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# Characterization of cyclodextrin glycosyltransferase immobilized on silica microspheres via aminopropyltrimethoxysilane as a "spacer arm"

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ABSTRACT

In this work, the enzymatic properties of immobilized cyclodextrin glycosyltransferase (CGTase) of *Thermoanaerobacter* sp. were investigated and compared with the soluble form of the enzyme. CGTase was immobilized on mesoporous silica microspheres synthesized using polyethylene glycol 400 as swelling agent, silanized with 3-aminopropyltrimethoxysilane (APTMS), and activated with glutaraldehyde prior to immobilization. This innovative approach for support preparation produced high yields of immobilization (83%) and activity recoveries (73%), which are the highest so far reported for CGTase. The soluble enzyme (CGTase) and its immobilized form (ImCGTase) showed similar values for the optimal pH activity, while optimal reaction temperatures were found to be 100 °C and 80 °C, respectively. The immobilized enzyme showed similar values for $K_m$ and thermal stabilities with the soluble form, while its $V_{max}$ was lower. The immobilized enzyme was tested in repeated batches in order to simulate recovery and reuse, keeping about 60% of the initial catalytic activity after 15 cycles, showing its good chemical and mechanical resistance.



## 1. Introduction

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a microbial enzyme capable to convert starch and related polymers into cyclodextrins (CDs) via cyclization reactions. Cyclodextrins have the ability of encapsulating hydrophobic molecules into their internal cavity, promoting the stabilization, solubilization, and the masking of odors and tastes of a wide variety of important compounds that are used in foods, pharmaceuticals, cosmetics, agricultural products and chemicals [1–10].

The industrial production of CDs has been chiefly carried out in batch systems, with soluble CGTase used directly into the reaction mixture. The use of immobilized CGTase has been proposed as a technical alternative to allow for its continuous and repeated use, avoiding enzyme solubilization and improving the stability of the biocatalyst [11]. However, it has been reported that activity recoveries of immobilized CGTases are usually very low due to diffusional resistances imposed by the structure of the macromolecular substrates, such as starches and dextrins [11–13]. To overcome these problems, the addition of spacers, or linkers, has been investigated to produce more flexible spatial arrangements of the biomolecules, providing higher mobility of the resulting immobilized enzyme, while enhancing the interaction between the enzyme and the macromolecular substrate [14,15].

A wide variety of materials have been used as immobilization supports for CGTase such as Eupergit C [7], commercial chitosan [16,17], glyoxyl-agarose [18], controlled pore size silica [16,19], among others. In particular, silica supports have been shown to be excellent materials for enzyme immobilization due to their excellent biocompatibility, rigidity, mechanical and operational stability, high thermal resistance, and nontoxicity [20–22]. It also shows remarkable resistance to microbial degradation and to solvent distortion [23]. Surface modification via functional groups provides the opportunity to change the interfacial properties, while retaining their basic geometry and mechanical strength of the solid matrix in cyclic reuse [21].

Several enzymes have been immobilized in a variety of silica matrices using different methods. In this study, it is demonstrated the use of an emulsion for preparing ordered silica microspheres with the PEG polymer as the swelling agent [24]. Chemical attachment of *Thermoanaerobacter* sp. CGTase by covalent coupling onto silica microspheres silanized with 3-aminopropyltrimethoxysilane (APTMS) was the chosen mechanism because it can generate a highly stable system [25–27]. In this system, a solution of APTMS

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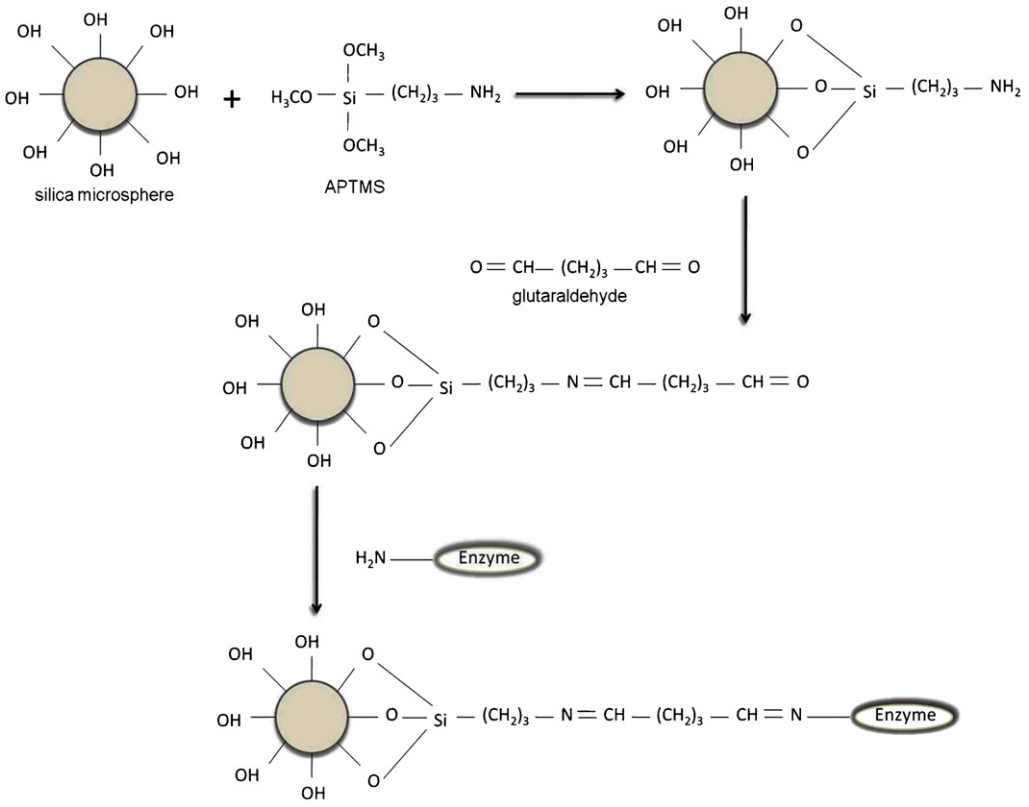


**Fig. 1.** Schematic diagram of the process for the preparation of CGTase immobilization on silica.

reacts with hydroxyl groups or silanol supports (the "silanization" process), introducing a spacer arm between the enzyme molecule and the support. This arrangement facilitates the access of the immobilized enzyme to its reactional substrate [28]. The effects of this immobilization process on pH reaction and temperature, the kinetic parameters, and the stability of ImCGTase were also investigated and compared with the soluble form of the enzyme.

## 2. Materials and methods

### 2.1. Enzyme and chemicals

CGTase from *Thermoanaerobacter* sp. (Toruzyme 0.2L) was kindly provided by Novozymes A/S (Bagsvaerd, Denmark). β-CD was purchased from Sigma–Aldrich (St. Louis, USA). All other chemicals were of analytical grade and purchased either from Fluka or Merck (São Paulo, Brazil).

### 2.2. Assay of β-CD forming activity

CGTase cyclization activity was determined using the phenolphthalein assay [29] with modifications as described by Pinto et al. [30], based on the ability of the formed cyclodextrin to form inclusion complexes with phenolphthalein. The reaction mixture, containing 26 mg of soluble starch in 0.65 mL of appropriate buffer, and 0.35 mL of commercial enzyme, was incubated at appropriated temperature for 15 min. The reaction was stopped with the addition of 4 mL of 0.04 mM phenolphthalein dissolved in 125 mM $Na_2CO_3$ solution. The absorbance was then measured at 550 nm. One unit of CGTase activity was defined as the amount of enzyme to produce 1 μmol of β-CD/min. The measurement of immobilized CGTase activity (ImCGTase) was performed using the reaction supernatant after centrifugation at 3500 × *g* for 15 min at 4 °C.

### 2.3. Synthesis and functionalization of silica–polyethylene glycol microspheres

The silica microspheres were prepared according to Yang et al. [22] with the following modifications. One aliquot of 5 mL of tetraethylorthosilicate (TEOS) was mixed with water at a molar ratio of 0.015:1 and acidified with HCl; the pre-hydrolysis of TEOS was then obtained by adding 0.5 mL polyethylene glycol 400 (PEG-400) to this mixture under vigorous stirring. When the emulsification was complete, 0.5 mL of the dicyclohexylamine catalyst was added in order to start the polycondensation reactions. The microspheres were produced in the form of hydrogel, which resulted in solid spheres by dehydration. After aging and drying for 15 days at room temperature, the hydrogel beads were successively washed with water and ethanol.

The silica microspheres were functionalized with the addition of 1 mL of aminopropyltrimethoxysilane (APTMS)/g of microspheres under reflux in toluene for 24 h. The resulting product was washed several times with toluene, ethanol, ether, and water, and finally dried under vacuum at 60 °C for 4 h.

The pore size distribution was estimated using the BJH method [31] and the specific surface area was determined by the BET multipoint technique [32] using adsorption of $N_2$ at −196 °C. Images of the support were taken by scanning electron microscopy (SEM; JEOL, model JSM 6060, Japan).

### 2.4. Immobilization on silica

Functionalized silica (0.5 g) was immersed in a solution of 0.5 mL of glutaraldehyde (volume fraction 25%) and 4.5 mL of 20 mM sodium phosphate buffer (pH 7.0); the mixture was stirred for 1 h at room temperature. The activated support was extensively washed with the same buffer and immediately incubated under agitation in an orbital shaker (200 rpm), added of 10 mL of CGTase solution

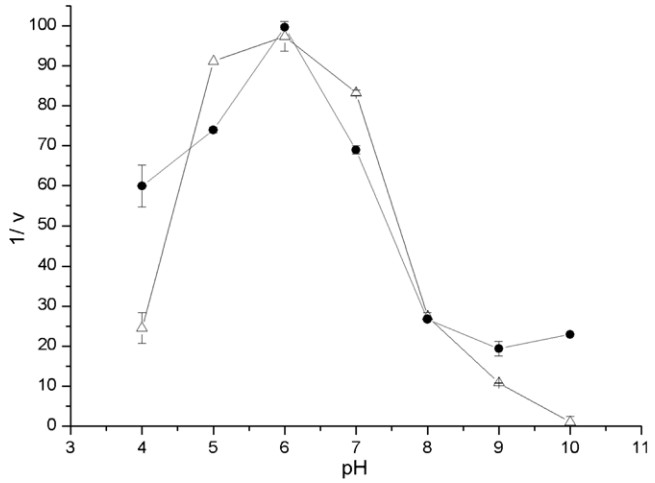


**Fig. 2.** CGTase relative activity as a function of pH (buffers 0.1 M sodium acetate for pH 4 and 5; 0.1 M sodium phosphate for pH 6, 7, and 8; and 0.1 M glycine–NaOH for pH 9 and 10) at 60 °C. (●) CGTase, soluble enzyme. (△) ImCGTase, immobilized. Results are the mean of triplicates.

(approximately 200 U/mL in 0.1 M sodium phosphate buffer, pH 6.0) at 8 °C for 15 h. The schematic representation of the immobilization process is shown in Fig. 1.

After immobilization, successive washings with buffer were used to remove the excess of enzyme until no activity could be detected in the washed fractions. The immobilized enzyme system was also washed with 1 M NaCl and ethylene glycol (volume fraction 30%) in order to eliminate any enzyme molecule non-covalently attached to the support.

### 2.5. Effect of pH and temperature on the CGTase activity

The effects of immobilization on the enzyme activities were measured for the range of pH varying from 4.0 to 10.0 at 60 °C, using either sodium acetate buffer 0.1 M (pH 4 and 5), sodium phosphate buffer 0.1 M (pH 6, 7 and 8), or glycine–NaOH buffer (pH 9 and 10). The effects of temperature on CGTase activities were measured in the presence of sodium phosphate buffer 0.1 M, pH 6.0, in the range varying from 50 to 120 °C. Reactions were carried out using the CGTase assay procedure described in Section 2.2.

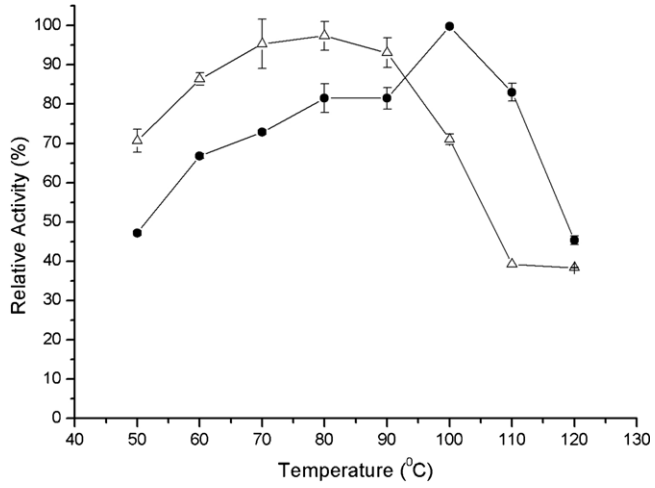


**Fig. 3.** CGTase activity as a function of temperature (50–120 °C) using 0.1 M sodium phosphate buffer pH 6. (●) CGTase, soluble enzyme. (△) ImCGTase, immobilized. Results are the mean of triplicates.

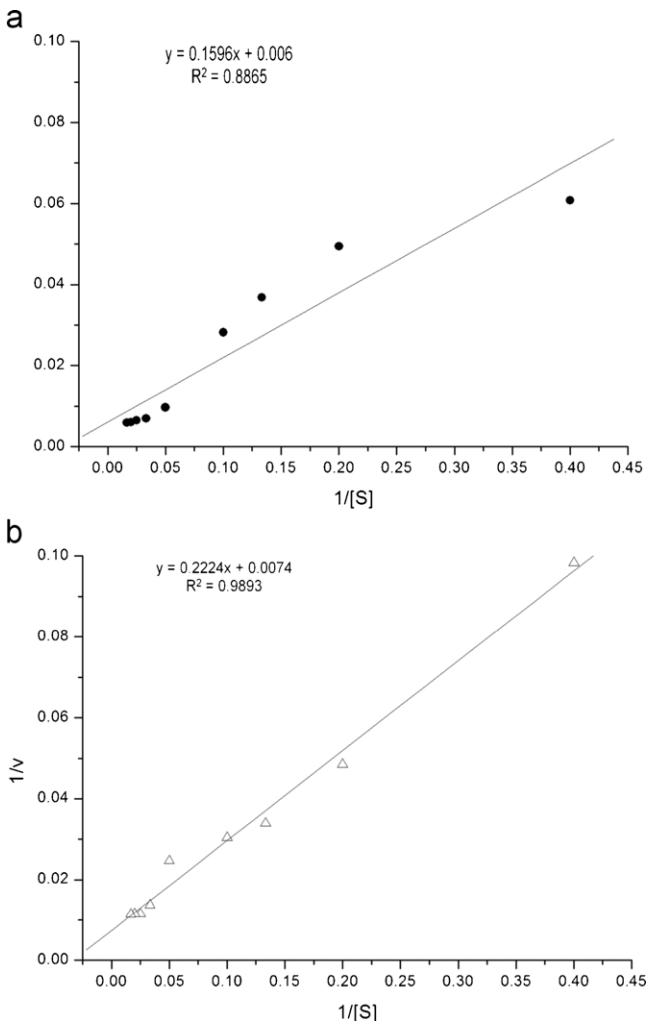


**Fig. 4.** Lineweaver–Burk plots of CGTase, soluble enzyme (A) and ImCGTase, immobilized (B). Results are the mean of triplicates.

### 2.6. Kinetic parameters of the soluble and immobilized CGTases

The kinetic parameters were determined by incubating the diluted enzyme (approximately 200 U/mL) with 2.5–60 mg/mL

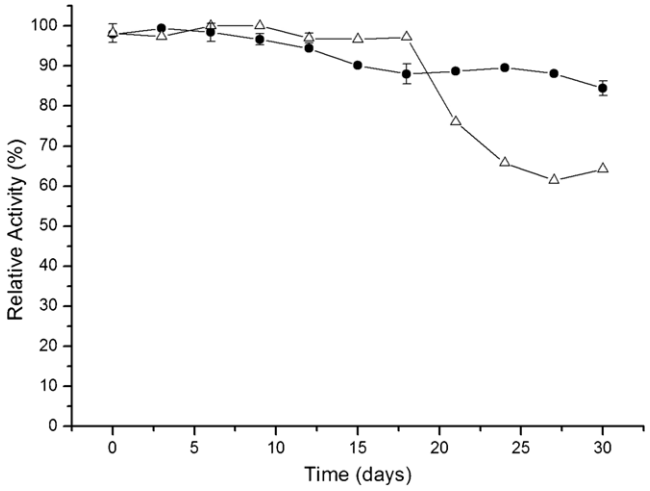


**Fig. 5.** Storage stability in fridge at 8 °C for 30 days. (●) CGTase, soluble enzyme. (△) ImCGTase, immobilized. Results are the mean of duplicates.

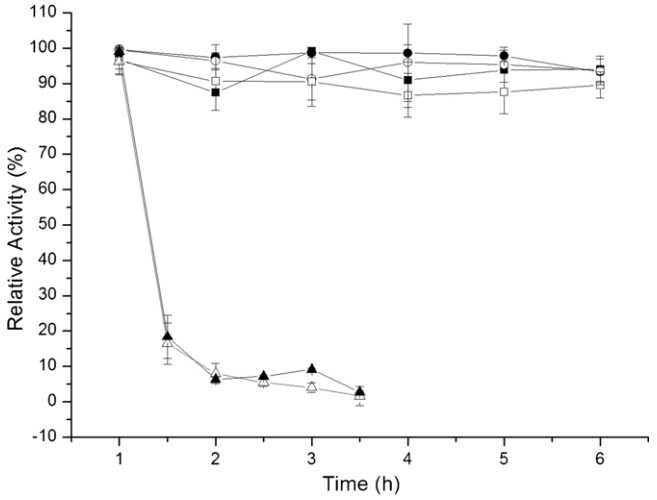


**Fig. 6.** Thermal stability at different temperatures. (●) CGTase at 60 °C. (○) ImCGTase at 60 °C. (■) CGTase at 80 °C. (□) ImCGTase at 80 °C. (▲) CGTase at 100 °C. (△) ImCGTase at 100 °C. Results are the mean of duplicates.

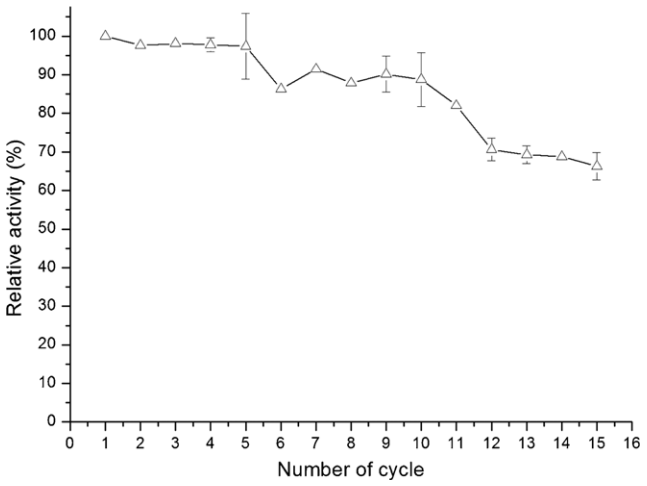


**Fig. 7.** Relative activities for the cyclization of starch with multiple uses. Cyclization reaction was performed in sodium phosphate buffer solution (0.1 M, pH 6) for 15 min at 80 °C and 200 rpm rotation. Results are the mean of triplicates.

solutions of soluble starch in 0.1 M sodium phosphate pH 6 at the optimal temperature for 15 min. The values of $K_m$ and $V_{max}$ were then determined using the Lineweaver–Burk plot.

### 2.7. Storage and thermal stability of immobilized CGTase

Immobilized and soluble enzymes were stored in the fridge at 8 °C for 30 days. The remaining enzyme activity was measured with phenolphthalein at optimal pH and temperature for 15 min as described above. Thermal inactivation assays were performed at 60, 80, and 100 °C in 0.1 M sodium phosphate pH 6. Aliquots were periodically taken and the activity was measured as described in Section 2.2.

### 2.8. Operational stability of the immobilized CGTase

For the reuse tests of the ImCGTase, the immobilized enzyme system was incubated under the optimal conditions (80 °C, pH 6) for CD production. At the end of each cycle, the immobilized enzyme was separated from the reactional product by centrifugation at 3500 × *g* for 15 min at 4 °C and the activity was measured in the supernatant. The ImCGTase was thoroughly washed with 0.1 M sodium phosphate (pH 6) and finally resuspended in a freshly prepared substrate solution to restart a new cycle.

## 3. Results and discussion

### 3.1. Immobilization of CGTase on silica microspheres and support characterization

Analysis of silica microspheres showed that the specific surface area of the support was 185.04 $m^2/g$ and the pore volume was 0.08 $cm^3/g$, with mesoporous structure (pore diameter 4.6 nm) being in accordance with the standards recommended by the International Union of Pure and Applied Chemistry.

The activated surface of these mesoporous microspheres was effective for enzyme immobilization. It can be observed that the yield of immobilization reached 83% and the recovery of activity was 73%. Table 1 shows the measured parameters for immobilization of CGTase on silica microspheres support. These results clearly show the high efficiency of silica microspheres immobilization system for the CGTase of *Thermoanaerobacter* sp. Compared with eupergit C and glyoxyl-agarose immobilized systems, the recoveries of activities obtained in this work were approximately seven and two-fold higher, respectively [7,18], being the highest ever reported for ImCGTase.

### 3.2. Effects of pH and temperature on the activities of CGTase and ImCGTase

Fig. 2 presents the results for the enzyme activities as a function of pH. Results show that at 60 °C, the optimal pH was 6.0, with no differences found for soluble and immobilized CGTases. The activities of soluble CGTases were kept elevated in acid pH, as it has been reported by other authors [11].

The optimal temperatures for CGTase and ImCGTase were evaluated in the range varying from 50 to 120 °C at the optimal pH 6 for the catalytic activity. The highest activity for the soluble enzyme was achieved at 100 °C, suggesting its excellent thermostability. On the other hand, the ImCGTase presented best activities between 70 and 90 °C, remaining constant in this range, as shown in Fig. 3. These high reactional temperatures are very important for the possible industrial applications of ImCGTase. Zhou et al. [33] showed that the optimal temperature for Toruzyme of *Thermoanaerobacter* sp. was 100 °C, while Unsworth et al. [34], reported that several thermophilic enzymes, such as α-amylases from *Pyrococcus furiosus* and *Methanococcus jannaschii*, have optimal reaction temperatures between 106 and 120 °C, respectively.

Conformational changes in the enzyme molecule could explain this change in the best temperature for optimal activity when soluble and immobilized CGTase are compared. Small decreases in the optimal temperature have also been reported for *Thermoanaerobacter* sp. ImCGTase by other authors [7]. Nevertheless, at this range of temperatures, ImCGTase could be used in industrial processes ensuring that no microbial contamination would take place. Moreover, at 80 °C, the solubility of substrates and products are high, possibly increasing productivity and yields, while reducing the costs of industrial applications.

### 3.3. Kinetic parameters

The kinetic parameters calculated using the Lineweaver–Burk plots are listed in Table 1 and shown in Fig. 4. The lower $V_{max}$ values for the immobilized enzyme might be due to the conformational changes caused by the fixation of the enzyme to the support. Alternatively, it might reflect the constraints for the substrate to access the active site of the enzyme [35]. Similar changes in $V_{max}$

**Table 1**
Parameters obtained for the CGTase immobilized onto silica microspheres compared with the soluble enzyme.

| | Offered activity (U/mL) | Non-linked activity (U/mL) | Immobilized yield (%) | Immobilized enzyme activity (U/mL) | Activity recovery (%) | $K_m$ (mg/mL) | $V_{max}$ (U/mL) |
|---|---|---|---|---|---|---|---|
| ImCGTase | 192 | 32 | 83 | 117 | 73 | 27 | 125 |
| CGTase | – | – | – | – | – | 27 | 167 |

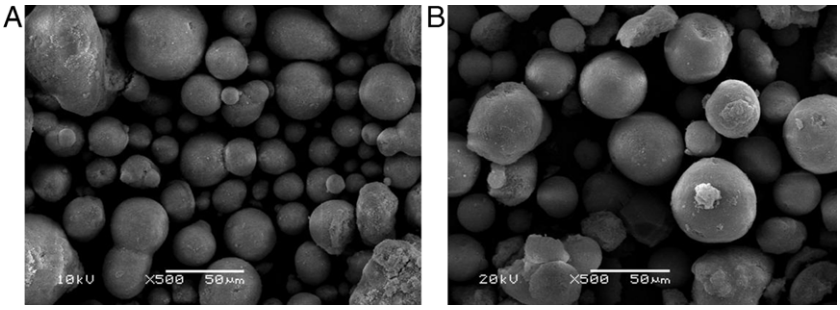


**Fig. 8.** Scanning electron micrograph (SEM). (A) Silica microspheres before activation with glutaraldehyde (10 kV; 500×); (B) silica microspheres after 15 batches (20 kV; 500×).

were observed for other immobilization systems using PVC [12] and alumina [35] as supports, with CGTases of different microbial sources.

### 3.4. Storage and thermal stabilities

The storage and thermal stabilities of immobilized enzymes are important parameters concerning their possible industrial applications [35]. Fig. 5 shows the results for the storage stabilities of the soluble and immobilized CGTases. The activity of ImCGTase was practically unchanged for the first 18 days of storage. However, after 30 days in the refrigerator, the remaining ImCGTase activity was 60%, lower than that of the soluble enzyme, which was 85% after this time.

Concerning the thermal stability, shown in Fig. 6, the ImCGTase presented practically the same characteristics of the soluble enzyme at 60 and 80 °C, with activities kept above 90% after 5 h of incubation. However, at 100 °C a fast decrease was observed, with 85% of activity loss in 0.5 h for both free and immobilized enzymes. Nevertheless, the immobilized system used in this work performs better than other immobilized CGTases. For instance, Amud et al. [11] reported that the sol-gel-encapsulated CGTase from *Thermoanaerobacter* sp., incubated at 60 °C and pH 6.0 (citrate buffer), was almost completely inactivated after 3 h of reaction. The same enzyme, when immobilized on Eupergit C, showed good thermostabilities at temperatures of 60 and 85 °C, but retained only 40% of its relative activity after 1 h of incubation at 95 °C [7].

### 3.5. Stability of ImCGTase over repeated batches processes

It is highly desirable from the industrial point of view that an enzymatic system could be reused several times without losing its activity, thus reducing process costs. The stability of the immobilized CGTase over repeated batches was evaluated in a 15 cycles process and the results are shown in Fig. 7. It was found that the immobilized enzyme produced a satisfactory yield of β-CDs after successive runs, retaining 60% of the initial catalytic activity at the end of the 15 cycles. Up to the 11th cycle, activity losses were not detected. Again, the silica microspheres system performed well compared with other systems. For instance, the operational stability of immobilized *Thermoanaerobacter* sp. CGTase on Eupergit C reported by Martín et al. [7] showed that after 8 cycles the system kept only 40% of residual activity.

The physical stability of the support is an important factor because it prevents the loss of the enzyme to the reaction medium, which would contaminate the product, making more difficult its purification [36]. In Fig. 8 is presented the scanning electron micrograph (SEM) of the structure of the silica microspheres after 15 cycles. The pictures clearly show the exceptionally good mechanical resistance of the system, with no structural modifications appearing in the microphotographies.

## 4. Conclusions

There are many important reasons for using immobilized enzymes, which includes the convenience of enzyme preparation, handling, and downstream separation of enzyme and its reactional products. Moreover, the possibility of enzyme reuse in successive batches, or in a continuous enzyme reactor, is of foremost importance. In particular, enzyme reuse provides cost advantages, which are often an essential prerequisite in order to establish an economically viable enzyme catalysis process [37,38]. In this work, it was possible to successfully immobilize *Thermoanaerobacter* sp. CGTase using a new approach on silica microspheres, showing high immobilization yields and activities recoveries, which could be used up to 11 batches reaction cycles almost without any decrease in its relative activity. The results obtained here show better performances of ImCGTase compared with other immobilization systems reported in the literature. The operational stability of *Thermoanaerobacter* sp. ImCGTase on silica microspheres presented in this work points out to its possible applicability in the industrial production of cyclodextrins, granting further research on the scaling-up of this enzymatic process.

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