

## Effect of Hydrolyzed Lima Protein (*Phaseolus Lunatus*) on The Quality of Yogurt Products

Pham Thi Hoan<sup>1\*</sup>, Phan Ngoc Thuy Tien<sup>2</sup>, Duong Tran Minh Tran<sup>3</sup>

<sup>1</sup> Ho Chi Minh City University of Technology and Education, Vietnam

<sup>2</sup> Tri Viet Phat PRO CO., LTD

<sup>3</sup> Suntory Pepsico Vietnam Beverage

\*Corresponding author. Email: [hoanpt@hcmute.edu.vn](mailto:hoanpt@hcmute.edu.vn)

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### ABSTRACT

This study was conducted to evaluate the quality of yogurt with partial replacement of hydrolyzed Lima protein (*Phaseolus lunatus*). The hydrolysis of phaseolus protein by Alcalase at the rate of 0.24 AU/g protein, at 50 °C, shaking speed of 150 rpm for 1 hour was used to receive the hydrolyzed protein. The analytical results showed that the hydrolyzed protein content had a great influence on the whey separation of yogurt. The higher the hydrolyzed protein content (0% - 2%), the higher the whey separation (39.12% - 46.6%). Besides, hydrolyzed protein also contributed to shortening yogurt fermentation duration. The results of rheological analysis of yogurt samples showed that the yogurts partially replaced with hydrolyzed Lima protein had higher viscosity and shear stress than the reference sample 1 (Ref1 - without protein replacement). Sensory evaluation results showed that yogurt samples replacing 1% hydrolyzed protein (YP1) were preferred over other replacement samples. Microbial transglutaminase (MTGase) of 1.5 IU/g protein formed a cross-link between protein molecules that improved the water holding capacity of yogurt better than the Ref1. Product YP1 has chemical composition and microbiological criteria that meet the standards of TCVN 7030:2009 (Codex stan 243-2003) about fermented milk. The technological process of the researched yogurt production is fully applicable at pilot and industrial scales.

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## 1. Introduction

Nowadays, with the development of society, people have easy access to different sources of information. In particular, people's awareness of nutrition in food is increasing. Consumers have higher requirements for technological food products. Moreover, it is always necessary to develop products and create diversity in categories. In Vietnam, the demand for milk and dairy products is increasing. According to Euromonitor's statistics [1], Vietnam's consumption of milk and dairy products reached 1.76 million tons (8.6% increase) in 2020. In which, the highest growth industries include drinking milk (+ 10%), yogurt (+12%), cheese (+11%), butter (+10%), and other dairy products (+8%). In general, consumers of dairy products, especially fermented milk, are increasingly demanding quality products, with health and sensory-friendly features. Desired sensory attributes include characteristics such as appearance, texture, color, taste and odor. However, syneresis is considered a primary disadvantage related to the sensory appeal of yogurt. Syneresis is a consequence of shrinking milk protein gel, which decreases the size of casein aggregates promoting whey separation. This phenomenon often occurs during the storage of yogurt in the refrigerator and is considered a technical error [2]. Some effective methods to reduce water separation for yogurt products are the use of additives, polymers, such as pectin, gelatin, carrageenan, protein... In addition, MTGase can be used as an enzyme to create cross-links between the protein molecules to improve whey separation in this group of products [3].

Legumes are a rich plant source of protein, with an almost complete amino acid profile, e.g. soya protein... Beside soy beans, Lima beans (*Phaseolus lunatus*) are a natural source of plant-based protein with a wide variety of essential amino acids [2], [3]. Lima protein contains the amino acids

phenylalanine and tyrosine (9.3g/100g protein) three times higher than soybean (3.28g/100g protein) [5]. With the desire to create a new line of yogurt products enriched with protein content, especially plant protein sources. The Lima protein was chosen to enrich this product line. However, legume proteins have low solubility and allergenic or anti-nutritional properties, as well as slow digestibility and assimilation [6]. The solution proposed to overcome the above drawback is the enzymatic hydrolysis of protein before partially replacing milk material in the yogurt production process. Hydrolysis offers many potential applications for plant proteins in food in order to adjust the appropriate physico-chemical and sensory properties without producing negative effects on nutritional quality or function. Hydrolysis may result in peptides with high biological activity, but it can also produce molecules with a smaller mass, which weakens the protein network, thereby reducing water retention [7].

Therefore, this study was conducted to evaluate the possibility of creating a yogurt product that partially replaces hydrolyzed Lima protein with milk material; also, with the use of MTGase, to evaluate the improvement of the water holding capacity of the yogurt product.

## 2. Materials and Methods

### 2.1. Materials

Whole milk powder originated in New Zealand was purchased from Dai Tan Viet Company at 145 Ton That Dam, Ben Nghe Ward, District 1, Ho Chi Minh City, Vietnam. Certificate of analysis of the product shows that whole milk powder has protein, fat and moisture contents of 24.29%, 26.23% and 3.19%, respectively. Total yeasts and molds < 1 CFU/g, *Enterobacteriaceae* < 10 CFU/g. Phaseolus protein concentrate (PPC) was obtained from the Lima bean according to the method of Hoan et al., 2022 [8].

Alcalase (2.5 LPF) is a hydrolytic enzyme originating from the Danish Novozymes Group, purchased from Phuong Tram Chemical and Agricultural Products Trading Co., Ltd., at address 69A Bui Quang La Street, Ward 12, Go District Vap, Ho Chi Minh City. Enzyme activity reached 2.5 AU/g, brown color in liquid form. Microbial Transglutaminase (MTGase) was purchased from My Australia Science and Technology Development Joint Stock Company at 783/40, CMT8 Street, Tan Binh District, Ho Chi Minh City, Vietnam. MTGase has an activity of 106 U/g without *Salmonella* and *E. coli*, Coliforms < 30 CFU/g.

The starter culture used to ferment yogurt was a starter mixture of three strains: *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus fermentum* and *Streptococcus thermophilus* (>10<sup>9</sup> CFU/g) which was purchased from OOO company “Vivo Induction”, 121087, Moscow, Russia.

### 2.2. Yogurt preparation

Yogurt was obtained following the flowchart (shown in Figure 1). First, PPC was hydrolyzed according to the method of Polanco-Lugo et al., 2014 [9] and Betancur-Ancona et al., 2009 [5], with some modifications. Lima protein powder was mixed with water at the ratio 2:100 (m/m), then Alcalase (0.24 AU/g protein) was added to the solution. The solution was hydrolyzed at 50 °C for 1 h, pH7.0, shaking speed was of 150 rpm. After hydrolyzation, the Alcalase was inactivated at 85 °C for 20 min (Nuñez-Aragón và cộng sự, 2019). Then, the yogurt production process was carried out. Accordingly, whole milk powder (15.46g) was reconstituted with water (84.54g, t = 50°C) in 30 minutes to obtain 100g of reconstituted milk solution (total solids were 15%) (Tetra Pak, 1995). The hydrolyzed protein solution was then replaced with milk powder in such proportions that the total dry matter content of the reconstituted solution was 15% (**Experiment 1**). Next, MTGase was added to the material mixture at the ratio of 0.5; 1.0; 1.5; 2 IU/g protein (**Experiment 2**), incubated at 40 °C for 2 h (Tarapatsky et al., 2019) to create cross-linking between proteins, thereby improving product structure. The milk mixture after incubation with MTGase was pasteurized at 85°C for 20 min (Johnson and Olson, 1985). Bacterial cultures were added into milk with a ratio of 0.03 % (w/w) at 42 ± 1 °C under aseptic conditions. Fermentation was carried out at 42 ± 1 °C until the pH reached 4.6 then stopped (Trejo et al., 2014). The final product (yogurt with protein - YP) was cooled and stored at 4-6 °C for 21 days (**Experiment 3**).

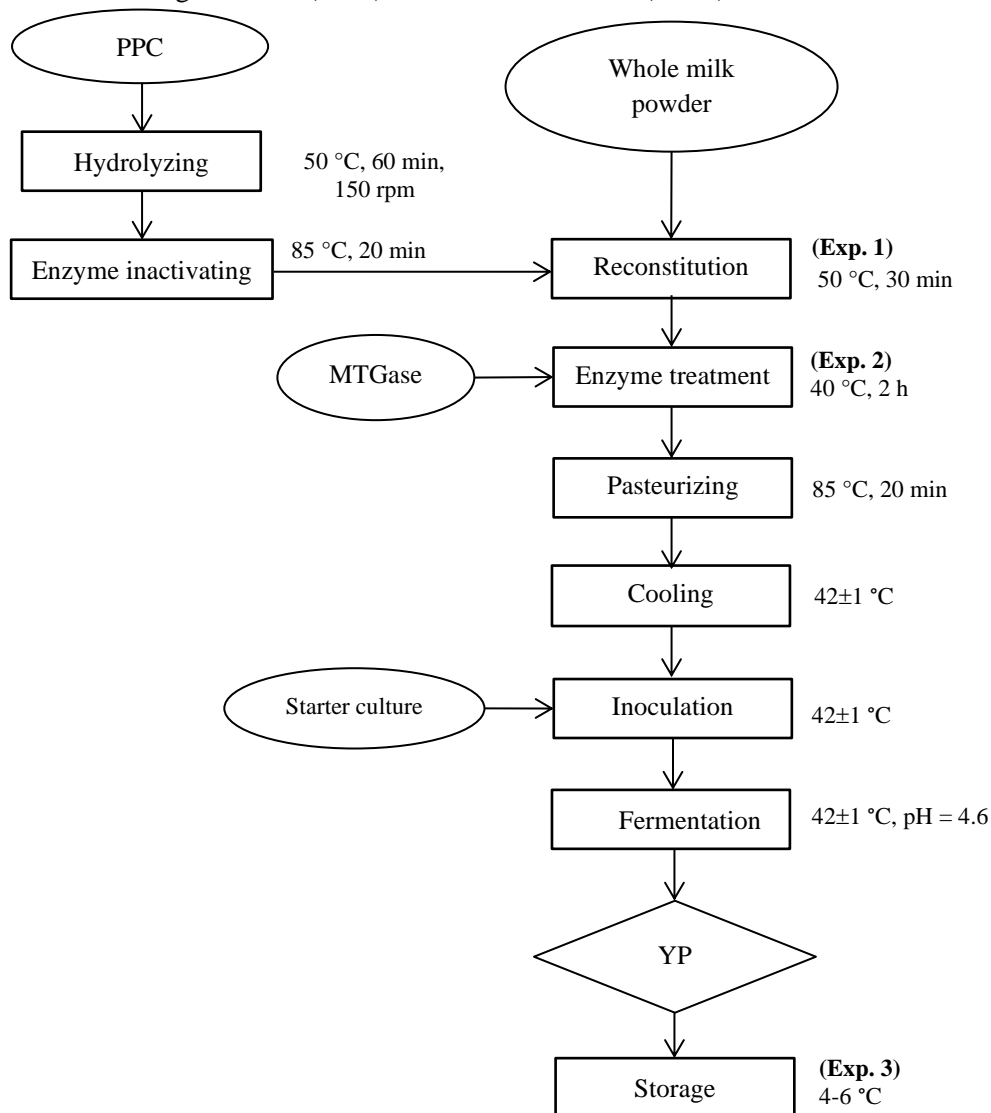
**Experiment 1:** Investigate the effect of substituted hydrolyzed PPC rates on the quality properties of fermented milk. Dry matter content in the mixture of milk and hydrolyzed PPC was of 15 %, MTGase

enzyme concentration was 1.5 (IU/g protein). Milk powder was reconstituted with different ratios (25%; 50%; 75%; 100%) of hydrolyzed protein solution and water. From there, we had the protein contents in reconstituted milk of 0.5%; 1%; 1.5% and 2 %, respectively. Corresponding to the coded samples in the list of acronyms: YP0.5; YP1; YP1.5; YP2.

**Experiment 2:** Investigate the effect of MTGase concentration on YPs products. The dry matter content in the milk mixture was 15 %, the hydrolyzed protein concentration of 1% with water was used to reconstitute milk powder. Then, MTGase was added to the mixture at concentrations of 0.5; 1; 1.5; 2 (IU/g protein). Corresponding to the coded samples in the list of acronyms: YE0.5; YE1; YE1.5; YE2.

**Experiment 3:** Investigate the change in quality criteria of yogurt products during storage. Yogurt samples were stored in glass jars, stored at temperature  $4 \pm 2$  °C in 21 days. Samples were evaluated for quality parameters (whey separation, titratable acidity, pH) at days 1, 7, 14 and 21 of storage.

In all of the above experiments, the reference samples were yogurt samples made from reconstituted milk without adding MTGase (Ref1) and/or with MTGase (Ref 2).



**Figure 1.** Technological flowchart of yogurt production with substituted hydrolyzed protein

### 2.3. Analytical methods

**Whey separation** (WS, %) reflects the degree of dehydration of the fermented dairy product. The whey separation of yogurt was measured by centrifugation [10]. Weigh 30g of yogurt sample and

centrifuge at 4500 rpm for 30 minutes to collect the supernatant. Then, collect the entire supernatant, weigh and calculate the whey separation according to the following formula:

$$WS (\%) = \frac{m_{wh}}{m_{sample}} \times 100 \quad (1)$$

Where,  $m_{wh}$  was the weight of separated whey,  $m_{sample}$  was the initial sample weight

**Titrateable acidity (TA, mmol/100g)** of yogurt samples was carried out according to TCVN 6509:2013 (ISO 11869:2012) [11]. Acidity is the amount, in milliliters, of 0.1 mol/L sodium hydroxide solution required to titrate 100 grams of product to pH  $8.3 \pm 0.1$ . The titrateable acidity (TA) was determined as following:

$$TA = \frac{V \times 10}{m} \quad (2)$$

Where,  $V$  was the volume of NaOH solution used for titration (mL);  $m$  was the mass of the titrated yogurt sample (g).

**The rheological properties** were analyzed using the Thermo Scientific HAAKE Rheometer to determine the liquid characteristics of the yogurt samples. The yogurt samples (2 g) were stabilized at 25°C for 30 minutes and placed in the sample position. The shear stress ( $\sigma$ , Pa) and viscosity ( $\eta$ , Pa.s) was measured when the shear rate ( $\dot{\gamma}$ , 1/s) run from 1 to 500  $s^{-1}$  (first cycle) and from 500 to 1  $s^{-1}$  (second cycle). The Herschel-Bulkley model (3) was used for determining the rheological type of yogurt: probe P35 TiL ( $\varnothing = 35$  mm) was used, distance between probe and base was 1 mm, temperature was 25 °C.

$$\sigma = k \times \dot{\gamma}^n + \sigma_0 \quad (3)$$

Where,  $k$ : consistency coefficient,  $\dot{\gamma}$ : shear rate 1/s,  $n$ : flow index,  $\sigma$ : shear stress,  $\sigma_0$  - threshold stress

This equation is suitable for many types of liquids in foods. Specifically, when  $0 < n < 1$ , the liquid has a pseudo-liquid state and the viscosity is inversely proportional to the shearing speed (Shear thinning fluid); when  $1 < n < \infty$ , the viscosity of the liquid is proportional to the shear rate (Shear thickening fluid) [12].

**Microstructure observation.** Scanning electron microscopy (SEM) images were taken to compare the differences in microstructures of yogurt products. Firstly, the yogurt samples (100 g) were frozen at -50°C for 24h. They were then freeze-dried by equipment Yamato DC-401 (Japan) at  $30 \pm 2$  °C, 10-20 Pa in 24h. The yogurt samples were mounted on a stub and photographed with TM4000Plus (Japan). Observation conditions were selected: Accelerating Voltage (Standard 5 kV Mode 2), Vacuum level was Standard (H), Detector was BSE.

**Sensory evaluation.** The sensory evaluation of yogurt samples was performed according to an acceptance test with the 9-point hedonic scale (1 – extremely dislike and 9 – extremely like) [13]. The test was evaluated by a group of 60 consumers, including 27 males and 33 females between the ages of 21-24. The sensory evaluation was carried out in a clean room without strange odors.

**Other analysis.** pH was measured by means of a penetration pH meter (Hanna HI 9124, USA). Moisture content, fat and protein contents of raw materials were estimated by ISO 5537:2004) [14], ISO/CD 9877|IDF 258 [15], ISO 1736:2008 [16], , ISO 1211:2010 [17] and Kjeldahl method ISO 8968 1:2014 [18], ISO 20483:2013 [19], respectively. Microbiological analysis of experimental samples was determined following specified criteria of TCVN 7030: 2009 (Codex stan 243-2003) [20].

**Statistical analysis.** Each experiment was done in triplicate. Data were expressed as means  $\pm$  standard deviation. Experimental data was statistically analyzed by ANOVA (Duncan test) that was used to study the difference between means with a significance level of  $\alpha = 0.05$  in SPSS software program.

### 3. Results and Discussion

#### 3.1. Chemical composition of raw materials

The initial chemical composition of whole milk powder was determined including moisture, protein and lipid contents. The results are presented in Table 1. Analytical results showed that, the whole milk powder had protein content of 23.42%, lipid content of 27.13%, moisture content of 2.7%. These values showed similarities with published data from the certificate of product analysis. In addition, the chemical composition of the reconstituted milk solution was also determined. Protein, lipid and moisture contents

were 3.45%, 3.45% and 84.28%, respectively. So that, the total solids of reconstituted milk were about 15%. The chemical composition of PPC powder was also determined with protein content of 63.85%, lipid content of 0.1% and moisture content of 9.69%.

**Table 1.** Chemical composition of raw materials

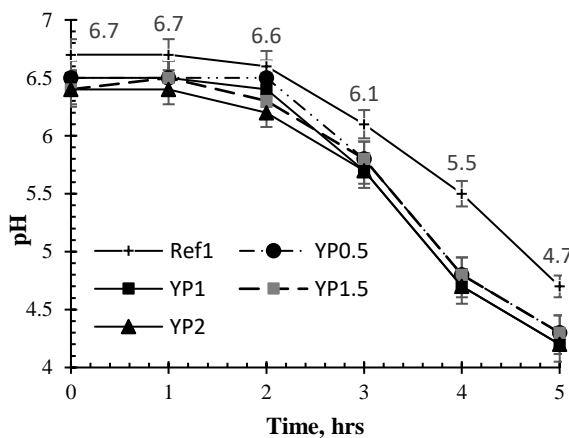
Material	Chemical composition (%)		
	Protein	Lipid	Độ ẩm
Whole milk powder	23.42 ± 0.04	27.13 ± 0.02	2.70 ± 0.04
Reconstituted milk	3.45 ± 0.04	3.45 ± 0.04	84.28 ± 0.04
PPC powder	63.85 ± 1.59	0.10 ± 0.05	9.69 ± 0.80

### 3.2. Effect of hydrolyzed protein concentration on the quality of yogurt products

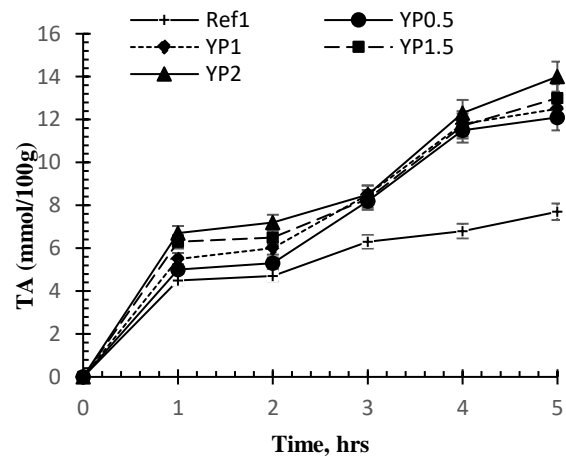
#### a) pH, titratable acidity during fermentation

The changes in pH and titratable acidity of the yogurt samples are shown in Figures 2 and 3. The analytical results (Figure 2) showed that, during the fermentation process, the pH of yogurt samples gradually decreased. In the first 2 hours, the fermentation process took place slowly, the pH almost did not change in all samples, the graph was in the form of a horizontal line. From hour 2 to hour 5 pH decreases rapidly, the graph in this range had a steeper slope. The pH of yogurt samples with partial replacement of hydrolyzed *Phaseolus* protein (YPs) was always lower than that of Ref1 samples at all times. The pH value at the starting point of the YPs samples fluctuated at pH = 6.5, while the pH of the reconstituted milk reached 6.7. The reference sample 1 (Ref1) had a slower pH reduction rate than the YPs samples, showed in the gentle slope line (Figure 2). After 04 hours of fermentation, the surface of the YPs products coagulated, the pH reached 4.7, while the pH of the reference sample Ref1 only dropped to 5.5. After another hour, the Ref1 sample completely coagulated, pH = 4.7.

In contrast to pH, titratable acidity resulted in a gradual increase in fermentation (Figure 3). During the first 2 hours of fermentation, the amount of lactic acid produced was low. However, in the period from 2 to 5 hours, the amount of lactic acid increased rapidly. There was a significant difference in the amount of lactic acid between the Ref1 and the remaining samples (YPs). Particularly, the Ref1 sample showed the lowest amount of lactic acid. The more hydrolyzed protein content substituted, the higher the titratable acidity. Our results were similar to those of Yao et al. (2006) [21] when the authors studied the change in acidity of yogurt products that partially replaced hydrolyzed soy protein. The authors concluded that, the higher the replacement of protein content, the faster the lactic acid production [21].



**Figure 2.** Change of pH in yogurt samples during fermentation at 42 °C



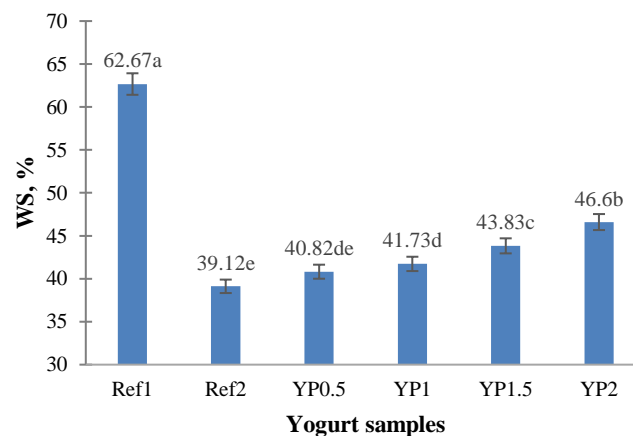
**Figure 3.** Change of titratable acidity in yogurt samples during fermentation at 42 °C

The explanation for this result is that the hydrolyzed proteins act as an exogenous nitrogen source to enhance the growth of lactic acid bacteria [22]. It means that hydrolysates, namely small, bioactive peptides, promote the growth of probiotic bacteria and increase the acidifying activity of these microorganisms [23]. Specifically, the original protein with a complex structure was hydrolyzed to simple structural peptides, which facilitated absorption and metabolism (Gómez et al., 2008; Darmawan et al., 2010). In addition, the hydrolyzed protein specifically supports the growth of the strain *Str. thermophilus* which is used in the dairy industry due to its properties of milk acidification and flavor development. However, *Str. thermophilus* is a fastidious organism and requires an exogenous source of amines or peptides for optimal growth [24]. Therefore, the partial replacement of hydrolyzed protein in yogurt could act on this role, thereby shortening the fermentation time of the product.

#### b) Whey separation

The degree of whey separation of yogurt samples partially replacing hydrolyzed protein at different ratios is shown in Figure 4. The analytical results showed that the replacement of hydrolyzed protein content in yogurt samples affected the degree of whey separation. The higher the hydrolyzed protein substituted, the higher the whey separation of the product ( $p < 0.05$ ). The whey separation level of the reference sample without MTGase (Ref1) had the highest value (62.67%), while the reference sample with MTGase (Ref2) had the lowest value (39.12%). All yogurt samples with hydrolyzed protein had a higher whey separation than that in the Ref2 sample but lower than that in the Ref1.

The usual yogurt gel texture is formed through coagulation of milk by lactic acid from fermented strains, in which casein micelles precipitate and agglomerate into a complex lattice structure and are capable of trapping water molecules inside this network [25]. Otherwise, because of shortening the protein length under hydrolyzation, the inclusion of smaller peptides in yogurt showed a lower water holding capacity than the control sample [22]. This also means that the hydrolysis has caused the disruption of the protein network, making the proteins reorganize in a different way, with less access to the water molecule. It means that the more hydrolyzed protein content is replaced, the looser the protein network becomes, so the higher the whey separation. Our experimental results were similar to those of Hu et al. (2020) when the author studied the addition of hydrolyzed soy protein to evaluate the quality of yogurt.



The different letters (a-e) indicate significant difference ( $p < 0.05$ )

**Figure 4.** Effect of hydrolyzed protein content on whey separation in yogurts

#### c) Rheological properties

The change in shear stress ( $\sigma$ , Pa) with shear rate ( $\dot{\gamma}$ , 1/s) of yogurt samples was shown in Figure 5. The regression equations showed the relationships between them are presented in Table 2. The analytical results (Figure 5) showed that in the 1-st cycle, the shear stress of yogurt samples increased as the shear rate increased. While in the 2-nd cycle, the shear rate decreased, shear stress also decreased. The analytical results (table 3) showed that the Herschel - Bulkley regression equations in both of cycles had no threshold stress ( $\sigma_0 = 0$ ) and a flow index ( $n$ ) of  $0 < n < 1$ . Thus, the pseudoplastic

nature of the products was not changed when partially replacing the raw milk powder with hydrolyzed Lima protein. All of the YPs samples had lower shear stress than those of the Ref2 but higher than those of the Ref1. In addition, the concentration of hydrolyzed protein substituted in yogurt also affected the shear stress. As this content increased, the shear stress decreased, resulting in a decrease of the strength between the bonds in the gel system of yogurt.

Hydrolyzed proteins can form gels, but the resulting gels are relatively weak, because the gel properties are affected by hydrophobicity and the resulting gels have few disulfide bonds [26], [27]. Felix et al. (2017) have shown that different contributions of ionic bonds, hydrogen bonds, disulfide bonds and hydrophobic interactions can alter the resulting gel network [28]. At the same time, at a certain degree of hydrolysis, the small peptide size is difficult to form a continuous network, so gelation is hindered [29]. However, the shear stress of samples with hydrolyzed protein was higher than that of Ref 1 (Yogurt without MTGase). It can be speculated that the presence of MTGase and protein has the effect of increasing the strength of the gel system because of cross-links forming between polypeptides [30].

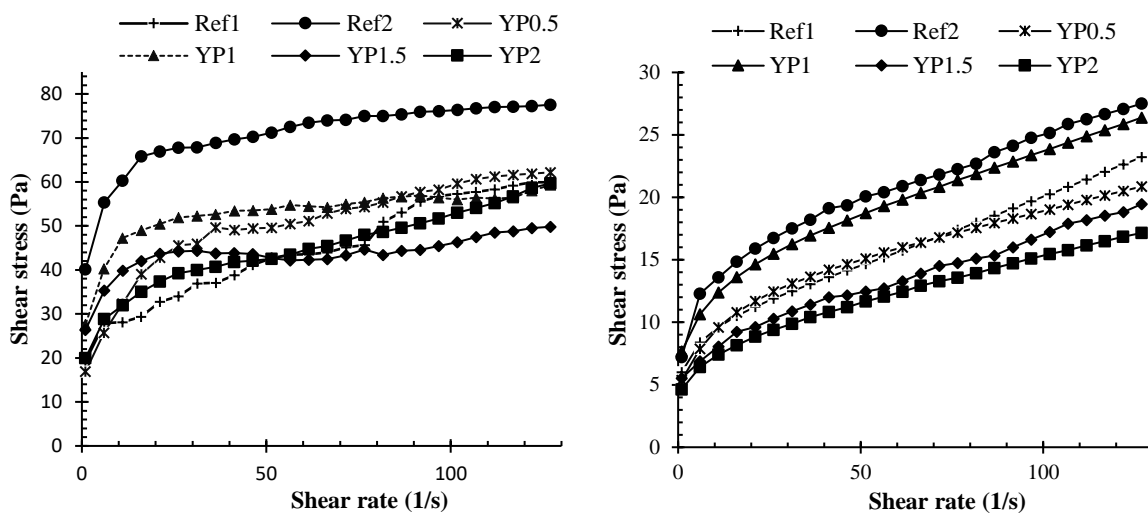


Figure 5. Change of shear stress of yogurt samples at the 1-st cycle (left) and the 2-nd cycle (right)

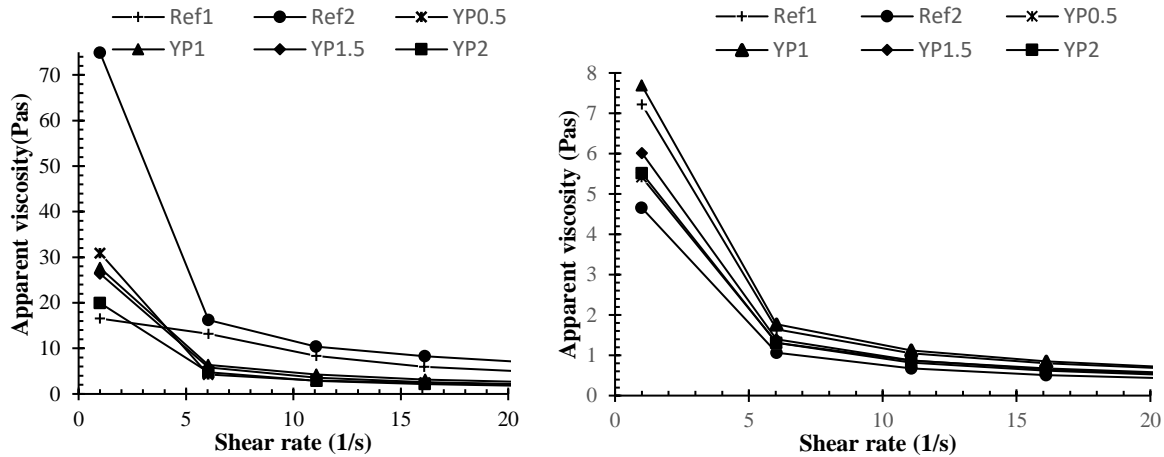
Table 2. Herschel - Bulkley regression equations of yogurt samples

Sample	The 1-st cycle			The 2-nd cycle		
	Function	R <sup>2</sup>	n	Function	R <sup>2</sup>	n
Ref1	$\sigma = 16.217\dot{\gamma}^{0.255}$	0.9281	0.2550	$\sigma = 4.9338\dot{\gamma}^{0.2939}$	0.9808	0.2939
Ref2	$\sigma = 44.814\dot{\gamma}^{0.1195}$	0.8823	0.1195	$\sigma = 7.0941\dot{\gamma}^{0.2696}$	0.9852	0.2696
YP0.5	$\sigma = 17.504\dot{\gamma}^{0.2668}$	0.9780	0.2668	$\sigma = 4.9269\dot{\gamma}^{0.2893}$	0.9939	0.2893
YP1	$\sigma = 33.066\dot{\gamma}^{0.1244}$	0.7637	0.1244	$\sigma = 6.7580\dot{\gamma}^{0.267}$	0.9808	0.2670
YP1.5	$\sigma = 29.345\dot{\gamma}^{0.1019}$	0.8097	0.1019	$\sigma = 4.3937\dot{\gamma}^{0.2825}$	0.9469	0.2825
YP2	$\sigma = 19.145\dot{\gamma}^{0.2165}$	0.9602	0.2165	$\sigma = 3.8985\dot{\gamma}^{0.2889}$	0.9729	0.2889

d) Apparent viscosity

The relationship between shear rate ( $\dot{\gamma}$ , 1/s) and apparent viscosity ( $\eta$ , Pa.s) of yogurt samples is shown in Figure 6. The analytical results showed that the rate of replacing protein affected the apparent viscosity of yogurt samples. As shear rate increased, the viscosity of yogurt products decreased. When the shear rate reached 20 s<sup>-1</sup>, the viscosity gradually reached a steady state (The data are not shown). Meanwhile, samples Ref 1 and YP2 had the lowest initial viscosities (16.54 and 19.94 Pa.s, respectively). The yogurt sample Ref 2 had the highest initial viscosity (74.89 Pa.s). The remaining YPs samples have viscosities ranging from 26.39 to 30.91 Pa.s. When a force was applied to the product, this changed the direction of the particles' movement. Changing the direction of movement of the particles would reduce the viscosity of the solution compared with randomly moving particles [31]. The viscosity

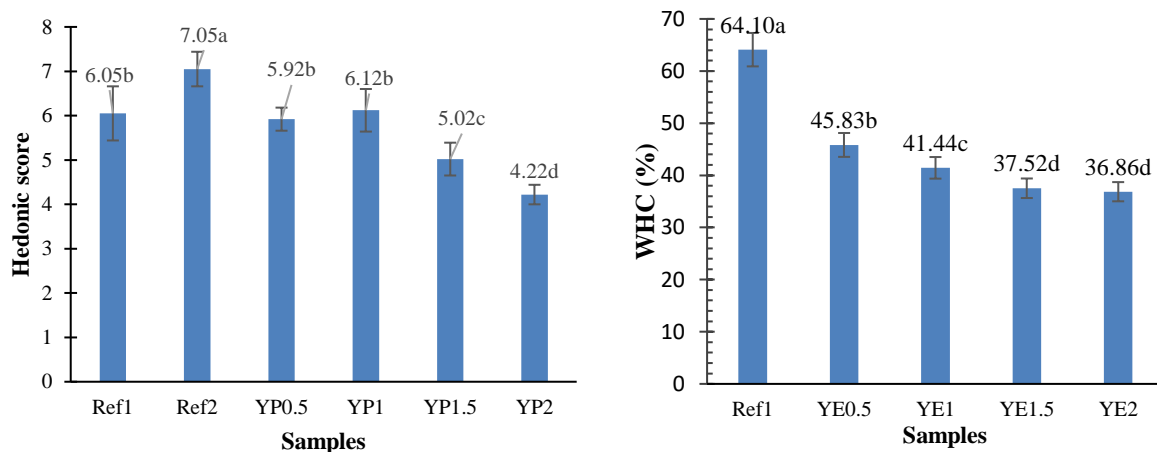
of YPs samples was higher than that of Ref1 samples ( $p < 0.05$ ). This result was attributed to the presence of MTGase and the formation of cross-links between amino acids in YPs products [32]. Furthermore, at the 2-nd cycle, the analytical results showed that after applying a force to the gel structure, the yogurt products could not restore the broken links. This represents the nature of pseudoplastics [33].



**Figure 6.** Change of apparent viscosity of yogurt samples at the 1-st cycle (left) and the 2-nd cycle (right)

e) Sensory evaluation

Yogurt samples were evaluated the acceptance using the rating method with a 9-point hedonic scale ranging from 1 (Dislike extremely) to 9 (Like extremely). The panel included 60 testers who had used this product category. The test was conducted in a room without strange odor, at 30°C and under fluorescent light. The analytical results (Figure 7) showed that yogurt products supplemented with MTGase (Ref2) were the most preferred (7.05); products Ref1, YP0.5 and YP1 were equally preferred; products YP1.5 and YP2 were least preferred. Our results were also similar to those of Franco-Miranda et al. (2017) in which the level of consumer acceptance decreased as the level of substituted protein increased [34]. However, with protein replacements of 0.5% and 1% also showed an acceptable organoleptic effect. Moreover, the higher the protein replacement content in the yogurt product, the higher the nutritional value of the product. Thus, we chose the replacement protein content of 1%.



Where Ref1, Ref2 were reference samples;

YP0.5-YP2 – yogurts product with different replaced ratio of Phaseolus protein (from 0.5-2%) with MTGase of 1.5 IU/g protein.

YE0.5-YE2 – yogurts product replaced 1% hydrolyzed Phaseolus protein with different concentrations of MTGase (form 0.5-2 IU/g protein)

The different letters (a-e) indicate significant difference ( $p < 0.05$ )

**Figure 7.** Hedonic score of yogurts samples

**Figure 8.** Effect of MTGase on the whey separation of yogurt samples

### 3.3. Effect of MTGase on the whey separation of yogurt samples

The purpose of adding MTGase to yogurt samples is to increase the texture characteristics of the product. The effect of MTGase on the whey separation of yogurt samples is shown in figure 8. The analytical results showed that the MTGase-fortified yogurt samples had lower whey separation than the reference sample (Ref1 which was without MTGase). When the concentration of MTGase was increased from 0 to 1.5 (IU/g protein), the whey separation decreased from 64.10% to 37.52%. However, continuing to increase the amount of MTGase from 1.5 to 2 (IU/g protein), the change in whey separation of yogurt was insignificantly different ( $p < 0.05$ ). This result is consistent with the results of many previous studies. In particular, the results of Farnsworth et al. (2006) showed that with increasing MTGase concentrations in yogurt made from goat's milk, the whey separation rate decreased by more than 40% compared to yogurt samples not supplemented with MTGase. The results reported by Setiadi and Ramdhani (2018) on the addition of MTGase to yogurt made from cow's milk also showed that the addition of MTGase significantly reduced the whey separation rate of the product in storage.

This is explained by the fact that the addition of MTGase has been reported to improve the water retention of yogurt samples. Casein is the main protein in milk and a good substrate for MTGase activity, especially  $\kappa$ -casein and  $\beta$ -casein [32]. In yogurt, milk gels are mainly stabilized by non-covalent bonds, i.e. electrostatic interactions, hydrogen bonds and hydrophobic bonds. And these bonds are often unstable, so yogurt often has some problems with whey separation. For this reason, acidification of the sample with the addition of MTGase results in a more solid gel and a stronger protein network. This is because MTGase creates more covalent bonds and introduces more stable bonds into the yogurt gel [35]. At this point, the gel is formed from the covalent bonds between the lysine and glutamine residues, creating cross- or intermolecular linkages that make the protein network stronger, helping these samples have better water retention than the control sample, thereby reducing the ability to separate whey (Chiya et al., 2001). On the other hand, the reduced whey separation may be due to the effect of MTGase on the gel pore size. As the gel pore size is reduced, a protein network is formed leading to a lower whey separation rate [36]. Moreover, the experimental products contained both plant and animal proteins, so MTGase could act as a bridge linking these two proteins. MTGase combines protein molecules with intermolecular covalent bonds to provide physicochemical and functional properties that are different from those of single protein molecules. Binding of plant and animal proteins using MTGase resulted in better rheological properties, water retention and emulsion stability [30].

The whey separation level of yogurt samples supplemented with MTGase with concentrations that increased from 1.5 to 2 (IU/g protein) showed no difference ( $p > 0.05$ ). This can be explained that at this time, all proteins have been completely and thoroughly linked together, so the process of forming cross-linking did not take place anymore. This result was similar to the results of Farnsworth et al. (2006) when increasing the concentration of MTGase from 2 to 4 (IU/g protein), there was no significant difference between the two samples [35]. Therefore, in terms of economy, we selected samples with an enzyme concentration of 1.5 IU/g protein to save on production costs but still ensure product efficiency.

In short, the experimental results on the effect of hydrolyzed protein on the quality properties of yogurt showed that the yogurt sample was produced with 1% hydrolyzed protein, with a MTGase content of 1.5. IU/g protein provided the appropriate qualities and it was consistent with the goals of the study.

### 3.4. The quality of yogurt products partially replaced hydrolyzed protein (YP1)

The chemical composition of YP1 including carbohydrates, fat, protein, moisture, ash contents and microbiological criteria was analyzed. The results are shown in Table 3. According to TCVN 7030:2009, it is required for the quality of yogurt that the fat-free dry matter content is not less than 8.2%, the fat content is more than 2%, the microbiological criteria (*Salmonella*, *E.coli*, *Coliforms*, *Staphylococcus aureus*) for undetectable results [20]. From the results of Table 3, it can be seen that the researched yogurt sample YP1 completely met the needs of chemical composition and microbiological criteria.

**Table 3.** Some quality indicators of sample YP1

STT	Indicators	Result (%)
<i>Chemical composition</i>		
1	Carbohydrates	10.30 ± 0.78
2	Lipid	3.84 ± 0.64
3	Protein	3.92 ± 0.57
4	Moisture	81.0 ± 1.23
5	Total ash	0.93 ± 0.06
<i>Microbiological criteria</i>		
1	<i>Salmonella</i>	Not detected
2	<i>E. coli</i>	Not detected
3	<i>Coliforms</i>	Not detected
4	<i>Staphylococcus aureus</i>	Not detected

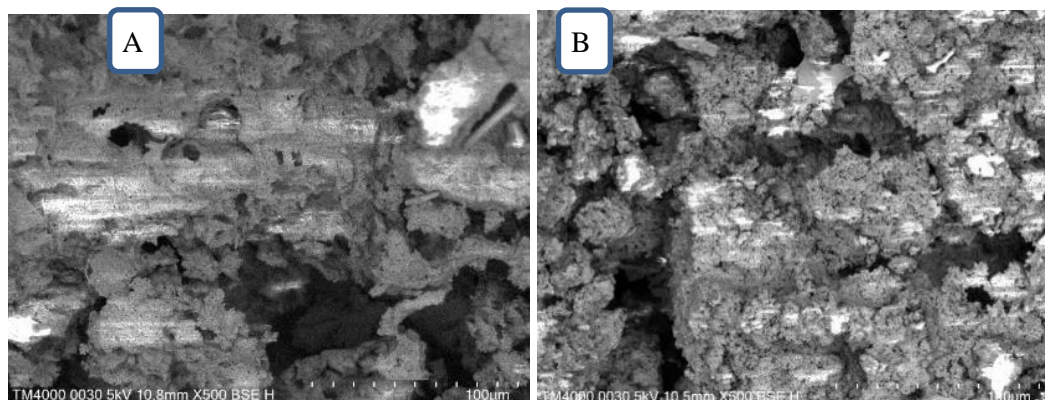
**Table 4.** Whey separation of yogurt products

Storage time	<i>during storage</i>	
	Whey separation (%)	
	Reference 1 (Ref1)	YP1
Day 1	63.67 ± 0.45 <sup>a</sup>	41.47 ± 0.17 <sup>a</sup>
Day 7	65.50 ± 0.79 <sup>b</sup>	45.87 ± 0.82 <sup>b</sup>
Day 14	67.12 ± 1.13 <sup>c</sup>	47.87 ± 0.50 <sup>c</sup>
Day 21	72.12 ± 0.31 <sup>d</sup>	50.09 ± 0.32 <sup>d</sup>

*a-d: In the same column, values with different letters are statistically different (p<0.05)*

### 3.5. Quality of yogurt products during storage

The whey separation of dairy products during storage is the result of the recombination of casein micelles. This is because of the flexible structure of the casein molecules. The casein molecules tend to form more compact micelle structures, resulting in whey separation, i.e. water release. Reaggregation is spontaneous and is interpreted as the contraction of the gel without the application of any external force, resulting in a rearrangement of the gel network and dissociation of the whey. This is an undesirable feature of this product category [2]. The whey separation of yogurt products during storage is presented in Table 4. The analytical results showed that, in all yogurt samples, whey separation increased gradually with storage time. However, the amount of whey separated from yogurt products that replaced 1% of the hydrolyzed protein (YP1) always gave better whey retention. This is thought to be the result of cross-links between proteins activated by MTGase, leading to a decrease in gel permeability. Reduced gel permeability causes a more compact and stable microstructure with smaller compartments in yogurt, and thus, multiple compartments attracting water into the yogurt gel network [37]. Scanning electron microscopy images also showed a more compact grain structure of the YP1 samples (Figure 9).



**Figure 9.** Scanning electron microscopy images of yogurt samples. A – Ref1; B – YP1

The change in titration acidity, pH of yogurt products is presented in Table 5. The analytical results showed that the titratable acidity increased gradually and pH decreased gradually in 21-day storage period. However, YP1 always had lower titratable acidity and higher pH than sample Ref1. The increase in acidity and the gradual decrease in pH during storage can be explained by residual activity of lactic acid bacteria in the product even under cold storage conditions (2 - 4 °C) [38]. Besides,

hydrolyzed proteins increased the number of beneficial bacteria in the initial fermentation stage. But it slowed down the post-fermentation acidification process [23], which led to the titratable acidity of the yogurt sample always being lower than that of the reference sample.

**Table 5.** Changes of titratable acidity and pH of yogurt products during storage

Storage time	TA (mmol/100g)		pH	
	Ref1	YP1	Ref1	YP1
Day 1	11.33 ± 0.001 <sup>a</sup>	10.17 ± 0.002 <sup>a</sup>	4.54 ± 0.04 <sup>a</sup>	4.64 ± 0.04 <sup>a</sup>
Day 7	12.17 ± 0.003 <sup>b</sup>	11.80 ± 0.004 <sup>b</sup>	4.36 ± 0.07 <sup>b</sup>	4.63 ± 0.03 <sup>b</sup>
Day 14	13.00 ± 0.004 <sup>c</sup>	12.33 ± 0.007 <sup>c</sup>	4.15 ± 0.05 <sup>c</sup>	4.44 ± 0.03 <sup>c</sup>
Day 21	13.05 ± 0.003 <sup>d</sup>	12.67 ± 0.002 <sup>d</sup>	3.96 ± 0.08 <sup>d</sup>	4.34 ± 0.04 <sup>d</sup>

*a-d: In the same column, values with different letters are statistically different (p<0.05)*

#### 4. Conclusions

In summary, Lima protein hydrolyzed by Alcalase with a concentration of 0.24 AU/g protein at 50 °C, shaking speed of 150 rpm for 1 hour can be used to partially replace whole milk powder in the process of yogurt production. The greater the amount of substituted hydrolyzed lima protein, the greater the whey separation of the final product. Partial replacement of hydrolyzed Lima protein also increased the rate of lactic acid production and the pH decrease during fermentation. Furthermore, the combination of protein substitution and the use of MTGase improved whey separation and rheological properties of the yogurt products. Sensory evaluation results showed that the yogurt sample replacing 1% hydrolyzed Lima protein (YP1) was preferred over other studied samples and was at the acceptable level of consumers (6/9 point). Using microbial transglutaminase with 1.5 IU/g protein helped to form a cross-link between protein molecules that improved the water holding capacity of yogurts during storage. YP1 product has chemical composition and microbiological criteria that meet the standards of TCVN 7030:2009. However, the limitations of this study are that: firstly, it has not been clarified how the substitution of hydrolyzed protein under different conditions affects the quality of yogurts; and, finally, the effect of restructuring of polypeptide chains between two sources of proteins under the action of MTGase has not been evaluated.

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**Pham Thi Hoan** received the BE degree in food technology from Tver State Technical University, Russia in 2010 and the Ph.D degree in Technology and commodity science of food products, functional and specialized products and public catering from Moscow State University of Food Production, Russia in 2014. From 6/2015 till now, she has been being a lecturer in Ho Chi Minh city University of Technology and Education, Ho Chi Minh city, Vietnam. Her research interest includes food processing; acquisition and application of active substances in food processing; application of enzymes in food processing.

Email: [hoanpt@hcmute.edu.vn](mailto:hoanpt@hcmute.edu.vn)

Tel.: (081) 97 220 93 96



**Phan Ngoc Thuy Tien** received the BE degree in food technology from Ho Chi Minh City University of Technology and Education in 2022. From 12/2022 till now she has been being an engineer in research and development department at Tri Viet Phat PRO CO.,LTD. Her research is about seasoning and flavour.

Email: [phanngocthuytien2000@gmail.com](mailto:phanngocthuytien2000@gmail.com)

Tel: 0916 643 453



**Duong Tran Minh Tran** received the BE in food technology from Ho Chi Minh City University of Technology and Education in 2022. Her research focuses on food processing and the application of substituting animal protein for vegetable protein. Since 2023, she has worked for Suntory Pepsico Vietnam Beverage.

Email: [trandtm0504@gmail.com](mailto:trandtm0504@gmail.com)

Tel: 0944.021.639