Lentikat®-based Biocatalysts: Effective Tools for Inulin Hydrolysis

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Original scientific paper
Received: June 1, 2009
Accepted: October 1, 2009

Dedicated to the memory of Professor Dr. Valentin Koloini

A commercial inulinase preparation from *Aspergillus niger* was immobilized into polyvinyl alcohol hydrogel lenticular particles (Lentikats®) and into hemispheric-shaped capsules, both based on the use of LentiKat® liquid. The characterization of the resulting biocatalysts, aiming at inulin hydrolysis to fructose, was performed, and the two methods of immobilization were compared. Temperature and pH profiles, as well as kinetic constants were determined, for both free and immobilized enzyme preparations. A broader-shaped curve was observed for the pH-activity profile when immobilized forms were compared to the free form. The apparent K_M of inulinase increased roughly 2-fold upon immobilization in either form of the support particles, suggesting diffusion limitations of inulin inside the gel. Long-term operation with immobilized enzymes proved unfeasible above 55 °C, due to the lack of mechanical stability of the supports tested. When the temperature of incubation was lowered to 50 °C, the hemispheric form of the immobilized enzyme displayed considerable long-term operational stability, since it allowed 20 repeated, consecutive batch-mode runs, with a final decay in product yield of 20 %. When inulinase immobilized in Lentikats® particles was used, the final decay in product yield was roughly 70 %.

Key words:
Inulinase, inulin hydrolysis, polyvinyl alcohol, enzyme immobilization, polyethylene glycol

Introduction

Inulin is a linear-linked fructose polymer that occurs as a reserve substrate in Jerusalem artichoke, chicory or dahlia tubers. This polymer is an acknowledged source for the production of fructose syrups, an alternative to multi-enzymatic starch hydrolysis, or to the less favored inverted sugar syrup production.[1] Inulin hydrolysis can also be performed chemically, but this approach leads to unwanted by-products and to colored compounds. Such shortcomings can be overcome if the enzymatic hydrolytic route, performed by inulinases, is used.[2] When inulin hydrolysis with inulinase is carried out under optimized conditions, a final concentration of fructose of 95 % can be expected, making this a promising approach for fructose production.[1,3] Within the scope of enhancing the enzymatic production of fructose from inulin, recent work has been presented, that focuses on the evaluation of different modes of operation, on detailed process modeling, and on the improvement or introduction of novel immobilization strategies.[4–7] The enzymatic approach is clearly favored if a suitable immobilization method can be used, since this strategy allows for high biocatalyst concentration in the bioreactor, hence favoring productivity, enables different bioreactor configurations and modes of operation, and simplifies downstream processing.[8] In the food industry, immobilization requires a readily available biocompatible support and simple immobilization method, in order to both cut down costs and complexity and comply with safety regulations.[9] Immobilization of inulinase for fructose production has mostly relied on covalent and ionic binding methods, although entrapment into alginate, chitin and casein has also been reported.[1,10] Polyvinyl alcohol (PVA) is a synthetic polymer that can be used to form hydrogels, and combines the biocompatibility of natural polymers with high mechanical strength.[11] Several methods have been developed to produce PVA capsules. Although these are mostly used for the immobilization of whole cells,[12] enzyme-polymer composites and enzyme aggregates have also been effectively immobilized in PVA beads.[13–15] The most commonly used methodologies for immobilization on PVA supports rely on mixing the biocatalyst suspension with a solution of PVA, and promoting the gelification by freezing and thawing[16] or by UV

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light,[17] or otherwise by extruding the mixture to a solution of boric acid or sodium sulfate, where the cross-linking between the PVA molecules is promoted.[18–26] One of the most recent approaches for immobilization in PVA relies on the controlled drying of a PVA-based hydrogel (LentiKat® liquid), and subsequent chemical stabilization, leading to lens-shaped particles.[27] This methodology has been applied to the hydrolysis of di- and oligosaccharides,[28,29] but not to the hydrolysis of polysaccharides such as inulin. In the present work, two methodologies for inulinase immobilization, based on the use of LentiKats® liquid, were evaluated: gelation of PVA by controlled drying, yielding lenticular shaped particles,[27] and a novel approach. In the latter, PVA is extruded into polyethylene glycol (PEG), where gelification occurs, and hemispheric-like capsules are formed. Inulin hydrolysis to fructose was used as the bioconversion model system. The kinetic parameters, pH and temperature profiles of free and both forms of immobilized inulinase were established, and the operational stability of the two forms of the immobilized biocatalyst was evaluated.

Materials and methods

Materials

Fructozyme L, a commercial preparation of inulinase from *Aspergillus niger*, was provided by Sigma (St. Louis, MO, USA), polyethylene glycol 600 (PEG 600) came from Fluka (Deisenhofen, Germany), LentiKat® liquid, a PVA-based material, and LentiKat® stabilizer came from GeniaLab (Braunschweig, Germany, further details on these materials at http://www.genialab.de/LentiDatasheet.php, assessed on 28 September 2009), and inulin from chicory (Fibruline Instant, with an average polymerization degree of about 10, and one terminal glucose unit) was a kind gift from Cosucra (Warcoing, Belgium). Acetic acid (96 %, for analysis) and acetic acid, sodium salt, anhydrous (99 %, for analysis) came from Acros Organics (Geel, Belgium). 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 Sigma (St. Louis, MO, USA).

Inulinase immobilization

Entrapment of inulinase in Lentikats® was performed according to the protocol provided by GeniaLab.[30] Briefly, the LentiKat® liquid was heated to 95 °C and then cooled to 40 °C. 0.5 mL of an enzyme preparation were added to 10 mL of the LentiKat® liquid and thoroughly mixed. The enzyme preparation consisted of a ten-fold diluted suspension of Fructozyme L. The dilution was performed in 100 mmol L^{-1} acetate buffer, pH 4.5. The resulting liquid was extruded through a needle (20 G x 1 1/4", Therumo Medical Corp., Somerset, NJ, USA) into Petri dishes to form small drops. These were left at room temperature, until roughly 28 % of the initial mass remained. LentiKat® stabilizer solution was then poured into the gel particles for re-swelling them to roughly the original size, and the whole stirred for 2 hours. The lens-shaped particles, with 3–4 mm diameter, and about 400 μm thickness, were harvested, thoroughly washed with acetate buffer, and soaked in the buffer for two hours, harvested and weighed after removal of excess buffer with qualitative filter paper, and either immediately used for bioconversion runs or stored at 4 °C until use. Alternatively, immobilized biocatalyst particles were prepared by extruding drop-wise the LentiKat® liquid containing inulinase into 150 mL of PEG 600, under gentle magnetic stirring. The LentiKat® liquid containing inulinase was prepared as described for the production of lens-shaped particles. After a 2-hour period, the formed hemispheric capsules, also with roughly 3–4 mm diameter, were harvested, thoroughly washed with acetate buffer, weighed after removal of excess buffer with qualitative filter paper, and either immediately used for bioconversion runs or stored at 4 °C until use. Immobilization yield was determined as the ratio between the specific activity of immobilized inulinase and that of the soluble inulinase, in a 30-minute run. Specific activity is defined as mg reducing sugars formed mg^{-1} protein s^{-1}. Runs were performed in 1.5 mL screw-capped, magnetically stirred (500 rpm) reactors, filled with 1.0 mL of 100 mmol L^{-1} acetate buffer (pH 4.5), containing 5.0 % (w/v) of inulin. All runs were performed at least in triplicate.

Determination of temperature and pH profiles, and kinetic parameters

Experiments for establishing the effect of temperature and pH in enzymatic activity were performed in a pH range of 4 to 6, and in a temperature range of 40 °C to 65 °C), using a 5.0 % (w/v) inulin solution (unless stated otherwise) in 1 mL of acetate buffer (100 mmol L^{-1}). For the determination of the kinetic parameters, enzymatic hydrolysis was performed over inulin solutions, with initial inulin concentrations ranging from 0.1 to 15.0 % (w/v). Runs were performed at pH 4.5 and 50 °C. In all cases, 50 mg of immobilized inulinase or 20 μL of a 40-fold diluted preparation of free inulinase were used. Runs were performed in 1.5 mL screw capped vessels, filled with 1.0 mL of inulin solution. Agita-

tion (500 rpm) was promoted by magnetic stirring. Samples (10 μL) were taken after 15 minutes and immediately assayed for reducing sugars. All runs were performed at least in triplicate. The concentration of the product formed during this incubation period was such that it allowed for the determination of initial reaction rates. This was established previously, and occasionally checked during the different bioconversion runs, by adapting the strategy presented by Doig and co-workers.[31] Briefly, four identical bioconversions were performed simultaneously, and samples were collected and analyzed at 0, 4, 8, 12 and 15 minutes. When single datum point per vessel was plotted for all vessels over time, the product evolution rate proved linear, as expected. Kinetic parameters were determined using the commercial software Leonora.[32]

Repeated batch hydrolysis

Reactions were performed in 1.5 mL screw-capped, magnetically stirred (500 rpm) reactors at either 50 °C or 55 °C. The reactors were filled with 1.0 mL of a 5.0 % (w/v) inulin solution in 100 mmol L^{-1} acetate buffer (pH 4.5), and with 50 mg of immobilized inulinase. Throughout each cycle (24-hours run), 10 μL samples were collected and assayed for reducing sugars. After each cycle, the immobilized biocatalyst was harvested, thoroughly washed with acetate buffer, 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. The quantification of protein was performed by the BCA method,[34] using a commercial kit from Pierce Biotechnology (Rockford, IL, USA). Particles of the immobilized biocatalyst were dissolved in distilled water heated at 70 °C, prior to protein quantification. Liquid supernatants, resulting from the immobilization procedures were also assayed for protein levels. The standard deviation from these determinations did not exceed 8 %, unless stated otherwise.

Results and discussion

Immobilization efficiency

Depending on the procedure for production of PVA-encapsulated inulinase, different shapes were obtained; the standard drying method leading to lens-shaped particles (Lentikats®), whereas extrusion of LentiKat® liquid into PEG 600 led to hemispheric particles. Lentikats® allowed slightly higher enzyme loading and activity retention upon immobilization, when compared to hemispherical beads, but the differences were not significant, considering the standard deviation (Table 1). No trace of protein was observed in the supernatants resulting from either immobilization method. Given the mild methodology used for immobilization, immobilization yields are most likely suggestive of diffusion limitations. Still, when immobilized biocatalysts were used, the final product yield after a 24-hour biotransformation period of a 5.0 % (w/v) solution of inulin was close to the one obtained with the free enzyme. This result clearly highlights the feasibility of this approach for producing highly performing immobilized biocatalysts.

Table 1 – *Immobilization of inulinase in Lentikat®-liquid based matrices*

Immobilized biocatalyst	Enzyme loading/ mg g^{-1} support	Immobilizaton yield/%	Final product concentration/ g L^{-1} [a]
Lentikats®	0.20 ± 0.05	27 ± 4	46 ± 3
Hemispheric particles	0.13 ± 0.02	21 ± 3	47 ± 2
Free enzyme	–	–	49± 4

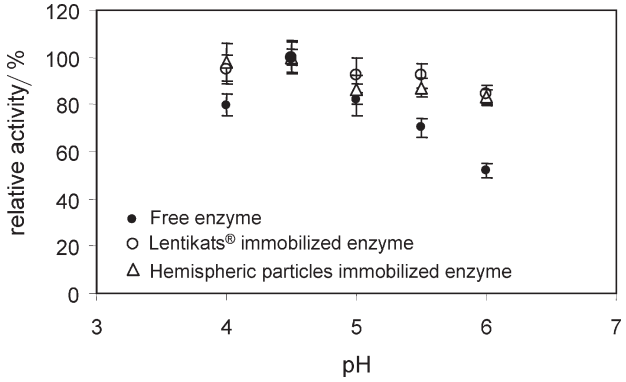
[a]Determined after a 24-hour biotransformation run using a 5.0 % w/v inulin solution in 100 mmol L^{-1} acetate buffer, pH 4.5 and 50 °C.

The activity retention upon immobilization in PVA matrices compares favorably, or at least matches data from other authors for the immobilization of inulinase in alginate and Dowex,[10] gelatin,[35] casein,[36] activated charcoal, porous silica beads, gelatin and gelatin-citrate.[37]

Effect of pH and temperature on enzyme activity

The influence of pH on the initial reaction rate of free and immobilized inulinase was evaluated in the pH range 4.0–6.0 at 50 °C (Fig. 1).

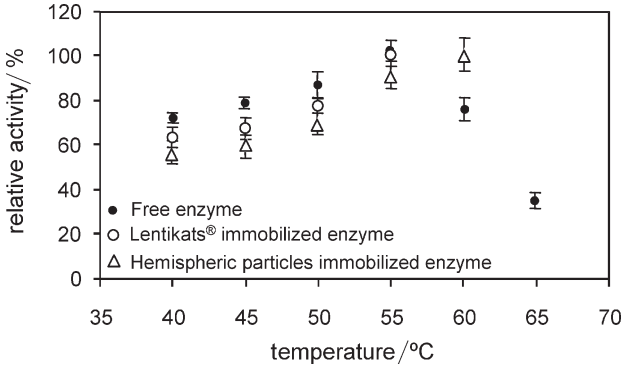
Both free and immobilized forms of the enzyme show an optimum pH for catalytic activity at 4.5. A much broader-shaped curve was nevertheless observed for the two immobilized forms of the biocatalyst, when compared to the free form, namely when the more acidic region of pH is considered. This trend can prove advantageous, since operation at lower pH values reduces the risk of microbial contamination. A broadened pH range upon immobilization was also reported for inulinase immobilization in Amberlite,[38] but was not observed for immobilization in DEAE-Sephacel or in QAE-Sephadex.[4] This effect was also reported for invertase immobilization in Lentikats®, and was



F i g . 1 – *pH profiles of free inulinase (●), inulinase immobilized in Lentikats® (○), and inulinase immobilized in hemispheric particles (△). Reactions were performed in 100 mmol L^{-1} acetate buffer containing 5.0 % (w/v) of inulin, at 50 °C. 50 mg of immobilized inulinase, or 20 μL of a 40-fold diluted preparation of free inulinase were used as biocatalyst. The maximum activity (mg fructose mg support s^{-1}) for free inulinase, inulinase immobilized in Lentikats® and inulinase immobilized in hemispheric particles was 1.8 (± 0.2), 0.51 (± 0.05) and 0.38 (± 0.06), respectively.*

tentatively ascribed to secondary interactions between the polymer and the enzyme and polymer matrix throughout the immobilization process.[29]

The influence of temperature on the initial activity of free and immobilized inulinase was evaluated at pH 4.5, in the range of 40 °C to 65 °C (Fig. 2).



F i g . 2 – *Temperature profiles of free inulinase (●), inulinase immobilized in Lentikats® (○), and inulinase immobilized in hemispheric particles (△). Reactions were performed in 100 mmol L^{-1} acetate buffer containing 5.0 % (w/v) of inulin, at pH 4.5. 50 mg of immobilized inulinase, or 20 μL of a 40-fold diluted preparation of free inulinase were used as biocatalyst. The maximum activity (mg fructose mg support s^{-1}) for free inulinase, inulinase immobilized in Lentikats® and inulinase immobilized in hemispheric particles was 2.0 (± 0.3), 0.62 (± 0.08) and 0.58 (± 0.06), respectively.*

Free inulinase displayed the maximum activity at 55 °C. The activity of the inulinase immobilized in Lentikats® steadily increased up to 55 °C. Above this temperature, the PVA-based matrix proved unstable and tended to melt. This behavior occurred almost immediately, once the lens-shaped particles were incubated at 60 °C. Such behavior, that clearly prevents the useful application of Lentikats® above 55 °C, was previously reported,[29] when the use of Lentikats® immobilized invertase for sucrose hydrolysis was assessed. Hemispheric shaped particles displayed slightly enhanced mechanical stability, since temperature-activity profile could be evaluated up to 60 °C, with biocatalytic activity also consistently increasing with temperature. Above 60 °C, however, the trend towards melting was also observed. Therefore, 60 °C was established as the temperature for maximum activity for inulinase entrapped in hemispheric beads. Under such conditions, it was possible to observe the increase of the optimum temperature for immobilized inulinase activity when compared to the free form.

Effect of immobilization in kinetic parameters

The trials for the determination of the constants of the Michaelis-Menten type kinetics, which fitted the enzymatic hydrolysis of inulin, were performed at pH 4.5 and 50 °C. Kinetic constants were determined from experimental data using Leonora software.[32] The apparent K_M for both forms of the immobilized enzyme was about 2-fold higher than that of the free inulinase (Table 2). This increase can most likely be ascribed to the impaired accessibility of the bulky inulin molecule to the active site of the immobilized enzyme, as a result of the diffusion barrier created by the matrix. The thin nature of the lenticular particles would be likely to lower diffusion hindrances, when compared to hemispheric particles. However, given the similarity of apparent K_M values for the two immobilized biocatalysts, such an effect could not be considered significantly noticeable. An increase in apparent K_M value, upon immobilization by entrapment in gelatin[35] or by adsorption in porous supports,[39] has also been reported. Immobilization also affected V_{max}, a roughly 4-fold decrease was observed, irrespective of the immobilization strategy, as compared to the free enzyme (Table 2).

T a b l e 2 – *Kinetics constants obtained for inulin hydrolysis with free and immobilized inulinase*

Biocatalyst	K_M/g L^{-1}	V_{max}/ g fructose g^{-1} enzyme s^{-1}
Free enzyme	26 ± 3	2.7 ± 0.1
Lentikats®	43 ± 8	1.1 ± 0.2
Hemispheric particles	49 ± 5	0.71 ± 0.02

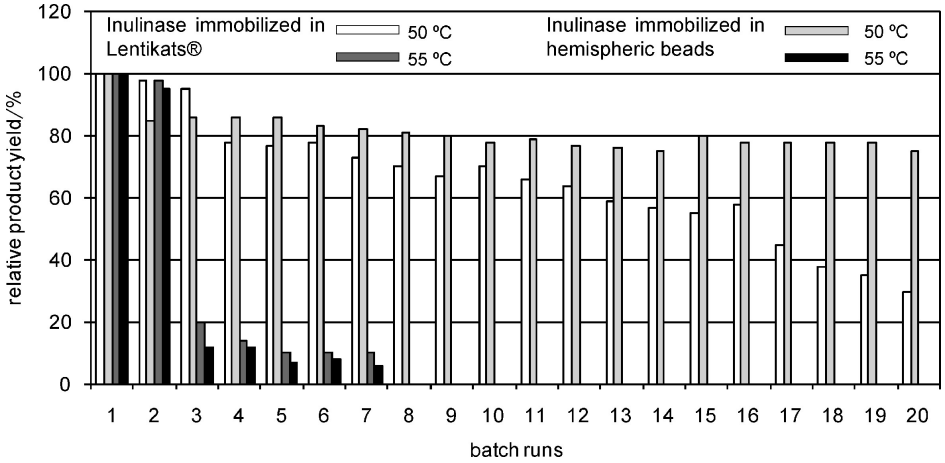


Fig. 3 – *Effect on the final product yield of the repeated use of inulinase immobilized in Lentikats® (□,■) and in hemispheric beads (■,■), for the hydrolysis of a 5 % (w/v) inulin solution. Batch runs (24 h) were performed at 50 °C (□,■) and 55 °C (■,■), at pH 4.5. Final fructose concentration in the first cycle at 50 °C was 46 (± 3) g L^{-1} and 47 (± 2) g L^{-1} for inulinase immobilized in Lentikats® and in hemispheric beads, respectively. Final fructose concentration in the first cycle at 55 °C was 47 (± 1) g L^{-1} and 48 (± 3) g L^{-1} for inulinase immobilized in Lentikats® and in hemispheric beads, respectively. In each run, 50 mg for the immobilized form of the biocatalysts was used. Standard deviation did not exceed 10 %.*

Biocatalyst reuse

The feasibility of the application of an immobilized enzyme system in a large-scale process clearly depends on its operational stability. In order to evaluate this key parameter in the presented system, repeated batch runs were performed at 50 °C and 55 °C, using both forms of the immobilized biocatalyst (Fig. 3). The reuse of both immobilized forms of the biocatalyst proved unfeasible at 55 °C, due to mechanical instability of the supports. This effect was also noticed in Lentikats® at 50 °C, which also seemed relatively sensitive to the shear stress induced by the magnetic stirring. Thus, along the repeated runs, some of the lens-shaped particles were partially broken. Hemispheric particles were successfully used at 50 °C throughout 20 cycles, displaying a final decrease in the relative product yield of only 20 %. In similar conditions, the final decrease in the relative product yield when Lentikats® were used was considerably higher.

Conclusions

Inulinase was effectively immobilized in polyvinyl alcohol particles, either as LentiKats® or in hemispheric beads, the latter obtained through extrusion of LentiKat® liquid into PEG 600. Immobilization broadened the pH-activity profile, paving the way for operation under relatively acidic conditions. The activity of free and LentiKats® immobilized enzyme reached a maximum at 55 °C, which was shifted to 60 °C when hemispheric beads were used. Still, in both immobilized preparations, a significant decay in mechanical strength was observed above 55 °C, which effectively limited to 50 °C their application in long-term operation. Diffusion limitations were observed as a result of entrapment. Hemispheric beads were successfully used throughout 20 consecutive batch runs with no apparent mechanical changes in the matrix and only a 20 % decrease in the final product yield. These results highlight the potential of inulinase immobilized in PVA-based particles obtained through extrusion into PEG as a suitable biocatalyst for application in large-scale processes for fructose production through inulin hydrolysis.

ACKNOWLEDGEMENTS

P. Fernandes acknowledges Fundação para a Ciência e Tecnologia (FCT) for contract under Programme Ciência 2007. M. P. C. Marques acknowledges Fundação para a Ciência e Tecnologia (Portugal) for financial support in the form of the PhD grant SFRH/BD/24433/2005.

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