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Hydrolysis of sucrose by invertase immobilized on nylon-6 microbeads

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Abstract

A commercial extracellular invertase (EC 3.2.1.26) from *Saccharomyces cerevisiae* has been inmobilized by covalent bonding on novel microbeads of nylon-6 using glutaraldehyde. The enzyme was strongly bound on the support, immobilized with an efficiency factor of 0.93. The biocatalyst showed a maximum enzyme activity when immobilized at pH 5.0, but optimum pH activity for both immobilized and free invertases was 5.5. The optimum temperatures for immobilized and free enzymes were 60 and 65 °C, respectively. Kinetic parameters were determined for immobilized and free invertases: V_{max} values were 1.37 and 1.06 mmol min^{-1} mg^{-1}, respectively. The K_m and K_i values were 0.029 and 0.71 M for immobilized invertase and 0.024 and 0.69 M for free invertase. It was found that the thermal stability of the immobilized invertase with regard to the free one increased by 25% at 50 °C, 38% at 60 °C and 750% at 70 °C. The immobilized biocatalyst was tested in a tubular fixed-bed reactor to investigate its possible application for continuous sucrose hydrolysis. The effects of two different sugar concentrations and three flow rates on the productivity of the reactor and on the specific productivity of the biocatalyst were studied. The system demonstrated a very good productivity up to 2.0 M sugar concentration, with conversion factors of 0.95 and 0.97, depending on sucrose concentration in the feeding. This approach may serve as a simple technique and can be a feasible alternative to continuous sucrose hydrolysis in a fixed bed reactor for the preparation of fructose-rich syrup.

Keywords: Enzyme immobilization; Invertase; Nylon-6; Sucrose hydrolysis; Fructose-rich syrup

1. Introduction

Invertase, from *Saccharomyces cerevisiae*, is an enzyme catalyzing the hydrolysis of sucrose. The invert sugar produced has been used as fructose-rich syrups, principally for beverage industries. The application of enzymes for industrial purposes may be increased if they can be immobilized in an active state, but their use is limited for various reasons: economic, continuous operation and enzyme reuse. Enzymes have been immobilized on different supports, using methods such as adsorption, entrapment, microencapsulation or covalent immobilization (Arroyo, 1998; D'Souza, 1989; Mosbach, 1987). The main use has been in covalent immobilization. This technique involves linkage of any reactive component, generally the amine group, on the enzyme to an activated support, to produce a system free of diffusion limitation (with no porous support) and provides very strong binding. We have previously reported the immobilization of *Klyveromyces lactis* lactase, for lactose hydrolysis, in the same support with the aid of glutaraldehyde as cross-linking agent (Ortega, Morales, Montes, & Magaña, 1993). However, few systems have successfully reached a commercial operation level.

The present paper describes a simple way of comparing the results obtained with the enzyme invertase, both in solution and immobilized on nylon-6 microbead surface, using glutaraldehyde and discusses the potential for its

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application to the hydrolysis of concentred sucrose solution for the preparation of fructose-rich syrups.

2. Materials and methods

2.1. Enzyme

A commercial extracellular invertase (β-D-fructofuranosidase) of *Saccharomyces cerevisiae* (Fluka, USA), was used without any additional purification.

2.2. Enzyme immobilization

Nylon-6 was obtained from Celanese Mexicana, SA (México) as 2.0 mm × 2.0 mm pellets. Microbeads were prepared from pellets as previously described by Magaña-Plaza (pat 168411 México, 1993).

Invertase was covalently immobilized on to nylon-6 microbeads, previously activated with glutaraldehyde, using polyethylenimine (PEI) as spacer (Salleh, 1982). The procedure was adapted and optimized for this enzyme (Ortega et al., 1993) as follows: 10 g of nylon microbeads were suspended in 50 ml of coupling buffer (0.05 M sodium acetate, pH 5.0, 0.005 M L-cysteine) and 15 mg of protein per gramme of polymeric support were incubated for 24 h at 4 °C. After this time, the microbeads were washed and stored at 4 °C in 0.05 M acetate buffer, pH 5.5. The amount of bound protein was estimated by the difference between the initial and final amounts of protein in the supernatant and determined by absorption at 260–280–320 nm.

2.3. Invertase activity assay

The invertase activity was assayed as follows: 50 μl of immobilized enzyme suspension or enzyme solution was added to 3.0 ml sucrose solution (0.3 M in acetate buffer, pH 5.5) and incubated for 1 min at 50 °C The reducing sugars produced by sucrose hydrolysis were measured by the DNS method (Miller, 1959). One international unit (IU) of activity was defined as 1.0 μmol of hydrolyzed sucrose per minute under the assay conditions.

2.4. Effectiveness factor (Fε)

The effectiveness factor ($F\varepsilon$), of an immobilized enzyme reflects the efficiency of immobilization procedure and represents the retained activity after immobilization (Ahmad, Anwar, & Saleemuddin, 2001). The effectiveness factor ($F\varepsilon$) was calculated as

$$F\varepsilon(\text{moles/mg protein}) = (A_i/A_o - A_e),$$

where A_i represent the activity of immobilized enzyme, A_o is the activity of the enzyme added for immobilization and A_e is the soluble enzyme activity of remaining enzyme after immobilization. The free and immobilized invertase activities were determined as described above.

2.5. Determination of optimum temperature, pH and kinetic parameters

Optimum temperature and pH were determined by individually changing conditions of the invertase activity assay (pH from 4.0 to 7.0, temperature from 20 to 75 °C and sucrose concentration from 0.025 to 2.0 M). V_{max} and K_m were determined under optimum conditions and calculated from Lineweaver-Burk plot; K_i (inhibition constant) was calculated by Dickensheets, Chen, and Tsao (1977) plot.

2.6. Thermal stability

The thermal stabilities of free and immobilized invertase were determined by measuring the residual activity of the enzyme exposed to three different temperatures (50, 60 and 70 °C) in acetate buffer, 0.05 M, pH 5.5 for 2.5 h. Every 15 min time interval, an aliquot of free and immobilized invertase was removed and assayed for enzymatic activity as described above. The first-order inactivation rate constants (K_d) were calculated according to a method described by Akgöl, Kaçar, Denizli, and Arica (2001).

2.7. Packed bed reactor and its operation

For the continuous hydrolysis of sucrose, a packed bed reactor was used with a fixed layer of biocatalyst (260 mm diameter and 60 mm high). The reactor was equipped with a jacket connected to a water bath circulator for temperature control (50 °C). A diagram of the reactor configuration is shown in Fig. 1. The biocatalyst bed (1.1 and 1.3 g.) was fixed between two adjustable piston-like closures covered with nylon cloth sieves. Sucrose solutions (1.2 and 0.8 M) dissolved in acetate buffer 0.05 M, pH 5.5, were fed to the reactor from the top to the bottom by a multipurpose peristaltic pump (Brinkmann STA, Germany) at 3.1, 3.7 and 5.3 ml min^{-1}. All experiments were done in duplicate. The efficiency of the hydrolytic process was evaluated by the quantity of reducing sugars liberated in the reactor in unit time (μmol min^{-1}).

2.8. Sucrose analysis

Samples taken from the reactor were diluted. Sucrose hydrolysis was analyzed by measuring the reducing sugars

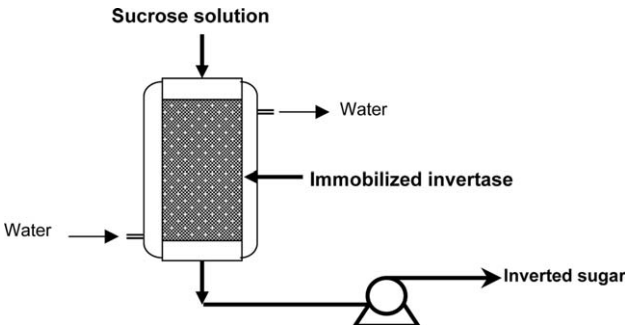

Fig. 1. Diagram of packed bed reactor.

using the DNS method (Miller, 1959). Equimolar mixtures of glucose and fructose were used as standards, prepared in solutions of different sucrose concentrations as controls. Total sucrose was analyzed by the same method after hydrolysis of residual sugar with HCl for 9 min in a boiling bath at 92 °C. Residual sucrose was defined as the difference between total and hydrolyzed sucrose.

2.9. Chemicals

White refined commercial sucrose from local sources was used as substrate. All other chemicals were of analytical grade.

3. Results and discussion

3.1. Invertase immobilization

The elaboration of methods for biocatalyst immobilization is conceived as one of the most important innovations in biotechnology. Enzyme coupling was completed after 24 h, 4.95 mg of protein per g of nylon-6 bonded. Fig. 2 shows that the coupling of invertase to activated nylon-6 microbeads is affected by pH. In order to study this dependence, the biocatalyst in a buffer with different pH values was examined. Preparations immobilized at pH 5.0 at 4 °C in 0.05 M acetate buffer showed the highest activity, 1200 IU mg^{-1} of protein coupling, under the assay conditions. The efficiency of immobilization, expressed as the efficiency factor ($F\varepsilon$) of 0.93, was obtained, which means that 93% of the immobilized protein retained its enzymatic activity. Ahmad et al. (2001) showed an ($F\varepsilon$) of 0.82, for invertase immobilized on lectin (*Cajanus cajan*), meaning that 82% of immobilized protein retained its enzymatic activity.

Immobilization of invertase, using alginate, was tried by various researchers, but it was found that alginate-immobilized invertase leaks out of beads and an additional process is required to prevent invertase leakage (Ro & Kim, 1991; Heichal-Segal, Rappoport, & Braun, 1995).

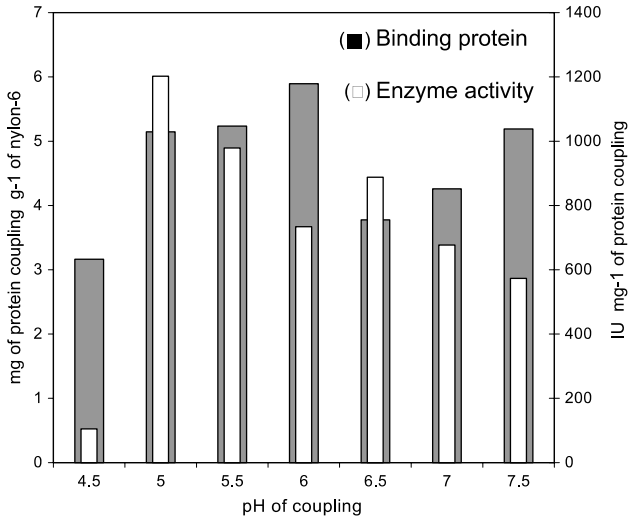

Fig. 2. pH coupling effect on invertase binding to nylon-6: (■) binding protein; (□) enzyme activity. For assay conditions, see Section 2.

3.2. Effects of pH and temperature

Characterization of the immobilized system, as related to temperature, pH and substrate concentration, was done and compared to the free system. The influence of pH on the sucrose hydrolysis ability of both free and immobilized invertase is shown in Fig. 3. There is a plateau of maximum activity in a wide range, from pH 5.0 to 7.0. However, the activity of both enzymes is 50% at pH values below 4.0. Fig. 4, shows the effect of incubation temperature on the sucrose hydrolysis. It is interesting to observe that the maximum activity of the immobilized invertase was found to be in a close range from 65 to 70 °C and 55 to 65 °C for the free enzyme. Both enzymes decreased their activity rapidly above 70 °C. Energy activation was calculated by Arrhenius plot in the temperature range from 20 °C to optimum. Activation energies were found to be 20.3 and 18.7 kJ mol^{-1} for free and immobilized invertase, respectively. These results

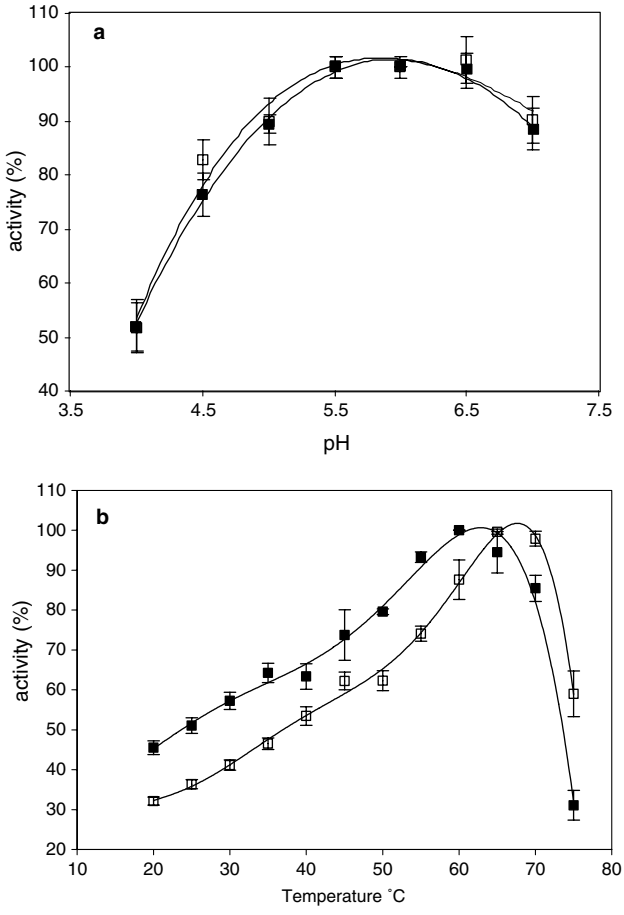

Fig. 3. (a) pH profiles of free and immobilized invertase. Free and immobilized enzymes were incubated in appropriate buffer. The buffers used for various pH ranges were acetate (pH 4.5–5.5), sodium citrate phosphate (pH 5.5–7.0). (b) Temperature profiles of free and immobilized invertase. Free invertase (□) and immobilized invertase (■).

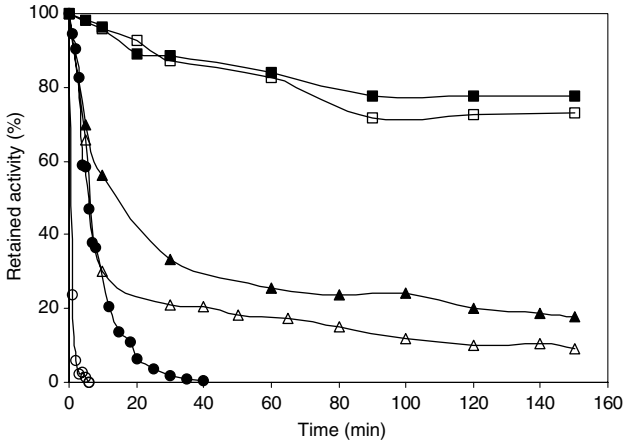

Fig. 4. Influence of temperature on the stability of free and immobilized invertase. Free invertase (□) and immobilized invertase (■) at 50 °C; free invertase (△) and immobilized invertase (▲) at 60 °C; free invertase (○) and immobilized invertase (●) at 70 °C.

suggest that the immobilized invertase is slightly more stable to temperature effects. Other researchers have obtained similar results upon immobilization of invertase and other enzymes (Arica, Denizli, Baran, & Hasirci, 1998; Erginer, Toppare, Alkan, & Bakir, 2000; Tien & Chiang, 1999).

3.3. Thermal stability of invertase immobilized on nylon-6

Fig. 4 shows the thermal inactivation of free and immobilized enzymes. At 50 °C, free and immobilized enzymes preserved 80% of their activities, but at higher temperatures, their activities decayed more than 60% in 10 min. These results show that the two enzyme forms have the same stability at high temperatures. In various immobilized systems, the immobilized form was inactivated at a slower rate than the free form (Germain & Crichton, 1986; Ulbrich, Shellenberger, & Damerau, 1986). The inactivation rate constants, K_d and half-life values for the free and the immobilized invertase at different temperatures are presented in Table 1.

3.4. Kinetic parameters

K_m and K_i values were not affected after covalent immobilization of invertase on nylon-6 with sucrose as substrate, but V_{max} value increased by approximately 30% with respect to the free after immobilization (Table 2). In the literature, there is an increase in K_m value for other immobilized invertases (Akgöl et al., 2001; Kotzelski & Staude, 1996; Tanriseven & Dogan, 2001; Tümtürk, Arslan, Disli, & Tufan, 2000). The results indicate that the immobilization method did not significantly affect this parameter.

Table 1
Half-lives ($t_{1/2}$) and inactivation rate constant (K_d) of the free and the immobilized invertase at three different temperatures

T (°C)	Free invertase		Immobilized invertase	
	K_d (min^{-1})	$t_{1/2}$ (min)	K_d (min^{-1})	$t_{1/2}$ (min)
50	1.0×10^{-3}	693	8.0×10^{-4}	866
60	4.3×10^{-2}	16	3.1×10^{-2}	22
70	8.8×10^{-1}	0.8	1.1×10^{-1}	6

Table 2
Properties of the free and immobilized invertase on nylon-6 microspheres

Parameter	Free invertase	Immobilized invertase
V_{max} (mol min^{-1})	1.06	1.37
K_m (M)	0.024	0.029
K_i (M)	0.71	0.69

3.5. Continuous production of fructose-rich syrup

For the continuous hydrolysis of sucrose, a packed bed system was fed continuously with inflow–outflow rates of 3.1, 3.3 and 5.3 ml min^{-1} of previously prepared substrate solutions. The temperature was maintained by pumping water in to the outer jacket using a thermoregulated water circulation system. Commercial sucrose dissolved in acetate buffer pH 5.0 was pumped into the column from the bottom using a peristaltic pump at the required flow rate. Less than 40 min was sufficient to hydrolyze more than 0.97 g of sucrose under the assay conditions, Sucrose concentration and flow rate were critical, as shown in Table 3. The column was thoroughly washed with acetate buffer and then again operated continuously under the same conditions. The use of nylon-immobilized invertase for the production of fructose-rich syrup has been demonstrated in this paper. Also, enzymatic hydrolysis using immobilized invertase avoids the production of coloured by-products generated by processes involving acidic hydrolysis.

3.6. Operational stability

The operational stability of the immobilized invertase in the packed bed reactor was monitored for 38 h. The operational temperature was set at 50 °C. The operational inactivation rate constant and operational half-life of the immobilization invertase under the assay conditions, were calculated as 4.5×10^{-5} min^{-1} and 1.54×10^{4} min (about 11 days). The operational stability could be increased if both a lower operational temperature and higher sucrose concentration were used. These results were also observed by Monsan, Combes, and Alemzadeh (1984).

3.7. Stored stability of immobilized enzyme

In general, enzymes are not stable during storage in solution and their activities are gradually reduced or lost through time. The storage stability of the immobilized invertases was determined under acetate buffer (0.05 M, pH 5.5) at 4 °C, and periodically sampled to determine their residual activity. They preserved 78% of their activity after 2.0 years with respect to its original activity. when the acetate buffer was changed to 4.5, the enzyme retained 95%

Table 3
Operation conditions of packed bed reactor

Immobilized invertase (g)	Sucrose concentration (M)	X	Rate flow (ml min^{-1})	Reducing sugar concentration (M)
1.1	1.2	0.95	3.1	2.28
1.1	0.8	0.97	5.3	1.55
1.3	1.2	0.95	3.7	2.28

X, convertion factor.

Table 4
Retained activity of immobilized invertase in different storage forms

Storage way	% Retained activity of immobilized invertase[a]
Sodium azide 0.015%[a]	76
Glycerol 20%[b]	80
Deionized water	85
Acetate buffer (0.05 M, pH 5.5)	78
Acetate buffer (0.05 M, pH 4.5)	95

In all cases, the samples were stored at 4 °C.
[a] After 2 years retention.
[b] Acetate buffer (0.05 M, pH 5.5) treated with sodium azide and glycerol.

of its activity under the same conditions. However, when the immobilized enzymes were stored in deionized water or in 20% glycerol, they preserved a good activity (more than 80%) as shown in Table 4. These results indicate that nylon-6 immobilization of invertase provided a stable environment and prevented the loss of activity that occurs when the enzyme was stored in solution at the temperature, and several enzymes can be immobilized using this immobilized method.

4. Conclusion

The immobilization of invertase on nylon-6 for the production of inverted syrups has been demonstrated in this paper. Invertase has been immobilized in several supports. Natural and synthetic supports have been chosen for their flow properties, low cost, non-toxicity and ease of availability. When compared to other supports, nylon-6 microbeads fulfil most of the above requirements. Lignocellulosic materials have gained importance as supports for enzyme immobilization because they are economic and are available as agro-inductrial byproducts (D'Souza & Melo, 2001; D'Souza & Godbole, 2002). The technique developed in this work could serve as a useful efficient covalent immobilization to yield functional groups on nylon-6 microbeads and the results obtained show an improvement in activity and stability when compared to the previously described ones, using other immobilization protocols (Akbulut, Sungur, & Pekyardimci, 1991; Akgöl et al., 2001; Arica, Thornener, Alaeddinodlu, Patýr, & Denizli, 2000; Krastanov, 1997; Marek, Valentino, & Kas, 1984; Monsan et al., 1984; Piedade, Gil, Cavaco, & Andrade, 1995; Tümtürk et al., 2000). This method could also be used for the immobilization of other enzymes. The invertase immobilized was stable over a wide range of pH's and temperatures and is similar to free enzyme. The immobilized enzyme was used in a packed bed reactor for 38 h without losing activity and could be used successfully in a continuous system for the production of inverted syrups from sucrose solutions. It could easily be recycled and recharged in column operation. A stable immobilized system and long storage life are good for a number of applications that would not be feasible with a soluble enzyme system. In addition, enzymatic hydrolysis using invertase avoids the production of coloured by-products generated by processes involving acidic hydrolysis. Moreover, not only pure sucrose solution but also sugar cane juice, can be inverted without difficulties and this work is in preparation at present in our laboratory.

In summary, the system described here satisfies many of the requirements for the sugar cane industry and could be operated before sugar crystallization. High efficiency and stability of the system and the reactor configuration are suitable for continuous operation. Studies to further characterize the packed bed reactor operated with sugar cane juice, are in progress.

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