

## Lactose conversion and the synthesis of galactooligosaccharides in a simultaneous lagged bioprocess using $\beta$ -galactosidases and probiotic microorganisms

*Conversão da lactose e síntese de galacto-oligossacarídeos em bioprocesso simultâneo defasado com  $\beta$ -galactosidases e microrganismos probióticos*

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### ■ Summary

Lactose conversion and the production of galactooligosaccharides (GOS) were evaluated in a simultaneous bioprocess using probiotic microorganisms and the addition of  $\beta$ -galactosidases, lagged or otherwise. The fermentation was carried out using normal yogurt cultures, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, associated with two probiotic microorganisms, *Bifidobacterium animalis* and *Lactobacillus acidophilus*. An enzymatic preparation containing  $\beta$ -galactosidases of distinct origins, obtained from *Kluyveromyces lactis* and *Aspergillus niger* was used. The simultaneous bioprocess was evaluated, varying the initial lactose and enzyme concentrations and also the time of addition of the enzymes, with a lag in the enzyme application in relation to the beginning of fermentation. The results indicated a positive impact on lactose conversion, reducing the disaccharide concentration by between 97.7 and 99.7%, as compared to 13.8 and 19.8% in fermentations without enzyme addition, obtaining between 2.7 and 4.2 g.L<sup>-1</sup> GOS in the lagged processes carried out with the highest initial lactose concentration and the addition of the lowest enzyme concentration.

**Key words:** Enzyme catalysis; Lactose conversion; Lactic fermentation; GOS.

### ■ Resumo

Em bioprocesso simultâneo conduzido por microrganismos probióticos e através da adição, defasada ou não, de  $\beta$ -galactosidases, foram avaliadas a redução da lactose e a obtenção de galacto-oligossacarídeos (GOS). A fermentação foi conduzida por culturas características de iogurte, *Lactobacillus bulgaricus* e *Streptococcus thermophilus*, associadas a dois microrganismos probióticos, *Bifidobacterium animalis* e *Lactobacillus acidophilus*. Foi utilizado um preparado enzimático contendo  $\beta$ -galactosidases de origens distintas, obtidas de *Kluyveromyces lactis* e de *Aspergillus niger*. O bioprocesso simultâneo foi avaliado variando as concentrações iniciais de lactose e de enzima, e o tempo de adição da enzima, caracterizando uma defasagem na aplicação da enzima em relação ao início da fermentação. Os resultados indicaram um impacto positivo na conversão da lactose, reduzindo entre 97,7 e 99,7% a concentração desse dissacarídeo, em comparação com 13,8 e 19,8% nas fermentações sem adição de enzima, bem como a obtenção de GOS entre 2,7 e 4,2 g.L<sup>-1</sup> nos processos defasados realizados com concentração inicial máxima de lactose e com concentração mínima de enzima adicionada.

**Palavras-chave:** Catálise enzimática; Conversão da lactose; Fermentação láctica; GOS.

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### 1 Introduction

Research on processes for lactose conversion using enzyme technology have always had the goal 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 crystallization of this carbohydrate (JURADO et al., 2002).

Lactose intolerance affects about 70% of the worldwide adult population with a significant impact in Africa, Asia, America and the native people of Oceania and their descendants, in which the incidence may be higher than 90% (SCHAAFSMA, 2008; SWALLOW, 2003).

In general, the range of action of the commercially available enzymes is near to neutral pH (7.0-6.5) and they are obtained from yeasts of the genus *Kluyveromyces*. Others function at acidic pH (5.5-4.5) and are obtained from moulds of the genus *Aspergillus*. Their application with the objective of reducing the lactose content of milk products is widely known (ABU-REESH, 2000; JURADO et al., 2004).

The addition of  $\beta$ -galactosidase in the production of low lactose fermented milks usually occurs in two stages. In the conventional process, enzymatic catalysis first occurs in a substrate maintained at a pH between 6.5 and 6.8. The substrate, now with a reduced lactose content then proceeds to the second processing stage, with the application of a lactic culture and the beginning of fermentation (Figure 1) (ROSALES, 2005).

An alternative that reduces the high contact time of the enzymatic stage in this process is to use systems that concentrate the lactose, generally by membrane filtration. The concentration of the substrate provides greater flexibility in comparison to the traditional process despite the use of multiple stages, but the investment in fixed assets is an important limitation to such systems (VALIO, 2009).

The main result sought for in simultaneous processes in which biocatalysis and fermentation are associated, is to overcome the drawbacks of the processes with two or more stages. In this study,  $\beta$ -galactosidases from distinct sources were used, associating the enzyme obtained from *Kluyveromyces lactis*, which operates preferably at a pH above 6, with the enzyme obtained

from *Aspergillus niger*, which acts preferentially at a pH below 5.5. The use of an enzymatic preparation containing  $\beta$ -galactosidases aimed to produce fermented milks with low lactose content and the formation of galactooligosaccharides.

According to Fooks and Gibson (2002), oligosaccharides are amongst the fibres providing a positive effect on the human intestinal microflora, when consumed in association with probiotic microorganisms such as *Lactobacillus* spp. and *Bifidobacteria* spp. According to Roberfroid (2007), amongst the oligosaccharides, only the fructooligosaccharides (FOS) and galactooligosaccharides (GOS) show evidence of prebiotic characteristics.

The GOS are produced from lactose via the transgalactosilation activity of the  $\beta$ -galactosidase enzyme. This enzyme is also responsible for lactose hydrolysis to form galactose and glucose.  $\beta$ -galactosidase has a similar affinity for both lactose hydrolysis and galactose transgalactosilation. GOS formation occurs in a lactose-rich substrate, especially milk, whey or a mixture of both. The addition of whey to a lactic substrate subjected to  $\beta$ -galactosidase action implies increasing the availability of lactose in the medium, by increasing the protein content and reducing the water activity. The lactose conversion rate increases when the substrate concentration increases, in order to reduce the effect of the water activity in the solution (MARTINS and BURKERT, 2009).

The decision to develop a process for the preparation of reduced lactose content fermented milks with possible symbiotic characteristics, allowing for the evaluation of the consortium existing between the prebiotics - in this case the GOS - and the probiotic cultures, was associated with market demands linked to the success of biomolecules of nutraceutical interest.

The aim of this study was to develop a low lactose probiotic yogurt containing GOS, and the behaviour of the process in which the enzyme was added after the beginning of fermentation, here called "Simultaneous Lagged Bioprocess" was also assessed. This new processing strategy was compared with the process in which the enzyme and lactic culture are added together, as well as fermentation without the addition of enzyme.

### 2 Material and methods

To obtain the probiotic yogurt, a 2<sup>3</sup> factorial experimental design was used. The variables studied were: the initial lactose concentration in the substrate ( $L_0$ ), the initial concentration of enzyme (E) and the time of enzyme addition (t). The responses evaluated were the final lactose concentration ( $L_F$ ) and the concentration of galactooligosaccharides (GOS). Table 1 shows the experimental matrix, and the experimental results were



**Figure 1.** Two-stage lactose conversion process for fermented milks: enzymatic catalysis and fermentation. Source: Martins (2009).

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analysed by the program Statistica 5.0 (StatSoft Inc., USA).

The substrate was obtained from a milk base containing skimmed milk powder and whey powder, both of the brand Elegê (BR Foods, Brazil). The skimmed milk powder was used in all experiments, reconstituted in the same proportion of 9% (w/v). The whey powder was added in different proportions to achieve the initial values for lactose concentration shown in Table 1.

The commercial enzyme Lactomax Flex (Prozyn, USA) was used, this being a liquid formulation composed of  $\beta$ -galactosidases produced from *Kluyveromyces lactis* and *Aspergillus niger*.

The commercial lactic culture used was ABY-3 (Chr Hansen, Denmark), a freeze-dried preparation provided in a single package containing the following microorganisms: *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium animalis* and *Lactobacillus acidophilus*, sufficient for use in 250 L of substrate. According to the manufacturer's recommendations, the contents of the package were added to 500 mL of UHT milk, and stored in previously sterilized bottles at 4 °C for up to two weeks, using 4 ml for each 2 L batch of substrate.

The substrate was heat treated at  $90 \pm 1$  °C for  $5 \pm 1$  min during the skimming process, and also to minimize the risk of microbiological contamination. Fermentation occurred in a 2 L Biostat fermenter (B Braun, Germany), the temperature being maintained at 43 °C throughout the processing time until a pH of 4.70 was reached, when the product was cooled.

The final lactose ( $L_F$ ) and galactooligosaccharide (GOS) concentrations were identified and quantified by high performance liquid chromatography with pulse amperometric detection (HPLC-PAD), using a CarboPac PA1 column (4 x 250 mm) with a PA1 guard column (4 x

50 mm), a temperature of 22 to 24 °C, a GP-50 gradient pump, an ED40 electrochemical detector and Peaknet software (Dionex, USA). The mobile phase was 20 mM NaOH and the flow rate 1.0 mL.min<sup>-1</sup> (MANERA et al., 2010).

### 3 Results and discussion

Table 1 shows the final lactose and galactooligosaccharide concentrations after fermentation of the controls (C01 to C03), to which enzyme was not added, and the trials (T01 to T11) of the experimental design matrix.

With respect to the lactose content, the variation took into account a report by Yadav et al. (2007), who identified an increase in GOS production in fermentations carried out by lactobacilli with a substrate concentration of about 9% (w/w) of this disaccharide.

The variation in enzyme concentration was suggested by the manufacturer, since there are no previous studies with this enzyme.

The time parameter was related to the interval observed between the addition of the lactic culture and the addition of the enzymes. The central idea of a lag in the addition of the enzymes was to reconcile the adaptation of the microorganisms to the medium (lag phase) without the presence of any exogenous enzymes, in order to allow them to synthesize the enzymes needed for the consumption of that specific substrate, and reduce any possible hydrolytic behaviour of the enzyme in relation to the GOS.

The final lactose concentration was evaluated from the analysis of variance presented in Table 2.

All the variables were significant and predictive. Figure 2 shows the contour curves and response surfaces obtained from the linear model.

**Table 1.** Factorial design matrix and its responses.

Test	$L_0$ (g.L <sup>-1</sup> )	E (g.L <sup>-1</sup> )	t (min)	$L_F$ (g.L <sup>-1</sup> )	GOS (g.L <sup>-1</sup> )
T01	-1 (59)	-1 (0.5)	-1 (0)	0.7	0
T02	+1 (91)	-1 (0.5)	-1 (0)	1.9	2.7
T03	-1 (59)	+1 (1.0)	-1 (0)	0.2	0
T04	+1 (91)	+1 (1.0)	-1 (0)	0.7	0
T05	-1 (59)	-1 (0.5)	+1 (60)	1.2	0
T06	+1 (91)	-1 (0.5)	+1 (60)	2.1	4.2
T07	-1 (59)	+1 (1.0)	+1 (60)	0.3	0
T08	+1 (91)	+1 (1.0)	+1 (60)	0.9	0
T09	0 (75)	0 (0.75)	0 (30)	0.5	0
T10	0 (75)	0 (0.75)	0 (30)	0.6	0
T11	0 (75)	0 (0.75)	0 (30)	0.6	0
C01	59	*	*	47.3	0
C02	75	*	*	63.6	0
C03	91	*	*	78.4	0

\*C01, C02 and C03 control samples (without enzyme addition).

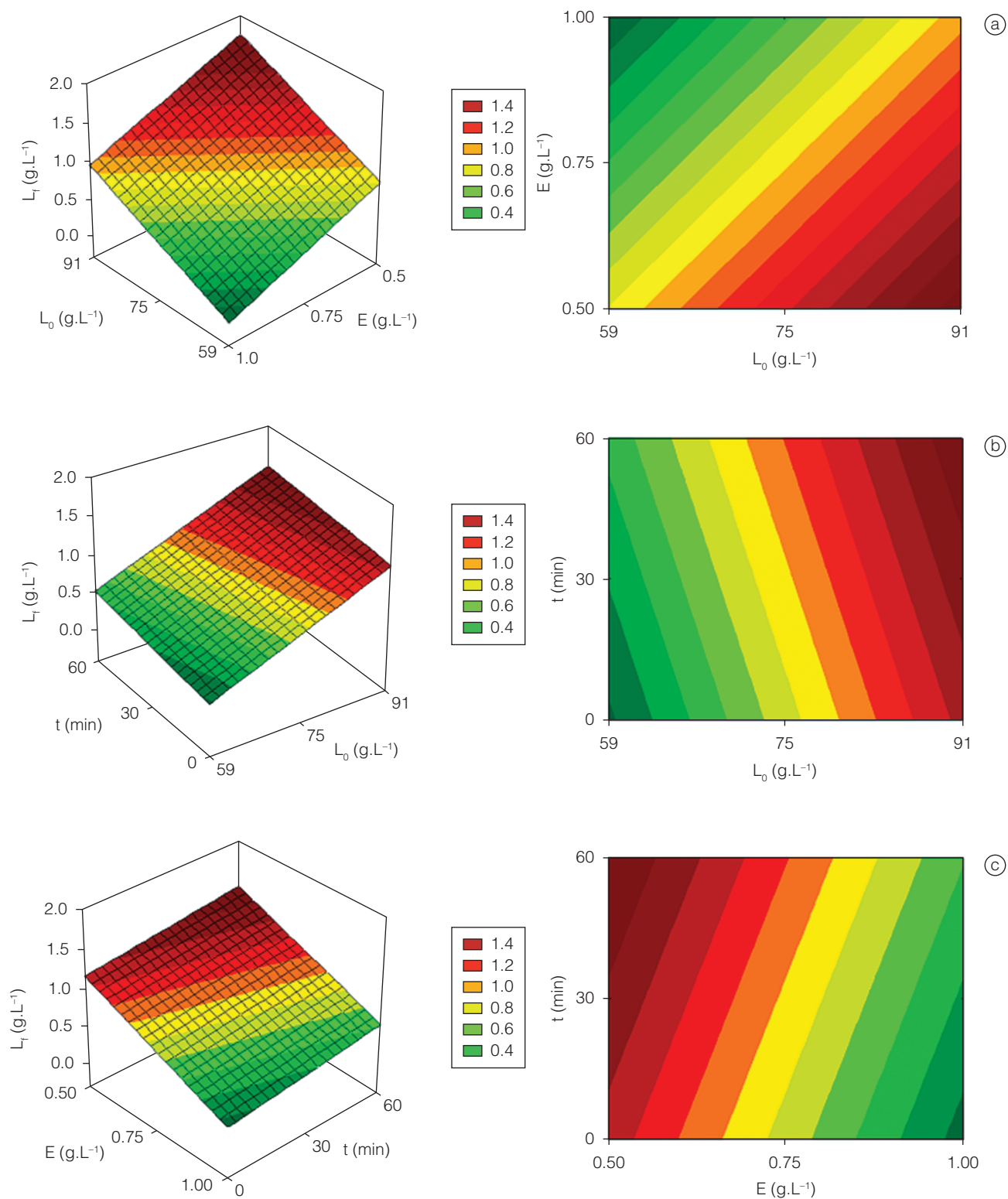
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**Table 2.** The analysis of variance of the final lactose concentration.

Variation source	Sum of squares	Degrees of freedom	Mean squares	F(calculated)
Regression	3.21	3	1.07	12.74
Residue	0.59	7	0.08	
Total corrected	3.80	10		

$F_{(tabulated) 3, 7, 0.05} = 4.35$ , correlation coefficient  $R = 0.92$ .



**Figure 2.** Contour curves and response surfaces for the final lactose concentration ( $L_f$ ).



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In the simultaneous bioprocess shown in Figure 2a, with variations in the concentrations of added enzyme (E) and initial lactose in the substrate ( $L_0$ ), it was found that the lowest lactose concentrations in the final yogurt were obtained with the highest enzyme concentrations and lowest initial lactose concentrations.

Figure 2b shows the lactose concentrations in the final product, with variations in the lactose concentration in the substrate ( $L_0$ ) and the time of addition of the enzyme (t), showing that the lowest possible final lactose concentration was obtained in the yoghurt with enzyme addition at the beginning of fermentation, together with the lactic culture, and a substrate with the lowest initial lactose concentration.

The variation in the final lactose concentration shown in Figure 2c, varying according to the concentration of enzyme added (E) and its time of addition (t), indicated that the best condition was when the highest enzyme concentration was added at the beginning of the process.

Toba et al. (1986) reported 64% of lactose conversion in yogurt obtained by a simultaneous bioprocess with  $\beta$ -galactosidase from *Aspergillus oryzae*.

More recently, Rodriguez et al. (2008) carried out simultaneous fermentation and catalysis using goat's milk as the substrate and an enzyme obtained from *Aspergillus oryzae*, applied together with the lactic culture. They obtained a conversion of up to 82.6% based on a substrate with an initial lactose concentration of 44.2 g.L<sup>-1</sup>.

In this study, values for lactose reduction between 97.7 and 99.7% were obtained using a simultaneous lactic fermentation bioprocess, as compared to values between 13.8 and 19.8% for fermentation without the addition of enzyme, for identical substrate compositions.

The responses obtained for GOS in the experimental design did not show normality, and could not therefore be treated by parametric statistical methods, as in the case of the final lactose concentration.

Therefore, non-parametric Artificial Neural Network (ANN) treatment was applied. The best fit obtained by the ANN module of the Statistic 5.0 was with the RBF (Radial Basis Function) topology, which resulted in a correlation of 0.998 and a standard error of 0.1. This is a progressive network with a radial basis function and a hidden intermediate level with 7 layers, which approximated the input variables of the response from a Gaussian type function. Table 3 compares the actual values, predicted values and the residues of the aforementioned model. It can be seen that the fit afforded by RBF function only underwent changes when the response returned to zero after passing through a value other than zero.

Using the above Gaussian type multidimensional activation function model, a response surface was

constructed, as also the rating input and output mapping, based on the data set obtained.

Figure 3 shows the response surfaces obtained from the artificial neural networks.

Figure 3a shows the variations in initial lactose concentration ( $L_0$ ) and enzyme concentration (E), indicating a trend towards more GOS production under conditions with higher initial lactose concentrations and lower enzyme concentrations in the substrate.

Figure 3b shows the surface generated for variations in initial lactose concentration ( $L_0$ ) and time of enzyme addition (t), indicating a trend towards more GOS production under conditions with higher initial lactose concentrations in the substrate and longer lag times between the start of fermentation and the addition of enzyme.

Figure 3c shows the surface generated for the variation in enzyme (E) concentration and its time of addition (t), indicating a trend towards greater GOS production under conditions with less added enzyme and an increase in lag time between the beginning of fermentation and the addition of  $\beta$ -galactosidase.

According to Pessela et al. (2003), with respect to the lactose conversion process, with higher initial concentrations of lactose, transgalactosylation is better than hydrolysis. On the other hand, lower enzyme concentrations probably mean less GOS hydrolysis, since it is also a  $\beta$ -galactosidase substrate.

The work of Toba et al. (1986) sought to obtain GOS by applying the enzyme obtained from *Aspergillus oryzae* in a simultaneous eight hour lactic fermentation. The authors found that 15% of the initial lactose had been converted to GOS by the second hour of processing. These values dropped by half at the end of fermentation, indicating that the enzyme itself tended to hydrolyze the GOS formed.

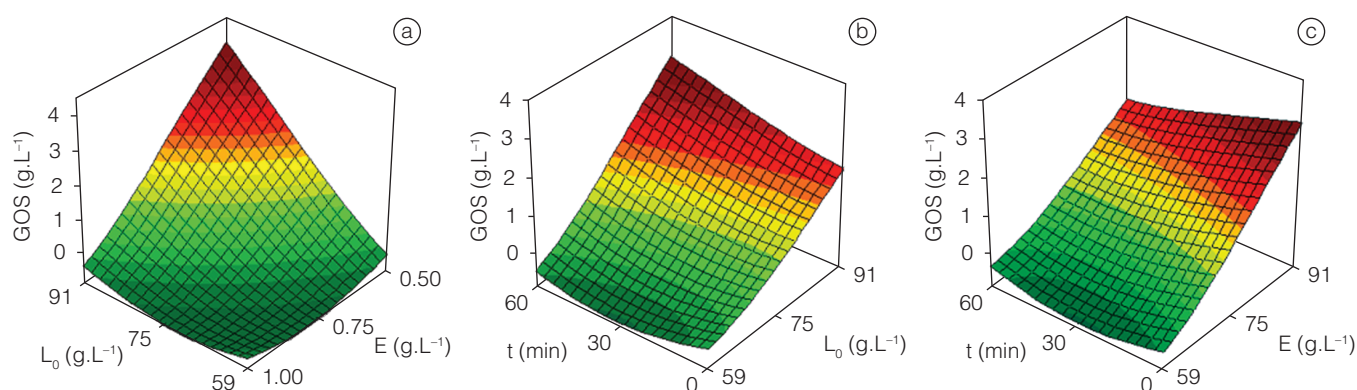
**Table 3.** Real values, predicted values and residue from the RBF model.

Test	Real values	Predicted values	Residue
1	0	0.00	0.0
2	2.7	2.70	0.0
3	0	- 0.13	- 0.13
4	0	0.00	0.0
5	0	0.00	0.0
6	4.2	4.20	0.0
7	0	- 0.33	- 0.33
8	0	0.00	0.0
9	0	0.00	0.0
10	0	0.00	0.0
11	0	0.00	0.0

Correlation coefficient: R = 0.998.

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**Figure 3.** Response surfaces for galactooligosaccharides (GOS).

Martínez-Villaluenga et al. (2008) observed the formation of GOS in commercial fermented milk, especially that containing bifidobacteria, indicating that the enzymes from lactic acid bacteria can also form and hydrolyze oligosaccharides. Yadav et al. (2007) found transgalactosylation activity in fermentations carried out using different lactobacilli.

The enzyme addition lag time, in relation to the application of lactic culture, can help to minimize the effect of GOS hydrolysis, indicating the possibility of establishing a Simultaneous Lagged Bioprocess (SLB) as a strategy to obtain low lactose fermented milks with GOS concentrations of commercial interest.

The Simultaneous Lagged Bioprocess (SLB) can be understood as a process in which enzyme addition occurs after fermentation has begun. On the other hand, according to Rodríguez et al. (2008), the term simultaneous bioprocess, sometimes referred to in the literature as Simultaneous Hydrolysis and Fermentation, is the process in which the addition of enzyme and microorganism occur at the same time.

The identification of a lag point for the enzyme addition time that allowed for the maximum production of GOS without compromising the texture of the fermented milk, was the critical item of the process developed in this study.

An appropriate definition of the time interval between the fermentation onset and enzyme addition will improve the advantages of the Simultaneous Lagged Bioprocess (SLB), combining fermentation and enzymatic catalysis to obtain biomolecules of nutraceutical interest in the development of fermented milks.

### 4 Conclusions

The addition of a mixture of  $\beta$ -galactosidases from different sources and their simultaneous action throughout the fermentation carried out by four different lactic acid bacteria, led to the following results:

- Positive impact on lactose conversion in the fermented milks, reducing the concentration of this carbohydrate by between 97.7 and 99.7%, which was directly proportional to the amount of enzyme added, resulting in final products made with raw milk and reduced lactose contents, indicating an important way of developing fermented milks for consumers that show intolerance to the aforementioned carbohydrate;
- GOS formation of between 2.7 and 4.2 g.L<sup>-1</sup> for the maximum lactose concentrations and minimum enzyme concentrations, showing the possibility of obtaining fermented milks containing prebiotics by processes involving enzyme catalysis and fermentation in a single processing step;
- Trend to obtain higher GOS concentrations with enzyme application after a lag period in relation to the start of fermentation, indicating that the Simultaneous Lagged Bioprocess (SLB) is a possible alternative to obtain these biomolecules in fermented milks; and
- The GOS produced is hydrolyzed with time, even if the fermented product is kept under refrigeration.

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