Statistically Defining Optimal Conditions of Coagulation Time of Skim Milk

[1,2] Mıthat Celebı*, [2,3] Zafer Omer Ozdemır, [4] Emre Eroglu and [5] Ibrahım Guney
[1]*Yalova University, Faculty of Engineering, Polymer Engineering, Yalova TURKIYE.*
[2]*Yildiz Technical University, Chemical and Metallurgical Faculty, Bioengineering Department, Istanbul, Esenler, TURKIYE.*
[3]*Kirklareli University, Faculty of Arts and Science, Department of Chemistry, Kirklareli, TURKIYE.*
[4]*Kirklareli University, Faculty of Arts and Science, Department of Mathematics, Kirklareli, TURKIYE.*
[5]*Bitlis Eren University, Faculty of Arts and Science, Department of Statistics, Bitlis, TURKIYE.*
mithat.celebi@yalova.edu.tr*

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Summary: Milk consist huge amount of largely water and different proteins. Kappa-kazein of these milk proteins can be coagulated by *Mucor miehei* rennet enzyme, is an aspartic protease which cleavege 105 (phenly alanine)-106 (methionine) peptide bond. It is commonly used clotting milk proteins for cheese production in dairy industry. The aim of this study to measure milk clotting times of skim milk by using *Mucor Miehei* rennet and determination of optimal conditions of milk clotting time by mathematical modelling. In this research, milk clotting times of skim milk were measured at different pHs (3.0, 4.0, 5.0, 6.0, 7.0, 8.0) and temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 ^{o}C). It was used statistical approach for defining best pH and temperature for milk clotting time of skim milk. Milk clotting activity was increase at acidic pHs and high temperatures.

Keywords: Milk clotting time, *Mucor miehei* Rennet, S curve, skim milk, optimization.

Introduction

Chymosin (calf rennet)– extracted from the fourth stomach (abamosum) of the young calves- has been used worldwide in cheese production for years and it has been preferred for its high quality. However, nowadays there has been a shortage of the chymosin in the world market, so proteolytic enzymes from different sources gained more importance [1].

Mucor miehei rennet is the most thermostable enzyme of *Mucor miehei* at acidic pH. It is an aspartic protease which cleavege 105 (phenly alanine)-106 (methionine) peptide bond in kappa-kazein (milk protein) [2, 3]. The aspartic proteinases (EC 3.4.23) are characterized by an optimum activity at acidic pH and contain two catalytically essential aspartate residues. A worldwide shortage of the aspartic proteinase chymosin, traditionally used in the production of cheese, has prompted a search for adequate substitues. Two alternatives are aspartic proteinases from fungi *Endothia parasitica* and *Mucor miehei.* Although *Mucor miehei* proteinase have high milk clotting potential relative to its capacity for general proteolysis, high termal stability has precluded its use as a chymosin substitute [4]. The stability of the casein micelle is dependent on the presence of κ-casein on the surface of the micelle where it functions as an interface between the hydrophobic caseins of the micelle interior and the aqueous environment. κ-casein is also involved in thiol-catalyzed disulfide interchange reactions with the whey proteins during heat treatments and, after rennet cleavage, in the facilitation of micelle coagulation. These functions of κ-casein are regulated by three–dimensional structure of the protein on the micelle surface [5, 6]. Rennet coagulation of milk is a two-stage process. In the first stage, casein micelles colloidal suspensions are destabilized by partial hydrolysis of their surface κ-casein, catalyzed by chymosin. Destabilized casein micelles spontaneously aggregate in the second stage as been considered as a diffusion-limited aggregation, according to the von Smoluchowsky model for unstable colloids coagulation [7, 8].

Mathematical models (nonlinear least squares, etc.) are very useful for defining optimal conditions in different study subjects [9, 12]. In curve fitting data, the prevailing assumption regarding model description is that the structure is linear in the model coefficients. In many areas of the physical, chemical, engineering, and biological sciences, knowledge about the experimental situation suggests the use of a less empirical, more theoretically based,

*To whom all correspondence should be addressed.

nonlinear model. The literature is quite rich in algorithms for the minimization of residual sum of squares in nonlinear model situations. In addition, there are many regression computer packages available that contain at least one nonlinear estimation method [11, 12].

In the literature, Ataci *et al* (2009) studied with *Mucor miehei* rennet at different pHs and temperature and they did only variance analysis [13]. Differently, optimum conditions of milk clotting times were investigated statistically with a mathematical equation (1) by using SPSS 18 program in our study and Fig. 2 was drawn according to this equation.

The objective of this study to measure coagulation time of skim milk by using Mucor miehei rennet at different pHs and temperatures, and determination of optimum milk clotting time with mathematical model depending on experimental data.

Results and Discussion

Milk solution coagulated before adding enzyme because of the high acidity at pH: 3.0. Therefore, milk clotting activity was not determined at this pH. In Fig. 1 was shown that *Mucor Miehei* Rennet displays high milk clotting activity at low pH values. Milk solution was coagulated as soon as possible at acidic pHs. However, milk clotting activity is low at pH 7.0 and pH 8.0.

Mathematical Modeling

There are many examples of nonlinear models. We offer but a few here as illustrations:

$$y = \alpha e^{\beta x} + \varepsilon \qquad (1)$$

Note that, in all of the models of Eq. (1) at least one of the parameters enters the model in a nonlinear way. As in the case of linear models, the analyst's initial task is to estimate the parameters involved. Again, the technique of least squares is used extensively.

Nonlinear Least Squares

The development of the least squares estimators for a nonlinear model brings about complications not encountered in the case of the linear model. This is easily illustrated in the case of the exponential regression model of Eq. (1). Given a set of data (y_i, x_i) for i = 1,2,…,n the estimators of a and β are found by minimizing.

$$SS_{Res} = \sum_{i=1}^{n} (y_i - \hat{\alpha} e^{\hat{\beta} x_i})^2 \qquad (2)$$

We differentiate the result of (2) with respect to $\hat{\alpha}$ and $\hat{\beta}$ and set each derivative to zero. This yields the following equations:

$$\sum_{i=1}^{n} (y_i - \hat{\alpha} e^{\hat{\beta} x_i})(-e^{\hat{\beta} x_i}) = 0$$
$$\sum_{i=1}^{n} (y_i - \hat{\alpha} e^{\hat{\beta} x_i})(-\hat{\alpha} e^{\hat{\beta} x_i} x_i) = 0 \qquad (3)$$

Equation (3) are nonlinear in the parameter estimators $\hat{\alpha}$ and $\hat{\beta}$ Thus we cannot compute estimates by elementary matrix algebra. Some type of iterative process must be used.

Graphics was drawned according to mathematical equation (1) by using SPSS 18 program in Fig. 2. All the results (except pH 8) obeyed S type curve. Because of this reason, we use S type curve for temperature and pH estimation. It was decided that S curve compliance with experimental observed milk clotting time values (Fig. 2).

Experimental

Materials

Mucor miehei rennet was purchased from Sigma. Skim milk powder was supplied by Pinar Co. Ltd. (İzmir, Turkey). Specifications of skim milk powder can be shown in the Table-1. Calcium chloride, 97 % powder, was obtained from Riedel-de Haen (Cat.: 12095). Other reagents were used of analytical grade.

Table-1: Specifications of milk powder (nutritional values for per 100 mg) from Pinar Co. Ltd.

Energy	363 kcal
Protein	36 g
Carbohydrate	52 g
Calcium	1256 mg
Fat	1.25 g

Preperation of Skim Milk Solution

Milk were prepared fresly from powder skim milk in a 2 L beaker. 50 g skim milk powder (10 %) was weighed and added to 500 mL buffer solution (acetate buffer pH: 5.0) than milk was mixed and than left for 30 min. to rest.

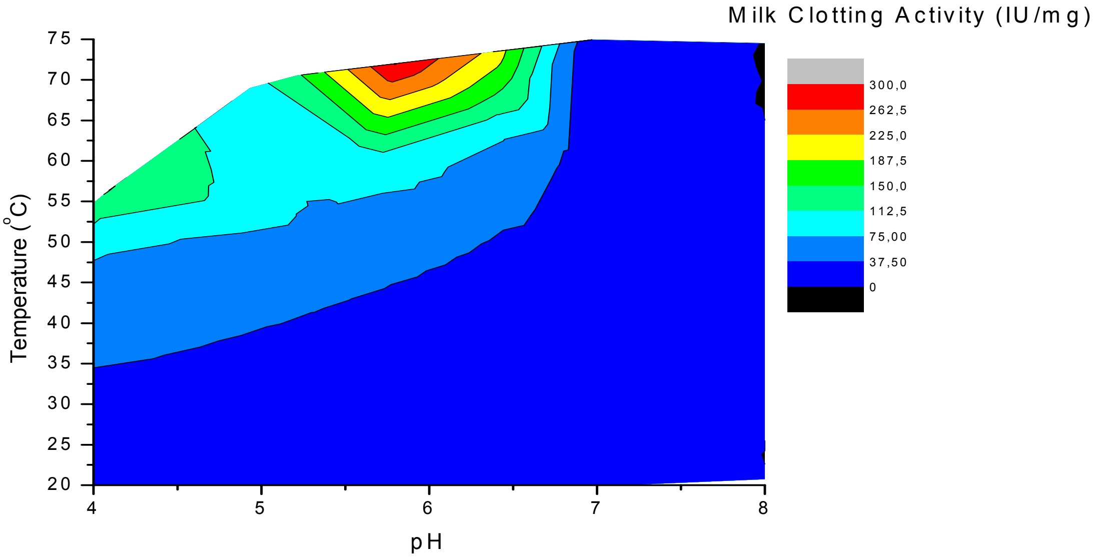

Fig. 1: Milk clotting activity (IU/mg) of *Mucor miehei* rennet at different pHs and temperatures

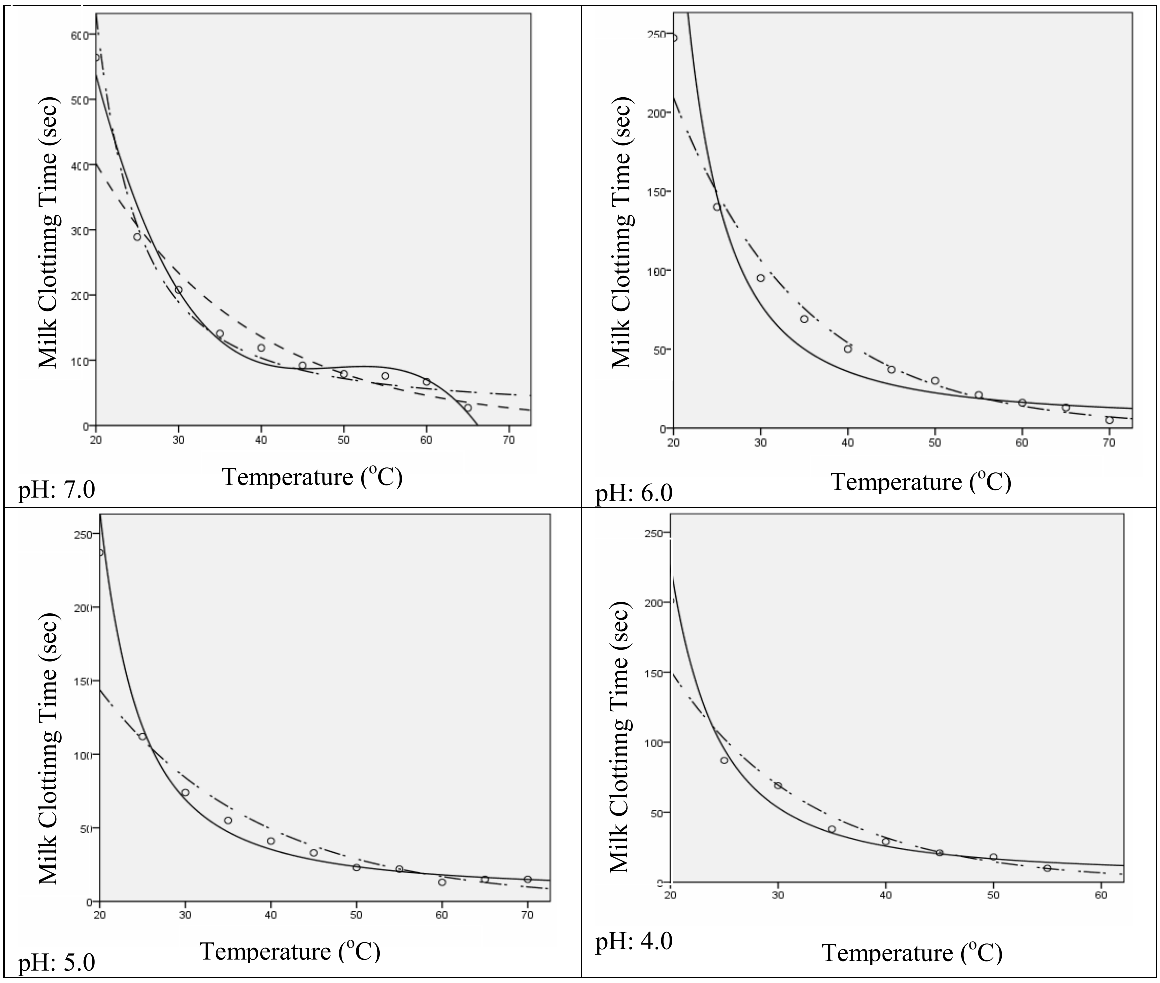

Fig. 2: Milk clotting times of *Mucor Miehei* Rennet at different pHs and temperatures.
(o experimental observed, _____ Cubic, ____ .S, ___ Exponential)

Determination of Milk Clotting Time

From fresh milk, 10 mL samples of skim milk were measured into a 25 mL beaker placed in a water bath at different temperature and pHs. After the addition of appropriate volume of rennet solution (0,04 mg mL^{-1}), the coagulation time was measured with the visual method described by Hostettler [14]. Aliquots (10 ml) of skim milk solution (at desired pH and temperature) were placed in separate beakers (each volume of them 25 mL) and tempered at desired temperature for 5 min. After 1 mL of enzyme solution were added to each beaker, the coagulation time was defined as the time required for the first appearance of clots on the surface of the glass walls of beaker in the milk solution [15]. Milk clotting times of *Mucor miehei* rennet were measured at different pHs and different temperatures. Results of milk clotting time of skim milk at different cicumstances can be shown in the Table-2. All milk clotting experiments were conducted with three replicates.

Table-2: Milk clotting times (sec.) of *Mucor miehei* rennet at different pHs and temperatures.

Temperature (°C)	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
20	201	237	247	564	'**'
25	87	112	140	289	'**'
30	69	74	95	208	539
35	38	55	69	141	299
40	29	41	50	119	244
45	21	33	37	92	218
50	18	23	30	79	211
55	10	22	21	76	223
60	'*'	13	16	67	413
65		15	13	127	'**'
70		15	5	'**'	
75		'*'	'*'		

* Milk solution was clotting by spontaneously without the addition of enzyme solution.
** Milk clotting was not observed after waiting 10 minutes. Each data point represents the average value of three independent experiments.

Milk Clotting Activity Determination

Milk clotting activity was calculated according to following the procedure method of Arima *et al*. [1, 16, 17] and expressed in terms of international units (IU). Description of briefly of method of Arima *et al*; 10 mL milk solution was contain 10 % skim milk in 0,0173 g $CaCl_2$. Skim milk solution was incubated at desired temperature to stabilize the temperature for 5 min. in a water bath and then milk clotting enzyme (Mucor Miehei Rennet) solution (1 ml - 0,04 mg mL^{-1}) was added to skim milk solution and then milk clotting time was determined by manually rotating the beaker occasionally and checking for first visible clot formation. Chronometer was launched once enzyme added to skim milk solution. One IU is defined as the amount of enzyme which clots 10 mL of milk solution containing 1 g skim milk powder and 0,01 M (0,0173 g) $CaCl_2$ in 1 min. at 35 °C.

Influence of pH and Temperature

The milk samples were prepared for desired pH at different buffers (0,05 M Sodium Acetate Buffer and 0,1 M Sodium Phosphate Buffer) and pH of milk solution were adjusted by slow addition of 1 M HCI or 1 M NaOH. The coagulation time was measured at temperatures over the range at 25 °C to 75°C.

Conclusion

Milk clotting can ocur two method; by acidification and enzyme. pH and temperature of skim milk were effect on milk clotting time. Milk solution was shortly clotting at low pHs and high temperature. In addition, increasing temperature also reduce milk clotting time and enhance milk clotting activity. Statistical approach was used for defining best pH and temperature for milk clotting time. Experimental data of this research are shown that equation for coagulation of milk is obeyed S curve. According to this research finding, researchers can be estimate milk clotting time for the same experimental conditions.

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