Evaluation of Milk Clotting and Proteolytic Activity of *Moringa oleifera* L.

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Abstract

Extracts from fruits and plants were used as milk coagulants in many areas of the world. The aim of the present study was to investigate the milk clotting and proteolytic enzyme activity of *Moringa oleifera* L. seed kernels with the aim of establishing the optimal conditions (pH, temperature and concentrations of enzyme) to be used for making cheese. In addition, the effects of calcium ions and cysteine on milk clotting and proteolytic enzyme activities were also studied. Moreover, the qualitative proteolytic enzyme activity was investigated utilizing the used X-ray film. The optimal conditions (pH, temperature and concentrations of enzyme) and effects of calcium ions and cysteine on proteolytic enzyme activities were determined by spectrophotometric method using Folin-Ciocalteu's reagent. Furthermore, the cheese was made by using the best optimal conditions of the enzyme solution of *Moringa oleifera* L.

Keywords: proteolytic, milk clotting, qualitative, optimal

Introduction

Proteolytic enzymes are widely used for manufacturing cheese. The milk coagulant for cheese production is animal rennet, which consists of aspartic protease, chymosin. The present research was carried out in order to extract milk clotting enzyme from *Moringa oleifera* L., which can be used in cheese preparation. *Moringa oleifera* L. (Drumstick) belongs to family of Moringaceae. *Moringa oleifera* seed kernels extract was also used as a milk-clotting agent (Tajalsir, 2014). *Moringa oleifera* seed kernels consist of several hydrolytic enzymes, in which proteases are the main enzymes (Ahmed, *et al*., 2012). In the present study, seed kernels of *Moringa oleifera* have been screened for milk clotting activity using skim milk as a substrate and for proteolytic enzyme activity using casein as a substrate. The extraction of crude enzymes from seed kernels of *Moringa oleifera* was described. The optimum conditions (pH, temperature and concentrations of enzyme) and effects of calcium ions and cysteine on milk clotting and proteolytic enzyme activities were investigated. In addition, the qualitative proteolytic enzyme activity was also studied by utilizing the used X-ray film. Furthermore, the cheese was made by utilizing the best optimal conditions of the enzyme.

Materials and Methods

Sample Collection

Moringa oleifera pods were collected from Mandalay Region, Myanmar and stored in a freezer at -20^{o}C.

Extraction of Crude Enzyme from *Moringa oleifera* Seed Kernels

Mature *Moringa oleifera* seeds were removed from pods. Seed coats were removed from seeds and dried for two days. After two days, seed kernels were grounded with blender to

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get fine powder. 100 g of *Moringa oleifera* fine powder were immersed in 500 mL of 0.05 M phosphate buffer pH 7. It was stirred in magnetic stirrer for 4 hours and filtered. The filtrate was centrifuged at 3000 rpm for 15 min and the supernatant solution obtained was stored in the refrigerator for analysis. The crude enzymes of the seed kernels were obtained (Maria, et al., 2017).

Determination of Milk Clotting Activity

0.25 g of skim milk powder was weighed and put into a test tube followed by 0.75 mL of 0.05 M phosphate buffer pH 7. The test tube was shaken until milk dissolved and placed in water bath for 10 min at 35ºC. After which, 1.0 mL of crude enzyme was added and the time taken for the milk to clot was taken as a measure of enzyme activity (Oseni and Ekperigin, 2013). Milk coagulating activity was calculated by using the following equation.

$$MCA = (2400 \times V)/(v \times t)$$

MCA = milk clotting activity
V = volume of milk in mL
t = clotting time in second
v = volume of crude enzyme solution in mL

Determination of Optimum pH of Milk Clotting Activity

0.25 g of skim milk powder was weighed and mixed with 0.75 mL of 0.05 M phosphate buffer pH(s) (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5). The test tube was shaken until milk dissolved and incubated in water bath for 10 min at 35ºC. After which, 1.0 mL of crude enzyme was added and the time taken for the milk to clot was taken as a measure of enzyme activity (Oseni and Ekperigin, 2013).

Determination of Optimum Temperature of Milk Clotting Activity

0.75 mL of 0.05 M phosphate buffer pH 7 was added into the 0.25 g of skim milk powder. The test tube was shaken and incubated in water bath for 10 min at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80ºC). After adding 1.0 mL of crude enzyme solution, the time taken for the milk to clot was recorded (Oseni and Ekperigin, 2013).

Determination of Enzyme Concentrations of Milk Clotting Activity

0.25 g of skim milk powder was mixed with 0.75 mL of 0.05 M phosphate buffer pH 7. The test tube was shaken well and incubated for 10 min at temperature 35ºC. After which, various concentrations (1.0, 1.5, 2.0, 2.5, 3.0 mL) of crude enzyme were added and the time taken for the milk to clot was noted (Oseni and Ekperigin, 2013).

Effect of Calcium Ions ($CaCl_2$) and Cysteine Concentrations on Milk Clotting Activity

0.25 g of skim milk powder was put into a clean test tube followed by 0.75 mL of 0.05 M phosphate buffer pH 7. The test tube was shaken and warmed for 10 min at 35ºC. After which, 1.0 mL of crude enzyme was added. The reaction mixtures were made to contain between (0.001 – 0.01 M) calcium ions concentration or (0.001 – 0.01 M) cysteine

concentration and the time taken for the milk to clot was taken as a measure of enzyme activity (Oseni and Ekperigin, 2013).

Examination of Qualitative Test for Proteolytic Enzyme Activity

A 1 g of used X - ray film was cut into small pieces of (1 cm × 1 cm) and placed in a beaker and 5 mL of crude enzyme were added into it. For another beaker, 1 g of used X-ray film was cut into small pieces of (1 cm × 1 cm) and placed in a beaker and 5 mL of distilled water was used instead of 5 mL of crude enzyme solution.

Preparation of Standard Tyrosine Solution

The stock solution (1 M) (1 mg/mL) of tyrosine was prepared by dissolving (1 mg) of tyrosine in 1 mL of 5% trichloroacetic acid. The tyrosine solution (0.552 mM, 0.276 mM, 0.138 mM, 0.069 mM and 0.035 mM) was prepared from the stock solution by dilution with appropriate amount of 5% trichloroacetic acid.

Construction of Calibration Curve for Standard Tyrosine Solution

A standard tyrosine solution 1 mL was added into a test tube containing 5 mL of 0.5 M sodium carbonate solution and 1 mL of 1 M sodium hydroxide solution. The mixture was shaken well. After 10 min, 0.5 mL of Folin-Ciocalteu's reagent was added into the mixture and blue colour appeared. The UV-Visible spectrophotometer was warmed up for 30 min. After 30 min, this mixture was placed in the sample cell of UV-visible spectrophotometer. Trichloroacetic acid (TCA) used as blank was placed in the reference cell.

Determination of Proteolytic Enzyme Activity

A 0.5 mL enzyme solution from *Moringa oleifera* seed kernels was added to a test tube containing 1 mL of 1% casein solution and 0.5 mL of 0.05 M phosphate buffer pH 7 solution. The mixture was shaken well and incubated at 35ºC for 30 min. After incubation time, 1 mL of 5% TCA was added. The mixture was centrifuged and 1 mL of supernatant was taken out. 5 mL of 0.5 M Na_2CO_3 solution and 1 mL of NaOH solution were added into the mixture and kept for 10 min and 0.5 mL of Folin-Ciocalteu's reagent was added. The tubes were incubated for 30 min, the blue colour appeared. A blank solution was prepared by carrying out the procedure as described above except that 0.5 mL of 0.05 M phosphate buffer pH 7 solution was used instead of 0.5 mL of enzyme solution. The absorbance of both test and blank solution was measured at 680 nm by using UV-visible spectrophotometer (Oseni and Ekperigin, 2013). The proteolytic enzyme activity was calculated by using the following equation.

$$\text{Proteolytic Activity} = \frac{\mu \text{ mole of Tyrosine liberated}}{\text{Reaction time} \times \text{Volume of enzyme solution}}$$

Determination of Optimum pH, Temperature, Enzyme Concentrations, Effect of Calcium Ions ($CaCl_2$) and Cysteine Concentrations of Proteolytic Enzyme Activity

A 0.5 mL of prepared enzyme solution was added to a test tube containing 1.0 mL of 1% casein solution and 0.5 mL of pH(s) (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and

8.5) phosphate buffer solution. Next, assays were performed as described in determination of proteolytic enzyme activity.

1.0 mL of 1% casein solution and 0.5 mL of pH 5.5 phosphate buffer solution were mixed with 0.5 mL of prepared enzyme solution. After the mixture was shaken well, the test tubes were incubated as fixed variously at20, 25, 30, 35, 40, 45, 50, 55, 60 and 65, 70, 75 and 80ºC under the same conditions described in determination of proteolytic enzyme activity.

Different enzyme concentrations, viz., (0.5, 1, 1.5, 2, 2.5 and 3 mL) were prepared and added to each test tube containing 1.0 mL of 1% casein solution and 0.5 mL of pH 5.5 phosphate buffer solution. The determination of enzyme concentrations on the activity was done by the same procedure as described in determination of proteolytic enzyme activity.

To determine the effect of Ca^{2+}concentration and cysteine concentrationson the enzyme activities, the assays were carried out as described in determination of proteolytic enzyme activity. The reaction mixtures were made to contain between (0.001 – 0.010 M) calcium ions concentration or (0.001 – 0.010 M) cysteine concentration.

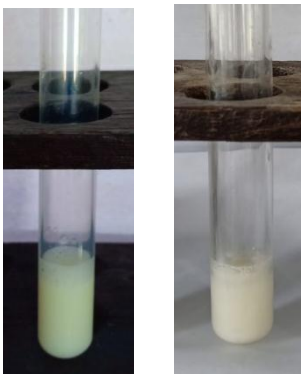
Cheese Preparation

Cheese making was carried out by preheating portions of 1 L of skim milk solution at 35ºC. Then, 100 mL of crude enzyme solution was added into the milk. After that, the solution was placed in water bath for 3 hours at 35ºC. Finally, the curd was placed in round-bottomed containers and cheeses were stored in polyethylene bags at 4ºC.

Results and Discussion

Determination of Milk Clotting Activity

The skim milk powder solution was added to crude enzyme solution in the test tube. The milk solution was placed in another test tube. By comparing these two test tubes, crude enzyme solution in the test tube was found to be milk clotting as shown in Figure (1 (b)). Therefore, crude enzyme solution from *Moringa oleifera* seed kernels was found to have milk clotting activity.



(a) **(b)**

Fig. 1 Qualitative Test for Milk Clotting Activity
(a) milk powder solution
(b) milk powder solution and crude enzyme solution

Determination of Optimum pH of Milk Clotting Activity

The pH values of sodium acetate buffer solution ranging from 3.0 and 8.5 were used to determine the milk clotting activity of the enzyme sample. The nature of the milk clotting

activity vs. pH curve of the enzyme was shown in Figure (2). According to the results, the optimum pH for milk clotting activity was attained at pH 7.

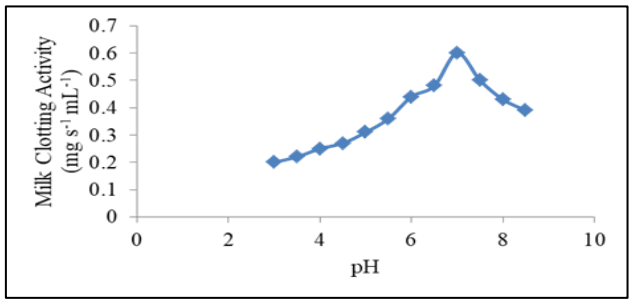


Fig. 2 Optimum pH of Milk Clotting Activity

Determination of Optimum Temperature of Milk Clotting Activity

In the present work, different temperatures ranging from 20 to 80ºC were used to determine the optimum temperature of milk clotting activity of the enzyme sample.

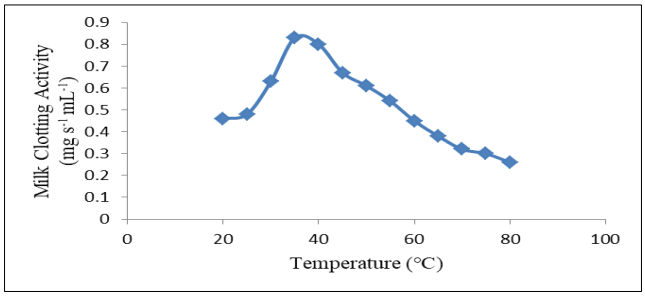


Fig. 3 Optimum Temperature of Milk Clotting Activity

According to the results, the optimum temperature for the milk clotting activity was found to be 35ºC.

Determination of Enzyme Concentrations of Milk Clotting Activity

The enzyme concentrations ranging from (0.5 - 3%) were used to determine milk clotting activity. The plot of milk clotting activity vs. enzyme concentration was shown in Figure (4).

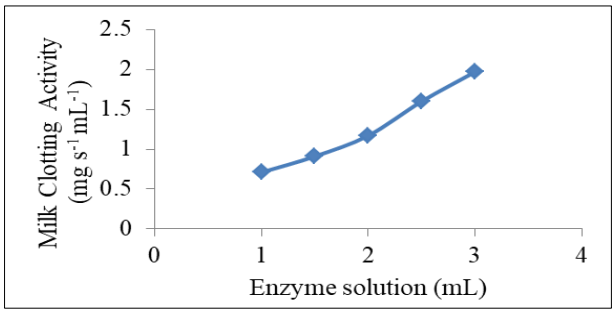
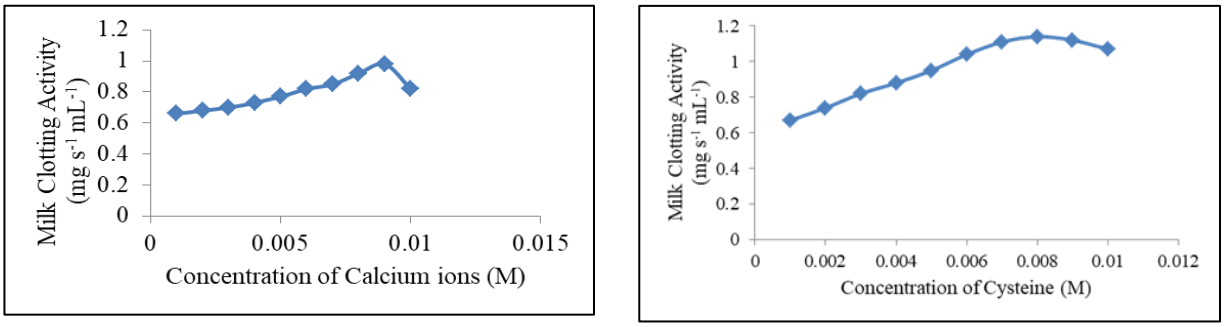


Fig. 4 Effect of Enzyme Concentration on Milk Clotting Activity

According to the results (Figure 4), the milk clotting activity was increased with the increase of enzyme concentrations.

Determination of Calcium Ions ($CaCl_2$) and Cysteine Concentration of Milk Clotting Activity

The effects of Ca^{2+} and cysteine concentrations ranging from (0.001 to 0.010 M) were used to determine milk clotting activity.



(a) (b)

Fig. 5 (a) Effect of Ca^{2+} Concentration on Milk Clotting Activity (b) Effect of Cysteine Concentration on Milk Clotting Activity

As shown in Fig. 5 (a) and (b), both calcium ions and cysteine were found to activate the enzymes with maximum activation at 0.009 M and 0.008 M for milk clotting activity.

Examination of Qualitative Test for Proteolytic Enzyme Activity

X-ray film is made by a plastic backing covered with an emulsion of AgCl in gelatin. Gelatin is a protein which can be hydrolyzed by a proteolytic enzyme. The used X-ray film and crude enzyme solution from *Moringa oleifera* seed kernels were placed in the beaker. After 6 hours, the black precipitates were found due to the proteolytic activity of crude enzymes of *Moringa oleifera* seed kernels (Figure (6(b)). In another beaker, used X-ray film and distilled water were placed. By comparing, the black precipitates were not found due to the absence of proteolytic enzyme. The results revealed that the crude enzyme solution can be hydrolysed the X-ray film.

(a) (b) (c)

Fig. 6 Qualitative Test for Proteolytic Enzyme Activity
(a) used X-ray film
(b) used X-ray film and crude enzyme solution
(c) used X-ray film and distilled water

Determination of the Wavelength of Maximum Absorption of Tyrosine

For the determination of wavelength of maximum absorption (λ_{max}), ultraviolet and visible absorption spectroscopy was employed. The absorption spectrum of tyrosine compound was recorded in the range from 420 to 800 nm.

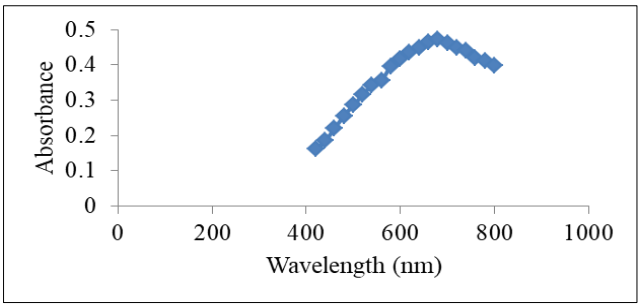


Fig. 7 Absorption Spectrum of Blue Colour Product for Tyrosine

As shown in Figure 7, the wavelength of maximum absorption tyrosine solution was found to be 680 nm.

Construction of Calibration Curve for Standard Tyrosine Solution

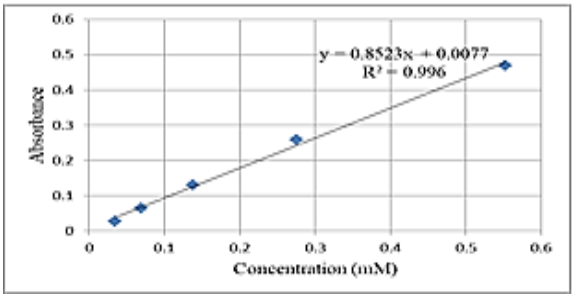


Fig. 8 Plot of Absorbance as a Function of Concentration of Standard Tyrosine Solution

According to the results shown in Figure (8), the calibration curve of absorbance vs. concentration of tyrosine revealed a straight line with a regression coefficient $R^2 = 0.996$.

Determination of Optimum pH of Proteolytic Enzyme Activity

The sodium acetate buffers at pH values ranging from 3.0 and 8.5 were used to determine the proteolytic activity of the enzyme sample.

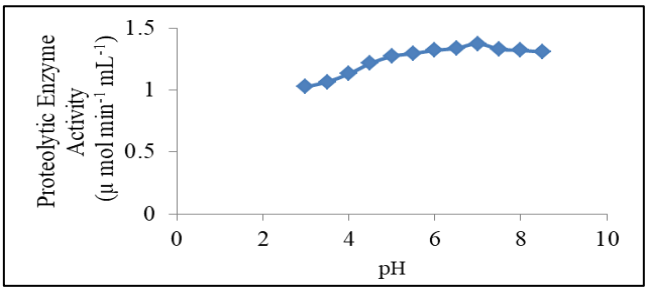


Fig. 9 Optimum pH of Proteolytic Enzyme Activity

The optimum pH was attained at pH 7 for the proteolytic enzyme activity.

Determination of Optimum Temperature of Proteolytic Enzyme Activity

The temperature ranging from 20 and 80ºC were used to determine the proteolytic activity of the enzyme sample.

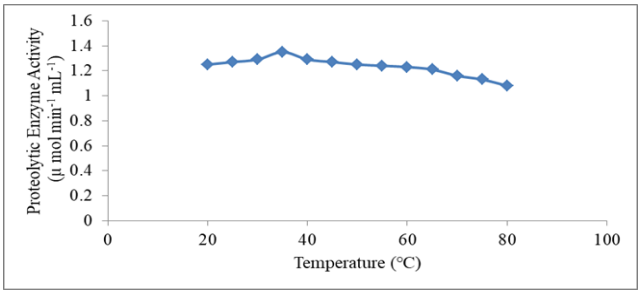


Fig. 10 Optimum Temperature of Proteolytic Enzyme Activity

According to the results illustrated in Figure (10), the optimum temperature was attained at 35ºC for the proteolytic enzyme activity.

Determination of Enzyme Concentrations of Proteolytic Enzyme Activity

The enzyme concentrations ranging from 0.5 to 3 mL were used to determine the proteolytic activity of the enzyme.

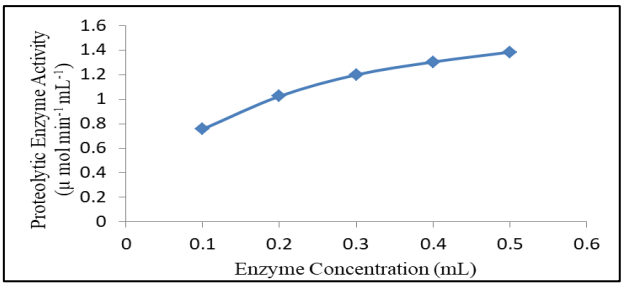
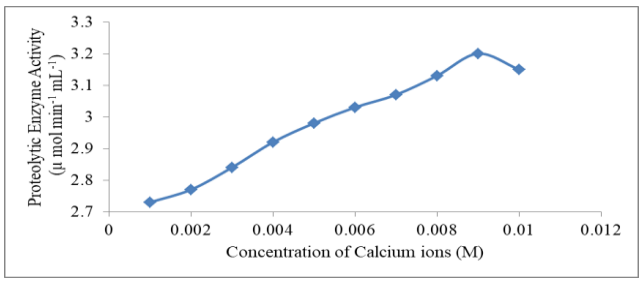


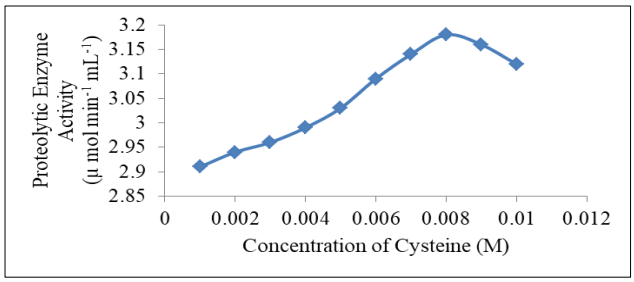
Fig.11 Effect of Enzyme Concentrations on Proteolytic Enzyme Activity

According to the results in figure (11), the proteolytic enzyme activity was increased with the increase of concentration of enzymes.

Determination of Calcium Ions ($CaCl_2$) and Cysteine Concentrations of Proteolytic Enzyme Activity

In the present work, Ca^{2+} and cysteine concentrations ranging from (0.001 - 0.010 M) were used to determine the proteolytic enzyme activity.





(a) **(b)**

Fig.12(a) Effect of Ca^{2+} Concentration (b) Effect of Cysteine on Proteolytic Enzyme Activity

According to the results in figure (4.12), calcium ions and cysteine were found to activate the enzymes with maximum activation at 0.009 M and0.008 M for proteolytic enzyme activity.

Cheese Preparation

The cheese was made using the optimal conditions (pH 3.5 and temperature at 35ºC) of the crude enzyme solution of *Moringa oleifera*. The resulting cheese was hard and crumbly.

Fig.13 Cheese

Conclusion

Plant rennet has been considered as substitutes for chymosin in the production of cheese. In this research work, the seed kernels of *Moringa oleifera* were selected for determination of milk clotting and proteolytic enzyme activities with the aim of establishing optimal conditions and preparation of cheese. The crude enzyme solution was extracted from the seed kernels of *Moringa oleifera*. The optimum conditions (pH, temperature and concentrations of enzyme) and effects of calcium ions and cysteine on milk clotting and proteolytic activity of crude enzyme solution were investigated. In addition, the qualitative test for proteolytic enzyme activity was investigated by utilizing the used of X-ray film. According to the results, X-ray film made with gelatin (protein) can be hydrolysed by crude enzyme solution. The optimum pH and temperature of the milk clotting and proteolytic enzyme activity was found to be 7 and 35ºC. In addition, the milk clotting and proteolytic enzyme activity increased with the increase of enzyme concentrations. Moreover, the calcium ions and cysteine were found to activate the enzymes, with maximum activation at 0.009 M (calcium ions) and 0.008 M (cysteine) for milk clotting and proteolytic activity. This means that the calcium ions and cysteine were found to activate the enzymes. Furthermore, the cheese was prepared by using optimal pH and temperature of enzyme solution of *Moringa oleifera*. The resulting cheese was hard and crumbly. The crude enzyme extract of *Moringa oleifera* seed kernels showed the high potential for use as milk coagulants in cheese making. However, it is required with some modification that can be later used in manufacturing cheese.

Acknowledgement

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