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Improving Galacto-Oligosaccharide Content in the Production of Lactose-Reduced Yogurt

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ABSTRACT
In a lactic fermentation process with probiotic microorganisms and the simultaneous addition of β-galactosidase, the reduction of lactose content and the formation of galacto-oligosaccharides were evaluated. The fermentation was promoted by lactic culture containing two probiotic microorganisms, *Bifidobacterium animalis* and *Lactobacillus acidophilus*, associated with the typical microorganisms of yogurt, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. An enzymatic preparation containing β-galactosidases from *Kluyveromyces lactis* and *Aspergillus niger* was used. A central composite rotational design (CCRD) and a total of 10 assays (2^2 assays plus axial points and two replicates at the central point) were conducted in order to evaluate the effects of enzyme concentration and the time of addition of the enzyme. Based on an experimental design, empirical models for the final lactose concentration and GOS concentration were proposed. The following conditions were established in order to maximize GOS concentration: enzyme concentration of 0.44 g/L and enzyme addition after 90 min from the beginning of fermentation. In these conditions a ten-fold increase in GOS concentration and a four-fold decrease in lactose concentration were observed in comparison with fermentation without enzyme addition.

Keywords **-** enzymatic catalysis, β-galactosidase, galacto-oligosaccharides, lactose hydrolysis, lactic fermentation.

I. INTRODUCTION

Research on lactose conversion processes using enzyme technology have always been for the purpose of developing products with a low content of this disaccharide, either for individuals who present intolerance to its consumption or to avoid unwanted technological characteristics, such as the crystallization of this carbohydrate [1].

Lactose intolerance affects a significant proportion of the worldwide adult population, with a major impact on the Asian, African, Amerindian and Aboriginal communities, at levels that may be in excess of 90% [2,3].

Commercial β-galactosidases obtained from *Kluyveromyces* yeasts have a range of action close to neutral pH (7.0-6.5), while β-galactosidases from *Aspergillus* act at an acidic pH (5.5 to 4.5). Their application to reduce the lactose content in milk products is widely known [4,5].

More recently, commercial preparations capable of acting on a wide range of pH have been available, with great potential for use in lactic fermentation because hydrolytic activity remains throughout the acidification process.

The addition of β-galactosidase in the manufacturing of low-lactose fermented milks usually occurs at two stages. In the first stage, enzymatic catalysis occurs in a substrate at a pH between 6.5 and 6.8 and temperatures between 4 and 6^{o}C, in order to minimize microbiological contamination. This temperature takes the contact time to at least 30 h. The substrate with low lactose content then continues to the second stage of processing, when the inoculation of lactic culture and early fermentation take place. A single-stage process with simultaneous enzymatic catalysis and lactic fermentation can overcome the drawbacks of the two-stage process [6].

On the order hand, β-galactosidase (EC 3.2.1.23) catalyzes both hydrolysis and transgalactosylation reactions of β-galactopyranosides and lactose. In the transgalactosylation mechanism, galacto-oligosaccharides (GOS) are produced. These carbohydrates are formed by a chain of 3-8 galactose units with a glucose end-cap [7,8].

Many positive physiological effects are ascribed to GOS. They are considered to be bifidogenic factors, since they are resistant to enzymatic digestion in the upper gastrointestinal tract and, thus, act as substrate for bifidobacteria, stimulating their metabolism and growth in the human intestine [8,9].

The beneficial effects to the gastrointestinal tract include the modulation of the immune system through anti-adhesive properties, which indicates possible reductions in the risks of colon cancer [10]. Moreover, there are nutritional benefits, such as the absorption of desirable minerals (especially calcium and magnesium) [11], and digestive advantages, including the regulation of intestinal transit [9].

In this study, the formation of GOS was evaluated during the obtainment of yogurt with low lactose content in a single-stage process.

This process involves simultaneous enzymatic catalysis promoted by the commercial β-galactosidase preparation Lactomax Flex, a mixture of *Kluyveromyces lactis and Aspergillus niger* β-galactosidases, and lactic fermentation promoted by the commercial lactic culture ABY-3.

A central composite rotational design (CCRD) was proposed in order to reconcile the reduction in lactose content and the increase in GOS concentration.

II. MATERIALS AND METHODS

2.1. Substrate

The milk base was obtained from whole milk powder (Cosulati, Brazil), in a single reconstitution with distilled water in order to obtain approximately 90 g/L lactose.

2.2. Enzyme preparation

The commercial enzyme Lactomax Flex (Prozyn, Brazil) was used, a liquid formulation composed of β-galactosidases produced by *Kluyveromyces lactis* and *Aspergillus niger*.

2.3. Lactic culture

The commercial freeze-dried ABY-3 lactic culture was used (Chr Hansen, Brazil), containing *Lactobacillus delbruekii*, *Streptococcus salivarius*, *Bifidobacterium animalis* and *Lactobacillus acidophilus*.

2.4. Lactic fermentation

As recommended by the manufacturer, the content of the lactic culture package was added to 500 mL of UHT milk and packaged in previously sterilized bottles. The inoculum (10^{11} CFU/mL) was stored under refrigeration and 4 mL dosed in each batch of 2 L of substrate.

The substrate underwent heat treatment at 90 ± 1ºC for 5 ± 1 min to denature the protein and minimize contamination risks. The fermentation processes were carried out in a Biostat B bioreactor (B. Braun Biotech International, Germany), with 2 L capacity. The temperature was maintained at 43ºC. The fermentation process was interrupted by cooling when the pH reached 4.70.

In a single-stage process with simultaneous exogenous β-galactosidase action and lactic fermentation, a CCRD with two central points was proposed, resulting in a total of 10 assays (Table 1) in order to evaluate the effects of the initial enzyme concentration (E) and the time of addition of the enzyme (t) on the final lactose concentration and GOS concentration. Furthermore, a control experiment, without enzyme addition, was conducted (Table 1). The data were treated using Statistica 5.0 software (Statsoft Inc., United States).

Lactose conversion (C_L) was calculated according to (1), where Lac_I and Lac_F, represent the initial and residual lactose concentrations:

$$L_C(\%) = \frac{Lac_I - Lac_F}{Lac_I} * 100 \quad (1)$$

2.5. Analytical determinations

The quantification of sugars (lactose, glucose, galactose and GOS) was carried out by ion exchange chromatography with pulsed amperometric detection (HPLC-PAD).

A Dionex (United States) chromatograph, supplied with a Carbopac PA1 (4x250 mm) column, a PA1 (4x50 mm) guard column, with a GP50 gradient pump, ED40 electrochemical detector and Peaknet software were used for the analyses. Sugars were eluted with 20 mM NaOH, at a flow rate of 1.0 mL/min. Before injection, the samples were diluted with water and filtered through 0.22 μm filters [12].

III. RESULTS AND DISCUSSION

In order to obtain a lactose-reduced yogurt with significant content of GOS, a 2^3 CCRD was proposed. The results regarding the final concentrations of lactose (Lac), galacto-oligosaccharides (GOS), glucose (Glu) and galactose (Gal) are shown in Table 1.

The best value for lactose conversion (a reduction of 63.1% in relation to the initial concentration) was achieved when the enzyme concentration was fixed at level +1 (0.44 g/L) and the time of the addition of the enzyme at level -1 (60 min).

This fact can be associated with a more effective action of the enzyme on the lactose, resulting from a combination of a higher enzyme concentration and a longer time for the enzyme to act.

Table 1. Matrix of experimental design and concentrations of carbohydrates at the end of fermentation

Run	E (g/L)	t (min)	Lac (g/L)	Glu (g/L)	Gal (g/L)	GOS (g/L)	C_L (%)
1	–1 (0.16)	–1 (60)	53.5	16.1	16.3	1.7	39.5
2	–1 (0.16)	+1 (90)	58.6	12.4	15.5	2.3	36.8
3	+1 (0.44)	-1 (60)	33.1	26.1	24.6	3.5	63.1
4	+1 (0.44)	+1 (90)	40.5	22.7	21.0	4.9	54.6
5	0 (0.30)	–1.41 (54)	36.9	24.0	23.0	3.2	58.5
6	-1.41 (0.10)	0 (75)	68.1	9.2	9.8	0.9	23.4
7	0 (0.30)	+1.41 (96)	49.2	17.3	18.2	4.7	45.4
8	+1.41 (0.50)	0 (75)	40.2	23.4	20.8	4.1	54.9
9	0 (0.30)	0 (75)	44.8	20.3	20.0	3.5	49.7
10	0 (0.30)	0 (75)	46.1	19.3	19.5	3.6	48.4
Control*	-	-	77.5	1.8	10.3	0.4	13.5

* Without enzyme addition.

In the work of Toba et al. [13], in which yogurt was obtained with the simultaneous addition of β-galactosidase from *Aspergillus oryzae*, 64% lactose conversion was achieved in 8 h of processing by adding the enzyme together with the starter culture. In the present work, we obtained similar values (Assay 3) in 4 h of processing.

According to Toba et al. [13], yogurt with 50% to 80% lactose depletion received the highest organoleptic scores. Therefore, the conditions corresponded to Assays 3, 4, 5 and 8, which were within this range (Table 1).

More recently, Rodriguez et al. [14] performed simultaneous fermentation and catalysis using goat milk as a substrate and an enzyme obtained from *Aspergillus oryzae* applied together with the dairy culture, obtaining a conversion of 82.6%, but with an initial substrate concentration of 44.2 g/L lactose.

Furthermore, it is worth noting that a considerably lower lactose concentration was achieved in all experiments (Table 1) in comparison with fermentation without the enzyme (13.5%).

Concerning the GOS concentration, the control assay showed that the starter culture is not capable of producing significant quantities of GOS (0.4 g/L) in comparison with the values reported for some commercial yogurts. In the work of Martínez-Villaluenga et al. [15], yogurts containing bifidobacteria had 357 to 585 mg GOS/100 g while conventional yogurts showed the lowest amounts (223 to 249 mg GOS/100 g). This fact enhances interest in increasing the content of GOS with the addition of the exogenous enzyme.

From the data presented in Table 1 (from Assay 1 to Assay 10), empirical coded models were proposed to describe final lactose concentration (2) and GOS concentration (3) as a function of the enzyme concentration and the time when the enzyme was added. Table 2 shows the analysis of variance (ANOVA) used to evaluate the adequacy of the fitted models ($p<0.05$).

Table 2. ANOVA for final lactose and GOS concentrations

Source of Variation	Sum of squares		Degrees of freedom		Mean squares		$F_{(calculated)}$	
	Lac	GOS	Lac	GOS	Lac	GOS	Lac	GOS
Regression	987.05	14.58	3	5	329.02	2.92	67.91	291.60
Residual	29.07	0.04	6	4	4.84	0.01		
Total	1016.12	14.62	9	9				

$F_{(tabulated)\ 3,\ 6,\ 0.05} = 6.94$; $R^2 = 0.97$ (Lac).

$F_{(tabulated)\ 3,\ 6,\ 0.05} = 6.94$; $R^2 = 0.99$ (GOS).

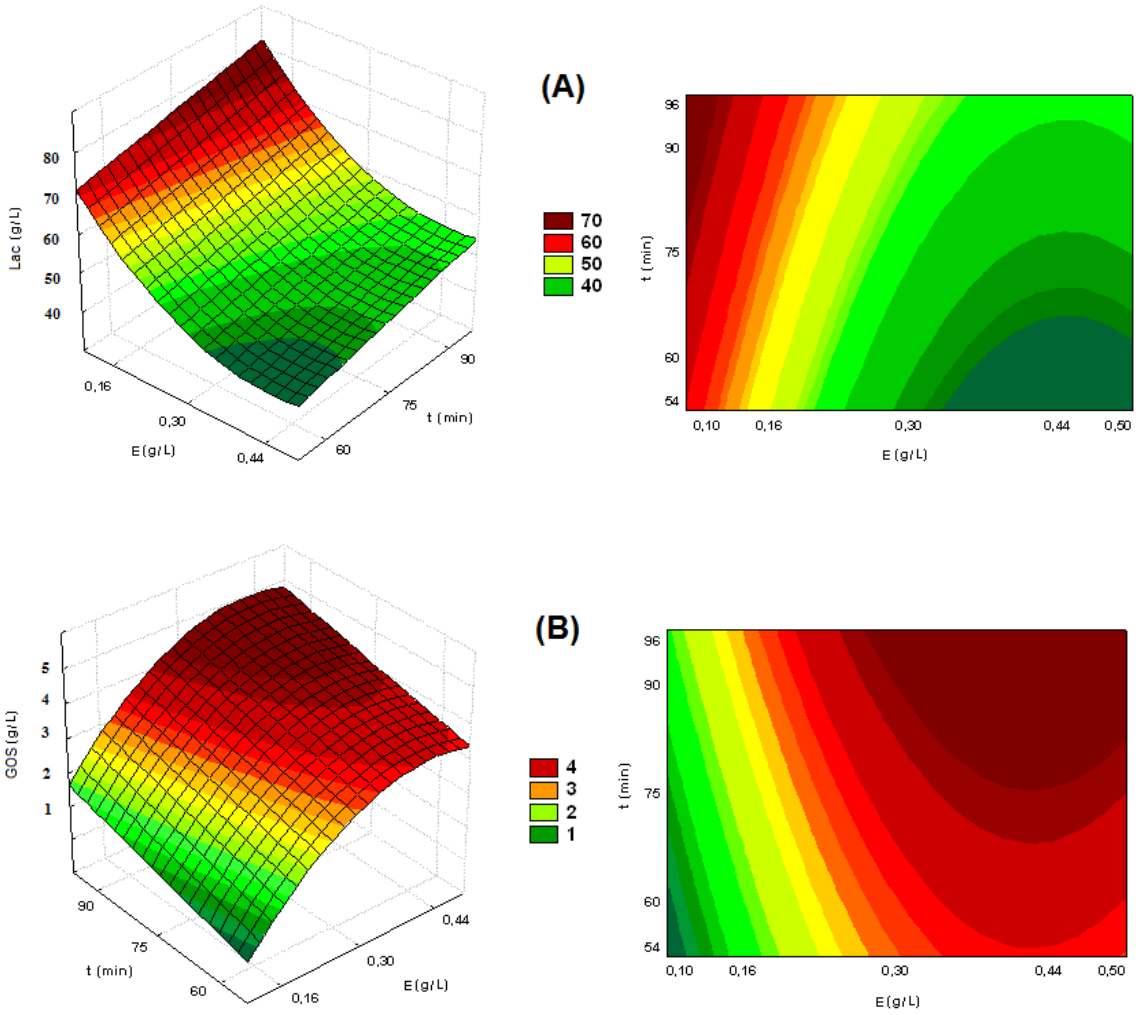

Figure 1. Response surfaces and contour diagrams for final lactose concentration (A) and GOS concentration (B) as a function of enzyme concentration (E) and time of addition of the enzyme (t).

The determination coefficient (0.97 and 0.99 for lactose and GOS concentration, respectively) and F test (9.8 and 42.0 times higher than the listed values, respectively, for lactose and GOS) were very good. Consequently, the coded models were considered predictive and can be used to generate the response surface for final lactose and GOS concentrations.

$$\text{Lac(g/L)} = 43.5 - 9.8E + 4.6E^2 + 3.7t \quad (2)$$

$$\text{GOS(g/L)} = 3.6 + 1.1E - 0.6E^2 + 0.5t + 0.2t^2 + 0.2(E \cdot t) \quad (3)$$

Regarding the final lactose concentration, the best result was obtained when there was a higher enzyme concentration and a lower time for the addition of the enzyme, as shown in Fig. 1-A.

However, in relation to GOS concentration, the best result was obtained when there was a higher enzyme concentration and a higher time for addition of the enzyme (Fig. 1-B).

The kinetic profiles of the experiments shown in Table 1 were obtained for the purpose of verifying the consumption of lactose and the formation of glucose, galactose, and GOS over time.

Fig. 2 shows the profiles of the most relevant assays for the production of GOS (Assays 4, 7 and 8) and the conversion of lactose (Assay 3).

For all the assays, a consumption of lactose and a release of glucose, galactose and GOS were observed over time. In Assays 3 and 8 (Fig. 2A and 2D), hydrolysis of GOS occurred during the fermentation, resulting in a depletion of GOS concentration of 23.3% and 43.5%, respectively, considering the maximum concentration and the final concentration.

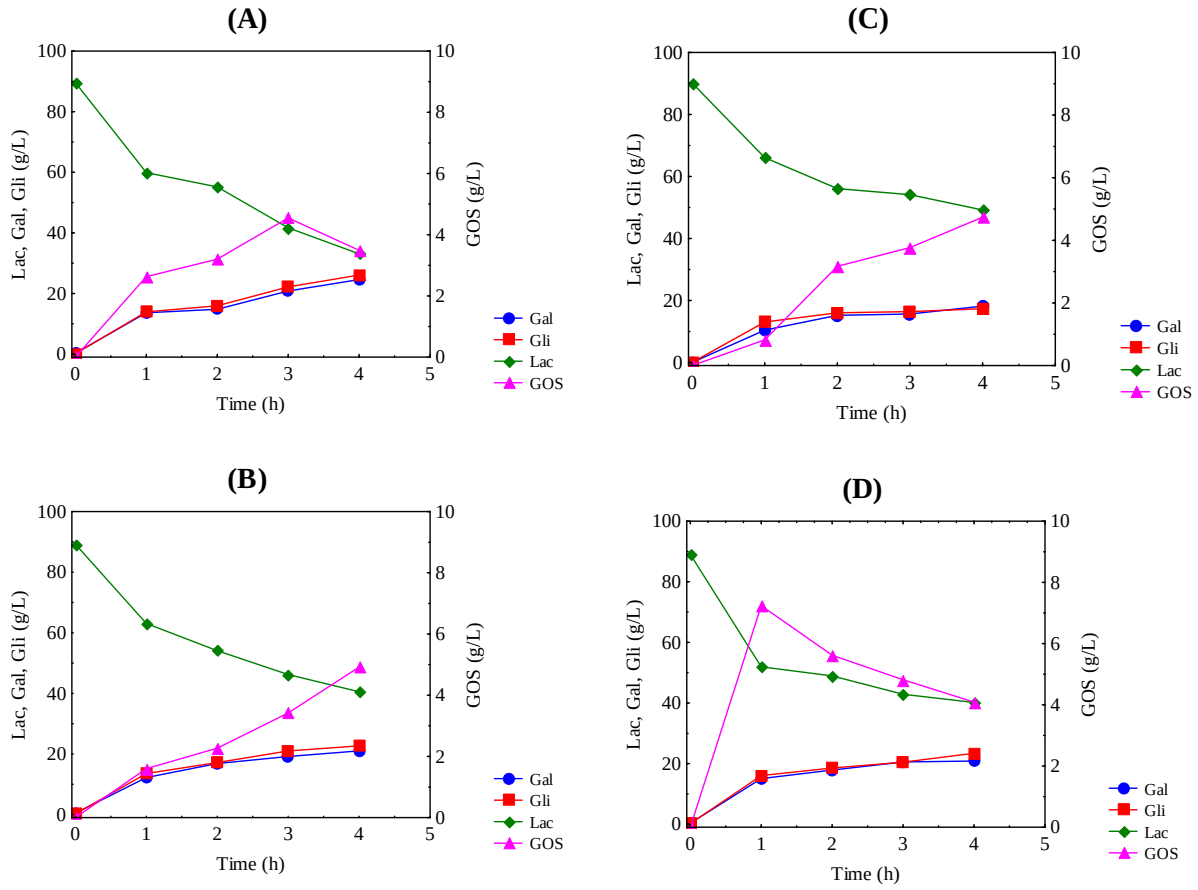

Figure 2: Carbohydrate composition changes during simultaneous lactic fermentation and enzymatic catalysis. (A) Assay 3; (B) Assay (4); (C) Assay 7; (D) Assay 8.

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However, in Assays 4 and 7 (Fig. 2B and 2C), GOS hydrolysis was not observed, probably due to the addition of the enzyme 90 min and 96 min after the process had begun, resulting in less time of action for the enzyme during fermentation.

IV. CONCLUSION

In the search for good conditions to obtain yoghurt with a reduction in lactose content and a higher GOS concentration, with simultaneous lactic fermentation and enzymatic catalysis, the enzyme concentration had a positive effect on the reduction of lactose content.

The late addition of the enzyme, relative to the beginning of fermentation, led to an increase in GOS concentration, but reduced the conversion of lactose. When 0.44 g/L of enzyme were used and it was added 90 min after the process had begun, it was possible to achieve a significant concentration of GOS and a decrease in lactose content without GOS hydrolysis during the fermentation process.

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