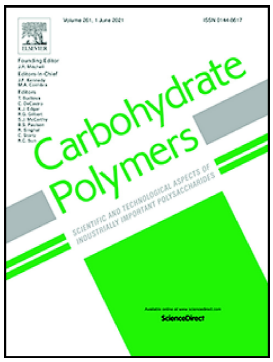

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PII: S0144-8617(22)00388-5

DOI: https://doi.org/10.1016/j.carbpol.2022.119483

Reference: CARP 119483

To appear in: *Carbohydrate Polymers*

Received date: 5 December 2021

Revised date: 30 March 2022

Accepted date: 10 April 2022

Please cite this article as: C.R. Hackenhaar, C.F. Rosa, E.E.E. Flores, et al., Development of a biocomposite based on alginate/gelatin crosslinked with genipin for β-galactosidase immobilization: Performance and characteristics, *Carbohydrate Polymers* (2021), https://doi.org/10.1016/j.carbpol.2022.119483

Development of a biocomposite based on alginate/gelatin crosslinked with genipin for β-galactosidase immobilization: performance and characteristics

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ABSTRACT

In this work, we studied the development of a biocomposite formulated with alginate and gelatin, crosslinked with genipin for application as support for β-galactosidase immobilization. Also, the biocomposites with the immobilized enzyme were characterized by thermal analyses and SAXS (size, density, and interconnectivity of alginate rods) for a detailed analysis of the microstructure, as well as the thermal and operational stabilities of the enzyme. The structural modifications of the biocomposite determined by SAXS demonstrate that the addition of both genipin and enzyme produced a significant reduction in size and density of the Ca(II)-alginate rods. Immobilized β-galactosidase could be stored for 175 days under refrigeration maintaining 80% of its initial activity. Moreover, 90% of its relative activity was kept after 11 reuses in a batch process of lactose hydrolysis. Thus, the biocomposite proved to be effective as support for enzyme immobilization.

Keywords: sodium alginate, gelatin, immobilization, genipin, lactose hydrolysis, *Aspergillus oryzae.*

1. Introduction

Enzyme technology is becoming gradually more important for many applications in several industrial areas. Not only for their versatility and efficiency but also environmental issues, since its use in industrial processes has been replacing conventional procedures that use chemical catalysts, generating environmentally friendly methods. Thereby, enzymatic immobilization is an excellent tool, due to the possibility to improve enzyme properties, including increased activity under adverse conditions, better specificity, and possible recovery and reuse, thus reducing costs (Bilal & Iqbal, 2019; Miletić et al., 2012). However, it is necessary to recognize the limitations in enzymatic immobilization such as decreased catalytic efficiency, enzyme inactivation in the presence of support due to multiple interactions (Basso & Serban, 2019), or even mass transfer limitations promoted by immobilization (Mateo et al., 2007).

Among different solid supports described in the literature for enzymatic immobilization (Eskandarloo & Abbaspourrad, 2018; Ricardi et al., 2018), biopolymers have attracted researchers due to their characteristics of biocompatibility, hydrophilicity, biodegradability, and adhesion properties (Bilal & Iqbal, 2019), being also compatible to immobilization with genipin (Amaro-Reyes et al., 2019; Díaz-Hernández et al., 2018; Gracida et al., 2019).

Alginate is an anionic polysaccharide extracted mainly from brown seaweed, and it is composed of repeated units of α-1,4-L-guluronate (G) and β-1,4-D-mannuronate (M). Its composition depends on the extraction source (Wang et al., 2019), as well as its physical-chemical characteristics, which are strongly dependent on the monomer composition (M/G ratio) and distribution, and molecular weight. One of the excellent properties of sodium alginate is its ionotropic gelation capacity in the presence

of divalent ions, which leads to the formation of an "egg-box" structure (Bennacef et al., 2021), leading to a rod-like interconnected network (Traffano-Schiffo et al., 2018).

Gelatin is a polymer derived from collagen by hydrolysis, is cheap and abundant as it is present in animal skin and bones. Hydrolytic depolymerization can be performed through acidic or basic pre-treatment, producing gelatin type A and B, respectively (Duconseille et al., 2015). These different treatments impact its isoelectric point, where gelatin type A has pI ~ 8.5 and gelatin type B has pI ~ 4.9, which can drastically affect its functionality (Smith et al., 2016). Also, gelatin has limited use due to high hygroscopicity and poor mechanical properties (Wang et al., 2019). To overcome these drawbacks, chemical crosslinking reagents, such as genipin, could be added into gelatin formulations to improve mechanical properties.

Moreover, mechanical properties of supports for enzyme immobilization can be improved by the crosslinking with agents as genipin. This substance is an iridoid, obtained from the fruit of *Genipa americana* L. and flowers of *Gardenia jasminoides* Ellis by direct extraction or after extraction and hydrolysis of the geniposide (Paik et al., 2001). Genipin can react spontaneously with primary amine groups of amino acids, proteins, or peptides through crosslinking (Ramos-de-la-Peña et al., 2016), and can be used as a natural crosslinking agent in some polymers, such as chitosan and gelatin. According Sung et al. (1999) the cytotoxicity of genipin in a studied in vitro with 3T3 fibroblasts resulted 10,000 times lower than that of glutaraldehyde, a conventional crosslinker.

β-galactosidase from *Aspergillus oryzae* is an extracellular monomeric enzyme and hydrolyze the (1→4) linkage of lactose [galactosyl (1→4) glucose] to glucose and galactose in several products such as milk and whey, giving rise to products with low lactose content, which benefit people intolerant to this sugar. Besides, in the presence of

concentrated lactose, this enzyme synthesizes galactooligosaccharides, a prebiotic ingredient (Klein et al., 2016).

In this context, this study aimed to develop a biocomposite formulated with alginate and gelatin using genipin as a crosslinker for the immobilization of β-galactosidase as a model enzyme. Besides a detailed analysis of the hybrid material microstructure using Small-Angle X-ray Scattering (SAXS), other techniques such as TGA, thermal stability, storage, and operational stabilities were made revealing details about molecular associations and structural conformations. Therefore, this research provides new insights and valuable information for future research, especially for food applications.

2. Materials and Methods

2.1. Materials

β-Galactosidase from *Aspergillus oryzae* and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Sigma-Aldrich (São Paulo, Brazil). Sodium alginate, molecular weight 198.11 g/mol, was purchased from Cromato Produtos Químicos Ltda (São Paulo, Brazil), and gelatin type A from porcine skin (GECOLL) was provided by the company Vêneto Mercantil Importadora LTDA (Flores da Cunha, RS, Brazil). A D-glucose determination kit was purchased from Labtest Diagnóstica SA (São Paulo, Brazil). Genipin from *Genipa americana* was obtained in our laboratory by enzymatic extraction (Bellé et al., 2018). All other chemicals were of analytical grade.

2.2. Preparation of alginate-gelatin biocomposite

The biocomposite of sodium alginate and gelatin were prepared by the ionotropic gelation method as described by Freitas et al. (2011), with slight modifications. For this, different concentrations of sodium alginate (1.5, 2.0 and 3.0% w/v) and gelatin (0, 1.5, 3.0, 7.5, 10.0, 20.0, 30.0% w/v) were evaluated. Sodium alginate was previously dissolved in 8 mL distilled water (final solution pH of 5.7), followed by the addition of gelatin (the volumetric ratio between the alginate solution and gelatin was 1:5). The mixture was heated to 50 °C in a magnetic stirrer (model C-MAG HS 7, IKA, Germany), under gentle stirring, until complete dissolution. Afterward, the solution was sonicated for 30 min to remove the air bubbles and it was slowly dripped with a peristaltic pump (model 120S, Watson-Marlow, Brazil) into a 0.2 M $CaCl_2$ cooled solution, under slow stirring (the volumetric ratio between the alginate-gelatin mixture and $CaCl_2$ solution was 1:5). The pump speed was 12 rpm. A plastic tip with a 1.0 mm inner diameter was used for the dropping, and the distance between the $CaCl_2$ cooled solution and the tip was 10 cm. The shape of the formed biocomposites was spherical. After alginate-gelatin (AG) biocomposite generation, they were left in the $CaCl_2$ solution for 2 h before being rinsed 10 times with distilled water to eliminate the excess of Ca^{+2}. Afterward, different concentrations of genipin solutions (0.05, 0.10, 0.15, and 0.25% (w/v)) prepared in 0.05 M Tris-HCl buffer pH 8.0 were tested. The AG biocomposite was left in the genipin solution for 24 h at 25 (±2) °C, followed by washes with distilled water until pH 7.0, obtaining the genipin crosslinked biocomposite (AGG – alginate-gelatin-genipin). The AGG was kept at 4 (±1) °C until use as a support for β-galactosidase immobilization.

2.3. Immobilization of β-galactosidase on alginate-gelatin-genipin biocomposite

After preparation of the support, 3 mL of β-galactosidase solution (prepared in 0.1 M sodium acetate buffer pH 4.5) were added to 30 AGG (10 mg of support on a dry basis) and incubated for up to 24 h at 25 (±2) °C, to produce the biocomposite containing the immobilized enzyme (AGG-β-gal). The effect of enzyme concentration was evaluated at 4.5, 7.0, 15.0, and 23.0 U mL^{-1}. Subsequently, the AGG-β-gal were successively washed with buffer to remove excess enzyme. Between these washes, the AGG-β-gal was washed with NaCl (1 M), and ethylene glycol (30% v/v) to eliminate ionic and hydrophobic interactions between enzyme and support, respectively. Experiments were performed in duplicate.

The evaluation of the immobilization procedure was performed through parameters as immobilization yield (Y) immobilization efficiency (IE), and activity recovery (AR), reported in Equations 1 to 3, respectively (Sheldon & van Pelt, 2013).

$$Yield\ (\%) = \frac{Immobilized\ activity}{Starting\ activity}\ x\ 100 \qquad (1)$$

$$Efficiency\ (\%) = \frac{Observed\ activity}{Immobilized\ activity}\ x\ 100 \qquad (2)$$

$$Activity\ Recovery\ (\%) = \frac{Observed\ activity}{Starting\ activity}\ x\ 100 \qquad (3)$$

In these equations, the *immobilized activity* is the difference between initial enzyme activity measured in the supernatant solution before immobilization, and the enzyme activity measured in the pooled supernatant and washing solutions of the biocomposite after immobilization. *Observed activity* is the activity measured in the immobilized enzyme, and the *starting activity* is the activity of the enzyme solution offered to the biocomposite.

2.4. Enzymatic activity assay

The enzymatic activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The reaction was performed as described in previous work (Klein et al., 2016). It has been defined that a unit of β-galactosidase activity (U) is the amount of enzyme that catalyzes the conversion of 1 μmol of ONPG to *o*-nitrophenol per minute under the established conditions. Assays were performed in duplicate.

2.5. Determination of optimal pH and temperature

The optimum pH of β-galactosidase activity was assayed of both free and AGG-β-gal in different buffers at 40 °C: 0.1 M sodium citrate (pH 3.0), 0.1 M sodium acetate (pH 4.0-5.5), 0.1 M sodium phosphate (pH 6.0-7.5) and 0.1 M Tris-HCl (pH 8.0-9.0). The optimum temperature of free and AGG-β-gal activity was determined at pH 4.5 in temperatures ranging from 25 to 80 (±0.3) °C using a controlled thermal bath (Dubnoff NT 232 - Novatecnica®, Brazil). Experiments and analysis were performed in duplicate.

2.6. Small-angle X-ray scattering

The microstructure characterization of AG, AGG, and AGG-β-gal samples was performed by Small-Angle X-ray Scattering (SAXS) at the LNLS SAXS1 beamline in the Brazilian Synchrotron Light Laboratory, Campinas, Brazil, working at λ=0.1488 nm. The wave vector (q) was selected in the range 0.142 nm^{-1}<q<5.035 nm^{-1}, as described by Traffano-Schiffo et al. (2018). Five parameters were analyzed: (i) α_1, the fractal dimension at distances greater than the characteristic size of the rods that

integrate the structure (D_1), which describes the degree of interconnection of the rods, at $q<0.29$; (ii) α_2, the fractal dimension at distances lower than R_1, at $q>0.6$, describing the degree of compactness within the rods; (iii) α_3, related to the connectivity between the associated polymer chains in the dimers (basic units that constitute the rods) at $q>1.7$; (iv) D_1, the outer diameter of the rods, related to the multiplicity of the domains of the junction zone in the alginate rods, given by $2R_1$, being $R_1=R_g\sqrt{2}$, with R_g being the mean gyration radius in the cross-section of the rods; and (v) D_2, indicating the size related to the outer diameter of the basic units of polymer dimers (Aguirre Calvo et al., 2018). Assays were performed in triplicate.

2.7. Thermogravimetric analysis (TGA)

The thermal stability of biocomposite (AG, AGG, and AGG-β-gal) was performed using a thermogravimetric analyzer PerkinElmer Pyris 1 TGA (Waltham, USA), where 7.1 mg of lyophilized samples were placed on a platinum pan and heated from room temperature up to 800 °C at a temperature gradient of 10 °C min^{-1}, with a nitrogen flow of 20 mL min^{-1}.

2.8. Storage stability of free and immobilized β-galactosidase

For storage stability, free and AGG-β-gal were studied for 75 and 175 days, respectively, at 4 °C, in activity buffer (pH 4.5). The AGG-β-gal prepared with different gelatin concentrations (0, 3.0, 7.5, 10.0, and 20.0% (w/v)) was evaluated to verify gelatin influence on stability, keeping alginate and genipin concentrations constant at 2.0% (w/v) and 0.15% (w/v), respectively. Also, the storage stability of the AGG-β-gal lyophilized biocomposite was studied. For this, the AGG-β-gal was frozen at -18 °C (conventional freezer) for 15 h followed by 24 h lyophilization performed in a

lyophilizer (model L101, Liotop, Brazil), operating at -54 °C and pressure below 0.1 mmHg. Then, the AGG-β-gal lyophilized biocomposites were stored for 124 days in a conventional freezer. The initial activity of β-galactosidase was considered 100%. Before each activity assay, the biocomposites were hydrated for 2 h with activity buffer (pH 4.5). Assays were performed in duplicate.

2.9. Thermal stability of free and immobilized β-galactosidase

The thermal stability of free and AGG-β-gal was measured in a thermostatic bath at 60 °C. The free β-galactosidase solution contained 5 mL of activity buffer, while for the AGG-β-gal was used 1.8 mg of dry support per 300 μL activity buffer. The concentration of both biocatalysts was equivalent (15 U mL^{-1}). At different time intervals (10 up to 360 min), samples were removed and immediately placed in an ice bath to stop thermal inactivation. For the AGG-β-gal, a thermal stability assay was conducted in the absence and presence of lactose solutions (5% and 40% (w/v), pH at 4.5). The remaining enzyme activity was determined as described above. Experiments and analysis were performed in duplicate.

2.10. Batch operational stability in lactose hydrolysis

The operational stability was evaluated by incubating 27 mg of support (on a dry basis) of the AGG-β-gal in Erlenmeyer flasks containing 3 mL of lactose solution (5% (w/v), 0.1 M, pH 4.5). Aliquots were taken periodically and assayed enzymatically for glucose formation. The reaction cycle time was fixed at 150 min with continuous stirring at 40 °C. After incubation time, the samples were washed 3 times with 0.1 M acetate buffer pH 4.5 and incubated in the same conditions of its first use. Experiments and analysis were performed in duplicate.

2.11. Operational stability in a continuous system during lactose hydrolysis

The column type packed-bed-reactor composed of a water-jacketed glass column (15.0 cm x 1.0 cm) packed with 223 mg of support (on a dry basis) of the AGG-β-gal was used for continuous lactose hydrolysis. The temperature was kept at 40 °C by a thermostatic bath, and the buffered lactose solution (5% (w/v)) was continuously fed from the bottom of the reactor using a peristaltic pump. The substrate was pumped into the column in different flow rates to determine the effect of flow rate on hydrolysis (0.19 mL min^{-1} up to 2.1 mL min^{-1}). After reaching steady-state, i.e., when the lactose hydrolysis reaction remained constant, samples were collected from the top of the reactor and assayed enzymatically for glucose formation. Analysis were performed in duplicate.

2.12. Statistical analysis

Statistical analyses were subjected to one-way analyses of variance (ANOVA), and the Tukey test was used to compare means at a level of 95% ($p<.05$) of significance using the software Statistica 10 (StatSoft, USA).

3. Results and discussions

3.1. Influence of sodium alginate, gelatin, genipin, and enzyme concentration in the immobilization parameters

Preliminarily, the effect of sodium alginate, gelatin, genipin, and enzyme concentration was investigated through immobilization parameters. According to Tables S1, S2, S3, and S4 (Supporting Information), in general, there is a decrease in yield and

activity recovered parameters with an excess in the concentration of sodium alginate, gelatin, genipin, and enzyme. This decrease in the parameters is associated with the smaller pore sizes of calcium alginate that may have caused substrate limitation to reach the active site of the enzyme (Rehman et al., 2013). Furthermore, a higher concentration of gelatin promotes excessive crosslinking in the biocomposite. Similar behavior occurs when the crosslinker concentration is too high, leading to excessive polymerization or even self-polymerization of genipin, decreasing the available groups to bind the enzyme (Flores et al., 2019). Finally, an excess of enzyme can lead to saturation of active support binding sites (limited by the selected concentration of genipin). Thus, for further experiments, the following concentrations were chosen: sodium alginate (2.0% w/v), gelatin (7.5% w/v), genipin (0.15% w/v), and enzyme (15 U mL^{-1}), because they gave the best performance on immobilization yield (84%), efficiency (22%), and activity recovered (18.2%). In this case, the biocomposite obtained has shown a specific activity of 32.1 units per mg protein (U mg^{-1}), and the free enzyme commercial preparation had a specific activity of 42.7 units per mg protein (U mg^{-1}), and both samples are equivalent to 100% of the relative activity. The low recovery can be due to steric hindrance between enzyme and support after immobilization (Schöffer et al., 2013). Similar results were found by Flores et al. (2019) when immobilizing β-galactosidase from *A. oryzae* on genipin-activated chitosan support. The authors reported a low recovered activity (19%) due to support saturation when using 20 U mL^{-1} of the enzyme and 1.5 mg mL^{-1} of genipin. A similar phenomenon was found by Gennari et al. (2022) in the development of a one-step purification and targeted immobilization of a β-galactosidase (Gal) from *Kluyveromyces sp*. in different magnetic cellulose supports. On the other hand, Ricardi et al. (2018) had relatively high recovered activity results (33%) for the immobilization of a β-galactosidase from *K. lactis* on

silica/chitosan composite support. In this case, the authors explained that chitosan offered a stronger protective effect on the enzyme.

3.2. Optima pH and temperature

Results for the effects of pH and temperature on the relative activity of free and AGG-β-gal are presented in Fig. 1A and 1B, respectively. As can be seen, no alterations of the optimum pH (4.5) were detected after the immobilization, as shown in Fig. 1A. However, AGG-β-gal retained higher activities in both acidic and alkaline conditions when compared with free β-galactosidase, which is mainly due to covalent binding of the enzyme to support, that provides a strong stabilization effect on enzymes (Lima et al., 2013). Also, Shen et al.(2011) suggest that in an alkaline solution, negatively charged alginate can repel OH^-, which probably reduces the pH around the catalytic site of the enzyme compared to the bulk solution, preserving the enzyme activity.

It can be observed in Fig. 1B that the effect of temperature on the activity is similar for free and AGG-β-gal, i.e., as the temperature increased, the activity increased. However, the optimum temperature for the AGG-β-gal was 65 °C while for free β-galactosidase it was 60 °C, which agreed with the findings of Freitas et al. (2011). This shift indicates that after immobilization, the enzyme may have its active conformation protected against damage caused by heat exchange (Zhang et al., 2010).

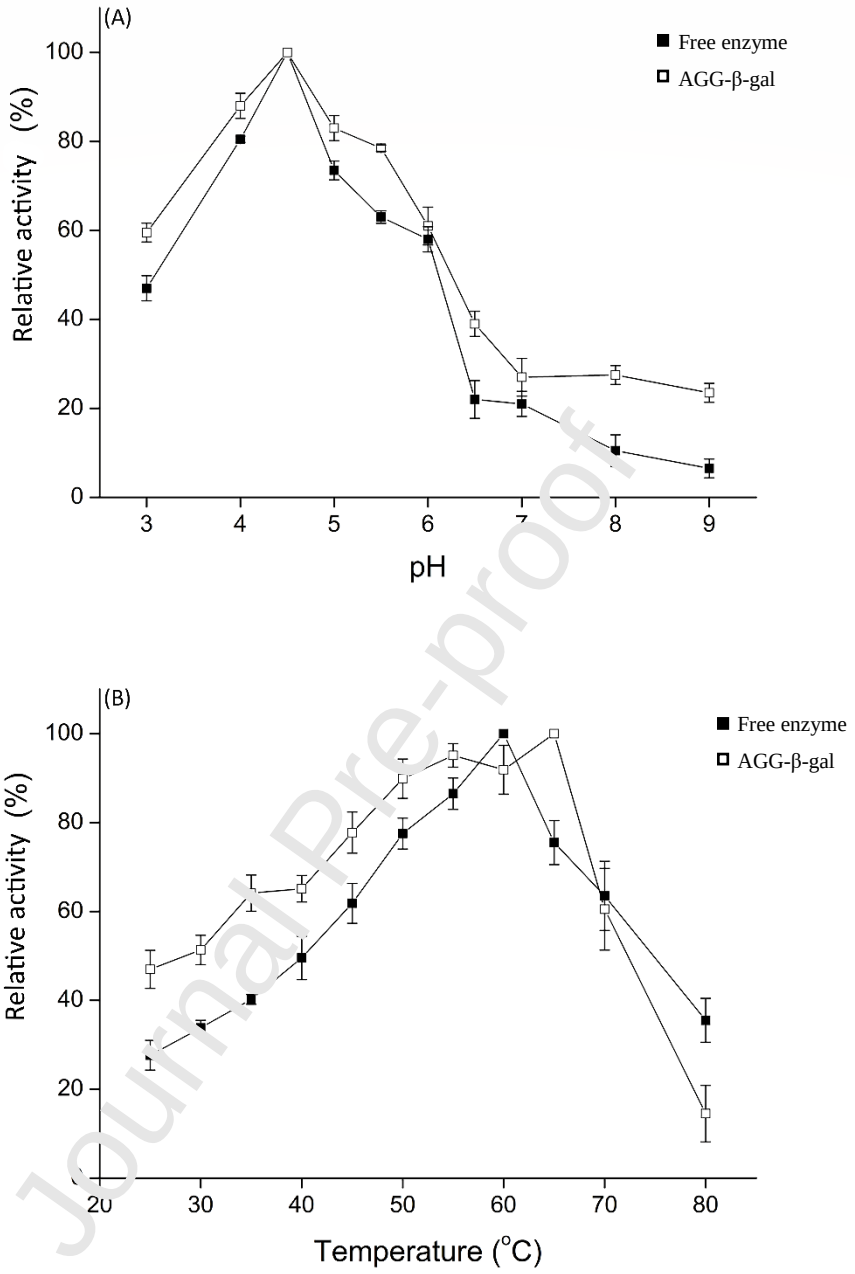

Fig. 1: Effect of pH (A) and temperature (B) on the relative activity of free ( ) and AGG-β-gal ( ). Mean and standard deviation are included.

3.3. Microstructure characterization by SAXS

The microstructure of the hydrated biocomposite was analyzed by SAXS at different scales, from the supramolecular arrangements of Ca(II)-alginate rods to the molecular arrangement of the alginate dimers. The log-log SAXS profile shown in Fig.

S1 (Supporting Information) identifies the three regions from which the microstructural information was obtained, based on previous works (Aguirre Calvo et al., 2018; Traffano-Schiffo et al., 2018). The interconnectivity of the rods (α_1) was obtained from the slope at low q values, the compactness within the rods at intermediates q values (α_2), and α_3 characterize the connectivity between associated polymer chains forming dimers. From the cross point of the correspondent slopes from these domains, the diameter of the rods (D_1) and the polymer dimer's basic units (D_2) can be obtained. Fig. S1 reveals several differences among the systems especially at intermediate q values, as will be further analyzed.

It is important to keep in mind that it is expected that the addition of proteins or other hydrocolloids produces certain changes in the microstructure of alginate plain beads, as observed in Traffano-Schiffo et al. (2018, 2020) for cowpea proteins (mainly low molecular weight globulins) and for arabic and guar gums and by Kuhn et al. (2021) for egg albumin and whey protein isolates. These changes revealed the interactions between the biopolymers, affecting from the molecular to the supramolecular scale. Then, in the present work the already complex Ca(II)-alginate network is now modified by i) the presence of gelatin, interacting with alginate; ii) by genipin, crosslinking gelatin (interacting in turn with alginate) and iii) by the addition of the enzyme, which can interact through genipin with gelatin (mainly) and alginate. Moreover, some microstructural changes could be induced during synthesis due to pH variations: all beads were synthesized at pH 5.7 and rinsed to pH 7.0 afterward; then, those crosslinked with genipin were placed at pH 8.0 for 24 h, then rinsed to pH 7.0; and the beads for immobilization with the enzyme were incubated at pH 4.5 and washed later at the same pH. Aguirre Calvo et al. (2018) and Zazzali et al. (2019) reported that there are no microstructural differences in the range of pH between 5.0 to 7.0; so, it

could expect a low effect of the pH of synthesis between alginate-gelatin with and without genipin. However, there are several differences between pH 3.8 and 5.0 (Zazzali et al., 2019) produced by the partial protonation of alginate chains due to their vicinity to their pK_a, showing an increment in the rod size and density as well as the interconnectivity.

The size (D_2) and density (α_3) of the basic units formed between two alginate chains are shown in Fig. 2A and 2B, respectively, for the lower scale analyzed (~1 nm). The density was unaffected by genipin nor the addition of the enzyme, indicating that once biocomposites were synthesized, the dimers exhibit low rearrangements at this scale. However, their size was significantly reduced by genipin and even more by the enzyme. It is noteworthy that SAXS profiles illustrate the overall sample in a high relative volume (around 1 mm^3) but did not account for inhomogeneities due to the calcium gradient. Then, it could be proposed that an increase in the junction zones promoted by genipin at this scale could account for an overall reduced size of the dimers. The further reduction observed for the AGG-β-gal is a consequence of the additional crosslink imposed between enzyme/genipin/gelatin/alginate or by a direct electrostatic interaction between the enzyme and alginate, considering that the pI of the protein (~8.5 (Smith et al., 2016)) is higher than the pH of the synthesis.

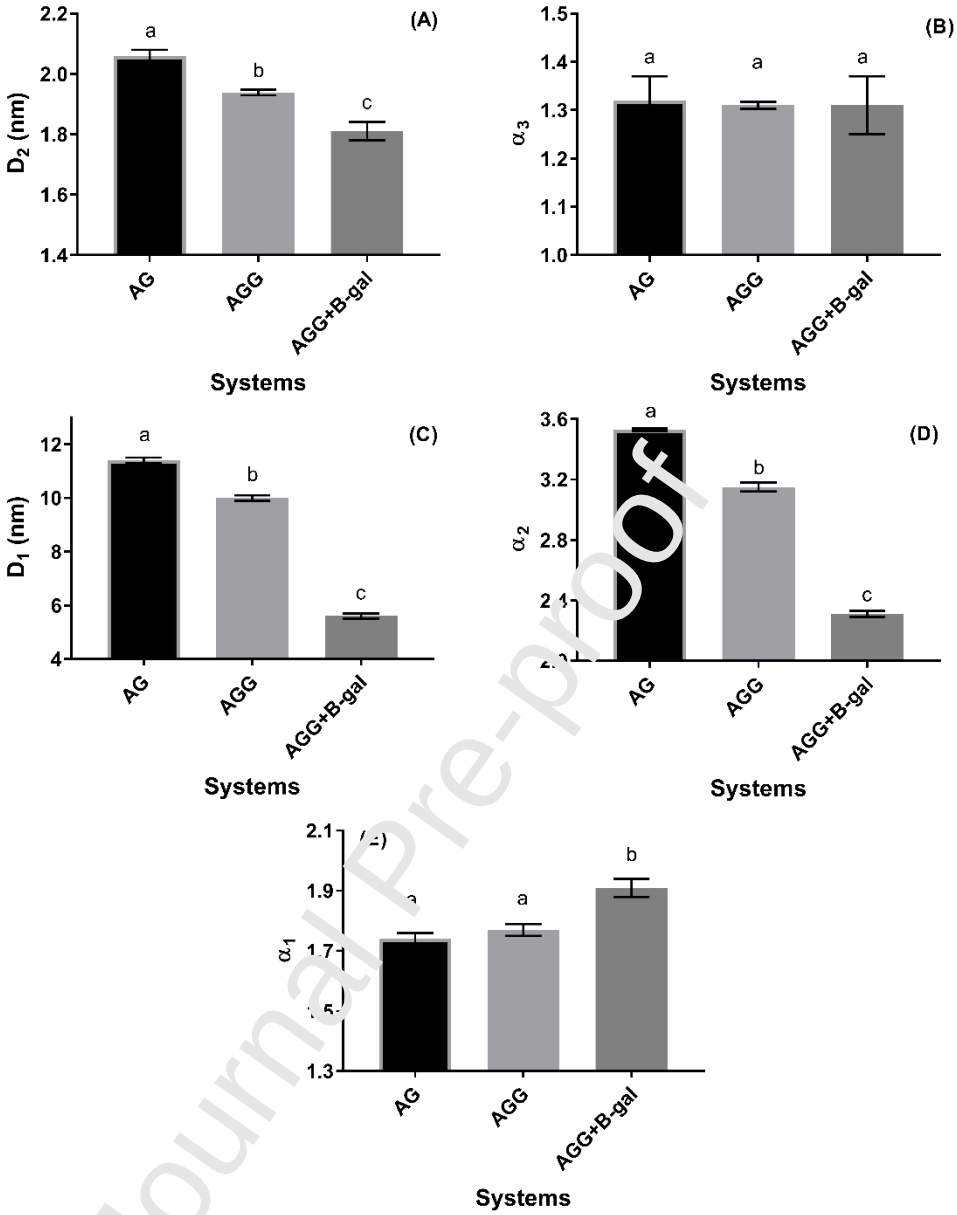

Fig. 2: Microstructure parameters of Ca(II)-alginate biocomposite with and without genipin and immobilized β-galactosidase. (A): Characteristic size of the Ca(II)-alginate dimers (D_2). (B): Fractal dimension at distances lower than D_2 defined as parameter α_3 of the microstructure derived from log-log SAXS profiles. (C): Diameter obtained for the outer radius of the cross-section of the rods (D_1). (D): Fractal dimension at distances lower than the characteristic size of the rods or parameter α_2 of the microstructure derived from log-log SAXS profiles. (E): Fractal dimension of the rod network - the

interconnectivity of the rods - or parameter α_1 of the microstructure derived from log-log SAXS profiles. Mean and standard deviation are included.

Considering the intermediate scale (~10 nm), the size (D_1) and density (α_2) of Ca(II)-alginate rods are shown in Fig. 2C and 2D, respectively. The addition of genipin produced a significant reduction in both parameters. The lower compactness within the rods due to genipin addition can be explained by considering that each rod became "perturbed" by the extra-coordination imposed by genipin, which can hinder the formation or the extension of formed junction zones; then, the smaller size of the rods could be a consequence of packing the same amount of dimer (of smaller size as shown in Fig. 2A) or fewer alginate dimers, as a consequence of the perturbation in the packing imposed by gelatin-genipin. The lower intensity observed in Fig. S1 could also account for this, revealing fewer scattering objects. Moreover, the addition of the enzyme produced a significant impact on both parameters, since the crosslinking through genipin with an object that has a similar size to the rod can further perturb the Ca(II)-alginate network.

Finally, at the higher analyzed scale (~100 nm), no differences were observed regarding the interconnectivity of the rods with the addition of genipin (Fig. 2E). Instead, the latter immobilization of the enzyme produced an increase in the interconnectivity. This could be related to two factors: the reduction of pH which leads to some degree of reduction in the electrostatic repulsion between alginate chains (being closer to pK_a) - as previously mentioned - and to the direct presence of the enzyme that could coordinate different (and smaller, as shown Fig. 2C) alginate chains that could rearrange forming a highly interconnected network.

3.4. Thermogravimetric analysis (TGA)

Fig. 3A shows the weight loss of the samples with increasing temperature. The first stage corresponds to the loss of water associated with the hydrophilic groups, where the loss of mass of AGG-β-gal was higher (19%) than other samples, due to the presence of clusters that form hydrophilic interactions. The second stage occurred between 230-400 °C and corresponds to depolymerization of the polysaccharides, i.e., the breaking off segments of D-mannuronic acid and L-guluronic acid of sodium alginate (Chen & Zhang, 2019). In this stage, more extensive degradation occurred, and it was 24%, 34%, and 38% for AG, AGG, and AGG-β-gal, respectively. The higher weight loss of the AGG and AGG-β-gal could be attributed to a lower density of crosslinking between genipin and gelatin, contributing to a reduction in its stability. This less thermo-resistant observed after the crosslinking process has been suggested in some studies (Flores et al., 2019; Klein et al., 2016; Rivero et al., 2013). The third stage occurs above 450 °C and corresponds to the complete carbonization of the polymer chains (Chen & Zhang, 2019).

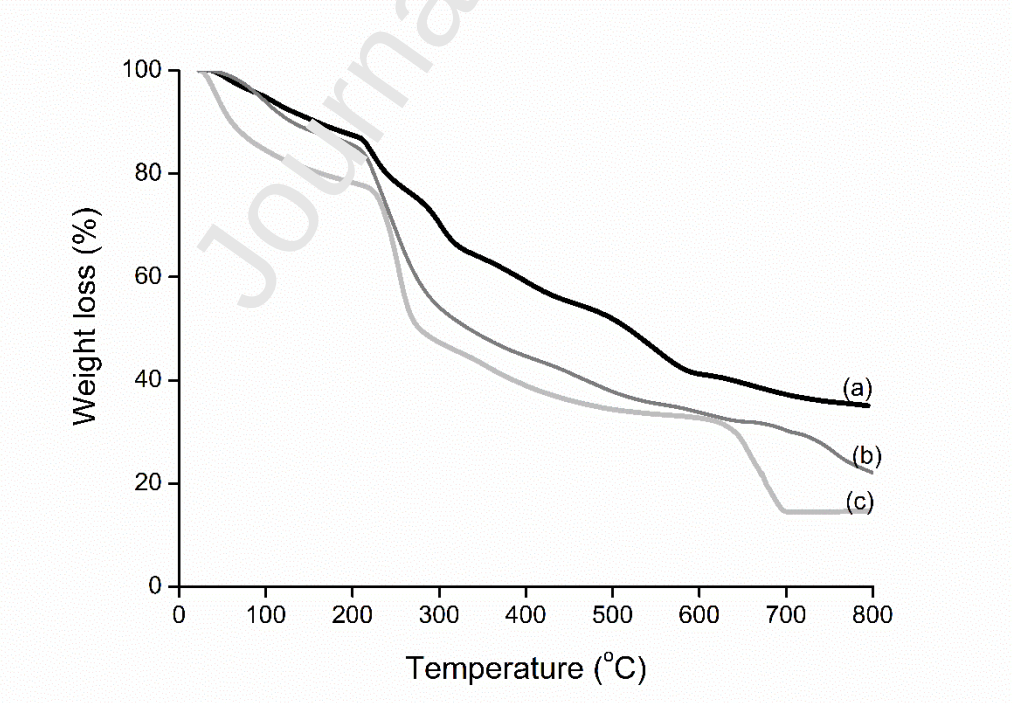

Fig. 3: Thermogravimetric analysis of (A) AG, (B) AGG, and (C) AGG-β-gal.

3.5. Storage stability

The storage stability of immobilized enzymes is important for practical purposes, the more stable the better. As can be seen in Fig. 4A, the free β-galactosidase exhibited 31 ± 2% of the original activity after 75 days of storage under refrigeration, while for the AGG-β-gal prepared using alginate 2.0% (w/v), genipin 0.15% (w/v), and gelatin 3.0% and 7.5% (w/v), the AGG-β-gal maintained approximately 80% of the initial activity after 175 days of storage, indicating an enzyme stabilization by the immobilization process. It was observed that when gelatin content increased (10.0 and 20.0% (w/v)), there was a decrease of almost 40% of its initial activity, because an excessive amount of gelatin leads to aggregation, thus destroying the compatibility between alginate and gelatin, which can open up the polymer chains (Wang et al., 2019).

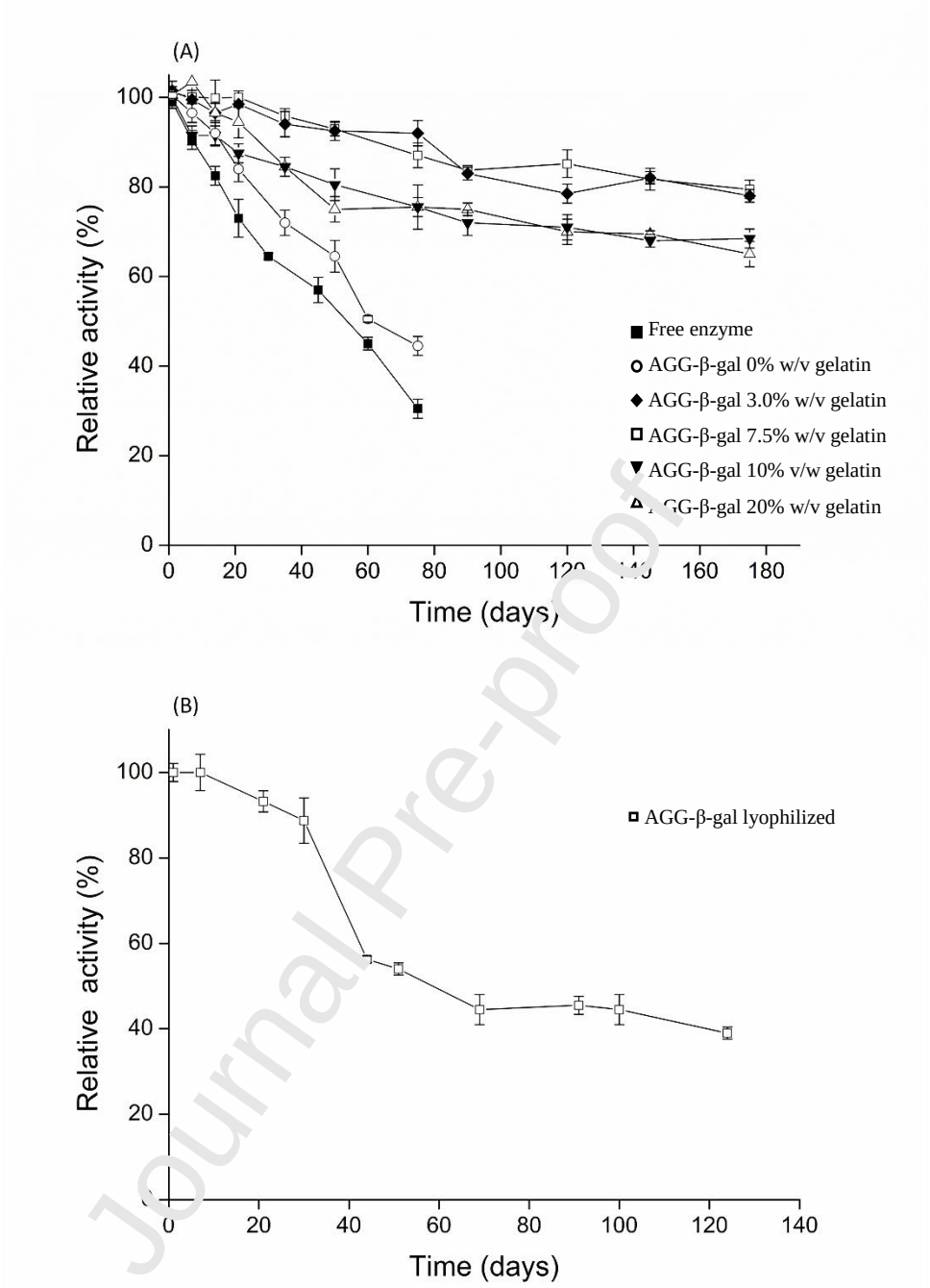

Fig. 4: Storage stability under refrigeration (A) of AGG-β-gal varying gelatin concentration and keeping alginate (2.0% (w/v)) and genipin (0.15% (w/v)) constant: free enzyme (■), AGG-β-gal 0% w/v gelatin (○), AGG-β-gal 3.0% w/v gelatin (◆), AGG-β-gal 7.5% w/v gelatin (□), AGG-β-gal 10.0% w/v gelatin (▼) and AGG-β-gal 20.0% w/v gelatin (△), and storage stability at conventional freezer (B) of AGG-β-gal lyophilized. Mean and standard deviation are included.

Immediately after the lyophilization process, the AGG-β-gal lyophilized loses approximately 17% of its initial activity due to the process. After that, the storage stability was performed (Fig. 4B), and it was considered as 100% of relative activity for further analysis. The AGG-β-gal lyophilized biocomposite remains stable for up to 30 days with ~ 88% of its activity. However, after that period, there was a progressive loss of enzyme activity. According to Santagapita, Mazzobre, and Buera (2012), lyophilized beads are much more fragile than vacuum or air-dried beads and show an irregular shape, which may have contributed to the loss of activity of the immobilized enzyme during storage in this study.

3.6. Enzyme thermal stability

A comparison of thermal stability of free and AGG-β-gal is shown in Fig. 5. After 75 min of reaction, the free and AGG-β-gal under non-reactive conditions, completely lose enzyme activity at 60 ºC. The lower thermal stability of AGG-β-gal (as demonstrated by the TGA) is due to the self-polymerization of genipin at high temperatures (Flores et al., 2015), which left the enzyme unprotected on the surface, making it susceptible to denaturation by heat.

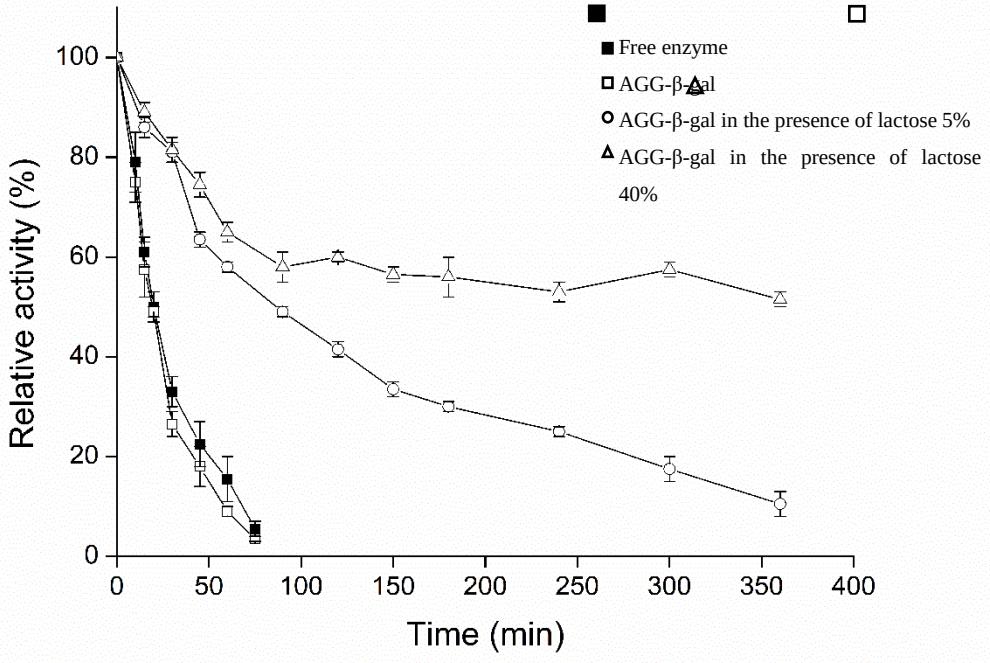

Fig. 5: Comparison of the ther

mal inactivation at 60 °C for ( ) free enzyme, and ( ) AGG-β-gal, ( ) AGG-β-gal in the presence of lactose 5% (w/v) and ( ) AGG-β-gal in the presence of lactose 40% (w/v). Mean and standard deviation are included.

However, the thermal stability of AGG-β-gal increased under reactive conditions (5 and 40% (w/v) of lactose solutions) as can be seen in Fig. 5. After 150 min of reaction at 60 ºC, in the presence of 40% (w/v) lactose buffered solution, the AGG-β-gal maintained 57 ± 1% of relative activity and in the presence of 5% (w/v) lactose solution, still showed 34 ± 2% of relative activity, which means that the presence of the substrate prevents the deactivation of the enzyme through generating an enzyme-substrate complex tolerant to high temperatures (Kovalenko et al., 2007), in addition to the preferential effect of hydration of the enzyme molecule (preferential exclusion of sugars from the surface of the protein) (Beg et al., 2018).

3.7. Batch operational stability in the lactose hydrolysis and operational stability of immobilized β-galactosidase in a continuous packed-bed reactor

The operational stability of AGG-β-gal in lactose hydrolysis was determined for 11 cycles of reuse at 40 °C in a repeated batch mode, with 69% of lactose conversion in 150 min of reaction in the first use. This conversion was defined as 100% for the following reuses. As shown in Fig. 6A, the AGG-β-gal demonstrates the reuse viability, maintaining 90 ± 4% of lactose hydrolysis in the last cycle of reuse. Shen et al. (2011) using Ca(II)-alginate capsules for β-galactosidase from *K. lactis* encapsulation, reported a loss of around 50% of its initial activity in the 11th reaction cycle. In contrast, using alginate–gelatin–calcium phosphate hybrid capsules (AGCaP), in the 11th repeated use, there was no loss of activity. The authors explain that this reported difference in activity may be due to the solid inorganic skeleton that could effectively restrict the expansion

of capsules caused by water absorption. The result obtained in our study shows that the AGG-β-gal presented satisfactory operational stability.

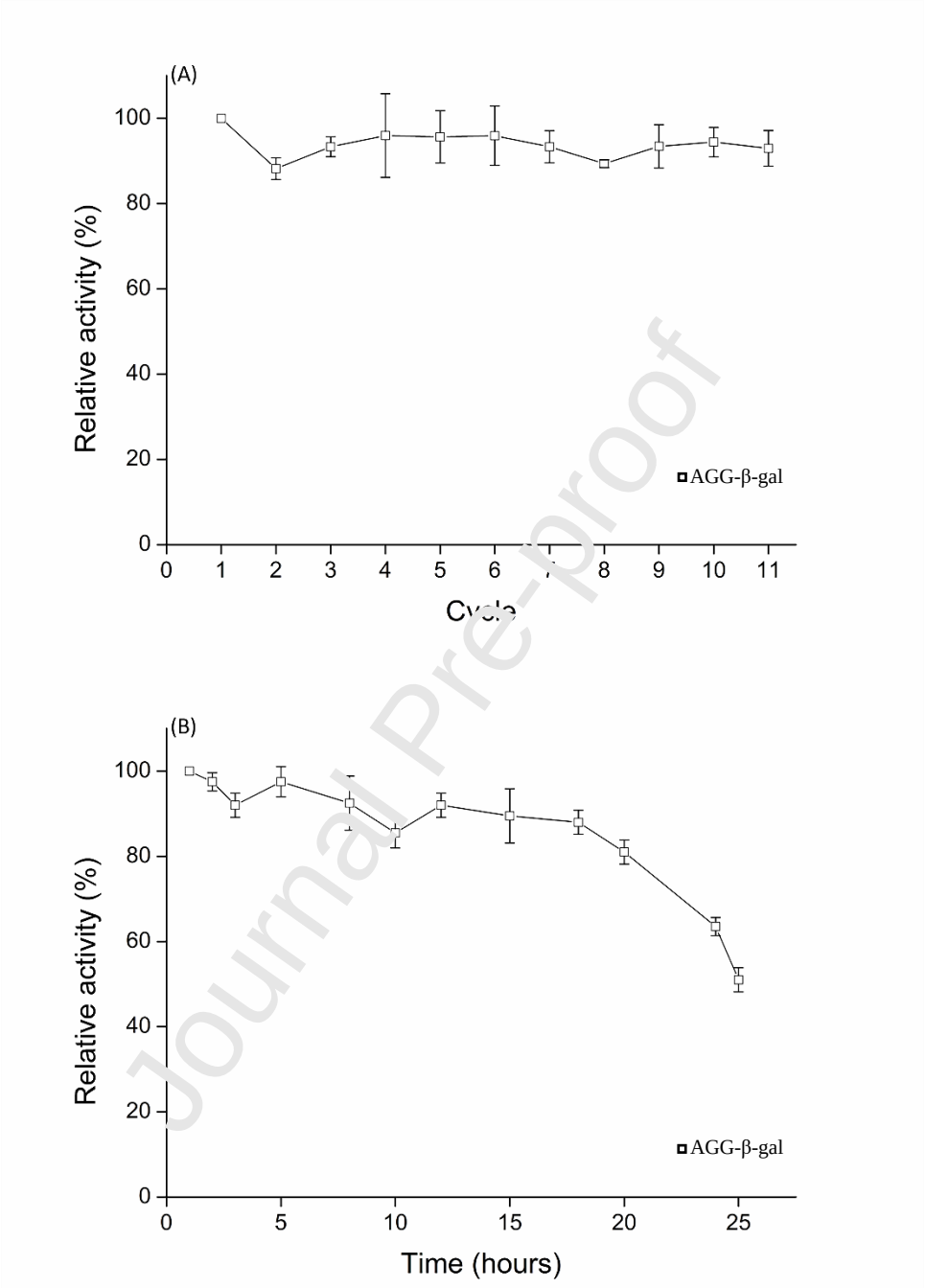

Fig. 6: Operational stability of AGG-β-gal on the lactose hydrolysis (A) and (B) in the continuous hydrolysis of lactose, using a 5% (w/v) buffered lactose solution with a flow

rate of 0.25 mL min^{-1}. The activity of the first batch was taken as 100%. Mean and standard deviation are included.

Fig. 6B shows the results of the relative activity as a function of the reactor's operating time. After reaching the stationary period, the flow rate was maintained at 0.25 mL min^{-1}, since it leads to the highest percentage of lactose hydrolysis (77%) and it was considered as 100% of relative activity. The other flows rate tested did not show satisfactory hydrolysis results (data not shown).

The continuous operation was performed for 25 h; however, in 20 h of reaction, the relative activity decreased 19%. This decrease in biocatalyst activity within the reactor might be due to degradation by mechanical stress observed in AGG-β-gal near to the reactor inlet, and could also be due to the gelatin was not completely crosslinked with genipin, probably leading to the release of gelation in the solution (Bigi et al., 2002). Furthermore, continued use may result in the inactivation of the enzyme molecule (Wahba, 2016).

Considering the flow rate of 0.25 mL min^{-1} (residence time, 19.37 min) and 223 mg of support (on a dry basis) of AGG-β-gal, the maximum productivity achieved in the continuous process was 0.21 g lactose hydrolyzed L^{-1} h^{-1} per mg of biocatalyst (on a dry basis). For the batch process, a maximum productivity achieved was 0.20 g lactose hydrolyzed L^{-1} h^{-1} per mg of biocatalyst (on a dry basis). Lactose hydrolysis in batch process can potentialize enzyme inhibition by glucose and galactose (Vera et al., 2011), thus affecting productivity. In the same way, a degradation of AGG-β-gal was observed in the inlet of the reactor in the continuous process, that can also affect enzyme activity and productivity.

4. Conclusions

A successful method for the immobilization of β-galactosidase from *Aspergillus oryzae* in biocomposites composed of alginate-gelatin and crosslinked with genipin was developed and showed good immobilization parameters. Although immobilization did not change the optimal pH, the AGG-β-gal was more resistant to alkaline conditions and more active at lower temperatures than the free enzyme. The structural characterization of biocomposites showed that the addition of genipin and β-galactosidase caused changes at the microstructural level, showing lower compactness within the rods (α_2) and rods with smaller cross-section radius (D_1) than Ca(II)-alginate. Through thermogravimetric analysis, it was observed that the AGG-β-gal is thermally stable up to 230 °C, above the temperature of most enzymatic reactions. In terms of stability, the AGG-β-gal shows thermal stabilization in operating systems and could be reused for at least 11 cycles, being an important parameter from an industrial point of view. Thus, compared to our previous works, the biocomposite developed is considered simple to prepare, making this material an interesting alternative for applications in the food industry and bioengineering.

Acknowledgments

The authors would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), from Brazilian government. This work was supported by the Brazilian Synchrotron Light Laboratory (LNLS, Brazil, proposal SAXS1-20190143), Agência Nacional de Promoción Científica y Tecnológica (ANPCyT PICT-2017-0569).

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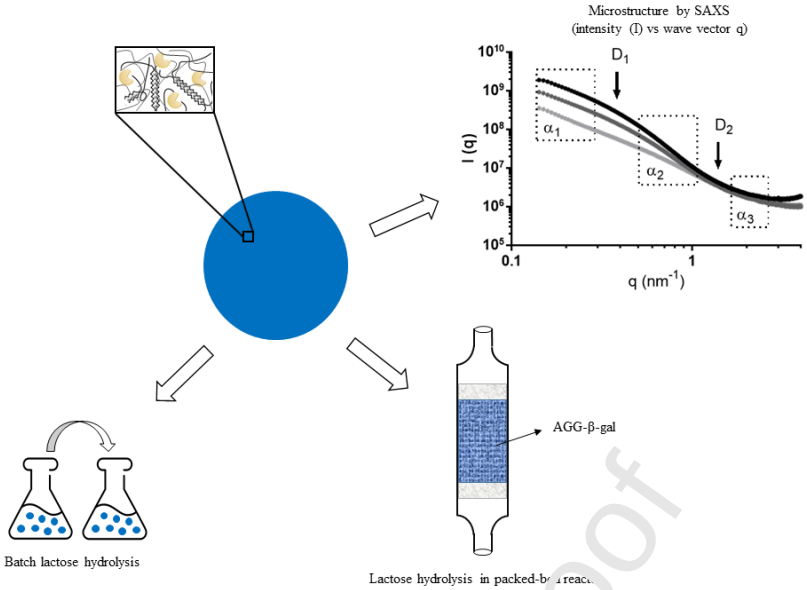
Credit authorship contribution statement

Camila Regina Hackenhaar: Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization. **Carolina Flores Rosa**: Investigation. **Elí Emanuel Esparza Flores**: Formal analysis, Investigation. **Patrício Román Santagapita**: Formal analysis, Resources, Writing – Review & Editing, Supervision. **Manuela Poletto Klein**: Methodology, Formal analysis, Resources, Writing – Review & Editing, Supervision. **Plinho Francisco Hertz**: Conceptualization, Methodology, Formal analysis, Resources, Writing – Review & Editing, Supervision, Project administration.

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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Graphical abstract