O9-4	**Characterization of β-glucosidase entrapped in LentiKats and in sol-gel supports**	

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INTRODUCTION AND OBJECTIVES

β-Glucosidases (β-glucoside glucohydrolases, EC 3.2.1.21) are responsible for the hydrolysis of β-glycosidic linkages in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glycosides, and oligo- or disaccharides. The interest in β-glucosidases has been growing due to its application in a wide array of biotechnology process, among them the production of aromatic compounds, the stabilization of juices and beverages, and the improvement of the organoleptic properties of food and feed products. These enzymes have also found applications in biomass degradation and production of fuel ethanol from cellulosic agricultural residues; and in the synthesis of alkyl- and arylglycosides from natural polysaccharides or their derivatives and alcohols, by reversed hydrolysis or trans-glycosylation. The resulting products have many potential applications in pharmaceutical, cosmetic, and detergent industries (Bhatia 2002). The immobilization of β-glucosidase in a solid carrier offers the prospect of cost savings by enabling enzyme recycling through multiple cycles of batchwise runs; allowing for continuous mode of operation; and easing downstream processing. Lentikats® technology is relatively recent, but has been proving effective for the production of biocatalysts targeted to the food and feed, and pharmaceutical industries (Grosová 2009). The implementation of sol-gel to the immobilization of biological molecules, among them enzymes, has been gaining relevance since the late 1990s (Avnir 2006). Neither of these methodologies has been tested for the immobilization of β-glucosidase, but given their array of applications in enzyme immobilization, they were considered promising candidates for said role. Thus, in the present work, entrapment in sol-gel and in Lentikats® is evaluated in order to assess the feasibility of such methodologies for the immobilization of a β-glucosidase from *Aspergillus* spp.

MATERIALS AND METHODS

Materials

Tetramethoxysilane (TMOS) and 4-nitrophenyl β-D-glucopyranoside (PNPG) were obtained from Sigma-Aldrich. Lentikats® was obtained from Genialab. A partially purified 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, was used as source of enzyme preparation. All other reagents were of analytical grade from different sources.

Enzyme immobilization

The enzyme preparation was diluted 1000-fold in 100 mM acetate buffer pH 4.5. Immobilization in Lentikats® was performed according to the protocol provided by GeniaLab (http://www.genialab.de/download/tt-english.pdf.), adding 0.1 mL of the diluted enzyme preparation in 1 mL LentiKat® liquid. The resulting solution was extruded to Petri dishes. After dehydration, under 30ºC, to 30 % (w/w) of the original weight, to allow for gelation, the lenses were incubated in 100 ml of a 15 gL^{-1} solution of LentiKat®Stabilizer for two hours at room temperature. The lenses were then washed and stored in 100 mM acetate buffer pH 4.5 at 4ºC until use. Immobilization in sol gel was performed as described elsewhere (Bernardino 2009). Briefly, 0.16 mL of the diluted enzyme preparation was mixed with a solution containing 100 µL TMOS (2.32 M) and 40 µL HCl (1.37 mM), which had been previously sonicated in a Transsonic T 460 sonicating water bath for 10 min. The resulting sol-gel solution 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 one week. The particles obtained were suspended in 1 mL of the same acetate buffer and stored at 4°C until use.

Biocatalyst activity

The determination of β-glucosidase activity of both free and immobilized biocatalyst was performed according to Matsuura et al. (1995). The spectophotometric method is based on the determination of *p*-nitrophenol released from the enzymatic hydrolysis of PNPG in acetate buffer based reaction medium. Data from the bioconversion runs were established based in the initial reaction rates of hydrolysis. Triplicates of each run were performed and the standard deviation for these determinations did not exceed 10%, unless if stated otherwise.

Protein concentration

The concentration of protein in the diluted enzyme preparation, in the supernatant and in the effluents from the washing steps was determined at 280 nm based on a calibration curve of β-glucosidase.

RESULTS AND DISCUSSION

Effect of immobilization in pH and temperature profiles

The effect of immobilization in the initial reaction rate of PNPG hydrolysis was evaluated within a given range of pH (Figure 1) and temperature (Figure 2). The immobilization in either sol-gel or Lentikats® hardly altered the

enzymatic pH-activity profile, as compared to the free form, with the pH optimum remaining unaltered at 4.5. Only the activity decay of the free enzyme was slightly more pronounced higher pH values. This can be ascribed to the microenvironment surrounding the biocatalyst.

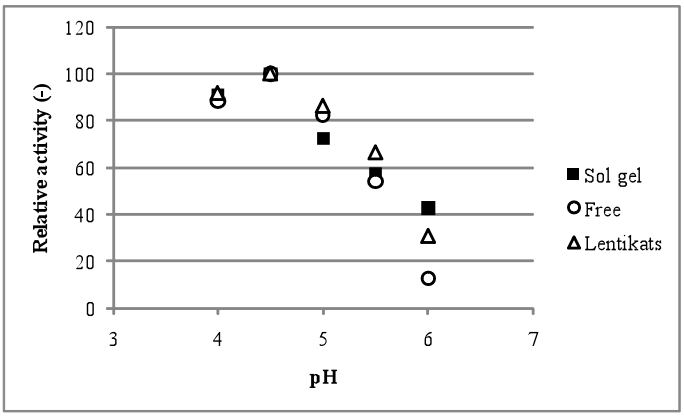

Figure 1 : Influence of pH on the activity of free (dots), sol-gel (closed squares) and Lentikats® (triangles) immobilized forms of the enzyme preparation. Bioconversion runs were carried out at 50◦C.

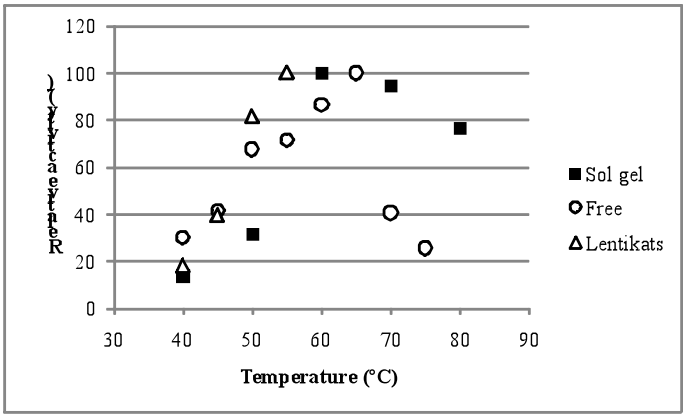

Figure 2 : Influence of the temperature on the activity of free (dots), sol-gel (closed squares) and Lentikats® (triangles) immobilized forms of the enzyme preparation. Bioconversion runs were carried out at pH 4.5.

The enzymatic temperature-activity profile displayed significant differences for the three forms of the biocatalyst. Lentikats® biocatalyst proved effective up to 55 ºC, with no enzyme leakage observed, but above this temperature, melting of the support was observed. This later behaviour was also reported by Rebros (2007) and prevents further evaluation for this support at higher temperatures, which could be considered given the optimum temperature of 65ºC for the activity of the free enzyme. Above this temperature, there is a sharp decay of activity for the free enzyme, unlike what is observed for the sol-gel entrapped enzyme, which still retains about 80 % of the initial activity at 80ºC.

Mass transfer limitations of immobilization

An increase in the apparent K_M (Michaelis constant) was observed following immobilization, which was more marked for Lentikats® than for sol-gel (Table 1). The decreased affinity of the enzyme towards the substrate is most likely the result of hindered substrate diffusion inside the matrix. Despite of the optimized geometry of the Lentikats®, this matrix still displayed the most significant diffusion limitations. Both forms of the biocatalyst could be used in more than 10 consecutive batch runs with no activity decay (data not shown).

Table 1 : Mass transfer limitations as result of immobilization

Biocatalyst	K_M (mM)
Free enzyme	2.5
Sol-gel	5.0
Lentikats®	12.0

CONCLUSIONS

Immobilization of β-glucosidase was effectively performed in sol-gel and in Lentikats®, leading to reusable biocatalysts. The later proved however more prone to diffusion limitations and temperature limited, unlike sol-gel, which increased the thermal stability of the enzyme. In the overall, sol-gel apparently presents a better option for β-glucosidase immobilization. Further work is underway for optimization and more thorough characterization of the immobilized biocatalyst.

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