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Sucrose hydrolysis by gelatin-immobilized inulinase from *Kluyveromyces marxianus* var. *bulgaricus*

Fabrício C. de Paula [a], Márcia Luciana Cazetta [a], Rubens Monti [b], Jonas Contiero [a,*]

[a] *Department of Biochemistry and Microbiology, Biological Sciences Institute, São Paulo State University-Unesp, Av. 24A 1515, Rio Claro 13506900, Brazil*
[b] *Department of Food and Nutrition, Faculty of Pharmaceutical Sciences, São Paulo State University-Unesp, Brazil*

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ABSTRACT

The crude cell-free medium from a culture of *Kluyveromyces marxianus* var. *bulgaricus* was immobilized in a gelatin-water support, with an immobilization yield of 82.60% for inulinase activity. The optimum pH for both free and immobilized inulinase was the same (3.5) and the optimum temperatures were 55 °C for the free and 60 °C for the immobilized enzyme. The Arrhenius plots were linear and activation energies were 56.20 (free enzyme) and 20.27 kJ/mol K (immobilized enzyme). The kinetic parameters were calculated by Lineweaver–Burk plots and the V_{max} and K_m were 37.60 IU/mg protein and 61.83 mM for the free inulinase and 31.45 IU/mg protein and 149.28 mM for the immobilized enzyme, respectively. The operational stability of the immobilized inulinase was studied in a continuous fixed-bed column reactor for 33 days, at the end of which the sucrose conversion was 58.12%.

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1. Introduction

Inulinases are 2,1-β-D-fructan fructanohydrolases (EC 3.2.1.7) which hydrolyze inulin, a fructose polymer found in many species of Compositae, e.g. in the tubers of Jerusalem artichoke and dahlia and roots of dandelion or chicory (Ettalibi & Baratti, 2001; Pandey et al., 1999; Vandamme & Derycke, 1983). This polymer, with its high content of D-fructose (>75%), is recognized as a raw material for the production of high fructose syrups, an alternative to the multi-enzyme hydrolysis of cornstarch or the less-favoured invert sugar production with invertases (Rocha, Catana, Ferreira, Cabral, & Fernandes, 2006; Vandamme & Derycke, 1983). However, the main inulin sources are plants sensitive to adverse weather conditions and their tubers are difficult to harvest (Guiraud & Galzy, 1990). Furthermore, inulin has a limited solubility at room temperature, and there is strong chance of microbial contamination at room temperature (Gill, Manhas, & Singh, 2006a).

The conversion of sucrose to high quality invert syrups is performed, in current practice, in acidic conditions, though enzymatic hydrolysis is preferable, to avoid unwanted by-products (Sturm, 1999). The yeast *Kluyveromyces marxianus* has been widely studied for the production of inulinase (Cruz-Guerrero, Garcia-Peña, Barzana, Garcia-Garibay, & Gomes-Ruiz, 1995; Vandamme & Derycke, 1983) and, besides inulin, its enzyme is able to hydrolyse sucrose, raffinose and levans (Guiraud & Galzy, 1990; Snyder & Phaff, 1960). Further, the inulinases from *K. marxianus* var. *bulgaricus* are thermostable and commercially available for industrial applications (Kushi, Monti, & Contiero, 2000). However, this approach is particularly effective if an immobilized enzyme is used, since this allows either biocatalyst reuse or continuous mode of operation and prevents contamination of the processed product (Rocha et al., 2006).

Inulinase immobilization has been evaluated by several authors (Ettalibi & Baratti, 1992; Gupta, Kaur, Kaur, & Singh, 1992; Nakamura, Ogata, Shitara, Nakamura, & Ohta, 1995; Wenling, Huiying, & Shiyuan, 1999; Yun et al., 2000) and the use of a cheap and simple, yet effective, immobilization method would be expected to play an important role in food industries in which fructose syrup is widely used (Katchalski-Katzir & Kraemer, 2000; Rocha et al., 2006; Schmid et al., 2001). Therefore, in this paper we report a simple method for the immobilization of inulinase in a gelatin matrix, a low cost immobilization support. We describe the properties of the free and immobilized enzyme and evaluate the gelatin-immobilized inulinase for continuous production of invert sugar syrup (equimolar fructose–glucose mixture) by hydrolysis of sucrose in a fixed-bed column reactor.

2. Materials and methods

2.1. Biocatalyst

The yeast strain *K. marxianus* var. *bulgaricus* (ATCC 16045), obtained from Department of Food Engineering, UNICAMP (Brazil), was grown in 2 L of a fermentation medium containing 1% sucrose, 0.5% yeast extract, 1% peptone, 0.5% KH_2PO_4, 0.15% NH_4Cl,

* Corresponding author. Tel.: +55 19 35264180; fax: +55 19 35264176.
E-mail address: jconti@rc.unesp.br (J. Contiero).

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0.12% KCl and 0.07% $MgSO_4 \cdot 7H_2O$ (w/v). The inoculum was incubated in a rotary shaker for 16 h at 30 °C and 180 rpm, in 200 mL of the fermentation medium. The batch culture was fed with a 20% (w/v) sucrose solution and maintained at 30 °C and pH 5.0 for 72 h, the pH being controlled automatically with 1 M KOH. The shaking and aeration rates were increased to 600 rpm and 1.0 vvm, respectively, during the fermentation. The fermented broth was centrifuged at 5600*g* for 20 min. The supernatant obtained was used as crude enzyme and for protein assays and enzyme immobilization.

2.2. Enzyme immobilization

The enzyme immobilization on gelatin support was carried out as described by Chatterjee, Kumar, and Sanwal (1990) with some modifications. Gelatin (500 mg) was dissolved in 5 mL of water at 50 °C, and thereafter allowed to cool to room temperature. To this, 0.5 mL (containing 1.69 mg of protein) of crude enzyme was added with constant stirring. After the suspension was gellified, 10 mL of 2.5% (v/v) glutaraldehyde was poured on the gel and left for 30 min. To remove excess glutaraldehyde, the resulting gel was washed with distilled water and cut into small pieces. Finally, the gel was suspended in 10 mL of 0.05 M citrate–phosphate buffer at pH 4.0.

2.3. Enzyme and protein assays

The enzymatic activity of the free and immobilized inulinase was determined according to Suzuki, Ozawa, and Maeda (1988), through the determination of reducing sugars formed by incubation of the 1 mL of enzyme in 2% (w/v) sucrose, citrate-phosphate buffer 0.05 M with pH 4.0, using the reagent 3,5-dinitrosalisylic acid, according to Miller (1959). Glucose (1 g/L) was used for the standard curve. One inulinase unit (IU) was defined as the amount of enzyme that released 1.0 μmol of reducing sugar per min under the above conditions. Protein content was measured as described by Lowry, Rosenbrough, Farr, and Randall (1951), using bovine serum albumin as standard. The protein content for immobilized enzyme was calculated from the protein concentration of the initial solution and the final concentration of unbound protein. The immobilization yield was calculated as the ratio of the specific activity of immobilized enzyme to that of the soluble enzyme. The enzyme and protein assays data represent a mean of three replicates.

2.4. Effect of pH and temperature on enzyme activity

The effect of pH on inulinase activity of the free and immobilized enzyme was determined in the pH range of 2.5–8.0, using 0.05 M citrate–phosphate buffer (pH 2.5–6.5) and 0.05 M phosphate buffer (pH 7.0–8.0), at 45 °C.

The effect of temperature on enzyme activity was measured at temperatures ranging from 25 °C to 70 °C, in 0.05 M citrate–phosphate buffer (pH 3.5). Thermal stability was estimated by measuring the residual activity after incubating the enzyme for various intervals up to 24 h at 45, 50 and 55 °C. The activation energies of the free and immobilized enzyme reactions were calculated from Arrhenius plots.

2.5. Estimation of kinetic constants

The Michaelis–Menten constants of the free and immobilized enzyme were determined from activity assays in the presence of sucrose at concentrations in the range 15–375 mM. The apparent values of K_m and V_{max} were calculated from the initial velocity rates by linear regression performed on Lineweaver–Burk plots.

2.6. Sucrose hydrolysis

A continuous fixed-bed reactor was operated in a column (15 mL) containing 12.5 g (dry weight) of the gelatin-immobilized inulinase for 782 h (33 days). The column was fed with 1% (w/v) sucrose solution in 0.05 M citrate–phosphate buffer (pH 3.5), at 45 °C, with a flow rate of 25 mL/h. The sucrose conversion (%) was measured by the amount of reducing sugars produced in the reaction.

2.7. Thin layer chromatography

Thin layer chromatography (TLC) on silica gel was used for qualitative analysis of the reaction products. Samples were spotted along the TLC plate and components were separated by a mobile phase of *n*-butanol:ethanol:water (5:3:2 v/v/v). The sugars were detected by heating after spraying the dried plates with a reagent solution containing 0.2% (w/v) orcinol:sulphuric acid:methanol (1:1:9 v/v/v). Fructose, glucose and sucrose were used as standard sugars.

3. Results and discussion

3.1. Immobilization of inulinase

The crude supernatant from the *K. marxianus* var. *bulgaricus* culture was immobilized in gelatin support by gel entrapment and then cross-linked by treatment with glutaraldehyde. The immobilization yield was 82.60% and the amount of bound protein was maximal for the tested support.

Several supports have been used to immobilize inulinase. The inulinase from *Aspergillus ficuum* was immobilized on porous glass beads of various porosities (Ettalibi & Baratti, 2001), which retained 70–77% of the enzyme activity. This data can be compared with inulinase from *Kluyveromyces fragilis*, which showed 22.5% and 54% activity retention when immobilized on tygon tube and amino ethyl cellulose, respectively (Kim, Byun, & Uhm, 1982), whereas the inulinase from *Fusarium oxysporum* exhibited 30–40% retention on DEAE–cellulose (Gupta et al., 1992).

Gill, Manhas, and Singh (2006b) immobilized the purified inulinase from *Aspergillus fumigatus* on chitin, casein and sodium alginate. They also reported the fraction of enzyme retained on the anionic exchangers DEAE–Sephacel (100%), QAE–Sephadex (100%), Dowex (63%) and Amberlite (39%) and also on the affinity matrix ConA-linked amino-activated silica beads (100%).

One disadvantage of most immobilization methods is that expensive supports are required. Further, it is necessary to use relatively large amounts of catalyst. Moreover, the covalent coupling techniques, commonly applied for enzyme immobilization, involve several reaction steps which promote chemical modifications of the biocatalyst. The enzymes immobilized in this fashion generally lose activity upon immobilization process (Woodward, 1985). The gelatin-immobilized inulinase presents relatively low mechanical resistance, when compared with amberlite and silica supports. However, the major advantage observed in this simple immobilization method is the low cost of the gelatin support associated with the high immobilization yield obtained (Assis et al., 2004). Furthermore, the gelatin gel has the advantage that the mass transfer resistance is relatively low compared to other entrapment methods (Woodward, 1985).

3.2. Effect of pH on free and immobilized inulinase

The effect of pH on enzyme activity was studied in the range of 2.5–8.0. The optimum pH for both the free and immobilized inulin-

ase was 3.5 (Fig. 1). The microbial inulinases are described as stable and active between pH 3.5 and 6.5 (Zittan, 1981). The pH optima of 4.0 and 4.75 were reported for free inulinase from *K. marxianus* var. *bulgaricus* by Cazetta, Martins, Monti, and Contiero (2005) and Kushi, Monti, and Contiero (2000), respectively. The maximum inulinase activity from *Kluyveromyces* sp. Y-85 was observed at pH 4.5 (Wenling et al., 1999). Pessoa and Vitolo (1999) described a high inulinase activity between pH 3.2 and 5.0. The immobilized inulinases from *F. oxysporum*, *A. fumigatus*, *Aspergillus niger*, *Aspergillus candidus*, *Aspergillus versicolor* and *A. ficuum* showed pH optima of 5.5, 5.5, 5.2, 5.5, 5.0 and 5.0, respectively (Ettalibi & Baratti, 2001; Gill et al., 2006b; Gupta et al., 1992; Kochhar, Gupta, & Kaur, 1999; Kochhar, Kaur, & Gupta, 1998; Nakamura et al., 1995).

3.3. Effect of temperature on free and immobilized enzyme

The effect of temperature on the activity of free and immobilized inulinase was studied in the range from 25 to 70 °C. The optimum temperature of the free inulinase was 55 °C, whereas the immobilized inulinase showed highest activity at 60 °C (Fig. 2). Kushi et al. (2000) and Cazetta et al. (2005), respectively, reported optimum temperatures of 55 °C and 60 °C for the free inulinase from *K. marxianus* var. *bulgaricus*. Wenling et al. (1999) obtained an optimum temperature of 50 °C for free inulinase and 55 °C for immobilized inulinase from *K. marxianus* Y-85. The maximum activity of immobilized inulinases from *F. oxysporum*, *A. niger*, *A. candidus*, *A. fumigatus* and *A. ficuum* was observed at 45, 50, 55, 60 and 70 °C, respectively (Gupta et al., 1992; Kochhar et al., 1999; Nakamura et al.; 1995; Gill et al., 2006b; Ettalibi & Baratti, 2001).

The Arrhenius plots were linear and the activation energy of the free enzyme was 56.20 kJ/mol K, whereas that of the immobilized inulinase was 20.27 kJ/mol K. Ettalibi and Baratti (2001) reported activation energies of 29.4 (free enzyme) and 26 kJ/mol (immobilized enzyme) for the inulinase from *A. ficuum*.

3.4. Thermal stability

The effect of temperature on enzyme stability was determined by monitoring the residual activities from 14.88 IU/mg protein (100%) for various intervals over a 24-h period of incubation. The enzyme immobilization resulted in an increase in thermostability at higher temperatures (Table 1). During the thermal stability assay, was observed an oscillation in the residual activity values up to 2 h of incubation. This might be due to the enzyme thermal adaptation. The residual activities of the free and immobilized enzyme were similar after 24 h at 45 °C, retaining 78.8% and 78.0% enzyme activity, respectively. However, the free inulinase lost its activity completely after 24 h at 50 °C. The same was observed after 2 h at 55 °C, whereas the immobilized enzyme retained 79.1% and 60.5% inulinase activity at these temperatures, respectively. Gill et al. (2006b) reported a higher thermal stability, with residual activity of 70% after 48 h at 55 °C, for the immobilized enzyme from *A. fumigatus*. The immobilized inulinase from *K. marxianus* retained 70% enzyme activity for 140 min at 60 °C (Bajpai & Margaritis, 1987). These data showed better values when compared with the immobilized enzyme from *A. ficuum*, which lost 50% activity after incubation for 60 min at 62 °C (Ettalibi & Baratti, 2001), and the immobilized enzyme from *F. oxysporum*, which retained 50% activity after 45 min at 50 °C (Gupta et al., 1992).

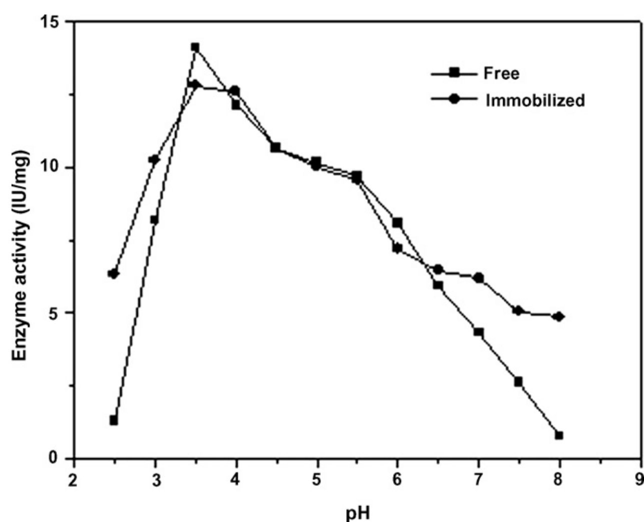

Fig. 1. Effect of pH on the activity of the free and immobilized inulinase from *K. marxianus* var. *bulgaricus*.

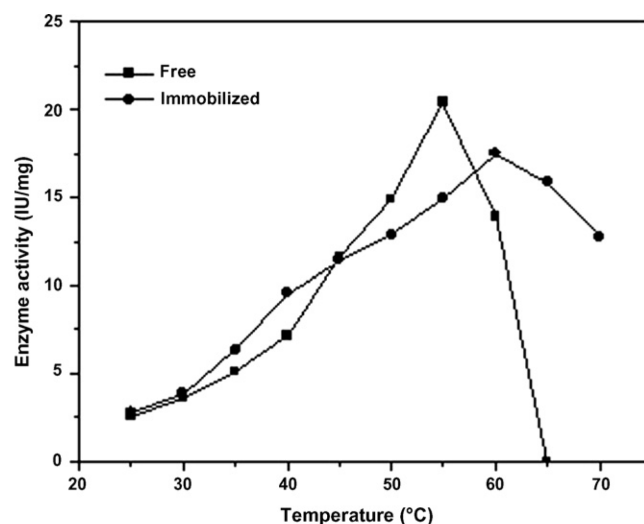

Fig. 2. Effect of temperature on the activity of the free and immobilized inulinase from *K. marxianus* var. *bulgaricus*.

Table 1
Residual activity of free and immobilized inulinase at different times during incubation at 45, 50 and 55 °C (the bioconversion medium contained 10 mL of 1 (w/v)% sucrose solution prepared in 0.05 M citrate–phosphate buffer at pH 3.5)

Time (h)	Residual activity (%)							
	0	0.25	0.5	1	2	4	8	24
45 °C								
Free	100	68.5	70.6	74.5	93.6	96.0	96.0	78.8
Immobilized	100	75.8	72.8	55.5	66.1	76.7	81.4	78.0
50 °C								
Free	100	58.2	56.6	56.6	61.9	46.2	21.3	0
Immobilized	100	74.7	73.8	87.5	84.9	78.1	75.8	79.1
55 °C								
Free	100	43.0	20.9	5.8	2.0	0		
Immobilized	100	66.7	73.9	72.3	69.6	66.3	61.4	60.5

3.5. Kinetic constants

The kinetic constants K_m and V_{max} were evaluated from Lineweaver–Burk plots (Fig. 3). The values obtained for V_{max} were 37.60 and 31.45 IU/mg of protein and the apparent K_m values were 61.83 and 149.28 mM, for the free and immobilized inulinase, respectively. These K_m values, observed with sucrose as substrate for both forms of the enzyme from crude extract of *K. marxianus*

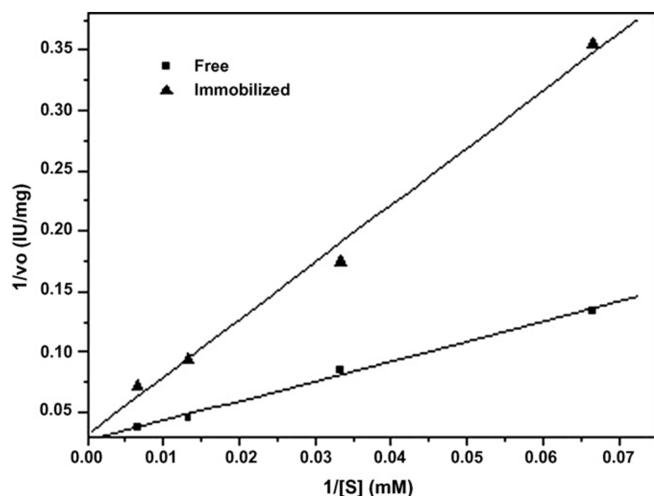

Fig. 3. Lineweaver–Burk plots of the free and immobilized inulinase from *K. marxianus* var. *bulgaricus*.

var. *bulgaricus*, were higher than those obtained with purified enzyme or with the substrate inulin, or with the inulinases from *A. fumigatus* (0.2–1.3 mM) (Gill et al., 2006b) and the immobilized enzymes from *F. oxysporum* (0.25–0.44 mM) (Gupta et al., 1992). For comparison, the immobilized inulinases from the *Kluyveromyces* genus showed K_m values in the range 7.7–10 mM for *K. fragilis* (Kim et al., 1982) and 13.3 mM for *K. marxianus* (Bajpai & Margaritis, 1987). However, the results obtained in this work are very similar to those of the free and immobilized inulinases from *A. ficuum*, for which K_m values of 60 and 148 mM, respectively, were reported (Ettalibi & Baratti, 2001).

3.6. Sucrose hydrolysis in continuous reactor

The fixed-bed column reactor filled with gelatin-immobilized crude enzyme was run continuously for 782 h (33 days) at 45 °C, with 1% (w/v) sucrose flowing at 25 mL/h (Fig. 4), during which time the sucrose conversion fell to 58.12% of the starting value. The half-life of the immobilized inulinase activity was estimated as 46 days. This data can be compared with inulinase from *A. fumigatus* immobilized on chitin, QAE–Sephadex and ConA-linked amino-activated silica beads, that exhibited half-lives of 35, 22 and 45 days, respectively (Gill et al., 2006b). The reaction products were analyzed by TLC (Fig. 5), which showed the hydrolysis of sucrose into reducing sugars (glucose and fructose) by immobilized inulinase.

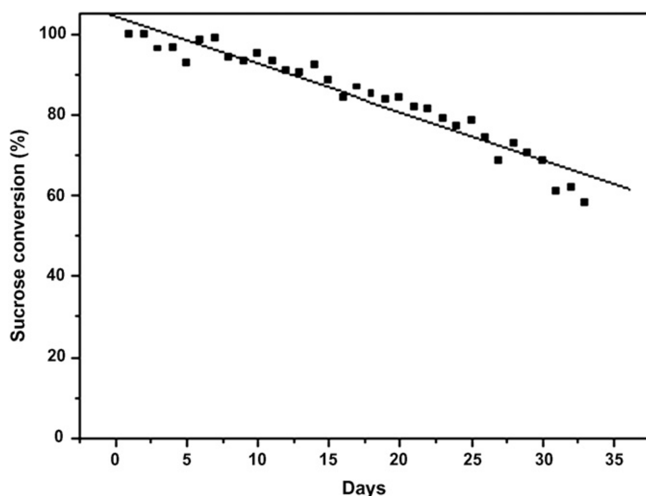

Fig. 4. Operational stability of the gelatin-immobilized inulinase in continuous production of reducing sugars up to 33 days.

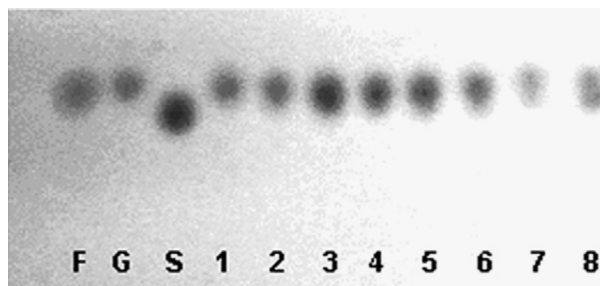

Fig. 5. Thin layer chromatography: qualitative analysis of the reaction products from sucrose hydrolysis by gelatin-immobilized inulinase after 52 (1), 104 (2), 205 (3), 302 (4), 425 (5), 518 (6), 639 (7) and 782 h (8) runs in a fixed-bed column reactor, at 45 °C. The standards were fructose (F), glucose (G) and sucrose (S).

4. Conclusion

The gelatin-immobilized crude inulinase from the culture medium of *K. marxianus* var. *bulgaricus* was assayed to obtain the optimum pH and temperature conditions, so as to maximize sucrose hydrolysis in a continuous fixed-bed column reactor. The optimum temperatures were 55 °C for the free inulinase and 60 °C for the immobilized inulinase. The optimal pH was 3.5 for both enzymes. The method of immobilization in gelatin followed by glutaraldehyde cross-linking resulted in an increase in thermal stability. The Michaelis–Menten constants were: K_m 61.83 (free enzyme) and 149.28 mM (immobilized enzyme) and V_{max} 37.60 (free) and 31.45 IU/mg protein (immobilized). The continuous reactor was operated for 33 days at 45 °C and the activity had a half-life of 46 days. To conclude, the gelatin is considerably cheaper than the most common immobilization supports and it is thus more suitable to the economic viability of the process. Summarizing, the results obtained in this study suggests a potential use of the gelatin-immobilized inulinase from *K. marxianus* var. *bulgaricus* in commercial processes for the production of high purity fructose syrups.

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