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Hydrolysis of UHT milk lactose by partially purified crude enzyme of β-galactosidase obtained from *Lactobacillus plantarum* B123 indigenous strain

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Abstract

Crude enzyme of β-Galactosidase obtained from *Lactobacillus plantarum* B123 indigenous strain after partial purification, by ammonium salt precipitation and membrane filtration, was investigated to hydrolyze lactose in UHT milk at batch process. The β-galactosidase activity contained in the enzyme at 4.8 and 7.2 U/mL milk were applied for the hydrolysis in two types of UHT milks, full cream and low fat milks, with their respective total fats 3.5 and 1.0% w/v. The highest degree of lactose hydrolysis in full cream milk was 60.0% of its initial lactose concentration, achieved by the application of 7.2 U β-galactosidase/mL milk for 9 h at 50°C. The hydrolysis in low fat milk could be at 81.1% under the same condition. Enzyme activity, hydrolysis time and total fat concentration in milk affected the lactose hydrolysis ($P \leq 0.05$). The β-galactosidase contained in the partially purified enzyme has a potential use for commercial production of low lactose milk.

Introduction

Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the milk industry because of some beneficial effects including the elimination of lactose in milk for people with lactose intolerance, prevention of lactose crystallization and contribution to the product sweetness (Jurado *et al*., 2002). Enzymatic hydrolysis by β-galactosidase in milk have been reported by Jokar and Karbassi (2011), Katrolia *et al.* (2011), Ansari and Husain (2012), Erich *et al*. (2012), and Matute *et al.* (2012). Lactose concentration in milk is 4.6% (w/w) in average (Walstra *et al.*, 2006). The content of lactose in low lactose milk is considered to be less than 20% of that in normal milk (Tuure and Korpela, 2004). This means that the concentration of lactose is lower than 1% in final product. This low concentration can be acceptable for people with lactose intolerance problem (Tuure and Korpela, 2004).

β-Galactosidase can be produced by lactic acid bacteria or LAB which has been widely recognized as a safe microorganism. Therefore, β-galactosidase produced by LAB can be used directly in food (Jokar and Karbassi, 2011). *Lactobacillus plantarum* is one of the LAB producing β-galactosidase (Iqbal *et al.* (2010), Schwab *et al.* (2010)). The optimum temperature of β-galactosidase from *Lactobacillus plantarum* was 56°C at pH 6.8 (Schwab *et al.*, 2010). This characterictic was possible to be applied in lactose hydrolysis of milk. Indigenous *Lactobacillus plantarum* B123 strain has been isolated from an Indonesian traditional fermented vegetable product. The use of such microorganism to produce β-galactosidase was expected to increase the number of value added products such as low lactose UHT milk. The objective of this research was to evaluate the potential of β-galactosidase in partially purified crude enzyme from *Lactobacillus plantarum* B123 strain to hydrolyze lactose in two types of milk, full cream and low fat. Analysis of the lactose, glucose and galactose composition in the two milks was also performed by High Performance Liquid Chromatography (HPLC) to characterize the lactose hydrolysis process.

Materials and Methods

Materials and reagents

Indigenous *Lactobacillus plantarum* B123 strain was obtained from a fermented vegetable from

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Indonesia, which had been molecularly identified by Sulistiani in 2012, and has become a collection of Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences, LIPI Cibinong Science Center, Cibinong. This strain has been maintained as a working culture. Full cream and low fat-UHT milks (liquid form) were purchased from the local supermarket in Bogor, Indonesia. Based on label information, protein, fat and carbohydrate content in full cream-UHT milk were 3.5% (w/v), 3.5% (w/v), and 7.5% (w/v) and in low fat-UHT milk were 3.0% (w/v), 1.0% (w/v), and 8.0% (w/v), respectively.

Indigenous *Lactobacillus plantarum* B123 strain was growed in MRSB (de Mann Rogosa Sharpe Broth) consisted of: 10 g/L peptone, 8 g/L beef extract, 4 g/L yeast extract, 10 g/L lactose, 1 g/L Tween 80, 5 g/L sodium acetate, 2 g/L triammonium citrate, 0.2 g/L magnesium sulphate ($MgSO_4.7H_2O$), 0.05 g/L mangan sulphate ($MnSO_4.4H_2O$) and 2 g/L disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$). All the media were obtained from DIFCO (Becton Dickinson Co, USA), whilst the chemicals were obtained from Merck (Merck Co., USA) and Sigma (Sigma Aldrich Chemie Gmbh., Germany). All chemicals used were of analytical grade.

The o-nitrofenil-β-D-galactopiranoside (oNPG), o-nitrofenol (oNP) reagent, lactose and D-galactose standard were obtained from Sigma Aldrich. Bovine serum albumin (BSA), phosphate buffer, Na2CO3, D-glucose standards, ethanol absolute, sulfuric acid, and asetonitrile for HPLC grade were obtained from Merck. Regenerated cellulose acetate membrane 0.45 µm and membrane filter 0.2 µm were purchased from Sartorius Stedim Biotech. Co. (USA).

Production of β-galactosidase crude enzyme with partial purification

A total of 2% inoculum of indigenous *Lactobacillus plantarum* B123 strain with 0.7 optical density (5.00 × 107 cfu/ml) was inoculated into the production medium (MRSB with 1% lactose content and pH 8 of the medium) which had been sterilized and incubated at 37°C. Cells were harvested after 24 h of incubation time. The suspension was then centrifuged at 9500 rpm (14330 × g) for 15 min at 4°C. The pellets were washed twice with 0.05 M sodium phosphate buffer (pH 6.5). Pellets obtained were suspended in 0.05 M sodium phosphate buffer (pH 6.5) with a ratio of 1:5, and then sonicated at 50 kHz and 4°C for 15 min. The resulted precipitate was mixed with its supernatant to re-suspend the cells. The cells suspension was then centrifuged at 9500 rpm (14330 × g) for 15 min at 4°C, subsequently re-sonicated and re-centrifuged. The supernatant obtained was a crude extract of β-galactosidase, called as crude enzyme. β-Galactosidase activity assay to the crude enzyme was determined by Lu *et al*. (2012) with a modification and protein content by the method of Bradford (1976).

Precipitation of enzyme was done by fractionation of 50-60% ammonium sulphate gradually. Ammonium sulphate salt was added gradually in crude extract of β-galactosidase to a concentration of 50% (0-50%) while stirring, until the salt was completely dissolved, and then let for 20 min at 4°C with stirring at 60 rpm. The mixture was then allowed to stand for 1 h at 4°C, and then centrifuged at 9500 rpm (14330 × g) for 15 min at 4°C. The resulted supernatant was further precipitated with ammonium sulphate salt by using the above procedure for 50-60% fractionation. All the precipitates were pooled and dissolved in 0.05 M sodium phosphate buffer (pH 6.5), then centrifuged at 9500 rpm and 4°C for 15 min. The supernatant is an enzyme solution from 0-50% salt fractionation combined with that of 50-60% salt. β-Galactosidase activity and protein concentration of the enzyme solution were measured by using the methods described above.

Partially purified enzyme from the above step was further filtered using a cellophane membrane for overnight at 4°C (with stirrer) as a dialysis process against 0.05 M sodium phosphate buffer (pH 6.5). During the dialysis process, the dialysis buffer was changed three times. β-Galactosidase activity and protein concentration of the dialysate were measured. The dialysate containing β-galactosidase was stored at 4°C prior to lactose hydrolysis experiments. After storage, the enzyme solution was filtered by 0.2 µm membrane filter and measured for β-galactosidase activity and protein concentration prior to lactose hydrolysis of UHT milk.

Optimum pH and temperature of partially purified crude enzyme

The optimum pH of the partially purified crude enzyme containing β-galactosidase was studied by measuring enzyme activity in 0.1 M of phosphate buffer with pH range of 5.0 − 8.0. The optimum temperature was determined by measuring enzyme activity in 0.1 M of phosphate buffer with temperature range of 30 − 60°C at optimum pH.

Hydrolysis of UHT milk by partially purified crude enzyme of β-galactosidase in batch process

The hydrolysis of full cream and low fat-UHT milk in batch process was performed at 50°C and 70 rpm in a shaking waterbath. The lactose hydrolysis was

Table 1. Partial purification of β-galactosidase crude enzyme from *Lactobacillus plantarum* B123 indigenous strain

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	152.55±2.11	13286.35±284.99	87.09	1.00	100
Ammonium sulphate fraction 50-60%	73.66±1.46	7721.01±364.72	104.82	1.20	58.11
Dialysis	25.20±2.26	4284.13±393.51	169.98	1.95	32.24

started by adding 4.8 and 7.2 U of β-galactosidase/ mL milk after pre-warm at 50°C for 10 min. After 0, 1, 3, 5, 7, and 9 h of enzymatic lactose hydrolysis, a portion of sample (2.25 mL) were taken. The reaction in the sample was stopped by heating at 80°C for 15 min. After inactivation, 1 mL of the sample was added with 9 mL of absolute ethanol, and then centrifuged for 60 min at 4°C and 3500 rpm (2270 × g). The supernatant was collected and diluted with distilled water to 10 mL volume in a 10 mL volumetric flask. This solution was subjected to HPLC analysis for determination of sugars composition.

Sugars composition analysis by HPLC

A serial solution of standard mixtures (lactose, glucose and galactose) was prepared at concentrations of 62.5, 125, 250, 500 and 1000 µg/mL to obtain linear curves for sugars quantification. Hydrolysed milk samples and standard solutions were filtered with 0.45 µm cellulose acetate membrane prior to HPLC analysis.

The HPLC analysis was carried out on a HPLC (1200 Series, Agilent Technologies, USA) coupled with refractive index detector and equipped with 20 µL sample loop. The instrument was performed at an isocratic condition using acetonitrile 5% in 5 mM H_2SO_4 as a mobile phase at 0.6 mL/min flow rate. For separating the sugars, Aminex® HPX-87H ion exclusion column (300 mm × 7.8 mm) from Bio-Rad was used.

Statistical analysis

Data were represented as the mean ± standard deviation of 2 hydrolysis experiments. Analysis of variance (ANOVA) with significant level 0.05 was used to determine the effects of enzyme concentration, lactose hydrolysis process time and milk fat concentration. If there was a significant difference ($P \leq 0.05$), then it was followed by the analysis using Duncan's test. All statistical analyses were done with IBM SPSS Statistics 20.

Results and Discussion

Partial purification of β-galactosidase crude enzyme

The results of partial purification of β-galactosidase crude enzyme from *Lactobacillus plantarum* B123 indigenous strain were presented in Table 1. The enzyme was partially purified corresponding to 1.95-fold increase of activity compared to the crude enzyme, and yielded 32.24% of the dry matter of crude enzyme. The β-galactosidase specific activity in the crude enzyme was 87.09 U/ mg protein; this increased to 169.98 U/mg protein after dialysis. The partially purified crude enzyme of β-galactosidase after dialysis was used in its free form to hydrolyzed lactose in UHT milk.

Optimum pH and temperature of partially purified crude enzyme of β-galactosidase

Effect of pH on the activity of crude enzyme after precipitation by ammonium salt and dialysis (dialyzed β-galactosidase crude enzyme) from indigenous *Lactobacillus plantarum* B123 strain evaluated at the pH range of 5.0-8.0. The dialyzed β-galactosidase crude enzyme showed its maximum activity at pH 6.5. This pH is suitable for application of the enzyme in sterilized milk, which has pH 6.6 (Walstra *et al*., 2006).

Effect of temperature on the activity of dialyzed β-galactosidase crude enzyme evaluated in the temperature range of 30°C-60°C.. The dialyzed β-galactosidase crude enzyme exhibited its maximum activity at 50°C. Schwab *et al.* (2010) have reported that the optimum temperature and pH of β-galactosidase from *Lactobacillus plantarum* were 56°C and pH 6.8, respectively, while Iqbal *et al.* (2010) have reported that the optimum temperature and pH of β-galactosidase from *Lactobacillus plantarum* WCFS1 with oNPG substrate were 55°C and 7.5. With this result, the partially purified enzyme was applied further in lactose hydrolysis of full cream and low fat-UHT milks at the optimum temperature

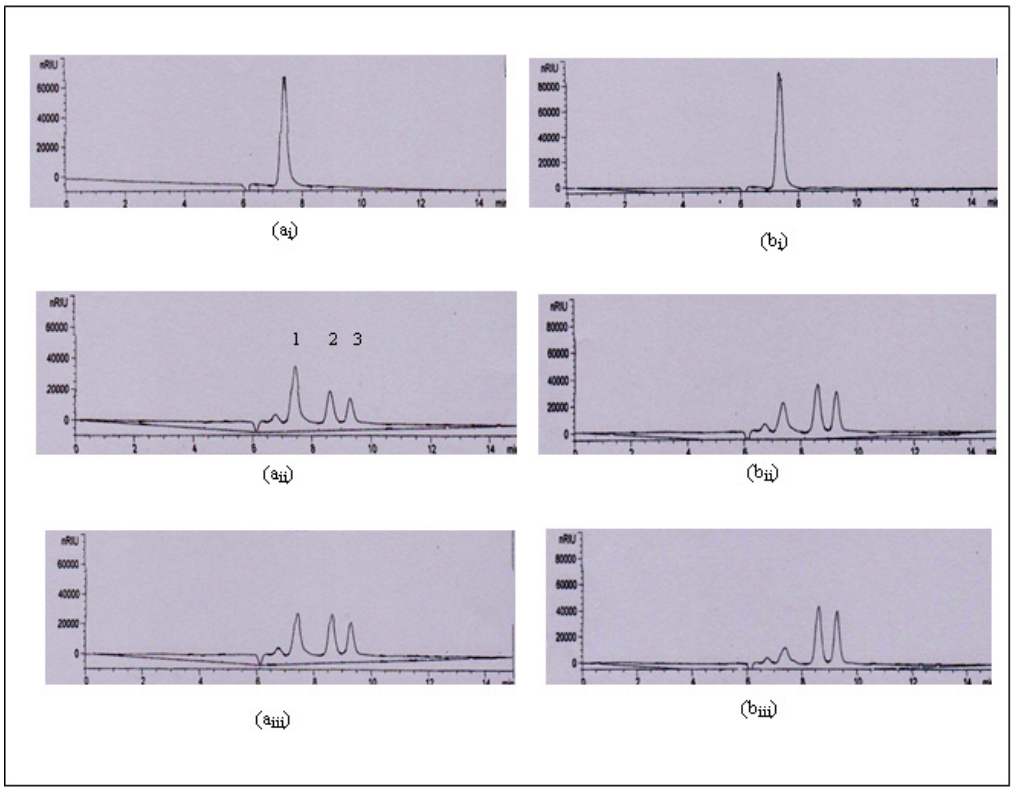

Figure 1. Chromatograms of lactose (1), glucose (2) and galactose (3) using HPLC with RI detector. In lactose-hydrolysed full cream-UHT milk: control or without lactose-hydrolysis (a_i), with the addition of partially purified crude enzyme of β-galactosidase at 4.8 U/mL milk (a_{ii}) and at 7.2 U/mL milk (a_{iii}). In low fat-UHT milk: control (b_i), with the addition of partially purified crude enzyme of β-galactosidase at 4.8 U/mL milk (b_{ii}) and at 7.2 U/mL milk (b_{iii}). Lactose hydrolysis in milk was conducted at temperature 50°C for 9 h.

and pH investigated in this study.

Lactose hydrolysis of full cream and low fat-UHT milks

Lactose hydrolysis in UHT milks by partially purified crude enzyme of β-galactosidase can be characterized by using HPLC analysis results. Chromatogram of lactose, glucose and galactose in samples showed the retention time of lactose, glucose and galactose at 7.4, 8.6, and 9.3 min, respectively. In general, the longer the hydrolysis process at 50°C and the higher β-galactosidase activity unit added in milk gave the lower peak of lactose and the higher peaks of glucose and galactose (Figure 1).

On the basis of HPLC analysis results, full cream and low fat-UHT milks, contained lactose at concentrations of 3.82 and 4.44% w/v respectively. These concentrations are lower than that reported by Walstra *et al*. (2006), 4.7% (w/v) in average. Fresh milk had an average lactose content of 4.91% w/v (Salavuo *et al.*, 2005). The effect of Maillard reaction between protein and reducing sugars, including lactose, on the decrease of lactose content during UHT milk process was reported by Siddique *et al.* (2010). Based on label information of the milk product, protein concentration in low fat-UHT milk was 3.0% (w/v), slightly lower than that in full cream-UHT milk (3.5% (w/v)).

Figure 1 illustrates hydrolysis of lactose in full cream and low fat-UHT milk by the addition of partially purified crude enzyme at 4.8 U/mL milk and 7.2 U/mL milk of β-galactosidase activity in batch mode at 50°C for 9 h. Incubation time for 9 h is considerably for the application in food industry. Jokar and Karbassi (2011) have reported that crude β-galactosidase from *Lb.* ssp. *Bulgaricus* (CHR Hansen Lb-12) with activity 20.9 U/mL and concentration 0.418 U/mL milk could hydrolyzed 78% of lactose after 6 h at temperature 50°C.

Concentrations of lactose, glucose and galactose in full cream-UHT milk after hydrolysis with the partially purified crude enzyme at β-galactosidase activity 4.8 U/mL milk for 9 h at temperature 50°C were 1.93% (w/v), 0.98% (w/v) and 0.75% (w/v) respectively (Figure 2). Concentrations of lactose, glucose and galactose in full cream-UHT milk after hydrolysis with the crude enzyme at higher activity 7.2 U/mL milk for the same time and temperature application were 1.52% (w/v), 1.25% (w/v) and 0.97% (w/v) respectively (Figure 2). Lactose

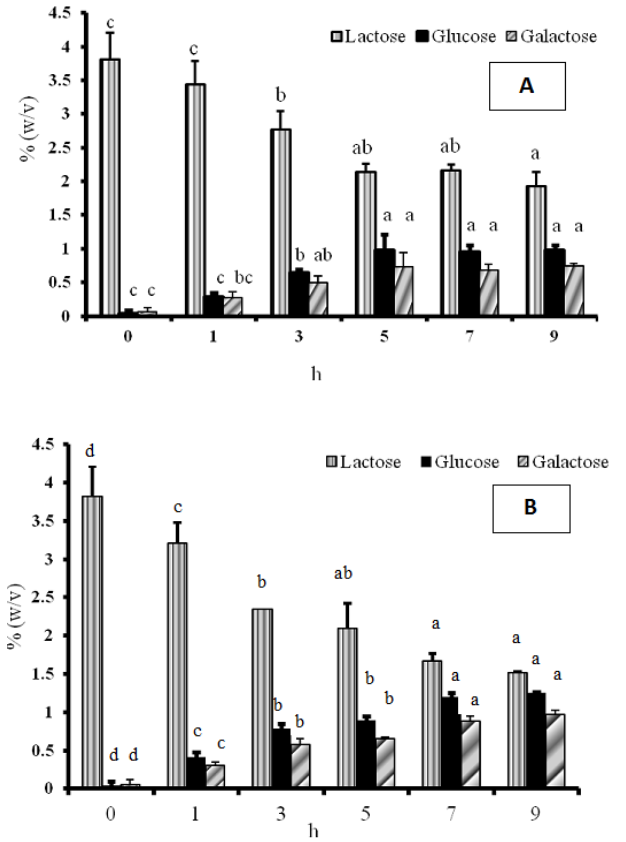

Figure 2. Concentration of lactose, glucose, and galactose in full cream-UHT milk with the addition of partially purified crude enzyme of β-galactosidase, incubation at temperature 50°C at enzyme activity 4.8 U/mL milk (A), 7.2 U/mL milk (B).

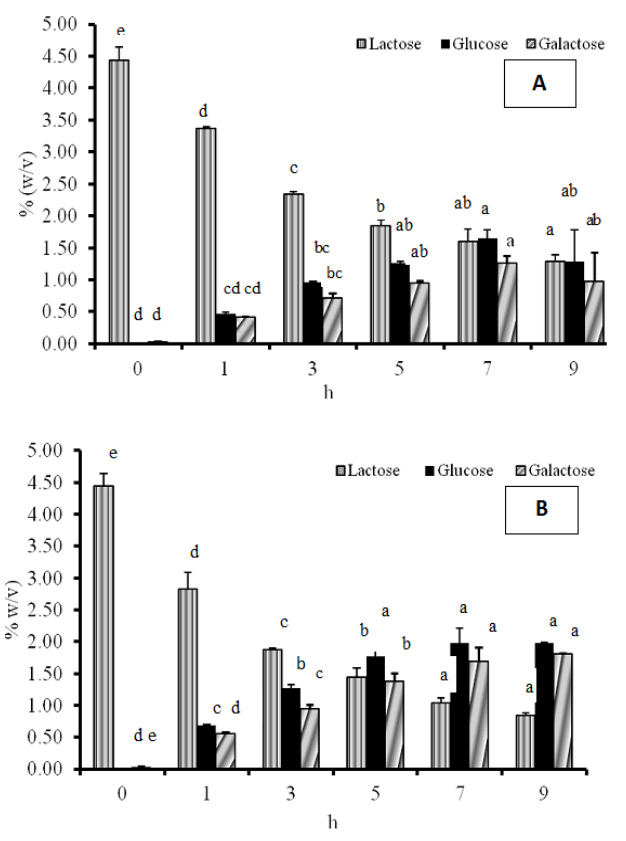

Figure 3. Concentration of lactose, glucose, and galactose in low fat-UHT milk with the addition of partially purified crude enzyme of β-galactosidase, incubation at temperature 50°C, at enzyme activity 4.8 U/mL (A), 7.2 U/mL milk (B).

could be decreased higher at the higher activity of β-galactosidase.

Concentrations of lactose, glucose and galactose in low-fat UHT milk after hydrolysis with the enzyme at activity 4.8 U/mL milk and hydrolysis time 9 h at temperature 50°C were 1.29% (w/v), 1.29% (w/v) and 0.97% (w/v) respectively (Figure 3). Concentrations of lactose, glucose and galactose in the same milk after hydrolysis with the enzyme at higher activity 7.2 U/ml milk and hydrolysis time 9 h at temperature 50°C were 0.84% (w/v), 1.98% (w/v) and 1.81% (w/v) respectively (Figure 3). Again, the higher decrease of lactose occurred in low fat milk with the application of higher activity of β-galactosidase. This result has been predicted in a review by Aehle (2012).

In full cream-UHT milk, the degree of lactose hydrolysis by β-galactosidase was 49.4% by the enzyme addition at 4.8 U/mL of milk and 60.0% at 7.2 U/mL milk. In lowfat-UHT milk, the hydrolysis degree was 71.1% by the enzyme addition at 4.8 U/mL milk and 81.1% at 7.2 U/mL milk (Figure 4). Statistically, the hydrolysis time and the concentration of enzyme significantly affected the degree of lactose hydrolysis ($P \leq 0.05$) in full cream and low fat-UHT milks.

Higher degree of lactose hydrolysis was seen in low fat-UHT milk as compared to that of full cream-UHT milk. This was probably caused by the presence of fat which could inhibit the activity of β-galactosidase to hydrolyze lactose in UHT milk. Statistically, fat content significantly affected the degree of lactose hydrolysis ($P \leq 0.05$).

From the data of glucose and galactose concentrations, the galactose concentrations were lower than glucose concentrations. This might be related to the formation of galactooligosaccharides (GOS). In the presence of highly concentrated lactose, β-galactosidase can produce GOS by transferring galactosyl residues to lactose molecules. Klein *et al*. (2013) have reported about GOS synthesis from immobilized β-galactosidase from *Kluyveromyces lactis*, experiments were performed using 4%(w/v) buffered lactose solution, pH 7 at 37°C. Colinas *et al,*. (2014) have reported that a commercial preparation of *K. lactis* (Lactozym pure) at low dosage (0.1% v/v) with a low content of lactose (2.1-2.7 g/l) is able to give a maximum GOS yield at 4 °C in 5 h.

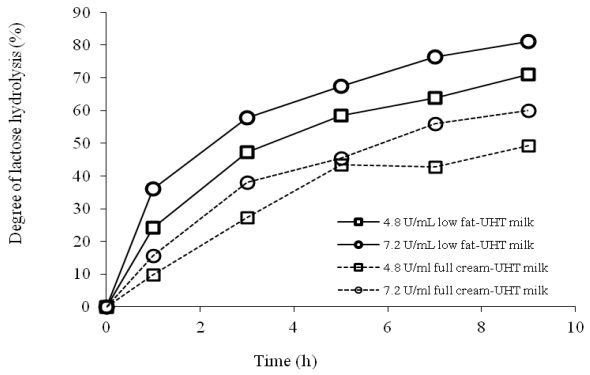

Figure 4. Degree of lactose hydrolysis in full cream and low fat-UHT milks with the application of partially purified crude enzyme of β-galactosidase from *Lactobacillus plantarum* B123 indigenous strain at temperature 50°C.

Conclusions

β-Galactosidase contained in partially purified crude enzyme of indigenous *Lactobacillus plantarum* B123 strain had a potential activity to hydrolize up to 60.0% of lactose in full cream-UHT milk and 81.1% in low fat-UHT milk at β-galactosidase activity 7.2 U/mL milk, with the condition of 9 h hydrolysis at 50°C.

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