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β-galactosidase from *Kluyveromyces lactis* in genipin-activated chitosan: An investigation on immobilization, stability, and application in diluted UHT milk

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ABSTRACT

The objective of this research was to evaluate the immobilization of the enzyme β-galactosidase in a genipin-activated chitosan support. The influence of the number of spheres and substrate concentration on immobilization yield (IY) and enzyme activity (EA) was analyzed using experimental design. Thermal, operational and storage stabilities were assessed, and the enzymatic derivatives were characterized by thermogravimetric analysis (TGA) and scanning electron microscopy (SEM). The TGA showed that the enzymatic derivatives kept their thermal behavior, and the SEM images revealed smooth surfaces in all the spheres. The optimized conditions for the immobilization process were 4.57 mg·mL^{-1} of spheres and a substrate concentration of 10 mM (IY = 84.13%; EA = 24.97 U·g^{-1}). Thermal stability was enhanced at 10 and 37 °C, enabling four successive cycles of lactose hydrolysis in diluted UHT milk. Therefore, the immobilized enzyme in genipin-activated chitosan has potential for lactose hydrolysis and applications in the food industry.

1. Introduction

Enzyme immobilization is used not only to increase stability and selectivity but also enable enzyme recovery and acquisition of specific products. In addition to kinetic stability, industrial applications also require a biocatalyst with adequate mechanical resistance and safety for applications in the food industry (Klein et al., 2016; Sheldon & van Pelt, 2013).

Chitosan has been widely used as a support for enzyme immobilization because it has desirable characteristics, e.g., non-toxic, inertness, biocompatible, biodegradable, bacteriostatic, inexpensive; moreover, it is considered to be a versatile material (Flores et al., 2019; Kaushal, Seema, Singh, & Arya, 2018). The structure of chitosan makes it a multifunctional support for enzyme immobilization (Verma, Kumar, Das, Randhawa, & Chamundeeswari, 2020). However, the low mechanical strength and crosslinking of chitosan require the use of agents to form more stable networks. Among the agents employed for this purpose, the best-known and most widely used is glutaraldehyde (Facin, Melchiors, Valério, Oliveira, & Oliveira, 2019). However, this agent should be avoided in food products owing to its cytotoxic effects at concentrations higher than 0.5 ppm (Hamedi, Moradi, Hudson, & Tonelli, 2018; Sung, Huang, Huang, & Tsai, 1999). Therefore, natural agents with lower toxicity are needed.

Genipin is a compound obtained from the fruits of *Genipa americana* and *Gardenia jasminoides* Ellis. Some studies show the genipin capacity to generate crosslinking between polymers and proteins. For this reason, the genipin is present activator character. Therefore, genipin plays a double role, as a support activating agent and as inter- or intramolecular crosslinker (Delmar & Bianco-Peled, 2015; Muzzarelli, El Mehtedi, Bottegoni, & Gigante, 2016; Tacias-Pascacio, García-Parra, Vela-Gutiérrez, Virgen-Ortiz, Berenguer-Murcia, Alcántara, & Fernandez-Lafuente, 2019). Genipin is 5,000 to 10,000 times less cytotoxic compared to glutaraldehyde (Bellé et al., 2018; Sung et al., 1999); therefore, it is a promising compound for enzyme immobilization systems with applications in the food industry. Moreover, genipin is reported to have antioxidant, anti-inflammatory, antitumor, antidepressant, antifungal, antiangiogenic, antidiabetic, and immunosuppressant activities (Hobbs et al., 2018; Lee, Kim, Oh, & Lee, 2018;

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Shanmugam et al., 2018).

The enzyme β-galactosidase is produced by the yeast *Kluyveromyces lactis* and plays an important role in the dairy industry by catalyzing the hydrolysis of lactose into glucose and galactose. This enzyme is essential for the development of new products with low lactose content and/or the production of prebiotics such as lactulose and oligosaccharides (Bellé et al., 2018). However, few studies in the literature have described the immobilization of this enzyme for application in these processes and products.

It is estimated that about 70% of the adult population has some degree of lactase deficiency (Rueda et al., 2016). To attend the growing number of individuals with lactose intolerance, the food industry has searched for ways to employ enzymes to benefit the health of consumers, improve the nutritional quality of foods, and obtain novel functional products (Balthazar et al., 2017). Evaluating the thermal stability of β-galactosidase in the presence of lactose (40% w/v), Klein et al. (2016) found that the enzyme immobilized in genipin-activated chitosan spheres had greater thermal stability compared to the free biocatalyst. The immobilized enzyme showed 63% of residual activity after 540 min of incubation at 60 °C, meaning that the enzyme is more stable in the immobilized form than in the free form under operational conditions. Thermal stability needs to be assessed, as both time and temperature are important aspects of the processing of zero-lactose foods and the production of prebiotics, such as lactulose. Previous studies reported that immobilized enzymes operate at different temperatures and times. Song, Lee, Park, and Kim (2013) investigated the production of lactulose and concluded that the temperature of immobilized β-galactosidase is 40 °C, and remains stable for 150 min. Thus, thermal stability is an advantage of immobilized enzymes over free enzymes, enabling greater industrial applicability.

Based on the satisfactory results achieved with chitosan as a support for β-galactosidase (Bellé et al., 2018; Flores et al., 2019; Kaushal et al., 2018) and considering the importance of improving bioprocesses in terms of food compatibility, the use of genipin as a crosslinking agent and natural activator may be a viable alternative for immobilization of β-galactosidase and subsequent application in lactose hydrolysis processes as well as in the synthesis of prebiotics (Guerrero, Vera, Serna, & Illanes, 2017). Therefore, the aim of this study was to evaluate the immobilization of the enzyme β-galactosidase from *Kluyveromyces lactis* in a genipin-activated chitosan support and to apply the enzymatic derivatives in the hydrolysis of ultra-high-temperature (UHT) diluted milk in lower temperatures. Genipin is less cytotoxic compared to activators commonly used for enzyme immobilization and, for this reason, it can be safely applied in the food industry (Bellé et al., 2018; Sung et al., 1999).

2. Materials and methods

2.1. Materials

β-Galactosidase from *Kluyveromyces lactis* (EC 3.2.1.23) was kindly donated by the Prozyn Company (Brazil), with enzymatic activity of 1121 U.mL^{-1} in O-nitrophenyl β-D-galactopyranoside (ONPG) substrate. Standard chitosan support was purchased from Polymar (Brazil). ONPG, 2-nitrophenol (ONP), and genipin (analytical grade), all with purity ≥ 98%, were purchased from Sigma-Aldrich Co. (Germany). For phosphate buffer solution, sodium phosphate monobasic and sodium phosphate dibasic were used (P.A., from Reactec - Brazil). Acetic acid P.A. and sodium hydroxide P.A. were acquired from Dinâmica (Brazil). All reagents were used as received.

2.2. Preparation of support

Chitosan spheres were prepared using the method proposed by Duarte et al. (2017), with some modifications. Chitosan (0.2 g) was solubilized in 10 mL of acetic acid solution 0.35 M and submitted to sonication for one hour in an ultrasound bath (Quimis, 135 W; frequency of 60 Hz – Brazil). The product was then dripped with the aid of a syringe and needle at a cold temperature (10 °C) for coagulation in an alcoholic sodium hydroxide solution (sodium hydroxide 1 M and ethanol 26% v/v) under magnetic stirring (250 rpm). The spheres remained motionless for two hours in a refrigerator at 10 °C, followed by washing with distilled water until neutrality and then submitted to dry in an experimental apparatus with the aid of a vacuum pump, operating at 100 mbar, connected to Büchner flask and funnel, using qualitative filter paper (Whatmann, number 40), for one hour at room temperature.

2.3. Crosslinking and activation of support

The simultaneous crosslinking and activation process were conducted in 0.5% genipin solutions in the presence of phosphate buffer 0.02 M, pH 7.0 (Klein et al., 2016). In test tubes, chitosan spheres measuring 2 mm were immersed in 0.4 mL of genipin solution. Activation was performed in a water bath (60 °C) for one hour without stirring. After activation, the spheres were washed in phosphate buffer solution (0.02 M, pH 7.0).

2.4. Immobilization of β-galactosidase

The activated spheres were used as support for enzyme immobilization, employing a covalent bond technique. For such, the spheres were placed in contact with 2 mL of enzymatic solution at a concentration of 56 U·mL^{-1} (Song et al., 2013) for 8 h at 25 °C in a rotary shaker Lucadema- LUCA-223 (Brazil) at 200 rpm.

2.5. Enzyme activity

2.5.1. Free enzyme

ONPG was used as the substrate to measure the activity of the free enzyme. 10 mL of potassium phosphate buffer 0.02 M (pH 7.0), ONPG 10 mM, and 25 μL of β-galactosidase solution were used. The reaction was conducted at 37 °C and 150 rpm for 10 min in a rotary shaker. To interrupt the reaction, 2 mL of sodium carbonate 1 M was added to the reaction medium. The reaction releases 2-nitrophenol (ONP), which was measured in a FEMTO Cirrus 80SA spectrophotometer (Brazil) at 410 nm and calculated by Equation (1). A standard ONP curve was used to calculate enzyme activity. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 mol of ONPG per minute under the reaction conditions (Song et al., 2013).

$$\textit{Free enzyme activity } (FE) = \frac{(R_{abs} - B_{abs})xVxf}{rtxV_{enz}} \quad (1)$$

where:

R_{abs}: absorbance read in the spectrophotometer after reaction with the free enzyme;
B_{abs}: absorbance of the blank;
V: reaction volume;
f: correction factor of the ONP calibration curve;
rt: reaction time;
V_{enz}: volume of enzyme used.

2.5.2. Immobilized enzyme

The activity of the immobilized enzyme was measured by adding 40 chitosan spheres (4.57 mg of support on a dry weight basis to 20 mL of phosphate buffer 0.02 M, pH 7.0, and 10 mM ONPG at 37 °C and 150 rpm. Aliquots of 1 mL were withdrawn every 10 min until the end of the reaction (60 min). The reaction was interrupted with 0.2 mL of sodium carbonate 1 M and enzyme kinetics was evaluated. The activity of the enzyme derivates was calculated using Equation (2):

$$\textit{Immobilized enzyme activity } (EA) = \frac{(Slope - Slope_B)xVxDxf}{m_s} \quad (2)$$

where:

Slope: angular coefficient of the enzyme kinetics curve;
$Slope_B$: angular coefficient of the blank enzyme kinetics curve;
V: reaction volume;
D: dilution;
f: correction factor of the ONP calibration curve;
m_s: mass of the support.

Immobilization yield (IY) was calculated on the basis of Sheldon and van Pelt (2013), using Equation (3):

$$Immobilization\ yield\ (IY) = \frac{Immobilized\ activity}{Initial\ activity} x100 \quad (3)$$

Immobilized activity was considered as the difference between the activity offered to the support and the activity determined in the supernatant (water used to wash the immobilized spheres). Initial activity was considered the activity of the enzyme offered to the support.

2.6. Optimization of enzyme immobilization

To optimize the enzyme immobilization process, the variables number of spheres submitted to immobilization and substrate concentration were evaluated, by using a central composite rotational design (CCRD) 2^2 with axial points and three repetitions at the central point, in a total of 11 assays carried out in duplicate. The independent variables were number of spheres ($mg{\cdot}mL^{-1}$) used in the reaction medium (X_1) and concentration of the ONPG substrate (mM) (X_2). The dependent variables were immobilization yield (%) and enzyme activity ($U{\cdot}g^{-1}$). These were performed with rinsed samples in order to avoid that the remaining substrate alter the enzyme activity data. The results underwent analysis of variance (ANOVA) with the aid of the Protimiza Experiment Design software (Brazil). The ranges of the number of spheres and substrate concentrations used in the experimental design were determined on the basis of previous descriptions from the literature (Flores et al., 2019; Klein et al., 2016; Song et al., 2013) and are shown in S1 (Supplementary Material).

2.7. Thermal stability of free and immobilized enzymes

To determine the thermal stability of the immobilized enzyme, 4.57 $mg{\cdot}mL^{-1}$ of spheres, with an enzymatic load of 56 $U.mL^{-1}$, was added to a tube containing phosphate buffer solution 0.1 M at pH 6. The assays for the free enzyme used the same protocol with concentration of 25 $\mu L{\cdot}mL^{-1}$ (corresponding an enzymatic activity of 56 $U.mL^{-1}$) at phosphate buffer solution 0.02 M at pH 7.0, as described in section 2.5.1. The temperatures evaluated were 10 °C, which is the temperature of milk and whey cooling, and 37 °C, the optimal temperature indicated by the enzyme manufacturer (PROZYN) for lactose hydrolysis and another studies with β-galactosidase from *Kluyveromyces lactis* (Lima et al., 2013). For the temperature of 10 °C, the tubes with the enzymes were incubated in a digital BOD incubator (Cienlab – Brazil). A digital water bath (SolidSteel – Brazil) was used for the assays at 37 °C. Stability was assessed by measuring enzyme activity (as described below in Items 2.5.1 and 2.5.2) until relative activity was less than 50%.

2.8. Operational stability

The operational stability of the immobilized enzyme was evaluated in discontinuous cycles, incubating the number of spheres optimized in the experimental planning (Item 2.6) in 1 mL of UHT milk and 9 mL of phosphate buffer 0.1 M, pH 7.0, based on literature report (Wolf, Belfiore, Tambourgi, & Paulino, 2019). The cycle was performed for 300 min at 37 °C under constant stirring (150 rpm). Aliquots were withdrawn at 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min for the analysis of enzymatic activity using an ANALISA kit to detect glucose (Gold Analisa - Brazil). Absorbance was read in a spectrophotometer (FEMTO Cirrus 80SA - Brazil) at 505 nm and a standard curve performed with 2-Nitrophenol was used for the calculation. After each cycle, the immobilized enzyme was withdrawn from reactional medium, washed with phosphate buffer (0.02 M, pH 7.0), and then submitted to dry in an experimental apparatus with the aid of a vacuum pump, operating at 100 mbar, connected to Büchner flask and funnel, using qualitative filter paper (Whatmann, number 40), for one hour at room temperature and placed in a new reaction medium. The percentage of lactose hydrolysis after the first cycle was defined as 100% and calculated using Equation (4). The determination was performed in reaction replicates, and the analyses were performed in triplicate.

$$Lactose\ hydrolysis\ (\%) = \frac{Sample_{abs}}{Standard_{abs}} x100 \quad (4)$$

2.9. Stability during storage

Stability during storage was analyzed using four different storage methods: (1) lyophilized spheres frozen at −18 °C, (2) lyophilized spheres stored in a refrigerator at 10 °C, (3) spheres in sodium phosphate buffer solution 0.1 M (pH 7.0) in a refrigerator at 10 °C, and (4) spheres frozen at −18 °C. After storage for 24 h, the spheres were analyzed, and activity was measured as described in Item 2.5.2. Subsequent analyses were performed after 7, 14, 21, and 30 days of storage.

2.10. Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed to determine the thermal degradation of the enzymatic derivatives in an N_2 atmosphere. The analysis was performed with a STA 449 F3 Jupiter (Netzsch - Germany). Samples of approximately 10 mg were heated from 30 to 700 °C at a rate of 20 $°C{\cdot}min^{-1}$ in a flowing nitrogen atmosphere (10 $mL{\cdot}min^{-1}$).

2.11. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to collect information on the morphological characteristics of the chitosan spheres, such as fissures, pores, and rugosities. The samples were fixed with double-sided tape on a stub and coated with a thin layer of gold. Scanning electron microscopy with field emission (HITACHI TM3030 - Japan) was performed, and the samples were analyzed with magnifications of 25×, 30×, 60×, and 80× at power emissions of 5 and 15 kV.

2.12. Textural characterization

Texture analysis of the dry spheres (DS), activated dry spheres (DS-A), immobilized activated dry spheres (DS-I), and immobilized activated frozen dry spheres (DS-F) was performed in a CT3 Texture Analyzer (Brookfield - USA) with an acrylic cylindrical probe measuring 25.4 mm, previously calibrated using a 50 kg load cell. The samples were submitted to 20% compression at a constant speed of 2 $mm{\cdot}s^{-1}$. Based on the force results, the quantified texture variable was hardness, which is the force required to achieve a given deformation of the material (Bellé et al., 2018).

2.13. Statistical analysis

The formulations were prepared in replicates and the analyses were carried out in triplicate. The results underwent analysis of variance (ANOVA) and Tukey's test using the R software (R Foundation-New Zealand), with statistical significance set at 5% ($p < 0.05$) and considering 95% confidence levels.

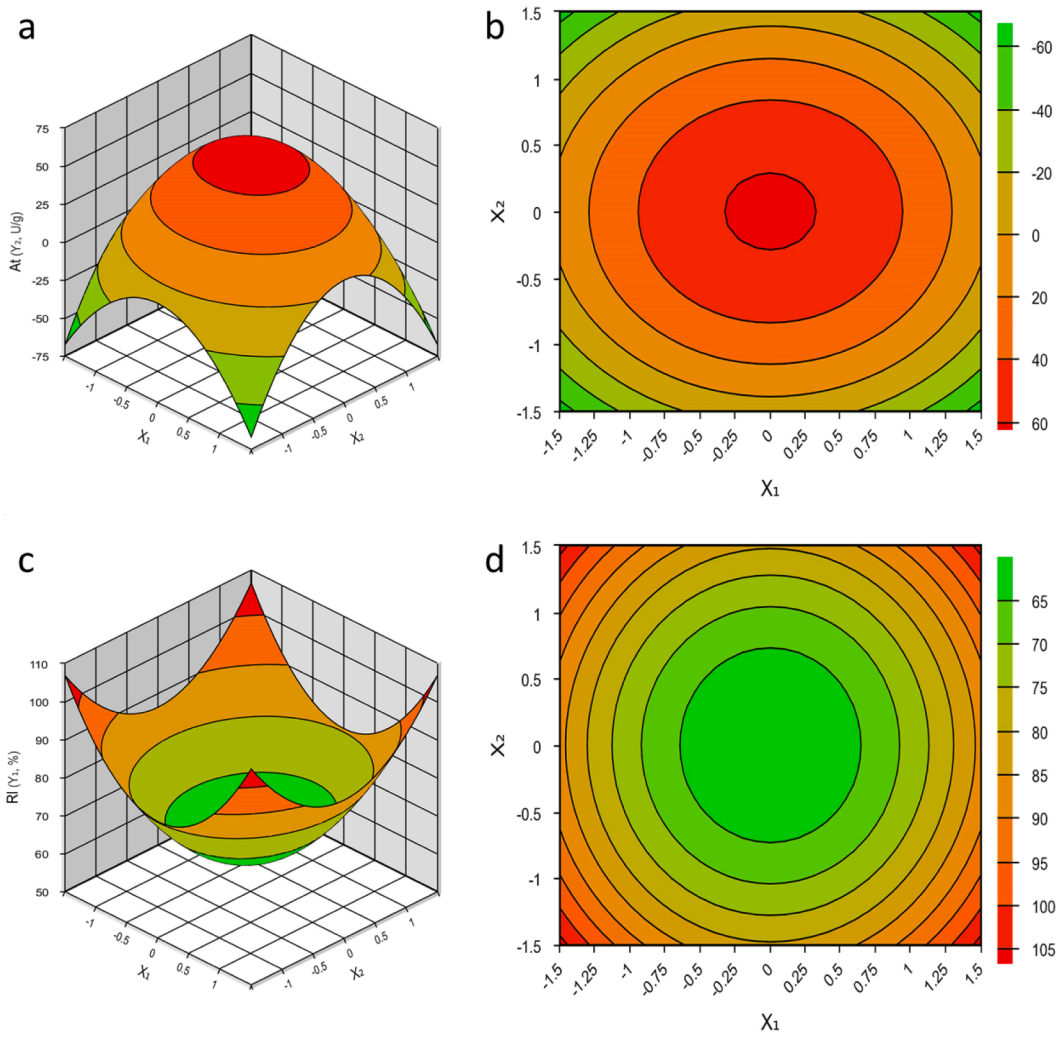

Fig. 1. Response surfaces and contour curves for enzyme activity (a, b) and immobilization yield (c, d) of β-galactosidase immobilized in the genipin-activated chitosan support according to number of spheres (mg·mL^{-1}) (X_1) and substrate concentration (mM) (X_2).

3. Results and discussion

3.1. Optimization of enzyme immobilization

Enzyme activity ranged from 0.67 ± 0.01 to 66.51 ± 0.24 U·g^{-1} and immobilization yield ranged from 58.98 ± 0.14 to 84.13 ± 0.14% (S1 – supplementary information), demonstrating considerable variability with the change in the number of spheres and substrate concentration. Flores et al. (2019) studied β-galactosidase immobilization in a genipin-activated chitosan support using 2 mg·mL^{-1} of spheres and 15 mM of substrate and found IY of 86.1%. Bellé et al. (2018) used 0.5 mg·mL^{-1} of support and 10 mM of substrate, resulting in an IY of 74%.

The models were validated using Fisher's test (F test) considering the degrees of freedom of the regression and residuals. Based on the ANOVA results shown in S2 (supplementary information), the models can be used to predict the enzyme activity and immobilization yield of β-galactosidase in the genipin-activated chitosan support, since F_{cal} values were higher than F_{tab} values. Moreover, regression was highly significant, as F_{cal}/F_{tab} was 20.87 for enzyme activity and 105.43 for immobilization yield. Therefore, the experimental data are well represented by the adjusted models, which can be employed for predictive purposes within the domain of the study factors.

Substrate concentration and number of spheres exerted an influence on the dependent variables. Below are the models of estimated enzyme activity (EA) (Equation (5)) and immobilization yield (IY) (Equation (6)) of β-galactosidase for the chitosan-based support as a function of significant encoded variables:

$$EA\left(U.g^{-1}\right) = 60.11 + 11.6X_1^2 + 9.09X_2^2 \quad (5)$$

$$IY(\%) = 62.74 - 25.47X_1^2 - 32.34X_2^2 \quad (6)$$

where:

X_1: number of spheres (mg.mL^{-1});
X_2: substrate concentration (mM).

After the validation of the models, response surfaces and contour curves were generated for enzyme activity (Fig. 1a, b) and immobilization yield (Fig. 1c, d) of β-galactosidase in the genipin-activated chitosan support.

The responses appear on the Y axis as EA (U·g^{-1}) and IY (%) as a function of the operational conditions: number of spheres (mg·mL^{-1}) (X_1) and substrate concentration (X_2). The saturation tones with a more intense color correspond to regions with the highest enzyme activity and immobilization yield. The optimal experimental conditions for EA were the central point of the experimental design (X_1 = 2.6 mg·mL^{-1} and X_2 = 10 mM). For IY, the axial points (X_1 = 4.57 mg·mL^{-1} and X_2 = 10 mM) were more satisfactory.

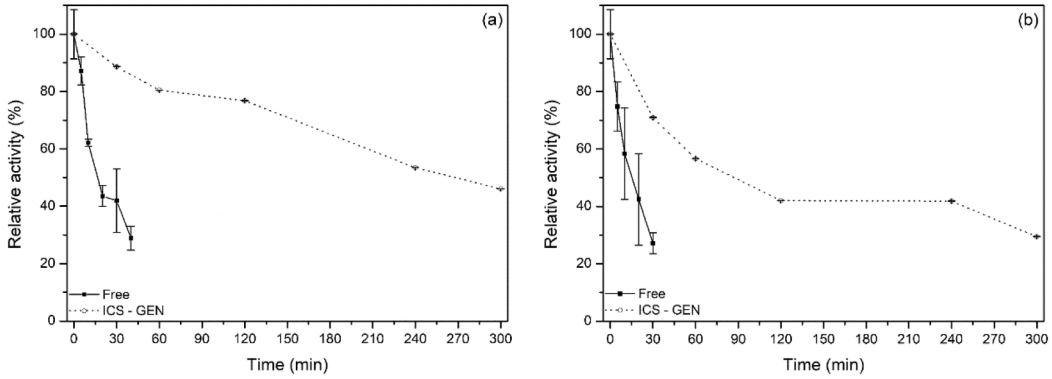

Fig. 2. Thermal stability of free and immobilized enzyme in genipin-activated chitosan spheres at 10 °C (a) and 37 °C (b).

Number of spheres (X_1) and substrate concentration (X_2) were significant for IY, as shown in Equation (6). The largest number of spheres resulted in higher IY and considerable EA. As the concentration of the activator was not altered in the assays, 4.57 mg·mL^{-1} of spheres enabled greater bond interfaces, increasing the total area for contact with the activating agent and enzyme (Klein et al., 2013) and, consequently, leading to a higher IY value compared to smaller numbers of spheres. Moreover, this number of spheres (4.57 mg·mL^{-1}) was satisfactory for the distribution of the enzyme within the macropores and its access to the active site, as demonstrated by EA (24.97 U·g^{-1}). The medium substrate concentrations (10 nM) conducted to higher EA and IY for largest number of spheres (2.6 and 4.57 mg·mL^{-1}). Probably due to the greater number of active sites being available, substrate access, and interaction between enzyme-substrate, evidenced by the values of enzymatic activity obtained.

In the study of EA, the two variables also exerted a significant influence ($p \leq 0.05$). However, the optimal conditions were found at the central point (2.6 mg·mL^{-1} of spheres and 10 mM of substrate). Despite the greater activity achieved in this assay, lower IY was found (60.68%) compared to assay 6 (84.13%), which may be due to the smaller area available for enzyme fixation. Previous studies conducted by Klein et al. (2013) indicate that immobilized enzymes can undergo random fixation of proteins on the surface of the particles, which can alter the conformation of the enzyme. Another possible explanation is the diffusional limitation in the immobilized enzyme, which leads to a reduction in IY (Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013). For this operational recycling enabled by the immobilized enzyme, high IY is important because enzyme fixation to the support is evaluated based on yield. Therefore, a larger amount of enzyme in the support (provided that it is activated and available) increases the likelihood of reuse in continuous cycles.

Considering the results achieved with the experimental design with maximum immobilization yield (84.13%) and satisfactory enzyme activity (24.97 U·g^{-1}) for β-galactosidase immobilized in a genipin-activated chitosan support, 4.57 mg·mL^{-1} of spheres (+1.41 axial point) and a substrate concentration of 10 mM (central point) achieved the best results. Therefore, assay 6 was selected for the subsequent experiments.

3.2. Thermal stability

Enzyme reactions and the velocities of these reactions are dependent on temperature. However, the decrease on enzyme activity may occur as a result of denaturation at some temperatures (Wolf et al., 2019). Industrial processes require enzyme stability owing to the variable conditions employed for different products. Thus, the thermal stability of the free and immobilized enzyme was analyzed at temperatures of 10 and 37 °C. The results of these experiments are shown in Fig. 2.

The immobilized enzyme exhibited greater thermal stability

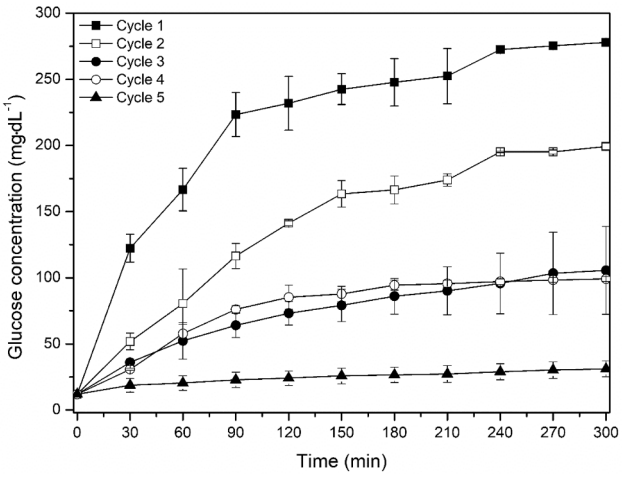

Fig. 3. Operational stability of immobilized enzyme during five successive cycles for 300 min (9 mg of support per mL of solution containing diluted UHT milk) at 37 °C and constant stirring (150 rpm).

compared to the free enzyme, indicating that immobilization in genipin-activated chitosan spheres had a positive effect on the thermal stability of β-galactosidase, protecting the active conformation of the enzyme from damage owing to heat exchange. The immobilized enzyme maintained more than 40% of its initial activity after 300 min of incubation at 10 °C, whereas the free enzyme exhibited less than 30% of activity after only 40 min.

Klein et al. (2016) reported greater stability and a more rigid structure of β-galactosidase after its immobilization in genipin-activated chitosan particles owing to the formation of multipoint covalent bonds between the enzyme and the immobilization support. Some studies reported that molecular confinement, which restricts movement, reduces the possibility of enzyme inactivation and increases its thermal stability (Klein et al., 2016). This phenomenon was observed in this study. Thus, immobilization in genipin-activated chitosan spheres created a more rigid structure of the enzyme and enabled it to maintain activity for a longer time at a low temperature (10 °C).

The thermal stability of an immobilized biocatalyst is key for industrial applications. It may depend on the support material, the immobilization process or the enzyme itself. The temperature of 37 °C was investigated in the present study because this is the optimal temperature of the immobilized enzyme and the temperature of the specification chart of β-galactosidase produced by *Kluyveromyces lactis* (PROZYN) and other studies with this same enzyme (Lima et al., 2013). The immobilized enzyme exhibited better stability than the free enzyme, maintaining 30% of its initial activity after 300 min of incubation,

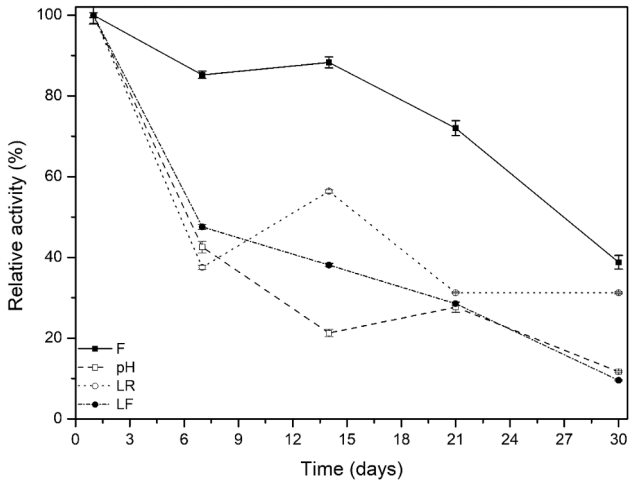

Fig. 4. Stability during storage of immobilized enzyme with different preservation methods: frozen (F), immersed in buffer solution (pH), lyophilized and refrigerated (LR) and lyophilized and frozen (LF).

whereas the free enzyme maintained only 27% after 30 min.

The greater thermal stability of the immobilized enzyme in comparison to the free enzyme may be related to the protection provided by the structure formed between the enzyme and genipin-activated chitosan support (Monajati, Borandeh, Hesami, Mansouri, & Tamaddon, 2018), which enables the conformation of the catalytic site to remain intact. Moreover, such stability may derive from the multipoint covalent bonds between the enzyme and support (Pereira et al., 2015).

3.3. Operational stability

Reuse capacity is an advantage of immobilized biocatalysts compared to free enzymes, leading to a reduction in operational costs (Flores et al., 2019). In this study, the immobilized enzyme was reused to assess the maintenance of the catalytic activity in successive hydrolysis reactions. The results are shown in Fig. 3.

We assessed five cycles with the immobilized enzyme in reactions using diluted UHT milk. The immobilized enzyme maintained a high degree of lactose hydrolysis for four cycles, possibly because the rigidity of the enzyme attachment on the support, preventing the catalytic site deformation (Flores et al., 2019). Moreover, the cofactors of the enzyme (Mn^{+2} and K^{+}) are present in the mineral composition of bovine milk and may have contributed to the good performance of the immobilized biocatalyst. Enzymatic cofactors, such as the ions Na^{+}, Mg^{2+}, and Mn^{2+}, contained in UHT milk assist in lactose hydrolysis by β-galactosidase (Plou, Polaina, Sanz-Aparicio, & Fernández-Lobato, 2017).

Immobilized β-galactosidase in genipin-activated chitosan spheres could be employed in four successive cycles of lactose-free/low-lactose milk with good performance. The reduction in activity that occurs after the reuse of immobilized enzymes could be due to weakening in the strength of binding between the matrix (alkylamine glass with glutaraldehyde) and enzyme, which might detach the enzyme from the matrix. Another reason could be the frequent encountering of substrate and the catalytic site, which might distort the regular three-dimensional arrangement and hence the catalytic efficiency either partially or completely, as observd by Kishore and Kayastha (2012), since the performance of the enzyme depends on several parameters, e.g., tertiary structure and enzyme characteristics, immobilization methods and matrix being used (Katrolia, Liu, Li, & Kopparapu, 2019).

In reactions for the hydrolysis of UHT milk with β-galactosidase immobilized in glutaraldehyde-activated chitosan, Wolf et al. (2019) found a concentration of 90 mg·dL^{-1} of formed glucose after five successive cycles. In this study, approximately 275 mg·dL^{-1} of glucose was formed in the first cycle. There was a decrease over the cycles, reaching

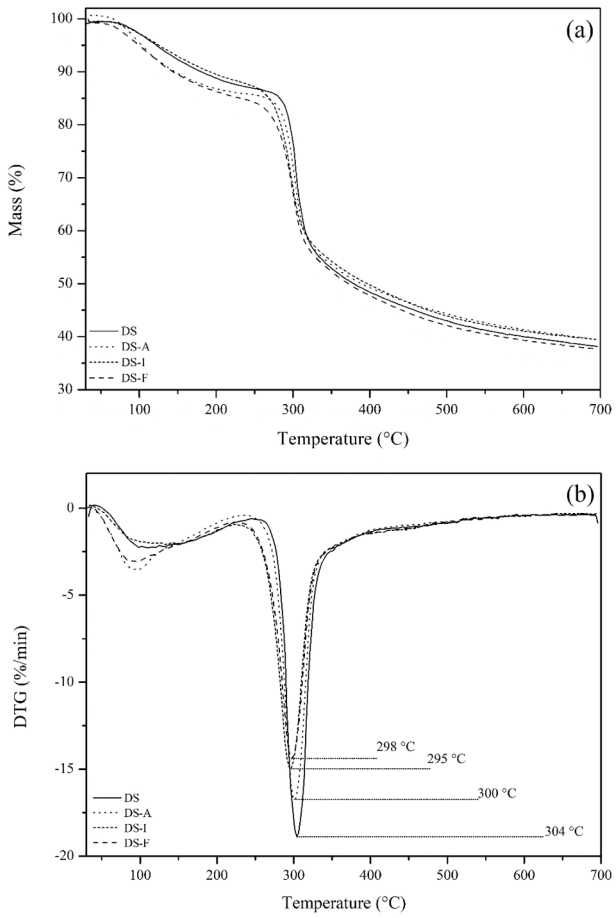

Fig. 5. Thermogravimetric analysis (a) and derivatives curves (b) of dry spheres (DS), activated dry spheres (DS-A), immobilized activated dry spheres (DS-I), and immobilized activated frozen dry spheres (DS-F).

approximately 96 mg·dL^{-1} in the fourth cycle of use. This difference shows the possibility of a higher degree of hydrolysis in diluted UHT milk with the enzyme immobilized in genipin-activated chitosan. The result shows the possibility of applying a natural agent, with lower toxicity compared to conventional ones.

3.4. Stability during storage

The stability of β-galactosidase immobilized in genipin-activated chitosan spheres was evaluated during storage for a period of 30 days using four different processes (Fig. 4). The results show that freezing was the most effective, as this method maintained relative activity greater than 70% for up to 21 days of storage. Such stability may be due to the rigid structure of the immobilized enzyme that was acquired after the formation of multipoint covalent bonds between the enzyme and the immobilization vehicle (Rodrigues et al., 2013). A more rigid enzyme is less prone to unfolding and denaturation after a long storage time (Zhang et al., 2017), and freezing seems to have been more effective at maintaining the structure of the immobilized enzyme. The rearrangement of the chitosan polymer network during the freezing process may have contributed to the formation and preservation of the porous structure.

The lyophilization process removes water or solvents from the structure, compresses the pores of polymer matrices, and can sometimes generate the self-degradation of the matrix. This degradation may lead to a reduction in the number of active sites, contributing to a reduction in activity (Risbud, Hardikar, Bhat, & Bhonde, 2000). Regarding the immobilized enzyme stored in the buffer solution, the temperature and

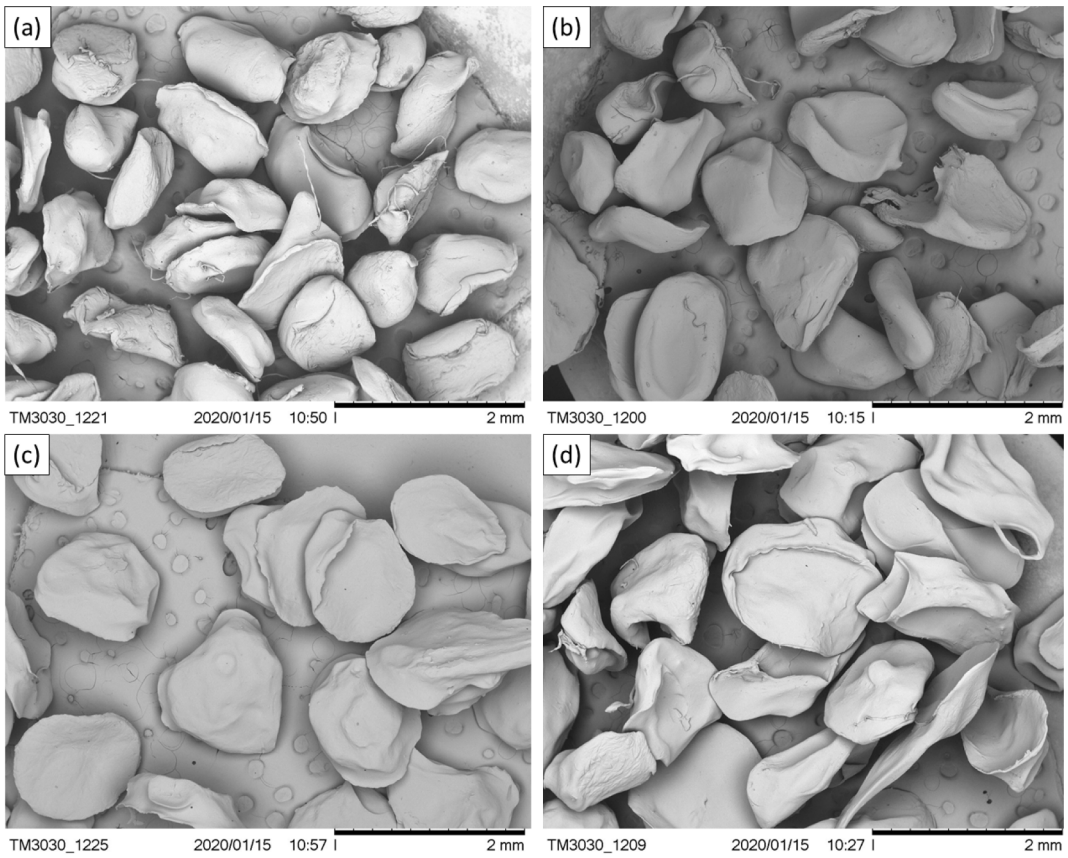

Fig. 6. Scanning electron microscopy images of the dry spheres (a), activated dry spheres (b), immobilized activated dry spheres (c), and immobilized activated frozen dry spheres (d) (magnification: 25×).

pH could be influencing the enzyme activity, reducing it throughout the storage period. Katrolia et al. (2019) reported that the pH optima of chitosan-immobilized enzymes shifted to more acidic pH values of pH 3.0 as compared to that of the free enzyme (pH 4.5). On the other hand, immobilization did not greatly affect the optimal pH of the yeast (*K. lactis*) enzymes. The pH optima of free and immobilized enzymes were similar at pH 7.0. So, at this work, the enzyme could have suffered the influence of genipin and, it had its activity decreased even at pH 7. Flores et al. (2019) pointed that the crosslinking reaction mechanisms for chitosan with genipin are different at each pH value. The increase on the pH values generates a bigger ionization of genipin and chitosan chains, producing multipoint attachments, which can possibly promote changes on tertiary conformational of the enzyme structure and, consequently, a decrease of activity recovery. Moreover, the low temperature (10 °C) could be influenced the activity of the enzyme stored in the buffer solution at refrigerated conditions, because the optimal temperature of this enzyme is 37 °C (Lima et al., 2013).

Based on these results, freezing was selected for the storage of β-galactosidase immobilized in chitosan spheres, as this method maintained adequate activity for up to 21 days, enabling the industry to use the immobilized enzyme for a longer time and store it until required for the biocatalysis of subsequent reactions.

3.5. Thermogravimetric analysis

Fig. 5 shows the results of the thermogravimetric analysis (TGA) of the dry spheres (DS), activated dry spheres (DS-A), immobilized activated dry spheres (DS-I), and immobilized activated frozen dry spheres (DS-F). The first degradation step is relative to the evaporation of absorbed water, owing to the hydrogen-bond with amino and the hydroxyl groups. The second degradation step occurred between 295 and 304 °C, as shown in the DTG image. This step indicated the depolymerization of the chitosan polysaccharide ring structure (C—O—C and C—C bonds) (Gámiz-González et al., 2017). As reported in the literature, neat genipin and neat chitosan degradation occurs around 220 °C and 304 °C, respectively. Therefore, if the degradation temperature of these two compounds together is around this value (closer to the degradation temperature of chitosan), indicates that there was effective crosslinking between components, and a more thermally stable chitosan structure (Mak & Leung, 2019). Also, as there were minimal differences in thermal degradation of the samples, it can be stated that activation and immobilization did not affect the thermal behavior of chitosan. Therefore, this biopolymer can be considered as appropriate for this specific use.

3.6. Scanning electron microscopy

Fig. 6 shows the scanning electron microscopy (SEM) images of the dry spheres (DS), activated dry spheres (DS-A), immobilized activated dry spheres (DS-I), and immobilized activated frozen dry spheres (DS-F). The activation of spheres can change the way genipin reacts and polymerizes, which can generate different structures, colors, and capacities (Neri-Numa, Pessoa, Paulino, & Pastore, 2017). Also, the use of crosslinker agents could have preserved the surface of chitosan spheres under vacuum and, therefore, they may have suffered less shrinking (Biró, Németh, Sisak, Feczkó, & Gyenis, 2008). Therefore, the spheres maintained their structure during activation with genipin and immobilization of β-galactosidase as well as during frozen storage in comparison to the dry spheres. The samples presented a smooth and homogenous surface, and there was no presence of wrinkles or fissures, enabling greater contact for activation and immobilization (Klein et al., 2013).

3.7. Textural characterization

Hardness of the genipin-activated chitosan spheres after activation and immobilization (S3 – supplementary information) was analyzed to determine the possibility of a change in structure. The chitosan spheres crosslinked with genipin showed improved hardness. The DS had the lowest hardness value (0.30 N). Crosslinking enables the formation of covalent bonds that maintain a rigid structure in chitosan spheres (Bellé et al., 2018). The samples DS-I and DS-F presented a hardness value of 2.53 N, while DS-A evaluation shown a 1.57 N of hardness.

Bellé et al. (2018) found a hardness value of 0.55 N for chitosan spheres and 2.5 N for spheres activated with 0.5% genipin, whereas the hardness of glutaraldehyde-activated spheres was only 1 N. Therefore, immobilization using genipin as the activator achieves better results in terms of improved structural quality compared to glutaraldehyde, offering advantages from the economic and toxicological standpoints.

In the specific case of chitosan, the addition of small amounts of genipin can significantly increase the elasticity and strength of the product under acidic conditions (Peng et al., 2014). In the immobilization process, β-galactosidase forms multipoint covalent bonds with the support through the reaction with the crosslinked chitosan. These bonds provide the enzyme with a more rigid structure (Rodrigues et al., 2013). Moreover, freezing does not seem to have affected the structure of the spheres, making this method a convenient choice for storage of the spheres.

4. Conclusions

The immobilization of β-galactosidase produced by *Kluyveromyces lactis* in a genipin-activated chitosan support proved to be a viable strategy for biocatalysis process. Genipin was efficient as an activating and crosslinking agent of the chitosan support, making the immobilized enzyme more stable at different temperatures. Also, this compound enabled mechanical resistance for the enzymatic derivatives. Thus, it was possible to obtain catalytic capacity, operational stability, and a differentiated conformation of the active and immobilized chitosan spheres. Furthermore, genipin has lower toxicity compared to other activators used in the immobilization process, offering an advantage in possible applications. The use of the immobilized enzyme was feasible, exhibiting potential use in free-lactose, prebiotic, and functional products, as shown by the results achieved in the reactions performed with diluted UHT milk. Moreover, the immobilized enzyme exhibited greater thermal stability than the free enzyme at a temperature of 10 °C, which is used in some milk-based food production processes. Therefore, the immobilization technique showed to be efficient and enables future applications of the enzymatic derivative obtained in this work.

CRediT authorship contribution statement

Pâmela Cristina Lima: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing - original draft. **Isadora Gazoni:** Investigation. **Alexandra Melissa Gonçalves de Carvalho:** Investigation. **Daniela Bresolin:** Methodology, Investigation, Writing - review & editing. **Darlene Cavalheiro:** Methodology, Writing - review & editing. **Débora de Oliveira:** Supervision, Conceptualization, Validation, Writing - review & editing. **Elisandra Rigo:** Project administration, Supervision, Conceptualization, Data curation, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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