Biocatalysts prepared by the entrapment of inulinase and penicillin G acylase in sol-gel

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INTRODUCTION

Enzyme immobilization is an important technique in chemical and biochemical industries. The immobilization of enzymes offers many advantages, such as multiple reuse, easy separation, and improved stability for efficient biotransformation and biodegradation. One promising method for the immobilization of enzymes is a sol-gel technique, in which enzymes are confined within a chemically inert sol-gel support that is prepared by the hydrolysis and polycondensation of organometallic precursors (O´Neil et al., 2002; Reetz et al., 1998). The advantages of the sol-gel method are: (a) simplicity, (b) the process occurs at near room temperature, and (c) the easy insertion of substituting groups into a silica matrix that provides the entrapped enzymes with beneficial microenvironments (Shin et al., 2007). A well-established sol-gel processing technique consists in hydrolyzing the adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to produce a hydrated metal or semi-metal oxide hydrogel. Removal of water from the wet gel, results in a porous xerogel (O´Neil et al., 2002; Reetz et al., 1998). The most common silica sol-gel encapsulated enzyme systems have been produced in crushed powder form from the dried xerogel state, or sol-gel coatings with enzymes affixed to various solid material surfaces. The crushing of the silica particles, however, yields irregular shapes and sizes and makes the process of scale-up very difficult (Lee et al., 2006). Recently a sol-gel emulsion technology has been developed that combines the emulsion and sol-gel technology and used to prepare small spherical silica particles which minimize diffusion limitations on the porous network. Surfactants and other non-surfactant organic molecules have been employed as pore-forming agents (Shin et al., 2007). In this work particular emphasis is given to advances in inulinase and penicillin G acylase (PGA) immobilization by entrapment in a sol-gel matrix. Penicillin acylases (penicillin amidohydrolase, EC 3.5.1.11) are a group of enzymes which are involved mainly in industrial production of 6-aminopenicillanic acid (6-APA), and 7-aminodesacetoxycephalosporanic acid (7-ADCA), which are, respectively, key intermediates in the synthesis of semi-synthetic penicillins (SSP), and semi-synthetic cephalosporins (SSC) (Kallenberg et al., 2005). Inulinases are a group of enzymes that typically include endoinulinase (EC 3.2.1.7) and exoinulinase (EC 3.2.1.80), the former promoting the endohydrolysis of 2,1-beta-D-fructosidic linkages in inulin, the latter presenting hydrolysis of terminal, non-reducing 2,1- and 2,6-linked β-D-fructofuranose residues in fructans, hence emulating invertase activity. Comercial preparations of inulinases are composed of a mixture of both enzymes can thus be used for inulin hydrolysis and sucrose hydrolysis.(Ricca et al., 2007)

MATERIAL AND METHODS

PGA solution (26.6 mg mL^{-1} protein, 35 U mg^{-1}) from *Escherichia coli*, Fructozyme L, a commercial preparation of inulinase from *Aspergillus niger*, tetramethoxysilane (TMOS) ≥ 99%, sodium dioctyl sulfosuccinate (AOT) were all purchased from Sigma-Aldrich (USA). Isooctane was supplied from Riedel de Haën (Germany). Penicillin G (PG) was obtained from Fersinca Gb (Mexico). Inulin from chicory (Fibruline Instant) was a kind gift from Cosucra (Belgium). All other reagents used were either laboratory or analytical grade.

PGA Sol-gel biocatalyst

A solution containing 100 μL TMOS (2.32 mol dm^{-3}) and 40 μL HCl (1.37 mmol dm^{-3}) was sonicated in a Transsonic T 460 sonicating water bath for 10 min until the hydrolysis reaction was complete, according to method described in Clark et al. (Bernardino et al., 2009). In a typical immobilization procedure, 75 μL of PGA (or 160 μL of 10-fold diluted Fructozyme L in pH 4.5 acetate buffer) were suspended in 100 mmol dm^{-3} phosphate or tris buffer, pH 7.6 - and then mixed with the sol solution. 300 μL of the sol-gel solution with enzyme was immediately added to 6 mL of 150 mmol dm^{-3} AOT/isooctane solution, before gelation. The resulting mixture was placed under vortex for 1 min, washed twice with 100 mmol dm^{-3} phosphate or tris buffer, pH 7.6 and aged at room temperature during one week, under controlled water activity (a_w = 0.75). A water activity controlled environment was established by incubating eppendorfs with sol-gel in a closed container with a saturated solution of sodium chloride (Greenspan et al., 1977). The micro-particles obtained were suspended in 1 mL of the same phosphate or tris buffer (PGA), or acetate buffer (inulinase). The particles are either used or stored at 2 – 8 °C.

Assay for protein concentration

The concentration of protein in the enzyme solution was determined as described elsewhere (Bernardino et al., 2009)

PGA activity assay

One unit of PGA activity (U) for the soluble and immobilized enzymes was defined according either to the pH STAT method or to the NIPAB method, as described elsewhere (Bernardino et al., 2009)

Repeated batch runs for inulin/sucrose hydrolysis

Consecutive 24-batch runs for the hydrolysis of 1 mL inulin (50 gL^{-1}) or sucrose (100 gL^{-1}) solutions in pH 5.0 acetate buffer were performed using 20 μL of sol-gel suspension containing inulinase. Runs were performed at 50ºC. Further details as described elsewhere (Fernandes et al., 2008). Biocatalyst particles were recovered by centrifugation between consecutive runs.

Effect of methanol

In order to evaluate the influence of methanol in the deactivation of PGA, a solution of free PGA was incubated in phosphate or tris buffer 100 mM with methanol 35% (v/v), pH 7.6 at 37 °C for 1 h and in the same buffer solution but without methanol. Activity was determined by NIPAB method at specific times.

Activity retention

Activity retention of PGA upon immobilization was defined as the ratio between the specific activity of the enzyme immobilized on the support and the specific activity of the free enzyme.

Quantification of reduing sugars in inulin/sucrose hydrolysis
Quantification of reducing sugars was performed by the DNS method (Miller, 1959)

SEM
Dry particles of biocatalyst were put on a double carbon tape and analyzed in a Field Emission Scanning Electron Microscope (Jeol JSM-7001F).

RESULTS AND DISCUSSION

With xerogels silica matrixes from tetrametoxisilane (TMOS) mechanically stable carriers were produced. Diffusion limitations are minimized given the average diameter (20 - 30 μm) of the micro-carriers obtained (Figure 1). Immobilization of PGA in these micro-carriers allowed for an immobilization yield of 95-100%, whereas the recovered activity was 45-65% at 37 ºC, as determined by the pH STAT method. This clearly exceeds results reported in a previous work on PGA immobilization in sol-gel where only 10 % of activity was recovered (Kallenberg et al., 2005). The results from thermal stability studies with free enzyme and sol-gel micro-particles performed in 35% methanol (v/v) in 100 mM phosphate ($t_{½free\ enzyme}$ = 8 min; $t_{½sol\text{-}gel}$ = 21 min) and in 100 mM Tris buffer ($t_{½free\ enzyme}$ = 38 min; $t_{½}$ = 197 min) at pH 7.6 (Figure 2), show that sol-gel immobilization is a sound strategy for PGA stabilization and that PGA is more stable when incubated in the presence of tris buffer, as compared to phosphate buffer. Crystal structures of PGA from *E. Coli* reveal a tightly bound calcium ion in the structure, which is not directly involved in the catalytic mechanism but assumed to be involved in the stabilization of the native state (Ignatova et al., 2005) and phosphates can sequester divalent cations such as Ca^{2+} and Mg^{2+}, which can affect PGA activity.
Immobilized biocatalyst was used repeatedly in several consecutive 24 h batch hydrolysis runs at 50 ◦C, with an initial inulin and sucrose concentrations of 5% and 10% (w/v), which is suggestive of high operational stability (Figure 3).

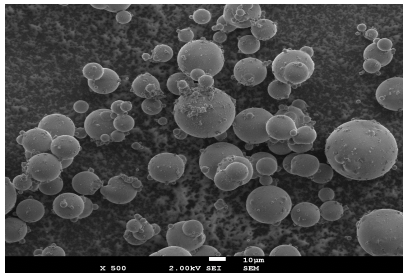

Figure 1: SEM micrograph of sol-gel micro-particles with encapsulated PGA (bar match 10 μm).

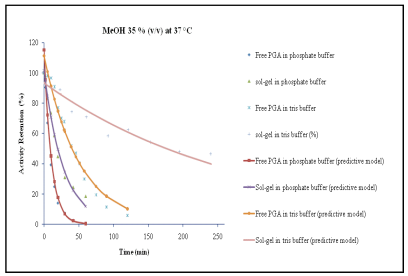

Figure 2: Thermal stability studies with free enzyme and sol-gel micro-particles performed in 35% methanol (v/v) in 100 mM phosphate and tris buffer at pH 7.6.

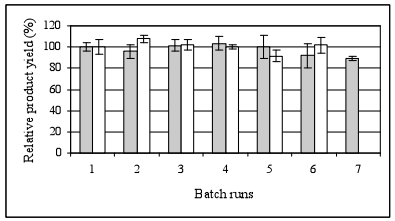

Figure 3: Effect on the final product yield of the repeated use of immobilized inulinase for the hydrolysis of 5 and 10.0% (w/v) inulin (white) and sucrose (grey) solutions. In the first cycle 45 (±) g L−1 and 92 (±7) gL^{-1} product titer was observed.

CONCLUSIONS

PGA and inulinase were effectively immobilized in a silica xerogel matrix. Ris bffer enhances the stability immobilized PGA. The use of an emulsion process allowed us to prepare relatively regular sized active microenzyme immobilized beads. Immobilized inulinase was recycled many-fold, which is a key asset for economic feasibility of processes. Similar performance is to be demonstrated for immobilized PGA.

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