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Establishing the Feasibility of Using β-Glucosidase Entrapped in Lentikats and in Sol−Gel Supports for Cellobiose Hydrolysis

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ABSTRACT: β-Glucosidases represent an important group of enzymes due to their pivotal role in various biotechnological processes. One of the most prominent is biomass degradation for the production of fuel ethanol from cellulosic agricultural residues and wastes, where the use of immobilized biocatalysts may prove advantageous. Within such scope, the present work aimed to evaluate the feasibility of entrapping β-glucosidase in either sol−gel or in Lentikats supports for application in cellobiose hydrolysis, and to perform the characterization of the resulting bioconversion systems. The activity and stability of the immobilized biocatalyst over given ranges of temperature and pH values were assessed, as well as kinetic data, and compared to the free form, and the operational stability was evaluated. Immobilization increased the thermal stability of the enzyme, with a 10 °C shift to an optimal temperature in the case of sol−gel support. Mass transfer hindrances as a result of immobilization were not significant, for sol−gel support. Lentikats-entrapped glucosidase was used in 19 consecutive batch runs for cellobiose hydrolysis, without noticeable decrease in product yield. Moreover, encouraging results were obtained for continuous operation. In the overall, the feasibility of using immobilized biocatalysts for cellobiose hydrolysis was established.

KEYWORDS: *glucosidase, immobilization, sol−gel, Lentikats, cellobiose*

INTRODUCTION

β-Glucosidase is an enzyme commonly found among plants, fungi, and bacteria. Furthermore, there are also some reports on the production of β-glucosidase from yeast (*Saccharomyces cerevisiae*, *Pichia etchellsii*) and mesophilic fungi (*Trichoderma harzianum* and *Aspergillus* sp.). Recent reports suggest that thermophilic fungi (*Thermoascus aurantiacus*, *Chaetomium thermophile*, *Humicola insolens*, *Sporotrichum thermophile*) and hydrocarbon utilizing novel fungus *Cladosporium resinae* are also good sources of β-glucosidase.[1−6]

β-Glucosidases (β-glucoside glucohydrolases, EC 3.2.1.21) are responsible for the hydrolysis of β-glucosidic linkages in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glucosides, and oligo- or disaccharides. β-Glucosidases constitute an important group of enzymes because of their potential use in several biotechnological processes.[7] β-Glucosidases belong to the cellulolytic enzyme complex that has a major role in biomass degradation, preventing cellobiose accumulation and controlling the overall rate of cellulose hydrolysis reaction.[3,8] β-Glucosidases can be thus used within the scope of biomass degradation and production of fuel ethanol from cellulosic agricultural residues.[9−12] Moreover, β-glucosidases can be used to synthesize alkyl glucosides that, in suitable formulations, are added to food products, detergents and cleaning agents, personal care products, fine chemicals, and pharmaceuticals.[13−17]

The use of immobilized biocatalysts offers several advantages as compared to the free form, when industrial applications are envisaged. Those include the repeated use of the enzyme, contributing for more cost-effective processes; a broader range of process design possibilities; eased separation of reaction products from the biocatalyst; and often the improvement of enzyme stability. There are many different strategies that have been proposed to immobilize enzymes, involving both support-based and carrier-free methods, as was thoroughly reviewed recently.[18−20]

In particular, β-glucosidase has been immobilized with several methods and supports, such as alginate, gelatin, Eupergit C, chitosan, kaolin, and silica gel.[21−25] Recently, while screening within an array of supports commonly used in the design of immobilized biocatalysts for a suitable support for the immobilization of β-glucosidase, using the artificial substrate 4-nitrophenyl β-D-glucopyranoside (PNPG), encouraging results were obtained when the enzyme was entrapped in sol−gel particles and in polyvinyl alcohol (PVA) formulations, Lentikats.[26] Sol−gel immobilization of enzymes has been shown to allow operation under harsher conditions than usual.[27,28] Sol−gel immobilization is a relatively simple immobilization method, which is performed at room temperature, thus minimizing the risks of thermal denaturation. As the outcome, enzymes remain entrapped in a silica-based, biocompatible host, which is resistant to microbial degradation and can be suitably doped to provide the more adequate microenvironment. On the other hand, mass transfer limitation can result from sol−gel immobilization. This drawback can be

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partially overcome through the use of pore-forming agents (surfactants), but these can have a deleterious effect on enzyme activity. Moreover, most methods require drying and grinding stages, which enhance the complexity and time span of the process.[29] PVA is a synthetic material that has emerged as a suitable alternative for the production of hydrogels, because it is cheap, innocuous, biocompatible, and mechanically and chemically robust.[30,31] The specific nature of Lentikats formulation further provides an easy and scalable method for enzyme immobilization; results in the formation of particles easily separated form the reaction medium; and diffusion limitation is overcome as a result of the thin-sized particles (200−400 μm thickness). On the other hand, enzyme leakage can occur, and operation above 55−60 °C is avoided due to particle melting.[32] In the present work, further characterization of the immobilized biocatalyst is conveyed, but using a natural substrate, cellobiose, which β-glucosidases are able to hydrolyze, alongside with other cellooligosaccharides, into glucose. This activity is relevant because it allows cellulolytic enzymes to perform more efficiently, by preventing cellobiose inhibition.[3] On the other hand, the affinity of β-glucosidases to cellobiose is lower than that to PNPG, and β-glucosidases are inhibited by glucose.[3,33,34] The outcome of the present work is thus expected to contribute to provide a more realistic perspective on the use of said biocatalysts within the scope of process design for cellulose hydrolysis. To comply with this goal, the immobilized enzyme preparations were characterized, for immobilization efficiency, optimum pH and temperature, kinetics, thermal, and operational stability. Moreover, the use a continuous mode of operation is tentatively assayed, and the effect of glucose formed in the performance of the biocatalyst is addressed.

MATERIALS AND METHODS

Materials. Cellobiose, tetramethoxysilane (TMOS), and PNPG were obtained from Sigma-Aldrich, U.S. Lentikats was obtained from Genialab, Germany. An extract with β-glucosidase activity, from an *Aspergillus* strain belonging to the culture collection of the Biochemistry and Food Laboratory, Faculty of Food Engineering, State University of Campinas, Brazil, was used as the source of enzyme preparation. All other reagents were of analytical grade from different sources.

Production of β-Glucosidase. An extract of β-glucosidase was obtained as described previously, from an *Aspergillus* sp. strain belonging to the culture collection of the Biochemistry and Food Laboratory, Faculty of Food Engineering, State University of Campinas, Brazil.[26] Briefly, spores were spread on potato dextrose agar in Petri dishes and incubated for 5 days at 30 °C. 10 mm discs were then taken from the Petri dish cultures, and 15 disks were transferred to a 500 mL Erlenmeyer flask containing 20 g of culture medium, composed of wheat bran (95%, w/v) and sugar cane bagasse (5%, w/v). The Erlenmeyer flasks were incubated for 5 days at 30 °C. A suspension was then prepared by adding 100 mL of distilled water to the Erlenmeyer flasks and shaking at 150 rpm for 20 min. The suspension was filtered through qualitative filter paper, and the filtrate was recovered for salting out. This was carried out by addition of an ammonium sulfate solution (80% of saturation) and storage at 3 °C overnight. The suspension was then centrifuged for 10 min at 10 000 rpm, and the precipitate was suspended in sodium phosphate buffer 0.05 M pH 7.0. This extract was lyophilized for 48 h and was stored in a refrigerator at 4 °C. Prior to further use, the lyophilized enzyme was suspended in 100 mM acetate buffer pH 4.5 to yield an enzyme solution with a titer of 0.1 g L^{-1} and an activity of 1.75 U mL^{-1}.

Enzyme Immobilization. The enzyme preparation was diluted 1000-fold in 100 mM acetate buffer pH 4.5, to yield an enzyme solution of 0.1 g L^{-1}. Immobilization in Lentikats was performed according to the protocol provided by GeniaLab.[35] Briefly, 0.1 mL of the diluted enzyme preparation was added to 1 mL of Lentikat liquid. The resulting solution was extruded to Petri dishes. After dehydration under 30 °C to 30% (w/w) of the original weight, to allow for gelation, the lens-shaped hydrogel particles were incubated in 100 mL of a 15 g L^{-1} solution of LentiKat Stabilizer for two hours at room temperature. The lens-shaped hydrogel particles were then washed and stored in 100 mM acetate buffer pH 4.5 at 4 °C until use. Immobilization in sol gel was performed as described elsewhere.[26] Briefly, 0.16 mL of the diluted enzyme preparation was mixed with a solution containing 100 μL of TMOS (2.32 M) and 40 μL of HCl (10 mM), which had been previously sonicated in a Transsonic T 460 sonicating water bath for 10 min. The sol−gel solution thus obtained was immediately added to 6 mL of 150 mM AOT/isooctane solution. The mixture was vortexed for 1 min, washed twice with 100 mM acetate buffer pH 4.5, and aged under room temperature and controlled water activity, a_w = 0.75, for 1 week. The particles obtained were suspended in 1 mL of the same acetate buffer and stored at 4 °C until use.

Encapsulation efficiency was determined on the basis of an activity balance, considering the initial activity of the diluted enzyme preparation and the activity in the effluents recovered from the immobilization procedures, as described elsewhere.[36]

Scanning Electron Microscopy (SEM). The beads were characterized regarding morphology and surface structure using scanning electron microscopy (SEM) images. SEM micrographs were taken with a JEOL model JSM 5800 LV (Tokyo, Japan). The lyophilized beads were placed using double-sided tape on brass stamps and covered with a thin spray coat of gold under vacuum in Sputter (Balzer model SCP 050). The acceleration voltage used was 10 kV with the secondary electron image as a detector.

Determination of Protein and β-Glucosidase Activity. Protein concentrations were determined by direct reading at 280 nm with reference to a calibration curve prepared with BSA (bovine serum albumin) standards. Samples were taken from crude and diluted enzyme extracts and from the supernatants recovered from immobilization procedures or from buffered/bioconversion media. Routine determination of β-glucosidase activity of both free and immobilized biocatalyst was performed using a spectrophotometric method as described elsewhere.[37] This spectophotometric method is based on the determination of *p*-nitrophenol released from the enzymatic hydrolysis of PNPG in acetate buffer-based reaction medium. Reaction mixtures contained 0.3 mL of 5 mM PNPG in sodium acetate buffer 0.05 M pH 5.0 and an appropriate amount of free or immobilized β-glucosidase in 0.3 mL of sodium acetate buffer. Reaction mixtures were incubated at 50 °C for 15 min with 400 rpm magnetic stirring, followed by addition of 0.3 mL of 0.5 M Na_2CO_3 solution, pH 12, to stop the reaction. These conditions were established after preliminary confirmation that the initial rate of product formation was linear, therefore allowing for a simple calculation of the initial reaction rate based on single datum point, according to a methodology suggested by Doig and co-workers.[38] Hydrolysis was determined by monitoring the release of *p*-nitrophenol at 410 nm with reference to a standard curve prepared using *p*-nitrophenol. Activity was expressed in International Units (IU), where 1 IU corresponds to the release of 1 μmol of *p*-nitrophenol per min. These determinations were used to establish the encapsulation efficiency, through a balance of the hydrolytic activity of the β-glucosidase preparation prior to immobilization procedures and the hydrolytic activity in the supernatants resulting from the different stages of the immobilization procedures. For the determination of pH and temperature profiles, of kinetic parameters, and of operational stability, through consecutive batch runs, β-glucosidase activity was established by monitoring the hydrolysis of cellobiose in 50 mM sodium acetate buffer. Glucose production was measured using an enzymatic glucose assay kit (Sigma-Aldrich, U.S.). To evaluate whether immobilization prevented the inhibitory effect of glucose in hydrolysis, the initial reaction rate was also assessed in the presence of initial concentrations of glucose up to 100 mM. Again, the single datum point approach was used to establish the initial reaction rate.[38]

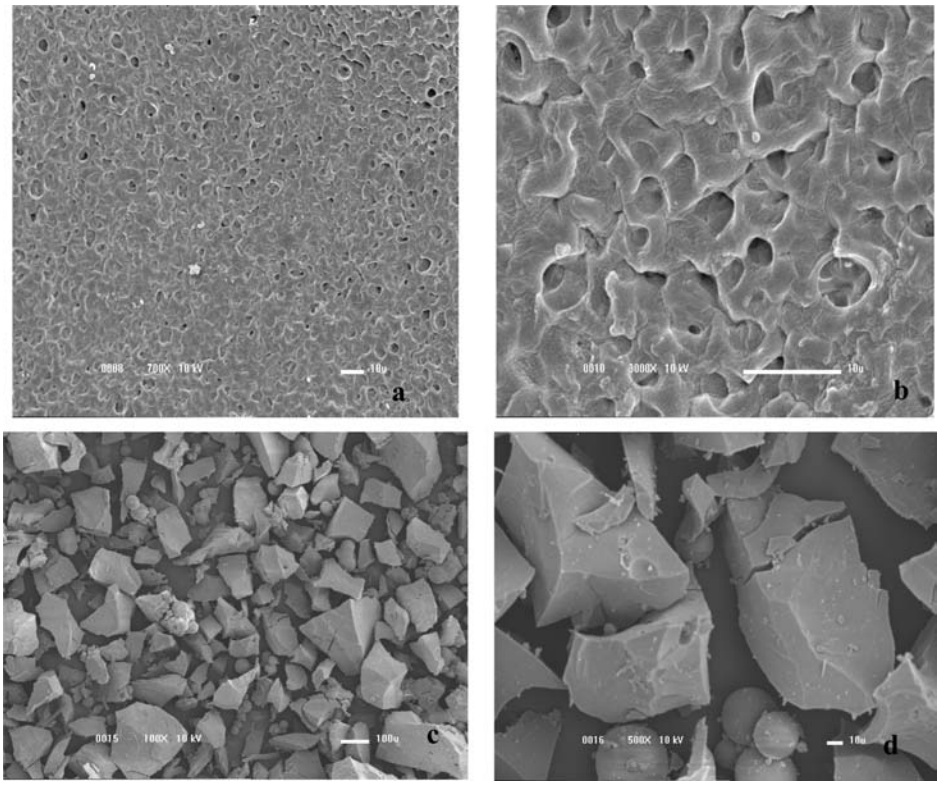

Figure 1. β-Glucosidase immobilized in Lentikats at magnification 700 (a) and magnification 3000 (b); β-glucosidase immobilized in sol−gel supports at magnification 100 (c) and magnification 500 (d).

Determination of Temperature and pH Profiles and of Kinetic Parameters. The effect of temperature on the activity of free and immobilized enzyme was established as follows. About 7 mg of immobilized enzyme preparation was incubated in 50 μL of acetate buffer pH 4.5, and the whole suspension was added to 50 μL of a 3 mM solution of cellobiose at pH 4.5. For assessing the activity of the free enzyme, 50 μL of a 0.1 g L^{-1} enzyme solution was used instead of the suspension. The reaction mixture was incubated at different temperatures within 40−80 °C for 30 min, after which glucose production was assessed. Triplicate runs were performed. The effect of pH on the activity of free and immobilized enzyme was performed similarly, except acetate buffer solution within 4.0−6.0 was used in the preparation of reaction media, and runs were performed at 55 or 70 °C, whether Lentikats or sol−gel preparation were assessed, respectively, alongside with corresponding runs with the free enzyme preparations. The effect of substrate concentration in the immobilized and free β-glucosidase activity was tested using solutions with different initial concentrations of cellobiose (0−10 mM). The assays were performed under optimal pH and temperature.

The K_M and V_{max} values for the Michaelis−Menten model were determined through a nonlinear method using the Solver Excel tool. The initial estimates for K_M and V_{max} for this least-squares nonlinear regression algorithm were obtained from the Lineweaver−Burk plots. Such approach reduces the risk of the algorithm failing to converge on an answer. The final values for K_M and V_{max} were those that minimized the sum of squares of the differences between experimental and predicted values.

Stability Experiments. The thermal and pH stabilities of free and immobilized enzymes were examined by measuring the activity of each enzyme formulation after incubation in buffered solutions for 1−3 h, at different temperatures (40−70 °C) and at different pH values (4.0−5.0). Experiments for the determination of thermal deactivation parameters were carried out at pH 4.5. From the experimental data on the effect of temperature on the activity of the enzyme formulations, the best fit for thermal deactivation was screened between the exponential decay and linear inverted models, according to standard methodologies as described in detail elsewhere.[39,40]

Repeated Batch Hydrolysis. Reactions were performed in a 2 mL screw-capped magnetically stirred (400 rpm) eppendorf at 50 °C, in 1.5 mL of 50 mM acetate buffer (pH 4.5), containing 1.5 mM of cellobiose and 10 mg of immobilized β-glucosidase. Throughout each 24 h batch run, 200 μL samples were collected and assayed for glucose. After each batch run, the immobilized biocatalyst was harvested, thoroughly washed with acetate buffer, and immediately used for the next run. The overall time of the experiment was of 456 h. All runs were performed in triplicate, at least.

Continuous Operation. The continuous process was carried out with 0.7 g of β-glucosidase enzyme immobilized in Lentikats, packed in a 1.0 cm^3 column. A cellobiose solution (1.5 mM) in 0.05 mM acetate buffer pH 5.0 was fed into the column, with a flow rate of 0.3 mL/min. Continuous operation was carried during 100 h, and aliquots were collected periodically from the effluent, for quantification of glucose concentration.

RESULTS

Encapsulation efficiencies of 58% and of 97% were observed for sol−gel and Lentikats immobilization supports, respectively.

SEM images of the β-glucosidase immobilized in Lentikats and sol−gel supports are shown in Figure 1. SEM examinations were performed to elucidate the structure and surface of both supports. On the basis of scanning electron microscopy (SEM), Lentikats support seems to present a more porous surface then the sol−gel support. Given this pattern, diffusion limitations are likely to be less pronounced in the case of Lentikats than in sol−gel particles, albeit this has to be balanced with the diffusivity and the diffusion path. In the case of Lentikats, the axial diffusion path, roughly of 100−200 μm,[41] is likely to exceed that of sol−gel particles. On the other hand, the more open structure of Lentikats is more prone to enzyme leakage.

Effect of Immobilization in pH and Temperature Profiles. The effect of immobilization in the temperature–activity profile was quite similar to the patterns observed previously, where PNPG was used as substrate source,[26] and is summarized in Figure 2. Similar trends on the effect of temperature in β-glucosidase activity when PNPG and cellobiose were used as substrates for characterization of β-glucosidases from *Aspergillus* spp. have been reported.[42] Entrapment in sol–gel particles, on the other hand, led to a shift of the optimal temperature from 60 °C (free enzyme) to 70 °C, and although activity decreased above this threshold, the biocatalysts still retained about 40% of the activity at the optimal temperature when challenged at 90 °C. On the other hand, the trend observed for the Lentikats biocatalyst closely matched the behavior followed by the free enzyme, with an optimum at 60 °C. Lentikats displayed some morphological changes when incubated at this temperature for prolonged time periods. Hence, further work with enzyme immobilized in Lentikats was performed under temperatures never exceeding 55 °C. The optimum pH remained unaltered, at pH 4.5, when β-glucosidase was immobilized in either sol–gel or in Lentikats (Figure 3).

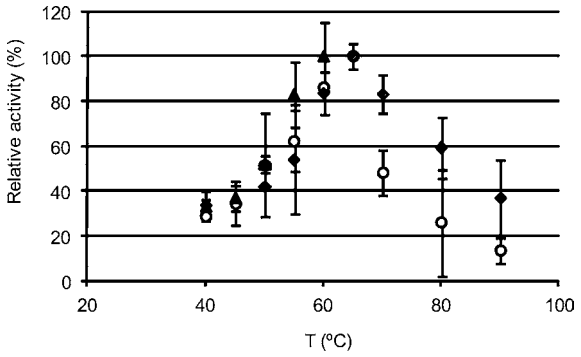

Figure 2. Influence of temperature on the activity of free (○), and Lentikats (▲) and sol–gel (◆) immobilized forms of the enzyme preparation.

Effect of Immobilization on Kinetics. The kinetic parameters of the free enzyme and immobilized by sol–gel and Lentikats methods were determined at pH 4.5 and 60 and 50 °C, respectively (Table 1). The predicted values for the kinetic parameters led to a model that provided a nice fit to the experimental data (Figure 4). Immobilization has occasionally been shown to reduce enzyme inhibition.[43] In the present case, however, no significant differences on enzyme activity were observed for the different enzyme formulations when in the presence of glucose, a recognized inhibitor of cellobiose hydrolysis[34,44] (Figure 5). This pattern is further highlighted when the apparent IC'_{50} values,[45] the concentration of inhibitor that reduces enzyme velocity by one-half, are compared, because these hardly change for the different enzyme formulations (Table 2).

Table 1. Kinetic Constants Obtained by Nonlinear Regression for Cellobiose Hydrolysis with Free and Immobilized β-Glucosidase[a]

biocatalyst	K_M (mM)	V_{max} (mM/min)	*F*-test value
free enzyme	1.0 ± 0.2	0.040 ± 0.007	0.938
Lentikats	3.0 ± 0.2	0.032 ± 0.002	0.951
sol–gel	1.1 ± 0.1	0.071 ± 0.003	0.999

[a]Nonlinear regression was performed using the Solver tool from Excel.

Thermal Stability. Both immobilized enzyme formulations displayed higher half-lives (Table 3) and energy of deactivation (Table 4) when compared to the free enzyme formulation. Such behavior is illustrative of enhanced thermal stability promoted by immobilization.[40] Thermal deactivation of both the free enzyme and the sol–gel biocatalyst followed the exponential decay model, whereas the linear inverted model provided a better fit for Lentikats biocatalyst (data not shown). Enzyme leakage was ruled out as no protein was detected in the supernatants under the operational conditions used.

Operational Stability. Immobilized biocatalyst was used repeatedly in several consecutive 24 h batch hydrolysis runs at 50 °C (Figure 6). The enzyme immobilized in Lentikats showed higher operational stability than the enzyme immobilized by sol–gel, with no significant loss of activity after 19 batch runs. The behavior presented by the sol–gel biocatalyst may be partially ascribed to some loss of carrier during manipulation (incomplete biocatalyst recovery after liquid–solid separation at the end of each batch run).

Given the encouraging results obtained with the enzyme immobilized in Lentikats, continuous operation was tentatively addressed, by flowing a 1.5 mM solution of substrate through a miniature tubular reactor packed with this immobilization support. After 96 h of continuous operation, glucose concentration in the outflow decreased to 1.2 (±0.2) mM, which corresponded to a product yield of 40% (Figure 7). Because, however, a low residence time was tested, it is expected that a decrease in the flow rate will allow for an

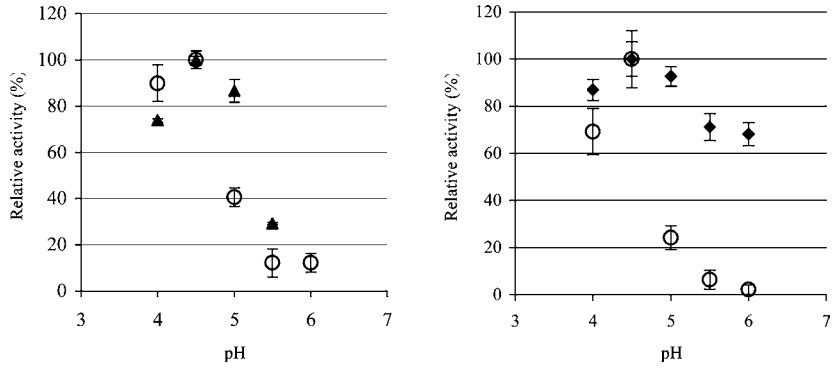

Figure 3. Influence of pH on the activity of free (○), and Lentikats (▲) and sol–gel (◆) immobilized forms of the enzyme preparation. Bioconversion runs were carried out at 55 °C (left side) or at 70 °C (right side).

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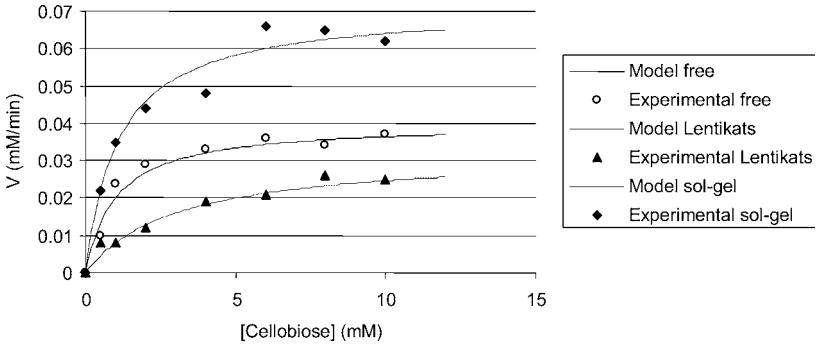

Figure 4. Experimental and predicted values of the initial reaction rate for cellobiose hydrolysis using free and immobilized β-galactosidase. Experimental data for free (○), Lentikats (▲) and sol−gel (◆) immobilized forms of the enzyme preparation are given. Predicted values for free (continuous line), Lentikats (dotted line) and sol−gel (line and dot) immobilized forms of the enzyme preparation were obtained on the basis of nonlinear regression using the Solver tool from Excel.

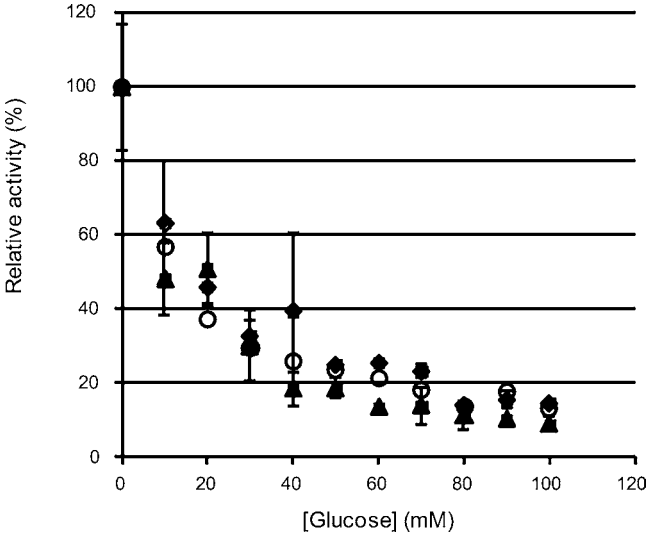

Figure 5. Inhibitory effect of glucose on the activity of free (○), and Lentikats (▲) and sol−gel (◆) immobilized forms of the enzyme preparation. The reference for relative activity is the initial rate of enzymatic hydrolysis in the absence of glucose.

Table 2. Effect of Immobilization on the Inhibitory Effect of Glucose As Expressed by IC'_{50} Values[a]

biocatalyst	IC'_{50} (mM)
free enzyme	17
Lentikats	11
sol−gel	16

[a] IC'_{50} values were determined on the basis of the following equation:[45] relative activity (%) = $(IC'_{50}/[\text{glucose}] + IC'_{50})$after linearization for computing the slopes, according to this equation:[45] $(1/(\text{relative activity (\%)}) = ([\text{glucose}]\cdot(1/IC'_{50}) + 1)$

Table 4. Energy of Deactivation of Free and Immobilized Preparations of β-Glucosidase

	free enzyme	Lentikats	sol−gel
energy of deactivation (kJ mol^{-1})	66.0	68.7	76.4

increase in the conversion yield up to the levels observed for repeated 24-h batch runs.[46]

DISCUSSION

Encapsulation efficiency data reported in the present work differ from the catalytic efficiency of these sol−gel and Lentikats biocatalysts, 85 ± 13% and 32 ± 11%, respectively, reported previously[26] because in the later case the specific activities of the immobilized and free enzyme formulations were compared.[47] Electrostatic interactions between the enzyme and gel have been shown to influence encapsulation efficiency,[36] and these may also be accountable for the different results observed in sol−gel and hydrogel, such interactions being more favorable in the later than in the former. On the other hand, the lower catalytic efficiency observed in case of the hydrogel may be due to some diffusion limitations often related to these supports, despite the mitigation provided by the lens-shaped nature of Lentikats.[41,48]

The shift of the optimum temperature for catalytic activity observed as an outcome of sol−gel immobilization can be ascribed to limitations in the thermal movement of the enzyme molecule, or to the molecular cage action of the support around the protein molecule, protecting the enzyme molecule from the deleterious action of the bulk temperature.[28,49,50] Sol−gel supports endured prolonged exposure even at the higher temperatures tested, as expected given their acknowledged thermal stability.[28] The protective effect of this support was further highlighted at such extreme temperature, because the

Table 3. Half-Lives of Free and Immobilized Preparations of β-Glucosidase

	half-life value (h)		
temperature	free enzyme	Lentikats	sol−gel
40	8.0	15	11
45	3.6	9.3	not determined
50	not determined	7.7	4.3
55	2.9	4.9	not determined
60	1.5	not determined	1.7
70	0	not determined	0.88

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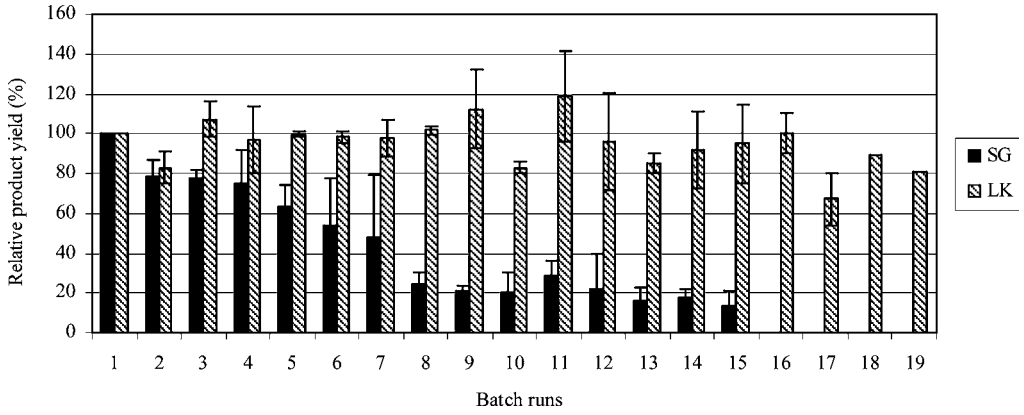

Figure 6. Cellobiose hydrolysis in consecutive 24-h batch runs using β-glucosidase immobilized in Lentikats (dashed bars) and sol−gel (filled bars) supports. Runs were performed at 50 °C, using as substrate 1.5 mM solution of cellobiose in acetate buffer pH 4.5. Product yield is given in normalized form, using as reference the product yield observed at the end of the first batch run, where full conversion was observed.

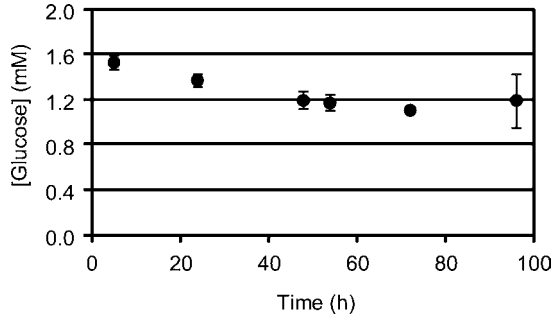

Figure 7. Continuous operation for hydrolysis of a 1.5 mM cellobiose solution (pH 4.5, 50 °C) in a tubular reactor packed with Lentikats immobilized enzyme. A flow rate of 0.3 mL/min was used.

activity retention exceeded that of the free enzyme under such conditions. This pattern has been reported in other works,[22,23,26,51,52] although this is not a consensual behavior.[53] Moreover, the sol−gel biocatalyst endured almost 1 h of incubation at 70 °C to retain one-half of its initial activity, whereas no noticeable activity was observed for the free enzyme (Table 3). This result compares favorably with data from Karagulyan and co-workers.[25] At this temperature, these authors reported half-lives of β-glucosidase immobilized in kaolin and in silica gel of 11 and 21 min, respectively,[25] The result obtained in the present work may not seem as impressive as the report of Nagatomo and co-workers[22] of a gelatin immobilized β glucosidase displaying unchanged activity after 8 h of incubation at 70 °C. The enzyme used by these authors displayed an optimum temperature of 90 °C for the free enzyme and 90−100 °C for the immobilized enzyme. On the other hand, Lentikats biocatalyst tended to melt after prolonged exposure to temperatures in excess of 60 °C, and melting becomes noticeable, a behavior that is in accordance with previously published data.[54,55] Because the activity of the Lentikats biocatalyst steadily increased up to the 60 °C threshold, this was assumed the optimum temperature, which actually matched that of the free enzyme. In either case, the protective effect of the support was further corroborated by the enhanced thermal stability observed for both enzyme formulations tested. The trend toward melting of the Lentikats support at higher temperatures prevented its use. Still, in the temperature range where its application is feasible, the half-life of the immobilized form bests that of the free enzyme by at least 1.7-fold. In addition, the increase in the energy of deactivation upon immobilization is representative of the enhanced stability of the immobilized enzymes, as compared to the free form.[56] Although thermal stability enhancement upon immobilization is common,[19,21−25,44] this pattern does not always occur.[57] Its occurrence in the selected systems is therefore illustrative of their potential for commercial applications.

No shifts in optimum pH were observed as an outcome of immobilization. However, the activity decay of the free enzyme was more pronounced at higher pH values when compared to the immobilized biocatalyst, for the sol−gel biocatalyst, a pattern also reported in other works focused on enzyme immobilization in sol−gel[51,52] and on gelatin immobilized glucosidase.[22] This behavior was more evident than that previously observed when PNPG was used as substrate source,[26] and can be tentatively ascribed to the higher temperatures used in the present work. This further highlights the protective role of the microenvironment surrounding the immobilized biocatalyst.[28] On the other hand, when a thermostable β-glucosidase from *Sulfolobus shibatae* was immobilized on silica gel using cross-linking with trans-glutaminase, the immobilized enzyme displayed a lower activity than the free enzyme for pH values under 5.5, but no further shifts were observed as an outcome of immobilization.[23]

Both immobilization methods resulted in mass transfer limitations, as suggested by the increase in the apparent K_M (Michaelis constant) in both immobilized biocatalysts. This feature is a typical outcome of immobilization.[24,55−59] Such diffusion limitations were more noticeable when the enzyme was entrapped in Lentikats, as compared to sol−gel, although scanning electron microscopy (SEM) results (Figure 1) apparently suggested that the former support presented a more porous surface then the later. The results may suggest favored partition and/or diffusion in the case of the sol−gel support as compared to the hydrogel, where diffusion is influenced both by the diffusion coefficient and by the length of the diffusion path. Still, the K_M shifts observed in the present work were less marked than that observed by Tu and co-workers, who reprted a 10-fold increase in K_M for cellobiose after β-glucosidase immobilization in Eupergit C.[24] Moreover, the more open structure of Lentikats could be more prone to enzyme leakage, a feature that could limit the application of such formulation.[60] However, no hints of significant enzyme

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leakage were observed in either case during the thermal stability studies, yet these were performed over a relatively short timespan. On the other hand, while assessing the operational stability of both enzyme formulation through consecutive batch runs, very small amounts of immobilized sol−gel biocatalyst were physically lost during the transfer procedures required for the reuse of the biocatalyst, as detected by visual inspection. As a result, the amount of biocatalyst used throughout the consecutive batch runs was not constant. This may have contributed for the decrease observed in the product yield under the operational conditions used. This feature has been reported for bioconversion processes involving sol−gel formulation.[61] In addition to such depletion, some enzyme leakage from the sol−gel may however have also occurred during the consecutive batch runs, which took place over a larger timespan than thermal stability studies. Such leakage was recently reported for sol−gel immobilized cellulose,[62] and led to a decay in residual activity to 20% of the original value after 6 cycles. This is less favorable than that obtained in the present work, for the sol−gel biocatalyst, and is clearly bested by the current Lentikats biocatalyst. Tu and co-workers also assessed the operational stability of β-glucosidase immobilized in Eupergit C over consecutive batch runs, but using acetic acid pulp, a lignocellulosic material, as substrate. No significant changes in residual activity were observed over six successive rounds of hydrolysis, over a total of experimental period of 288 h.[24] This is somehow less than the experimental period of 456 h corresponding to the repeated use of Lentikats biocatalyst with no decay in residual activity reported in the present work.

In hydrogel particles, V_{max} decreased as compared to the free enzyme, whereas in the case of sol−gel immobilization an increase in V_{max} was observed. The former behavior is typical of immobilization and may be ascribed to interactions with the immobilization matrix that lead to adverse modifications in the enzyme structure.[55,59] On the other hand, and although an increase in V_{max} as a result of immobilization is less common, it has been reported.[24,48,63] The mechanisms underlying such behavior require further investigation, although the pattern may again be related with favorable interaction with the support resulting in structural changes of the enzyme or in favored mass transfer.[57,64]

The continuous hydrolysis of cellobiose, using a miniature tubular reactor packed Lentikats immobilized β-glucosidase, was implemented with relative success, for a product yield of 53% was achieved. It is foreseen to decrease the flow rate as an operational strategy to increase product yield up to the levels observed for repeated 24-h batch runs. Multireactor operation will be also considered to overcome deactivation while maintaining a high product yield. This later approach involves the use of a set of packed bed reactors in series, where fresh enzyme is added to the last reactor.[46]

Characterization of β-glucosidase entrapped in either sol−gel or Lentikats particles was performed using cellobiose hydrolysis as bioconversion system. The latter support was shown to present a macroporous nature as opposite to the former. Immobilization in sol−gel led to a 10 °C increase of the temperature optimum as compared to the free form, which suggests a protective effect of the support by limiting the thermal motion of the enzyme. On the other hand, Lentikats melted at temperatures over 60 °C, and hence could not be used. The activity of the Lentikats biocatalysts increased steadily with temperature up to 60 °C. Accordingly, this was considered the optimal temperature for activity for this formulation, which was the same as that for the free enzyme. Still, both methods were shown to improve thermal stability. Immobilization did not change the optimal pH for activity. Still, decrease of activity under exposure to less acidic environments was mitigated when either form of the immobilized biocatalyst was assayed, as compared to the free form. Entrapment in sol−gel introduced negligible mass transfer limitations, which were more severe when Lentikats were used, as compared to the free form. Under consecutive batch-run operation, Lentikats biocatalyst outmatched the sol−gel formulation, because the former could be used in up to 19 runs with no marked decay in the product yield. Continuous hydrolysis of cellobiose using Lentikats biocatalyst was tentatively assessed with encouraging results, but dedicated efforts to optimize such approach are needed and are in the pipeline. Information thus gathered suggests that the setup for cellobiose hydrolysis promoted by β-glucosidase entrapped in Lentikats may provide a sound option for large application.

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ABBREVIATIONS USED

AOT, sodium bis-(2-ethyl-1-hexyl) sulfosuccinate; PNPG, 4-nitrophenyl β-D-glucopyranoside; PVA, polyvinyl alcohol; SEM, scanning electron microscopy; TMOS, tetramethoxysilane

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