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Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from *Aspergillus aculeatus*

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ABSTRACT

The enzymatic synthesis of fructooligosaccharides (FOS) was carried out using a partially purified β-fructofuranosidase from the commercial enzyme preparation Viscozyme L. Partial purification of β-fructofuranosidase from Viscozyme L was done by batch adsorption using ion-exchange resin DEAE-Sepharose, showing a 6-fold increase in specific activity. The biocatalyst was then covalently immobilized on glutaraldehyde-activated chitosan particles. Thermal stability of the biocatalyst was evaluated at 50 °C and 60 °C, being around 100 times higher at 60 °C when compared to the free enzyme. The immobilized biocatalyst was reused 50 times for FOS production (100 min per batch at 50 °C and pH 5.5) without significant loss of activity. The average yield (grams of FOS per grams of initial sucrose) was 55%. The immobilization process combined with partial purification method resulted in a derivative with activity of 1230 U_t/g, which is among the best for FOS production.

1. Introduction

Fructooligosaccharides (FOS), also known as oligofructose, are prebiotic food ingredients. This classification refers to its non digestibility, fermentation by intestinal microflora and selective stimulation of intestinal bacteria that contribute to a better health and well-being (Roberfroid, 2007). They can be obtained by inulin hydrolysis, using endoinulinase, or by synthesis, using β-fructofuranosidase (E.C.3.2.1.26, E.C.2.4.1.9). The FOS obtained from sucrose (β-D-fructofuranosyl(2→1)α-d-glucopyranoside) has a shorter chain than those from inulin, having more sweetening power, and may be used as a sweetener by diabetics (Mabel, Sangeetha, Platel, Srinivasan, & Prapulla, 2008).

Although the synthesis of FOS from sucrose is economical because sucrose is cheaper than inulin, the use of enzymes as catalysts for large-scale industrial processes is costly and once they have been made soluble, their recovery for reuse is not economically practical. The immobilization of enzymes allows an easy reuse and other advantages, for example, better stability, continuous operation and reducing overall costs (Liese & Hilterhaus, 2013; Sheldon & Van Pelt, 2013).

Chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan] has been used as a carrier for enzyme immobilization since the 70s (Kasumi, Tsuji, Hayashi, & Tsumura, 1977; Muzzarelli, Barontini, & Rocchetti, 1976). In recent studies, protocols for enzyme immobilization on chitosan were developed, resulting in stable biocatalysts, for β-galactosidase (Klein et al., 2012), invertase (Valerio, Alves, Klein, Rodrigues, & Hertz, 2013) and for chitinase (Seo, Jang, Park, & Jung, 2012). Obtained from chitin [(1→4)-2-acetamido-2-deoxy-β-D-glucan], that is subjected to N-deacetylation followed by purification procedures, the production of chitosan is an economically attractive use for crustacean shells (Krajewska, 2004).

The commercial enzyme preparation from *Aspergillus aculeatus* (Pectinex Ultra SP-L) has been the aim of several studies for FOS production in both free and immobilized form (Csanádi & Sisak, 2006; Ghazi et al., 2007; Tanriseven & Aslan, 2005). Recent study has shown that Viscozyme L, other preparation from *A. aculeatus* has even more β-fructofuranosidase activity than Pectinex Ultra SP-L (Vega-Paulino & Zúniga-Hansen, 2012). It is interesting to note that both enzyme preparations have other key enzyme activities apart from β-fructofuranosidase. Pectinex Ultra SP-L is marketed as polygalacturonase that hydrolyzes (1→4)-α-D-galactosiduronic linkages in pectate and other galacturonans, while Viscozyme L is marketed as endo-β-glucanase that hydrolyzes (1→3)- or (1→4)-linkages in β-D-glucans.

Thus, the aim of this work is to prepare a highly stable and active derivative of β-fructofuranosidase from *A. aculeatus* on chitosan particles for FOS synthesis. Firstly, the commercial

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preparation Viscozyme L was partially purified to improve the β-fructofuranosidase activity, and the immobilized preparation was evaluated for optimal pH and temperature, thermal stability, and operational stability in FOS synthesis.

2. Materials and methods

2.1. Materials

Viscozyme L, a commercial enzyme preparation from *A. aculeatus*, produced by Novozymes, was kindly donated by LNF Latino Americana. Centrifugal Filter Unit (30 kDa) was acquired from Millipore. DEAE-Sepharose fast flow® was acquired from Sigma-Aldrich. Sucrose was acquired from Vetec Química Fina Ltda (Brazil). Enzymatic glucose (D-glucose) determination kit was from In Vitro Diagnóstica Ltda (Brazil) and glutaraldehyde 25% was from Nuclear (Brazil). The high liquid chromatography (HPLC) column used in these tests was an Aminex® HPX-87C. All other chemicals were analytical or HPLC grade obtained from readily available commercial sources.

2.2. Enzyme activity

The enzymatic activity assay was carried out using a substrate solution of 100 g/L of sucrose in 50 mM sodium acetate buffer, pH 5.5 at 50 °C. Samples were taken at regular intervals and the reaction was stopped by the addition of 0.1 M sodium carbonate buffer, pH 10.0. Glucose release was measured with the enzymatic glucose determination kit. One unit of enzymatic activity measured (U) was defined as the amount of enzyme that releases 1 μmol of glucose from sucrose per minute at test conditions. All the activities of immobilized enzymes were carried out under agitation.

2.3. Enzyme purification

Viscozyme L was diluted in 20 mM sodium phosphate buffer (pH 5.8) to a final protein concentration of 20 mg/mL and then applied to flasks containing DEAE-Sepharose previously equilibrated with the same buffer. Desorption was carried out washing the resin with the same buffer containing increasing concentrations of sodium chloride. Most of enzyme was desorbed at 0.4 M of sodium chloride solution. After desorption, protein solution was filtrated using a centrifugal filter (30 kDa; 4000 × *g*; 40 min; 4 °C) to concentrate the β-fructofuranosidase and to remove the salt.

2.4. Preparation and activation of chitosan particles

The chitosan particles were prepared using the protocol described by Klein et al. (2012). Briefly, a chitosan solution was added dropwise into an alkaline coagulation solution, and the activation was performed by incubating the chitosan particles obtained with a glutaraldehyde solution (4%, v/v) prepared in 0.1 M phosphate-sodium buffer (pH 7.0), at room temperature for 3 h in an orbital shaker at 120 rpm with 12 mm of orbital diameter. The activated support was exhaustively washed with 50 mM sodium acetate buffer (pH 5.5) to remove the excess glutaraldehyde.

2.5. Enzyme immobilization

Chitosan particles were incubated with partially purified enzyme solution previously diluted in 50 mM of sodium acetate buffer (pH 5.5), for 3 h at room temperature and under gentle shaking. The immobilized enzyme was washed with, buffer, NaCl (1.0 M) and ethylene glycol (30%, v/v) to eliminate unbounded and ionic and hydrophobic bounded enzymes.

The immobilization yield (IY) and immobilization efficiency (EF) were calculated by the same equations previously described by Valerio et al. (2013).

2.6. Thermal stability

Soluble and immobilized enzymes were incubated at 50 °C and 60 °C for up to 9 days. Samples were withdrawn at intervals, placed in ice bath to stop thermal inactivation and enzyme activity was then measured.

In general, thermal inactivation of enzymes can be described by a first order reaction,

$$\frac{A}{A_0} = \exp(-kt) \quad (1)$$

where A is the enzyme activity at time t, A_0 the initial enzyme activity, t is the treatment time, and k is the constant inactivation rate at the studied temperature. In this work, k was determined by fitting the first order model to the data of the residual enzyme activity plot (A/A_0) versus time (min) using nonlinear regression (Statistica 7.0, StatSoft, Inc., Tulsa, OK, USA).

The enzyme half-life ($t_{1/2}$) represents the time required for the enzyme to decay to 50% of its initial value of activity and was calculated from the values of k, following this equation:

$$t_{1/2} = \frac{\ln(2)}{k} \quad (2)$$

2.7. Determination of optimal pH and temperature

The optimal pH and temperature were assayed for both partially purified (soluble) and immobilized enzymes. The optimal pH tests ranged from 3 to 8, maintaining the temperature constant at 50 °C and the optimal temperature was assayed from 42 °C to 80 °C maintaining pH at 5.5. The buffers (50 mM) used were glycine–HCl pH 3.0, sodium acetate pH 4.0, 5.0 and 5.5, and sodium phosphate pH 6.0, 6.5, 7.0, 7.5 and 8.0.

2.8. Fructooligosaccharides production

The FOS production was carried out at 50 °C using a solution of sucrose 600 g/L in 50 mM sodium acetate buffer pH 5.5. Fifty chitosan particles (22 U) were incubated in 2 mL of sucrose solution for 160 min. Samples were taken at 15 min intervals, for kinetic evaluation.

Reactions were carried out for 100 min at the same conditions, for operational stability evaluation. The particles were washed between batches with 50 mM sodium acetate buffer pH 5.5 to remove any trace of product. Samples were taken after each batch.

The samples were assayed for reducing sugars by the DNS method (Miller, 1959) and enzymatically for glucose. Fructose (D-fructose) was calculated as the difference between the concentrations of reducing sugars and glucose, FOS molar concentration was calculated as difference between the molar concentrations of glucose and fructose.

The samples were also analyzed by an HPLC system (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C (300 mm × 7.8 mm) column. Ultrapure water was used as eluting solvent at a flow rate of 0.6 mL min^{-1}, at 85 °C. The concentration of saccharides [sucrose, glucose, fructose, kestose (β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1)α-D-glucopyranoside) and nystose (β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1)α-D-glucopyranoside) was determined by interpolation using external standards. HPLC analysis was carried out to confirm the assessment method and for direct

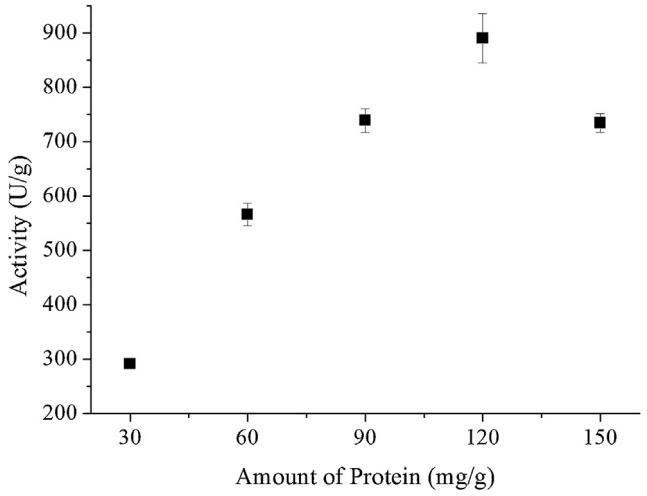

Fig. 1. Activity (■) measured on the immobilized biocatalyst for each amount of protein tested on immobilization.

measurement of transfructosylation activity of the immobilized biocatalyst. Transfructosylation activity (U_t) was defined as the amount of enzyme that produces 1 μmol of kestose or nystose from sucrose per minute.

3. Results and discussion

3.1. Enzyme partial purification

Crude Viscozyme L has a β-fructofuranosidase specific activity of 4.2 U/mg and volumetric activity of 500 U/mL, at 50 °C and pH 5.5. The purification process resulted in an enzyme solution with a specific activity of 25.0 U/mg resulting in a purification fold of 6.0 with an activity recovery of 60%. After centrifugal filtration, the enzyme was concentrated to over 330 U/mL.

This purification is only partial, and can be improved as shown by Ghazi et al. (2007), that purified a β-fructofuranosidase from *A. aculeatus* (Pectinex Ultra SP-L) and obtained a purification fold of 107, obtaining a specific activity of 2635 U/mg, which is 105 times higher than the activity obtained in our work. On the other hand, the activity recovery was only 36.8% after four purification steps, which is smaller than 60% obtained in our work by one-step purification. For the purification of Pectinex Ultra SP-L, a two step process resulted in a purification fold of 7, recovering 22.3% of initial activity (Nemukula, Mutanda, Wilhelmi, & Whiteley, 2009). The increase in enzyme purity can improve the immobilization conditions. However, enzyme isolation and purification can be expensive and time consuming (de Carvalho, 2011), and the enzyme immobilization should be a relatively simple operation not requiring a highly pure enzyme preparation that not always is commercially available (Sheldon & Van Pelt, 2013).

3.2. Enzyme immobilization

The results of the different enzyme load applied to the support are shown in Fig. 1. The best biocatalyst activity was obtained with an amount of 120 mg of enzyme per gram of dry support. This amount resulted in an immobilization yield of 90% and immobilization efficiency of 33%. The biocatalyst obtained has shown a hydrolytic activity of 880 U per gram of dry support, equivalent to 0.44 U per chitosan particle.

As reported for other authors, the low immobilization efficiency could be due to linkages with groups related to active site and those responsible for maintaining the tertiary structure of the enzyme

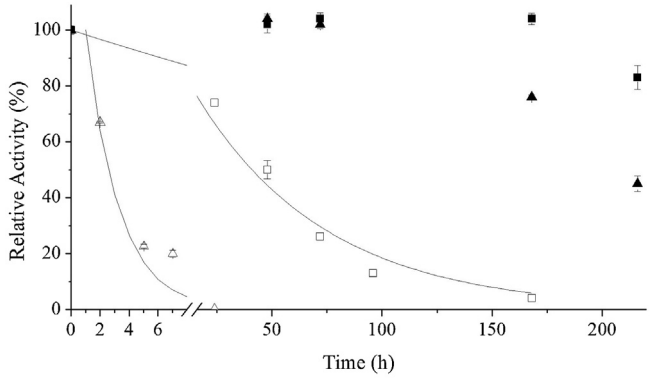

Fig. 2. Relative activity of the free enzyme at 50 °C (□), immobilized enzyme at 50 °C (■), free enzyme at 60 °C (△) and immobilized enzyme at 60 °C (▲).

(Tanriseven & Aslan, 2005). One possible solution, is the immobilization in the presence of substrate that can protect the active site of the enzyme, improving efficiency (Cadena et al., 2010). In this work, the presence of sucrose at 100 g/L and 600 g/L in the immobilization solution did not improve the immobilization efficiency, as also reported by Valerio et al. (2013) for the β-fructofuranosidase from *S. saccharomyces*. Moreover, other reports showed similar behavior for different enzymes (Csanádi & Sisak, 2006; Ghazi et al., 2005; Platková, Polakovič, Štefuca, Vandáková, & Antošová, 2006; Tanriseven & Aslan, 2005).

3.3. Thermal stability

The kinetic inactivation for both free and immobilized β-fructofuranosidase is shown in the Fig. 2 where the symbols refer to the average experimental values, while lines correspond to fitting Eq. (1) for the experimental data. The inactivation rate constant (k) and the half-life ($t_{1/2}$) for the free enzyme at 50 °C and 60 °C were given in Table 1 with the determination coefficient r^2.

It was not possible to quantify the half-life of the immobilized enzymes, because after 200 h the prepared biocatalyst presented 83% of its initial activity at 50 °C, and 50% at 60 °C, as depicted in the Fig. 2. According to Gardossi et al. (2010) the experiment must be carried out until 10% of residual activity to allow clear distinction of kinetic inactivation model and proper half-life determination.

Due to the high thermal stability, 10% of residual activity would be reached only after longer times. Fig. 2 clearly shows that the immobilized enzyme is much more stable than the free enzyme at both temperatures, proving that the immobilization onto chitosan particles has a positive effect in the thermal stability of the enzyme.

An increase in the number of enzyme-supports bonds was suggested as the most likely explanation for the stabilization achieved in the immobilized enzymes (Pedroche et al., 2007). However, when using supports at non-alkaline pH values, the immobilization occurs only via the amino terminal group (Mateo et al., 2005). Since in this work the enzyme is a dimeric glycoprotein (Ghazi et al., 2007), such high stability could be due to chitosan attachment of amino terminal group of both monomers of the enzyme, and then stabilizing the enzyme quaternary structure.

This high thermal stability provides several advantages for the biocatalyst, such as: the sucrose is easier solubilized at higher

Table 1
Kinetic parameters of thermal inactivation of free β-fructofuranosidase at 50 and 60 °C.

Biocatalyst	k (min^{-1})	$t_{1/2}$ (h)	r^2
Free β-fructofuranosidase at 50 °C	0.0169	41.01	0.98
Free β-fructofuranosidase at 60 °C	0.2221	3.12	0.9627

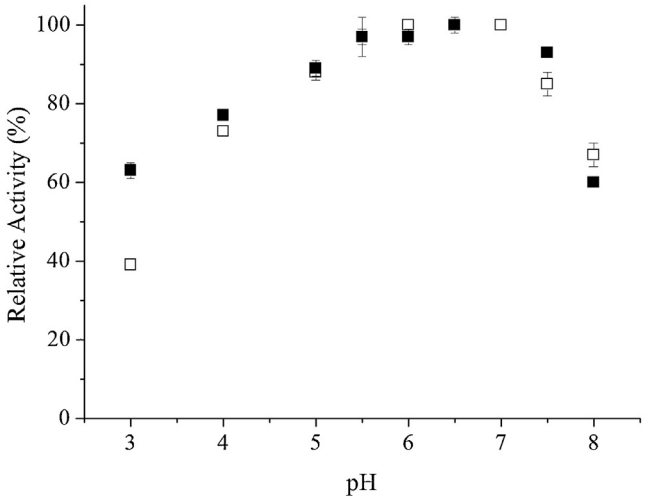

Fig. 3. Effect of pH on the free (□) and immobilized (■) enzyme at 50 °C.

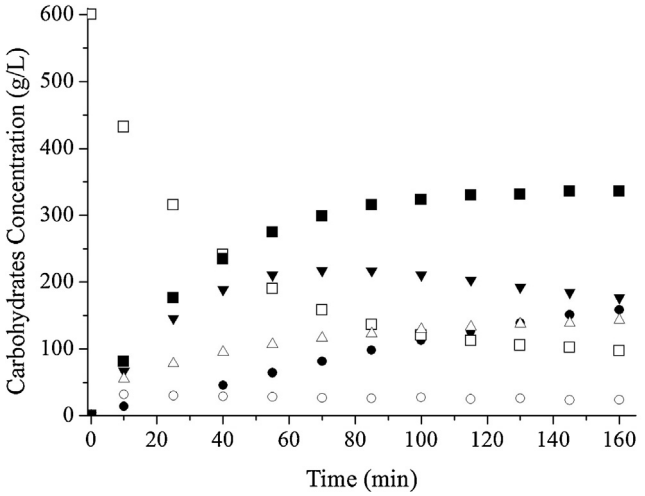

Fig. 5. Kinetic evaluation of carbohydrates: nystose (●), kestose (▼), sucrose (□), glucose (△), fructose (○), total FOS (■), during reaction with a new biocatalyst.

temperatures (Young & Jones, 1949); microbial growth of pathogenic microorganisms such as *Staphylococcus aureus* (Schmitt, Schuler-Schmid, & Schmidt-Lorenz, 1990), *Escherichia coli* and *Salmonella enterica* (Bronikowski, Bennett, & Lenski, 2001) is avoided at 50 °C; in addition, thermal stability of enzymes can limit the long term application of the biocatalyst in the process (Liese & Hilterhaus, 2013).

3.4. Determination of optimal pH and temperature

The results of optimal pH and temperature are shown in Figs. 3 and 4, respectively. It was not observed changes in the optimum pH comparing immobilized and free enzyme. However, the immobilized enzyme is slightly more active at lower pH values (pH 3.0). Sometimes the support may be an ionic exchanger, and may behave as a buffer, generating a pH inside the biocatalyst bead that may greatly differ from the pH value in the reaction medium (Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013). However, this explanation could be discarded since chitosan is a polyaminosaccharide without ionic strength and the enzyme molecules probably get immobilized mostly in their outer surfaces (Klein et al., 2012). This wider pH range may be probably due to the stabilization of enzyme 3D structure by the attachment on the support.

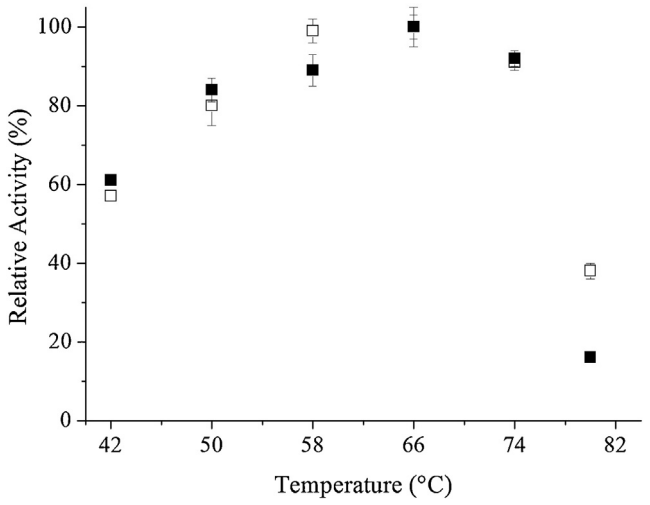

Fig. 4. Effect of the temperature on free (□) and immobilized (■) enzyme at pH 5.5.

The optimum temperature for free enzyme was between 58 °C and 66 °C, presenting 100% of relative activity on both temperatures. Nemukula et al. (2009) reported an optimal temperature of 60 °C in a reaction of 40 minutes; on the other hand, Tanriseven and Aslan (2005), reported an optimal temperature of 65 °C in a 1 min reaction. These differences of optimal temperatures found for the same enzyme can be explained by different reaction times. The optimal temperature measured will be higher for shorter reaction times since thermal inactivation occurs during enzymatic reaction.

As can be seen on Fig. 4, the immobilized enzyme showed a higher relative activity at 66 °C than at 58 °C. This result could be probably attributed to the higher thermal stability of the immobilized enzyme compared to free enzyme. The thermal stability is a key factor for studying the operational temperature. The enzyme is more active at 66 °C, however, is more stable at 50 °C. Then, for long-term use, it was selected 50 °C for the experiments of FOS production.

3.5. Fructooligosaccharides production

Fig. 5 shows the kinetics of FOS production from sucrose catalyzed by the chitosan-immobilized enzyme. The maximum yield of 55% of FOS (including kestose and nystose) was achieved at 100 min. It can be also noted that after 100 min, the concentrations of kestose decrease and nystose increase while the sum of them remained constant. This can be due to the fact that kestose is used as a substrate to produce nystose (Jung, Yun, Kang, Lim, & Lee, 1989). The stabilization on FOS overall concentration, and reduced rates of sucrose transfructosylation observed at the end of reaction can be due to the fact that glucose is a competitive inhibitor of the transfructosylating reaction of sucrose and kestose (Alvarado-Huallanco & Maugeri-Filho, 2010). The glucose concentration increased constantly and reached to 145 g/L after 160 min of reaction.

Fig. 6 shows the operational stability of the biocatalyst. It can be noted that the yield of FOS varied from 40% to 65% and no significant losses of activity were observed over the batches. After 50 uses, a new complete kinetic was performed, where samples were taken at 15 min intervals, as for the first batch, and carbohydrates concentration were assayed on HPLC. The comparison between those batches showed similar concentrations for FOS and other sugars, and only small differences were observed, which means that there was not activity change or loss after 50 reuses of the biocatalyst.

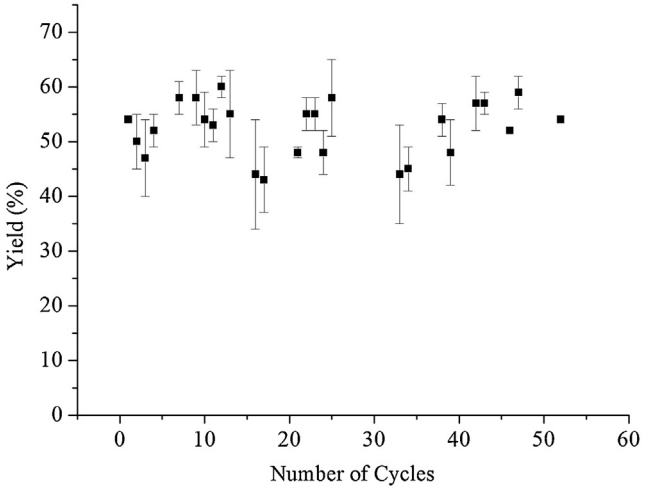

Fig. 6. Operational stability of β-fructofuranosidase immobilized on chitosan particles in the FOS synthesis.

The transfructosylation activity was 0.61U_t per chitosan particle and 1230U_t per g of dry support. An immobilized biocatalyst presenting a transfructosylation activity of 1230U_t/g of dry support is the best found in literature so far. Tanriseven and Aslan (2005) obtained a biocatalyst with transfructosylation activity of 129U_t/g, in a preparation using Eupergit C, an epoxy-activated acrylic bead, as support. Csanádi and Sisak (2006) obtained an immobilized biocatalyst with 14.8 U_t/g on Amberlite IRA900 Cl, an anion exchange resin. Recently, Fernandez-Arrojo et al. (2013) obtained a biocatalyst with 40.7 U/mL using a calcium alginate bead produced from sodium alginate SG300, which has similar properties than chitosan particles. A possible explanation for our high transfructosylation activity is that the enzyme initially used for immobilization had higher specific activity than the other works, since it was partially purified.

4. Conclusions

The immobilization of β-fructofuranosidase on glutaraldehyde-activated chitosan by covalent attachment produced a high active and stable biocatalyst, with a good immobilization yield and efficiency. Combined with the partial purification method, the immobilization process resulted in a derivative with activity of 1230U_t/g, which is among the best found in literature for FOS production. The biocatalyst also showed a high operational stability allowing its use for at least 50 times without significant losses of activity. The obtained biocatalyst presents great potential for industrial production of fructooligosaccharides from sucrose.

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