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From Inulin to Fructose Syrups Using Sol–Gel Immobilized Inulinase

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Abstract The present work aims to provide the basic characterization of sol–gel immobilized inulinase, a biocatalyst configuration yet unexploited, using as model system the hydrolysis of inulin to fructose. Porous xerogel particles with dimensions in slight excess of 10 μm were obtained, yielding an immobilization efficiency of roughly 80%. The temperature– and pH–activity profiles displayed a broader bell-shaped pattern as a result of immobilization. In the latter case, a shift of the optimal pH of 0.5 pH units was observed towards a less acidic environment. The kinetic parameters estimated from the typical Michaelis–Menten kinetics suggest that immobilization in sol–gel did not tamper with the native enzyme conformation, but on the other hand, entrapment brought along mass transfer limitations. The sol–gel biocatalyst displayed a promising operational stability, since it was used in more than 20 consecutive 24-hour batch runs without noticeable decay in product yield. The performance of sol–gel biocatalyst particles doped with magnetite roughly matched the performance of simple sol–gel particles in a single batch run. However, the operational stability of the former proved poorer, since activity decay was evident after four consecutive 24-hour batch runs.

Keywords Sol–gel · Inulinase · Inulin hydrolysis · Enzyme immobilization · Applied biocatalysis

Introduction

Carbohydrate processing is a field where the use of enzymes is clearly established, even when industrial applications are considered [7]. This pattern can be ascribed to the suitable

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combination of several issues: a) the high selectivity and specificity of biocatalysts; b) their ability to operate under mild conditions; c) the large number of enzymes available (about 3,000), albeit the number of commercialized (and cheap) industrial enzymes is roughly in excess of 100; d) enzymes are usually considered natural products and are hardly hazardous to the environment, as compared with traditional chemical catalysts [7, 40]. In the whole, these features also allow the design of environmentally friendly processes, since there is a reduction in emissions (including greenhouse gases), in industrial chemical waste, and in energy consumption, by doing away with the need to operate in the typically severe chemical environments [7, 17]. The designer of production processes must take into consideration that end products must present no enzyme activity, a requirement which is of particular relevance in the food and feed sectors [1, 17]. This can be achieved through enzyme immobilization, an approach that often contributes to increase the operational performance [49], enables re-use of the enzyme and continuous mode of operation, and may actually be needed to render the process economically feasible [6, 54]. For some decades, considerable attention has therefore been given to research on various aspects of enzyme immobilization and several developments in recent years suggest a renewed interest in this particular field [6, 52]. Immobilization may be performed through binding to a solid surface, cross-linking of enzyme aggregates or crystals to form carrierless macroparticle biocatalysts or entrapment [49]. The latter technique typically relies in hydrogels, but developments in sol–gel fabrication methodologies that occurred in the late twentieth century, namely allowing the formation of doped inorganic glasses in aqueous, room-temperature environment, have pushed forward inorganic gels as viable matrices for the encapsulation of enzymes [23, 39]. This methodology, anchored in the slow hydrolysis of a given precursor, mostly ester derivatives of silicic acid, can lead to the production of robust biocatalysts, with properties suitably tuned, regarding porosity, hydrophobicity and substrate partitioning [23, 39]. Silicic acid-like derivatives are favored as precursors since silica, with its high specific area and controllable pore diameter, which can be tailored to produce mesoporous materials, has been shown to provide a stable matrix for enzyme immobilization [5, 23]. Sol–gel immobilized enzymes have been widely used as catalysts in several reactions [27], but within these, the successful application to carbohydrate processing is rather scarce [39], exceptions include: the hydrolysis of sucrose with invertase entrapped in cellulose acetate:zirconium alkoxide [35], germania [42] and tetramethoxysilane (TMOS) matrices [37]; the hydrolysis of cellobiose by β-glucosidase entrapped in a TMOS matrix [37]; and the hydrolysis of soluble starch by α-amylase entrapped in either tetraethoxysilane (TEOS) or hybrid methyltriethoxysilane (MTES):TMOS matrices [55]. The present work aims to further establish the feasibility of sol–gel materials as suitable enzyme encapsulation matrices for the production of biocatalysts targeted for carbohydrate processing. In this particular work, inulinase was immobilized in a TMOS-based sol–gel matrix. The reaction system chosen, the hydrolysis of inulin to fructose, is a reaction with particular interest for the food and feed sectors [41, 45].

Materials and Methods

Materials

A commercial preparation of inulinase from *Aspergillus niger*, Fructozyme L, tetramethoxysilane (TMOS) ≥99%, iron (II,III) oxide nanopowder, <50 nm particle size

(magnetite nanopowder) and sodium dioctyl sulfosuccinate (AOT) were provided by Sigma (St. Louis, MO, USA). Inulin from chicory (Fibruline Instant, with an average polymerization degree of about ten, and one terminal glucose unit) was a kind gift from Cosucra (Warcoing, Belgium). Acetic acid and sodium acetate (both p.a. grade) were purchased for Acros Organics (Geel, Belgium). Isooctane was supplied by Riedel de Haën (Germany). Fructose, (D-)-Fructose, 98.5–101.2% (by anhydrous basis) was from VWR (VWR International—Material de Laboratório, Lda, Carnaxide, Portugal). All solutions were prepared in distilled water. All other chemicals used were of analytical grade from various suppliers.

Biocatalyst Immobilization

Inulinase immobilization was performed based on the methodology described by Bernardino et al. [3]. Briefly, a solution composed of 40 μl of HCl (10 mM) and 100 μl of TMOS was sonicated in a Transsonic T 460 sonicating water bath for 10 min, for TMOS hydrolysis. To the thus processed solution were added 160 μl of a ten-fold diluted suspension of Fructozyme L. Occasionally, when preparation of magnetic supports (magnetic sol–gel support) was aimed at, 100 μl of a 6.25-fold diluted suspension of Fructozyme L was suspended in 60 μl of 20% (*w*/*v*) magnetite solution in 0.1 M acetate buffer, pH 4.5. Dilutions of Fructozyme L were performed in this same buffer. The resulting sol–gel solution containing the enzyme was directly added to 6 mL of a 150-mM AOT/isooctane solution. The mixture was vortexed for roughly 1 min and centrifuged at room temperature (10 min at 4,000 rpm, in a B. Braun Sigma centrifuge, model 2-15, with rotor 11192). The supernatant was discarded, and the precipitate washed twice with 0.1 M acetate buffer pH 4.5. Each washing step was performed with 6 ml of buffer, and in any case, the precipitate was recovered by centrifugation, as described before. The sol–gel was aged at room temperature under controlled water activity (a_w=0.75) for 1 week. The intended water activity environment was established through the incubation of Eppendorf tubes, containing the sol–gel particles, in a closed container, which was filled with a saturated solution of sodium chloride [3]. The ensuing microparticles were re-suspended in 1 ml of the same acetate buffer and either immediately used or stored at 4 °C until use. Immobilization yield, Y, was calculated as the ratio of the total activity of the immobilized preparation to the total activity of the soluble enzyme taken for immobilization [34]. Triplicate 30-minute runs were performed in water-jacketed, magnetically stirred (400 rpm, unless stated otherwise) vessels, using as substrate 5.0 ml of a 5.0% (*w*/*v*) inulin solution in 0.1 M acetate buffer pH 4.5. Stirring was promoted by micro stirrer bars (2 mm diameter × 5 mm length). Periodically, 10-μl samples were taken for determination of the reducing sugars formed, and activity was determined based on the initial reaction rate. Runs for establishing immobilization yield were also occasionally performed using as substrate a 10.0% (*w*/*v*) sucrose solution in 0.1 M acetate buffer pH 4.5. Otherwise similar conditions were used.

Determination of Temperature and pH Profiles and of Kinetic Parameters

The performance of free and immobilized inulinase was evaluated as previously described [10]. Thus, and unless stated otherwise, runs were performed in 1.5 ml screw-capped and magnetically stirred (400 rpm) reactors, filled with 1.0 ml of a 5.0% (*w*/*v*) inulin solution in 0.1 M acetate buffer (pH range of 4.0 to 6.0), and in a temperature range of 45 to 65 °C. For the determination of the kinetic parameters, inulin solutions with concentrations ranging

from 0.2% to 20.0% (*w*/*v*) were used. In a typical run, about 20 μl of either a 100-fold diluted Fructozyme L suspension or of the sol–gel suspension, yielding equivalent amounts of protein, was used as a biocatalyst. Specific activity is defined as milligram-reducing sugars formed per milligram protein per second. All runs were performed at least in triplicate, and a "sacrificial well" approach was used. The latter was elaborated based on preliminary runs that allowed establishing that the rate of product formation is linear over the time span considered [15, 32]. Samples (10 μl) were taken after 15 min and directly assayed for reducing sugars.

Evaluation of Operational Stability of the Immobilized Biocatalyst

The operational stability of the immobilized biocatalyst was assessed by carrying out consecutive batch runs, based on a methodology described elsewhere [21–23]. Reactions were performed in 2 ml Safe-Lock Eppendorf Microtubes (VWR International—Material de Laboratório, Lda, Carnaxide, Portugal) with magnetic stirring (400 rpm) at 50 °C, filled with 1.0 ml of a 5.0% (*w*/*v*) inulin solution in 0.1 M acetate buffer, pH 5.0. In a typical run, about 45 μl of a 50-fold diluted Fructozyme L suspension or 20 mg of the sol–gel suspension, yielding equivalent amounts of protein, was used as a biocatalyst. A single 24-hour run with the free enzyme was performed. Throughout each 24-hour run, 10 μl samples were collected for the quantification of reducing sugars. After each cycle, the immobilized biocatalyst was harvested by centrifugation at room temperature (12,000 rpm, Hermle Microlitre Centrifuges, model Z 233M-2, Hermle Labortechnik, Germany), thoroughly washed with 0.1 M acetate buffer pH 5.0, and used for the next run. All runs were performed at least in triplicate.

Analytical Methods

The quantification of reducing sugars was performed by the DNS method [33] using as reference a calibration curve from fructose standards. The quantification of protein in the enzyme preparation and in the liquid supernatants, resulting from the immobilization procedures was performed by the BCA method [51], using a commercial kit from Pierce Biotechnology (Rockford, IL, USA). The standard deviation from these determinations did not exceed 10%.

Scanning Electron Microscopy

Dry particles of the immobilized biocatalyst were placed on a double carbon tape and analyzed in a Field Emission Scanning Electron Microscope (Jeol JSM-7001F), as described elsewhere [4].

Gel Electrophoresis

SDS-PAGE was performed using 10% polyacrylamide gel with tricine and glycerol as described elsewhere [48] using a Bio-Rad Kit Mini-PROTEAN® 3 Cell (Bio-Rad Life Science Research, California, USA). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. Molecular weight standards were also from Bio-Rad, namely Precision Plus Protein™ Standards All Blue.

Results and Discussion

Immobilization Efficiency

The methodology used allowed the production of stable, inulinase entrapped xerogel particles with significant catalytic activity, namely presenting immobilization yields (%) of 81±3 and of 98±5, whether inulin or sucrose were used as substrates, respectively, and a protein retention (%) of 93±7. The latter was determined by mass balance, considering the concentration of protein in the enzyme solution before immobilization and both in the supernatant and in the effluents from the washing steps. The sol–gel particles displayed an average diameter slightly in excess of 10 μm, as assessed by scanning electron microscopy (SEM). A typical picture of the resulting micro-particles of biocatalyst is given in Fig. 1. The approach undertaken for sol–gel encapsulation of inulinase proved a marked improvement when compared to the immobilization yield (1.2%) of inulinase entrapped in alginate-silicate capsules, assessed for sucrose hydrolysis [9]. The immobilization yield also surpasses, or at least matches data reported for inulinase immobilization in different supports, targeted for inulin or sucrose hydrolysis [24, 38], namely: polyvinyl alcohol (PVA)-based matrices (less than 30%) [10]; calcium alginate (30% to 35%); Duolite A568 (about 36%) [50]; macroporous anion exchange AV-16-GS resin synthesized by polycondensation of polyethylene polyamine, epichlorohydrine, and pyridine (30% to 85%) [30]; metal-link chelation method on cellulose (40% to 46%) [25, 29]; Amberlite (roughly 40% to 50%) [14, 24]; glutaraldehyde-treated 2-aminoethyl-cellulose (54%) [28]; amino-controlled pore glass (60% to 77%) [19, 20]; Dowex (about 63%) [24]; chitin (66%) [36]; gelatin (about 82%) [13, 38]; ConA linked-amino activated silica beads, QAE-Sephadex and DEAE-Sephacel (100%) [24]; and Sepabeads (80% to 120%) [44].

Effect of pH and Temperature on the Biocatalyst

The influence of the temperature on the initial reaction rate of free and immobilized biocatalyst was evaluated within 40 to 85 °C. The temperature optimum was not shifted, when free and sol–gel entrapped inulinase are compared (Fig. 2).The influence of pH on the

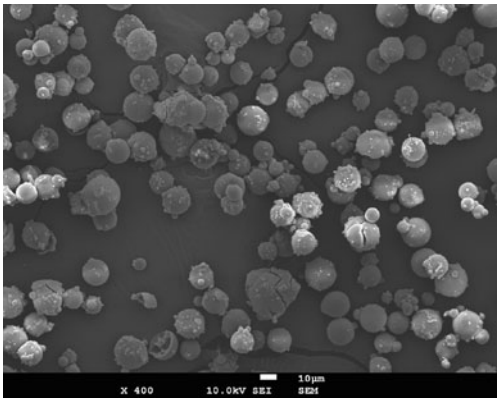

Fig. 1 SEM micrograph of sol–gel micro-particles with encapsulated inulinase (bar match 10 μm)

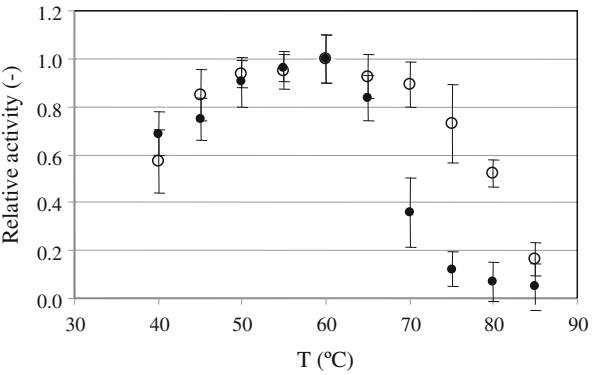

Fig. 2 Activity–temperature profiles of free (*black circle*) and sol–gel immobilized inulinase (*white circle*). Reactions were performed using a 5.0% (*w*/*v*) of inulin in 100 mM acetate buffer pH 4.5, within 40 to 85 °C. The maximal activity ($g_{reducing\ sugars}\ l^{-1}\ min^{-1}$) for free and immobilized inulinase was of 0.47 (±0.03) and 0.34 (±0.04), respectively

initial reaction rate of free and sol–gel encapsulated inulinase was evaluated within a pH range of 4.0 to 6.0 at 50 °C (Fig. 3). A trend towards a slightly less acidic pH optimum (0.5 pH units variation) can be observed as a result of immobilization, a pattern also observed when inulinase was immobilized onto activated Amberlite [47] or onto activated chitosan [36], but also for carboxymethyl cellulase entrapped in tetraethoxysilane-based sol–gel [16] and for lactase covalently linked to carrageenan–chitosan supports [18], among others. The observed shifts can be the outcome of a decrease in the inner matrix pH when compared to the bulk phase, hence affecting proton partitioning [22, 53]. However, both temperature–activity and pH–activity profiles of the immobilized enzyme display a broader-shaped curve than the free form. This pattern was not marked for inulinase immobilized in chitosan activated supports, ConA-linked amino activated silica beads or DEAE-Sephacel, although immobilization allowed for retention of enzymatic activity at temperatures where the free enzyme was inactive [24, 36]; however, it can be observed for inulinase immobilized in Amberlite, AV-16-GS resins, QAE-Sephadex and gelatin [13, 24, 30, 47]. Such feature may be ascribed to a protective microenvironment resulting of immobilization, which renders the enzyme less sensitive to the operational conditions in the bulk phase. Furthermore, the less drastic decay of activity under pH and temperature other than the optimal as a result of immobilization, makes the use of sol–gel immobilized inulinase more feasible under non-optimal conditions for activity (determined on the basis of initial reaction rates), but more amenable when: more prolonged operation is envisaged (viz. sub-optimal temperature for carrying out consecutive batches

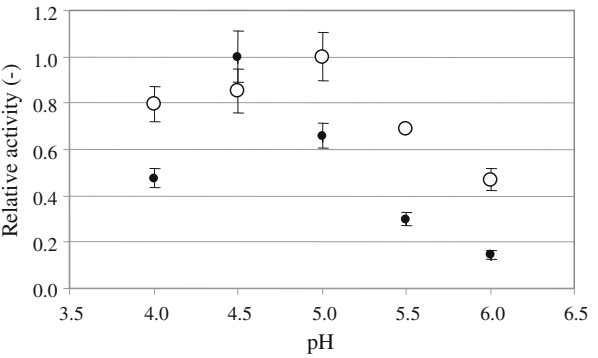

Fig. 3 Activity–pH profiles of free (*black circle*) and sol–gel immobilized inulinase (*white circle*). Reactions were performed using a 5.0% (*w*/*v*) of inulin in 100 mM acetate buffer within a pH range of 4.0 to 6.0, at 50 °C. The maximal activity ($g_{reducing\ sugars}\ l^{-1}\ min^{-1}$) for free and immobilized inulinase was of 0.42 (±0.02) and 0.29 (±0.03), respectively

or continuous operation) [46]; or minimization of microbial contamination under more drastic pH environment.

Effect of Immobilization on Kinetics

Immobilization typically brings along mass transfer limitations but it may also interfere with enzyme conformation, in a more or less drastic manner, hence affecting catalytic activity [12, 26]. Such effects are highlighted when the kinetics of the heterogeneous reaction system is compared with the homogeneous one, namely through modification in the kinetic parameters of the Michaelis–Menten-type equation [26]. The estimation of the kinetic parameters, V_{max} (maximal reaction rate) and K_M (Michaelis constant) was performed by linearization of the Michaelis–Menten hyperbolic curve using both Hanes–Woolf and Lineweaver–Burk plotting methods, the former being considered more adequate since it overrides the effects observed at lower substrate concentrations, when the Lineweaver–Burk method is used [36]. Kinetic parameters were also estimated by non-linear regression using the Solver tool from Excel. The outputs are given in Table 1. V_{max} and K_M predicted by the different methods provide a good fit to the experimental data, as can be observed in Fig. 4. Still, a much closer similarity can be observed for the estimated values for the free enzyme when compared to those for the immobilized form. In the latter case, the predicted values for both K_M and V_{max} using the Solver tool and the Hanes–Wolf linearization are closer than those predicted by Lineweaver–Burk linearization. As a whole, the highest degree of linear relationship between experimental and predicted values for the reaction rate at a given initial substrate concentration was observed when the Solver approach was used, according to the Pearson's correlation coefficient (Table 1), albeit the differences in this particular work are quite minute when the three methods are compared. It can be thus suggested that the Solver tool can be used in the estimation of kinetic parameters, as an alternative to the commonly used linearization methods.

The enhancement in the apparent K_M value observed for the sol–gel biocatalyst when compared to the free form, roughly 2.75 as predicted by non-linear regression, is suggestive of mass transfer/partition limitations resulting from the encapsulation, despite of the small particle size of the sol–gel capsules and of the nature of the silane precursor, which is the least hydrophobic among those typically used [31]. Preliminary runs performed allowed to establish that under the hydrodynamic conditions used the external limitations to mass transfer were negligible (data not shown). An increase in the apparent K_M for inulin as a

Table 1 Kinetics parameters obtained for the hydrolysis of inulin with free and sol–gel immobilized inulinase

Biocatalyst	K_M (g l^{-1})			V_{max} ($g_{reducing\ sugars}$ l^{-1} min^{-1})			Model	Pearson's correlation coefficient	
								Free	Immobilized
	Solver	L–B	H–W	Solver	L–B	H–W	Solver	0.99854	0.9951
Free	39	40	39	0.79	0.79	0.79	L–B	0.99853	0.9943
Immobilized	110	91	104	0.85	0.76	0.82	H–W	0.99854	0.9950

Constants were obtained by nonlinear regression analysis of experimental data using the Solver tool from Excel (Solver), using as criterion the minimization of the sum of the squares of the differences between experimental and predicted data, and by linearization of the Michaelis–Menten equation using Hanes–Woolf (H–W) and Lineweaver–Burk (L–B) plotting. Standard deviation never exceeded 10% of the individual data

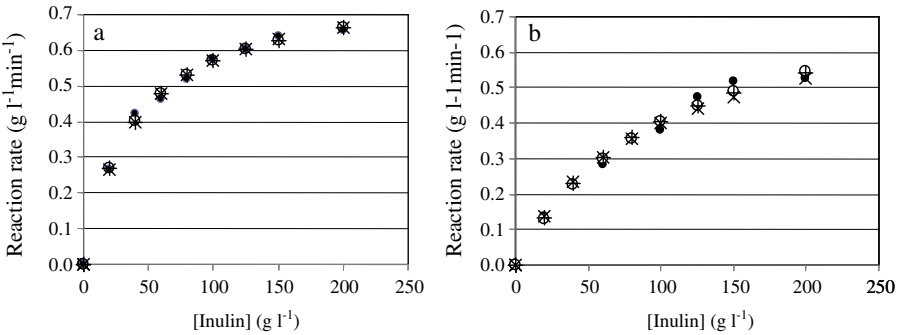

Fig. 4 Experimental (*black circle*) and predicted values of the initial reaction rate for inulin hydrolysis using free (**a**) and immobilized inulinase (**b**). Predicted values were obtained based on non-linear regression using the Solver tool from Excel (*white circle*) and by linearization of the Michaelis–Menten equation using the Lineweaver–Burk (×) or the Hanes–Wolf (+) approaches

result of immobilization was also reported for the immobilization of inulinase in PVA-based supports (1.7 to 1.9-fold) [10]; in alginate beads grafted with polyelthylenimine and glutaraldehyde (roughly 6-fold) [11]; and for the immobilization of whole cells with inulinase activity in gelatin (1.2-fold) [1]; in barium alginate (3.5-fold) [2]. On the other hand, V_{max} was roughly unaffected by sol–gel immobilization, suggesting no alterations of the native conformation of the enzyme resulting thereof, unlike reported data on the same parameter after immobilization on PVA-based supports [10], on Amberlite [47], on barium alginate [2] and on Sepabeads [43], where a decrease in said parameter was observed.

Biocatalyst Reuse

The likelihood of the successful implementation of a bioconversion process anchored in the use of an immobilized biocatalyst depends on the operational stability of the latter. In order to assess such feature, consecutive hydrolysis runs were performed using sol–gel immobilized biocatalysts. The possibility of using sol–gel particles doped with magnetite, thus providing a magnetic nature to the biocatalyst and potentially easing its separation

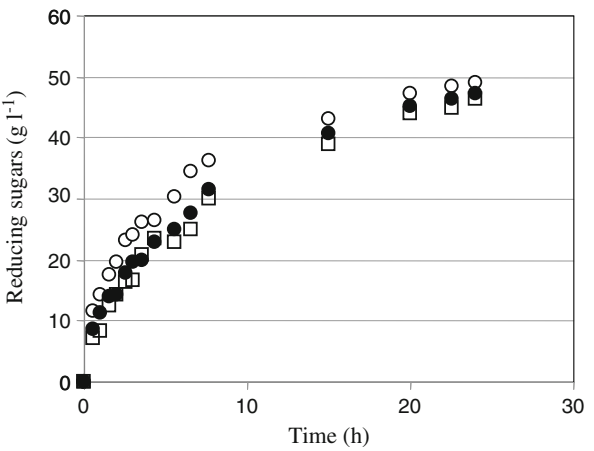

Fig. 5 Typical biotransformation batch run for the hydrolysis of a 5% (*w*/*v*) inulin solution in 100 mM acetate buffer pH 5.0 at 50 °C, using free inulinase (*white circle*), sol–gel immobilized inulinase (*black circle*) and magnetic sol–gel immobilized inulinase (*white square*)

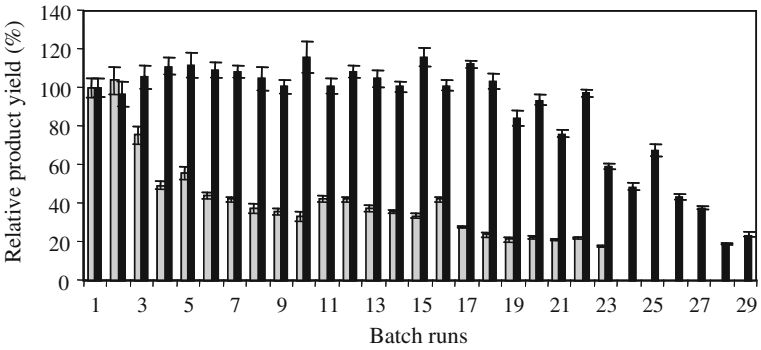

Fig. 6 Effect on the final product yield of the repeated use of inulinase immobilized in either sol–gel (*black bars*) or in magnetic sol–gel (*grey bars*) particles. A 5% (*w*/*v*) inulin solution in 100 mM acetate buffer pH 5.0 was used. Batch runs (24 h) were performed at 50 °C. Final fructose concentration in the first cycle at 50 °C was 47 (±4) and 46 (±2) g l^{-1} for inulinase immobilized in sol–gel and in magnetic sol–gel particles, respectively

from the biotransformation medium [3], was evaluated. The time course of typical hydrolysis runs was fairly similar for the two types of sol–gel biocatalysts, allowing for high product yield in 24-hour runs (Fig. 5). Simple sol–gel biocatalyst could be successfully reused for a little over 20 consecutive batch runs (Fig. 6), hence matching or exceeding the performance of similar runs performed with inulinase immobilized in Amberlite and in PVA-based supports [8, 10]. On the other hand, magnetic sol–gel particles displayed a gradual decay in activity throughout the consecutive batch runs, starting from the second consecutive run. From the visual inspection of the Eppendorf tubes where reactions were performed, a gradually increasing display of a grey shade was evident, suggesting magnetite leakage and eventually damaged biocatalyst particles. The latter feature was also reported when magnetic sol–gel particles were repeatedly used in 2-hour batch runs for penicillin G hydrolysis [3]. SEM images and SDS-PAGE analysis of the supernatant after

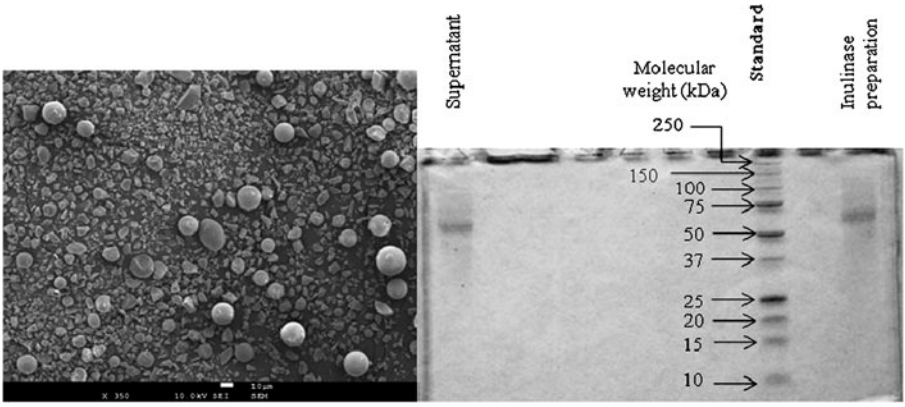

Fig. 7 SEM micrograph of magnetic sol–gel micro-particles with encapsulated inulinase (bar match 10 μm) after the fifth consecutive run (**a**), highlighting abrasion effects, and SDS-PAGE of the supernatant recovered from said run (**b**), evidencing the presence of inulinase

runs performed with magnetic particles validated such hypotheses (Fig. 7). Similar patterns started to be noticeable for the sol–gel particles after the 20th run (data not shown). The whole suggests that the use of a less aggressive bioreactor configuration (eventually plug-flow like) could be considered for continuous operation, since otherwise the operational stability of the sol–gel biocatalyst is promising.

Conclusions

A commercial inulinase preparation was entrapped in a silica xerogel matrix, based in a tetramethoxysilane precursor, leading to an immobilized biocatalyst with hydrolytic activity over inulin. Immobilization resulted in broadened temperature– and pH–activity profiles. In the latter case, a slight shift towards a less acidic environment was observed. Kinetic parameters were effectively evaluated using the Solver tool from Excel. Taking into consideration the said kinetic parameters of both free and immobilized forms of inulinase, it can be concluded that the native enzyme conformation was not apparently affected by the entrapment, although mass transfer resistances resulted thereof. The sol–gel biocatalyst was reused for more than 20 consecutive 24-hour batch runs without decay in the product yield. Doping the sol–gel with magnetite, aiming for eased separation from the biotransformation medium, apparently led to particles more susceptible to abrasion, hence hampering operational stability mostly due to mechanical susceptibility. The data gathered suggests the feasibility of developing a process for the production of fructose syrups using as substrate inulin, anchored in a sol–gel biocatalyst.

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