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Temperature and pH Effect on Milk Clotting Time of *Mucor miehei* Rennet

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In present studies, some data on the *Mucor miehei* rennet milk clotting time at different pH and temperature has been studied. Milk clotting time of *Mucor miehei* rennet from 30 to 70 ºC of temperature in pH range of 5, 6, and 7 were obtained. Effects of temperature, pH and the interaction of temperature and pH on milk clotting time of *Mucor miehei* rennet were changed significantly. Effects of temperature and pH were investigated with 2 factors factorial design. Multiple comparsions of parameters were made by the homogenous subsets. The homogenous subsets of the temperature and pH were determined through post hoc test. Statistical evaluation of the results showed that the assay results are in good agreement.

Key Words: Milk clotting time, *Mucor miehei* rennet, Post hoc tests.

INTRODUCTION

Calf rennet, which is a milk clotting enzyme derived from the mucosal lining of calf stomach has traditionally been used in cheese making industry. However due to the growing demand in the cheese industry, several acceptable substitutes have been developed for calf rennet, e.*g.*, porcine and bovine pepsins and proteinases from the fungi *Mucor miehei*, *Mucor pusillus* and *Endothia parasitica*[1].

Mucor miehei rennet is aspartic proteinase (E.C.3.4.23) and contain 2 catalytically essential aspartate residues in active site of enzyme. The aspartic proteinases are charactherized by an optimum activity at acid pH (pH < 6)[2]. Aspartyl proteinases successfully clot milk by its specificity toward the phenil alanin$_{105}$-methionine$_{106}$ bond of kappa kazein of the casein micelles[3].

$$S + E \rightarrow P + E + G$$

S, E are kappa kazein and enzyme and P, G are para kappa kazein and glycomacropeptide[4]. The glycomacropeptide moiety (resiues 106-109) is hydrophilic and soluble and leaves the micelle after the reaction, whereas the para kappa casein moiety (1-105) is hydrophobic and remains in the micelle.

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Mucor miehei rennet contains *ca.* 6 % carbohydrate that prolong the active life of a glycoprotein by stabilizing conformation and by protecting protein from proteolytic attack[5]. In industry thermostability of *Mucor miehei* rennet is critical in the selection of chymosin replacement[6].

In the experiments reported here, the authors investigated milk clotting time of *Mucor miehei* rennet in different pH and temperature.

EXPERIMENTAL

Pure microbial protease "*Mucor miehei*" was obtained from Sigma-Aldrich. Milk powder was obtained from Pinar in Turkey. Sodium acetate trihydrate, sodium dihydrogen phosphate, calcium chloride dihydrate, glacial acetic acid (AR grade) were purchased from Merck.

The reagents are 1 L sodium acetate pH: 5.0 buffer (0.05 M), 1 L phosphate pH:6.0 buffer (0.05 M), 1 L phosphate pH: 7.0 buffer (0.05 M), stock calcium chloride 500 g/L and 0.5 g/L $CaCl_2$ daily fresh cloride solution from stock calcium chloride.

Clifton model water bath and Cyber Scan 500 model pH meter were used in experimental process. Distilled water obtained from Elix Millipore device was used throughout the work statistical analysis were calculated by NCSS 2000 computer programme.

Enzyme preparation: Pure enzyme "*Mucor miehei* rennet" solution was prepared in 3 different pH as pH: 5, 6 and 7. So, 0.5 g of pure enzyme was weighed accurately at room temperature and transferred into a 25 mL standard flask and diluted to the mark with buffer.

Substrate preparation: For each buffer, 110 g of non-fat dry milk powder was weighed at room temperature and transferred into 1000 mL baker. After the addition of 800 mL of 0.05 M buffers and 100 mL of 0.5 g/L $CaCl_2$, it was diluted to mark with buffers.This substrate solutions were manually mixed until the milk was homogeneous.The milk was settled in darkness for 0.5 h at room temperature. It was prepared daily fresh prior to each use.

Determination of milk clotting time of enzyme: Enzyme and substrate preparing in pH: 5, 6 and 7 is presented as follows: 50 mL of substrates prepared in pH buffer with 0.05 g/L $CaCl_2$ solution were equilibrated for 10 min from 30 to 70 ºC at 5 intervals of temperature in the water bath. 1 mL of enzyme solution prepared in pH buffer was added in 50 mL milk suspension and then stirred manually until observed the coagulation of milk. Assay was performed twice[7].

Statistical analysis of milk clotting time of enzyme: The main effects of temperature and pH and interaction effect of them were investigated with analysis of variance for fixed two factorial design using two-way ANNOVA. The factors are temperature with 5 levels such as 30, 35, 40, 45 and 50 ºC and 3 different pH 5, 6 and 7. All measurable milk clotting time data were measured for statistical analysis.

RESULTS AND DISCUSSION

In this study, the objective was to determine pure *Mucor miehei* rennets clotting time in different pH and temperature. Experimental data is shown in Table-1.

TABLE-1
EXPERIMENTAL DATA OF MILK CLOTTING TIME OF MUCOR MIEHEI RENNET IN DIFFERENT pH AND TEMPERATURE

Temp. (ºC)	Milk clotting time (s)								
	pH 5.0			pH 6.0			pH 7.0		
	MCT_1	MCT_2	Average	MCT_1	MCT_2	Average	MCT_1	MCT_2	Average
30	35.0	39.4	37.2	49.4	50.8	50.1	161.6	169.6	165.6
35	27.0	26.9	26.9	36.6	37.2	36.9	95.4	104.6	100.0
40	22.6	22.0	22.3	18.4	22.4	20.4	76.2	74.0	75.1
45	21.4	22.3	21.8	23.2	25.2	24.2	77.0	71.0	74.0
50	18.8	18.2	18.5	17.8	19.9	18.8	76.4	68.4	72.4
55	16.0	15.6	15.8	17.4	18.8	18.1	–	–	–
60	14.8	17.6	16.2	98.2	104.6	101.4	–	–	–
65	35.2	32.4	33.8	–	–	–	–	–	–
70	–	–	–	–	–	–	–	–	–

MCT_1 = First determination; MCT_2 = Second determination;
– = 20 min no milk clotting detection.

The relationship of increasing heat with increasing pH was showed in Table-1. At pH: 7.0 there was not enzyme activity (MCT) at 55 and 60 ºC. However in pH: 5.0 and 6.0 enzyme activity was observed at the same temperatures. Activity of *Mucor miehei* rennet in neutral pH was decreased against high temperature. The heat stability of *Mucor miehei* rennet increased with decreasing pH. Milk clotting time of *Mucor miehei* rennet is the best in pH 5 and thermal stability is the highest. In 70 ºC temperature at each pH points enzyme activity is not detectable. pH: 6.0 having more acidic effect according to pH: 7.0 and enzyme is indicate activity as a coagulation of milk at 55 and 60 ºC. Furthermore pH 5.0, having the best acidic effect than other each pH points and milk clotting activity of enzyme is detectable until 65 ºC. The pH and temperature interaction is evidently followed in pH 7.0, since it is clearly seen from Table-1 that enzyme dramatically lost its milk clotting activity and thermal stability against high temperature in pH: 7. This condition should based on milk clotting enzymes heat resistance increased as pH decreased results were obtained earlier, proteins tend to have optimum resistance to heat denaturation at or near their isoelectric points (pI). Oortwijn and Venema[8] earlier stated that clotting activity is not a property of enzyme only but it also depends on factors such as pH and temperatures.

Statistical analysis: The main effects of temperature and pH were investigated with analysis of variance for fixed 2 factorial design (twoway ANOVA). The results applying to analysis of variance for fixed two factorial design statistical analysis method is shown in Table-2. The factors are temperature with five levels 30, 35, 40, 45, 50 ºC and pH with three levels 5, 6, 7. All measurable milk clotting time data were choiced for statistical analysis.

TABLE-2
ANALYSIS OF VARIANCE FOR MILK CLOTTING TIME DATA

Source of variation	Sum of squares	df	Mean square	F	P
Corrected model	46976.499[a]	14	3355.464	334.309	0.000
Intercept	77897.456	1	77897.456	7761.030	0.000
Temperature	9528.635	4	2382.159	237.338	0.000
pH	32494.485	2	16247.242	1618.735	0.000
Temperature × pH	4953.379	8	619.172	61.689	0.000
Error	150.555	15	10.037	–	–
Total	47127.054	29	–	–	–

[a]R squared = 0.997 (Adjusted R squared = 0.994).

Table-2 indicates that main effects of temperature and pH and interaction between this two parameter were found statistically significant ($p < 0.001$).

Since there is significant differences at levels of both factors, the post hoc tests according to Tukeys, Duncan, LSD and Bonferroni were used to identify pairs of levels that differed significantly[9]. The homogeneous subsets based on Tukey and Duncan were used to make multiple comparisons.

The results of Tukey HSD test and means for groups in homogeneous subsets according to Duncan fort the levels of temperature and pH are displayed in Tables 3 and 4, respectively. The same results of hyphothesis tests are achieved using the other post hoc tests such as LSD, Bonferroni and Tukey B.

TABLE-3
HOMOGENOUS SUBSETS FOR TEMPERATURE LEVELS

Temperature (ºC)	N	Subset		
		1	2	3
50	6	36.5833	–	–
40	6	39.2667	–	–
45	6	40.0167	–	–
35	6	–	54.6167	–
30	6	–	–	84.3000

TABLE-4
HOMOGENOUS SUBSETS FOR pH LEVELS

pH	N	Subset		
		1	2	3
5	10	25.3600	–	–
6	10	30.0900	–	–
7	10	–	97.4200	–

It is clearly seen (Table-3) that the different three groups (subsets) for temperature were obtained according to Duncan test. The levels of temperature at 40, 45 and 50 ºC have statistically same effect on clotting time of enzyme while levels of 35 ºC (subset 2) and level of 30 ºC (subset 3) were statistically different from subset 1 ($p < 0.001$).

As a result the temperature gets higher above 40 ºC , the effect of temperature on clotting time reach homogeneous.

In Table-4, it is seen that the different two groups (subsets) for pH were obtained according to Duncan test. The levels of pH at 5.0 and 6.0 (subsets 1) have statistically same effect on clotting time while pH, 7.0 (subset 2) were statistically different from "subset 1" ($p < 0.001$). It should be emphasized that effect of neutral pH is different than others.

In summary, it is stated that according to experimental data confirmed by statistical analysis methods, neutral pH differs milk clotting time and thermal resistance of *Mucor miehei* rennet and the effect of increasing pH (from pH:5 to pH: 7) on milk clotting of *Mucor miehei* rennet are particularly observed at lower (30-35 ºC) and higher level (55-65 ºC) temperatures.

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