peptide bonds between amino acids and proteins. Proteases consist of about 60% of the enzymes present in the global market [5]. Different processes, like softening of meat, brewing, treatment for cancer, clotting of milk, digestion, and viral illnesses, involve the usage of proteases such as bromelain, ficin, and papain [6]. These types of enzymes are also used in bakery items [7] to reduce the consistency of dough, improve the texture and flavor, enhance the mixing process, and control the strength of gluten in bread [8]. Proteases are included in formulations of food items to improve their digestibility [9].

Protease is often purified by chromatography and salt precipitation [10], extraction by homogenizing in Tris-HCl buffer followed by purification in ammonium sulfate [11], three-phase partitioning (TPP) [12], extraction using phosphate buffer, and subsequent purification in acetone precipitation [13], and extraction using phosphate buffer and subsequent purification with acetone with TCA precipitation [14]. In ammonium sulfate precipitation, precipitation proceeds when high concentrations of small, highly charged ions such as ammonium sulfate are added. These groups compete with the proteins to bind to the water molecules. This removes the water molecules from the protein and decreases its solubility, resulting in precipitation [15]. Acetone prevents the dispersion of proteins among water-based solvents, causing protein aggregation and precipitation. A mixture of TCA and sodium deoxycholate enhances the precipitation of very small amounts of proteins as deoxycholate binds to the hydrophobic parts of proteins [16].

Starfruit (*Averrhoa carambola*), also known as carambola, belongs to the Oxalidaceae family and is widespread in tropical Southeast Asian regions [17]. Starfruit is sweet, mildly acidic, juicy, sugary, and has a unique flavor. The fruit is commonly consumed as fresh juices or used for flavoring [18]. The flesh and skin of this star-shaped fruit are crispy and juicy, and it bears waxy skin with plenty of smooth brown seeds [19]. The skin of the starfruit changes from dark green to orange during the process of maturation, with the dark green color referring to an immature starfruit and the orange color representing a highly mature starfruit [20]. The maturity of starfruits can be divided into seven categories, namely index 1 to index 7. The starfruit with the lowest maturity (unripe) is represented by index 1, while index 7 shows the starfruit with the highest maturity (ripe) [20].

Although proteases have demonstrated remarkable effectiveness in various industries, there is still room for optimization in their extraction process. In this study, we focused on optimizing two key factors: temperature and pH, as they are known to significantly influence protease activity and stability. Temperature and pH were chosen as primary variables based on prior research showing that starfruit proteases exhibit activity in the pH range of 6-8 and thermal stability between 40-80$^{\circ}C$. While factors like material-to-liquid ratio and incubation time also affect protease extraction, these were held constant in the current study, based on preliminary experiments and for simplicity in design. Future work will explore these additional factors for further optimization.

Because of their effectiveness, proteases continue to establish high standards in various sectors, and research in this area is rapidly growing [6, 21]. Proteases are widely used in food processing to enhance safety and value [3].The use of plant-derived enzymes, like those from starfruit, is gaining popularity, although their application is still limited. Starfruit is an evergreen plant, flowering year-round and producing fruit for much of the year. Despite its availability, starfruit is underutilized and not widely processed or consumed. Therefore, enzyme extraction presents a promising opportunity to enhance the utilization of starfruit in the food industry.

2 Materials and Methods

2.1 Raw materials

Starfruit of different maturity stages, were procured from the local cultivator of Chitwan, Nepal. It was stored at refrigeration temperature ($<10^{\circ}C$) until used. Selection criteria for different maturity are based on [22]:

1. Unripe fruits were firm to the touch, predominantly green in color, and lacked any noticeable sweetness

2. Semi-ripe starfruits at this stage had a yellow-green to yellow color and a slightly

softened texture.

3. Ripe fruits were fully yellow or golden in color, with a soft texture and a sweet flavor

2.2 Extraction of starfruit juice

The starfruit of the required maturity stages were cut, and the seeds were removed before the extraction of juice in the extractor (Model: Havel's max grind 14000). The juice was then filtered through a layer of muslin cloth and stored at $<4°C$.

2.3 Precipitation of starfruit juice

Three partial purification methods were used to purify the crude extract, comprising acetone, ammonium sulfate, and acetone with TCA precipitation.

2.3.1 Ammonium sulfate precipitation

Ammonium sulfate to a concentration of 40%, 50%, or 60% saturation (w/v) was followed by a 12 hr incubation at $4°C$ to precipitate the protease. This was followed by centrifugation at 10,000×g for 10 min using a centrifuge (Model D3024R DLAB, UK). After that, the precipitate was dissolved in a 50 mM phosphate buffer with a pH of 7.2. It was then collected and stored at $4°C$ for further use [23].

2.3.2 Acetone precipitation

Cold acetone (-20$°C$) was slowly added to the crude extract at a ratio of 3:1 (acetone to crude extract), followed by gentle stirring to allow precipitation for 12 hours. The incubation time (12 hours) and material-to-liquid ratio (3:1) were fixed based on preliminary trials to standardize conditions and ensure effective precipitation. The mixture was then centrifuged (Model: D3024R DLAB, UK) at 10,000×g for 15 minutes. The supernatant was removed, and the precipitate was dissolved in 50 mM phosphate buffer with a pH of 7.2 [24].

2.3.3 Acetone with Tri-Chloro Acetic Acid (TCA) precipitation

Cold acetone (-20$°C$) was gradually added to the crude extract at a ratio of 3:1 (acetone to crude extract), followed immediately by the addition of 10% TCA. The mixture was gently stirred and allowed to precipitate for 12 hours. The incubation time (12 hours) and material-to-liquid ratio (3:1) were standardized based on preliminary trials to ensure consistency and reproducibility in results. The mixture was centrifuged at 10,000×g for 15 minutes using a centrifuge (Model D3024R DLAB, UK). The precipitate was dissolved in 50 mM phosphate buffer with a pH of 7.2 and stored at $4°C$ until further use [16].

2.3.4 Specific activity

Specific activity is a measure of enzyme purity and efficiency, defined as the amount of enzyme activity (U) per milligram of total protein in a sample. It represents the catalytic ability of an enzyme independent of sample concentration. It is determined by [25], the calculation equation is as follows:

$$\text{Specific activity} = \frac{\textbf{proteolytic}\ activity(\text{units/mL})}{\textbf{protein}\ content(mg/ml)} \quad (1)$$

2.4 Proteolytic activity determination

Protease activity was determined using a method given by [26]. It may be used as a standardized procedure to determine the activity of proteases. In this assay, casein acts as a substrate. Tyrosine and other amino acids, along with peptide fragments, are released when the protease breaks down casein. The primary reaction of Folin and Ciocalteus phenol, or Folin's reagent with free tyrosine, is the production of a blue-colored chromophore that can be quantified and recorded on a spectrophotometer as an absorbance value. Chromophores are produced in greater quantities, and the protease's activity becomes stronger when casein releases more tyrosine. Absorbance values obtained due to the activity of the protease are compared to a standard curve created by reacting a known amount of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. Protease activity can be calculated from the standard curve in terms of 'Units', which is the quantity in micromoles of tyrosine equivalents liberated per minute from the casein.

One protease unit can be defined as the amount of casein hydrolyzed to produce color equivalent to 1.0 μmole (181 μg) of tyrosine per minute at pH 7.5 and $37°C$ (color by Folin's reagent) and was calculated by the following standard formula:

$$\text{Protease activity (units/mL)} = \frac{(\text{mol Tyrosine}) \times V_T}{V_E \times t \times V_C} \quad (2)$$

where, V_T is the total assay volume in mL, V_E is the volume of the enzyme used in mL, t is the reaction time in min, and V_C is the volume used in the colorimetric reaction in mL.

2.5 Protein assay

The protein content of the starfruit juice was determined using the procedure given by [27]. It is a sensitive colorimetric protein assay with respect to the binding property of the dye Coomassie Brilliant Blue G-250 to the protein to be assayed. It is used to estimate protein concentration. For this, 1 mL of enzyme extract was mixed with 5 mL of alkaline copper solution in a test tube. An alkaline copper solution was prepared by mixing 50 mL of reagent A (2% Na_2CO_3 in 0.1 N NaOH) and 1 mL of reagent B (0.5% copper sulphate in 1% sodium potassium tartarate). The contents of the tubes were allowed to stand for 10 min, and 0.5 mL of diluted Folin's reagent (1:1 mixture of Folin's reagent and 0.1 N NaOH) was added and mixed quickly, and the tube was incubated at room temperature in the dark for 30 min. The contents of the tube were read in a spectrophotometer at 595 nm and compared with a calibration curve. 0.2, 0.4, 0.6, 0.8, and 1 mL of working standard solution (200 μg/mL of Bovine Serum Albumin (BSA)) were pipetted out in labeled test tubes. A tube with 1 mL of distilled water serves as a blank. The volume in each test tube was made up to 1 mL with distilled water. 5 mL of alkaline copper solution was added to each test tube, including the blank. The contents of the tubes were mixed by shaking them and were let to stand for 10 min. 0.5 mL of diluted Folin's reagent was added rapidly with immediate mixing, and the tubes were incubated at room temperature in the dark for 30 min. The absorbance of all the tubes was measured in a spectrophotometer at 595 nm, and a standard curve was plotted by taking the concentration of BSA along the X-axis and the absorbance at 595 nm along the Y-axis.

2.6 Experimental design

The experimental design, data analysis, and model building were performed using "Design Expert" software (Version 13, Stat-Ease Inc., USA). The optimization of the starfruit enzyme was prepared with variations in (a) pH and (b) temperature, as shown in Tables 1 and 2. The independent variables and their levels were selected based on literature and preliminary experiments. Starfruit proteases show activity in the pH range of 6-8 [28] and thermal stability between 40-80$^{\circ}C$ [29]. In the experiment, pH was adjusted by using different buffers (sodium phosphate at pH levels in the acidic range and Tris-HCl at pH levels in the alkaline range) [30]. A two-factor central composite design was employed. The response variable was proteolytic activity (PA). While temperature and pH were chosen as the primary variables for this study based on their significant influence on protease activity, other factors such as material-to-liquid ratio and incubation time, which may also impact the extraction process, were held constant in the current study. These factors will be considered for further optimization in future research.

Table 1. Different constraints for optimization of starfruit protease enzyme.

Name	Goal
Temperature of enzyme	In the range 40-90$^{\circ}C$
pH of enzyme	In the range 3.5-9.5
Proteolytic activity (PA)	Maximum

2.7 Analysis of data

The independent process variables were correlated using a second-order quadratic model. Multiple regression analysis with Design Expert® software was used to determine the second-order polynomial coefficient for each term of the equation. The statistical significance of the terms was investigated using analysis of variance for the response after the data was fitted to the selected model. R^2 (coefficient of determination- the amount of variation around the mean explained by the model), adjusted R^2 (a measure of the amount of variation around the mean explained by the model adjusted for the number of terms in the model), predicted R^2 (a measure of how well the model predicts a model value) and Fisher's F-test were used to test the adequacy of the model. The coefficient of determination R^2 is a measure of degree of fit, as it is the ratio of explained variation to the total variation. A better empirical model fits the actual data when R^2 approaches unity. The smaller the value of R^2, the less relevance the dependent variables in the model have in explaining the behavior variation. Then the effect of predictors on the response was interpreted using the models.

2.8 Model validation

Model validation was identified on the basis of the equation provided by Design Expert® and calculated data.

2.9 Storage stability

The storage stability of the protease enzyme was determined by storing the solution of an enzyme at a temperature below 10$^{\circ}C$ and measuring the activity for 7 days using the standard method for protease determination.

Table 2. Responses by variables of enzymes from starfruit (*Averrhoa carambola*).

Std	Run	Space type	Factor 1 A: pH	Factor 2 B: Temperature	Response 1 Proteolytic activity (units/mL)
1	1	Factorial	4.5	40	0.3822
2	2	Factorial	8.5	40	0.4003
6	3	Axial	8.5	65	0.822
9	4	Centre	6.5	65	0.8523
13	5	Centre	6.5	65	0.8116
5	6	Axial	4.5	65	0.763
10	7	Centre	6.5	65	0.8623
8	8	Axial	6.5	90	0.4364
4	9	Factorial	8.5	90	0.3682
12	10	Centre	6.5	65	0.7216
11	11	Centre	6.5	65	0.7313
3	12	Factorial	4.5	90	0.3527
7	13	Axial	6.5	40	0.5817

2.10 Statistical analysis

The data was statistically processed by IBM SPSS Statistics version 26. The mean values of the data were separated to determine whether they were significant or not by using Tukey's HSD method at a 5% level of significance. The data calculated was presented in the form of mean ± standard deviation.

3 Results and discussion

3.1 Comparison of precipitation methods across different maturity stages

3.1.1 *Selection of the best saturation level for ammonium sulfate precipitation*

The proteolytic activity of starfruit protease for different saturation levels (40, 50, and 60%) was found to be 0.2645±0.00734, 0.1934±0.00469 and 0.1170±0.00554, respectively, as shown in Figure 1 Ammonium sulfate with 40% saturation was found to be more effective for precipitation than other saturation levels. Ismail et al. [29], Banik et al. [31], and El-Beltagy et al. [32] also preferred a 40% saturation level for protease extraction.

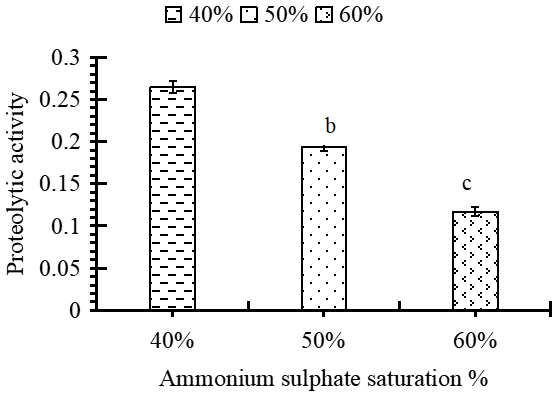


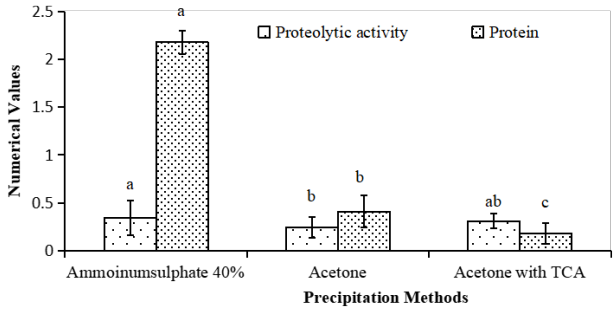
Figure 1. Ammonium sulfate saturation level versus PA.

3.1.2 *Precipitation methods and maturity stage (unripe)*

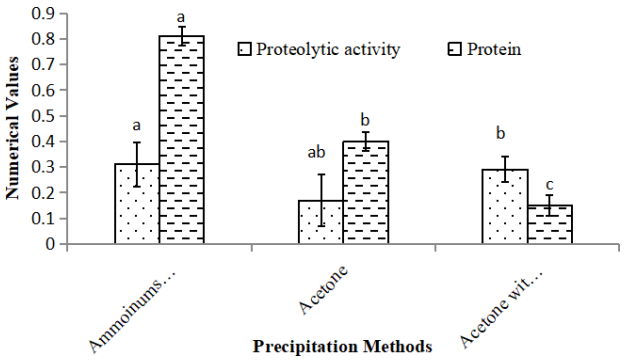
Three different precipitation methods were used: ammonium sulfate (40%), acetone, and acetone with TCA precipitation for protein precipitation. Proteolytic activity and protein assays were determined spectrophotometrically. Figure 2 (a) shows the effect of the precipitation method on maturity stage (unripe) proteolytic activity and protein assay. The mean value of proteolytic activity was found to be significantly higher ($p < 0.05$) for ammonium sulfate (40% saturation), followed by acetone with TCA and acetone precipitation. Whereas, the mean value of the protein assay was found to be highest for ammonium sulfate (40% saturation), followed by acetone and TCA-acetone precipitation. Ismail et al. [29] found a similar result of higher yield in unripe starfruit than in other maturity stages. Ismail et al. [11] purification using 40% ammonium sulfate precipitation is also recommended to partially purify proteases from *Averrhoa bilimbi*, especially from the unripe stage.

3.1.3 *Precipitation methods and maturity stage (semi-ripe)*

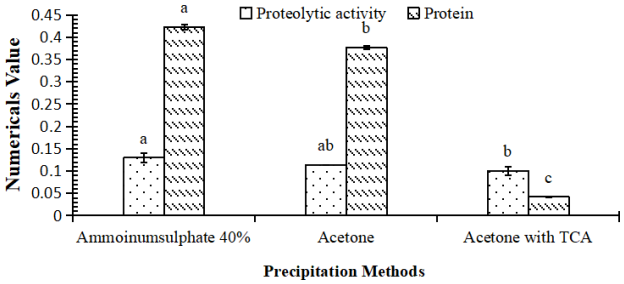
The mean value of proteolytic activity was found to be significantly higher ($p < 0.05$) for ammonium sulfate (40% saturation), followed by acetone with TCA and acetone precipitation. Whereas the mean value of the protein assay was found to be highest for ammonium sulfate (40% saturation), followed by acetone and TCA-acetone precipitation. Figure 2 (b) shows the effects of the precipitation method on maturity stage (semi-ripe) proteolytic activity and protein assay. In a study by [33], bromelain derived from partially ripe fruits yielded more protein



(a) Effect of the precipitation method on maturity stage (unripe) proteolytic activity and protein assay.



(b) Effects of precipitation method on maturity stage (semi-ripe) proteolytic activity and protein assay.



(c) Effect of precipitation method on maturity stage (ripe) proteolytic activity and protein assay.

Figure 2. Combined analysis of proteolytic activity across different maturity stages using varied precipitation methods.

than bromelain extracted from completely ripe fruits. According to their findings, bromelain activity reduces as ripening continues.

3.1.4 Precipitation methods and maturity stage (ripe)

The mean value of proteolytic activity was found to be significantly higher ($p < 0.05$) for ammonium sulfate (40% saturation), followed by acetone with TCA and acetone precipitation. Figure 2 (c) shows the effect of the precipitation method on maturity stage (ripe) proteolytic activity and protein assay.

3.1.5 Specific activity

The specific activities of 40% ammonium sulfate, acetone, and TCA were analyzed at three ripening stages: unripe, semiripe, and ripe, as shown in Figure 3. Ammonium sulfate activity increased significantly from the unripe stage (0.158 U/mg) to the semiripe stage (0.380 U/mg), but then slightly decreased at the ripe stage (0.3073 U/mg). In contrast, acetone activity showed a slight decline throughout ripening, decreasing from 0.439 U/mg at the unripe stage to 0.2921 U/mg at the ripe stage, suggesting a reduction in acetone-related enzymatic processes. TCA activity exhibited a continuous increase, rising from 1.722 U/mg at the unripe stage to 2.380 U/mg at the ripe stage, indicating an enhancement of TCA cycle-related enzyme activity during ripening. These results align with previous studies on enzyme activity dynamics during fruit maturation, demonstrating distinct changes across the ripening stages. For instance ,research on olive fruit β-glucosidase demonstrated that enzyme activity can vary with ripening stages, with optimal activity observed at different temperatures depending on the substrate used [34].

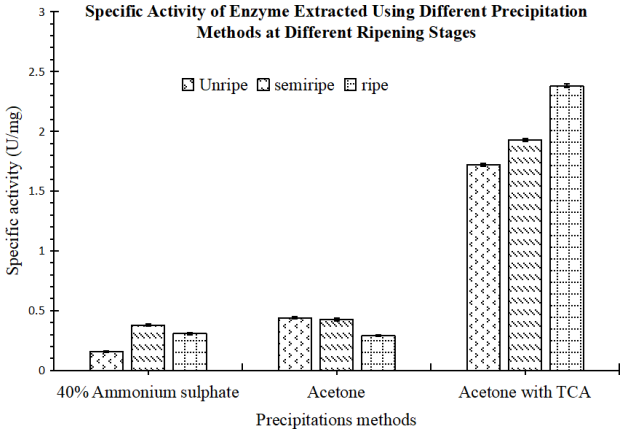


Figure 3. Specific activity of enzyme extracted using different precipitation methods at different ripening stages.

3.2 Proteolytic activity and maturity stages

The results for the effect of the maturity stage on PA were calculated using an equation deduced from the standard curve. Figure 4 shows the effect of maturity stage on PA. Statistical analysis at the 5% level of significance shows that all their stages were significantly different from each other. The highest value for unripe (0.34 ± 0.01) and the lowest for ripe (0.12 ± 0.01). This may occur because, during ripening, there is a decrease in the amino acid profile of tropical or climacteric fruits [35].

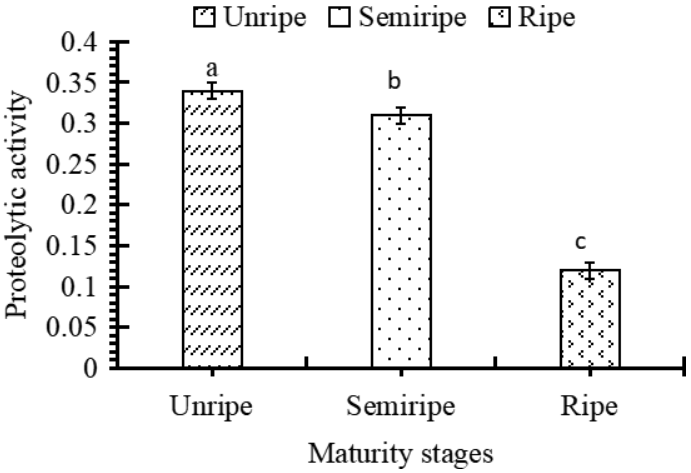


Figure 4. Effect of maturity stages on ammonium sulfate (40%) extracted PA.

3.3 Optimization of pH and temperature

3.3.1 Numerical optimization for protease activity (PA)

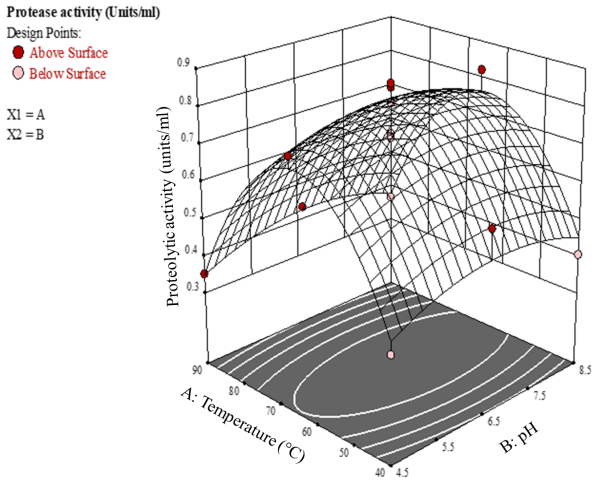
Effects of pH and temperature on PA: The parameter range was conducted with the help of a design expert, and proteolytic activity was identified. The response and contour plots are shown in Figure 5 (a) and (b), while the interaction graph is shown in Figure 6. The proteolytic activity of starfruit protease ranged from 0.352 to 0.8632 units/mL. The coefficients of the model and other statistical attributes of PA are shown in Tables 3 and 4. The model F-value of 19.71 implies the model is significant. There is only a 0.05% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case, B^2 is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. The lack of fit F-value of 1.22 implies that the lack of fit is not significant relative to pure error. There is a 41.05% chance that a lack of fit F-value this large could occur due to noise. A non-significant lack of fit is good, so it can be used to fit models. The predicted R^2 of 0.7027 is in reasonable agreement with the adjusted R^2 of 0.8863, i.e., the difference is less than 0.2. Adequate precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable, so a ratio of 9.854 indicates an adequate signal. This model can be used to navigate the design space. Considering all the above criteria, the model was selected for representing the variation of pH and temperature for PA and further analysis. The coded equation for proteolytic activity are given as:

$$\text{Proteolytic activity} = 0.8136 + 0.0161 \times A - 0.0358 \times B - 0.027 \times AB - 0.0655 \times A^2 - 0.3490 \times B^2 \quad (3)$$

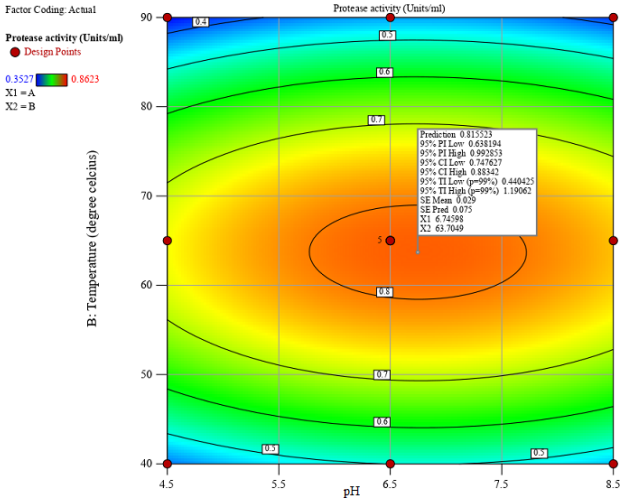
$$\text{Proteolytic activity} = -2.21972 + 0.224557 \times A + 0.071506 \times B - 0.000054 \times A \times B - 0.016388 \times A^2 - 0.000558 \times B^2 \quad (4)$$

where A and B are pH and temperature ($^\circ C$), respectively.

In quadratic equation (4), for the starfruit protease, PA had a significant ($P > 0.05$) positive effect of pH (A) but a non-significant ($P > 0.05$) negative effect of temperature (B) at a 95% level of confidence.



(a) Response surface plot of proteolytic activity of starfruit protease as a function of pH and temperature of enzyme.



(b) Contour plot of proteolytic activity of starfruit protease as a function of pH and temperature of enzyme.

Figure 5. Analysis of proteolytic activity in starfruit protease: Response surface and contour plots showing pH and temperature effects.

Solution with expected response: The selection of optimized temperature and pH was based on desirability and optimum protease activity, determined using the central composite response

Table 3. Analysis of variance (ANOVA) for proteolytic activity.

Source	Sum of squares	df	Mean square	F-value	P-value
Model	0.4730	5	0.0946	19.71	0.0005
A-pH	0.0016	1	0.0016	0.3234	0.5874
B-Temperature	0.0077	1	0.0077	1.61	0.2457
AB	0.0000	1	0.0000	0.0060	0.9406
A^2	0.0119	1	0.0119	2.47	0.1598
B^2	0.3364	1	0.3364	70.09	<0.0001
Residual	0.0336	7	0.0048	-	-
Lack of fit	0.0161	3	0.0054	1.22	0.4105
Pure error	0.0175	4	0.0044	-	-
Cor. total	0.5066	12	-	-	-

Table 4. Model summary statistics for proteolytic activity.

Source	Sequential p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2
Linear	0.9119	0.0071	-0.1782	-1.0651
2FI	0.9823	0.0052	-0.3089	-3.8358
Quadratic	10.0001	0.4105	0.863	Suggested
Cubic	0.6316	0.1812	0.8627	Aliased

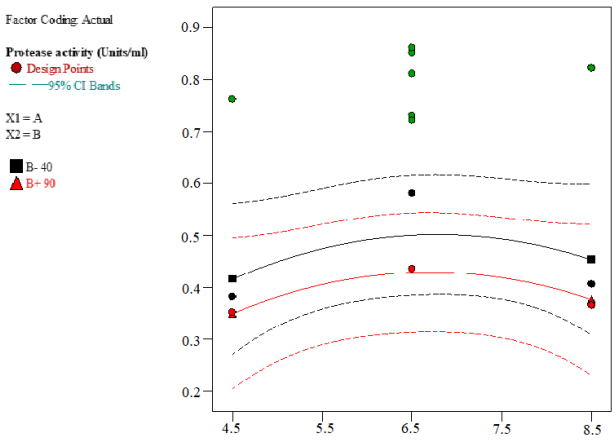


Figure 6. Multiple interaction graph of PA of Starfruit protease for both factor: A represents the pH and factor, B represents the temperature.

surface method, with constraints set as outlined in Table 2, and Figure 7 illustrates the optimization results for protease activity as a function of pH and temperature, pinpointing the ideal conditions the ideal conditions for maximizing protease activity while quantifying the predicted response and desirability. The top panels depict the explored design space: the top left panel shows the combined effects of pH (4.5 to 8.5) and temperature ($40°C$ to $90°C$) on predicted protease activity, while the top right panel highlights the temperature effect with the optimal corresponding pH value indicated. The bottom panel presents the protease activity (0.3527 to 0.8623) alongside its associated desirability. The red dot in the top panels and the blue dot in the bottom panel mark the optimal solution, predicting a maximum protease activity of 0.815523 at a pH of 6.74784 and a temperature of 63.7115, corresponding to a desirability score of 0.908. The label "Solution 1 out of 1" signifies that this is the single optimal solution identified by the optimization process. Overall, it highlights the successful identification of near-neutral pH and moderate temperature as the optimal conditions for maximizing protease activity, offering essential parameters for future experimental validation.

3.3.2 Optimization of starfruit protease

A numerical response optimization technique was applied to determine the optimum combination of temperature and pH of the starfruit protease enzyme for its proteolytic activity. Different constraints for optimization of starfruit protease are shown in Table 5. Under the assumptions of Design Expert (version 13), the optimum conditions (pH and temperature) for starfruit protease activity were 6.5 and $65°C$. The response predicted by the software for these optimum conditions reported proteolytic activity of 0.813 units/mL.

Verification of the model: Within the scope of the variables studied in Central Composite Design, additional experiments with different processing

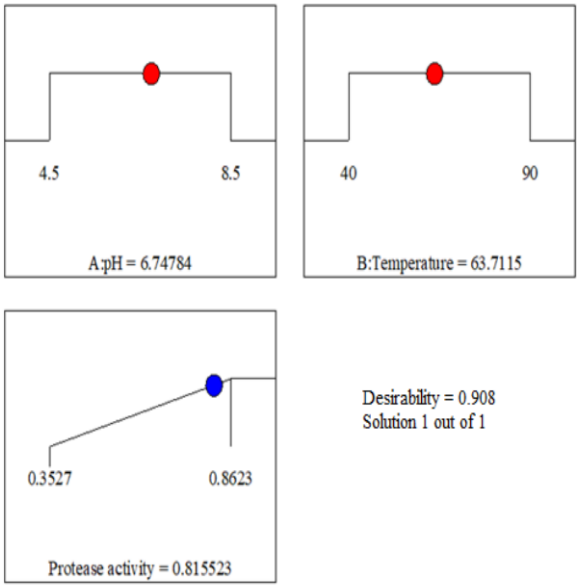


Figure 7. Solution with expected response.

Table 5. Different constraints and predicted PA for optimization of starfruit protease.

Name	Goal
Temperature of enzyme	In the range 40-90
pH of enzyme	In the range 3.5-8.5
Proteolytic activity (PA)	Maximum 0.813

conditions were conducted to confirm the adequacy of the model equations. Crude proteases were subjected to protease activity determination at an enzyme concentration of 1%, a pH of 6.5 and a temperature of $65°C$. The results for protease activity were calculated using an equation deduced from the standard curve. The conditions and the results of the confirmatory experiments are presented in Table 6. The predicted R^2 of 0.7027 is in reasonable agreement with the adjusted R^2 of 0.8863, i.e., the difference is less than 0.2.

Table 6. Actual values of the responses at the optimized condition.

Condition	Mean observed value (PA)
Temperature $65°C$, pH 6.5	0.862

Model Validation: Model validation was performed using the variables identified through Response Surface Methodology (RSM), which optimized factors like pH, temperature to predict proteolytic activity (PA). The model's accuracy was evaluated by comparing the predicted PA with the actual experimental PA. Figure 8 shows a scatter plot of predicted vs. actual PA, where a perfect prediction would align with the 45-degree line (Predicted PA = Actual PA). Deviations from this line indicate discrepancies, with small deviations suggesting good model accuracy and larger ones pointing to potential model improvements. The predicted proteolytic activities demonstrated a strong positive correlation with actual values ($R^2 = 0.796, r = 0.892, p < 0.001$), indicating good predictive performance.

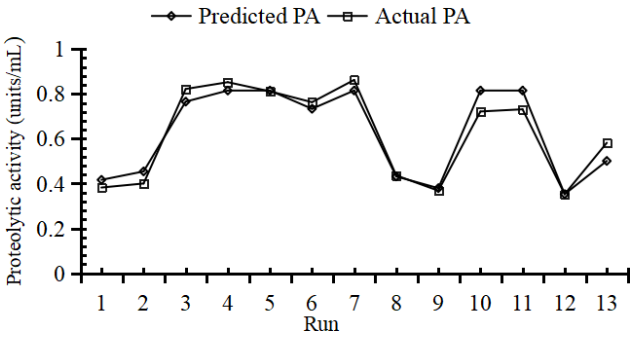


Figure 8. Predicted PA vs Actual PA.

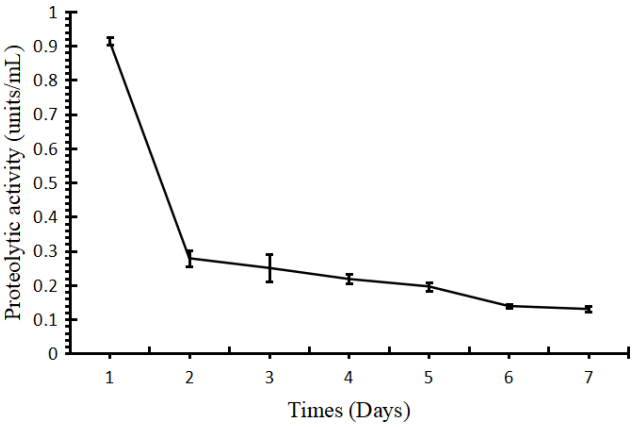


Figure 9. Proteolytic activity of starfruit protease of ammonium sulfate during 7-day storage.

3.4 Storage stability of starfruit protease

Starfruit proteases of the unripe maturity stage were incubated for one week at a temperature below $10°C$, and the proteolytic activity was determined, as shown in Figure 9. The proteolytic activity of protease partially purified with 40% ammonium sulfate rapidly decreased during the first day of storage and slowly decreased thereafter until the storage stability was approximately 1/9 at day seven. When stored at a lower temperature ($< 0°C$), its stability can be increased. Vallés et al. [36] reported that when preserved for 180 days at $-20°C$, the protease's enzyme activity from *Bromelia antiacantha's* ripe fruits was 100%. If starfruit proteases were held at temperatures below $4°C$, their relative activity would most likely be restored, and the rate of degradation

would be slower.

4 Conclusion and recommendations

This study demonstrated that protease extracted from *Averrhoa carambola* exhibited notable stability within an alkaline pH range, with optimal activity observed at pH 6–8 and a temperature range of 60–70°C. The most optimal conditions were identified as 65°C and pH 6.5. In terms of protease yield and efficiency, the unripe stage of the fruit showed higher protein concentration and proteolytic activity compared to the semi-ripe and ripe stages. However, despite lower total protein content, the higher specific activity in the ripe fruit indicates that the protease enzymes in this stage are more active and efficient. Specifically, TCA precipitation exhibited increasing specific activity as the fruit ripened, while ammonium sulfate and acetone methods showed varying trends across ripening stages. These results highlight the importance of considering both total protein content and specific activity when selecting the optimal stage for protease extraction. This study also emphasizes the critical role of storage conditions, as purified protease activity declined significantly over a 7-day period. Storing the enzyme at temperatures below 0°C may enhance its stability, which warrants further investigation. The findings underscore the need to carefully consider the ripeness stage and precipitation method for maximizing protease activity and industrial applications. While this study primarily focused on optimizing pH and temperature for protease extraction, other factors, such as material-to-liquid ratio and incubation time, were not included in the optimization process. Preliminary trials indicated that these factors did not significantly affect protease yield under the conditions tested, but further research could explore their influence in greater detail. The incorporation of these additional factors may further optimize the extraction and stability of starfruit proteases. These findings highlight the importance of selecting appropriate precipitation techniques and considering fruit ripeness when isolating proteases from starfruit. Additionally, further investigations into the purification process and the broader potential applications of starfruit-derived protease in industries such as food processing and pharmaceuticals are recommended.

Data Availability Statement

Data will be made available on request.

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Conflicts of Interest

The authors declare no conflicts of interest.

Ethical Approval and Consent to Participate

Not applicable.

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