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ORIGINAL ARTICLE

Modeling and optimization of enzymatic and fermentation reactions in the production process of a symbiotic fermented milk beverage

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Abstract

Symbiotic fermented kinds of milk are products with probiotic microorganisms and a simultaneous prebiotic effect. The presence of fibers or oligosaccharides causes it. This work aimed to evaluate and model fermented milk beverage production with probiotic microorganisms and *in situ* Galactooligosaccharides (GOS) synthesis. GOS enzymatic production was maximized through the mathematical model, and finally, the mathematical prediction was experimentally validated. For this purpose, the enzymatic kinetics of GOS production from *Aspergillus oryzae* β-galactosidase enzyme was experimentally evaluated for different initial lactose concentrations (30%, 35%, and 40% w/v) and enzyme/lactose ratio (R: 0.2%, 0.4%, and 0.6%). In addition, the fermentative kinetics of lactic acid and biomass production were evaluated using the probiotic strain *Lactobacillus acidophilus*. The parameters correlation for both models was then performed. A unified model determined the process conditions that maximize GOS concentration in the fermented milk beverage. According to the model, the operating values that maximized the GOS production were 40% of initial lactose, R: 0.15%, 20 min of enzymatic reaction, and 17 h of fermentative reaction. The final fermented milk drink showed a GOS content of 2.9 g/portion of 200 g, guaranteeing the recommended daily values of the prebiotic with the consumption of two servings.

Keywords: Galactooligosaccharides; Enzyme kinetics; Fermentation kinetics; Fermented milk drink; β-galactosidase.

Highlights

- An enzymatic reaction in the first part of the symbiotic milk beverage process generated galactooligosaccharides
- Enzymatic product rich in Galactooligosaccharides is part of the substrate of the fermentation reaction to produce the symbiotic milk beverage
- Fermented milk beverages could be classified as prebiotic beverages according to their galactooligosaccharide content

1 Introduction

Prebiotics are non-digestible food ingredients that affect the host positively by selectively stimulating the growth and activity of one or more beneficial gastrointestinal bacteria and improving health (Ma et al., 2020; Badui Dergal & Valdés Martínez, 2006). In the food industry, various types of prebiotics such as galactooligosaccharides (GOS), lactulose, tagatose, lactitol, and glucono-δ-lactone (GDL) are synthesized through different chemical and biochemical reactions (hydrolysis, transgalactosylation, isomerization, fructosyl transfer, reduction, and oxidation), as well as microbial fermentation processes using raw-milk whey or isolated lactose as raw material (Nath et al., 2017).

GOS are complex carbohydrates and essential raw materials for developing functional foods (Catenza & Donkor, 2021; Scott et al., 2016). Commercial GOS production is performed enzymatically through the β-galactosidase enzyme (EC 3.2.1.23); in addition, GOS are subsequently separated by purification techniques. The most widely used commercial enzyme is obtained from the yeast *Kluyveromces lactis* to produce lactose-free products. In contrast, the β-galactosidases used for the industrial production of GOS are obtained from *Bacillus circulans*, *Aspergillus oryzae*, and *K. lactis* (Botvynko et al., 2019).

Enzymatic GOS synthesis results from a dual and simultaneous function: the hydrolysis of lactose and the formation of the oligomers from the simple sugars present, and this reaction is called transgalactosylation (Rico-Rodríguez et al., 2021).

Because GOS can be incorporated into milk beverages, combining them with probiotic strains, such as *Lactobacillus acidophilus*, is feasible to achieve a symbiotic food. It is essential to differentiate a dairy beverage from other fermented dairy foods such as yogurt; in the dairy beverage, milk-whey can be added and joined with milk as substrate in the fermentation process (Tirado et al., 2015). This product results from evaluating a new way to obtain a symbiotic product like the ones in the market but with the advantage of the *in situ* generation of prebiotic GOS from cheese whey, a low-cost dairy byproduct. It is an example of a circular economy in the dairy industry, increasing the sustainability indexes.

This work aimed to evaluate and model fermented milk beverage production with probiotic microorganisms and *in situ* GOS synthesis. GOS enzymatic production was maximized through the mathematical model, and finally, the mathematical prediction was experimentally validated.

2 Materials and methods

2.1 Materials

Enzeco® Fungal Lactase from *A. oryzae*, was provided by Enzyme Development Corporation® and is characterized by an enzymatic activity of 14997 μmol ONP g^{-1} min^{-1}; *L. acidophilus* (LA3) LYOFAST® used for fermentation, was provided by SACCO®; food-grade lactose and milk-whey powder were acquired in Centro Agrolechero (Bogotá).

For High Performance Liquid Chromatography-Infrared Spectroscopy (HPLC-IR) quantification, distilled, deionized and degassed water was used. HPLC standards of glucose, galactose, lactose and rhamnose (Sigma-Aldrich), were used to build calibration curves (r^2>0.99), which were provided by the Laboratorio de Ingeniería Química, Universidad Nacional de Colombia.

2.2 Assessment of the enzymatic kinetics

Media were prepared with different initial concentrations of lactose: 30%, 35%, and 40% (w/v). Besides, a previous heating at 90 °C was applied to solubilize lactose according to Vera et al. (2012). Subsequently, the media was adjusted at 50 °C and pH: 4.5, then the enzyme was added at different concentrations, to reach an enzyme-lactose ratio, R: 0.2%, 0.4%, and 0.6% w/w. R (%) = grams of enzyme/grams of lactose*100.

The enzymatic treatment was carried out for 1h at constant temperature, and 3 mL samples were taken at 0, 10, 20, 30, and 60 minutes. Then those were heat-treated in a water bath at 90 °C for 10 min to inactivate the β-galactosidase (Iwasaki et al., 1996; Klein & Sant'Ana et al., 2018).

For quantification of carbohydrates obtained by the enzymatic reaction, samples were centrifuged for 30 min at 3000 rpm, afterward, these samples were diluted 1:20 ratio, using distilled and deionized water; then the samples were filtered with cellulose acetate syringe filters (0.22 µm), finally, they were frozen until the HPLC analysis.

The content of glucose, galactose, lactose, and GOS were determined with a Benson column (Polystyrene divinylbenzene sulfonated Na+) at 80 °C, operated with an HPLC system (Thermo) equipped with a Shodex RID-101A refractive index detector (Castro et al., 2021). Distilled, deionized, and degassed water was used as the mobile phase at a flow rate of 0.5 mL/min, using an injection volume of 20 µL and a retention time of 18 min. The concentration of GOS was determined by mass balance, based on the concentration of the other substances.

2.3 Modeling of the enzymatic reaction

The model parameters for the enzymatic reaction were determined using experimental data by minimizing the discrepancy between the observed and computed values (Equation 1) through the utilization of a diploid genetic algorithm (DGA) programmed in MATLAB®. The system of differential equations was solved employing the MATLAB® tool ODE15S.

$$F = \sum_k^D \sum_i^T \left(C_{ik}^{exp} - C_{ik}^{calc}\right)^2 \quad (1)$$

In Equation 1, F is the objective function result, D is the component (six), T is the time evaluated (eight), subscripts k corresponds to each component, and i corresponds to each time, and exp and calc represent the experimental and calculated compositions, respectively.

2.4 Evaluation of fermentation kinetics

The onset of the fermentation process marked the conclusion of the enzymatic reaction responsible for GOS synthesis. The resulting product from this phase constituted a portion of the fermentation medium (33.3%). This product was combined with whole milk powder (13.4%) and water (53.3%) to attain a final solution with a nutritional composition closely resembling that of whole cow's milk (3.8% protein and 3.7% fat). This strategic composition ensured the final product's compliance with regulatory standards (Food and Agriculture Organization, 2003).

A pitching rate of 200 mg/L of the probiotic culture *L. acidophilus* (LA3) SACCO®, was introduced to the fermentation medium. The fermentation process was conducted at a consistent temperature of 40 °C until a lactic acid concentration of 0.75% to 1.5% was achieved. Process monitoring encompassed the collection of 25 mL aliquots at 0 h, 17 h, and 18 h for carbohydrate analysis. Additionally, 20 mL aliquots were taken periodically until the desired level of acidity was attained. This allowed for the quantification of both acidity and biomass (Instituto Colombiano de Normas Técnicas y Certificación, 2001).

The biomass of *L. acidophilus* was quantified by ascertaining the dry weight, achieved through protein denaturation. This denaturation was induced by the addition of 0.1N HCL, followed by subsequent centrifugation (Álvarez et al., 2010; Sharma & Mishra, 2014).

2.5 Modeling of the fermentation reaction

The concentrations of the target compounds (GOS, lactic acid, and biomass) obtained over time were correlated using the Monod model and the simple production model. These models are commonly employed for fermentation systems and have also found application in enzymatic reactions (Álvarez-Ramirez et al., 2019). Specifically, the Monod model was utilized for GOS, while the simple production model was employed for lactic acid.

2.6 Optimization of the models

After establishing the kinetic constants and parameters of the fermentative model, the enzymatic model was incorporated to optimize the yield of GOS produced through the enzymatic process. Subsequently, the same procedure was replicated for the fermentative model.

The critical factors affecting enzymatic kinetics to optimize GOS content included initial lactose concentration, initial enzyme concentration, and reaction time. Additionally, for the fermentative process, key variables for maximizing GOS content were the lactic acid concentration in the fermented milk beverage and the reaction time.

For global optimization, the diploid genetic algorithm was employed, and it was implemented using MATLAB 2019b. The algorithm utilized 20 individuals and underwent 2000 generations for both scenarios (Xing & Gao, 2014).

2.7 Experimental validation of the model

To achieve the third objective, experimental validation of the correlation model was conducted. This validation involved evaluating the optimal values of the crucial variables using the procedures for assessing enzymatic kinetics and fermentation kinetics that were previously reviewed. The percentage error between the modeled and experimental data was calculated as a validation criterion.

The statistical analysis consisted of graphical and statistical comparisons between the concentrations of GOS, lactic acid, and biomass predicted by the model and the data obtained from the experiments. The methodologies outlined by Prada (2014) were employed to facilitate this analysis.

2.7.1 Goodness-of-fit statistics

The calculation of the sum of squares of errors (SSE), the R2 coefficient, the adjusted R2 coefficient, and the Root Mean Square Error (RMSE) were employed as primary methods to assess the goodness of fit.

2.7.2 Residual analysis

The variance between the experimental and calculated data was examined, aiding in the graphical assessment of the model's precision within the confidence intervals, depicting the model's capability to describe the experimental data accurately.

2.8 Sensory evaluation

Furthermore, a sensory evaluation was conducted on the resultant fermented milk beverage (FMB) compared to a Control sample produced without enzymatic treatment. This evaluation was carried out by a sensory panel consisting of 50 non-expert consumer judges selected from the employees and operators of AVESCO SAS. The evaluation employed hedonic acceptance and preference tests. Each judge was required to complete a form to assess the product's pleasantness or unpleasantness based on sensory attributes such as flavor, aroma, color, consistency, and overall appearance, using a nine-point hedonic scale. In the preference test, panelists were tasked with choosing between two samples (Conti-Silva & Kelli de Souza-Borges, 2019).

The statistical analysis of the samples was conducted using IBM SPSS Statistics 25® software. The acceptance data was subjected to a non-parametric test (Mann Whitney U), while the preference tests employed the cumulative binomial statistic. This data allowed for comparing preference proportions, enabling the determination of statistically significant differences. P-values <0.05 indicated meaningful distinctions between the samples (Tzavaras et al., 2022).

3 Results and discussion

3.1 Enzymatic process

Given that enzymatic GOS production necessitates the utilization of relatively elevated substrate concentrations, a solution comprising cheese whey and lactose was employed. This approach was adopted to avoid using pure lactose as the initial material, which could be both feasible and potentially more economically advantageous.

Figures 1 to 4 present concentration profiles of lactose, glucose, galactose, and GOS over time.

In Figure 1, the solution with a 30% w/v initial lactose concentration exhibited the most substantial reduction in lactose content, achieving a 72% reduction by the conclusion of the enzymatic reaction with an enzyme/substrate ratio (R) of 0.6%. Notably, there is a proportional relationship between R and the initial lactose concentration. Increasing R from 0.2% to 0.4% resulted in an average lactose reduction increase of 12%, and further raising R from 0.4% to 0.6% yielded an additional average increase of 7%. These lactose reduction outcomes mirror those reported by Wang et al. (2021), Castro (2020), Albayrak & Yang (2002), and Vera et al. (2012). They observed reductions of 35%, 48%, 40%, and 44% in lactose content at 60 minutes in a β-galactosidase enzyme reaction from *A. oryzae*, with initial lactose concentrations of 500 g/L, 400 g/L, 400 g/L, and 500 g/L, respectively. Differences could be attributed to variations in enzyme concentration and initial lactose content, with the lowest value corresponding to the highest lactose reduction.

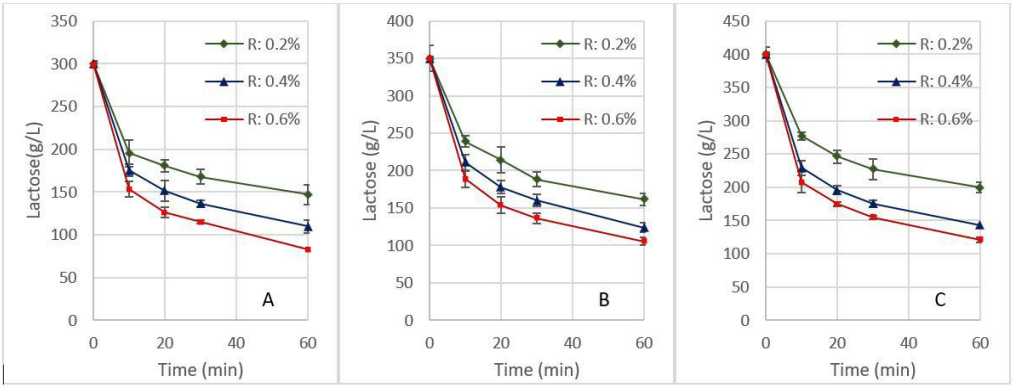

Figure 1. Lactose concentration vs time, produced in media containing 30% (A), 35% (B), and 40% (C) of initial lactose.

Regarding the glucose content, the highest production occurred in the medium with a 40% (w/v) initial lactose concentration, obtaining a glucose concentration of 140 g/L (Figure 2). Similarly, the highest galactose production was in the medium with a 40% (w/v) initial lactose concentration, in which the galactose concentration was 95 g/L after 60 minutes of enzymatic reaction (Figure 3). Galactose production was directly proportional to the initial lactose concentration.

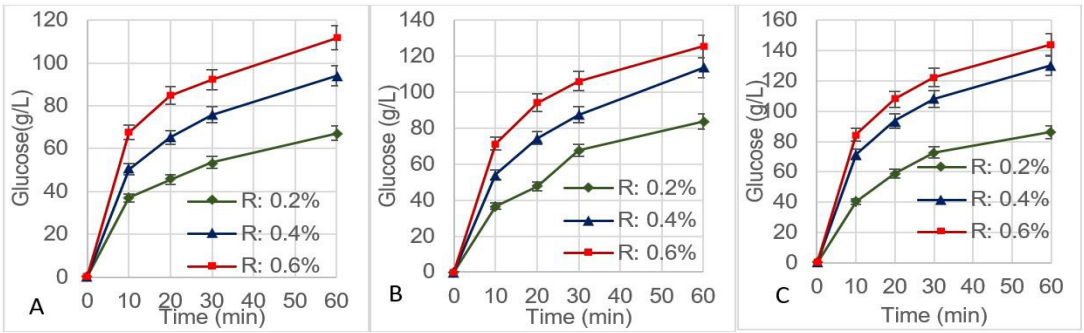

Figure 2. Glucose concentration vs time, produced in media containing 30% (A), 35% (B) and 40% (C) of initial lactose.

Additionally, glucose and galactose production in media with 35% and 40% w/v initial lactose and R: 0.2% are like those reported by Vera et al. (2012), who obtained a production of 80 g/L of glucose and 38 g/L of galactose after 60 minutes of reaction with the β-galactosidase enzyme from *A. oryzae*. González-Cataño et al. (2017) produced 100 g/L and 50 g/L of glucose and galactose, respectively, after 60 minutes, using an enzyme from *K. lactis* at a concentration of 6 U/mL. Compared with the results of Albayrak & Yang (2002), the amounts of glucose obtained were equal to those reported by them; they achieved 13% glucose (130 g/L). This work reached the same value at 40% initial lactose and R=0.4%. Albayrak & Yang (2002) reached 3% galactose (30 g/L); in this study, 35 g/L was achieved at 30% initial lactose and R=0.2%.

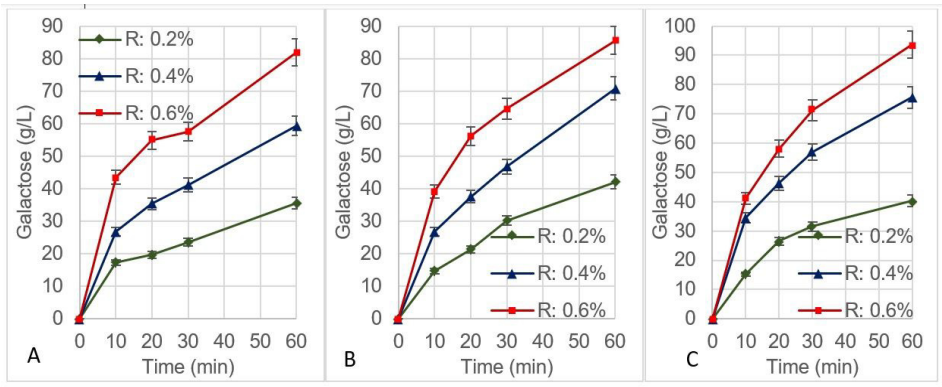

Figure 3. Galactose concentration vs time, produced in media containing 30% (A), 35% (B) and 40% (C) of initial lactose.

The maximum GOS production was 70+/-3.5 g/L, with 40% (w/v) initial lactose, R =0.2%, since minute 10 up to 60, this maximum had no significant differences (Figure 4).

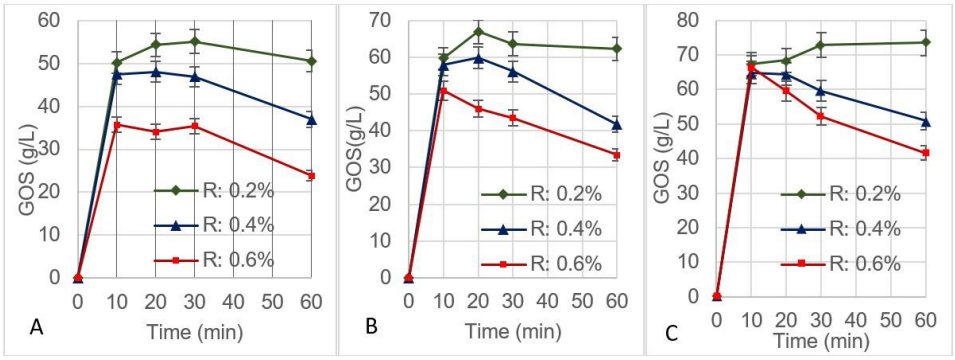

Figure 4. The concentration of GOS vs time, produced in media containing 30% (A), 35% (B), and 40% (C) of initial lactose.

Comparing Figures 2 to 4 and considering that the lactose molecule is composed of a glucose molecule bound to one galactose molecule, it is assumed that lactose hydrolysis produces equal amounts of glucose and galactose. However, the amount of glucose obtained at any given time was consistently higher than that of galactose. This disparity may be attributed to the transgalactosylation reactions wherein a glucose molecule binds to two or more galactose molecules, forming GOS. These reactions lead to a more significant decrease in galactose concentration in the medium.

The GOS concentration in the product of this study was 74 g/L. Castro (2020) achieved 104 g/L after 60 minutes of reaction in a medium with 40% initial lactose and a pH of 4.5. Similar results were reported by Vera et al. (2012), who obtained a GOS concentration of 110 g/L using the same enzyme and under the same conditions.

Additionally, as shown in Figure 1, lactose concentration in the medium decreased exponentially within the first 10 minutes. This reduction in GOS yield observed in Figure 4 after 10 minutes can be explained as initial lactose concentrations higher than 30% (w/w) are required to favor GOS synthesis (Wang et al., 2020).

Figure 4 shows that the GOS content initially increases, reaching a point where its content begins to decrease in most cases and sometimes remains at the same level. The decreasing behavior could be attributed to the fact that these compounds are intermediates in the enzymatic reaction and could be hydrolyzed by the enzyme β-galactosidase when the remaining lactose content is low (Botvynko et al., 2019).

3.2 Fermentation process

The fermentation process was carried out with the solution obtained from the enzymatic reaction performed for 20 minutes in a medium with 40% (w/v) initial lactose and R: 0.15%, conditions defined by the developed model. The fermentation time was 26 hours. Acidity and biomass concentration were monitored (Figure 5).

According to Figure 5, there was an increase in the acid content, reaching a value of 1.2% w/w (expressed as lactic acid) after 26 hours of reaction time due to the action of LAB on lactose, producing lactic acid. Generally, this process is carried out industrially for yogurt until its concentration reaches 0.7% to 1.5% w/w (expressed as lactic acid), as stipulated by the Health Ministry of Colombia (Colombia, 1986).

Industrially, mixed microorganisms are used in the fermentation process, as is the case with yogurt, where a symbiosis between two bacteria, *S. thermophilus* and *L. bulgaricus*, promotes their development. Moreover, this interaction considerably reduces the fermentation time (4-6 hours), and the resulting product has peculiarities that distinguish it from products fermented by a single bacterial strain (Spreer, 1991). However, because one of the objectives of the research was to develop a comprehensive model of the reactions that occur in the process of obtaining symbiotic fermented milk, it was decided to use a single strain to avoid greater complexity in the data modeling.

Figure 5 also demonstrates an ascending trend in biomass during fermentation. The latency phase lasts approximately 6 hours; from hour 8, the production rate increases until reaching 5% w/w of dry weight biomass (total dry weight of 13%w/w), equivalent to 6 g/L, at 26 hours of fermentation reaction in a medium with an initial lactose concentration of 147 g/L. These results suggest that this product has probiotic potential.

The quantity of biomass obtained differs from Rezvani et al. (2017) results, which reported values of 3 g/L after 30 hours for *L. delbruekii* and *L. fermentum*. Álvarez et al. (2010) achieved a value of 4.3 g of biomass/L in a 25-hour reaction time. The strain they used was *L. casei* var. *rhamnosus*, and the medium had a lower lactose content (35-70 g/L).

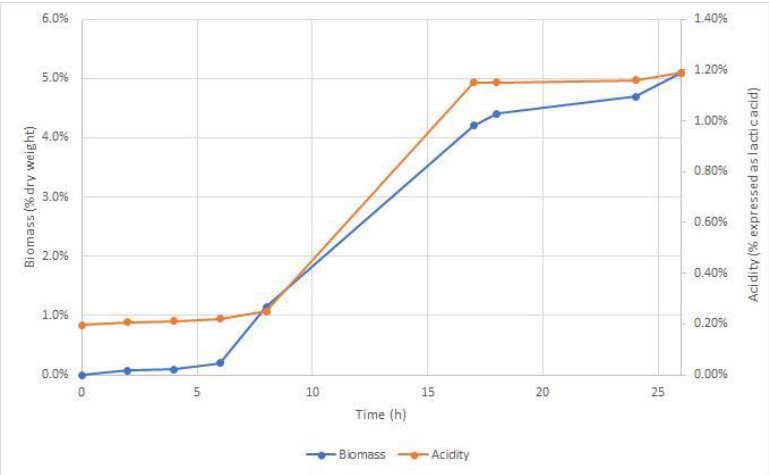

Figure 5. Biomass concentration and acidity vs time for fermentation reaction.

According to the Food and Agriculture Organization of the United Nations (FAO), probiotics are considered GRAS (Generally Recognized as Safe) substances, providing specific beneficial effects on consumers' health (Food and Agriculture Organization, 2006). The minimum daily amount of probiotics provided should ensure the minimum viable number of each probiotic strain at the end of its shelf life. As Romero Sánchez (2016) stated, a probiotic product should contain viable cells of >106-108 CFU/g or >108 -1010 CFU/product dose.

During fermentation, the GOS content decreased from 22.56 g/L at the beginning to 13.85 g/L after 18 hours of reaction. This behavior may be due, as mentioned above, to the fact that these compounds are intermediates in the enzymatic reaction and could be hydrolyzed by the same enzyme β-galactosidase (synthesized by the *lactobacillus*) when the remaining lactose is at a low concentration.

Martínez-Villaluenga et al. (2008) estimated GOS concentrations in Spanish commercial yogurts of three denominations: regular yogurts, yogurts with bifidobacteria, and yogurts with *L. casei*. They found concentrations ranging from 300 to 550 mg of GOS per 100 g product. In most cases, GOS-3 predominated with more than 50% presence, except in conventional yogurt. This concentration is consistent with the findings of the study by Mosquera-Martínez et al. (2023), which determined GOS concentrations of 5.52 g/L in a fermented milk product added with a nanofiltration retentate of an enzymatic lactose hydrolysate.

In this study, after diluting the enzymatic reaction product 1:2 with whole milk powder and water, the composition was as follows: GOS 22.53 g/L, lactose 147.1 g/L, glucose 17.86 g/L, and galactose 10.8 g/L.

During fermentation, the decrease in GOS content was likely caused by enzymatic breakdown reactions of GOS 4 to GOS 3 and GOS 3 to GOS 2/lactose mixture. HPLC analysis separated samples by size; thus, it was impossible to differentiate the components of this mixture.

A decrease in GOS and increase in lactose content were observed regarding the reaction time. GOS concentration reached 13.85 g/L after 18h. This change might be due to the hydrolysis of GOS 3 to GOS 2/lactose mixtures, as observed in the enzymatic reaction.

L. acidophilus strains produce the enzyme β-galactosidase, and its activity is lower at refrigeration temperatures (Brashears & Gilliland, 1995). Therefore, it is assumed that when the product is stored at refrigeration temperatures, the GOS concentration remains almost constant. Vénica et al. (2015) quantified the formation of GOS while manufacturing various yogurt varieties and analyzing their stability during storage. They found that GOS concentration remained stable in the product during storage due to the inability of the added cultures to metabolize them and because of the inactivation of the β-galactosidase enzyme at the acidic pH values of the yogurt. However, they mentioned that the stability of GOS could be different in yogurts made with other β-galactosidase enzymes that have their optimum pH in acidic media (Vénica et al., 2015), as observed in the present study.

3.3 Modeling and optimization of the enzymatic reaction

Table 1 presents the parameters of the mathematical model corresponding to enzymatic kinetics. The correlation coefficient between the experimental data and the model predictions was 0.946 (r^2), indicating a strong correlation. In Table 1, the formation and decomposition rates of the enzyme-lactose (E-L) complex were high, as indicated by the K1 and K2 parameters. Similarly, the constants K3 and K4 were comparable and related to the formation of GOS and monosaccharides, respectively. Additionally, it was observed that the K5 constant associated with GOS decomposition was the highest, leading to significant hydrolysis. Finally, K6 was the lowest, indicating the challenge of oligomerization from GOS 3 to GOS 4.

Comparing these results with those obtained by Palai et al. (2012), who employed β-galactosidase extracted from *Bacillus circulans* at a concentration of 525 g/L, with an enzyme/lactose ratio of 0.3% and a specific enzyme activity of 1300 μmol oNP min^{-1} g^{-1}, they reported K1, K3, and K5 constants of high orders, with the K3 constant being in the order of 10^11 mM h^{-1} (10^9 mol min^{-1} L^{-1}).

Table 1. Parameters of the mathematical model of the enzyme kinetics.

Constant	Value	Units
K_1	6160468.410	L/mol E^{-1} min^{-1}
K_2	4566077.603	L/mol E-L^{-1} min^{-1}
K_3	4396.097	L/mol E-L^{-1} min^{-1}
K_4	5410.835	L/mol E-M^{-1} min^{-1}
K_5	108411589.200	L/mol E^{-1} min^{-1}
K_6	5.859	L/mol E-M^{-1} min^{-1}
R^2	0.946	

E = enzyme; E-L = Enzyme-lactose complex; E-M = Enzyme-monosaccharide complex.

Figure 6 illustrates the correlation matrix among the parameters of the mathematical model. There is minimal correlation between parameters, except for the constants K2 and K5. These constants are directly associated with the complex formation (E-L) reversibility and the transgalactosylation reaction. Although K3 in the complex formation (E-M) can be linked to the transgalactosylation reactions, the kinetic constants K4, K5, and K6 are inversely proportional. This fact implies that an increase in the formation of this complex will predispose the reaction to enhance hydrolysis through K2.

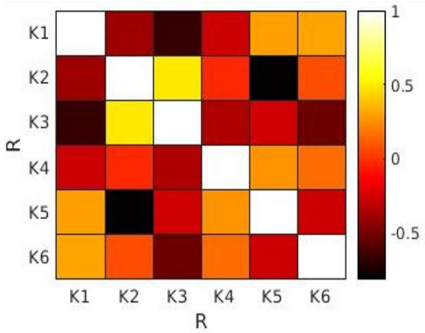

Figure 6. Correlation matrix between enzyme kinetics parameters.

Figure 7 illustrates the proposed model, where the GOS concentrations are predominantly close to the 45° straight line. Additionally, the glucose + galactose curve exhibits a significant number of high residues, mainly because errors in each of these compounds accumulate, making these concentrations the most challenging to optimize in the model. In contrast, the lactose curve initially shows a low accumulation of residues between the experimental and calculated concentrations. Nevertheless, it is noteworthy that as the reaction progresses, lactose concentrations decrease. Consequently, the frequency of residuals increases. Furthermore, there are abnormal residuals at the base and tip of the graph, indicating that the model's monitoring necessitates more data points between 0 and 10 minutes.

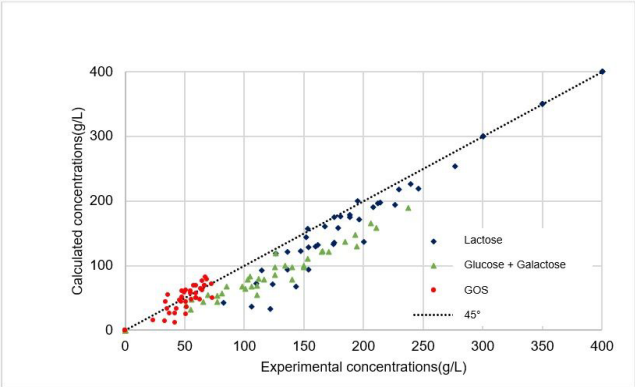

Figure 7. Comparison of enzymatic model concentrations.

According to Figure 8, there is a direct proportion between GOS concentration and lactose concentration for both experimental and modeled results. Regarding enzyme concentration at low lactose concentrations (300 to 350 g/L), there is a direct proportion between GOS and enzyme concentrations. This behavior contradicts the experimental and literature results (Milisavljević et al., 2016), which show increased GOS content at similar enzyme concentrations.

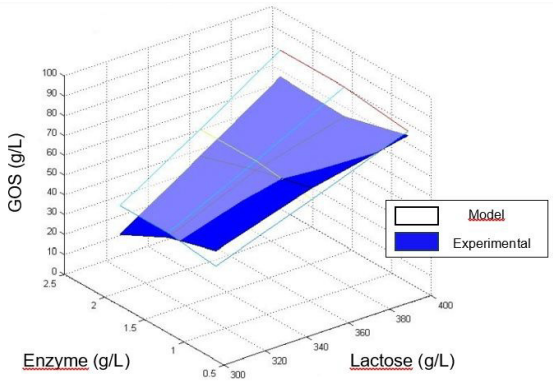

Figure 8. GOS concentration vs Enzyme and Lactose concentrations (g/L) for model and experimental results.

Table 2 compares the maximum point reached by the model to the experimental data. The model predicts higher concentrations of GOS, especially at low enzyme concentrations, indicating a better performance than the experimental data. This behavior is attributed to the two scenarios' differences in reaction dynamics and sequences.

Table 2. Enzymatic reaction peaks.

Variable	Model	Experimental
Optimal time	20 min	60 min
Optimal Concentration of GOS	80.94 g/L	73.6 g/L
Initial rate (Enzyme/Lactose)	0.15%	0.2%
Initial lactose (g/L)	400	400
GOS performance (% carbohydrates)	19.47%	18.4%

According to the fermentation model, the optimum reaction time was 17 hours. After this period, an acidity of 1%, expressed as lactic acid, a biomass content of 4.7% (dry weight), and a GOS concentration of 14.319 g/L were achieved.

3.4 Validation of the enzymatic and fermentative model

The GOS content obtained (14.32 g/L) was higher than that reported by Vénica et al. (2015), who inoculated *L. acidophilus* strains into yogurt made from milk treated with commercial β-galactosidase and generated GOS at concentrations ranging from 3.6 g/L to 6.2 g/L. However, the enzyme they used was from *K. lactis*, and the lactose concentration of the solutions was 50 g, 100 g, and 200 g/L, whereas in the present work, it was 400 g/L. It is essential to take into account that enzyme source affects GOS concentration; β-galactosidases from *K. lactis* have high hydrolytic and low transgalactosylation activities compared to β-galactosidases from *A. oryzae* (Botvynko et al., 2019). According to Resolution 333 of 2011 of the Colombian Social Protection Ministry (Colombia, 2011), one serving of a milk drink contains 200 g. The amount of GOS obtained in this work at the end of the fermentation process per one serving of fermented milk drink was 2.9 g of GOS (14.32 g/L), allowing it to be classified as a prebiotic food (Davis et al., 2010).

3.5 Sensory evaluation

Figure 9 presents the means for all attributes. The analysis was conducted after normalizing the test values (z) and (p) presented in Table 3. Among the samples analyzed, BLF corresponds to the fermented milk drink in which the solution with GOS obtained by the enzymatic reaction was used. Control corresponds to the fermented milk drink prepared without enzymatic treatment.

Table 3 and Figure 9 show that only the attributes of odor and flavor are significantly different. The control sample obtained a higher median value for these attributes. Abdel Wahab et al. (2024) showed a decrease in diacetyl concentration in yogurt, one of the main contributors to its flavor when applied β-galactosidase in raw milk for yogurt production; it is caused by the more comprehensive carbohydrate profile in the product of the enzymatic reaction, that changes fermentation products. The sensory characteristics evaluated are considered acceptable with values higher than 5. Accordingly, attributes below this value, such as the BLF sample's flavor and the Control sample's consistency, received lower ratings. The behavior of the consistency could be related to the contribution of GOS, owing to its water-holding capacity that increases viscosity.

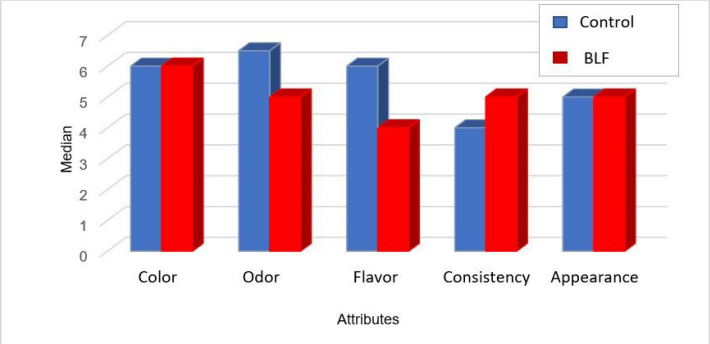

Figure 9. Median values for the attributes of color, odor, flavor, consistency and appearance.

Table 3. z-values and p-values for the different attributes evaluated.

Attributes	z	p
Color	-0.219	>0.05
Odor	-2.457	<0.05
Flavor	-4.524	<0.05
Consistency	-1.364	>0.05
Appearance	0.180	>0.05

Regarding consumer preference for each sample, the Control was significantly preferred by the evaluators, with a preference rate of 68%. The BLF obtained a preference rate of 32%. This fact suggests the possibility of continuing the study of the product to improve its flavor, odor, and appearance characteristics.

4 Conclusions

- The experimental evaluation of enzymatic transgalactosylation kinetics using β-galactosidase from *A. oryzae* resulted in a yield of 73.55 g/L of GOS after 60 minutes of reaction time, with 40% initial lactose and R: 0.2% ([g Enzyme/g lactose] *100);
- The fermentation kinetics showed an initial acidity (expressed as lactic acid) of 0.16%, which increased to 1.18% after 17 hours and reached 1.2% after 26 hours of reaction time. A biomass content of 5% w/w was achieved using a starter of 20 g of *L. acidophilus* per 100 L of the initial milk drink;

- The unified model predicted a maximum GOS production of 2.9 g per portion (200 g of fermented beverage) using a 40% lactose starter, R: 0.15%, 20 minutes of enzymatic reaction, and 17 hours of fermentative reaction. Experimental validation of the enzymatic reaction resulted in an error percentage of 19.7% in the GOS content compared to the calculation from the proposed model. In comparison, the fermentation reaction had an error percentage of 0.0069%;
- Unlike odor and flavor, attributes such as color, consistency, and appearance did not show significant differences between samples. Regarding product acceptance, 32% of consumers preferred the fermented milk drink with enzymatic treatment;
- The resulting fermented milk drink contained 2.9 g of GOS per serving, ensuring that two servings provide the recommended daily GOS intake.

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