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Extraction, Partial Purification and Utilization of Milk Coagulating Enzyme from Kiwifruit (*Actinidia Deliciosa*) in Fresh Cheesemaking

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Abstract

The study aimed to determine the potential of kiwifruit milk clotting enzyme in cheesemaking. The kiwifruit crude enzyme, extracted with sodium phosphate buffer (pH 7.0), was partially purified by 30-80% ammonium sulfate precipitation. 50% ammonium sulfate saturation exhibited maximum milk clotting activity (MCA), along with 1.56 purification fold, and 78.84% activity recovery. From SDS-PAGE analysis, the partially purified protease showed two bands with molecular mass of 24 kDa and 23 kDa respectively. The optimum conditions (temperature and pH of milk) for minimum time of coagulation (TOC) and maximum MCA were determined by response surface methodology (RSM). From numerical optimization study, the optimum conditions for cheesemaking were pH 6.5 and temperature 55 degree C, having 0.94 desirability. The cheese prepared by kiwifruit protease had significantly ($p<0.05$) higher moisture, ash, calcium content, and yield than rennet cheese, while significantly ($p<0.05$) lower fat content and acidity were observed in kiwifruit protease coagulated cheese. However, non-significant ($p>0.05$) difference in protein content was obtained between both cheeses. This study highlighted that kiwifruit protease has the ability to be used as efficient milk clotting enzyme in fresh cheesemaking.

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1. Introduction

Cheese is the inclusive term for a class °of fermented dairy-based food products, available in a wide range of forms and flavors. The basic goal of cheese making is to preserve the principal components of milk (Fox & McSweeney, 2017). It is produced by coagulating milk casein with rennet or similar enzymes in presence of lactic acid, from which a portion of the moisture has been removed by cutting, cooking and pressing, followed by shaping in a mould and ripening at appropriate temperature and humidity (Walstra et al., 2006). The source of rennet is the ruminant stomach, especially that of calf. Rennet contains chymosin (EC 3.4.23.4) as main enzyme component, which is responsible for specific cleavage between κ-casein Phe_{105}–Met_{106} bond, leading to the disruption of casein micelles and milk coagulation (Fox et al., 2017a). However, the need for alternative milk coagulating sources is rising due to the increase in cheese production and decrease in the rennet supply (Chazarra et al., 2007). Other factors such as increasing calf rennet price, ethical issues regarding the manufacture of such enzyme, religious belief, ban on genetically modified foods as well as vegetarianism are driving up the search for rennet substitutes (Roseiro et al., 2003).

The milk coagulation can be achieved by a number of proteolytic enzymes from various sources, such as microbial proteases (*Rhizomucor miehei*, *Cryphonectria parasitica*) and plant proteases (Andrén, 2011). Plant proteases have high catalytic activities, and can clot milk under wide range of pH and temperature conditions (Mazorra-manzano et al., 2018). Some plants having milk clotting proteases are *Cynara cardunculus* (Gomes et al., 2019), *Ficus carica* (Hachana et al., 2021), *Withania coagulans* (Salehi et

al., 2017), *Zingiber officinale* (Gagaoua et al., 2015), *Wrightia tinctoria* (Rajagopalan & Sukumaran, 2018), *Calotropis gigantea* (Rajagopalan et al., 2014), *Ananus comosus* (Kartawiria et al., 2019), *Carica papaya* (Maskey & Shrestha, 2020; Hafid et al., 2020), and *Actinidia deliciosa* (Mahdian Dehkordi et al., 2021; Nicosia et al., 2022). Plant proteases, rather than animal proteases and genetically modified organism-derived enzymes, have the advantage of being high source of bioactive compounds, antioxidant properties, and binding characteristics (Park et al., 2014; Gupta et al., 2015).

Generally most of the enzymes employed as milk coagulants are aspartic proteases, but enzymes from other categories, such as cysteine and serine proteases, have also been used (Shah et al., 2014). Kiwifruit (*Actinidia deliciosa*) contains milk clotting enzyme, actinidin (EC 3.4.22.14). Actinidin is a cysteine protease which has molecular mass of around 24 kDa, and it consists of 220 amino acid residues (Carne & Moore, 1978).. The pH and temperature profile for its optimum activity is suitable with conditions applied during cheesemaking process, and β-casein is the first target substrate during casein hydrolysis, followed by κ-casein at Arg_{97}-His_{98} and Lys_{111}-Lys_{112} (Lo Piero et al., 2011). Even in the presence of up to 5% cream fat, actinidin is able to hydrolyze casein fractions (Puglisi et al., 2012). Kiwifruit extract exhibits promising milk clotting activity to proteolytic activity ratio, only 30% less than that of rennet (Grozdanovic et al., 2013). Also, the texture of curd made by using crude kiwifruit extract is comparable to that obtained with chymosin (Mazorra-manzano et al., 2013). Hence, the objective of this present work was to partially purify kiwifruit protease, and utilize it in fresh cheesemaking, as well as compare the quality of prepared cheese with rennet cheese.

2. Materials and Methods

2.1 Materials

Raw cow milk of local breed was purchased from Dharan, Nepal. Chymosin (Chr. Hansen) was procured from Trishul Trade Links, Kathmandu. Ripe kiwifruit (*Actinidia deliciosa*) was purchased from departmental store in Dharan. Skim milk powder, purified casein, bovine serum albumin, ammonium sulfate, L-cysteine, tricine, tris buffer, and dialysis membrane were procured from HiMedia Laboratories Pvt. Ltd., India. Trichloroacetic acid, acrylamide, and protein ladder were procured from Thermo Fisher Scientific Pvt. Ltd., India. Sodium dihydrogen phosphate, disodium hydrogen phosphate, and sodium dodecyl sulfate were procured from Merck, India.

2.2 Methods

2.2.1 Kiwifruit crude protease extraction

The kiwifruit was peeled and cut into small pieces. It was mixed with 20 mM sodium phosphate buffer (pH 7.0) in the ratio of 1:1 (w/v) and homogenized in a blender (Model Havel's max grind 14000). The homogenate was stirred for 30 min under cold condition and filtered through cheese cloth. The obtained juice was centrifuged at 5000 rpm (Model DLAB D3024R) for 10 minutes at 4°C. The obtained supernatant was labeled as crude extract (CE). The CE was stored at 4°C for further purification process (Mazorra-manzano et al., 2013).

2.2.2 Partial purification

Partial purification of crude extract was carried out using ammonium sulfate precipitation method as described by Sharma & Vaidya (2018). The CE was subjected to $(NH_4)_2SO_4$ saturations using different concentrations of 30, 40, 50, 60, 70, and 80%. The respective saturations were centrifuged at 15000 rpm for 10 min at 4ºC. The obtained pellets were then dissolved in the phosphate buffer containing 10 mM L-cysteine, and dialyzed overnight through membrane (molecular weight cut off: 12 KDa) with three changes of buffer (pH 7.0) at 4°C. After dialysis, the partially purified enzyme was subjected to milk clotting activity, proteolytic activity and protein content determination.

2.2.3 Milk clotting activity

The milk clotting activity (MCA) of both CE and partially purified enzyme were determined following the method described by the International Dairy Federation (IDF, 2007). Reconstituted milk was prepared by mixing 5.5 g skim milk powder in 50 ml 0.5% $CaCl_2$ solution (w/v). The milk pH was maintained to 6.5 at temperature 35-40°C. 2 ml of milk was incubated in vial at 37°C and 200 µl enzyme was added. The vial was checked regularly at 10 s interval for any sign of clot. 1 unit of MCA is defined as the amount of enzyme which coagulates 10 ml of

reconstituted skim milk within 40 min at 37°C (Berridge, 1952).

$$\text{MCA}\left(\frac{\text{U}}{\text{ml}}\right) = \frac{2400 \times \text{V}_\text{S}}{\text{T} \times \text{V}_\text{E}}$$

where T = time taken for formation of clot (s); V_S = volume of milk (ml); V_E = volume of enzyme (ml).

2.2.4 Proteolytic activity

The proteolytic activity (PA) of CE and partially purified enzyme were determined following the method described by Ladd & Butler (1972). 1% casein (w/v) dissolved in 20 mM sodium phosphate buffer (pH 7.0) was used as substrate. Equal volume (1 ml) of substrate and diluted enzyme was incubated at 37°C for 30 min. 3 ml of 10% trichloroacetic acid was added to the mixture for termination of reaction, and incubated for 1 h under cold condition. It was then centrifuged at 5000 rpm for 10 min. The absorbance of the supernatant was measured at 280 nm by a spectrophotometer (Model Agilent Cary 60 UV-Vis). 1 unit of PA is defined as the amount of enzyme, which release 1 μg tyrosine under standard assay condition.

2.2.5 Protein content

The protein content of CE and purified enzyme was determined using Bradford protein-dye binding method (Bradford, 1976). The absorbance readings were measured at 595 nm by a spectrophotometer. The protein content was calculated by using bovine serum albumin (BSA) standard curve.

2.2.6 Molecular weight

For determination of molecular mass of enzyme, Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed (Model SE 400 vertical unit, GE Healthcare) (Schägger, 2006). 10% separating gel and 4% stacking gel were used for this analysis. The samples were mixed with reducing sample buffer, and the mixture was heated at 95°C for 5 min. 10 μg of protein was loaded in the well and the electrophoresis was carried out at 100 V. Thermo Scientific pageruler plus prestained protein ladder (10-250 kDa) was used in electrophoresis for molecular weight determination using GelAnalyzer 19.1 software.

2.2.7 Experimental design

The experimental design, data analysis, as well as model building were carried out using Design Expert software (Version 13, Stat-Ease Inc., Minneapolis, MN). The three coded levels were -1, 0 and +1 as shown in Table 1. The influence of pH of milk and temperature of milk during enzyme addition in cheesemaking was analyzed using response surface methodology (RSM) with central composite design (CCD) as given in Table 2. The fixed factor was enzyme concentration (0.5%) which was optimized according to the MCA of the enzyme. Similar result was reported by Sharma & Vaidya (2018). The design consisting of face centered one alpha, involved five center points along with one replicate of both factorial and axial points. According to Dhiman et al. (2021), the optimal temperature and pH range of purified kiwifruit protease was 35-55°C and 6.0-7.0 respectively.

Table 1: Range of factors for RSM

Factors	-1	0	+1
pH	6	6.5	7
Temperature (°C)	35	45	55

Time of coagulation (TOC) and milk clotting activity (MCA) were response variables. The TOC and MCA for various experimental combinations were related to coded variables (X_i, i=1 and 2) by second-degree polynomial equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 . X_2 + \varepsilon$$

The polynomial coefficients were expressed by β_0 (constant); β_1, β_2, (linear effects); β_{12} (quadratic effects); β_{11}, β_{22} (quadratic effects); and ε (random error). Multiple regression analysis was used for data modelling, while analysis of variance (ANOVA) for statistical significance of terms.

2.2.8 Cheesemaking

Two types of cheeses were prepared following the method mentioned by Walstra et al. (2006) with slight modifications. The cheese prepared using chymosin by direct acidification was marked as cheese A, while cheese prepared using partially purified kiwifruit protease as cheese B. Cow milk having 2.6% fat and 7.8% SNF was first heated to a temperature of 75-

80°C. For cheese A, chymosin was added at the rate of 2.5g/100L after the milk attained pH 6.0 by the addition of 2% food grade GDL (glucono-delta-lactone) at 37°C. For cheese B, pH of milk was adjusted to 6.5 at 55°C before the addition of enzyme (0.5%). The milk was stirred and allowed to coagulate for 40 min. Cutting of curd was then carried out by stainless steel wire cheese cutter, followed by cooking at 50°C for 1 h. After drainage of whey through cheese cloth, the curd cubes were moulded and pressed for 6 h, followed by brining overnight.

2.2.9 Physicochemical analysis

Fat: Fat content of cheese samples was estimated by the method mentioned in AOAC (2005).

Protein: Protein content of cheese samples was estimated by Kjeldahl method as mentioned in AOAC (2005).

pH: The pH of cheese samples was determined by the method mentioned in AOAC (2005).

Moisture: Moisture content of cheese samples was estimated by oven-drying method as described in AOAC (2005).

Ash: Ash content of cheese samples was estimated by dry ashing method as mentioned in AOAC (2005).

Acidity: The acidity of cheese samples was estimated by titrimetric method as described in AOAC (2005).

Calcium: The calcium content of cheese samples was determined following the method described by Ranganna (1986).

Yield: The yield of cheese samples was calculated using the equation as per Mahdian Dehkordi et al. (2021).

$$\text{Yield (\%)} = \frac{\text{Weight of cheese (kg)}}{\text{Weight of milk (kg)}} \times 100$$

2.2.10 Statistical analysis

The obtained data were analyzed by IBM SPSS statistics (version 26) using independent-samples t-test at 5% level of significance. The graph was constructed in Microsoft Excel (2019). The data pertaining to optimization study (RSM/CCD) were analyzed in Design Expert software using analysis of variance (ANOVA) and fit statistics for the significant model and R^2.

Table 2: Experimental design plan using RSM/CCD

Run	Factor 1	Factor 2
	A: pH of milk	B: Temperature of milk (°C)
1	6	55
2	6.5	45
3	6	45
4	6.5	45
5	6.5	45
6	6.5	35
7	6.5	55
8	7	45
9	6.5	45
10	7	35
11	7	55
12	6	35
13	6.5	45

3. Results and Discussion

In this study, protease was extracted from kiwifruit pulp and partially purified by ammonium sulfate precipitation. The molecular mass of purified enzyme was identified by Tricine SDS-PAGE. The effect of pH of milk and temperature of milk on the time of coagulation (TOC) and milk clotting activity (MCA) was optimized by response surface methodology (RSM). The cheeses, prepared from chymosin (A) and partially purified kiwifruit protease (B), were analyzed for both sensory as well as physico-chemical properties.

3.1 Partial purification of crude kiwifruit extract

The kiwifruit crude extract (CE) was partially purified by using different ammonium sulfate saturations (30-80%). The protease precipitated at 50% saturation exhibited maximum MCA (Table 3). Similar result in the purification of kiwifruit protease was mentioned by Sharma & Vaidya (2018). Gagaoua et al. (2015) also reported higher activity of protease extracted from *Zingiber officinale* Roscoe rhizomes, precipitated with 50% ammonium sulfate. Hydrophobic surface of protease might be unaffected by sulphate ion at lower salt concentration, while irreversible protease denaturation might occur at higher salt concentrations.

3.1.1 Purification profile

The purification profile of kiwifruit protease precipitated at 50% (w/v) ammonium sulfate precipitation is shown in Table 4. The purification fold of 1.56 along with 78.84% yield was obtained with 50% ammonium sulfate fractionation, which is in agreement with Sharma & Vaidya (2018). Gul et al. (2021) also reported the highest purity and yield percentage of pineapple crow bromelain, precipitated at 50% concentration. The activation of enzyme during ammonium sulfate precipitation, and removal of interfering substances during centrifugation under cool condition might have contributed to higher purity and activity recovery also.

3.1.2 SDS-PAGE

The gel electrophoretic analysis by Tricine SDS-PAGE demonstrated that the partially purified protease showed 2 bands with molecular mass of 24 kDa and 23 kDa respectively (Figure 1). Carne & Moore (1978) and Lo Piero et al. (2011) reported the similar molecular mass of kiwifruit protease (~24 kDa). But Dhiman et al. (2021) reported molecular mass of 27 kDa on SDS-PAGE analysis. The relative levels of protein in different kiwifruit varieties as well as the diversity might be reason for this variation.

Table 3: MCA of protease precipitated at different salt concentrations

Ammonium sulfate concentration (%)	TOC (s)	MCA (U/ml)
30	45 ± 1.0	533.33 ± 11.86
40	35 ± 0.58	685.71 ± 11.64
50	28 ± 0.57	857.14 ± 18.32
60	36 ± 1.15	666.67 ± 22.64
70	47 ± 1.52	510.64 ± 16.92
80	92 ± 2.51	260.87 ± 7.37

Note. Values are means ± standard deviation (SD) of the triplicates.

3.2 Numerical optimization of TOC and MCA by RSM

The measured values of TOC and MCA for partially purified kiwifruit protease varied from 17-80 s and 300-1411.76 units respectively. The change in TOC and MCA within designed space was represented by the equation (1) and (2) respectively.

$$TOC=31.72+8.67\times A-22.50\times B-5.75\times AB+1.97\times A^2 +9.47\times B^2 \quad (1)$$

$$MCA=776.52-151.54\times A+420.95\times B-57.94\times AB -35.12\times A^2+50.91\times B^2 \quad (2)$$

Where A and B are coded values of pH of milk and temperature of milk; AB, A^2 and B^2 model terms.

Table 4: Purification profile of kiwifruit protease

Purification step	PA (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
CE	189	3.40	55.59	1	100
Ammonium sulfate precipitation (50%)	149	1.72	86.63	1.56	78.84

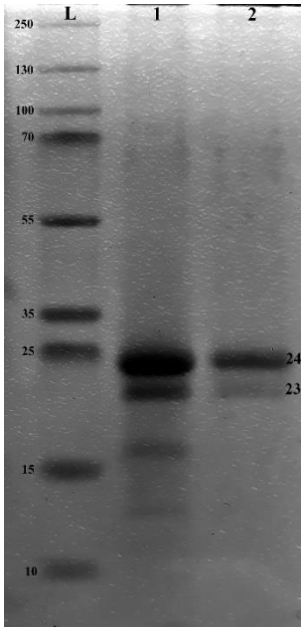

Figure 1: Tricine SDS-PAGE: lane L, protein ladder; lane 1, crude extract; and lane 2, partially purified kiwifruit protease with two bands of molecular mass

In quadratic equation (1), pH of milk (A) had significant ($p<0.05$) positive effect on TOC, and temperature of milk (B) had significant ($p<0.05$) negative effect at 95% level of confidence. Similarly, the interaction term of A and B (AB) had significant ($p<0.05$) negative effect. But the quadratic term of A (A^2) had non-significant ($p>0.05$) positive effect, and quadratic term of B (B^2) had significant ($p<0.05$) positive effect on TOC (Table 4). The variation in TOC by the significant negative effect of interaction term AB is shown in Figure 2. TOC increased gradually with the increase in pH, while TOC decreased with the increase in temperature.

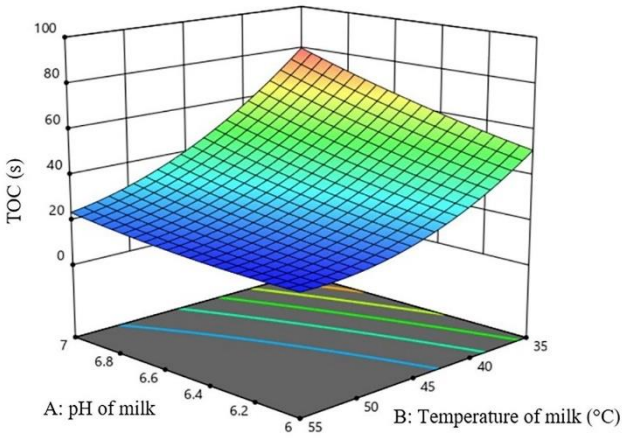

Figure 2: Response surface plot for TOC of purified kiwifruit protease

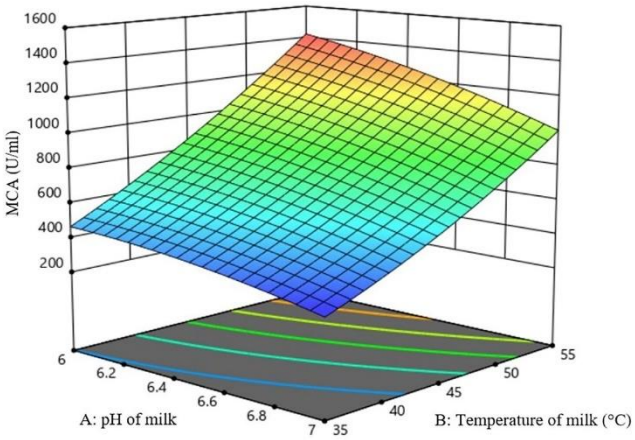

Figure 3: Response surface plot for MCA of purified kiwifruit protease

The ANOVA (Table 5) indicated that the F value for regression model is significant (p=0.0002) and the lack of fit is insignificant, indicating that the regression model can be fitted well. This was supported by the value of R^2 (0.9487) and adjusted R^2 (0.9120), along with the lower value of CV (12.40%).

Table 5: ANOVA for quadratic model of TOC

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	3968.16	5	793.63	47.14	<0.0001	Significant
A	450.67	1	450.67	26.77	0.0013	
B	3037.50	1	3037.50	180.43	<0.0001	
AB	132.25	1	132.25	7.86	0.0264	
A^2	10.67	1	10.67	0.6338	0.4521	
B^2	247.46	1	247.46	14.70	0.0064	
Residual	117.84	7	16.83			
Lack of fit	8.64	3	2.88	0.1055	0.9525	not significant
Pure error	109.20	4	27.30			
Cor Total	4086.00	12				

In quadratic equation (2), A had significant ($p<0.05$) negative effect on MCA, and B had significant ($p<0.05$) positive effect. AB had non-significant ($p>0.05$) negative effect. Likewise, A^2 had non-significant ($p>0.05$) negative effect, and B^2 had non-significant ($p>0.05$) positive effect on MCA (Table 5). The variation in MCA by negative non-significant effect of AB is shown in Figure 3. MCA increased with increase in milk temperature, and decreased with increase in pH of milk.

The ANOVA (Table 6) indicated that the F value for regression model is significant ($p=0.0002$) and the lack of fit is insignificant, indicating that the regression model can be fitted well. This was supported by the value of R^2 (0.9487) and adjusted R^2 (0.9120), along with the lower value of CV (12.40%).

3.2.1 Optimization

Numerical optimization method was used for determination of optimum combination of pH of milk and temperature of milk for minimum TOC and maximum MCA (Table 7). The optimum combinations were found to be 6.5 milk pH and 55°C of milk temperature, along with 0.94 desirability. The TOC of 18.69 s, and MCA of 1248.39 units were obtained under this optimum condition.

Table 6: ANOVA for quadratic model of MCA

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	1222000	5	244500	25.89	0.0002	significant
A	137800	1	137800	14.59	0.0065	
B	1063000	1	1063000	112.58	<0.0001	
AB	13428.17	1	13428.17	1.42	0.2719	
A^2	3406.71	1	3406.71	0.3607	0.5670	
B^2	7159.59	1	7159.59	0.7581	0.4128	
Residual	66106.78	7	9443.83			
Lack of fit	2919.09	3	973.03	0.0616	0.9774	not significant
Pure error	63187.7	4	15796.92			
Cor Total	1288000	12				

3.2.2 Model verification

In post analysis, three runs were carried out for the confirmation of adequacy of model equations. The results of confirmatory runs are summarized in Table 7. Since responses predicted through regression models and observed from experiments were in close agreement, the optimal conditions (pH 6.5 and temperature 55°C) were selected for cheesemaking. Dhiman et al. (2021) reported similar findings in the characterization of actinidin. Previous study by Gagaoua et al. (2015) on characterization of zingibain also expressed the similar outcomes.

3.3 Physicochemical properties of cheese

The physicochemical characteristics of cheeses, prepared from chymosin (A) and partially purified enzyme (B) are given in Table 9.

The moisture content of cheese A is in line with the data (40-50%) given by Fox et al. (2017b). But the cheese B had significantly ($p<0.05$) higher moisture than cheese A. Similar moisture content in the cheeses prepared by plant proteases was reported by Mahajan & Chaudhari (2014). Higher moisture content of cheese prepared by kiwifruit protease (i.e., 68%) was reported by Sharma & Vaidya (2018). This variation might be due to change in the enzymatic activity of

both coagulants. Higher moisture content in cheese destabilizes the protein network, resulting a smooth texture. Hence cheeses made from plant proteases are often soft. The cheese A had significantly ($p<0.05$) higher fat content than cheese B. The similar result in rennet cheese was reported by Maskey & Shrestha (2020). The fat level of cheese B is similar to the finding of Ordiales et al. (2014). The lower value of fat in cheese B might be due to fat loss during drainage of whey. Due to strong proteolytic activity of plant protease, the casein network breakdown in cheese results in higher fat losses in whey (Nuñez et al., 1991).

Table 7: Different parameters for optimization

Parameters	Goal	Lower limit	Upper limit
pH of milk	target = 6.5	6	7
Temperature of milk (°C)	in range	35	55
TOC (s)	Minimize	17	80
MCA (U/ml)	Maximize	300	1411.76

The flavor, texture and mouthfeel of cheese are significantly influenced by the fat content of cheese. The protein content of both cheeses is in line with the findings of Rajagopalan & Sukumaran (2020). In contrast, Sharma & Vaidya (2018) reported only 15.52% of protein in cheese, coagulated by kiwifruit protease. The variation in protein content might be attributed to milk quality and processing technique. The ash content of cheese B was significantly ($p<0.05$) higher than that of cheese A. Similar findings were reported by Maskey & Shrestha (2020). Ash content of cheese depends on minerals present in cheese, and amount of salt used in brining of cheese. Cheese B had significantly ($p<0.05$) higher pH value than cheese A. Khan & Masuq (2013) also reported similar findings. The variation in pH values may be attributed to the difference in initial pH of coagulants used, and their protease activity. The acidity of cheese B was significantly ($p<0.05$) lower than that of cheese A. Maskey & Shrestha (2020) also expressed similar result. However, Khan & Masuq (2013) observed slight increase in acidity of cheese. This variation might be due to change in optimum pH of milk at the time of enzyme addition. Cheese B had significantly ($p<0.05$) higher calcium content than cheese A. Previous study by Karki & Ojha (2018) also expressed similar outcomes. The yield of cheese B was significantly ($p<0.05$) higher than that of cheese A. The increase in moisture content of cheese may be contributed to the higher percentage of yield (Johnson et al., 2001).

Table 9: Physicochemical properties of fresh cheeses

Parameters	Cheese A	Cheese B
Moisture (%)	$49.63^{a} \pm 0.06$	$52.07^{b} \pm 0.14$
Fat % (wb)	$27.8^{b} \pm 0.43$	$25.3^{a} \pm 0.35$
Protein % (wb)	$19.46^{a} \pm 0.02$	$19.18^{a} \pm 0.02$
Ash % (wb)	$2.77^{a} \pm 0.13$	$3.20^{b} \pm 0.06$
pH	$5.78^{a} \pm 0.005$	$6.45^{b} \pm 0.01$
Acidity (% lactic acid)	$0.21^{b} \pm 0.017$	$0.16^{a} \pm 0.01$
Calcium (mg/100 g)	$627.43^{a} \pm 1.22$	$637.33^{b} \pm 2.44$
Yield (%)	$12.17^{a} \pm 0.38$	$13.58^{b} \pm 0.52$

Note. Values are means ± SD of three determinations. Values in row having different superscript are significant different at 5% level of significance.

Table 8: Predicted and observed values of the responses

Response	Optimal conditions		Predicted value	Mean observed value
	pH of milk	Temperature of milk (°C)		
TOC	6.5	55	18.69	19.67 ± 0.58
MCA	6.5	55	1248.39	1221.05 ± 36.46

Note. Observed values are expressed as means ± SD of three independent experiments.

4. Conclusion

On the basis of the findings of this study, kiwifruit protease exhibits potential as effective milk coagulating agent, and it can be used as efficient alternative to chymosin in fresh cheese making. The kiwifruit crude extract was partially purified to 1.56 purification fold, and 78.84% activity recovery by using 50% ammonium sulfate saturation. The protease had two bands with molecular mass of 24 kDa and 23 kDa. The optimum conditions for cheesemaking were pH 6.5 and temperature of 55°C. The cheese prepared by kiwifruit protease had significantly ($p<0.05$) higher moisture, ash, calcium, and yield than rennet cheese. However, detail biochemical and sensory analysis studies of cheese prepared by purified enzyme is required for its applicability.

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Conflicts of Interest

The authors declare that there do not have any conflict of interest.

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