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Effect of transglutaminase on quality properties of fresh cheese

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ABSTRACT

Graph Microbial transglutaminase (MTGase) is an enzyme widely used in the food industry. In this study, MTGase had been applied to produce fresh cheese made from whole milk powder. To evaluate the impact of factors in reconstituted milk under the treatment of MTGase, a set of 18 experiments was conducted. Besides, a response surface methodology (a central composite design) was applied to evaluate the quality properties of fresh cheese according to the objective functions of hardness, yield, protein content, total solid content, and sensory evaluation score. In detail, enzyme concentration (0.6-3.0U/g protein), reaction temperature (30-60°C), and reaction time (1.5-6.0h) were three factors used in this model. The results showed that all these functions reached the optimal values at treated temperature, reaction time, and enzyme concentration of 36.14°C, 4.53h, and 2.59U/g protein, respectively. Furthermore, scanning electronic micrographs also showed that the network structure of the experimental products became more uniform under enzymatic treatment. The quality properties of fresh cheese (sensory evaluation score, syneresis, acidity, and the total number of lactic acid bacteria) met the CODEX STAN 243-2003 revised 2010 for the fermented milk products. Generally, following 28 days of storage, the quality properties of fresh cheese samples are stable.

1. Introduction

Since consumers have perceived enzymes to be more 'natural' than chemicals, the use of them to modify the functional properties of foods has attracted food scientists. Therefore, over the last few years, many enzymatic treatment applications in food technology have increased. Microbial transglutaminase (MTGase) has recently received great attention for its ability to generate cross-linkages in protein-based products (Duarte et al., 2020).

Transglutaminase (EC 2.3.2.13, protein-glutamine γ-glutamyl transferase) catalyzes *in vitro* cross-linking reaction in whey proteins, soy proteins, wheat proteins, beef myosin, casein, and crude actomyosin refined from mechanically deboned poultry meat (Duarte et al., 2020). In recent years, this enzyme has also been used to gelatinize various food proteins through the formation of cross-links resulting in the improvement of the functional properties of food. Basically, the targets of transglutaminase reaction may be (a) modification of texture, (b) protection of lysine in food proteins from various chemical reactions, (c) encapsulations of lipids and/or lipid-soluble materials, (d) formation of heat- and water-resistant films, (e) prevention of gelation under heat processing, (f) improvement of elasticity and water-holding capacity, (g) modification of solubility and functional properties, and (h) production of highly nutritional protein-based products (Duarte et al., 2020; Quaglia and Gennaro, 2003).

Several applications of MTGase in the production of milk and dairy products have been extensively studied. Yüksel and Erdem (2010) investigated the effect of the MTGase on yogurt properties due to cross-linking of milk proteins. The study was conducted on skimmed milk and reconstituted whole milk (14% non-fat solids concentration) with different enzyme treatment conditions. Actually, MTGase was an effective treatment in the production of low-fat yogurt without the addition of additives. Furthermore, MTGase contributed to the shelf-life of products. Sanli (2015) evaluated the effects of using MTGase on many yogurt properties such as acidity, viscosity, gel strength, and microstructure. The

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addition of enzymes contributed to the increase in gel durability and the reduction in whey separation of the product. According to Metwally et al. (2018), the MTGase was the only covalent binding enzyme which is available to improve the quality of dairy products. Cross-linking reactions could lead to changes in protein properties such as solubility, emulsification, foaming, and gel formation. For example, in Quark cheese, the MTGase led to lower hardness, less grain structure, and finer texture.

The current Vietnamese domestic raw milk has only been able to fulfill 30-40% of consumer demand, and has met the only production of drinking milk. Most of the cheese in the Vietnamese market today is imported products from other nations. Besides, there had not many studies focusing on the effect of MTGase addition on physicochemical and sensory properties of fresh cheese made from milk powder. Based on practical needs and current trends in domestic production, in this research, we built a process of producing fresh cheese using whole milk powder. Under MTGase treatment, effects of factors (enzyme concentration, temperature, and reaction time) on responses (hardness, yield, protein content, total solid content, and sensory evaluation score) were investigated using response surface methodology. The quality properties (whey separation, titratable acidity, the total count of lactic acid bacteria, and sensory evaluation) of fresh cheese samples were assessed within 28 days of storage.

2. Materials and methods

2.1. Materials

Microbial transglutaminase (MTGase, EC 2.3.2.13, Activa® MP) was derived from spore-forming bacteria *Streptoverticillium mobaraense* was supplied by Ajinomoto, Malaysia. The enzymatic powder has a specific enzymatic activity of 36 units (U) per gram powder.

Freeze-dried yogurt starter culture, a mixed strain of *Streptococcus thermophilus* CHCC 3534 and *Lactobacillus delbrueckii ssp. bulgaricus* CHCC 3984, was obtained from Chr. Hansen, Denmark.

Whole milk powder was supplied by Fonterra Ltd, New Zealand with a moisture content of 2,85%, protein content of 23.69%, lipid content of 28.32%, lactose content of 39.00%, and an ash content of 5.80% according to the product's certificate of analysis.

2.2. Fresh cheese preparation

Whole milk powder (15.44g) was added to distilled water (100ml). Then, reconstituted milk (300ml) was heated (85°C, 30min) to eliminate bacteria and inactivate enzyme existing in raw material, then cooled. Afterward, MTGase was added at experimental enzyme-treated temperature. The conditions for the enzymatic reaction (enzyme concentration, temperature, and reaction time) were determined by experimental design using a central composite design (CCD). After enzymatic treatment, starter culture (5%, w/v) was added at 43±1°C for the coagulation (4-5h, pH reached to 4.6). The curd was then transferred into a plastic tube (ϕ=56 mm) and was slightly pressed to achieve a final height of 2.8 cm. Fresh cheese (M1) was weighed to determine the yield of production and then was stored (4±2°C, 28d).

Control samples (M2) were made from whole milk powder undergoing the same procedure as above without transglutaminase treatment. To evaluate the quality of experimental samples (M1), fresh cheese products made from raw milk with and without enzyme treatment (M3 and M4) were also prepared following the above-mentioned process of preparation. During the storage period of 28 days, the quality properties (whey separation, titratable acidity, total LAB count, and sensory evaluation) of the samples were determined.

2.3. Determination of cheese hardness

Cheese hardness was measured by a CT3 Texture Analyzer (Ametek Brookfield, America). Parameters for measurement were: (a) a cylinder force (TA-AACC36) with diameter of 3.6 cm; (b) test speed of 3.0 mm/s; (c) pretest speed of 2.0 mm/s; recovery time of 5.0 s; Trigger load of 5.0 g and target distance of 8.0 mm (Baraka, 2015; Benjamin et al., 2018).

2.4. The yield of fresh cheese production

The yield (H, %) was determined by a formula:

$$H = \frac{m_1}{m_o} \times 100 \quad (1)$$

where m_1 was the weight of fresh cheese (g); m_o was the weight of reconstituted milk solution (g).

2.5. Protein content and total solid content

Protein content (%) and total solid content (%) were determined following procedures of ISO 13580:2005 and ISO 8968-1:2014, respectively.

2.6. Sensory evaluation

Sensory properties of cheese samples were evaluated by a panel of 7 deeply trained assessors consisting of 6 females and 1 male with the age of 20-21. The sensory test was taken according to the ISO 22935-3: 2009 with a scale of 0 to 5 points using commercial products as reference samples. Evaluated attributes were appearance, texture, and flavor. The appearance was judged by the color and smoothness of the cheeses. The texture was evaluated through hardness (defined as the force required to bite entirely through a sample placed between molar teeth) and firmness (defined as the amount of resistance to compression offered by a 1

cm thick slice of cheese when pushed between the thumb and the index finger until fingers touch each other) (Clark et al., 2009). The term "flavor" has many definitions but within this study, this term will be defined as the "impressions perceived via the chemical senses from a product in the mouth" (Clark et al., 2009). Sensory evaluation sessions were conducted in the individual booth under fluorescent light. The samples were coded with three random digit numbers and presented monadically. The testing room was cleaned without a strange odor.

2.7. Whey separation

Whey separation was determined according to a method by Dmytrów et al. (2010). Cheese samples (25g) were weighed and placed into zip-lock packages. The whey leached out of from samples at 25°C was weighted after 20 hours. Percentage of whey separation (Wh, %) was calculated by the formula:

$$Wh = \frac{m_1}{m_o} \times 100 \quad (2)$$

where m_1 was the weight of separated whey from the sample (g); m_o was the initial weight of the sample (g).

2.8. Microstructure observation

Scanning electron micrograph (SEM) was taken according to a method of Lobato-Calleros et al. (2001). Cylindrical cheese samples of 0.5cm in diameter by 0.5cm in height were fixed in 2% buffered glutaraldehyde (0.1M phosphate buffer, pH 7.2, 6h), and subsequently dehydrated in increasing concentrations of aqueous ethanol solutions (50, 60, 70, 80, 90 and 100%, 30 min per each one) and placed in pure acetone in 1 hour. Samples were then dried in a vacuum dryer (50°C, 50mmHg, 6h). The cheese samples were mounted on a stub and coated with a thin layer of gold in a Fine Coat Ion Sputter JFC 1100 (Jeol Ltd., Akishima, Japan) before taking a photograph.

2.9. Fat content (FC), titratable acidity (TA), and total lactic acid bacteria count

Fat content (%) and titratable acidity (mM NaOH per 100g of product) of cheese samples were determined in accordance with the ISO 1736:2008/IDF 9:2008 and ISO 11869:2012, respectively. In addition, total counts of lactic acid bacteria (CFU/mL) were determined in accordance with ISO 15214:1998.

2.10. Color space measurements

The color space for the fresh cheese samples was determined by a CR-400 chroma meter (Minolta, Japan) according to Mokrzycki and Tatol (2011). The color parameters of MTGase-treated samples were compared with those of the non-enzyme treated cheese. The L, a, and b values represent white/black, red/green, and yellow/blue, respectively. The difference in color (ΔE) was calculated using Eq. 3:

$$\Delta E = \sqrt{((L_i - L_0)^2 + (a_i - a_0)^2 + (b_i - b_0)^2)} \quad (3)$$

where, (L_o, a_o, b_o) and (L_i, a_i, b_i) were color parameters of the non-enzyme treated cheese, and the enzyme-treated samples, respectively. Based on the ΔE value, the difference in color between the samples was expressed as $0<\Delta E<1$ (the observer did not notice the difference in color); $1<\Delta E<2$ (only experienced observers were able to notice the difference in color); $2<\Delta E<3.5$ (inexperienced observers might notice color differences); and $\Delta E>3.5$ (there was a clear color difference between the two samples).

2.11. Response surface methodology

A response surface methodology (RSM) was used to determine the optimum parameters of the enzyme treatment process including enzyme concentration, reaction temperature, and time-based on the objective functions (hardness, yield, protein content, total solid content, and sensory evaluation score of fresh cheeses). The levels of input factors were determined via literature overview and primarily experiment results (Table 1).

According to a central composite design (CCD)–an RSM–described by Box and Wilson (NIST, 2012) the number of experiments was found equaled 18 (8-factor points, 6 axial points, and 4 central points). A combination of these 18 experiments with variations of the input variables was designed following Table 2.

Table 1: Levels of variables

Variable	Levels of variables				
	-α (-1.414)	-1	0	+1	+α (1.414)
x_1, MTGase concentration, (U/g protein)	0.10	0.60	1.80	3.00	3.50
x_2, Reaction temperature (°C)	23.80	30.00	45.00	60.00	66.20
x_3, Reaction time (h)	0.57	1.50	3.75	6.00	6.93

Eighteen (18) fresh cheese samples were done, the values of objective functions (experimental response) were measured (Table 2). On the obtained data, a regression analysis of responses was performed and fitted into an empirical second-order polynomial model (NIST, 2012):

$$Y = b_0 + \sum_{j=1}^{k} b_j x_j + \sum_{j,i=1;i\neq j}^{k} b_{ji} x_j x_i + \sum_{j=1}^{k} b_{jj}\left(x_j^2 - \lambda\right) \quad (4)$$

where, Y–the predicted response, b_0–the model constant, b_j–the coefficients of the linear effects, b_{ji}–the coefficients of interaction between the factors, b_{jj}–the coefficients of the quadratic effects, x_i, x_j–the independent actual variables, λ–coefficient, k–the number of variables considered. The coefficients of the regression models (b_0, b_j, b_{ji}, b_{jj}) were obtained if the p-value≤0.05, R^2 >0.9, lack-of-fit>0.05.

The Design-Expert software program (version 11.10.1) was used for statistical calculation of the second-order polynomial equations from experimental data (p<0.05). Experimental data were analyzed statistically by one-way ANOVA (p<0.05) with the Minitab (version 16) software program.

Table 2: CCD with the independent variables and their experimental responses (n=3)

Run test	Variable			Experimental response*				
	x_1	x_2	x_3	Cheese hardness, g (Y_1)	Yield, % (Y_2)	Protein content, % (Y_3)	Total solid content, % (Y_4)	Sensory evaluation score (Y_5)
1	0.60	30.00	1.50	139.17 ± 13.87^{a}	21.87 ± 0.57^{b}	12.10 ± 0.20^{ab}	28.20 ± 0.26^{b}	9.89 ± 1.69^{abcd}
2	3.00	30.00	1.50	136.67 ± 18.58^{a}	25.28 ± 0.48^{d}	12.12 ± 0.39^{abc}	29.61 ± 0.23^{de}	11.11 ± 1.54^{de}
3	0.60	60.00	1.50	335.00 ± 16.58^{c}	20.27 ± 0.49^{a}	12.60 ± 0.41^{bcd}	25.63 ± 0.58^{a}	9.11 ± 1.54^{abc}
4	3.00	60.00	1.50	296.83 ± 31.85^{bc}	25.50 ± 0.53^{d}	12.50 ± 0.42^{abcd}	29.71 ± 0.31^{de}	8.44 ± 1.42^{a}
5	0.60	30.00	6.00	332.00 ± 22.85^{c}	23.97 ± 0.67^{c}	12.21 ± 0.22^{abc}	29.25 ± 0.40^{cd}	10.11 ± 1.9^{bcde}
6	3.00	30.00	6.00	247.17 ± 25.90^{b}	26.54 ± 0.31^{f}	13.14 ± 0.39^{efg}	31.85 ± 0.22^{g}	10.44 ± 1.42^{cde}
7	0.60	60.00	6.00	462.33 ± 84.03^{e}	22.77 ± 0.25^{b}	12.31 ± 0.27^{abc}	28.10 ± 0.37^{b}	8.89 ± 1.27^{ab}
8	3.00	60.00	6.00	323.17 ± 35.04^{c}	23.76 ± 0.70^{c}	12.89 ± 0.13^{de}	29.11 ± 0.27^{c}	10.89 ± 1.62^{de}
9	0.10	45.00	3.75	487.83 ± 45.18^{f}	22.50 ± 0.87^{b}	12.32 ± 0.18^{abc}	27.78 ± 0.19^{b}	9.89 ± 0.78^{abcd}
10	3.50	45.00	3.75	240.00 ± 44.86^{b}	26.38 ± 0.71^{ef}	12.64 ± 0.32^{cd}	31.65 ± 0.18^{g}	12.56 ± 0.88^{fg}
11	1.80	23.80	3.75	118.50 ± 12.29^{a}	25.28 ± 0.17^{d}	12.57 ± 0.28^{bcd}	30.74 ± 0.26^{f}	10.44 ± 1.33^{cde}
12	1.80	66.20	3.75	353.83 ± 15.18^{cd}	22.67 ± 0.70^{b}	13.66 ± 0.17^{h}	28.22 ± 0.20^{b}	9.89 ± 1.05^{abcd}
13	1.80	45.00	0.57	436.67 ± 18.06^{ef}	24.56 ± 0.58^{cd}	12.02 ± 0.07^{a}	29.81 ± 0.12^{e}	11.56 ± 1.74^{ef}
14	1.80	45.00	6.93	413.00 ± 54.21^{de}	26.57 ± 0.23f	13.01 ± 0.22^{def}	31.79 ± 0.36^{g}	10.78 ± 1.30^{df}
15	1.80	45.00	3.75	615.00 ± 32.25^{g}	27.35 ± 0.42^{f}	13.73 ± 0.21^{h}	33.78 ± 0.21^{i}	13.44 ± 0.73^{g}
16	1.80	45.00	3.75	705.67 ± 63.29^{h}	26.55 ± 0.22^{f}	13.62 ± 0.09^{gh}	33.85 ± 0.21^{i}	12.67 ± 1.58^{fg}
17	1.80	45.00	3.75	699.50 ± 56.33^{h}	27.15 ± 0.19^{f}	13.46 ± 0.42^{fgh}	33.12 ± 0.08^{h}	13.33 ± 1.50^{g}
18	1.80	45.00	3.75	809.17 ± 61.83^{i}	27.07 ± 1.05^{f}	13.49 ± 0.09^{fgh}	33.42 ± 0.26^{hi}	13.11 ± 1.27^{g}

*Superscripts in each column indicated the significant differences (p<0.05)

3. Results and discussions

3.1. Response surface methodology

Eighteen cheese samples with input variables (x_1, x_2, x_3) (Table 2) were performed following the procedure described in section 2. The quality parameters of the fresh cheese products (objective functions including Y_1–hardness, Y_2–yield, Y_3–protein content, Y_4–total solid content, and Y_5–sensory evaluation score) were practically analyzed. The mean value±standard deviation (n=3) is shown in Table 2.

Experimental responses (Table 2) were entered in the CCD model of the Design-Expert software program (version 11.10.1). Analytical results showed that the MTGase concentration (x_1, U/g protein), reaction temperature (x_2, °C) and reaction time (x_3, h) were correlated with the objective functions (Y_1–hardness, Y_2–yield, Y_3–protein content, Y_4–total solid content, and Y_5–sensory evaluation score of fresh cheese). This correlation was shown in regression equations (5-9). Regression models in terms of actual factors:

$$Y_1 = -2042.72 + 85.81x_2 - 90.02x_1^2 - 0.86x_2^2 - 19.68x_3^2 \quad (5)$$

$$Y_2 = 5.53 + 5.04x_1 + 0.56x_2 + 2.07x_3 - 0.24x_1x_3 - 0.83x_1^2 - 0.01x_2^2 - 0.13x_3^2 \quad (6)$$

$$Y_3 = 8.18 + 1.19x_1 + 0.10x_2 + 0.82x_3 + 0.07x_1x_3 - 0.33x_1^2 - 0.09x_3^2 \quad (7)$$

$$Y_4 = 8.96 + 5.18x_1 + 0.67x_1 + 2.37x_3 - 1.16x_1^2 - 0.01x_2^2 - 0.22x_3^2 \quad (8)$$

$$Y_5 = -2.04 + 2.48x_1 - 0.63x_1^2 - 0.01x_2^2 - 0.18x_3^2 \quad (9)$$

Obviously, all the above equations showed the correlation between variables and responses (equations 5-9). With the exception of the interaction between x_1 (MTGase concentration) and x_3 (reaction time) on Y_2 (yield) and Y_3 (protein content), all variables did not interact with each other on responses. The polynomials well explained response variations (R^2>0.9, p-value<0.003, and lack-of-fit>0.08). The predicted values are close to the experimental values for all response functions (R^2>0.9, data not shown). These correlations demonstrated that the models were adequate in reflecting the expected optimization.

The results of one-objectively optimization (Fig. 1, Table 3) predicted that there was no defined condition to satisfy all five response functions. Specifically, the maxima of yield (Y_2), protein content (Y_3), total solid content (Y_4), sensory evaluation score (Y_5) were reached at ranges of MTGase concentration (x_1, from 2.01 to 2.71), temperature (x_2, from 41.54 to 52.18°C), reaction time (x_3, from 3.61 to 4.37 h).

The prediction of one set of five response functions (5-9) was also done by using the desirability function with a scale of 0 to 1, where 0 represents a completely undesirable response, and 1 represents the most desirable response. The results of multi-objectively optimization were statistically defined with desirability of 0.913. At this desirability the fresh cheese made under enzyme treatment condition (MTGase concentration (x_1), reaction temperature (x_2) and enzyme reaction time (x_3)

were 2.59U/g protein, 36.14°C, and 4.53h, respectively) can reach the optimized values (Table 4). In fact, the maximum hardness (Y_1) of our cheese (480.5g) was almost the same as that of commercial reference samples (tvorog "Savushkin Khutorok"–a Belarus fresh cheese). Furthermore, following the above multi-objectively optimized variables, a practical experiment was done to verify the predicted values (Table 4). Obviously, the verified and predicted values were insignificant differences. As a result, the RSM model was fitted to practical results.

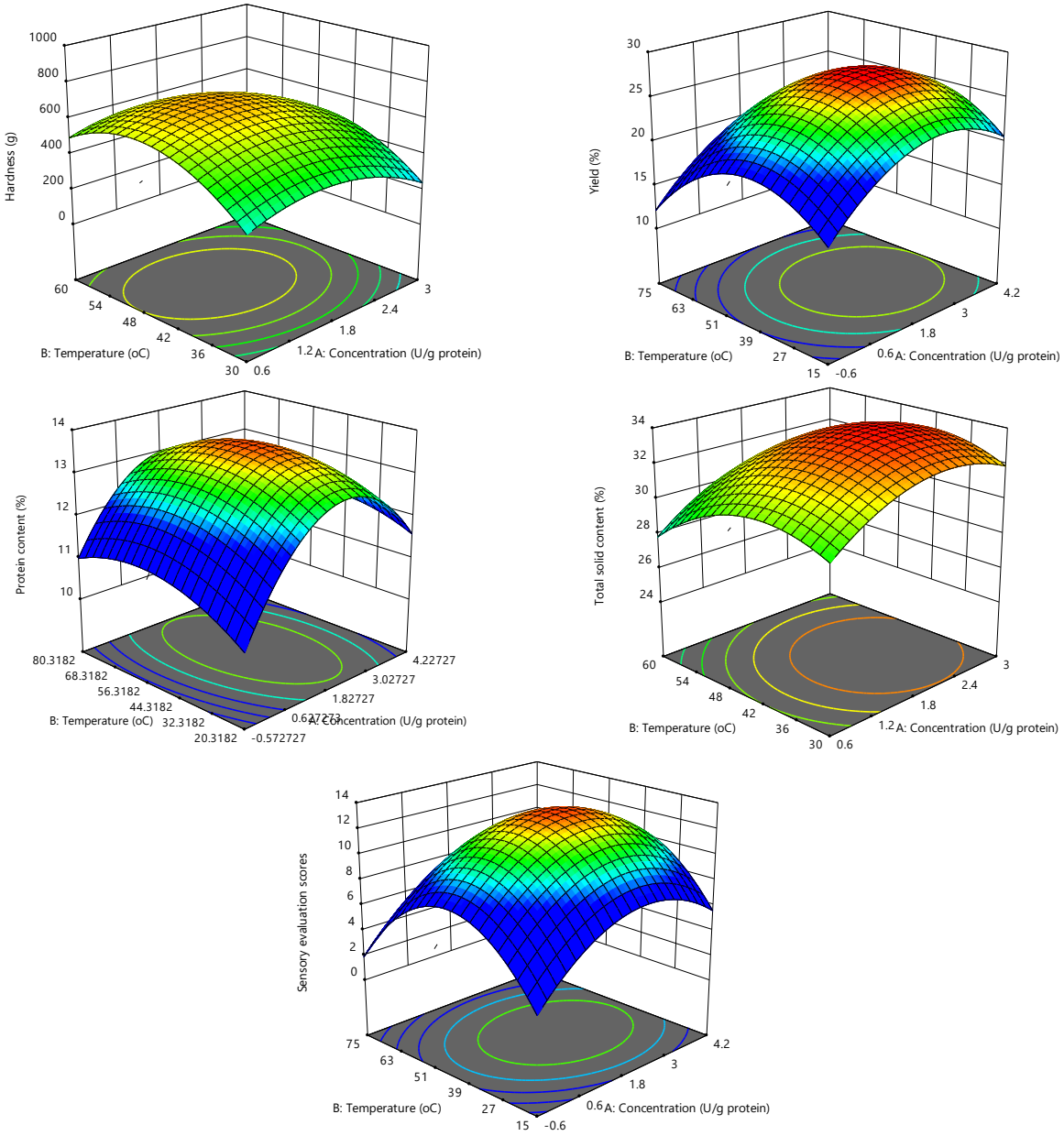

Fig. 1: Response surface and contour plots of objective functions

Table 3: The solutions for each function of responses

Functions	Optimized value	Variables		
		x_1, U/g protein	x_2, °C	x_3, h
Y_1 – Hardness, g	480.499	2.94	41.53	3.30
Y_2 – Yield, %	27.465	2.71	42.53	3.61
Y_3 – Protein content, %	13.610	2.01	52.18	4.22
Y_4 – Total solid content, %	33.776	2.20	41.54	4.37
Y_5 – Sensory evaluation score	13.223	2.18	42.86	3.84

Table 4: The multi-objectively optimized responses of experimentally verified samples

	Hardness (Y_1), g	Yield (Y_2), %	Protein content (Y_3), %	Total solid content (Y_4), %	Sensory evaluation score (Y_5)
Optimized (predicted) values	480.500	27.396	13.372	33.341	12.721
Verified values	471 ± 14.91	26.93 ± 0.62	13.76 ± 0.40	32.78 ± 0.70	11.81 ± 1.01

3.2. Influence of MTGase on quality properties of fresh cheese

Fresh cheese samples were produced under the multi-objectively optimized condition of MTGase treatment. Practically, the protein, lipid, and total solid contents (%) of this cheese were 13.76, 16.00, and 33.20, respectively. As a result, whole milk powder (raw material) caused the high-fat content of the cheese.

To characterize the influence of MTGase on the quality properties of fresh cheese, four samples under optimized conditions were done. Two of the samples were made from whole milk powder with (M1) and without MTGase (M2). The others were made from raw milk with (M3) and without MTGase (M4). The microstructure properties of the samples were determined (Fig. 2).

Obviously, the microstructural components of M1 were smaller than those of M2 products (Fig. 2). It seems that M1 had a more porous structure than M2 cheese. Interestingly, similar phenomena have been found in cheese made from fresh milk (M3 and M4). Changes in the microstructure of fresh cheese manufactured with MTGase could be explained by the creation of cross-linking between protein molecules. In the enzyme-free samples, the components were separated by large gaps, whilst the microstructure of the enzyme-treated gel was more homogeneous. Thus, MTGase-treated cheese contained not only a collection of small components linked together but also smoother networks with smaller constituent chains and gaps. Basically, cross-linking with MTGase helps prevent the separation of phases. Actually, this phenomenon was similar to research by Schorsch et al. (2000). In this study, fresh cheese samples (M3, M4, which were made from fresh milk), had less tight structure, smaller particle sizes, and fewer pores than those made from whole milk powder (M1, M2).

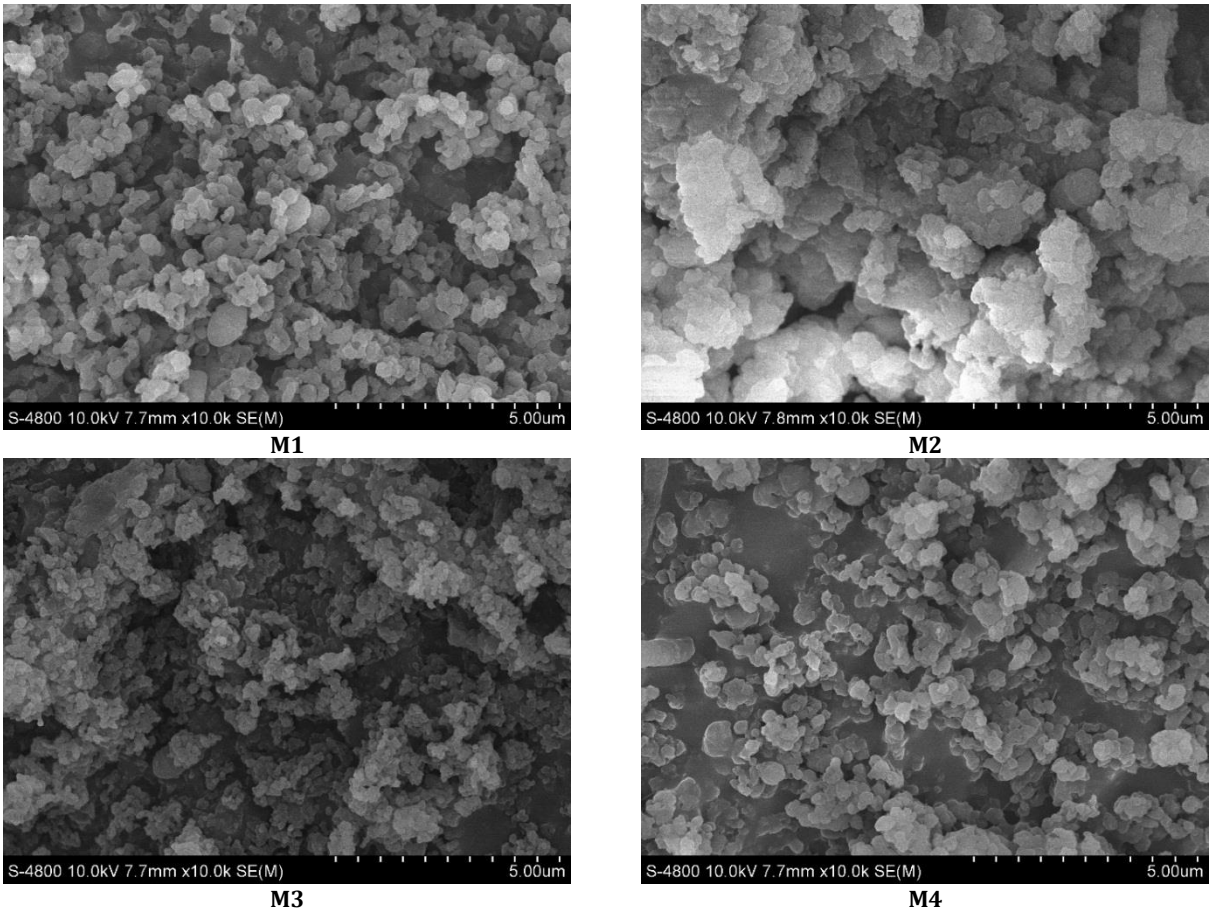

Fig. 2: SEM images of cheese samples

3.3. Sensory evaluation

Fig. 3 showed sensory evaluation scores of fresh cheeses (M1-M4) after 1, 7, 14, 21, and 28 days of storage time. In general, changes in the sensory properties of all product models are generally similar. The appearance characteristic of all cheeses did not change significantly during 28 days of storage. The scores of texture and flavor properties decreased significantly after three weeks of storage. Texture evaluation results showed that MTGase-fortified fresh cheeses made from whole milk powder (M1) and from fresh milk (M3) had a firmer structure after 21 days of storage. The increase in the perception of hardness can be explained by the fact that cross-linking is still possible and moisture is lost during cold storage in whey separation. In addition, fresh cheeses without enzyme treatment

have also been shown to have increased hardness during storage but to a lesser extent. A similar result in the study of Özer et al. (2013) showed an increase in the hardness of white-brined cheese using transglutaminase during the first 30 days of storage.

The sensory evaluation score of the flavor properties (Fig. 3) showed that taste of enzyme-treated fresh cheese samples made from whole milk powder (M1) and fresh milk (M3) was better than that of enzyme-free products (M2, M4) during storage. This could be explained by the fact that, during storage, the acidification of cheese samples resulted in increased titratable acidity. Apparently, the increase in sour taste reduced the acceptance of assessors.

3.4. Whey separation

Whey separation (%) of samples during the storage period were illustrated in Table 5. Analytical results showed that the whey separation increased with storage time for all samples. MTGase-treated cheese (M1, M3) had less whey separation (around 4% after 28 days of storage) than that of the others (M2, M4). Interestingly, cheese samples made from milk powder tend to have a lower water-holding capacity than those made from raw milk.

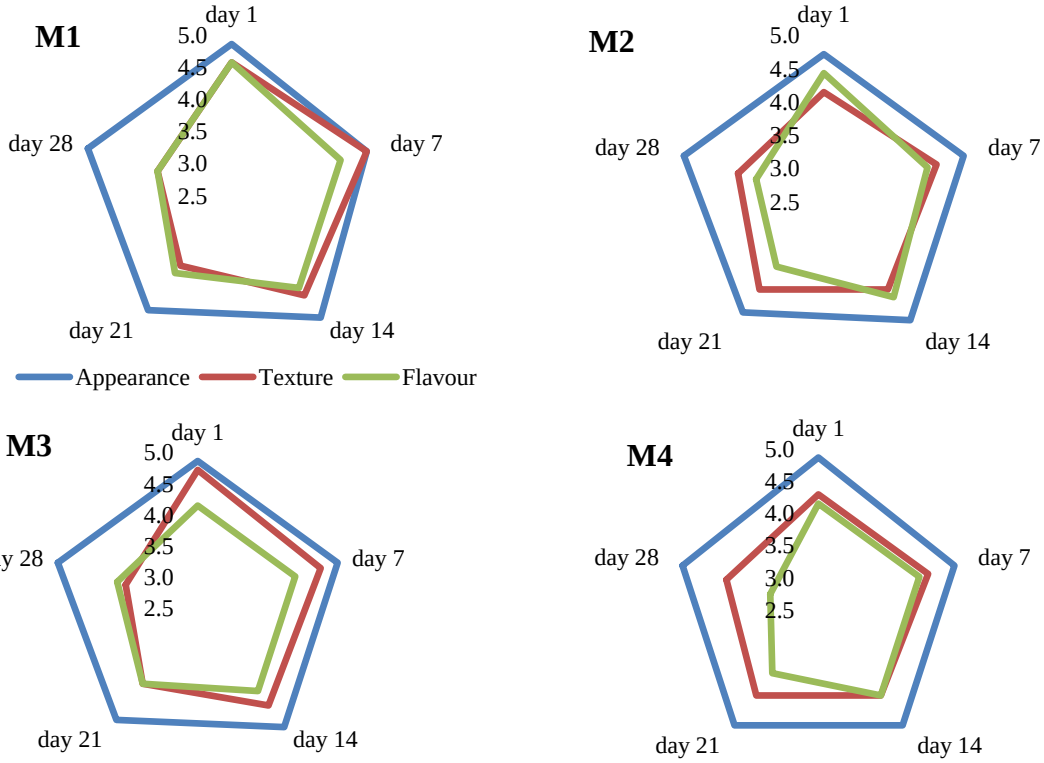

Fig. 3: Change in the sensory evaluation score of fresh cheese samples during storage time

Table 5: Whey separation and titratable acidity of samples during storage at 4±2 ℃

	Sample	Whey separation, %				
		1st day	7th day	14th day	21st day	28th day
Whey separation, %	M1	0	0.95± 0.1^{b}	2.33± 0.07^{c}	3.86± 0.34^{d}	4.27± 0.28^{e}
	M2	0.89± 0.11^{a}	2.70± 0.1^{b}	6.90±0.05^{c}	8.26±0.48^{de}	8.70±0.26^{de}
	M3	0	0.82±0.13^{b}	1.89±0.08^{c}	3.21±0.56^{d}	3.86±0.29^{e}
	M4	0	1.15±0.12^{b}	6.59±0,26^{c}	7.56±0.81^{de}	7.88±0.38^{de}
Titratable acidity (mmol NaOH/100g)	M1	11.76 ± 0.16^{a}	10.52 ± 0.38^{b}	11.67 ± 0.13^{a}	11.74 ± 0.19^{a}	12.95 ± 0.62^{c}
	M2	11.97 ± 0.54^{a}	12.10 ± 0.4^{a}	12.45 ± 0.32^{a}	13.00 ± 0.21^{bc}	13.30 ± 0.19^{c}
	M3	13.67 ± 0.60^{a}	12.81 ± 0.48^{b}	13.87 ± 0.05^{a}	14.80 ± 0.27^{c}	15.58 ± 0.60^{c}
	M4	14.68 ± 0.27^{a}	15.22 ± 0.17^{a}	15.27 ± 0.15^{a}	16.32 ± 0.28^{b}	17.48 ± 0.61^{c}

*Superscripts in each row indicate the significant differences ($p < 0.05$)

During storage, whey separation of cheese was the result of dense aggregates. Because the molecules of casein were extremely flexible, even if they were denatured. It tended to form more compact micelle structures that would lead to whey separation. The dense aggregates were spontaneous and interpreted as gel contraction without applying any external force, resulting in the reorganization of the gel network and the separation of whey (Han et al., 2002). However, the cross-linked molecules formed by MTGase have increased the stability of the gel network and water-holding capacity. As reported by Chen et al. (2019), due to its high flexibility, simple structure, and ease to be cross-linked by MTGase, the gel network structure was formed mainly by casein micelles. They could therefore minimize the whey separation of the products. Han et al. (2002) showed similar results in the case of

MTGase-treated cream cheese. Furthermore, casein micelles covalently bound by MTGase were not disassociated during acidification. This inhibits the rearrangement of these molecules, which leads to the minimization of whey separation.

3.5. Titratable acidity

The change of titratable acidity (TA) of samples is demonstrated in Table 5. The analytical results showed that the TA of the samples made from milk powder (M1, M2) was lower than that of the others made from fresh milk (M3, M4) during the storage period. TA of cheese samples without enzyme treatment (M2, M4) remained constant after the first 2 weeks of storage. Enzyme-treated fresh cheese (M1, M3) had a slight decrease in TA values during the first week of storage. Actually, this reflected the formation of isopeptide bonds between γ-carboxamide groups (-(C=O)NH_2) of glutamine residue side chains and the ε-amino groups (-NH_2) of lysine residue side chains with subsequent release of ammonia (NH_3). After one week, the TA of these samples was increased. Practically, the TA of M3 tends to increase faster than that of M1 samples. TA of all products increased after 28 days of storage. The increase of TA during storage can be explained by the fermentation of lactic acid even under cold storage (2-4°C). These results were found during quality evaluation of Tvorog samples with and without MTGase treatment in the storage period (Dmytrów et al., 2010). Furthermore, Yerlikaya and Özer (2014) reported these changes in the TA of fresh cheese fermented by *Str. thermophilus* during 28 days of storage. These previous studies have pointed out that an increase in the enzyme concentration could lead to a delay in the reproduction of the bacteria and could lead to a slower increase in acidity.

3.6. Total lactic acid bacteria count

The change in the total number of lactic acid bacteria in fresh cheese samples during storage is shown in Fig. 4. The results showed that the bacterial count of fresh cheese samples decreased significantly during storage (from 8.3lg (CFU/g) to 7.38lg (CFU/g). Thus, it can be seen that during storage, the number of bacteria is always higher than 10^7CFU/g, and therefore conforms to CODEX STAN 243-2003 revised 2010. Fig. 5 shows a change of *b* value in storage time and Table 6 shows the change in color of fresh cheese products (CODEX STAN, 2010).

The study results (Fig. 4) also showed that the total lactic acid bacteria count in the M2 and M4 samples decreased rapidly after the first week of storage. The number of lactic acid bacteria observed in fresh cheese samples made from raw milk (M3, M4) was always higher than that of samples made from milk powder (M1, M2). In the early days of storage, the number of lactic bacteria in enzyme-treated cheese samples (M1, M3) was lower than that of enzyme-free samples (M2, M4). According to Özer et al. (2007), there was no toxic effect of MTGase on yogurt bacteria. The only possible effect is the delayed growth of lactic acid bacteria due to the low molecular weight peptides and/or amino acids needed for *Str. thermophilus* was cross-linked to MTGase and became a part not available for *Str. thermophilus.* Our results are similar to studies of Ramdhani and Setiadi (2019).

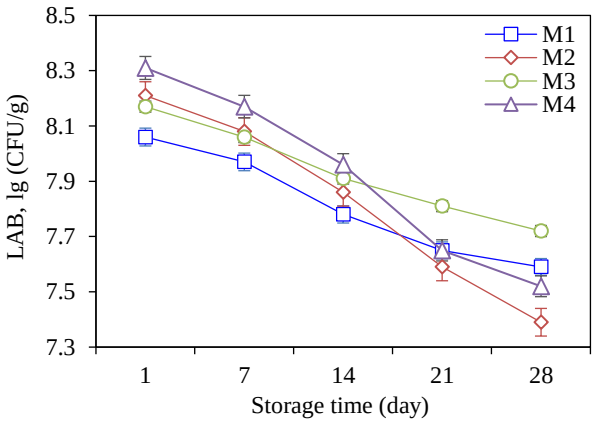

Fig. 4: Change of total lactic acid bacteria count in storage time

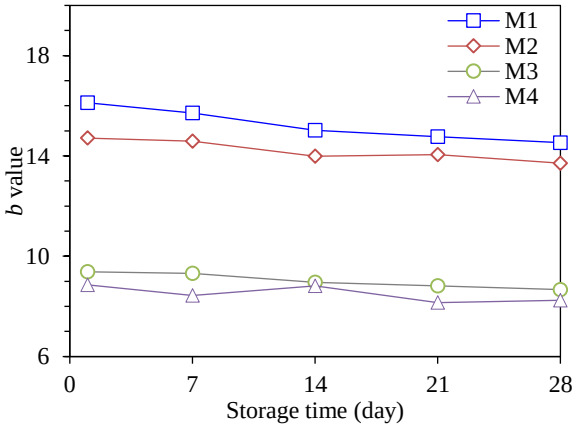

Fig. 5: Change of *b* value in storage time

The results of these studies showed that, after 2 weeks storage period, the number of lactic acid bacteria in the samples non-treated with transglutaminase was relatively higher than the MTGase-treated samples. Subsequently, the number of lactic bacteria in enzyme-untreated samples was lower than the MTGase-treated samples. The authors suggested that the addition of enzymes to milk would cause imbalances in the growth of lactic bacteria by a cross-linking reaction.

3.7. Color space measurements

The color space of samples were presented in Table 6 and Fig. 5. The analytical results showed that the color differences between the enzyme-treated samples (M1, M3) and the non-treated samples (M2, M4) were small (ΔE<1.5). Thus, MTGase treatment has little effect on the color of fresh cheeses. Additionally, the *b* values are greater than zero,

which means that the products tend to be yellow in color. Table 6 showed that cheese (made from whole milk powder) was more yellow than the others (made from fresh milk). This difference may be due to the original color of raw materials. Milk powder was darker in color due to its high-temperature drying process.

Table 6: The change in color of fresh cheese products

Day	Value	Sample			
		M1	M2	M3	M4
1st	*L*	90.31 ± 0.22^{d}	90.25 ± 0.15^{c}	92.06 ± 0.31^{c}	92.24 ± 0.40^{c}
	a	-1.64 ± 0.22^{a}	-1.74 ± 0.01^{a}	-0.85 ± 0.10^{a}	-1.04 ± 0.06^{a}
	b	16.12 ± 0.04^{b}	14.71 ± 0.35^{c}	9.38 ± 0.26^{b}	8.86 ± 0.06^{c}
	ΔE	0	1.41	0	0.58
7th	*L*	89.88 ± 0.25^{cd}	90.07 ± 0.12^{c}	91.88 ± 0.25^{c}	92.17 ± 0.55^{c}
	a	-1.48 ± 0.07^{a}	-1.46 ± 0.05^{b}	-0.80 ± 0.02^{a}	-0.87 ± 0.06^{b}
	b	15.71 ± 0.21^{b}	14.59 ± 0.39^{bc}	9.32 ± 0.20^{b}	8.43 ± 0.08^{b}
	ΔE	0	1.14	0	0.93
14th	*L*	89.29 ± 0.97^{bc}	88.76 ± 0.06^{b}	90.54 ± 0.31^{b}	90.51 ± 0.11^{b}
	a	-0.94 ± 0.05^{b}	-1.44 ± 0.04^{b}	-0.74 ± 0.03^{ab}	-0.83 ± 0.02^{b}
	b	15.02 ± 0.42^{a}	13.99 ± 0.21^{ab}	8.96 ± 0.05^{a}	8.82 ± 0.08^{c}
	ΔE	0	1.26	0	0.17
21st	*L*	88.73 ± 0.34^{ab}	88.03 ± 0.33^{a}	90.35 ± 0.15^{ab}	89.63 ± 0.05^{a}
	a	-0.87 ± 0.02^{b}	-0.93 ± 0.03^{c}	-0.67 ± 0.04^{bc}	-0.67 ± 0.06^{c}
	b	14.76 ± 0.42^{a}	14.05 ± 0.36^{ab}	8.82 ± 0.06^{a}	8.15 ± 0.11^{a}
	ΔE	0	0.99	0	0.98
28th	*L*	87.89 ± 0.35^{a}	87.93 ± 0.20^{a}	90.10 ± 0.09^{a}	89.25 ± 0.31^{a}
	a	-0.80 ± 0.08^{b}	-0.90 ± 0.02^{c}	-0.59 ± 0.09^{c}	-0.65 ± 0.03^{c}
	b	14.53 ± 0.07^{a}	13.71 ± 0.42^{a}	8.67 ± 0.32^{a}	8.25 ± 0.11^{a}
	ΔE	0	0.82	0	0.96

*Superscripts in each column indicate the significant differences ($p < 0.05$)

4. Conclusion

On the strength of this study, the application of the response surface methodology was effective in optimizing the parameters for the microbial transglutaminase treatment in fresh cheese products made from whole milk powder. The enzymatic treatment under optimized conditions (MTGase concentration of 2.59U/g protein at 36.14°C and enzyme treatment time was 4.53 hours) improved product recovery efficiency, protein content, and sensory evaluation of products due to the high-molecular-weight polymers formed during the cross-linking reaction.

SEM images also showed that the samples undergoing with MTGase treatment had a more homogeneous microstructure network with smaller elements than those of the fresh cheese samples without MTGase. The results of the quality properties analysis showed that the quality of fresh cheese samples made from whole milk powder was within the allowed level. The application of researching technology to produce fresh cheese using MTGase may limit the use of other additives such as stabilizers, thickeners, gelatin, agar, etc.

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Compliance with ethical standards

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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