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Immobilization of inulinase for sucrose hydrolysis

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Abstract

A commercial inulinase preparation immobilized on various supports was used for sucrose hydrolysis. Entrapment and encapsulation in Ca-alginate and entrapment in an alginate–silicate sol–gel matrix were evaluated. Best results were obtained with Ca-alginate beads. The influence of sodium-alginate concentration on the immobilization yield was assessed. Inulinase entrapped in Ca-alginate beads displayed high activity in the range 50–60 °C, whereas the optimum for the free enzyme was 60 °C. The optimum pH of the immobilized enzyme was slightly more acidic (4.0) than the one observed for the free form (4.5). The apparent K_M for sucrose of the immobilized inulinase was 184 mM, as compared to 82 mM for free inulinase, as a result of diffusion resistances.

Keywords: Biotransformation; Ca-alginate entrapment; Enzyme immobilization; Inulinase

1. Introduction

The current industrial approach for the production of inverted sugars syrups is based on the acidic hydrolysis of sucrose. This method can produce caramelized, off flavored and colored invert sugar (Arruda & Vitolo, 1999; Chen, Kang, Neoh, & Tan, 2000). Sucrose enzymatic hydrolysis provides an adequate tool to overcome such drawbacks, since the process is carried out under milder pH (4–5) and temperature (35–50 °C) as compared to pH 2 and 75 °C used in the chemical approach (Arruda & Vitolo, 1999; Ettalibi & Baratti, 2001; Sturm, 1999). Research work, aiming at improving the efficiency of this bioconversion process has revolved around the use of immobilized invertases (Arruda & Vitolo, 1999; Belcarz, Ginalska, & Lobarzewski, 2002; Emregul, Sungur, & Akbulut, 1996; Ginalska, Belcarz, Lobarzewski, & Wolski, 1999; Tanriseven & Dogan, 2001) different approach, relying on the use of a biocatalyst other than invertase, has been suggested. This is based on the activity for sucrose hydrolysis evidenced by crude enzyme preparations of inulinases from *Aspergillus ficuum* (Ettalibi & Baratti, 2001). Still, the use of an immobilized form of the biocatalyst is advised, due to the many advantages this approach provides. In this work, a screening of immobilized forms of a commercial inulinase preparation, Fructozyme L, for sucrose hydrolysis was performed. The highest immobilization yields were obtained with inulinase entrapped in Ca-alginate beads, a method compatible with the requirements of the food industry. Increasing the concentration of Ca from 1.5% to 4% led to a 15-fold increase in the immobilization yield. Shifts of the optimal pH and temperature from 5 to 4 and from 60 to 55 °C, respectively, were observed as a result of immobilization. Substrate inhibition was observed for sucrose concentrations above 0.7 M. The apparent K_M of sucrose hydrolysis was affected by immobilization, suggesting overall diffusion control.

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2. Experimental

2.1. Biocatalyst

Novozymes provided Fructozyme L, a commercial preparation of inulinases from *Aspergillus niger*.

2.2. Enzyme immobilization

Sodium alginate from *Laminaria hyperborea*, a material rich in guluronic acid residues, with guluronic- and mannuronic-block fractions of 60% and 40%, respectively, and a viscosity of 0.25 Pa s (in an aqueous polymeric solution 2% weight/volume), was obtained from BDH, UK.

Encapsulation: sodium alginate solutions of 1% and 1.5% were used. For the preparation of the cationic solution, xanthan was dissolved in a 4% calcium chloride solution, to give a final xanthan concentration of 0.4%. The enzyme preparation was added to the cationic solution as to achieve a final concentration of 21.3 $\mu g\,ml^{-1}$. About 2.2 ml of the cationic solution was then added drop wise to 50 ml of the sodium alginate solution. A gelation time of 30 min was observed. The capsules were rinsed with 100 mM acetate buffer pH 5.5 and hardened in 2% calcium chloride for 2 h, at 4 °C. The capsules were rinsed again with acetate buffer and incubated in 1.25% glutaraldehyde and 6% sucrose for 30 min at 4 °C. Beads were rinsed again with acetate buffer and stored in glycerol overnight. Prior to use for bioconversion trials, the capsules were thoroughly rinsed with acetate buffer.

Entrapment in alginate: entrapment of inulinase in Ca-alginate beads was basically performed as described by Gonçalves, Cabral, and Aires-Barros (1996). Thus, 6 ml of a 4% sodium alginate solution containing 600 µl of a solution of Fructozyme L diluted 100-fold in 0.1 M acetate buffer pH 5.5, was added drop wise to a 5.5% calcium chloride solution, except if stated otherwise. The gelling was allowed to proceed for 30 min at 4 °C. Beads were thoroughly rinsed with acetate buffer and used for bioconversion trials.

Entrapment in sol–gel: the procedure for inulinase immobilization was as described by Heichal-Segal (1995).

2.3. Sucrose hydrolysis

Bioconversion trials were performed in a magnetically stirred reactor, with 25 ml volume. Reactions were started by adding 1 ml of 1000-fold diluted solution of Fructozyme L to 14 ml of a sucrose solution in 0.1 M acetate buffer, or a given amount of immobilized inulinase. Bioconversion runs were performed in a temperature range of 30–80 °C, in a pH range of 3.5–6.5 and in a sucrose concentration range of 0.42–44 $g\,l^{-1}$. Samples (100 µl) were taken periodically, up to 12 min, and immediately assayed for quantification of reducing sugars. The activity immobilization yield, Y_{imm}, was calculated as the ratio of total activity yield of the immobilized preparation to the total activity of the soluble enzyme taken for immobilization (Munjal & Sawhney, 2002). Duplicate runs, at least, were performed.

2.4. Analytical methods

Quantification of reducing sugars was performed by the DNS method (Miller, 1959). Protein was quantified by the Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951).

3. Results

Formation of reducing sugars from sucrose in the experimental conditions used depended on the concentration of the biocatalyst (Fig. 1). Low immobilization yields were observed when both adsorption and sol–gel entrapment were used as immobilization methods (Table 1). This could be ascribed to low protein retention, in the former, or to enzyme deactivation due to exposure of the enzyme to the chemicals used for immobilization (e.g., tetramethoxy-*ortho*-silicate and hexane used to complete the polymerization process in sol–gel entrapment). Enzyme encapsulation did not provide a significant enhancement of the immobilization yield, as compared to entrapment in alginate beads, for similar sodium alginate concentration. Such low yields could be related to enzyme leakage through the capsule or through the pores of the gel, as a consequence of the low molecular weight of inulinases from *Aspergillus* spp. (roughly under 80 kDa) (Arand et al., 2002; Cho & Yun, 2002; Nakamura, Nagatomo, Hamada, Nish-

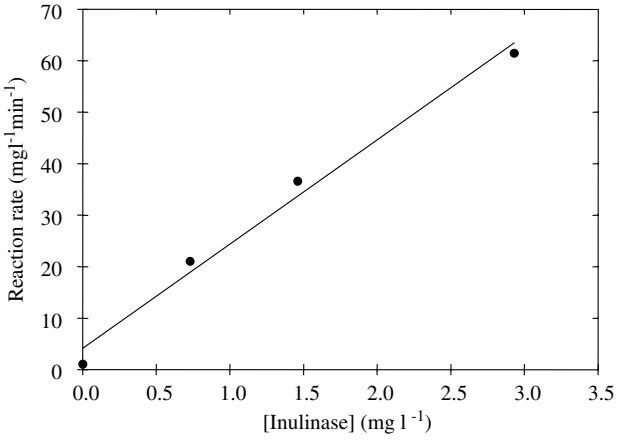

Fig. 1. Effect of inulinase concentration on the hydrolysis of a 25-$g\,l^{-1}$ sucrose solution.. Bioconversion runs were carried out at 55 °C and pH 5.5.

Table 1
Immobilization yields of Fructozyme L in different supports

Immobilization method	Alginate concentration (%)	Y_{imm} (%)
Encapsulation	1.0	2.2
	1.5	2.9
Entrapment	1.5	2.6
	2.0	3.2
	4.0	3.4×10^1
Sol–gel	1.5	1.2

Experiments were performed at 55 °C, in acetate buffer pH 5.5, and sucrose concentration of 3.33 g l^{-1}.
Standard deviation did not exceed 10%.

ino, & Ohta, 1994; Uhm et al., 1999). The entrapment of enzymes in hydrogels is often characterized by some diffusion of the biocatalyst from the support, particularly for enzymes with molecular weight less than 300 kDa (Tanaka, Kurosawa, Kokufuta, & Veliky, 1984; Tanriseven & Dogan, 2001). Still, this methodology has been effectively used for the immobilization of cutinase, a low molecular weight (22 kDa) enzyme (Gonçalves et al., 1996). Increasing to 4% the concentration of the sodium alginate solution used in bead preparation led to a significant increase in immobilization yield. This effect was ascribed to a decrease in enzyme leakage, as monitored during the time course of the bioconversion runs (data not shown), which could be related to a decrease in the gel pore diameter. Characterization of the immobilized system as related to temperature, pH and substrate concentration was performed, and compared to the free system. A shift in the optimum pH from 5 to 4 could be observed as a result of immobilization (Fig. 2). This pH shift could result from the H^+ partition between the inner part of the alginate beads and the bulk of the solution, a phenomenon often observed when an enzyme is immobilized in an inert matrix (Lartigue, 1975). Furthermore, a much broader-shaped curve resulted when the immobilized biocatalyst was assayed as compared to the free enzyme, as a result of the different microenvironment of the former system. An optimum pH of 5 was reported for inulinases from *A. ficuum* (Ettalibi & Baratti, 2001). A broader-shaped curve was also observed for the temperature-activity profile from 50 to 60 °C of the immobilized enzyme preparation, as compared to the free form (Fig. 3). The activity of the immobilized enzyme form was shifted from 60 to 55 °C as compared to the free form. A substrate inhibition model provided the best fit to the experimental results relating enzyme activity and substrate

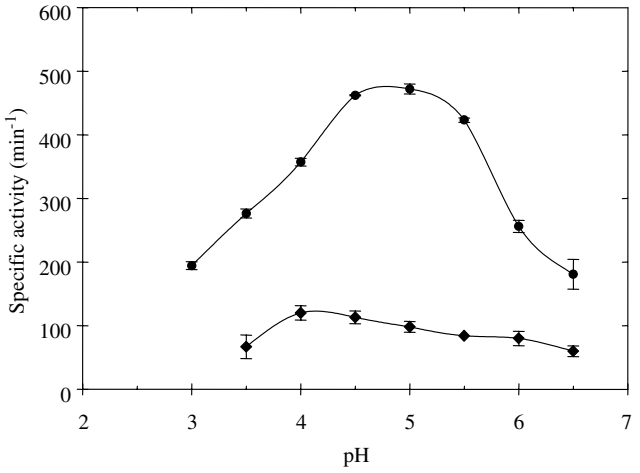

Fig. 2. Influence of pH on the activity of free (•) and immobilized (♦) forms of the enzyme preparation. Bioconversion runs were carried out at 55 °C. A 3.33 g l^{-1} sucrose solution was used.

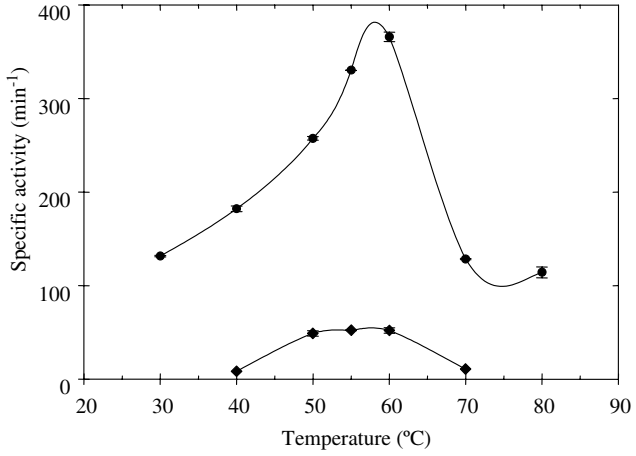

Fig. 3. Influence of temperature on the activity of free (•) and immobilized (♦) forms of the enzyme preparation. Bioconversion runs were carried out at pH 4. A 3.33 g l^{-1} sucrose solution was used.

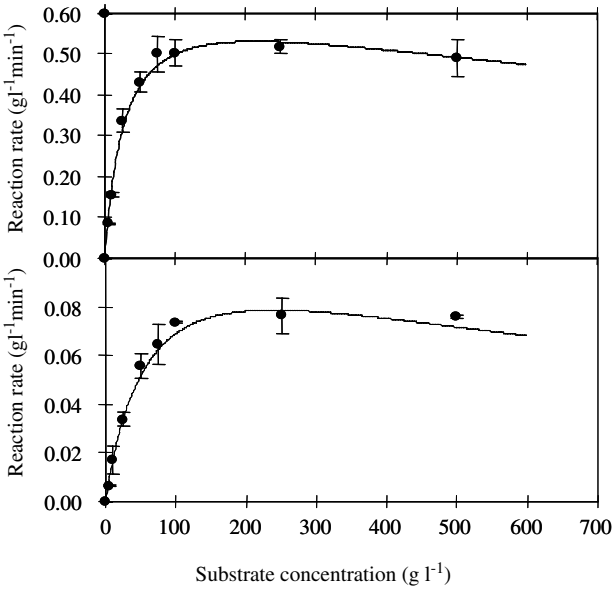

Fig. 4. Influence of the substrate concentration on the activity of free (a) and immobilized (b) forms of the enzyme preparation. Bioconversion runs were carried out at pH 4 and 55 °C.

concentration (Fig. 4(a) and (b)). A similar trend was reported for immobilized inulinases from *A. ficuum* onto porous glass beads (Ettalibi & Baratti, 2001). These authors reported K_M values for free and immobilized enzyme of 0.06 M (20.5 g l^{-1}) and 0.148 M (50.6 g l^{-1}), respectively. In the present work, the K_M values for free and immobilized enzyme were 0.082 M (28 g l^{-1}) and 0.184 M (63 g l^{-1}), respectively, suggesting significant diffusion restrictions. Inhibition constants K_I of 4.68 M (1600 g l^{-1}) and 2.67 M (912 g l^{-1}) for the free and immobilized enzyme were calculated.

4. Conclusions

Inulinases from *A. niger* were effectively entrapped in alginate beads, provided a 4% sodium alginate solution was used. The immobilized inulinase preparation showed less sharpened temperature- and pH-profiles as compared to the free form. Shifts of the optimum pH and temperature from 5 to 4 and from 60 to 55 °C were observed when free and immobilized enzyme preparations, respectively, were assayed. Substrate inhibition type kinetics provided the best fit for the experimental results, but diffusion resistances hampered the performance of the immobilized enzyme preparation.

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