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Effect of Starter and Enzyme Additions on Clotting Time of Milks for Mish Manufacture

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Abstract: ***Cows' milk samples were subjected to changes in their physical and chemical properties when different additions of Starter, Rennet, Starter/ Rennet combinations and their effects on the rate of coagulation were studied. The milk samples were first pasteurized and then coagulated with different additions of enzyme and acid starter combinations. Time from the enzyme and starter additions to the onset of gelation as indicated by the first visible floccules was measured. The results showed that the longest clotting time of milk recorded (22.7 min) was in samples containing 0.25 ml of rennet enzymes solution (1ml = 20 mg rennet), while the shortest time was (4.65 min) in samples containing 1.5 ml of enzyme. Other samples ranged intermediate. Using starter alone, the longest clotting time of milk recorded (19.69 min) was in the samples containing 0.25 ml inoculum of lactic acid starter mother culture, while the shortest time (6.87 min) was in samples containing 1.5 ml of starter. Other samples ranged intermediate. Furthermore, the additions of different mixtures of starter and rennet significantly (P≤ 0.05) affected the clotting time of milk. The longest clotting time recorded (10.05 min) was in the sample containing a mixture of 0.5 ml starter and 0.5 ml rennet, while the shortest clotting time (9.43 min) was in the samples containing a mixture of 0.5 ml starter and 1.0 ml rennet enzyme. The longest clotting time (13.55 min) was when renetting the samples at pH (5.9), while the shortest clotting time (6.87 min) was at pH (5.7). Viscosity changes in the coagulated milk samples were followed during the coagulation process. At zero time, the viscosity was (4.1) Poise and was highest (49.8) Poise at the end of incubation time using the mixture of enzyme and starter. These experiments were done to establish a guideline for Mish manufacture, a popular Sudanese fermented milk product.***

Keywords: Rennet, Starter, Viscosity, Coagulation, Clotting Time, Mish

1. Introduction

Fermented milk products are known for their taste, nutritive value and therapeutic properties. Dairy starters are the 'heart' of fermented milk products, the most crucial component in the manufacture of high-quality fermented milks. In the dairy industry, some enzymes are required for the production of cheeses, yogurt, and other dairy products, while others are used in a more specialized fashion to improve texture or flavor (Bezie and Regasa, 2019). Milk coagulation and formation of rennet-induced gel (coagulum) is the most important and the most sensitive process in the production of the rennet curd cheese varieties. It is principally manifested through the action of numerous factors, which control both biochemical and physico-chemical processes during coagulation and directly influence rheological characteristics of rennet-induced casein gel (Jovanović et al., 2002). Enzymatic Coagulation of milk can be divided into two phases: the primary (or enzymic) phase, during which a proteolytic enzyme such as rennet cleaves a phenylalanine – methionine bond of ƙ-casein creating a metastable state of the casein micelle, and the secondary (or non-enzymic) phase, where the milk subsequently gels and forms a clot (TSE/BSE 2002; Kevany et al. 2015) . The primary phase exhibits normal characteristics of an enzymic system, such as pH, concentration of enzyme and substrate and temperature dependant. Starter culture changes the milk sugar, lactose, into acid (lactic acid) so that the milk becomes sour (fermented or cultured). (Lucey and Singh, 1997; Rajiv, 1998). A wide variety of acidified milk products are produced. Some of the main products are fresh acid-coagulated cheese as stated above and yoghurt. The manufacture and technologies engaged in the production of fresh acid cheese varieties have been reviewed (Kosikowski and Mistry, 1997; Lucey, 2002) .

'Mish', as a fermented manufactured dairy product, is known to all regions in Sudan with different names and is now available in the markets of urban areas in Sudan. The product is fermented for two or more days before consumption. In modern dairy industry, it is made from whole cows or skim milk by adding starter culture and after curdling spices such as black cumin, fenugreek, garlic and sometimes hot or green pepper are added. It is then packaged and left for 24 hours to ripen and develop a smooth texture (Mamoon and Ali, 2021). The objective of the present study was to investigate the use of a unique combination of acid and enzyme in curd formation of Mish. This required solid findings which could solve numerous problems associated with the coagulation of milk in which co-aggregates were formed. Hence, creating optimum coagulation conditions by using different starter and enzyme combinations additions to form gel with good rheological properties and consumer acceptability in milks for mish production.

2. Materials and Methods

2.1 Materials

Source of Milk
Milk was obtained from local farms in Khartoum North, Sudan.

Source of Rennet Enzyme
A certified Rennet powder was obtained from CHR. HANSEN of Denmark and dissolved in distilled water in a concentration of 1ml= 20 mg rennet.

Source of Starter Culture
A Commercial starter culture (a blend of *Lactobacillus bulgaricus* and *Stretococcus thermophilus*) was obtained from a local supplier and prepared (1 g /1000 ml milk) via incubation at 22 ^{o}C for 4 hrs. The starter was subsequently maintained by propagating and growing in fresh portions of milk and used as ready to use activated inoculums.

2.2 Methods

Preparation of Milk Samples
150 ml of pasteurized milk samples were heated to 42 ^{o}C and kept in a water bath till clotting time was recorded. Rennet was dissolved in Distilled water (1ml = 20 mg rennet); 0.5 ml was then added to 150 ml milk samples. Time was recorded and triplicate samples of 15 ml each in test tubes were rotated slowly till tiny clots were seen.

Milk acid coagulation test was carried out using 0.5 ml inoculums added to 150 ml of milk samples. Time was recorded and triplicate samples of 15 ml each in test tubes were rotated slowly till tiny clots were seen.

Milk Clotting Time (MCT)
The measurements of MCT (min) of the samples were carried out following the method described by (Jovanović *et al*., 2002). A stop watch was used to take the time from mixing of each aliquot of Rennet and/or Starter to the sign of sudden breakdown of the clot on the test tube walls. All experiments were replicated three times.

pH value
The pH values of milk samples were carried out using a pH-meter (Sentron 1001, Netherland).

Viscosity
Viscosity was done according to (ISO 2555, 2018) using a Thermo Scientific™ HAAKE™ Viscotester™ E (USA).

Statistical Analysis The significance of differences between means were compared at each time point using Duncan's multiple range test after ANOVA for one-way classified data (Snedecor and Cochran, 1989).

3. Results and Discussion

Enzyme coagulation of milk
The results obtained for the Milk Clotting Time (MCT) as influenced by coagulation factors (starter and rennet additions) were shown in **Table (1) and Figure (1)**. The longest clotting time of milk (22.7 min) was in the sample with 0.25 ml concentration of Rennet, while the shortest time (4.65 min) was in the sample containing 1.5ml of Rennet. Other samples ranged intermediate. The results as shown were significantly different ($P \leq 0.05$). Enzymatic coagulation of milk is the modification of casein micelles via limited hydrolysis of casein by rennet, followed by calcium-induced micelle aggregation (Fox & McSweeney, 1998; Andrén, 2002). Coagulation is enhanced by decreasing pH, increasing calcium concentration and temperature (no aggregation below 20°C). Syneresis is augmented by increasing temperature, pH and applied pressure, e.g. stirring. (Walstra, *et al.*, 2006). It was obvious from the results that by increasing the amounts of rennet the MCT decreased significantly. ($P \leq 0.05$).

Table 1: Milk Clotting Time (MCT) at different additions of Rennet enzyme

Rennet Additions (ml) (1ml =20mg enzyme)	MCT(min)*
1.50	4.6 ± 0.18^{e}
1.25	10.1 ± 0.017^{d}
1.00	17.0 ± 0.02^{c}
0.50	19.3 ± 0.06^{b}
0.25	22.7 ± 0.16^{a}

*Mean values ± SE within the column having different superscripts letters are significantly different ($P \leq 0.05$).

Acid coagulation of milk
The results in **Table (2) and Figure (1)** show the effect of the additions of lactic acid on clotting time (min) of milk. The longest clotting time recorded (19.69 min) was by the additions of 0.25 ml lactic acid bacteria starter, while the shortest time found (6.87min) was by the samples containing 1.5 g of the starter. Other samples ranged intermediate with significant differences ($P \leq 0.05$). In the acid coagulation of milk, casein micelle properties are altered by a lowered milk pH (Lucey & Singh, 1997). This causes dissociation of electrons from the micelles and the negative charges in the casein micelles are neutralized, with aggregation occurring as the isoelectric point of the casein micelle (pH 4.6) is approached. (McKenzie *et al.*, 1971; ; Haque & Kinsella, 1988; Guyomarc'h *et al.*, 2003). Dannenberg & Kessler, (1988) stated that, the increased curd firmness was due to an increased number and strength of bonds of the acid gel as denatured whey proteins associated with casein micelles interacted with each other. Further, the concentration of protein in the gel network will be increased because of the active participation of the denatured whey protein in the structure formation. (Lucey& Singh, 1997). The joint effect of starter and enzyme on clotting time could be due to the specificity of rennet enzyme and the optimum pH reached by the starter. In milk coagulation, the rennet pH zone (6.5 – 5.3) is broad and the acid zone (5.3 – 4.6)) is narrow. This is influenced by other factors mainly Ca^{++}. (FAO, 1987; Dejmek & Walstra, 1993; Fox & Mc Sweeney, 2004). These findings were adopted in creating a unique process model for the manufacture of the new Sudanese Mish product. (Mamoon and Ali, 2021)

Table 2: Effect of Starter Additions on Milk Clotting Time (MCT) (min)

Starter Additions (ml)	MCT (min)*
1.50	6.8 ± 0.16^{d}
1.00	8.4 ± 0.05^{c}
0.50	16.2 ± 0.05^{b}
0.25	19.7 ± 0.2^{a}

*Mean values ± SE within the column having different superscripts letters are significantly different ($P \leq 0.05$).

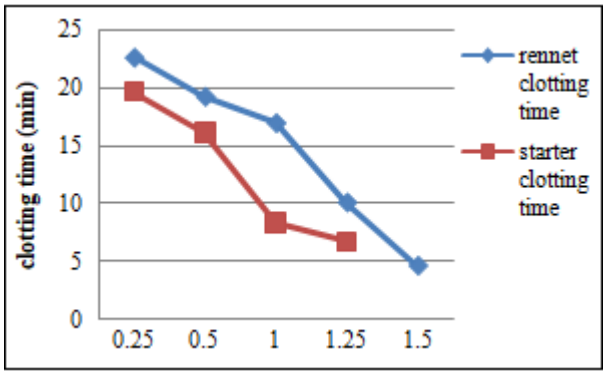

Figure 1: Acid and Rennet Milk Clotting Time with different additions of Starter and Rennet

Effect of Starter and Rennet combined additions on MCT

The results in **Table (3) and Figure (2)** show the combined effect of starter and rennet additions on MCT. The longest clotting time was (10.05 min) in samples containing a mixture of 0.5ml starter and 0.5 ml rennet, while the shortest clotting time (9.43 min) was in samples containing a mixture of 0.5 ml starter and 1.0 ml rennet. The transformed lactose (the milk sugar) into lactic acid helped the rennet's work. (Bezie and Regasa, 2019). The time needed for the fermentation may be as short as 2.5 h for the classic yoghurt starter culture (Knut, 2001). As could be expected on the basis of the chemical acidification reaction that underlies the fermentation process, pH dropped during the 3-5 hrs to values of 4.6. Moreover, it was observed that samples produced by strains with low proteolytic activity had sigmoidal pH decrease, but starter cultures with high proteolytic activity showed different acidification profiles and the fermentation times had been longer (Jovanović *et al*., 2002; Imdakim *et al*., 2015; Elahe *et al*., 2016). This fact was again crucial in establishing the intended new Process Model for the Sudanese Mish manufacture. (Mamoon and Ali, 2021).

Table 3: The effect of Starter and Rennet combination effect on clotting time (min)

Mixture of Rennet and Starter Additions	MCT (min)*
0.5 starter and 1ml enzyme at same time	9.40 ± 0.09^{c}
0.5 starter and 1ml enzyme after 10 min	2.14 ± 0^{d}
0.5 starter and 0.5 ml enzyme at same time	10.00 ± 0.04^{a}
0.5 starter and 0.5 ml enzyme at 10 min	5.03±0.05b

*Mean values ± SE within the column having different superscripts letters are significantly different (P≤ 0.05).

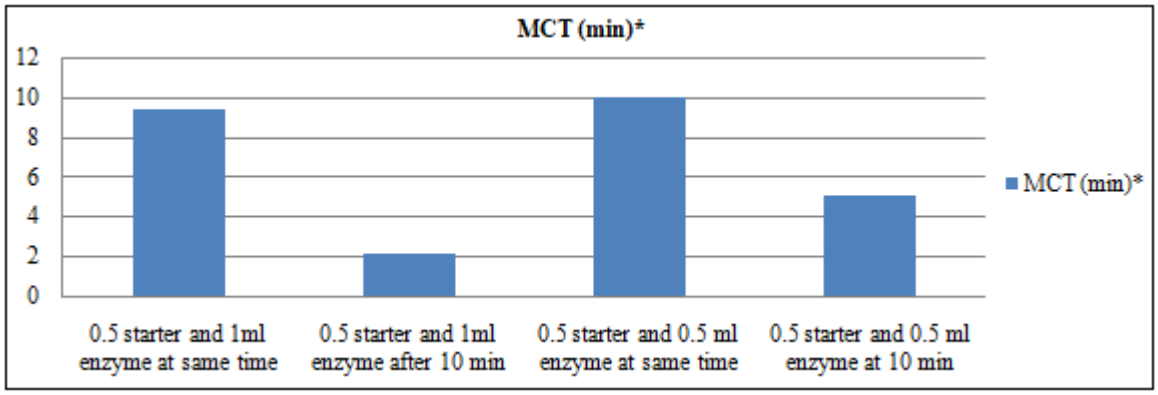

Figure 2: Starter and Rennet combination effect on clotting time (min)

MCT at different starter Incubation Periods (IP) :

The results in **Table (4)** show the effect of adding 0.5 g/ml of starter before incubating the milk samples for different incubation periods of 5 ,10, 15, 20 minutes followed by adding rennet enzyme. The pH values were recorded at each addition. The fastest clotting time was (6.91 min) at pH 5.7, the slowest clotting time was (13.55 min) at pH 5.9. The decrease in the pH of milk from 7.0 to 5.2 caused a decreases in the clotting time; pH optimum for the hydrolysis of k-casein being 5.1–5.3. Similar results were obtained by Eck, and Ernstrom, (1990). The most important effects of lowering the pH of the milk are the solubiliation of micellar calcium phosphate as well as, the decrease in the net charge of the casein molecule and the dissociation of casein from micelles (Dalgleish & Law, 1988; Desobry-Bannon, 1991). It has also been reported that lowering the pH causes an increase in the curd firming rate (Daviau et al., 2000).

Table 4: Milk Clotting Time (min) at different Starter Incubation Periods (IP)

Incubation Periods (IP), min	pH at end IP	MCT (min)*
5	5.9	13.55 ± 0.31^{a}
10	5.8	11.37 ± 0.12^{b}
15	5.8	10.36 ± 0.4^{c}
20	5.7	8.34 ± 0.08^{d}
25	5.7	6.91 ± 0.29^{e}

*Mean values ± SE within the column having different superscripts letters are significantly different (P≤ 0.05)

Curd viscosity at different times after the addition of starter, enzyme and starter and enzyme mixture

The results in **Table (5)** and **Figure (3)** show the changes in viscosity during coagulation process when adding starter, enzyme and starter and enzyme mixture. At zero time the viscosity was 4.1 Poise. At the end of incubation time the viscosity of the enzyme and starter mixture was the highest. The highest viscosity in the added starter and enzyme together was due to the starter making optimum pH for the rennet enzyme activity. Again such findings were used to establish the Sudanese Mish process model. (Mamoon and Ali, 2021). The lowest viscosity obtained using starter alone

was due to the fact that lactic acid bacterial needed long time to make lactic acid and decrease the pH to 4.6 to precipitate casein. Similar result was obtained by (Soukoulis et al 2007) who successfully described the joint pH decline and viscosity development during the fermentation process. The viscosity and incubation time data were also fitted to linear models against pH.

Table 5: Change of Viscosity (Poise) during coagulation time by starter, Rennet and Starter/ Rennet combinations

Time (min)	Starter	Rennet	Rennet + Starter
0	4.1	4.1	4.1
15	4.1	19.2	27.3
30	4.2	14.9	40.0
45	4.2	14.8	48.5
60	4.2	17.6	48.0
75	4.2	21.9	48.2
90	4.2	23.8	49.0
105	4.2	25.2	49.8

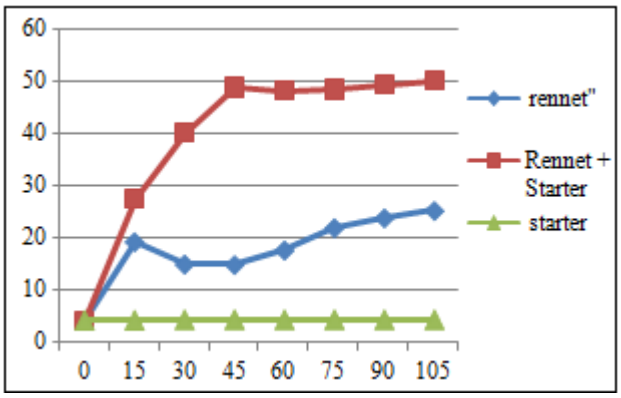

Figure 3: Changes of Viscosity (Poise) during coagulation time by starter, Rennet and Starter/ Rennet combinations

4. Conclusion

Enzymatic and acid coagulation of milk is the modification of casein micelles via limited hydrolysis of casein by rennet or reaching isoelectric point forming a network or micelle aggregation. To get the best curd formation in an attempt to obtain a firm body for the required fermented product (Mish), clotting should not be fast or slow. This would allow acid coagulation proceed slowly before the enzymatic clotting and hence giving a unique final product with good quality and better consumer acceptability.

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