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In-house Production of Lactose-hydrolysed Milk by Beta-galactosidase from *Lactobacillus bulgaricus*

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ABSTRACT

Crude Enzyme (beta-galactosidase) Extract (CEE) was produced by *Lactobacillus* ssp. *bulgaricus* CHR Hansen Lb-12 and was applied in sterile milk which had been processed through Ultra High Temperature method (UHT milk), for hydrolyzing lactose. Lactose-hydrolyzed milk was also produced by a pure and commercially available beta-galactosidase (Maxilact). Optimum quantities of CEE and Maxilact enzyme, for producing lactose-hydrolyzed milk, during 6 hours of processing, were 0.418 and 0.512 U ml^{-1}, respectively. Using more than 0.418 U ml^{-1} CEE resulted in unacceptable acidity. Acidity of lactose-hydrolyzed milk produced through 0.418 U ml^{-1} of CEE was significantly increased from 15 to 17 °D, while enhancement of acidity in lactose-hydrolyzed milk produced through Maxilact enzyme was not significant. Total count of lactose-hydrolyzed milk by 0.418 U ml^{-1} CEE, after 6 hours of processing was significantly increased from 5 to 30 CFU (Colony Forming Unit). Sensory evaluation of lactose-hydrolyzed milk and ordinary UHT milk (as control) did not show any significant differences with respect to acceptability of sweetness, taste, aftertaste and color.

Keywords: Beta-galactosidase, Crude enzymatic extract, *Lactobacillus bulgaricus*, Lactose hydrolysis.

INTRODUCTION

Lactose is a disaccharide, found in mammalians' milk. Cow milk and its various side products, are among human main nutritious foods. Therefore, lactose contributes mainly to the daily intake of carbohydrates. Some people cannot tolerate and digest lactose due to a lack of beta-galactosidase in their intestine. Consuming milk and dairy products by these people leads to cramp, flatulence, vomiting, etc [4]. So a valuable source of food would be unavailable for more than half of the people in the world due to their lactose intolerance [21]. Scientists suggested several methods to make many milk products available for lactose intolerant people namely: (1) consuming beta-galactosidase itself during consumption of lactose-containing foods, (2) consuming limited quantities of milk and dairy products, (3) consuming low lactose containing foods in which lactose has been hydrolyzed by beta-galactosidase, and (4) consuming dairy foods that contain viable bacteria to produce beta-galactosidase in consumer's intestine. Showing the importance of the subject, during 1974-1984 approximately 1000 scientific papers were published in contribution to lactose intolerance [18]. The main industrial application of beta-galactosidase is converting lactose to glucose and galactose. Lactose hydrolysis benefits from several advantages of : (1) rapid fermentation of glucose, (2) higher sweetness of the liquid in which lactose has been hydrolyzed, (3) higher solubility of glucose and galactose, (4) high stability of lactose-hydrolyzed dairy products, such as frozen condensed milk, (5) rapid fall of pH in cheese,

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made from lactose-hydrolyzed milk and in consequence rapid development of cheese flavor and texture [10], and (6) using beta-galactosidase in whey, eliminates technological problems (such as sandiness in whey powder and ice cream), while improving the nutritional quality of whey and whey powder. It also leads to the development of novel products and the production of new sweeteners [13]. Another useful application of beta-galactosidase is producing galacto-oligosaccharides, non-digestible food ingredients that advantageously affect the host by selectively stimulating the proliferation of bifidobacteria and lactobacilli in the intestine, considered to be beneficial to human health and which can be synthesized from lactose when the sugar acts as the acceptor and transgalactosylation is catalyzed through beta-galactosidase [4 and 9].

The main sources of beta-galactosidase are microorganisms. In the present research, *Lactobacillus delbrueckii* ssp. *bulgaricus*, has been used to obtain beta-galactosidase. This bacterium is one of the main microorganisms in yoghurt making it highly resistanct to acidity and temperature. So its enzyme can be applied in high temperatures. Lactic acid bacteria are generally recognized as safe, so their enzymes can be used directly in foods without the need for any purification [5, 6, 12, and 21].

Wendorff *et al*. (1971) applied beta-galactosidase from *Saccharomyces fragilis* in milk and dairy products. They concluded that total solids of the medium, except lactose, inhibit enzyme activity, so whey is the best medium for hydrolyzing lactose, as it has low total solids and a high level of lactose. Dalqvist *et al*. (1977) reported that maximum hydrolysis of lactose was obtained when $MgCl_2$ (with 0.1 Molarity, thereafter abbreviated M) and K_2SO_4 (0.1 mM) were added to the medium. Beta-galactosidase in sterile milk and under sterile conditions was stable without any diminition in enzyme activity [7]. Guy and Bingham (1978) found that hydrolyzing lactose in condensed skim milk (40% total solids) was 15% lower than that in ordinary skim milk.

The purpose of this research is to offer a simple way of producing and applying CEE in milk, and also to determine the optimum amount of CEE for controlling the quality of milk.

MATERIALS AND METHODS

CEE was produced by *Lactobacillus* ssp. *bulgaricus* (CHR Hansen Lb-12). For propagation of *Lactobacillus* ssp. *bulgaricus* whey permeate which is a by-product of ultrafiltration of milk, (procured from Pegah Dairy Plant in Shiraz, Iran) was used as the basic medium. One and a half percent sweet whey powder (procured from Ramak Dairy Plant in Shiraz, Iran), 3% yeast extract (Merck Company) and 2% wheat steep liquor were added to it as nutrient supplements. Acidity (pH) of whey permeate was around 6.45, but after adding whey powder and yeast extract, it decreased to 5.8, which we had to adjust. Acidity (pH) of the medium was adjusted to 6.8 through phosphate buffer (pH= 12, 0.1M). Jokar and karbassi (2009) reported the developed medium, as mentioned above, as having the best effect on producing beta-galactosidase. Other researchers have also reported that the best carbohydrate, and protein source to produce beta-galactosidase are lactose and yeast extract, respectively [1, 2, and 11]. CEE was produced according to the following procedure:

Lactobacillus ssp. *bulgaricus* propagated in KM culture (1% skim milk powder, 0.5% glucose, 2.5% yeast extract) was kept in 43°C for 12 hours [17]. One ml of propagated *Lactobacillus* ssp. *bulgaricus* inoculated into 250 ml of the developed medium and incubated at 43°C for 12-13 hours. Microorganisms were collected by centrifugation (Sorvall Rc-5 super speed refrigerated centrifuge) of the medium in 5°C and 4000×g for 10 minutes. Fifty ml of UHT milk was added to the precipitant, then bacterial cell walls were lysed by ultra sonication, (Schoeller and Co Frankfurt am main-sud TG125) at 65% intensity for 10 minutes. Samples were cooled using ice water

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bath to prevent activity loss during sonification. Enzyme activity was determined immediately after sonication according to the procedure of Hestrin *et al.* (1976).

According to this method, 0.1 ml of CEE was diluted by use of 3 ml of phosphate buffer (pH= 7.2, 0.15M), then 1 ml of Orthonitrophenyl beta-D-galactopyranoside (ONPG, N1127- 5G, 120K5307, Sigma-Aldrich, Canada) solution (0.01M) was added to the tubes. Enzymatic reactions were immediately stopped by adding 1 ml of $NaCO_3$ 1M after 5 minutes at 40°C. Using standard calibration curve, concentration of released Orthonitrophenyl (ONP) was determined from the absorbance at 420 nm through spectrophotometry (Jenway 6405 UV/VIS Spectrophotometer).

One unit of activity was defined as the amount of the enzyme, which hydrolyzes 1 µmole of ONPG within 1 minute.

In order to determine the optimum level of CEE for production of lactose-hydrolyzed milk, 2, 3, 5 and 10% (v/v) CEE were added to 250 ml of UHT milk. Temperature and the rate of shaking milk were adjusted to 52°C and 200 rpm in a shaker, respectively (New Brunswick Scientific N.J., U.S.A). Fifty two °C was chosen to reduce proteolytic activity of CEE, which had been at the lowest rate in 50-55°C in comparison with 38-42°C [20]. Lactose-hydrolyzed milk was also produced by use of Maxilact enzyme (DSM Food Specialist Maxilact l2000, France) following exactly the same procedure as CEE. Different levels of Maxilact enzyme (0.01, 0.03, 0.04, and 0.08% v/v) were employed. The rate of lactose hydrolysis was determined at intervals (2, 4, 6, and 8 hours following processing). Lactose hydrolysis was indirectly determined through assessment of glucose content. According to the method of Worthington Biochemical Cooperation [24], 1 ml of milk was transferred into a tube, for the clarification of which, 2 ml of $Ba(OH)_2$ 1.8% w/v (BDH) and 2 ml of $ZnSO_4$ 2% w/v (BDH) were added to the tube, respectively. After filteration of the liquid, glucose content of 0.1 ml of clarified liquid was determined through glucose oxidase kit (Man Company, Tehran, Iran). Initial lactose content of UHT milk was determined through phenol-sulfuric acid method.

Quality Control of Lactose-hydrolyzed Milk

Acidity according to Dornic degree method ($^{\circ}$D) (Titration by NaOH 1.9 Normal) and total count of microorganisms (surface culture by standard method agar) were determined in lactose-hydrolyzed milk before and after processing [15].

Sensory Evaluation

According to the method of Jellinek (1990), the related sensory thresholds of several people were initially determined. Different solutions of four basic tastes (Sweetness, Bitterness, Sourness, and Saltiness) were offered to tasters to see if they can effectively distinguish the different degrees of the tastes. Four basic solutions were prepared according to Table 1, as offered by Jellinke (1990).

Twelve people, the thresholds of whose sensory, met the requirements were invited. Sensory was evaluation performed in Hedonic 5-point Scaling Test. Nonparametric Kruskal-Wallace Test was employed for analysis of sensory evaluation by Mstatc software (Michigan State University).

All experiments and analyses were carried out in triplicate. Statistical significance of differences ($P< 0.05$) was determined by ANOVA and *F*-test where appropriate. All given values are therefore means arrived at out of 3 replicates. Statistical analyses were done through Costat software (CoHort software).

Table 1. Basic solutions prepared and offered for determining the thresholds of tastes' of the panel.

Basic tastes	Gr 100 ml^{-1} solution
Sweetness (Sucrose)	0.33
Saltiness (NaCl)	0.14
Sourness (Citric acid)	0.027
Bitterness (Caffeine)	0.0048

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RESULTS AND DISCUSSION

Unit activity of CEE in UHT milk was 20.9 U ml^{-1} following lysing of lactose. The optimum level of CEE for hydrolyzing lactose in UHT milk which resulted in 78% of lactose hydrolysis was 0.418 U ml^{-1}. Unit activity of Maxilact enzyme was 1,280 U ml^{-1}. Using 0.512 U ml^{-1} Maxilact in UHT milk resulted in 90% of lactose hydrolysis.

Lactose hydrolysis through different quantities of CEE is presented in Table 2. Initial lactose content in UHT milk was 4.78%. As seen, using CEE more than 0.418 U ml^{-1} resulted in clotting of milk. UHT milk has not been clotted when the milk processed using Maxilact enzyme. Clotting of milk has mainly been related to the enhancement of acidity due to the growth of viable thermophile bacteria in CEE, which was about 50 CFU ml^{-1}. Although the activity of other enzymes, especially proteases is very low at the temperature of processing, but clotting may be related to minor activity of these enzymes for a duration of 6 hours. This issue calls for more investigation.

Variations of acidity and total counts of microorganisms in milk before and after being processed by beta-galactosidase are shown in Tables 3 and 4. As indicated in Tables 3 and 4, the increase of acidity and total counts of microorganisms in milk as a result of CEE is significant.

It seems that the number of bacteria in 0.418 U ml^{-1} of CEE and the level of proteolytic enzymes were not enough to increase acidity in the milk. It may please be noted that the quality of UHT milk before processing is very important.

Vasiljevic and Jelen (2002) reported enhancement of bacteria during processing of milk by crude beta-galactosidase. They concluded that the remaining viable cells contained in the CEE (50 CFU) may present a potential for undesirable growth, which could affect the quality of a final product. Milk as a suitable medium, provides all the necessary nutrients for dairy cultures and the availability of glucose from lactose hydrolysis and the other possible products of proteolytic activity would further create suitable conditions to enhance the active growth of *Lactobacillus bulgaricus*.

Lactobacillus delbrueckii ssp. *bulgaricus* is a thermophile bacterium, so the enzymes, which are related to this bacterium, are active in high temperature ranges (49-57°C). Thermal stability of beta-galactosidase from thermophilic bacteria would make the hydrolysis of lactose possible in high temperature ranges, in which mesophilic bacteria cannot grow. However, in the present research it was found that the initial number of microorganisms in CEE was critical.

Using different quantities of Maxilact enzyme in UHT milk did not present any problems, because acidity and viable bacteria did not increase significantly in the milk. The results of using different quantities of Maxilact enzyme in UHT milk are presented in Figure 1.

Using 0.512 U ml^{-1} of Maxilact enzyme, 90% of lactose content was hydrolyzed, while 75% of the lactose content was hydrolyzed by using 0.384 U ml^{-1} during 6 hours of processing.

The optimum times (Figures 2 and 3) for processing UHT milk through CEE and Maxilact enzyme were 6 hours. Processing of milk for more than 6 hours did not practically increase lactose hydrolysis. The rates of lactose hydrolyzing at different intervals by Maxilact and CEE are shown in Figures 2 and 3. After 6 hours of processing, Maxilact and CEE hydrolyzed 90% and 78% of lactose content, respectively. Levels 0.418 U ml^{-1} of

Table 2. Lactose hydrolysis due to different quantities of CEE in UHT milk.

CEE (U ml^{-1})	Processing time (hr)	Lactose hydrolysis (%)
0.418	6	78 %
0.627	6	Milk was clotted. 80%
1.045	6	Milk was clotted. 85%
2.09	5	Milk was clotted. 95%

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Table 3. Variation of acidity during UHT milk processing by CEE and Maxilact enzyme.

Product	Acidity (°Dornic)	
	Starting point	6 hours after processing
Milk with 0.512 U ml^{-1} Maxilact enzyme	14^a	15^a
Milk with 0.418 U ml^{-1} CEE	15^b	17^a
UHT milk	14^a	15^a

Different letters are indicative of the significant difference (P< 0.05).

Table 4. Variation of total counts of microorganisms during processing of milk through CEE and Maxilact enzyme.

Product	CFU per 1 ml milk	
	Starting point	6 hours after processing
Milk with 0.512 U ml^{-1} Maxilact enzyme	3^a	3^a
Milk with 0.418 U ml^{-1} CEE	5^b	30^a
UHT milk	2^a	5^a

Different letters are indicative of the significant difference (P< 0.05).

CEE, and 0.384 U ml^{-1} of Maxilact hydrolyzed nearly 75% of lactose. More CEE was used up for hydrolysis of the same amount of lactose. As shown, acidity increased during processing of UHT milk through CEE, this can be a reason for lower hydrolysis of lactose through CEE, because the extent of beta-galactosidase activity is lower in solutions with a high level of acidity. This issue reflects the same results as those by other researchers (Ladero *et al*., 2006; Pessela *et al*., 2003).

In the present research, 0.512 U ml^{-1} of Maxilact enzyme and 0.418 U ml^{-1} of CEE were employed to hydrolyze lactose content in 0.1 liter of milk during 6 hours. Pastore and Morisi (1976) used immobilized beta-galactosidase on fiber. After 20 hours, 75% of lactose content of milk (milk flow was 7 L min^{-1}) was hydrolyzed. The amount of the enzyme, which was necessary for hydrolyzing 75% lactose in 7 liters of milk for a duration of 20 hours, was around 750 unit activity. Zhou and Chen (2001) increased the rate of hydrolyzing lactose from 1.1 to 7.7 by immobilizing the enzyme on graphite surfaces. It is undrestood that immobilized enzyme increases the yield of conversion. CEE has different enzymes and proteins, so its immobilization is in need of further research.

Sensory Evaluation

According to the reports of the taste

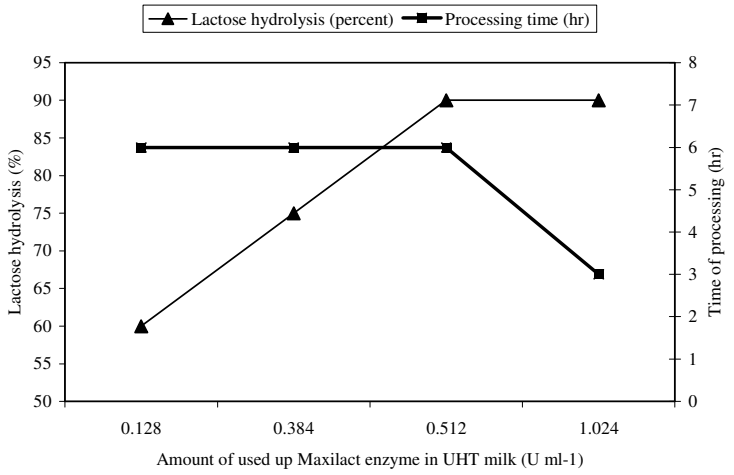

Figure 1. Lactose hydrolysis by different quantities of maxilact enzyme in UHT milk.

Uml^{-1})

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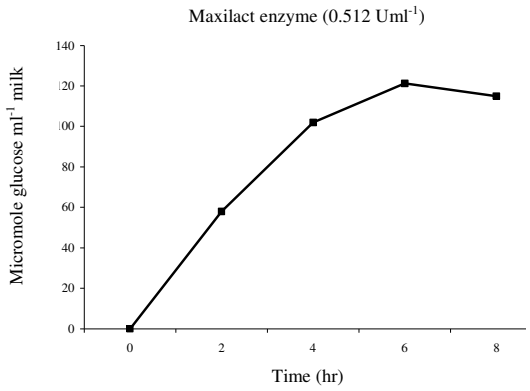

Figure 2. Lactose hydrolysis by different quantities of CEE at the indicated intervals. Values are means resulted from 3 replicates.

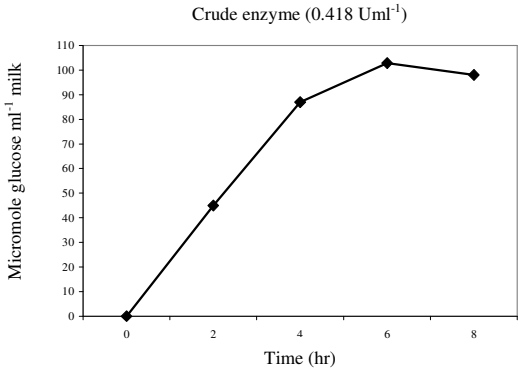

Figure 3. Lactose hydrolysis by different quantities of CEE at the presented intervals. Values are means calculated, using 3 replicates.

panel, lactose-hydrolyzed milk obviously tasted sweeter than ordinary UHT milk. Lactose-hydrolyzed milk, produced by 0.512 U ml^{-1} Maxilact enzyme got the lowest score in respect of aftertaste, however it was selected as the best sample with respect to its sweetness and taste. Lactose-hydrolyzed milk produced through 0.418 U ml^{-1} of CEE had the lowest score with respect to taste. UHT milk was the most acceptable sample with regard to its aftertaste and the least acceptable regarding its sweetness. However, the statistical analyses of these data (Tables 5-7) did not show any significant differences ($P< 0.01$) among the overall acceptability of the three samples.

CONCLUSIONS

In-house production of crude beta-galactosidase prepared from thermophilic lactic acid bacteria could be technologically and economically feasible. Due to the extraordinary costs of purifying enzymes, we can apply CEE directly in sterile milk. If milk is processed at 52°C, around 80% of lactose content will be hydrolyzed by using 0.418 U ml^{-1} of CEE without any significant problems

Table 5. Scores and results of Kruskal-Wallace Test for taste.

Test criterion: 1.1428	Probability: 0.5647											
sample 1	4	3	4	4	4	4	3	4	4	2	3	3
sample 2	1	4	4	5	5	3	3	3	5	4	4	3
sample 3	4	3	3	3	3	3	4	3	4	3	4	4

Table 6. Scores and results of Kruskal-Wallace Test for aftertaste.

Test criterion: 0.1671	Probability: 0.9198											
sample 1	4	3	3	4	4	4	2	4	4	3	4	3
sample 2	2	3	4	4	5	4	3	4	3	5	4	2
sample 3	5	4	3	4	3	3	3	4	4	4	3	4

Table 7: Scores and results of Kruskal-Wallace Test for sweetness.

Test criterion: 0.7169	Probability: 0.6978											
sample 1	5	3	4	4	3	3	4	5	4	5	4	2
sample 2	3	5	3	4	4	3	2	4	4	5	5	3
sample 3	3	4	3	4	4	3	5	3	3	4	4	3

Sample 1: UHT milk.
Sample 2: Lactose hydrolysed milk through 0.418 U ml^{-1} of CEE.
Sample3: Lactose hydrolysed milk through 0.512 U ml^{-1} of Maxilact.

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with regard to protein decomposition, enhancement of acidity and bacterial content. Lactose-hydrolyzed milk produced through CEE and Maxilact enzyme does not show any significant differences in comparison with UHT milk with respect to sensory characteristics. Further research to immobilize CEE is highly recommended.

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تولید خانگی شیر با لاکتوز هیدرولیز شده توسط آنزیم بتا-گالاکتوسیداز

ا. جوکار و ا. کرباسی

چکیده

آنزیم بتا گالاکتوسیداز ناخالص توسط باکتری *Lactobacillus* ssp. *bulgaricus* CHR Hansen Lb-12 تولید و به منظور هیدرولیز لاکتوز در شیر فرا دما (UHT) به کار گرفته شد. شیر با لاکتوز هیدرولیز شده توسط آنزیم بتا-گالاکتوسیداز ناخالص و یک نوع آنزیم خالص و تجارتی به نام Maxilact نیز تولید شد. هیدرولیز لاکتوز با اندازه گیری مقدار گلوکز شیر اندازه گیری گردید. مقادیر بهینه آنزیم بتا-گالاکتوسیداز ناخالص و آنزیم Maxilact در طی ۶ ساعت فرآوری به ترتیب برابر با ۲ و ۰/۰۴ درصد بود. به کار گرفتن بیشتر از ۲ درصد بتا-گالاکتوسیداز ناخالص منجر به افزایش غیر قابل قبول در اسیدیته شیر گردید. اسیدیته شیر با لاکتوز هیدرولیز شده توسط آنزیم ناخالص به طور قابل توجهی از ۱۵ به ۱۷ درجه دورنیک افزایش یافت. در حالیکه افزایش اسیدیته در شیر تولیدی توسط Maxilact قابل ملاحظه نبود. شمارش کلی شیر تولیدی با آنزیم ناخالص، پس از ۶ ساعت فرآوری به طور قابل توجهی از ۵ به ۳۰ CFUافزایش یافت. هر ۲ نوع شیر با لاکتوز هیدرولیز شده و شیر معمولی فرا دما (به عنوان نمونه کنترل) از نظر مقبولیت شیرینی، طعم، پس طعم و رنگ هیچ تفاوت قابل ملاحظه ای نداشتند.

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