Carbohydrate Polymers 137 (2016) 184–190

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Chitosan crosslinked with genipin as support matrix for application in food process: Support characterization and β-D-galactosidase immobilization

Manuela P. Klein[a,b], Camila R. Hackenhaar[b], André S.G. Lorenzoni[b], Rafael C. Rodrigues[b], Tania M.H. Costa[c], Jorge L. Ninow[a], Plinho F. Hertz[b,*]

[a] Departamento de Engenharia Química e Alimentos, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil
[b] Laboratório de Enzimologia, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91501-970, Brazil[1]
[c] Laboratório de Sólidos e Superfícies, Instituto de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91501-970, Brazil

ARTICLE INFO

Article history:
Received 17 June 2015
Received in revised form 14 October 2015
Accepted 19 October 2015
Available online 23 October 2015

Keywords:
Immobilization
Genipin
Chitosan
β-D-Galactosidase
Lactose hydrolysis
Galactooligosaccharides

ABSTRACT

In order to develop safer processes for the food industry, we prepared a chitosan support with the naturally occurring crosslinking reagent, genipin, for enzyme. As application model, it was tested for the immobilization of β-D-galactosidase from *Aspergillus oryzae*. Chitosan particles were obtained by precipitation followed by adsorption of the enzyme and crosslinking with genipin. The particles were characterized by Fourier transform infrared (FTIR) spectroscopy and thermogravimetric analysis (TGA). The immobilization of the enzyme by crosslinking with genipin provided biocatalysts with satisfactory activity retention and thermal stability, comparable with the ones obtained with the traditional methodology of immobilization using glutaraldehyde. β-D-Galactosidase–chitosan–genipin particles were applied to galactooligosaccharides synthesis, evaluating the initial lactose concentration, pH and temperature, and yields of 30% were achieved. Moreover, excellent operational stability was obtained, since the immobilized enzyme maintained 100% of its initial activity after 25 batches of lactose hydrolysis. Thus, the food grade chitosan–genipin particles seem to be a good alternative for application in food process.

1. Introduction

In recent years, the advances in biotechnology now make possible to manipulate most enzymes so that they exhibit the desired properties (Bornscheuer et al., 2012; Burton, Cowan, & Woodley, 2002; Sheldon & van Pelt, 2013). Various methods including protein engineering, medium engineering and immobilization of biocatalysts can provide suitable enzyme stability, specificity and activity, which is often the limiting factor in most bioprocesses (de Barros, Fernandes, Cabral, & Fonseca, 2010). Immobilization of enzymes is a relatively simple methodology and offers many benefits, for example: efficient reuse of the enzyme, continuous operation, enhanced stability, under both storage and operational conditions, facile separation from the medium reaction, thereby minimizing or eliminating protein contamination of the product, low or no allergenicity, since an immobilized enzyme cannot easily penetrate the skin, among others (Sheldon & van Pelt, 2013).

Beyond kinetic stability, industrial application also requires a biocatalyst with mechanical stability and safety, the latter being essential in food and pharmaceutical industries. As a support for enzyme immobilization, chitosan [(1 → 4)-2-amino-2-deoxy-β-D-glucan], offers a number of desirable characteristics including nontoxicity, biocompatibility, physiological inertness, biodegradability to harmless products and remarkable affinity to proteins. The solubility in acidic solutions and aggregation with polyanions impart chitosan with excellent gel-forming properties (Krajewska, 2004). Moreover, mechanical properties of supports obtained from chitosan can be easily improved by crosslinking with glutaraldehyde, genipin and others reagents (Cauich-Rodriguez, Deb, & Smith, 1996; Muzzarelli, 2009).

Currently, genipin can be obtained from the fruits of *Genipa americana* and *Gardenia jasminoides* Ellis. After extraction, the geniposide is hydrolyzed into the aglycone genipin with β-D-glucosidase in a microbiological process involving *Penicillium nigricans* (Butler, Ng, & Pudney, 2003; Muzzarelli, 2009). The use

* Corresponding author.
E-mail address: plinho@ufrgs.br (P.F. Hertz).
[1] www.ufrgs.br/bbb.

http://dx.doi.org/10.1016/j.carbpol.2015.10.069
0144-8617/

of genipin as crosslinker with chitosan has been proposed for several purposes. For example, the creation of a polymer network formed by chitosan/gelatin for dye adsorption (Cui et al., 2015), the crosslink electrospun of chitosan fibers to improve wet durability (Li et al., 2015), and for crosslinking a blend of chitosan/poly-L-lysine to create biomaterials for tissue engineering applications (Mekhail, Jahan, & Tabrizian, 2014). Moreover, it was reported that genipin might be about 5000–10,000 times less cytotoxic than glutaraldehyde (Sung, Huang, Huang, & Tsai, 1999).

β-D-Galactosidases have an important role in dairy industries. This enzyme catalyzes the hydrolysis of lactose (β-D-galactopyranosyl-(1 → 4)-D-glucopyranose) into D-glucose and D-galactose, allowing the consumption of dairy products by lactose intolerant people. Moreover, in the presence of concentrated lactose, this enzyme can transfer the β-D-galactosyl moiety from lactose hydrolysis to another lactose molecule, thus synthesizing galactooligosaccharides (GOS), an important prebiotic food ingredient, naturally present in human milk (Grosova, Rosenberg, & Rebros, 2008).

Recent works (Klein et al., 2012; Klein et al., 2013; Lorenzoni, Aydos, Klein, Rodrigues, & Hertz, 2014; Schöffer, Klein, Rodrigues, & Hertz, 2013; Valerio, Alves, Klein, Rodrigues, & Hertz, 2013) have reported the successful immobilization of enzymes on chitosan particles using glutaraldehyde, resulting in biocatalysts with high thermal and operational stability. Based on the satisfactory results presented on chitosan as support for enzyme immobilization, and the importance of the improvement of bioprocess from the safety point of view, we are proposing the preparation of chitosan particles, with food compatibility, using the naturally occurring crosslinking reagent genipin to immobilize enzymes for food applications. Chitosan particles were prepared and crosslinked with genipin and compared with the crosslinking using glutaraldehyde. Particles were characterized by FTIR and TGA. β-D-Galactosidase from *Aspergillus oryzae* was used as enzyme model for immobilization, and the changes that chitosan crosslinked with genipin can impart to the immobilized enzyme was verified. The effects of the immobilization approach on the activity retention, thermal stability, operational stability, as well as the galactooligosaccharides synthesis were also evaluated.

2. Materials and methods

2.1. Materials

A. oryzae β-D-galactosidase, genipin, chitosan (from shrimp shells, ≥75% deacetylated), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), D-glucose, D-galactose, lactose, raffinose (β-D-fructofuranosyl α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside), and stachyose (β-D-fructofuranosyl α-D-galactopyranosyl-(1 → 6) α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside) were obtained from Sigma–Aldrich (St. Louis, USA). A D-glucose determination kit was purchased from Labtest Diagnóstica SA (São Paulo, Brazil). All solvents and other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of β-D-galactosidase immobilized on genipin-crosslinked chitosan particles

Chitosan particles (CS) were prepared by the precipitation method as described in a previous work (Klein et al., 2012). Then, 100 chitosan particles (0.5 g) were incubated with β-D-galactosidase solution (2 mL, 20 U mL^{-1}) prepared in 0.02 M of sodium phosphate buffer (pH 7.0), during 8 h at room temperature. Crosslinking of chitosan particles with genipin (CS-GEN) was performed by adding 500 μL of 0.5% (w/v) genipin solution (pH 7, sodium phosphate 0.02 M) and it was allowed to react during 15 h at room temperature. After crosslinking, successive washings with acetate buffer (pH 4.5, 0.1 M), NaCl (1 M) and ethylene glycol (30%, v/v) were carried out to eliminate ionic and hydrophobic interactions between enzyme and support.

Chitosan particles with adsorbed β-D-galactosidase followed by glutaraldehyde crosslinking (CS-GLU) were prepared to compare the influence of the crosslinking agents on some properties of the immobilized β-D-galactosidase, following the methodology proposed by Lopez-Gallego and co-workers (2005), with some modifications: 100 μL of glutaraldehyde 25% (v/v) was added to the chitosan particles previously incubated with 2 mL of β-D-galactosidase solution, at room temperature, during 1 h.

2.2.2. Characterization of genipin-crosslinked chitosan particles

Changes on the molecular structure of chitosan particles were determined before and after genipin crosslinking by Fourier transform infrared (FTIR) spectroscopy with a Varian 640-IR spectrometer. Samples previously lyophilized were crushed and thoroughly mixed with powdered KBr and then pressed to form a transparent pellet (1%, w/w). The spectra were obtained at room temperature with 40 accumulative scans and 4 cm^{-1} of resolution. The thermogravimetric analysis (TGA) was performed using a Shimadzu thermal analyzer Model TA50, at a heating rate of 10 °C min^{-1}, from room temperature up to 600 °C under argon atmosphere.

2.2.3. Activity assay of β-D-galactosidase

β-D-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. For the free enzyme the measurements were performed in 0.5 mL of 0.1 M sodium acetate buffer (pH 4.5) containing ONPG 15 mM and an adequate amount of free enzyme. After incubation (40 °C for 2 min), the reaction was stopped by adding 1.5 mL of 0.1 M sodium carbonate buffer (pH 10) and the absorbance was measured at 415 nm. The above quantities were doubled for measurements with the immobilized enzyme. One unit (U) of β-D-galactosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to o-nitrophenol and galactose per min at the defined assay conditions.

The enzyme activity adsorbed was calculated from the difference between the applied and recovered enzyme activities in the supernatant before and after adsorption. The immobilization efficiency (IE) were calculated by Eq. (1), previously described in Sheldon and van Pelt (2013):

$$\mathrm{IE}\,(\%) = \frac{\text{Observed Activity}}{\text{Immobilized Activity}} \times 100 \quad (1)$$

2.2.4. Optimal pH and temperature for free and immobilized β-D-galactosidase

The optimum pH of β-D-galactosidase activity was studied by monitoring enzyme activity of both free and immobilized β-D-galactosidase in different buffers, at 40 °C: 0.05 M glycine–HCl (pH 2.3–3), 0.1 M Na-acetate (pH 4.0–5.5), 0.1 M Na-phosphate (pH 6.0–7.0) and 0.1 M Tris–HCl (pH 8.0). The optimum temperature was determined by measuring the activity between 20 °C and 75 °C at pH 4.5.

2.2.5. Thermal stability of the immobilized β-D-galactosidase

For thermal stability studies, the immobilized enzyme was incubated in sealed tubes, in thermostatically controlled water bath at 60 °C. Thermal stability was performed in activity buffer (pH 4.5), with 40% (w/v) buffered lactose solution, to simulate operational conditions of galactooligosaccharides synthesis. At defined

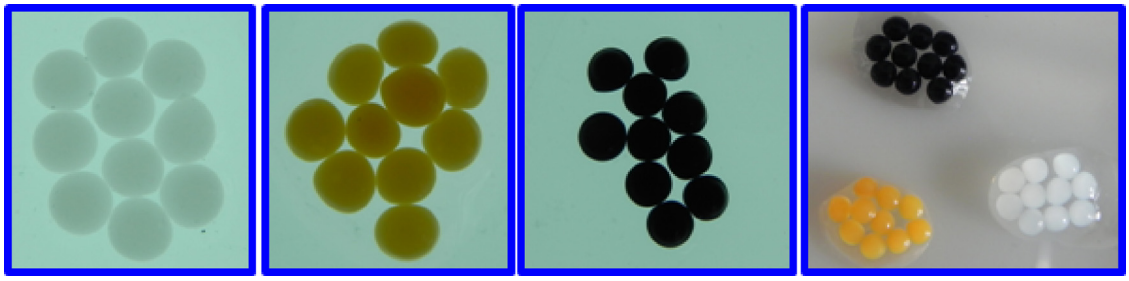

Fig. 1. Pictures of CS particles (~2 mm; translucent white particles), crosslinked with glutaraldehyde (yellow particles) and with genipin (dark blue particles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

time intervals, the immobilized enzyme was withdrawn, chilled immediately and tested for enzyme activity using routine assay.

2.2.6. Operational stability of immobilized β-D-galactosidase in the lactose hydrolysis

Lactose hydrolysis in batch was performed with β-D-galactosidase immobilized on genipin-crosslinked chitosan particles incubated in Erlenmeyer flasks containing 5% (w/v) of buffered (pH 4.5) lactose solution. Samples were withdrawn periodically and analyzed enzymatically for glucose formation. After its first use, the immobilized enzyme was incubated repeatedly in the same conditions described above to evaluate its operational stability in the successive hydrolysis batches.

2.2.7. Galactooligosaccharides synthesis

Synthesis of galactooligosaccharides from lactose was studied with the immobilized enzyme in different conditions of lactose concentrations (30, 40 and 50%, w/v), pH values (4.5, 5.25, and 7), and temperatures (40, 47.5 and 55 °C). Samples were taken at appropriate time intervals to obtain the complete reaction profile, filtered using 0.22 μm cellulose acetate membranes, diluted and analyzed for sugar content by high performance liquid chromatography (HPLC).

2.2.8. Analytical procedures

Lactose and products from the transgalactosylation reaction (GOS, D-galactose and D-glucose) were analyzed by HPLC (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C column (300 mm × 7.8 mm). Ultra-pure water was used as eluting solvent at a flow rate of 0.6 mL min^{-1}, at 85 °C. The concentration of saccharides was calculated by interpolation from external standards. Authentic standards were used for lactose, D-glucose, and D-galactose. GOS concentration was calculated as raffinose and stachyose equivalents from external raffinose and stachyose standards, respectively, as described by Gosling, Stevens, Barber, Kentish, and Gras (2011). The yield (%) of GOS synthesis was defined as the percentage of GOS produced compared with the weight of initial lactose in the reaction medium.

3. Results and discussion

3.1. Characterization of chitosan particles

Fig. 1 shows the chitosan particles without crosslinking (CS, translucent white particles), crosslinked with glutaraldehyde (CS-GLU, yellow particles) and with genipin (CS-GEN, dark blue particles). After crosslinking with genipin, the particles turned dark blue, due to oxygen radical-induced polymerization of genipin (Bi et al., 2011), and they showed to be resistant to acid pH solutions, unlike the non-crosslinked chitosan. Moreover, no swelling effects were observed in the CS-GEN particles during more than 4 months of refrigerated storage at pH 4.5. It was reported that the numerous interchain interactions formed by crosslinking inhibit swelling, since most of the amino groups of chitosan must have reacted with the crosslinker (Berger et al., 2004). Indeed, the lower swelling ability of chitosan gel is attributed to the increased intermolecular or intramolecular linkage of the $-NH_2$ sites in chitosan, which is normally achieved by a more complete crosslinking reaction (Mi, Sung, & Shyu, 2001).

3.2. FTIR analysis

Spectra of chitosan particles (CS), chitosan particles crosslinked with genipin (CS-GEN) and CS-GEN with immobilized β-D-galactosidase are presented in Fig. 2. The spectrum of CS (a) shows absorptions at 1650 cm^{-1} and 1585 cm^{-1}, characteristics of N—H bending vibrations of primary amines (Lambert, 1987) present on chitosan structure. The peak at 1376 cm^{-1} was attributed to —C—O—H stretching of a primary alcoholic group in chitosan. The absorption bands between 1000 cm^{-1} and 1100 cm^{-1} were attributed to C—O and C—N stretching vibrations, and C—C—N bending vibrations (Lambert, 1987). The three spectra showed a broad band between 3000 cm^{-1} and 3600 cm^{-1} that was attributed to the O—H stretching vibration, mainly from water, which probably overlaps the amine stretching vibrations (N—H) in the same region (Lambert, 1987), and the bands between 2800 cm^{-1} and 3000 cm^{-1} were attributed to the C—H stretching vibration (Colthup, Daily, & Wiberley, 1975). The crosslinking of genipin with chitosan involves a fasten reaction that is the nucleophilic attack by the amino group of chitosan on the olefinic carbon atom at C-3 of genipin which results in the opening of the dihydropyran ring and the formation of a tertiary amine, i.e. a genipin derivative linked to a glucosamine unit. The subsequent slower reaction is the formation of amide through the reaction of the amino group on chitosan

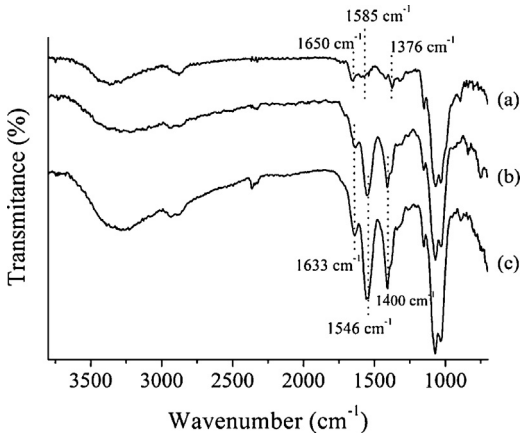

Fig. 2. FTIR spectra of (a) CS, (b) CS-GEN and (c) CS-GEN with immobilized β-D-galactosidase.

with the ester group (by C-11) of genipin (Mi et al., 2001). At the same time, polymerization can take place between genipin molecules already linked to amino groups of chitosan, which could lead to the crosslinking of amino groups by short genipin copolymers (Butler et al., 2003; Muzzarelli, 2009). Then, after crosslinking with genipin (b), the amide II band at 1546 cm^{-1}, characteristic of N—H deformation (Lambert, 1987), is probably due to the formation of secondary amides as a result of the reaction between the genipin ester and hydroxyl groups and the chitosan amino groups. The peak at 1633 cm^{-1} was attributed to C=O stretch in secondary amides (Lambert, 1987). Furthermore, the increase observed in the peaks at around 1400 cm^{-1} and 1000 cm^{-1} can be assigned to absorptions from C—N stretching vibrations and C—OH stretching vibrations (Lambert, 1987), respectively, more numerous after crosslinking with genipin. The spectra of CS-GEN with immobilized β-D-galactosidase (c) showed no changes in comparison with the spectra of CS-GEN because the mechanisms involved in the crosslinking reaction in the presence of the enzyme are the same involved in the crosslinking of chitosan particles (CS). The increase in the intensity of characteristic bands is presumable due to the increase of amino groups available (from the adsorbed enzyme), which reacts with genipin, which, in turn, contributes to the increase of groups from crosslinking, as amide linkages.

3.3. Support thermal stability

The thermal stability of chitosan particles was measured using thermogravimetric analysis. The changes in sample weight with the increase of the temperature are shown in Fig. 3. In all samples, there is a weight loss up to 100 °C due to adsorbed water elimination. It can be seen that chitosan particles (CS) show a lower weight loss in this region indicating lower hydrophilic character compared to the CS-GEN particles. It was also observed that chitosan is thermally stable up to 250 °C, and from 270 °C up to 500 °C, it showed a significant weight loss. This decomposition step can be assigned to the complex dehydration of the saccharide rings, depolymerization, and pyrolytic decomposition of the polysaccharide structure with vaporization and elimination of volatile products (Penichecovas, Arguellesmonal, & Sanroman, 1993; Zohuriaan & Shokrolahi, 2004). However, for the CS-GEN particles and CS-GEN with immobilized enzyme it was observed a continuous weight loss from 100 °C up to 270 °C, being of 25.8% and 30.8%, respectively, indicating a lower thermal stability compared to CS. These high values for the weight loss at this range of temperatures can be ascribed to a possible weakening of part of the chitosan structure caused by the crosslinking with genipin. It is important to note that the total weight loss increased for CS-GEN and CS-GEN with immobilized β-D-galactosidase, confirming the crosslinking and the enzyme immobilization, respectively. Moreover, TGA curves indicated that the obtained chitosan particles would be thermally stable at the temperature range used in most enzymatic reactions (up to 100 °C).

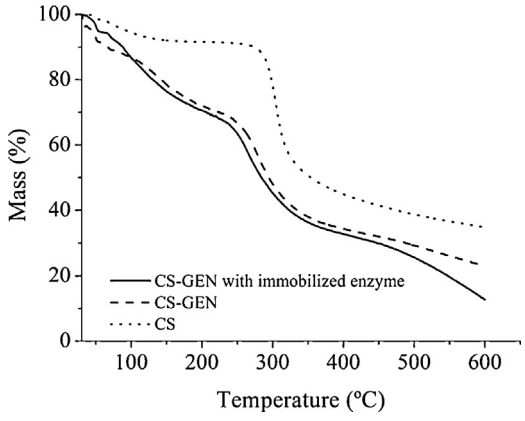

Fig. 3. TGA curves of chitosan particles (CS), chitosan particles crosslinked with genipin (CS-GEN) and CS-GEN particles with immobilized β-D-galactosidase.

3.4. Enzyme immobilization

As stated before, genipin is a naturally occurring crosslinking reagent compatible for food applications. In this sense, it would be a good alternative for the traditional crosslinker glutaraldehyde (Barbosa et al., 2014). Although glutaraldehyde is the most used reagent for crosslinking of proteins, it is also known by its toxicity, since glutaraldehyde can also crosslink DNAs and functional proteins in body, under physiological conditions, thus inducing cytotoxicity or carcinogenicity (Liu, Xu, Mi, Xu, & Yang, 2015; Mitra, Sailakshmi, & Gnanamani, 2014; Wang, Gu, Qin, Li, Yang, & Yu, 2015), limiting its application in food process.

The enzyme seemed to be affected in a distinct way by the two different methodologies of immobilization (using genipin or glutaraldehyde), since values of immobilization efficiency (IE %) were higher for the immobilized enzyme using genipin (66%) than the IE % of the immobilized enzyme using glutaraldehyde (36%) (Table S1). Fujikawa, Yokota and Koga (1988) reported slight differences using different crosslinking reagents, since 50% and 63% of activity effectiveness was found for β-glucosidase immobilized in alginate gel crosslinked with glutaraldehyde and genipin, respectively. In another study, Wang, Jiang, Zhou, and Gao (2011) reported very high activity recoveries (98.67% and 90.33%) for lipase immobilized on two different mesoporous resins by crosslinking with genipin. The same authors pointed out that highest activity recoveries was achieved after 6 h of reaction, and longer crosslinking time gave the immobilized lipase a good strength, however leads to more loss of activity. Then, immobilization by crosslinking with genipin (or glutaraldehyde) should be a compromise between adequate mechanical strength combined with relatively high enzyme activity. Moreover, using genipin as crosslinking agent, it was possible to increase the activity per gram of support in more than 50% (Table S1), which results in a more active and useful biocatalyst than that made using glutaraldehyde.

3.5. Optima pH and temperature

The effect of pH on the relative activity of free and immobilized β-D-galactosidase was evaluated in the range of 2.3–8.0 (Fig. 4A). The optimum pH for the free enzyme was found to be around 4.5–5.0, which agreed with others works reporting the effect of pH on the activity of β-D-galactosidase from *A. oryzae* (Guerrero, Vera, Araya, Conejeros, & Illanes, 2015; Mohy Eldin, El-Aassar, El-Zatahry, & Al-Sabah, 2014). After immobilization on chitosan particles, the optimum pH shifted toward a more acidic region, being pH 4 considered the optimum for both, CS-GLU and CS-GEN. Moreover, both immobilized enzymes showed to have higher activity also at pH 3, preserving more than 90% of its activity, when compared to the free enzyme.

Generally, binding of the enzyme to a polycationic support would result in an acidic shift in the pH optimum (Goldstein, Levin, & Katchals, 1964). The pKa of the amino group of glucosamine residue on chitosan is about 6.3, hence chitosan is polycationic at acidic pH values, being extremely positively charged at pH 4.5 (Hwang & Damodaran, 1995; Shahidi, Arachchi, & Jeon, 1999). Close to neutrality or at higher pHs, chitosan has free positive charges in smaller amounts (Berger et al., 2004). Then, it could be inferred that positive free charges can influence in the changes of pH optimum observed after immobilization. Indeed, according to Chibata (1978), charged supports shift the enzyme activity/pH profile toward lower pHs when the concentration of hydroxyl ions in the immediate

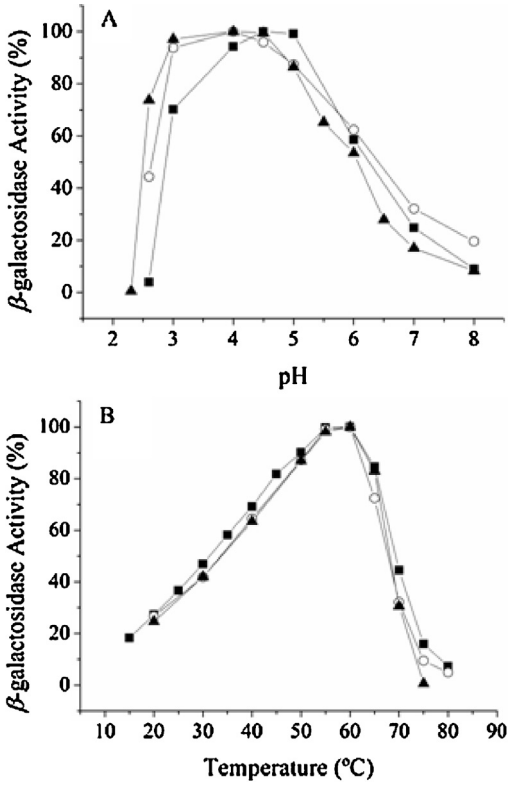

Fig. 4. Effect of pH (A) and temperature (B) on the activity of free (■) and immobilized β-D-galactosidase on (○) CS-GLU and (▲) CS-GEN.

vicinity of the support surface is higher than in the bulk solution, attracted by the positive free charges (that is the case of chitosan) or toward higher pH values when the contrary occurs.

Fig. 4B shows the effect of reaction temperature on the residual activities, in the range of 15–80 °C, for free and immobilized β-D-galactosidase. The optimum temperature for free *A. oryzae* β-D-galactosidase was found to be around 55–60 °C. This result agrees with the findings of Mohy Eldin et al. (2014). After immobilization, the optimum temperature for the enzyme immobilized in both CS-GLU and CS-GEN was also found to be around 55–60 °C, indicating that immobilization did not alter the optimum temperature of β-D-galactosidase.

3.6. Enzyme thermal stability

Fig. 5 shows the residual activity of the different biocatalysts. After 60 min of incubation under non-reactive conditions, the CS-GEN and CS-GLU presented 34% and 44% of residual enzyme activity. It is noteworthy that all immobilized preparations were more stable than the free enzyme, which presents 16% of residual enzyme activity after 60 min of incubation in the same conditions. The mechanism of immobilization using glutaraldehyde is generally simple and involves the amino terminal group from the enzyme (Chiou & Wu, 2004). On the other hand, the crosslinking with genipin involves many distinct reactions, and provide a different gel structure compared to glutaraldehyde (even less thermostable, as demonstrated by the TGA); a factor that can leads to unwanted reactions at high temperatures, which can explain its lower enzyme thermal stability.

Sugars and other osmolytes can improve the thermal stability of enzymes by reducing the enzyme movement due to the preferential exclusion of the osmolytes from the protein backbone, thus avoiding unfolding and denaturation (Kumar, Attri, & Venkatesu, 2012; Liu, Ji, Zhang, Dong, & Sun, 2010). Fig. 5 also shows that, in the

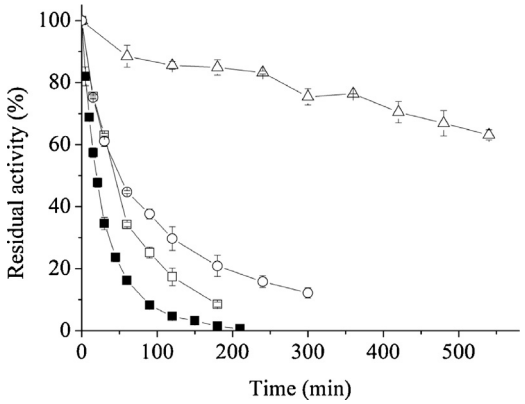

Fig. 5. Thermal inactivation at 60 °C of (■) free and immobilized *A. oryzae* β-D-galactosidase on (□) CS-GEN, (○) CS-GLU and (△) CS-GEN in the presence of lactose 40% (w/v).

presence of lactose buffered solution (40%, w/v), the immobilized enzyme on CS-GEN particles presented increased thermal stability. After 540 min of incubation at 60 °C the immobilized enzyme still presented 63% of residual enzyme activity, which means that, under operational conditions, the enzyme is much more stable than in buffer solution. It is important to evaluate β-D-galactosidase thermal stability in the presence of lactose, because it gives information about the real potential of this enzyme for dairy industry application. Moreover, it avoids underestimate enzyme stability.

3.7. Operational stability in the lactose hydrolysis

Operational stability of the CS-GEN biocatalyst was evaluated in the hydrolysis of buffered lactose solutions (5%, w/v; pH 4.5) at 40 °C. Lactose hydrolysis performed with 25 CS-GEN particles in 1.5 mL of lactose resulted in 70% of lactose conversion in 6 h for its first use (Fig. S1). Repeated batch hydrolysis of buffered lactose solutions by the immobilized enzyme allowed 25 repeated cycles with maximum activity. From these results, it can be concluded that *A. oryzae* β-D-galactosidase immobilized on chitosan by crosslinking with genipin shows satisfactory operational stability in the lactose hydrolysis.

3.8. Galactooligosaccharides synthesis

3.8.1. Effect of lactose concentration

To determine the influence of substrate concentration on GOS synthesized by immobilized *A. oryzae* β-D-galactosidase on CS-GEN particles, experiments were performed with increasing lactose concentration 300, 400, 500 g L^{-1} at 45 °C and pH 5.25, following a time course of reaction up to 420 min. Fig. 6 shows that GOS synthesis increased with increasing lactose concentration. The maximal GOS concentrations for initial lactose concentrations of 300 g L^{-1}, 400 g L^{-1} and 500 g L^{-1} were 75 g L^{-1}, 114 g L^{-1} and 146 g L^{-1} after 180 min, 300 min and 420 min, respectively. In fact, β-D-galactosyl groups should have a higher probability of attaching to lactose than water at increasing lactose concentrations (Iwasaki, Nakajima, & Nakao, 1996). For the initial lactose concentration of 300 g L^{-1} and 400 g L^{-1}, the GOS synthesis decreased after achieving the maximum. This fact is attributed to a preferential hydrolysis rather than GOS synthesis (Neri et al., 2009). The same reduction was not observed using an initial lactose concentration of 500 g L^{-1}, at the same reaction time, since there is more lactose to be hydrolyzed and to serve as acceptor for β-D-galactosyl groups. In terms of GOS yield, the values increased for the increasing lactose concentrations (25%, 28.5% and 29%, respectively). Huerta, Vera, Guerrero, Wilson, and Illanes (2011) also found yields of around 28% on the

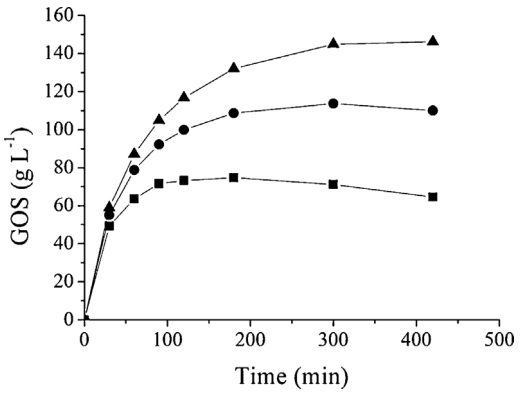

Fig. 6. Effect of lactose concentration: (■) 300 g L^{-1}, (●) 400 g L^{-1}, (▲) 500 g L^{-1} on the GOS synthesis using β-D-galactosidase immobilized on CS-GEN.

synthesis of GOS from lactose 500 g L^{-1} using distinct concentrations of the enzyme (*A. oryzae* β-D-galactosidase) immobilized on glyoxyl-agarose.

3.8.2. Effect of pH

The effect of pH on the GOS synthesis was investigated at 45 °C for pH values of 4.5, 5.25 and 7, at an initial lactose concentration of 400 g L^{-1}. Fig. 7 shows the time course of GOS synthesis at different pH values. The rate of the transgalactosylation reaction increased as the pH decreased, since the maximum GOS concentration was achieved in less time at pH 4.5 (116 g L^{-1} in 180 min), than at pH 5.25 (114 g L^{-1} in 300 min) and at pH 7 (121 g L^{-1} in 420 min). The corresponding yields are 29% at pH 4.5, 28.5% at pH 5.25, and 30% at pH 7. Since the optimum pH was found to be between 3.5 and 4.5 (Fig. 4A), it seems clear that lactose hydrolysis occurs faster at acidic conditions. In these conditions there is more D-galactose liberated from lactose hydrolysis that will serve as substrate for the transgalactosylation reaction, than increasing its rate. At pH 7, the opposite occurs: since hydrolysis activity is not favored, the rate of liberated D-galactose is slower and the maximum GOS synthesis is achieved in longer times. The reaction at pH 4.5 has the advantage of provide higher productivity (38.7 g L^{-1} h^{-1}) than at pH 7 (17.3 g L^{-1} h^{-1}).

It is noteworthy that the maximum GOS concentration achieved at pH 7 was slightly higher than the GOS concentration found at pHs 4.5 and 5.25. This behavior was already described by others researchers using β-D-galactosidase from *A. aculeatus* (Cardelle-Cobas, Martinez-Villaluenga, Villamiel, Olano, & Corzo, 2008; Cardelle-Cobas, Villamiel, Olano, & Corzo, 2008), and it is possible explained by the higher solubility of lactose at pH 7 (380 g L^{-1}) than at pH 4 (147 g L^{-1}) at 45 °C (Brito, 2007).

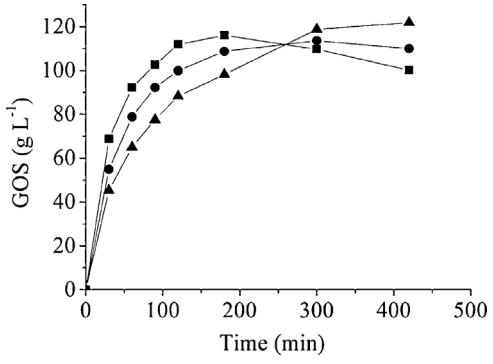

Fig. 7. Effect of pH 4.5 (■), pH 5.25 (●), pH and pH 7 (▲) on the GOS synthesis using β-D-galactosidase immobilized on CS-GEN.

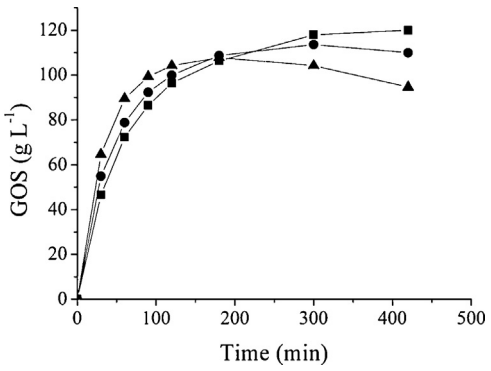

Fig. 8. Effect of temperature: (■) 40 °C, (●) 47.5 °C, (▲) 55 °C on the GOS synthesis using β-D-galactosidase immobilized on CS-GEN.

3.8.3. Effect of temperature

To determine the influence of temperature on GOS synthesis, experiments were performed at 40, 47.5 and 55 °C at initial lactose concentration of 400 g L^{-1} and pH 5.25, following a time course of reaction up to 420 min. Temperature normally has a pronounced effect on enzyme reaction rates but showed to have a minimal effect on GOS yield. From Fig. 8, it can be seen that the maximum GOS concentration, at 40 °C, 47.5 °C and 55 °C was 120 g L^{-1}, 114 g L^{-1} and 108 g L^{-1} after 420 min, 300 min and 180 min, respectively. These concentrations represent GOS yields of 30%, 28.5% and 27% at 40 °C, 47.5 °C and 55 °C, respectively. In terms of productivity, the GOS synthesis at 55 °C is advantageous since the productivity was of 36 g L^{-1} h^{-1} in comparison to the productivity at 40 °C (17.1 g L^{-1} h^{-1}). However, although the immobilized enzyme presented good thermal stability in the presence of concentrated lactose (Fig. 5), it was slowly inactivated during the reaction. Thus, from these results, we could suggest that an adequate range of temperature for GOS synthesis with the obtained biocatalyst is around 47 °C, since it gives good productivity (22.8 g L^{-1} h^{-1}) and allows more numbers of reuses. Vera, Guerrero, and Illanes (2011) also reported that the transgalactosylation activity of *A. oryzae* β-D-galactosidase increased with temperature in the range of 40–55 °C, and this is reflected in the corresponding increase in productivity for GOS synthesis.

4. Conclusions

Chitosan is widely used as support for enzyme immobilization, and usually, glutaraldehyde, a very toxic reagent, is employed as crosslinker agent, limiting the application in food process. For such case, the support used should be cheap and safe. The biocatalyst obtained in the present work satisfies these requirements, since it was prepared from chitosan, which is a cheap and non-toxic polysaccharide, and crosslinked with genipin, a safe and naturally occurring bi-functional crosslinking reagent, instead of glutaraldehyde. From a kinetic point of view, the β-D-galactosidase immobilized on this support showed to have an activity higher than the activity of the biocatalyst prepared with glutaraldehyde. Moreover, it presents thermal stability, reusability on the lactose hydrolysis, and good yields on the synthesis of galactooligosaccharides. From a practical point of view, the obtained particles were resistant to acid pH, easy to handle and more resistant mechanically than the particles prepared with glutaraldehyde, hence no fractures were observed in all batches of lactose hydrolysis or galactooligosaccharides synthesis.

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), by the Fundação de

Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) of the Brazilian government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2015.10.069.

References

Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Torres, R., Rodrigues, R. C., & Fernandez-Lafuente, R. (2014). Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization. *RSC Advances*, *4*(4), 1583–1600.

Berger, J., Reist, M., Mayer, J. M., Felt, O., Peppas, N. A., & Gurny, R. (2004). Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics*, *57*(1), 19–34.

Bi, L., Cao, Z., Hu, Y., Song, Y., Yu, L., Yang, B., et al. (2011). Effects of different cross-linking conditions on the properties of genipin-cross-linked chitosan/collagen scaffolds for cartilage tissue engineering. *Journal of Materials Science: Materials in Medicine*, *22*(1), 51–62.

Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C., & Robins, K. (2012). Engineering the third wave of biocatalysis. *Nature*, *485*(7397), 185–194.

Brito, A. B. N. (2007). *Estudo de cristalização de lactose em diferentes solventes. Programa de Pós-Graduação em Engenharia Química* (Tese de Doutorado). pp. 152. São Paulo: Universidade Federal de São Carlos.

Burton, S. G., Cowan, D. A., & Woodley, J. M. (2002). The search for the ideal biocatalyst. *Nature Biotechnology*, *20*(1), 37–45.

Butler, M. F., Ng, Y. F., & Pudney, P. D. A. (2003). Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *Journal of Polymer Science Part A: Polymer Chemistry*, *41*(24), 3941–3953.

Cardelle-Cobas, A., Martinez-Villaluenga, C., Villamiel, M., Olano, A., & Corzo, N. (2008). Synthesis of oligosacccharides derived from lactulose and Pectinex Ultra SP-L. *Journal of Agricultural and Food Chemistry*, *56*(9), 3328–3333.

Cardelle-Cobas, A., Villamiel, M., Olano, A., & Corzo, N. (2008). Study of galacto-oligosaccharide formation from lactose using Pectinex Ultra SP-L. *Journal of the Science of Food and Agriculture*, *88*(6), 954–961.

Cauich-Rodriguez, J. V., Deb, S., & Smith, R. (1996). Effect of cross-linking agents on the dynamic mechanical properties of hydrogel blends of poly(acrylic acid)–poly(vinyl alcohol vinyl acetate). *Biomaterials*, *17*(23), 2259–2264.

Chibata, I. (1978). *Immobilised enzymes-research and development*. New York: John Wiley and Sons, Inc.

Chiou, S. H., & Wu, W. T. (2004). Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. *Biomaterials*, *25*(2), 197–204.

Colthup, N. B., Daily, L. H., & Wiberley, S. E. (1975). *Introduction to infrared and raman spectroscopy*. New York: Academic Press.

Cui, L., Xiong, Z., Guo, Y., Liu, Y., Zhao, J., Zhang, C., et al. (2015). Fabrication of interpenetrating polymer network chitosan/gelatin porous materials and study on dye adsorption properties. *Carbohydrate Polymers*, *132*, 330–337.

de Barros, D. P. C., Fernandes, P., Cabral, J. M. S., & Fonseca, L. P. (2010). Operational stability of cutinase in organic solvent system: Model esterification of alkyl esters. *Journal of Chemical Technology and Biotechnology*, *85*(12), 1553–1560.

Fujikawa, S., Yokota, T., & Koga, K. (1988). Immobilization of beta-glucosidase in calcium alginate gel using genipin as a new type of cross-linking reagent of natural origin. *Applied Microbiology and Biotechnology*, *28*(4–5), 440–441.

Goldstein, L., Levin, Y., & Katchals, E. (1964). A water-insoluble polyanionic derivative of trypsin. 2. Effect of polyelectrolyte carrier on kinetic behavior of bound tripsin. *Biochemistry*, *3*(12), 1913.

Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E., & Gras, S. L. (2011). Effect of the substrate concentration and water activity on the yield and rate of the transfer reaction of beta-D-galactosidase from *Bacillus circulans*. *Journal of Agricultural and Food Chemistry*, *59*(7), 3366–3372.

Grosova, Z., Rosenberg, M., & Rebros, M. (2008). Perspectives and applications of immobilised beta-D-galactosidase in food industry – A review. *Czech Journal of Food Sciences*, *26*(1), 1–14.

Guerrero, C., Vera, C., Araya, E., Conejeros, R., & Illanes, A. (2015). Repeated-batch operation for the synthesis of lactulose with β-D-galactosidase immobilized by aggregation and crosslinking. *Bioresource Technology*, *190*, 122–131.

Huerta, L. M., Vera, C., Guerrero, C., Wilson, L., & Illanes, A. (2011). Synthesis of galacto-oligosaccharides at very high lactose concentrations with immobilized beta-galactosidases from *Aspergillus oryzae*. *Process Biochemistry*, *46*(1), 245–252.

Hwang, D. C., & Damodaran, S. (1995). Selective precipitation and removal of lipids from cheese whey using chitosan. *Journal of Agricultural and Food Chemistry*, *43*(1), 33–37.

Iwasaki, K., Nakajima, M., & Nakao, S. (1996). Galacto-oligosaccharide production from lactose by an enzymic batch reaction using beta-galactosidase. *Process Biochemistry*, *31*(1), 69–76.

Klein, M. P., Fallavena, L. P., Schöffer, J. D. N., Ayub, M. A. Z., Rodrigues, R. C., Ninow, J. L., et al. (2013). High stability of immobilized β-D-galactosidase for lactose hydrolysis and galactooligosaccharides synthesis. *Carbohydrate Polymers*, *95*(1), 465–470.

Klein, M. P., Nunes, M. R., Rodrigues, R. C., Benvenutti, E. V., Costa, T. M. H., Hertz, P. F., et al. (2012). Effect of the support size on the properties of beta-galactosidase immobilized on chitosan: Advantages and disadvantages of macro and nanoparticles. *Biomacromolecules*, *13*(8), 2456–2464.

Krajewska, B. (2004). Application of chitin- and chitosan-based materials for enzyme immobilizations: A review. *Enzyme and Microbial Technology*, *35*(2–3), 126–139.

Kumar, A., Attri, P., & Venkatesu, P. (2012). Effect of polyols on the native structure of alpha-chymotrypsin: A comparable study. *Thermochimica Acta*, *536*, 55–62.

Lambert, J. B. (1987). *Introduction to organic spectroscopy*. New York: Macmillan.

Li, Q., Wang, X., Lou, X., Yuan, H., Tu, H., Li, B., et al. (2015). Genipin-crosslinked electrospun chitosan nanofibers: Determination of crosslinking conditions and evaluation of cytocompatibility. *Carbohydrate Polymers*, *130*, 166–174.

Liu, F.-F., Ji, L., Zhang, L., Dong, X.-Y., & Sun, Y. (2010). Molecular basis for polyol-induced protein stability revealed by molecular dynamics simulations. *Journal of Chemical Physics*, *132*(22.)

Liu, P., Xu, H., Mi, X., Xu, L., & Yang, Y. (2015). Oxidized sucrose: A potent and biocompatible crosslinker for three-dimensional fibrous protein scaffolds. *Macromolecular Materials and Engineering*, *300*(4), 414–422.

Lopez-Gallego, F., Betancor, L., Mateo, C., Hidalgo, A., Alonso-Morales, N., Dellamora-Ortiz, G., et al. (2005). Enzyme stabilization by glutaraldehyde crosslinking of adsorbed proteins on aminated supports. *Journal of Biotechnology*, *119*(1), 70–75.

Lorenzoni, A. S. G., Aydos, L. F., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2014). Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from *Aspergillus aculeatus*. *Carbohydrate Polymers*, *103*, 193–197.

Mekhail, M., Jahan, K., & Tabrizian, M. (2014). Genipin-crosslinked chitosan/poly-L-lysine gels promote fibroblast adhesion and proliferation. *Carbohydrate Polymers*, *108*, 91–98.

Mi, F. L., Sung, H. W., & Shyu, S. S. (2001). Release of indomethacin from a novel chitosan microsphere prepared by a naturally occurring crosslinker: Examination of crosslinking and polycation–anionic drug interaction. *Journal of Applied Polymer Science*, *81*(7), 1700–1711.

Mitra, T., Sailakshmi, G., & Gnanamani, A. (2014). Could glutaric acid (GA) replace glutaraldehyde in the preparation of biocompatible biopolymers with high mechanical and thermal properties? *Journal of Chemical Sciences*, *126*(1), 127–140.

Mohy Eldin, M. S., El-Aassar, M. R., El-Zatahry, A. A., & Al-Sabah, M. M. B. (2014). Covalent immobilization of β-D-galactosidase onto amino-functionalized polyvinyl chloride microspheres: Enzyme immobilization and characterization. *Advances in Polymer Technology*, *33*, 21379. http://dx.doi.org/10.1002/adv.21379

Muzzarelli, R. A. A. (2009). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*(1), 1–9.

Neri, D. F. M., Balcão, V. M., Costa, R. S., Rocha, I. C. A. P., Ferreira, E. M. F. C., Torres, D. P. M., et al. (2009). Galacto-oligosaccharides production during lactose hydrolysis by free *Aspergillus oryzae* β-D-galactosidase and immobilized on magnetic polysiloxane–polyvinyl alcohol. *Food Chemistry*, *115*(1), 92–99.

Penichecovas, C., Arguellesmonal, W., & Sanroman, J. (1993). A kinetic-study of the thermal degradation of chitosan and a mercaptan derivative of chitosan. *Polymer Degradation and Stability*, *39*(1), 21–28.

Schöffer, J. d. N., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2013). Continuous production of beta-cyclodextrin from starch by highly stablecyclodextrin glycosyltransferase immobilized on chitosan. *Carbohydrate Polymers*, *98*, 1311–1316.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosans. *Trends in Food Science & Technology*, *10*(2), 37–51.

Sheldon, R. A., & van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: Why, what and how. *Chemical Society Reviews*, *42*(15), 6223–6235.

Sung, H. W., Huang, R. N., Huang, L. L. H., & Tsai, C. C. (1999). In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. *Journal of Biomaterials Science, Polymer Edition*, *10*(1), 63–78.

Valerio, S. G., Alves, J. S., Klein, M. P., Rodrigues, R. C., & Hertz, P. F. (2013). High operational stability of invertase from *Saccharomyces cerevisiae* immobilized on chitosan nanoparticles. *Carbohydrate Polymers*, *92*(1), 462–468.

Vera, C., Guerrero, C., & Illanes, A. (2011). Determination of the transgalactosylation activity of *Aspergillus oryzae* beta-galactosidase: Effect of pH, temperature, and galactose and glucose concentrations. *Carbohydrate Research*, *346*(6), 745–752.

Wang, W., Jiang, Y., Zhou, L., & Gao, J. (2011). Comparison of the properties of lipase immobilized onto mesoporous resins by different methods. *Applied Biochemistry and Biotechnology*, *164*(5), 561–572.

Wang, X., Gu, Z., Qin, H., Li, L., Yang, X., & Yu, X. (2015). Crosslinking effect of dialdehyde starch (DAS) on decellularized porcine aortas for tissue engineering. *International Journal of Biological Macromolecules*, *79*, 813–821.

Zohuriaan, M. J., & Shokrolahi, F. (2004). Thermal studies on natural and modified gums. *Polymer Testing*, *23*(5), 575–579.