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High operational stability of invertase from *Saccharomyces cerevisiae* immobilized on chitosan nanoparticles

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ABSTRACT

Invertase (E.C.3.2.1.26) from *Saccharomyces cerevisiae* was covalently immobilized on chitosan nanoparticles prepared by the ionotropic gelation method and activated with glutaraldehyde. The support was characterized and it was studied its load capacity, the influence of the presence of substrate during immobilization, and determined the biocatalyst kinetic parameters and stabilities. The light scattering analysis (LSA) and transmission electron microscopy (TEM) techniques indicated a mixture of chitosan nano and aggregated nanoparticles, providing high superficial area for enzyme immobilization. The thermal and storage stabilities, the optimal pH and temperature of the enzyme were not altered. K_m increased 3-fold, while V_{max} remained unaltered. The immobilized biocatalyst was reused for 59 batches with maximal invertase activity, the highest operational stability so far described in the literature. These results fulfill some important aspects for the enzyme immobilization: the simplicity of the protocols, the conservation of the enzyme activity, and the high operational stability.

1. Introduction

Invertase or β-D-fructofuranosidase (E.C. 3.2.1.26) from *Saccharomyces cerevisiae* is an enzyme that has major specificity for sucrose hydrolysis. This reaction results in an equimolar mixture of α-D-glucose and β-D-fructose, known as invert syrup. As a consequence, the crystallization phenomenon is avoided and, as fructose is sweeter than sucrose, the sweetness of the product is higher. The prevention of crystallization justifies the main food industrial application of the enzyme in the manufacture of fillings of sweets, keeping the softness. Also, invertase can be applied in fermentation process, when sucrose is the substrate, in the manufacture of artificial honey, humectant agent for candies production, besides other applications as in cosmetic, paper and drug industry (Kotwal & Shankar, 2009).

The solubility of free enzymes makes their uses for large-scale relatively costly since in a mixture containing the substrate, products and other components, their recoveries are difficult, being economically unattractive (Kotwal & Shankar, 2009). Nevertheless, biocatalysts are increasingly being employed because of their high selectivity and potential as a greener alternative to chemical catalysts (Polizzi, Bommarius, Broering, & Chaparro-Riggers, 2007), which could result in the formation of undesirable color and flavoring agents.

The interest for enzymatic processes is over growing, which also reflects the great interest for biocatalysts immobilization. Taking into account distinct applications, there are diverse immobilization techniques with different methods of linkages among enzymes and supports. It is noteworthy that each protein and support has typical characteristics, so there is no universal ideal method of immobilization. For example, when the support for the immobilization is solid and non-porous, the size of the particles needs to be as small as possible, considering that nanoparticles provide a reasonable enzyme load capacity (Garcia-Galan, Berenguer-Murcia, Fernandez-Lafuente, & Rodrigues, 2011). In general, the enzyme immobilization aims at keeping or increasing storage, temperature, pH, and operational stabilities.

Chitosan has been used as a support for enzyme immobilization (Klein et al., 2012; Kuo et al., 2012; Li, Cai, Zhong, & Du, 2012; Muzzarelli, 1980; Orrego et al., 2010). Chitosan, isolated from chitin, is the linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli, 1977, 2012; Muzzarelli et al., 2012). Certain chitosan salts are soluble in water, thus they form gels and polyelectrolyte complexes, in particular with proteins (Krajewska, 2004). Invertase was encapsulated in chitosan microbeads (Siso et al., 1997), and immobilized covalently via carbohydrate moiety (Hsieh, Liu, & Liao, 2000). The enzyme was also modified with chitosan and immobilized in sodium alginate-coated chitin support, and in hyaluronic-acid-coated chitin support (Gomez, Ramirez,

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Cabrera, Simpson, & Villalonga, 2008; Gómez, Ramírez, Villalonga, Hernández, & Villalonga, 2006). Chitosan was also prepared as films and utilized for the biocatalyst immobilization (Teodor, Radu, Dan, & Stanciu, 2006). Biró, Németh, Sisak, Feczkó, and Gyenis (2008) described protocols for the preparation of chitosan support suitable for biocatalysts immobilization, applying to β-galactosidase. The ionotropic gelation method proposed for nanoparticles production, modified from the one described by Berthold, Cremer, and Kreuter (1996), showed the highest enzyme activity, serving as model for the chitosan preparation described in the present study. Recently, it was studied the effect of support size for enzyme immobilization on chitosan (Klein et al., 2012). Macro and nanoparticles of chitosan were prepared and characterized for β-galactosidase immobilization. The authors found a high operational stability for lactose hydrolysis for both preparations.

Thus, the objective of this work was to immobilize the invertase from *S. cerevisiae* on chitosan nanoparticles. The evaluation of the enzyme properties and thermal and operational stability was also studied.

2. Materials and methods

2.1. Materials

Invertase from *S. cerevisiae* (Maxinvert L 10000, batch 409200451) was kindly donated by DSM Food Specialties (The Netherlands). Chitosan from shrimp shells (>75% deacetylated) was purchased from Sigma–Aldrich (St. Louis, USA). Sucrose and all other chemicals (acetic acid, dinitrosalicylic acid, ethylene glycol, glutaraldehyde, magnesium chloride, polysorbate 80, potassium phosphate, sodium and potassium tartrate, sodium chloride, sodium hydroxide and sodium sulfate) were of analytical grade and purchased either from Merck or Fluka (São Paulo, Brazil). All the following tests were performed in duplicate.

2.2. Preparation of chitosan particles

Chitosan support was prepared by ionotropic gelation according to a procedure already described (Berthold et al., 1996) with some modifications: 0.5 mL of sodium sulfate aqueous solution (1.4 M) were added dropwise into 9.5 mL of chitosan (0.25%, w/v) dissolved in 0.35 M acetic acid containing Tween 80 (1%, v/v) under sonication (30 min, 40 kHz, 25 °C). This suspension was magnetically stirred for 2 h (500 rpm) and the particles formed were collected by centrifugation (3500 × *g*, 15 min, 4 °C). The particles obtained were washed with distilled water and activated by suspending them in 10 mL of glutaraldehyde (1.25%, v/v) in 0.1 M phosphate–potassium buffer (pH 7.0) under agitation during 30 min. The glutaraldehyde excess was removed with successive washings using the same buffer.

2.3. Dry weight and support size determination

Support dry weight was performed by lyophilization, after activation with glutaraldehyde. The further immobilization results are expressed as units of enzyme activity by dry mass of support.

Two different techniques were used for support size determination, considering the procedures involved in the preparation of the samples for analysis and the sensitivity of each equipment. Results of the mean particle size before activation were performed using filtered sample (membrane of 45 μm of diameter) in light scattering analysis (LSA) on a Brookhaven Instruments standard setup (BI-200M goniometer, BI-9000AT digital correlator) with a He–Ne laser (k = 632.8 nm) as light source. Using a JEOL JEM 1200ExII transmission electron microscope (JEOL, Tokyo) operating at 120 kV, the transmission electron microscopy (TEM) was done for attainment morphological images of chitosan particles before and after activation. The support was suspended in distilled water and after homogenized with ultrasonic bath for 5 min, the samples were negatively stained with uranyl acetate solution (0.047 M). At room temperature the samples were air-dried.

2.4. Invertase covalent immobilization

Covalent immobilization of invertase on chitosan beads was carried out incubating 0.05 g (dry weight) of the chitosan activated support with 10 mL of each enzyme solution in acetate buffer (0.1 M; pH 4.5) overnight under gentle stirring, at room temperature. Then, the immobilized enzymes were centrifuged (15 min, 4 °C, 3000 × *g*) and washed with solutions of acetate buffer (0.1 M; pH 4.5), sodium chloride (1 M), ethylene glycol (5.38 M), and again with acetate buffer, until activity was no longer detected in the washing solutions. The immobilization yields were calculated using Eq. (1):

$$\mathrm{IY} = \frac{U_i - (U_3 + U_w)}{U_i} 100 \quad (1)$$

where IY is the immobilization yield, U_i is the initial enzyme activity in the solution, U_s the activity in the supernatant after immobilization, and U_w the activity in the washing solutions.

The efficiency of immobilization was defined as:

$$\mathrm{Ef} = \frac{U_i}{\mathrm{IY}} 100 \quad (2)$$

where Ef is the immobilization efficiency, U_i the activity in the immobilized enzyme, and IY the immobilization yield.

Except for free enzyme, all aliquots were submitted to the washing solutions.

2.5. Enzymatic activity assay

Based on a described method (Bryjak, Liesiene, & Stefuca, 2008), activities of free and immobilized enzyme were assayed by the addition of invertase suspension (1 mL of final volume) in acetate buffer (0.1 M; pH 4.5) to 2 mL of sucrose (8% in the same buffer). The samples were incubated during 5 min at 55 °C in a water bath (with agitation for the immobilized derivatives), and ice bath (5 min) was used to stop the reaction. An aliquot of 100 μL was withdrawn and analyzed with 1 mL of DNS reagent by DNS method (Miller, 1959), in order to quantify released reducing sugars by the sucrose hydrolysis. The absorbance was measured at 540 nm and the results were calculated using glucose as standard. The enzyme activity unit (U) was defined as the amount of enzyme liberating 1 μmol of reducing sugars per minute under the assay conditions. All samples were analyzed in duplicate.

2.6. Determination of optimal pH, temperature and kinetic parameters

For the determination of optimal pH and temperature of free and immobilized invertase, each parameter was individually changed (pH from 3.0 to 7.0; temperature from 40 °C to 70 °C). The buffers concentrations were 0.1 M, and except for pH 3 (citrate buffer) and for pH 7 (phosphate buffer), the others measurements were performed with acetate buffer.

The Michaelis–Menten constant (K_m) and V_{max} were calculated under optimal conditions, using Lineweaver–Burk plot.

2.7. Thermal stability

The thermal stability of free and immobilized invertase was measured at 55 °C and 65 °C. Samples were collected periodically

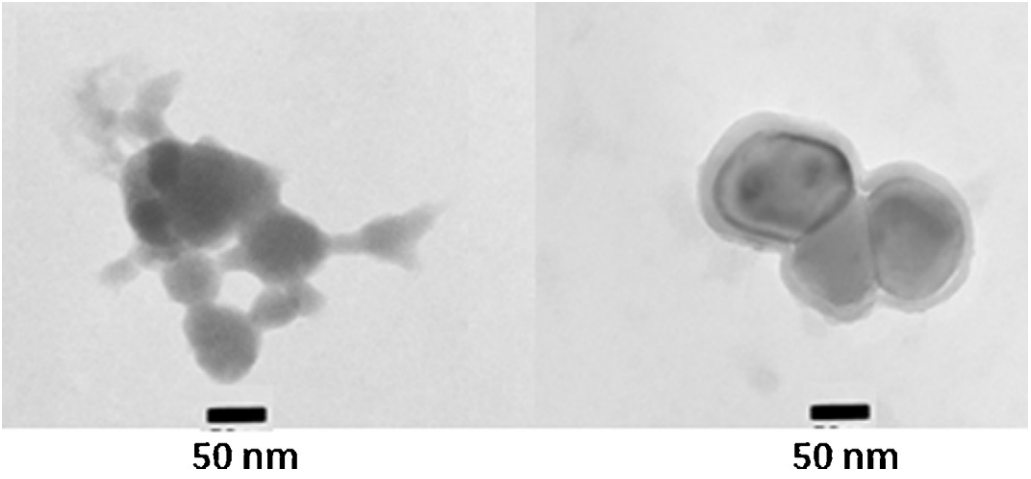

Fig. 1. Transmission electron microscopy of non-activated chitosan particles.

until the residual activity was 10%. Each sample was immersed in ice bath to stop the temperature damage and analyzed by previous enzymatic activity assay also being determined the half-life time ($t_{1/2}$).

2.8. Storage stability

Free and immobilized enzymes were stored at 8 °C in acetate buffer (0.1 M; pH 4.5), and their stability was measured once a week, during 25 weeks.

2.9. Operational stability

The operational stability of the immobilized enzyme was obtained by sucrose hydrolysis at 55 °C. The batches run up during 30 min, under gentle stirring, using 20 mL of substrate (8% in acetate buffer) and 0.05 g of support (200,000 U/g support). At the end of each batch, homogenous samples were taken, centrifuged (15 min, room temperature, 3000 × *g*), and the supernatant obtained utilized for reducing sugars determination. The precipitated pellet was recovered and used in a new batch (support washings among batches were not necessary).

3. Results and discussion

3.1. Support size determination

Light scattering analysis (LSA) indicated that the mean particle size of non-activated support was 410.9 nm, with a low polydispersity of 0.266 (parameter related to the particle size homogeneity, being higher as unequal is the sample). In the absence of stabilizing agents the nanoparticles form unstable systems with a tendency to aggregation. These events are addressed in a recent review (Zhang et al., 2011), in which are discussed various aspects of the production of nanoparticles providing particular reference to their influence on stability and size. Fig. 1 shows the transmission electron microscopy (TEM) pictures of the support not activated, indicating aggregated nanoparticles even before the utilization of glutaraldehyde. The TEM results after cross-linking (data not shown) indicate more particle aggregation phenomena, also showing the support with uneven surfaces and part of the samples reaching a micro size (Klein et al., 2012). The formation of larger support particles occurs because since the glutaraldehyde is a bifunctional agent, through its aldehyde groups it can crosslink two chitosan units from different polymeric chains (Monteiro & Airoldi, 1999). In a study for trypsin immobilization on linolenic acid modified chitosan, increasing the glutaraldehyde concentration for crosslinking, the particle size increased from 523 to 1372 nm (Liu, Desai, Chen, & Park, 2005).

LSA after activation (data not shown) without sample filtration presented higher values for polydispersity, showing a wide distribution of the aggregated particles, where it is not possible to determine the mean particle size formed. By other side, performing LSA after activation with the filtration step, considerable part of the support was retained. Thus, because of these factors this technique was used only before cross-linking.

Each additional unit operation for the support preparation represents an additional cost, being less attractive for use in large-scale process. In order to minimize the number of steps for support preparation and the possible material losses inherent in each step, in this work filtration step for particle size classification was not performed. So, it can be concluded that the invertase was immobilized on a chitosan support constituted by nano and aggregated nanoparticles, which have high superficial area for enzyme immobilization.

3.2. Support load capacity

Different enzyme solutions were prepared diluting Maxinvert L 10000, for the determination of the support load capacity. Immobilization yields and efficiencies for each concentration tests are presented in Table 1. Activity detected in the support increased with invertase concentrations, although not proportionally.

The aim of this study involves only covalent linkages, so through the washing solutions it was possible to remove the non-covalent attached enzymes (mainly adsorptions). One-point covalent immobilization in a non-porous support provides a system free of diffusion limitation (Amaya-Delgado, Hidalgo-Lara, & Montes-Horcasitas, 2006). However, when high enzyme concentrations are immobilized diffusion limitation phenomena can happen, as result of the blocking effect caused by neighboring proteins, in part because there are random enzyme orientations (Wong, Khan, & Micklefield, 2009), reducing the accessibility of substrate to the catalytic site (Giacomini, Villarino, Franco-Fraguas, & Batista-Viera, 1998). Besides the considerations about the support load and the enzyme concentration, it is important to regard the uneven surfaces from the aggregated nanoparticles that, in part, resemble a porous structure. This suggested 'porous nature' (Biró et al., 2008) a characteristic sustained by particles swelling ability (Shu & Zhu, 2002), could be considered as another factor for limitation diffusion effects. These indications may explain the lower efficiency values for samples 4 and 5, compared to the others. Considering the

Table 1
Enzyme solutions for estimating support load capacity.

Sample	Offered free enzyme (U/g support)	Immobilized enzyme (U/g support)	Immobilization yield (%)	Immobilization efficiency (%)
1	10,400	9200	91.8	96.36
2	45,800	45,800	100	100
3	140,600	119,200	90.3	93.89
4	453,600	207,600	74.3	61.6
5	1,121,400	223,200	59.87	33.24

enzyme concentration offered to immobilization and the results of immobilization yield and efficiency, sample number 4 presented the better relationship among the tested conditions, since it was possible to immobilize a high load of enzyme on the support and to obtain interesting immobilizations yield and efficiency. Thus, sample number 4 was selected for further experiments.

Once defined the enzyme concentration for following studies, the immobilization kinetics was evaluated (Fig. 2a). It was demonstrated (Cadena et al., 2010), that immobilization of invertase on polyurethane in the presence of sucrose, to protect the catalytic site, improved the load of immobilization protein. Thus, it was performed experiments maintaining sucrose concentration at 8% in the immobilization solution, keeping the catalytic site saturated (Fig. 2b).

Immobilization occurred in 5 h, when around 50% of the offered enzyme was immobilized in the support. It was defined 5 h as the immobilization time, since activity in the supernatant and support kept unaltered after this time. The presence of substrate (sucrose) in the immobilization solution did not improve enzyme immobilization, in contrast to another study (Cadena et al., 2010), in which is shown the increased covalent immobilization of invertase to an organic polymer in a sucrose solution (10%).

3.3. pH, temperature and kinetic parameters

Results for the effects of pH and temperature on the activity of free and immobilized invertase are presented in Figs. 3 and 4, respectively. Activities are related to the highest value, which was considered as 100% activity.

Although it is possible to change the optimal pH, temperature and kinetic parameters for an immobilized derivative as a result of the immobilization method, support structure and conformation change of the enzyme after bounded, making the catalytic site more or less accessible to substrate (Bayramoglu et al., 2009), in our work no alterations in pH and temperature were detected.

Fig. 3 shows the same optimal pH (4.5) for both enzymatic preparations, indicating that there is no conformational alteration by immobilization process in the monomeric invertase. Other authors (Bahar & Tuncel, 2002; Chen, Kang, Neoh, & Tan, 2000) also covalently immobilized the enzyme on polymeric supports and did not find shifts in optimal pH after immobilization. These authors considered this as a positive factor to the preservation of the biocatalyst during the immobilization process.

It can be observed in Fig. 4 that the behavior for free and immobilized invertase at different temperatures is similar, and 55 °C is the optimal temperature for both enzymatic forms. As stated in other studies (Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007; Rodrigues et al., 2009) the one-point covalent immobilization of the enzyme suggests that the immobilized biocatalyst presented the same rigidity than the soluble form. For this reason, the immobilization did not present rigidification and protective effects, as can be seen in multipoint or multisubunit covalent immobilization examples, where there are improvements in the stability of the enzymes (Garcia-Galan et al., 2011; Mateo et al., 2007).

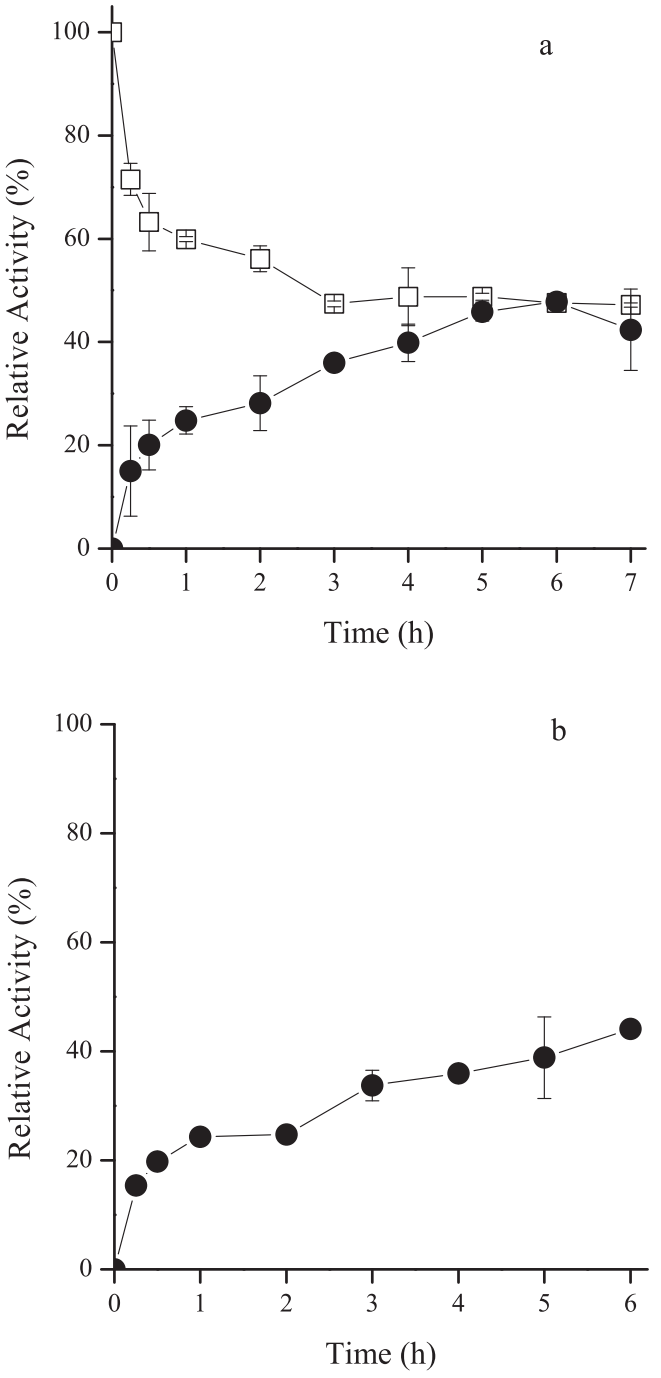

Fig. 2. Supernatant of immobilization solution (□) and support activity (●) during invertase immobilization process. (a) Absence of sucrose. (b) In the presence of sucrose (8%).

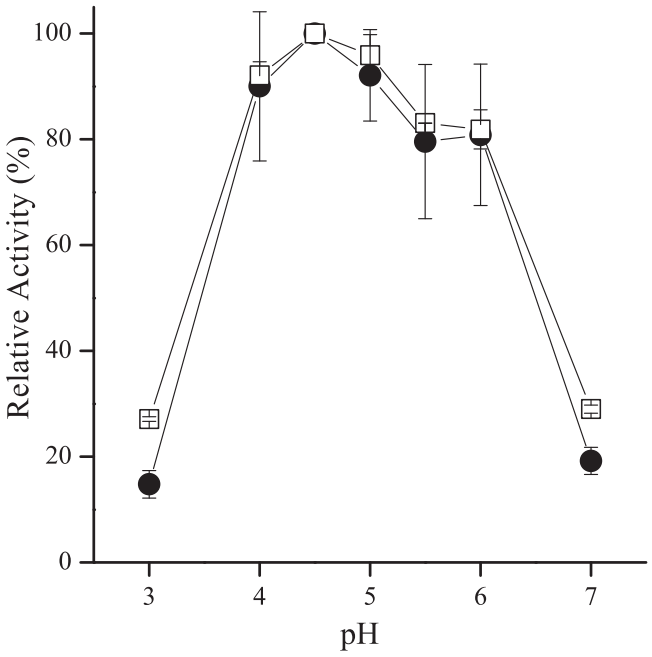

Fig. 3. Effect of pH on free (□) and immobilized (●) invertase activity at 55 °C.

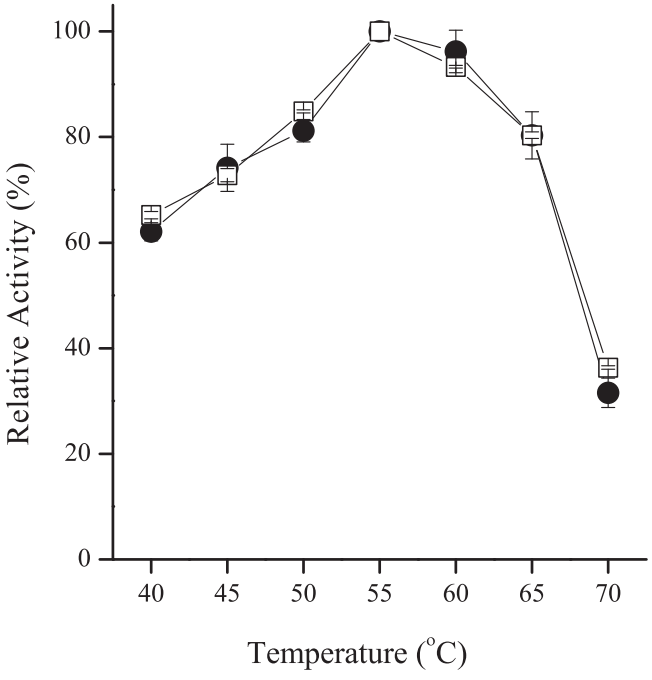

Fig. 4. Effect of temperature on free (□) and immobilized (●) enzyme at pH 4.5.

The determination of enzyme kinetic parameters is important because it is possible to measure the reaction rate and how it is influenced by physical and chemical conditions. The kinetic parameters for free and immobilized invertase (Table 2) were obtained by measuring the enzyme activities at sucrose concentration from 29.2 mM to 292.2 mM for free and immobilized invertase, in acetate buffer (0.1 M, pH 4.5) at 55 °C.

Table 2
Kinetic parameters for free and immobilized invertase.

Enzyme form	K_m (mM)	V_{max} (U/mL)	Catalytic efficiency[a]
Free	65.7	1670.0	25.4
Immobilized	205.7	1830.0	8.9

[a] V_{max}/K_m.

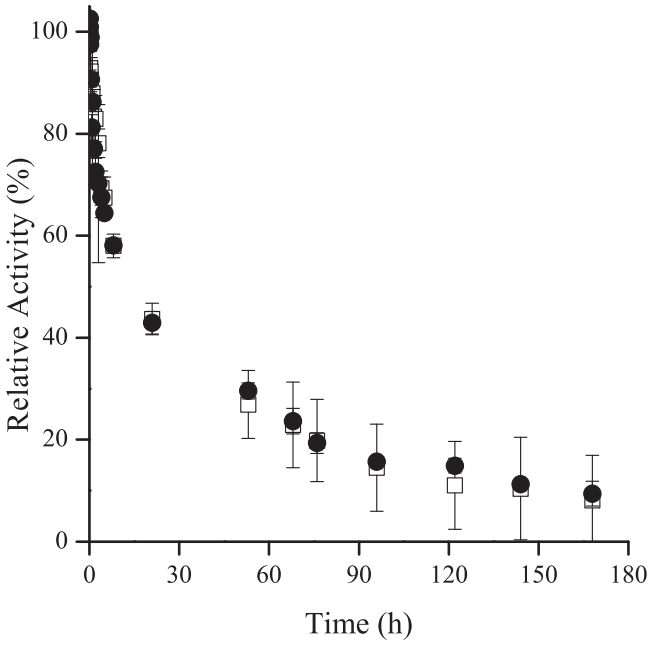

Fig. 5. Influence of temperature on the stability of free (□) and immobilized (●) invertase at 55 °C and pH 4.5.

K_m for the biocatalyst after immobilization increased approximately 3-fold. The catalytic efficiency shows higher value for the free enzyme. Similar results were found for invertase K_m (46 mM and 203 mM, for free and immobilized form, respectively) in a study using a polymeric membrane as support (Mazi, Emregul, Rzaev, & Kibarer, 2006). This considerable reduction in the affinity of the enzyme for the substrate, could be due to the uneven surface of the support, and also attributed to the high concentration of protein that was immobilized, generating diffusion effects. On the other hand, V_{max} did not change, showing the conservation of the maximal conversion of sucrose into its monosaccharides, since the invertase conversion capacity after immobilization was kept.

3.4. Thermal stability

One of the different targets of immobilization is the improvement of the enzyme stability (Garcia-Galan et al., 2011), which is quite dependent of the immobilization technique, besides the enzyme and support characteristics. Considering 55 °C the optimal temperature obtained for free enzyme and immobilized form, and the fact that many immobilization techniques can improve enzyme stability (Mateo et al., 2007), thermal stability of free and immobilized invertase were carried out at optimal conditions (Fig. 5) and at 65 °C. The enzymatic forms were incubated in the absence of sucrose.

At 55 °C free biocatalyst and immobilized derivative showed very similar profile, with no difference between the half-life times before and after immobilization. At 65 °C (data not shown) the half-life times also did not change between free and immobilized invertase, but sharply decreased for few minutes of incubation in relation to the results obtained for optimal temperature. Kumar, Chauhan, and Nahar (2008) also observed significant decrease in immobilized derivative activity at temperatures higher than optimal, and obtained similar result for free invertase at similar conditions, since Gómez, Ramírez, and Villalonga (2000) increased this parameter for immobilized derivative, using an immobilization

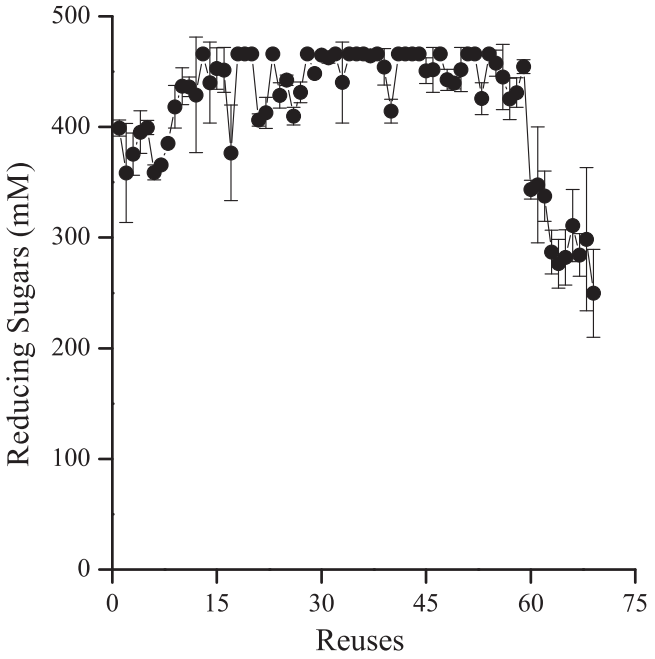

Fig. 6. Residual enzyme activity using invertase immobilized in 0.05 g support in a sucrose solution (20 mL, 8%) during 69 batches (each batch of 30 min, 55 °C, gentle stirring).

protocol that induced enzyme sugar moiety oxidation by sodium periodate. The possible explanation is that nonpolar surface area of proteins indirectly induces the enzyme inactivation in vitro (Turková, 1999), therefore oriented immobilization protocols, which couple the biocatalyst to the support through carbohydrate moiety, can contribute to derivative stabilization.

The results from the present work agreed with previous reports, which showed that one-point attached enzymes to the support, with no diffusion limitations, have similar profiles to free biocatalyst (Garcia-Galan et al., 2011). Although there was not an improvement in the invertase thermal stability, the conservation of whole enzyme activity observed after immobilization is important because of the possibility of reusing the immobilized biocatalyst is dependent upon the residual enzyme activity.

3.5. Storage stability

For practical applications, storage stability of immobilized derivatives is important, the more stable the better. Free and immobilized invertase did not lose their activities after 25 weeks of storage at 8 °C and optimal pH, showing that there were neither biocatalyst modifications nor stabilization by the immobilization process.

3.6. Operational stability

Due to the high cost of enzymes, they should be reused for several times to be competitive with chemical catalysts. Ideally, the immobilization of enzymes should provide, besides thermal and solvent stabilities, a high operational stability (Bayramoglu et al., 2009). The results for repeated uses of the immobilized invertase in the sucrose hydrolysis are presented in Fig. 6.

Fig. 6 shows the expressive number of 69 reuses of the immobilized invertase, keeping the sucrose hydrolysis near to 100% (465.93 mM) until reuse 59. Immobilizing invertase onto nanogels, just remained 11.03% of initial activity at the ninth cycle (Raj, Chauhan, Azmi, Ahn, & Manuel, 2011). The immobilized derivative (hydrogel-clay) was reused for 26 successive batches retaining about 50% of residual activity (Oztop, Hepokur, & Saraydin, 2010). In other study the authors observed the enzyme hydrolysis during 30 cycles keeping at the last one 70% of activity using a mineral as support (Sanjay & Sugunan, 2008).

Probably the decrease in the activity shown at the final batches is a consequence to different factors as the progressive decay in mechanical resistance of the preparation (support-enzyme), inherent wastes by the material transfer, but mainly due to the natural denaturation of the biocatalyst. Nevertheless, since there was not found in literature any work for sucrose hydrolysis by immobilized invertase with similar number of reuses, the results obtained in this work represents the highest operational stability so far described.

4. Conclusions

The immobilization of invertase from *S. cerevisiae* on nano and aggregated chitosan nanoparticles presented high recoveries of activities, with good immobilization yields and efficiencies. After the immobilization the enzyme presented similar parameters of the soluble form (optimal pH, temperature, thermal and storage stabilities). V_{max} did not change, while K_m presented a sharp increase (from 65.7 mM to 205.7 mM), showing the reduction of the affinity of the enzyme for sucrose, yet keeping the invertase maximal conversion capacity. In terms of operational stability, the immobilized preparation could be reused for 59 batches at maximal enzyme activity. These results fulfilled some important requirements for the invertase immobilization because the protocols for support preparation and enzyme immobilization are simple, the enzyme activity and optimal conditions were conserved, and it was obtained the highest operational stability for sucrose hydrolysis so far described in the literature.

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