Effect of pH on Clotting Properties from *Moringa Oleifera* Seeds with Focused Microwave-Assisted Soxhlet Extraction Method as Milk Coagulant

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Abstract. This study aimed to determine the optimal pH for *Moringa oleifera* seeds extract as a milk casein coagulant in terms of milk-clotting activity (MCA), caseinolytic activity (CA), ratio MCA/CA, and coagulation time. *Moringa oleifera* seeds extraction was obtained using the Focused Microwave-Assisted Soxhlet Extraction (FMASE) method. Milk-clotting activity was analyzed using skim milk. Caseinolytic activity was assessed using sodium caseinate as substrates. The effects of pH (5–8) on Moringa oleifera seeds enzyme activities were determined using experimental design of Completely Randomized Design (CRD) method with 5 treatments, maceration extract (P0), FMASE extract at pH 5 (T_1), 6 (T_2), 7 (T_3) and 8 (T_4) with 3 times replications. The significant influence will be tested using Duncan test. The results showed that the difference in pH atmosphere had a very significant effect ($P < 0.01$) on MCA, CA, MCI, and coagulation time. The pH 5 setting on the addition of *Moringa oleifera* seeds extract as a milk coagulant showed the optimum results at MCA 69.68 U/ml and MCI 3209.09, low yields at CA 0.02. U/ml, and gives a short time in the milk coagulation process of 14.35 s. The conclusion of this study is that adjusting the pH atmosphere in an increasingly acidic atmosphere will increase the work of the protease enzyme in *Moringa oleifera* seeds extract. pH 5 and 6 *Moringa oleifera* seeds extract can work optimally and will decrease at pH 7.

Keywords: *Moringa oleifera* seeds · FMASE · pH · milk casein coagulant

1 Introduction

Milk coagulation is the one of the critical steps in cheese making. Milk coagulation process occurs due to the clumping process of the milk protein called casein to separate

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the curd and whey. Coagulation of milk casein occurred by adding the coagulant agent. Generally, the coagulant agent often used is rennin enzyme derived from the abomasum of young ruminants because high specificity Milk-Clotting Activity (MCA) which is able to break the Met_{105}-Phe_{106} chain in k-casein and low general proteolytic activity causes this type of coagulant agent to be widely used in cheese production [1]. Milk-clotting activity is on important parameter to determine the ability of an enzyme to hydrolyze casein during the milk coagulation process. Due to increasing public demand for dairy products such as cheese, cause the reduction of natural calf rennet supply and the increase in the price of calf rennet. In addition, the use of animal rennet on cheese making has begun to be limited due to several reasons such as religious issues (Judaism and Islam), food safety (*bovine spongiform encephalopathy*) and vegetarianism of some consumers [2]. According to Garcia, plant-based proteases can be consumed by vegetarian consumers and can also be certified Kosher and Halal, causing plant-based proteases to overcome religious issues and vegetarianism. The use of coagulants from plant extracts such *as Ananas comosus, Carica papaya, Calotropis procera, Ficus carica, Calm viscera, Cynara cardunculus, Cynara scolymus*, and *Solanum dubium* in cheese making has long been used in the Mediterranean, West Africa and southern European countries [3]. Coagulant agents from plant extracts are rarely used, due to the low organoleptic characteristics of the resulting cheese such as bitter taste and cheese texture which are less attractive to consumers due to the high value of its proteolytic activity. For this reason, it is necessary to further search for other plant extracts that are suitable for use as coagulant agents in cheese production without affecting its organoleptic characteristics [4].

Moringa oleifera Lam. is a plant that has many benefits for human health and for other fields, one of them as a coagulant and flocculant in the water purification process. *M. oleifera* has a fast growth and high adaptability, it makes this plant has spread in various tropical and subtropical areas up to an altitude of 2000 m. Ismail stated that *M. oleifera* is a newest protease source that has the potential to be used as a coagulant in cheese production, due to the promotion of extensive hydrolysis of k-casein and low degradation of α- and β-casein compared to other plant coagulants [5]. The results of the study by Tajalsir the highest milk-clotting activity was found in the extract of *M. oleifera* seeds and other parts (leaves, stems, flowers, and fruit) showed very low milk-clotting activity. Milk-clotting activity, protease enzyme activity and the ratio of milk-clotting activity to protease enzyme activity of purified *M. oleifera* seeds extract were 13407.8 units/ml, 2.50 units/ml, 5279 units/ml, respectively, these results higher than calf rennet of 249.6 units/ml), 0.05 units/ml, 4992 units/ml [2].

M. oleifera seeds can be extracted using a method that has been developed, namely FMASE (Focused Microwave-Assisted Soxhlet Extraction). FMASE combines a Soxhlet extractor (SE) with a microwave to maintain the advantages of conventional SE while overcoming limitations such as long extraction times, robust non-quantitative extraction of retained analytes, and unsuitability for automation. The distillation of the extractant in FMASE is achieved by electric heating, which is independent of the polarity of the extractant, and recycling thereby saving 75%-85% of the total extractant volume [6]. The protease enzymes of each type of coagulant have different optimum coagulation activities which are influenced by temperature, pH, substrate concentration, enzyme

concentration, activator and inhibitor [7]. The pH is one that affects the work of the enzyme itself, where the more appropriate the pH value, the more optimal the work of the enzyme. Each enzyme produced or used to make mozzarella cheese has a different pH to produce optimal enzyme work. The decrease in pH in the manufacture of mozzarella cheese will occur if it is added with acidic ingredients [8]. The pH needed or in accordance with the enzymes produced by the stomach can work optimally in milk coagulation in the pH range of 4–6.5 (Pereira and Gimenez, 2017). According to thus condition the objective of this research to determine the optimal pH for Moringa oleifera seeds extract with FMASE method as a milk casein coagulant on clotting properties.

2 Materials and Methods

2.1 Materials

Moringa oleifera seeds samples were supplied by local farmer at Blora Regency, Central Java, Indonesia. All fresh seeds samples were dried for two days under the sunlight and separated from the hard seeds shell. Thereafter, samples of *M. oleifera* seeds kernel was ground using blender and sieved using 40 mesh, then stored in closed containers at room temperature. All chemical used were of analytical grade.

2.2 Extraction *M. Oleifera* Seeds with Maceration Method

M. oleifera seeds extraction by maceration method refers to [9] as follows. *M. oleifera* powdered seeds (40 mesh) was macerated with 70% ethanol (1:10 w/v) at room temperature without light for 48 h. Stirring is done every 6 h for 5 min [10]. The macerated extract was filtered using Whatman No. Filter paper. 1, then soaked again for 48 h and filtered again using Whatman filter paper No.1. The obtained filtrate is concentrated with a Rotary Vacuum Evaporator at 45 °C to about 10% of the original volume, samples were stored at 4 °C until use.

2.3 Extraction *M. Oleifera* Seeds with FMASE Method

M. oleifera seeds extraction preparation refers to [11] as follows. *M. oleifera* powdered seeds (40 mesh) was weighed as much as 5 g, then wrapped in filter paper and placed into a thimble Soxhlet. One neck flask with a volume of 60 ml was connected to a Soxhlet thimble and filled with 80 ml of sodium phosphate buffer pH 7 through a Soxhlet thimble.

M. oleifera seeds extraction by FMASE method refers to [12] which have been modified are as follows. Thimble Soxhlet which already contains *M. oleifera* seeds powder simplicial is connected to a neck flask containing solvent and placed in the microwave irradiation zone, then on the outside of the microwave zone a condenser is installed. The assembled FMASE tool can extract Moringa seeds powder simplicial for 6 min with 100% power. Extraction was carried out with a 1 min flame system and a 2 min pause. *Moringa oleifera* seeds extract was dissolved in ammonium sulfate with a saturation of 60% (w/v) and centrifuged at 4,000 rpm speed for 30 min. Moringa seeds extract samples were stored in a freezer at temperature −20 °C until used.

2.4 Determination of Milk-Clotting Activity

Determination of Milk Clotting Activity (MCA) according to [5] using 10% (w/v) skim milk as a substrate. Heated skim milk at 50 °C. Dissolved skim milk in distilled water and 10 mM $CaCl_2$ at pH 6.5 for *M. oleifera* seeds extract with Maceration (T_0), FMASE extract at pH 5,6,7 and 8 (T_1, T_2, T_3, and T_4) and heated the solution at 50 °C using magnetic stirrer. Milk solution in different pH treatment of 10 ml was incubated with 1 ml of *M. oleifera* seeds extract at 37 °C and the coagulation time was observed by curd formation. The first point of the visible separation of curd and whey was recorded. One unit of milk coagulation is defined as the amount of enzyme that can coagulate 10 ml of milk in 180 s. Milk Clotting Activity can be calculated using the formula according to [13] below:

MCA (U/ml) = "Volume of substrate x 100(dilution factor)" /"coagulation time x volume of enzyme".

2.5 Determination of Caseinolytic Activity

Determination of caseinolytic activity (CA) according to [14] using 0.6% (w/v) sodium caseinate as a substrate. Dissolved 100 μl extract from *M. oleifera* seeds with 300 μl buffer sodium phosphate (0.1 M) at pH 7.5 for *M. oleifera* seeds extract with maceration (T_0), *M. oleifera* seeds extract with FMASE at 5, 6, 7 and 8 (T_1, T_2, T_3 and T_4) containing 0.6% sodium caseinate as a substrate (w/v), the mixture was added with 100 μl tritone (X-100) 0,1% (v/v). The solution was incubated at 37 °C for 1 h. To stop the reaction was added with 200 μl trichloroacetic acid (TCA) 10% (w/v) and incubated at 4 °C for 30 min, then centrifuged at 9000 rpm speed for 10 min and the absorbance of the supernatant was measured using spectrophotometer UV-Vis at 366 nm. The calculation of Caseinolytic Activity using the formula according to [15] below:

CA (U/ml) = (λ 366 nm x 10 x dilution factor)/ (volume of enzyme x reaction time).

2.6 Determination of Ration MCA/CA

The ratio of milk clotting activity to caseinolytic activity can be obtained using the milk clotting index (MCI) formula. Milk clotting index is a formula to determine the ratio of milk clotting activity to caseinolytic activity. Determination of ratio MCA/CA according to [16] using the formula below:

$$\text{ratio MCA/CA} = \frac{\text{value of milk-clotting activity}}{\text{value of caseinolytic activity}}$$

2.7 Determination of Coagulation Time

Determination of coagulation time followed to [5] procedure using 10% (w/v) skim milk as a substrate. Heated skim milk at 50 °C. Dissolved skim milk in distilled water and 10 mM $CaCl_2$ at pH 6.5 for *M. oleifera* seeds extract with Maceration (T_0), FMASE extract at pH 5,6,7 and 8 (T_1, T_2, T_3, and T_4) and heated the solution at 50 °C using

magnetic stirrer. Milk solution in different pH treatment of 10 ml was incubated with 1 ml of *M. oleifera* seeds extract at 37 °C and the coagulation time was observed by curd formation using stopwatch. The time of first point of the visible separation of curd and whey was recorded.

2.8 Statistical Analysis

Each determination was carried out and analyzed in triplicate and the figures were then averaged. The variation was assessed by the analysis of variance (ANOVA) and the significant influence will be tested using Duncan's test.

3 Result and Discussion

3.1 Milk Clotting Activity of *M. Oleifera* Seeds Extracted by FMASE Method at Varying pH

Milk Clotting Activity (MCA) is a test conducted to determine the coagulation activity of casein in milk as a result of the activity of a number of enzymes which are usually expressed in SU (Soxhlet units) per ml or Units per ml. Milk casein coagulation occurs by adding a coagulant agent such as a protease enzyme to the extract of *M. oleifera* seeds, which causes the hydrolysis of the peptide bond between the two amino acids, phenylalanine (Phe_{105}) and methionine (Met_{106}) in k-casein (casein protein that stabilizes micelle structure), which thickens casein micelles into curds [17]. The higher the MCA value of a coagulant agent indicates that the activity or the enzyme works more optimum, this is because by adding a coagulant agent such as a protease enzyme in *M. oleifera* seeds, it can hydrolyze the amino acid chain of milk k-casein specifically so that the two milk proteins are separated in the form of curds and whey. According to [18], the activity of milk coagulation enzymes also depends on pH, the coagulation activity of casein will increase as the pH decreases. This is in accordance with the results of our study, the results of the analysis of variance showed that the effect of pH treatment on the MCA value of *M. oleifera* seeds extract extracted by the FMASE method gave a very significant difference ($P < 0.01$) (Table 1). Table 1 shows the highest MCA value was shown in an acidic atmosphere or pH 5 (T_1) with an average value of 69.68 ± 0.95^{e} U/ml while the lowest MCA value was found in an alkaline atmosphere or pH 8 (P4) with an average value of 6.28 ± 0.04^{a} U/ml. And control treatment or maceration extract with pH 6.5. Figure 1 shows that the MCA value of *M. oleifera* seeds extracted by the FMASE method will decrease with increasing pH. This shows that the protease enzyme in *M. oleifera* seeds extracted by the FMASA method works optimally in an acidic environment or pH 5–6.

The high value of MCA in the treatment of pH 5 (T_1) indicated that the protease enzyme presents in the seeds extract of *M. oleifera* with the FMASE method worked optimally in an acidic environment which caused the activity of the protease enzyme in the hydrolysis of milk casein to form curd rapidly. [19] stated that the protease enzyme works actively in the pH range of 4–6 and reaches a maximum value at pH 5 and the coagulation activity value of milk decreases significantly at pH 7 and when it is at pH

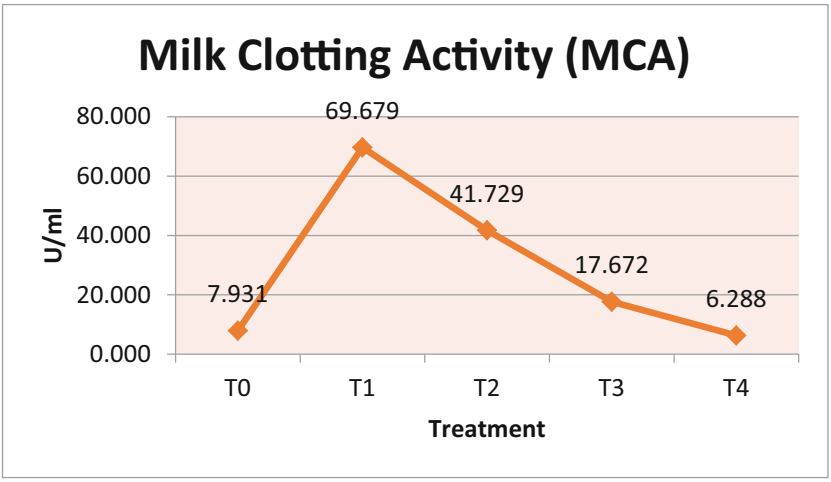

Fig. 1. Milk clotting activity of *M. oleifera* seeds extracted by maceration and FMASE method

8 the enzyme is inactive. This is in accordance with the results of this study. This is in accordance with the results of research by [20] which stated that pH 5.5 in Moringa seeds extract produced the highest MCA value when compared to pH 4.5 and 6.5 at 3.66 U/ml, 2.75 U/ml and 2.34 U/ml, respectively. These results showed that the protease enzyme in *M. oleifera* seeds could not work under alkaline conditions. [15] stated that the highest MCA value was shown by the purified *M. oleifera* seeds extract at pH 5, temperature of 65 °C and a concentration of 10% of the substrate volume, with values of 35.36% U/ml and 12.88 U/ml, respectively. According to [21], the coagulation activity of milk is highly dependent on the pH and temperature of milk. The pH setting in the MCA test using $CaCl_2$, the more $CaCl_2$ concentration used, the lower the pH of the substrate. [22] used $CaCl_2$ with different concentrations (0.01–0.05%) to determine the optimal $CaCl_2$ concentration for MCA testing on *M. oleifera* coagulant, the results showed that the MCA value was directly proportional to the concentration of $CaCl_2$ used so as to produce activity enzymes in the optimal extract of *M. oleifera* require the appropriate concentration of $CaCl_2$.

3.2 Caseinolytic Activity of *M. Oleifera* Seeds Extracted by FMASE Method at Varying pH

Caseinolytic activity (CA) or protease activity of an enzyme is expressed in units of activity unit (U), namely the number of proteases that cause an increase in one unit of absorbance at 366 nm. According to [23], protease activity or caseinolytic activity plays an important role during cheese ripening, through the release of amino acids that can reduce bitter taste by hydrolyzing bitter peptides, changing texture, increasing pH and increasing water binding capacity in cheese. The results of the analysis of variance showed that the effect of pH treatment on the CA value of *M. oleifera* seeds extract extracted by the FMASE method gave a very significant difference ($P < 0.01$) (Table 1). The highest mean CA value was shown in the control treatment (T_0) or maceration

Table 1. The effect of pH on MCA, CA and ratio MCA/CA at *M. oleifera* seeds extracted by maceration and FMASE method

Treatment	MCA (U/ml)	CA (U/ml)	Ratio MCA/CA
T_0	$7{,}93 \pm 0{,}06^{b}$	$0{,}51 \pm 0{,}018^{d}$	$15{,}66 \pm 0{,}560^{a}$
T1	$69{,}68 \pm 0{,}95^{e}$	$0{,}02 \pm 0{,}003^{a}$	$3209{,}09 \pm 0{,}43^{e}$
T2	$41{,}73 \pm 0{,}99^{d}$	$0{,}10 \pm 0{,}002^{c}$	$407{,}47 \pm 0{,}82^{d}$
T3	$17{,}67 \pm 0{,}29^{c}$	$0{,}06 \pm 0{,}001^{b}$	$306{,}37 \pm 0{,}85^{c}$
T4	$6{,}28 \pm 0{,}04^{a}$	$0{,}05 \pm 0{,}0006^{b}$	$126{,}01 \pm 0{,}75^{b}$

Notes: $^{a, b, c, d, e}$ Different superscripts in the same column indicate that different pH of the *M. oleifera* seeds extract extracted by the FMASE method gave a very significant difference ($P < 0.01$) to the milk clotting activity (MCA), activity caseinolytic (CA) and MCA/CA ratio. (Maceration extract pH 6.5), (FMASE extract pH 5), (FMASE extract pH 6), (FMASE extract pH 7), (FMASE extract pH 8)

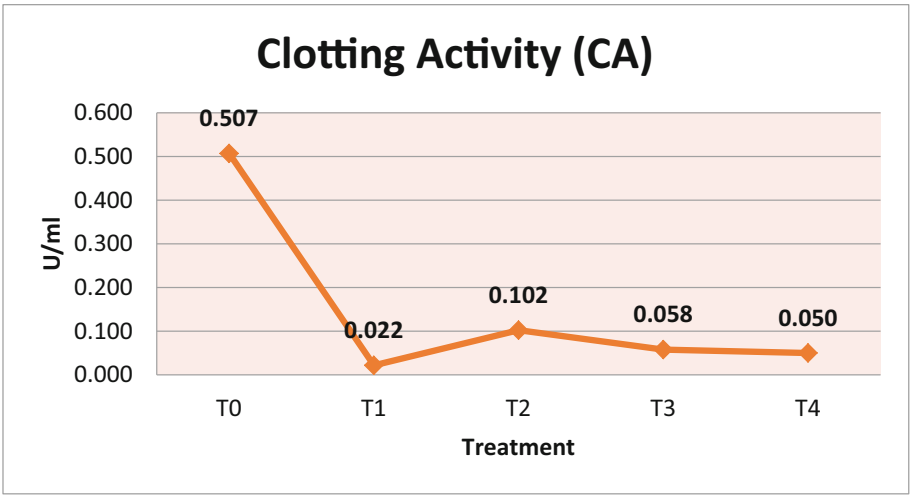

Fig. 2. Casein lytic activity of *M. oleifera* seeds extracted by maceration dan FMASE method

extract of *M. oleifera* seeds with a substrate pH of 7.5 with an average value of 0.51 ± 0.018^{d} followed by treatment T_2 or *M. oleifera* seeds extract using the FMASE method at a substrate pH of 6 with an average value 0.10 ± 0.002^{c} U/ml while the lowest CA value was found in the treatment T_1 or *M. oleifera* seeds extracted by FMASE method at a substrate pH of 5. This is in line with the opinion of [24] which states that the factors that affect the activity of the enzyme are temperature and pH. [25] also added that the enzyme has an active site that matches the casein substrate and causes the formation of an enzyme complex with a maximum substrate at the optimum pH.

The low value of CA in the treatment of *M. oleifera* seeds extract with the FMASE method with a substrate pH of 5–8 compared to the control treatment (P_0) or the macerated extract of *M. oleifera* seeds with a substrate pH of 7.5 suggests the participation of ammonium sulfate in the *M. oleifera* seeds extract using the FMASE method. The low protein content in the *M. oleifera* seeds extract required the use of ammonium sulfate in the *M. oleifera* seeds extract using the FMASE method, which aims to concentrate protein in the *M. oleifera* seeds extract. The results of [14] found that the protein content of *M. oleifera* seeds extracted by maceration method with sodium acetate pH 5 was 0.88 mg/ml. Due to the low protein content of *M. oleifera* seeds, 60% ammonium sulfate participation is required to concentrate the caseinolytic and milk-clotting activity from *M. oleifera* seeds. According to [14], protein concentration using ammonium sulphate has three main advantages: it is a rapid and inexpensive method, it does not affect the structure and function of proteins, and the salt can be easily removed from the protein solution by dialysis.

CA values in all treatments were lower than the results of the study by [22] showed that the CA value of the *M. oleifera* extract with the participation of 20–40% ammonium sulfate was 2.47 U/ml. This difference in results could be due to the different concentrations of ammonium sulfate used, the type of substrate used in our study, the CA test was carried out using sodium caseinate, while in the study of [22] the substrate used is azocasein. Figure 2 shows the optimum pH for the enzyme incubation of *M. oleifera* extract to hydrolyze casein at pH 6–7. The results of [14] found the highest CA value in the precipitated protein fraction from *M. oleifera* flowers, namely incubation pH pH 7.0 at 35.5 U/ml. It is known that pH can affect the shape, nature of the charge, correct position of the substrate and ionization of amino acid side chains, at both the active site and throughout the enzyme [26].

3.3 Ratio MCA/CA of *M. Oleifera* Seeds Extracted by FMASE Method at Varying pH

The ratio of milk clotting activity (MCA) to alkaline caseinolytic activity (CA) is called the milk clotting index (MCI). The MCA/CA ratio of an enzyme is a very useful indicator to determine the quality of a coagulant agent in cheese making [27]. The results of the analysis showed that the effect of pH treatment on the MCA/CA ratio of *M. oleifera* seeds extract extracted using the FMASE method gave a very significant difference ($P < 0.01$) (Table 1). The highest mean value of the MCA/CA ratio was shown in the treatment T_1 or *M. oleifera* seeds extract by FMASE with substrate pH of 5 method was 3209.09 ± 0.43^{e}, while the lowest mean MCA/CA ratio was in the T_0 or maceration extract of *M. oleifera* seeds treatment was 15.66 ± 0.560^{a}. The higher the MCI value on coagulant agent means the quality of the coagulant in forming curd will be better. This is in accordance with the opinion of [28] which states that coagulant agents with high MCI values are usually able to form curd with higher yield and less bitter peptide development during cheese making. However, on the contrary, the MCI value which can result in lower raw cheese yield, poor curd elasticity, and the occurrence of bitter peptides during cheese ripening can affect the sensory attributes of the final product which are less attractive to consumers. The results of the study by [2] showed that the highest value of the MCA/CA ratio was shown by purified *M. oleifera* seeds extract,

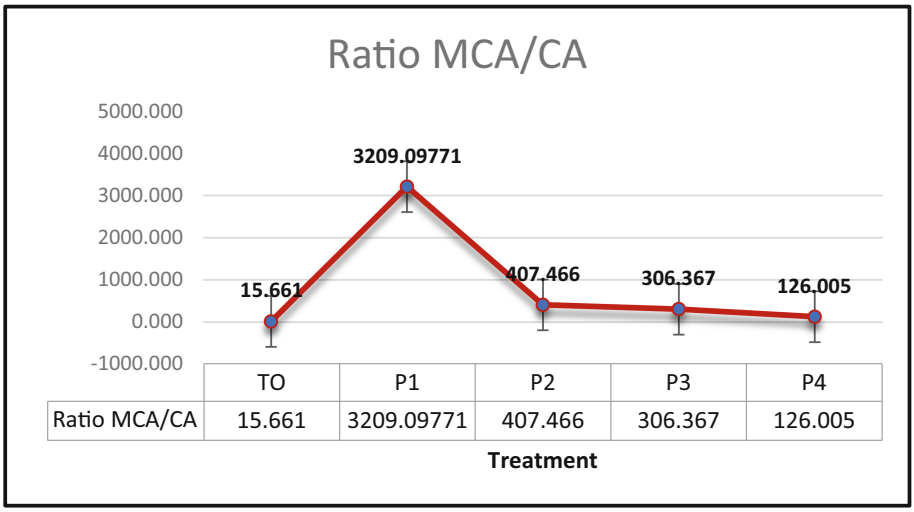

Fig. 3. Ratio MCA/CA of *M. oleifera* seeds extracted by maceration dan FMASE method

the value MCA/CA ratio of purified *M. oleifera* seeds extract was 5279 compared to the other coagulant enzymes such as calf rennet, dubium and papain.

Figure 3 shows a graph of the MCA/CA Ratio of *M. oleifera* seeds extracted by maceration and the FMASE method. It can be seen in Fig. 3 that the higher the pH, the lower the MCI value of the coagulant *M. oleifera* seeds extracted by the FMASE method. This shows that the protease enzyme in *M. oleifera* seeds extracted by FMASE works optimally at an acidic atmosphere or pH 5. A high average value of milk clotting activity and a low average value of caseinolytic activity causes a high milk clotting index value at a pH 5 (T_1) atmosphere. Based on Fig. 3, it can be seen that the MCA/CA ratio value experienced a significant increase at pH 5 (T_1) compared to the control treatment or *M. oleifera* seeds extract from the maceration method and also experienced a significant decrease continuously at pH 6 (T_2) to pH 8 (T_4). The higher the value of milk clotting activity, the greater the effect on the formation of curd or the occurrence of coagulation in milk. This is in accordance with the statement of [29] which stated that the high MCI value, coupled with high curd production, could make protease purification of *M. oleifera* seeds useful as a substitute for commercial calf rennet.

3.4 Coagulation Time of *M. Oleifera* Seeds Extracted by FMASE Method at Varying pH

Coagulation time is defined as the period between the addition of enzymes to the milk until coagulation or curd formation occurs. According to [27], substrate source, substrate pH, and type of coagulation enzyme used to influence cheese yield and curd formation time. Based on research on the coagulation time of milk with the results of the analysis showed that the effect of treatment on the coagulation time of *M. oleifera* seeds extract extracted by the FMASE method gave a very significant difference ($P < 0.01$) (Tables 1 and 2).

Table 2. The effect of pH on coagulation time at *M. oleifera* seeds extracted by maceration and FMASE method

Treatment	Coagulation time (second)
T0	126.09 ± 0.93^{e}
T1	14.35 ± 0.19^{a}
T2	23.97 ± 0.57^{b}
T3	56.60 ± 0.96^{c}
T4	159.03 ± 0.99^{d}

Notes: $^{a, b, c, d, e}$ Different superscripts in the same column indicate that different pH of the *M. oleifera* seeds extract extracted by the FMASE method gave a very significant difference ($P < 0.01$) to coagulation time

The average value of milk casein coagulation time is shown in Table 1, the average value of milk casein coagulation time was shown to be shown on T1 treatment or *M. oleifera* seeds extracted by FMASE with substrate pH 5 method for 14.35 ± 0.19a seconds, while the longest coagulation time in the T4 treatment or *M. oleifera* seeds was extracted by the FMASE method with a substrate pH of 8 for 159.03 ± 0.99d seconds. This is in accordance with [30] coagulation time in the study of Mucor miehei rennet showed pH 5 and 6 had a short coagulation time of 37.2 and 50.1 s, respectively. The results of [27] who observed the substrate coagulation time of 10 g/l at pH CaCl2, it was found that the coagulation time of the protease enzyme in *M. oleifera* seeds extract was 5.47 ± 0.33 s. According to [20] the coagulation time for Moringa seeds extract with pH 6.5 was $25.33 \pm 0.58^{a}{}_{b}$ seconds, faster than the leaf ($30.33 \pm 0.58^{a}{}_{c}$ second) and flower ($30.33 \pm 0.58^{a}{}_{c}$ second) extract of *M. oleifera.* This indicates that the more acidic the pH, the faster the coagulation time required. This is because the nature of the protease enzymes present in Moringa seeds can work optimally in acidic conditions. Optimal prosthetic enzyme action causes hydrolysis of k-casein and the time required is relatively short.

Figure 4 shows a graph of the effect of pH treatment on the coagulant agent of M. oleifera extract using the FMASE method on the coagulation time. It can be seen in Fig. 4 that pH treatment can affect the speed of enzymes in coagulating milk casein. The higher the pH or the more alkaline the substrate atmosphere, the longer the enzyme needed in the M. oleifera extract using the FMASE method to coagulate milk casein. So that the optimum pH treatment of M. oleifera extract with the FMASE method to produce the fastest coagulation time is treatment T1 or substrate pH 5 and T2 or substrate pH 6. This is in accordance with [18], milk coagulation enzyme activity also depends on pH, activity Casein coagulation will increase as the pH decreases. [31] added that most plant protease enzymes such as active aspartic protease enzymes at acidic pH have preferential specificity cleavage on peptide bonds between hydrophobic amino acid residues.

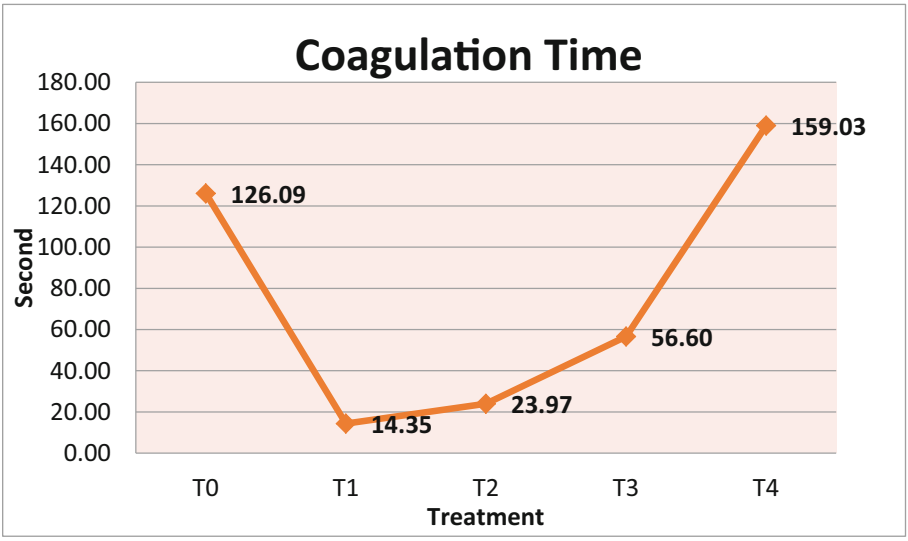

Fig. 4. Coagulation time of *M. oleifera* seeds extracted by maceration dan FMASE method

4 Conclusion

M. oleifera is a newest protease source that has the potential to be used as a coagulant in cheese production, due to the promotion of extensive hydrolysis of k-casein and low degradation of α- and β-casein compared to other plant coagulants. M. *oleifera* seeds can be extracted using a method that has been developed, namely FMASE (Focused Microwave-Assisted Soxhlet Extraction). However, pH is one that affects the work of the enzyme itself, where the more appropriate the pH value, the more optimal the work of the enzyme. The results showed the effect of pH treatment on clotting properties such as MCA, CA, ratio MCA/CA, coagulation time value of *M. oleifera* seeds extracted by the FMASE method gave a very significant difference ($P < 0.01$). The highest yield was shown in the treatment of T_1 or *M. oleifera* seeds extract using the FMASE method at a substrate pH of 5 with MCA values (69.68 ± 0.95^{e} U/ml), CA (0.02 ± 0.003^{a} U/ml), MCA/CA ratio (3209.09 ± 0.43^{e}) and coagulation time (14.35 ± 0.19^{a} second). This shows that *M. oleifera* seeds extracted by the FMASE work optimally at an acidic atmosphere or pH 5. Further research is recommended for moringa seed extract to be purified so as to obtain optimal caseinolytic activity values.

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