Scientific Research Publishing

Food and Nutrition Sciences, 2021, 12, 1243-1254
https://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

Effect of Temperature, pH and Amount of Enzyme Used in the Lactose Hydrolysis of Milk

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How to cite this paper: Popescu, L., Bulgaru, V. and Siminiuc, R. (2021) Effect of Temperature, pH and Amount of Enzyme Used in the Lactose Hydrolysis of Milk. *Food and Nutrition Sciences*, **12**, 1243-1254.
https://doi.org/10.4236/fns.2021.1212091

Received: November 25, 2021
Accepted: December 19, 2021
Published: December 22, 2021

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Abstract

Lactose intolerance is becoming a health state that requires the restriction of dairy products in the diet of people suffering from this condition. But milk and dairy products, due to a well-balanced composition in the main macro and micronutrients, cannot be missing from the diet of the consumer of any age. For these reasons, in recent years, in the milk processing industry, the production of low-lactose or lactose-free dairy products is explored. To reduce the lactose content of dairy raw materials, various industrial and biotechnological methods were used: enzymatic hydrolysis of lactose, baromembranous methods, bioconversion of lactose by lactic bacteria and others. The most widely used lactase enzymes in the industry are mesophilic enzymes from filamentous fungi (*Aspergillus spp.*) and yeasts (*Kluyveromyces spp.*). Therefore, the aim of this study was to evaluate the effect of the commercial enzyme *β*-galactosidase on the hydrolysis of cow's milk at different enzyme concentrations, temperatures and pH. Two commercial enzymes *β*-galactosidase obtained from *Bacillus licheniformis* and *β*-galactosidase obtained from *Kluyveromyces lactis*, were used in this study, according to information provided by the manufacturer. The thermal stability of lactose, the effect of milk pH, the effect of temperature, duration of hydrolysis and the amount of enzymes on the lactose hydrolysis degree and the sweetness degree of milk were determined. Research has identified the optimal parameters for obtaining a high degree of lactose hydrolysis in the use of these enzymes. Therefore, to ensure a high lactose hydrolysis degree (over 80%), the following lactose hydrolysis regimens were identified: temperature 4˚C - 6˚C, 0.3% *Bacillus licheniformis* enzymes, duration 4 hours; temperature 4˚C - 6˚C, 0.3% enzymes from *Klavyromyces lactis*, duration 12 hours and temperature 38˚C - 40˚C, 0.15% enzymes from (*Bacillus licheniformis* or *Klavyromyces lactis*), duration 2 - 3 hours. The results obtained allow the efficient use of *Bacillus licheniformis* and *Klavyromyces lactis* enzymes in industrial processes for the manufacture

of "lactose-free" or "low-lactose" drinking milk and fermented dairy products for people with lactose intolerance.

Keywords

Lactose Intolerance, β-Galactosidase Hydrolysis, Thermal Stability

1. Introduction

Lactose intolerance is a common health problem that causes gastrointestinal symptoms and avoidance of dairy products by affected people [1] [2]. Milk and dairy products are important suppliers of proteins, vitamins and minerals (especially calcium) so complete avoidance of these products is not recommended [3] [4] [5] [6]. Adherence to a lactose-free diet leads to an economic burden for patients, as lactose-free products available on the market have much higher prices compared to lactose-containing products. Thiele *et al.*, conducted a study to analyze the price variation of milk and dairy products with and without lactose and reported that all lactose-free products available on the market were more expensive compared to lactose-containing foods [4] [7]. The market for lactose-free and low-lactose dairy products in the Republic of Moldova remains an undervalued segment, a small assortment of lactose-free milk being provided only by import. Therefore, for the local dairy producers, the lactose-free segment seems to be an expanding market, extremely interesting and profitable. For the dairy industry as a whole, there is a great need to develop nutritious and economical foods without lactose, taking into account the exclusion of lactose-containing raw materials, the choice of an alternative source of milk, the sensory properties of lactose-free foods, improving the nutritional quality of food, safety and food labeling [8] [9]. These considerations will help to develop healthy, lactose-free, nutritionally complete and safe foods for people with lactose intolerance. To reduce the lactose content of dairy raw materials, various industrial and biotechnological methods were used: enzymatic hydrolysis of lactose, baromembranous methods, water treatment methods, bioconversion of lactose by lactic bacteria in the case of fermented dairy products, production of multicomponent dairy products with low lactose-free and lactose-free by mixing various micro- and macro-components with milk proteins isolated by ultrafiltration and diafiltration [10]. The most common way of reducing lactose in industry is lactose hydrolysis with an enzymatic product of lactase-β-galactosidase (E. C. 3.2.1.23). The most widely used lactase enzymes in the industry are mesophilic enzymes from filamentous fungi (*Aspergillus spp.*) and yeasts (*Kluyveromyces spp.*) [11] [12] [13] [14] [15]. Fungal sources, with acid-optimum pH, are effective for the hydrolysis of whey lactose, while yeast sources, with neutral pH-optimum, are more efficient for the hydrolysis of milk lactose [16].

In this context, the knowledge of the best working conditions of this enzyme extracted from different microorganisms is of great importance, aiming at more

efficient commercial applications, especially in the dairy industry. Therefore, the aim of this study was to evaluate the effect of the commercial enzyme β-galactosidase on the hydrolysis of cow's milk at different enzyme concentrations, temperatures and pH in order to establish the optimal lactose hydrolysis regimens and to identify the accessible lactose determination method in low-lactose dairy products. The results of the research can be applied in milk processing enterprises from the Republic of Moldova, in order to manufacture lactose-free and low-lactose dairy products.

2. Materials and Methods

2.1. Enzymes

Two commercial enzymes β-galactosidase obtained from *Bacillus licheniformis*, activity 5500 BLU·g^{-1} NOLA Fit 5500 (Chr. Hansen, Denmark) and β-galactosidase obtained from *Kluyveromyces lactis*, activity 5000 NLU·g^{-1} Maxilact LGi 5000 (Sedim Cedex, France), were used in this study, according to information provided by the manufacturer.

2.2. Thermal Stability of Lactose

The research was carried out in the temperature range 4˚C - 70˚C, the duration of the hydrolysis process 4.0 hours. Milk with a pH of 6.55, a 1.5% fat content and 4.75% lactose content were used for the lactose hydrolysis. 100 ml of milk was mixed with each enzyme (0.45%) and transferred to 250 ml Erlenmeyer flasks. The flasks were incubated in water baths at temperatures of 4˚C, 10˚C, 15˚C, 20˚C, 25˚C, 30˚C, 35˚C, 40˚C, 45˚C, 50˚C, 55˚C, 60˚C, 65˚C, 70˚C for 4 hours. The D-glucose and lactose content were determined in each sample. All analyzes were performed in triplicate.

2.3. Effect of Milk pH on the Lactose Hydrolysis Degree

Adjustment of the pH value was achieved by adding monobasic potassium phosphate buffer and sodium acetate buffer. The tested variable was the pH value (4.0; 4.5; 5.0; 5.5; 6.0 and 7.0). For the lactose hydrolysis, 100 ml of milk with a temperature of 4˚C - 6˚C were used according to the conditions described above. The D-glucose content and the lactose content were determined in each sample. All analyzes were performed in triplicate.

2.4. Effect of Temperature, Hydrolysis Duration and Amount of Enzymes on the Lactose Hydrolysis Degree

Research on determining the optimal amount of enzyme was performed at two regimes: 4˚C - 6˚C/30 hours and 38˚C - 40˚C/5 hours, pH milk in both cases being 6.55. The tested variables were temperature (4˚C - 6˚C and 38˚C - 40˚C), hydrolysis duration at 4˚C - 6˚C (0.5; 1; 2; 3; 4; 5 hours) and temperature 38˚C - 40˚C (3, 4, 5, 6, 12, 22, 24 and 30 hours). The lactose enzymatic hydrolysis processes were performed using concentrations of 5.5, 8.25 and 16.5 BLU/ml for

β-galactosidase obtained from *Bacillus licheniformis* and 5.8, 15 NLU/ml for *β*-galactosidase obtained from *Kluyveromyces lactis*. These concentrations corresponded to 0.10%; 0.15% and 0.30% (w/v). For the lactose hydrolysis, 100 ml of milk were used according to the conditions described above. The D-glucose content and the lactose content were determined in each sample. All analyzes were performed in triplicate.

2.5. Analytical Determination

The concentration of free D-glucose, as well as the D-glucose component of lactose was determined by glucose spectrophotometric method using the lactose test kit (k-LOLAC, Megazyme). The method includes pre-treatment steps to clarify and deproteinate samples and also to remove the high levels of free D-glucose in the samples.

The determinations were performed according to the method for the measurement of lactose in low-lactose and lactose-free products under Standard Method Performance Requirement (SMPRVR) 2018.009 [17]. The absorbance reading of the samples was performed at wavelength at 340 nm using UV Vis Shimadzu UV-1900 spectrophotometer. The analyses were performed in triplicate.

The amount of D-glucose was determined based on the relationship:

$$D-gl = 0.1673 \times F \times \Delta A_{D-glucose}, \text{g/L} \quad (1)$$

The amount of lactose was determined based on the relationship:

$$L = 0.3233 \times F \times \Delta A_{lactose}, \text{g/L} \quad (2)$$

where: *F*-dilution factor;

$\Delta A_{D-glucose}$ -substract the absorbance difference ($A_2 - A_1$) of the blank from the absorbance difference ($A_2 - A_1$) of the sample;

$\Delta A_{lactose}$ -substract the absorbance difference ($A_3 - A_2$) of the blank from the absorbance difference ($A_3 - A_2$) of the sample.

The initial lactose concentration and the concentration of D-glucose released were used to calculate the hydrolysis efficiency.

$$E = \frac{D-Gl_p \times MM_{lac}}{L_i \times MM_{gl}} \times 100, \% \quad (3)$$

where: $D-Gl_p$ -glucose concentration of the sample, g/L;

MM_{lac} -molar mass of lactose, g/mol;

L_i -initial lactose concentration, g/L;

MM_{gl} -molar mass of glucose, g/mol.

The degree of milk sweetness at different lactose hydrolysis stages was determined by the relation 4, [18]:

$$S = D-Ga \times 65 + D-Gl \times 72 + L \times 16, \text{units} \quad (4)$$

where: $D-Ga$ -galactose concentration of the sample, %;

$D-Gl$ -glucose concentration of the sample, %;

L-lactose concentration of the sample, %;

65, 75 and 16-sweetening power of galactose, glucose and lactose, respectively, units.

3. Results and Discussion

3.1. Determination of the Carbohydrates Sweetness and the Milk Sensory Characteristics According to the Lactose Hydrolysis Degree

Acceptance of dairy products is mainly due to the food sensory characteristics. Lactose-free ultrapasteurized milk is characterized by a more intense boiling aroma and a sweeter taste compared to lactose-free ultrapasteurized milk [19] [20]. This could be an obstacle to the consumption of lactose-free dairy products by the lactose intolerant population. More industry efforts are needed to develop higher quality lactose-free products and to educate consumers on lactose-free dairy products [4]. Therefore, before developing such products, it is important to analyze the sensory characteristics of lactose-free milk compared to normal milk.

According to the literature, for the production of milk with low lactose content, it is sufficient to achieve a degree of lactose hydrolysis of 70% - 80%, which is an optimal correlation between lactose intolerance and obtaining dairy products with high sensory characteristics. A degree of lactose hydrolysis of more than 90% is required only for lactose-free dairy products [21]. The dependence of the sweet index on the lactose hydrolysis degree is represented in **Figure 1**.

With the increase of the lactose hydrolysis degree, the intensity of the sweet taste of milk also increases, which leads to the milk sensory characteristics modification as a whole. Enzymatic hydrolysis of lactose in milk under the action of the enzyme *β-galactosidase* to the monosaccharides glucose and galactose leads to the appearance of sweet taste in milk, with increasing hydration increases the intensity of milk sweet taste.

To determine the influence of lactose hydrolysis on the milk sensory properties, the enzyme product *β-galactosidase* from *B. licheniformis* was used. Hydrolysis of lactose in milk was performed at 4˚C - 6˚C for 12 hours until a

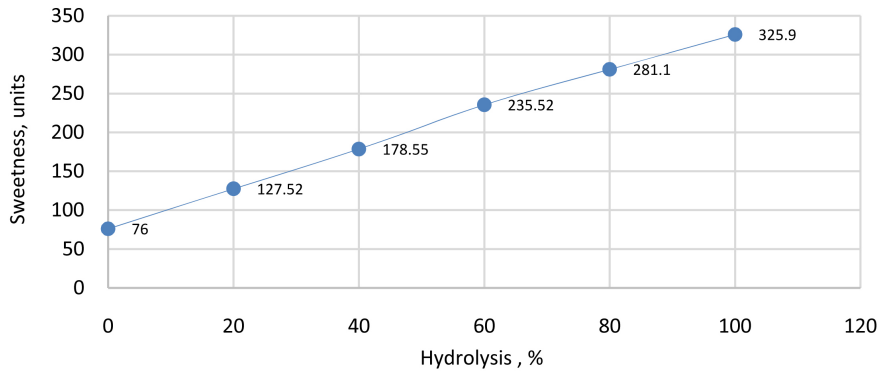

Figure 1. The dependence of the sweet index on the lactose hydrolysis degree.

hydrolysis degree of more than 95% was provided. The milk samples taste was performed in a group of 5 evaluators. The taste of sweet, boiled, caramel and butter was appreciated (Table 1).

The results of the sensory analysis of milk according to the hydrolysis degree of lactose showed that the increase of the lactose hydrolysis degree by more than 30% leads to the appearance in milk of the weakly noticeable sweet sensation. The sweet sensation becomes intense at a lactose hydrolysis degree of 70%. And at a hydrolysis degree higher than 70%, the milk boiling taste intensifies and a caramel nuances appears. Similar results have been reported by other authors [22] [23]. Therefore, we can mention that the lactose hydrolysis has a significant influence on the sensory properties of milk, especially on taste.

3.2. Thermal Stability of the Enzyme

The research was performed in the temperature range of 4˚C - 70˚C, the duration of the hydrolysis process 4.0 hours, the milk-raw material pH of 6.55. For lactose hydrolysis, 100 ml of milk with 1.5% fat content of and 4.75% lactose content were taken.

According to the data presented in Figure 2, the higher inactivation rate of *β*-galactosidase from *K. lactis* was directly proportional to the increase in temperature, especially from 40˚C ± 2˚C and *β*-galactosidase from *B. licheniformis* of 45˚C ± 2˚C which corresponds to the technical characteristics of the studied enzymes. Subsequent increase in temperature leads to decreased enzyme activity. Thus, at a temperature of 60˚C ± 2˚C the hydrolysis degree is approximately 50% for the *β*-galactosidase enzyme from *B. licheniformis* and 30% for the *β*-galactosidase enzyme from *K. lactis*. Complete inactivation is attested at a

Table 1. Sensory characteristics of milk obtained by lactose hydrolysis.

Hidrolysis duration, hours	Hydrolysis, %	Content, %		Sensory characteristics
		D-glucose	Lactose	The taste
-	-	-	4.75 ± 0.08	Slightly sweet characteristic of milk, without foreign nuances
1	21.3 ± 0.02	0.53 ± 0.04	3.71 ± 0.02	Slightly sweet characteristic of milk, without foreign nuances
2	32.5 ± 0.03	0.81 ± 0.02	3.17 ± 0.07	Sweetish, easy to boil, without foreign nuances
3	73.2 ± 0.04	1.83 ± 0.05	1.18 ± 0.03	Sweetish, easy to boil, without foreign nuances
4	87.6 ± 0.05	2.19 ± 0.07	0.47 ± 0.03	Sweet, boiled, light caramel, without foreign nuances
5	93.8 ± 0.04	2.35 ± 0.03	0.16 ± 0.05	Sweet, boiled, light caramel, without foreign nuances
12	97.06 ± 0.04	2.43 ± 0.04	0.01 ± 0.02	Sweet, boiled, light caramel, without foreign nuances

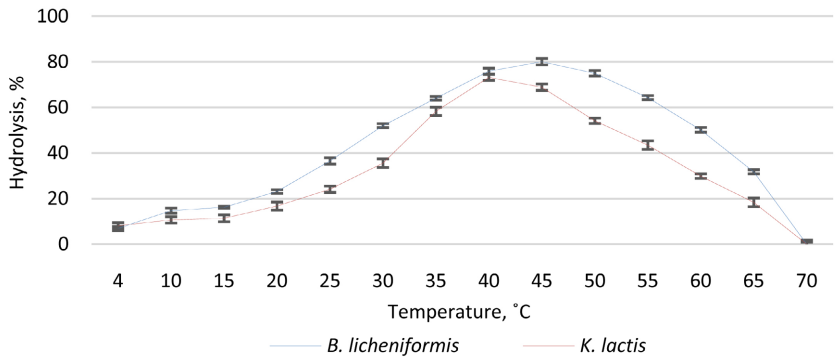

Figure 2. Influence of temperature on the lactose hydrolysis degree.

temperature of 70˚C ± 2˚C for both types of enzymes. In general, the enzyme from *K. lactis* was sensitive to temperatures above 40˚C, with complete inactivation at 55˚C at all studied pH ranges. The thermal stability results of commercial *β*-galactosidase enzymes have shown that they are inactivated during pasteurization/sterilization of milk, so there is no residual enzymatic activity in the final product, which is an advantage for food regulation and labeling. These data are consistent with literature data on microbial *β*-galactosidase [22] [24] and with the technical characteristics of these enzymes.

3.3. The Influence of pH on the Lactose Hydrolysis Degree

The pH value was adjusted by adding monobasic potassium phosphate buffer and sodium acetate buffer (**Table 2**).

Commercial *β*-galactosidase from *K. lactis* and *B. licheniformis* showed optimal activities under different reaction conditions (**Figure 3**).

At a pH of 6.5 the lactose hydrolysis degree for the enzyme from *K. lactis* is maximum (77.6%), the decrease of the pH below the value of 6.5 leads to the inactivation of the enzyme, demonstrating sensitivity to low values of pH. For the enzyme from *B. licheniformis* the maximum degree of lactose hydrolysis (85.2%) was reached at pH values of 5.5 - 6.0, the decrease of the pH below the value of 5.5 leads to inactivation of the enzyme.

3.4. Influence of Temperature, Hydrolysis Duration and Enzymes Quantity on the Lactose Hydrolysis

The degree of lactose hydrolysis is determined by the temperature, the hydrolysis duration and the enzyme amount.

Enzymatic hydrolysis of lactose can be done by incubating milk with lactase before pasteurization in cold conditions (familiar to the batch process for milk) or adding lactase together with the starter culture after pasteurization of milk (familiar for yogurt making) [7]. The batch process has a number of advantages, among which we can list: low temperatures prevent the growth of microbial, the enzyme is inactivated during pasteurization/sterilization of milk and milk is characterized by higher sensory properties compared to the continuous method of hydrolysis of lactose [7] [25].

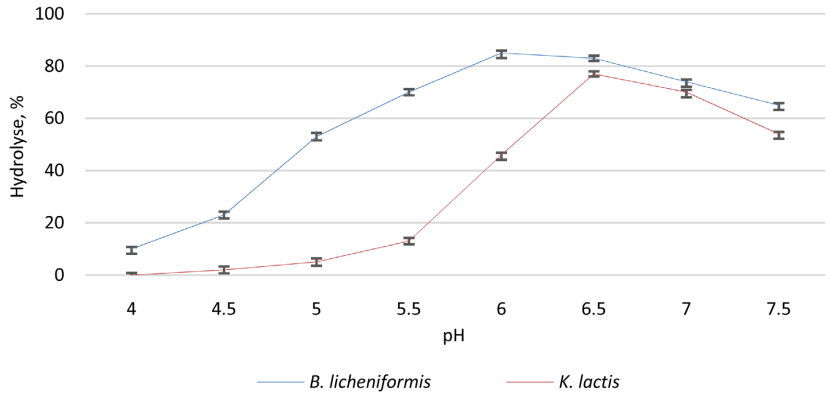

Figure 3. The influence of pH on the lactose hydrolysis degree.

Table 2. Experimental conditions to evaluate the thermal stability of the enzymes from *K. lactis* and *B. licheniformis*.

	K. lactis		*B. licheniformis*	
pH	7.0	4.0; 4.5; 5.0; 5.5; 6.0	7.0	4.0; 4.5; 5.0; 5.5; 6.0
Buffer solution	Potassium phosphate	Sodium acetate	Potassium phosphate	Sodium acetate
Temperature, ˚C	4 ± 2			
Duration, ore	4			
Quantity of enzymes, % (w/v)	0.15			

If referring to the process of adding lactase together with the starter culture after pasteurization of milk, most yogurt producers opt for this, because predigestion seems to inhibit the activity of some yogurt cultures [7] [26].

Therefore, research on determining the optimal amount of enzyme product was performed at two regimes: 4˚C - 6˚C/30 hours and 38˚C - 40˚C/5 hours, the pH of milk in both cases being 6.55. For the lactose hydrolysis, 100 ml of milk with 1.5% fat content and 4.75% lactose content were taken. The milk was previously heated at 63˚C - 65˚C for 10 - 15 seconds.

Figure 4 shows that with the increase of the fermentation time and the amount of enzymes, the hydrolysis degree increases. For example, at an enzymes amount from *B. licheniformis* of 0.1% after 6 hours, only 35.02% of the initial amount of lactose was hydrolyzed, when the amount of enzymes was increased to 0.3% in the same period of time 95.25% of lactose was hydrolyzed. The use of a quantity of 0.15% in the cold lactose hydrolysis process (temperature 4˚C - 6˚C) is not justified, because the hydrolysis duration process increases up to 22 hours, the degree of hydrolysis reaching the value of 80.75%. When using an amount of 0.1% in general, an optimal value of the hydrolysis degree is not reached (minimum of 70% - 75%).

When using the enzyme from *K. Lactis*, the maximum value of the cold lactose hydrolysis is established after 12 hours at an enzymes amount of 0.3%.

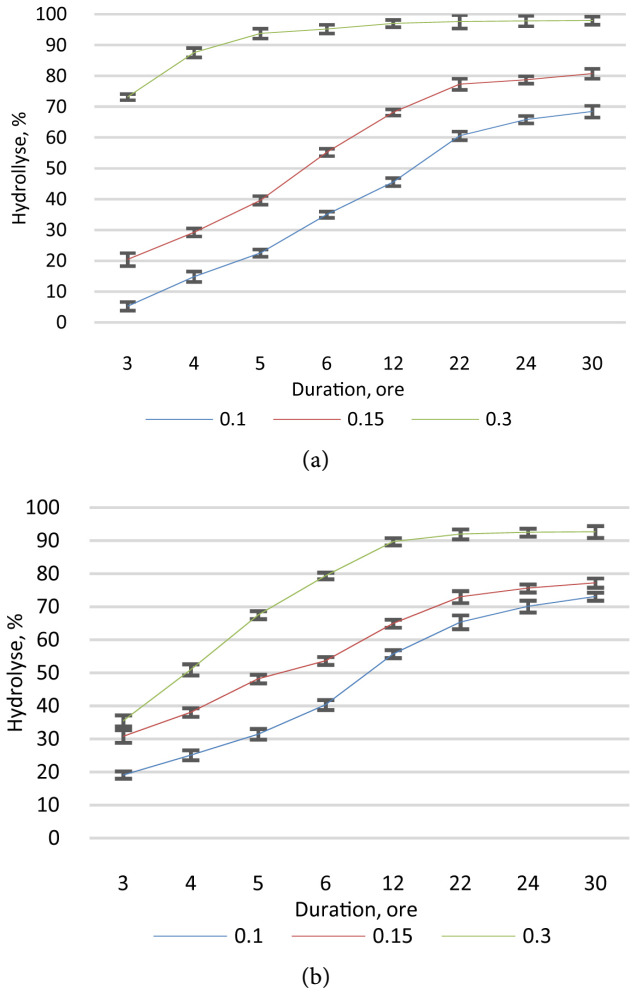

Figure 4. The influence of the enzymes amount on the lactose hydrolysis degree at a temperature of 4˚C - 6˚C and an average pH of 6.55: (a) The enzyme from *B. licheniformis*; (b) Enzyme from *K. Lactis*.

Figure 5 shows the same legitimacy for lactose hydrolysis under the action of the *β-galactosidase* enzyme at temperatures of 38˚C - 40˚C and hydrolysis at a temperature of 4˚C - 6˚C, namely the increase of the hydrolysis degree with the increase of the enzymes amount and hydrolysis duration. Therefore, there is a significant increase in the rate of hydrolysis, when use of enzyme from *B. licheniformis* in the amount of 0.3%, an increased hydrolysis degree (over 90%) is already recorded after 2 hours of hydrolysis. For the enzyme from *K. lactis*, the optimal regime identified is temperature of 38˚C - 40˚C, the amount of enzyme 0.15% and the duration of hydrolysis 3 - 4 hours.

The pH of the milk subjected to the lactose enzymatic hydrolysis under the action of *β-galactosidase* at the regimes of 4˚C - 6˚C/30 hours and 38˚C - 40˚C/5 hours practically did not change. No sensory defects were identified in the lactose hydrolysis process, such as defects due to the breakdown of fats or proteins. Therefore, we can say that the level of microorganisms activity in milk during the lactose hydrolysis process is very low.

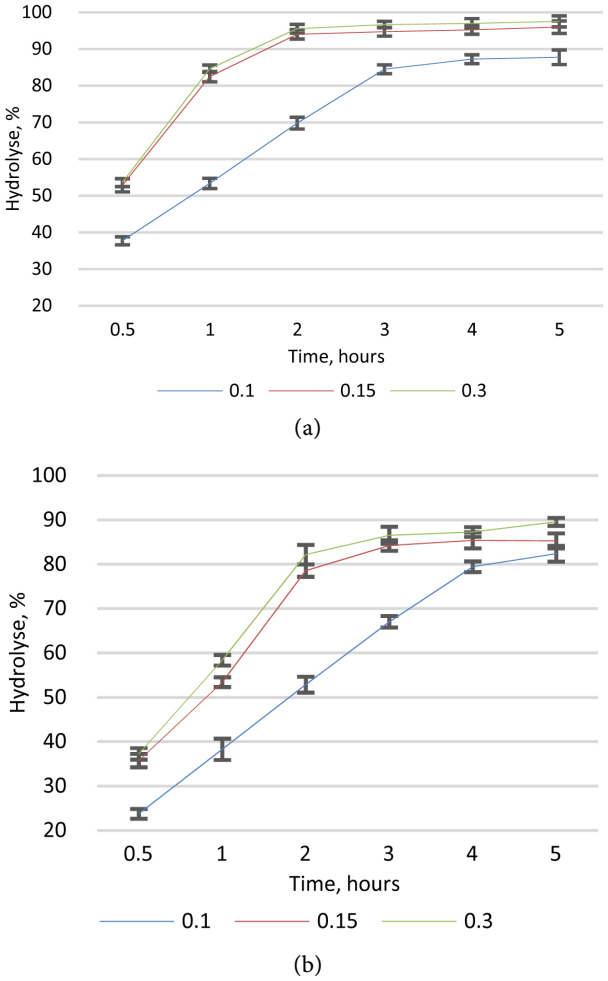

Figure 5. The influence of the enzymes' amount on the lactose hydrolysis degree at a temperature of 38˚C - 40˚C: (a) Enzyme from *B. licheniformis*; (b) Enzyme from *K. Lactis*.

4. Conclusions

The results of the study provide information on the ability of commercial enzymes to hydrolyze lactose in milk. Thus, to ensure a high lactose hydrolysis degree (over 80%), it is recommended that cold lactose hydrolysis be performed at a temperature of 4˚C - 6˚C, 0.3% enzyme from *B. licheniformis*, for 4 hours or temperature 4˚C - 6˚C, 0.3% of the enzyme from *K. lactis*, duration 12 hours. The residual amount of lactose enzyme is inactivated starting with 70˚C, so in the pasteurization process.

Hydrolysis of lactose at temperatures of 38˚C - 40˚C, when using the enzyme from *B. licheniformis* in the amount of 0.3%, an increased hydrolysis degree (over 90%) is registered already after 2 hours. For enzyme from *K. lactis*, the optimal regime identified is the temperature of 38˚C - 40˚C, the amount of enzyme 0.15% and the hydrolysis duration 3 - 4 hours.

These findings allow assuming that in the manufacture of yogurt with low lactose or free lactose, the enzyme β-galactosidase can be introduced together with the yogurt starter culture. A maximum lactose hydrolysis degree, at fermentation

temperature 38˚C - 40˚C, will be achieved in the first hours of fermentation, so in the lag phase of the growth curve of microorganisms during fermentation.

Funding

The research was funded by State Project 20.80009.5107.10, nr. PS-62 "personalized nutrition and intelligent technologies for my well-being", running at Technical University of Moldova.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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