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Cell wall invertase immobilization within calcium alginate beads

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Abstract

Yeast cell wall invertase (CWI) was immobilized within calcium alginate beads. The result of entrapment was the complete immobilization of all the added CWI. Three types of biocatalysts were prepared: CWI-S, CWI-155 and CWI-157. The optimum pH values were 4.5 and 5.0 for free and immobilized invertase, respectively. The optimum temperature was 60 °C, for both free CWI and CWI-S immobilizate. 80 °C was the optimum temperature for CWI-155 whereas the optimum temperature for CWI-157 ranged from 50 °C to 80 °C. Immobilized CWI was more stable than was free CWI above optimum activity temperatures. The activation energies were 32 kJ/mol for free CWI and 45, 21 and 25 kJ/mol for CWI-S, CWI-155 and CWI-157, respectively. The K_m values of free and immobilized CWI-157 were 28.4 mM and 72 mM, respectively. The V_{max} values were estimated as 4.5 mM/min and 0.42 mM/min, respectively. Immobilized CWI-157 was tested in a batch reactor using 70% sucrose (w/v). Complete sucrose conversion was achieved after 55 h. After 40 consecutive cycles, CWI-157 retained 90% of its activity.

Keywords: *Saccharomyces cerevisiae*; Cell wall invertase; Immobilization; Alginate

1. Introduction

Saccharomyces cerevisiae is an important source of enzymes that have found many applications in the food and drink industry (Buchholz, Kasche, & Bornscheuer, 2005). The enzyme invertase, which catalyses the hydrolysis of sucrose, is commercially produced from baker's yeast. Enzymatic hydrolysis of sucrose results in the formation of an equimolar mixture of glucose and fructose, known as invert sugar. The latter is sweeter and easier to incorporate in industrial preparations than is granular sucrose. It is widely used in the production of non-crystallising creams, jams and artificial honey and to a lesser extent in the industrial production of liquid sugar (Emregül, Sungur, & Akbulut, 2006; Hartmeier, 1988).

The application of enzymes for industrial purposes may be increased by their immobilization in an active state. Enzyme immobilization offers technical and economical advantages, such as cost-reduction of biocatalysts (as they can be reused many times), easy separation from reaction mixtures and the possibility of using higher enzyme activity per volume in the reactor, compared to soluble enzyme preparations (White & Kennedy, 1985).

Invertase, as well as whole yeast cells immobilized on different supports, has been used for invert sugar production. Invertase has been immobilized by adsorption, entrapment, microencapsulation and covalent immobilization (Amaya-Delgado, Hidalgo-Lara, & Montes-Horcasitas, 2006) on various insoluble carriers, including ion-exchange resins, synthetic polymers, animal charcoal, microporous glass, magnetite particles and other inorganic materials, and DEAE-cellulose, corn stover, triacetate-cellulose fibres, gelatin, hen egg-white, gluten proteins, wool fibres and different hydrogels (Bahar & Tuncel, 2002; Danisman, Tan, Kaçar, & Ergene, 2004; Emregül et al., 2006; Krastanov, 1997; Melo & D'Souza, 1992; Prodanović, Simić, & Vujčić, 2003; Prodanović, Jovanović, & Vujčić,

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2001; Sanjay & Sugunan, 2006; Tomotani & Vitolo, 2004). Various hydrogels have been used but calcium-alginate is considered to be the best matrix for invertase immobilization because it does not affect enzyme activity or its structure (Nakane, Ogihara, Ogata, & Kurokawa, 2001).

The main disadvantage of using soluble invertase for immobilization within alginate beads is loss of the enzyme in the gel-forming solution (Tanriseven & Doğan, 2001). Unfortunately, the immobilization yield decreases if the enzyme load during the immobilization process is increased. Therefore, the specific activity of a biocatalyst containing soluble invertase is rarely higher than 10–20 U/g (Arruda & Vitolo, 1999; Tanriseven & Doğan, 2001). Additional chemical treatments of invertase or alginate, in order to ensure complete invertase capture, are often too expensive and not applicable in the food industry. One such procedure is the attachment of soluble invertase to DE 32 (De Gooijer, Hens, & Tramper, 1989). Unfortunately, it was recognised a long time ago that ion-exchangers decrease the stability of immobilized invertase (Woodward & Wiseman, 1978).

Enzymes with molecular weights of 300 kDa or less, such as soluble invertase, leak out of alginate beads, resulting in over 80% loss (Ro & Kim, 1991). In some cases, leakage has been prevented by coupling invertase to concanavalin-A prior to entrapment within alginate beads (Husain, Iqbal, & Saleemuddin, 1985). Higher retention (66%) of invertase within alginate beads has been achieved using higher pH (Arruda et al., 1999), by combining immobilization with high pH and glutaraldehyde treatment (Tanriseven & Doğan, 2001) or subsequent chitosan treatment (Huguet, Neufeld, & Dellacherie, 1996).

Entrapment of whole yeast cells is another approach for overcoming this problem. However, by using this method it is impossible to obtain alginate beads with high enzyme activity as the whole cells have 1.5–5 times lower activity than has cell wall invertase (Hasal, Vojtíšek, Čejková, Kleczek, & Kofroňová, 1992; Rodríguez, Aguilar, & Padilla, 2000). Only whole yeast cell immobilization on materials treated with polyethylenimine could be considered in order to avoid diffusion problems. However, reactors with such a type of material have limited volumetric activity and a short life (8–16 cycles) (D'Souza & Melo, 2001). As whole cells are exposed in such biocatalysts, cell leakage from the reactor during harsh mixing conditions presents a serious concern, particularly if cell-free effluents are desired for the food industry.

To date there has been only one unsuccessful attempt to immobilize cell wall invertase (CWI) (Hasal et al., 1992). CWI was immobilized onto a reactive soluble polyethylenimine polymer crosslinked with glutaraldehyde. However, the obtained catalyst was very unstable during repeated batch sucrose hydrolysis cycles (Hasal et al., 1992).

In this work, CWI was immobilized within calcium alginate beads. A biocatalyst with a high enzyme load, without enzyme loss in the gel forming solution, without enzyme leakage and without chemical treatment of the alginate, was obtained. Various properties (pH and temperature optima and kinetic, thermal and storage stability parameters) of the biocatalyst were examined. In addition, the potential of the biocatalyst (its ability to hydrolyse a concentrated sucrose solution) for the preparation of invert sugar was tested in a repetitive batch process.

2. Materials and methods

2.1. Chemicals

Sodium alginate, sucrose, glucose and fructose were from Sigma (St. Louis, MO, USA). Sodium alginate Grindsted® Alginate FD 155 and 157 were generous gifts from Danisco (Copenhagen, Denmark). Bakers yeast was obtained from a local market. All other chemicals were analytical grade (or higher) and were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2. CWI preparation

Autolysis of baker's yeast was performed at 37 °C, using 3% toluene and 1% Na_2CO_3 for 4 h with occasional shaking. The obtained slurry was diluted with an equal volume of 1% sodium chloride and mixed for 30 min. The yeast cell wall was pelleted by centrifugation at 4000*g* for 15 min at 4 °C. The pellet was resuspended in five volumes of distilled water and the procedure was repeated until proteins could no longer be detected in the supernatant. The resulting solid material was defatted and dried using cold acetone. The pellet was left to dry overnight at room temperature. Dried CWI was kept at 4 °C until used (Espinosa, Schebor, Nudelman, & Chirife, 2004). The average yield (from various preparations) was 12 ± 1%, with a specific activity of 8000 IU/g.

2.3. Immobilization of CWI

Calcium alginate gel beads were prepared by dropping a mixture containing 10 ml of 2% sodium alginate and 10 ml of CWI suspension (in a range of 6–534 mg/ml) into 100 ml of 2% $CaCl_2$ with continuous stirring. The beads were left to harden for 2 h in the same $CaCl_2$ solution before being washed with 50 ml of 2% $CaCl_2$ and stored at 4 °C in 70% (w/v) invert sugar prior to use. Three types of biocatalysts were prepared, using different types of sodium alginate. Biocatalyst CWI-S was prepared using sodium alginate (Sigma). Biocatalysts CWI-155 and CWI-157 were prepared using Grindsted® Alginate FD 155 and FD 157 (Danisco), respectively.

2.4. CWI activity assay

The assay was performed in a batch reactor at 25 °C. 50 μl of free CWI suspension (1 mg/ml) was mixed with 450 μl of sucrose solution (0.3 M in 50 mM acetate buffer pH 4.5). After 5 min, the reaction was stopped by the addition of DNS reagent (500 μl). For immobilized enzyme

activity, 10 beads were taken (100 ± 3 mg) and mixed with 5 ml of sucrose. After 3 min, 500 μl of product mixture was mixed with 500 μl of DNS reagent. Reaction mixtures were heated in a boiling water bath for 5 min and then immediately cooled to room temperature. After dilution with 4 ml of water, the amount of reducing sugars was determined spectrophotometrically at 540 nm (Bernfeld, 1955) using a Philips UV/VIS/NIR PU 8630 spectrophotometer (L G. Philips-Displays, Blackburn, UK). One international unit (IU) of enzyme activity is defined as the amount of enzyme that hydrolyses one micromole of sucrose per minute under assay conditions.

2.5. Determination of optimum pH

The effect of pH was studied at 25 °C by varying the pH of the reaction mixture in the range of 2.0–8.0 (0.05 M glycine-HCl buffer at pH 2.0 and 2.5 was used, 0.05 M acetate buffer in the range of 3.0–5.6 was used and 0.05 M phosphate buffer in the range of 5.7–8.0 was used). The reaction conditions were as described in Section 2.4.

2.6. Determination of optimum temperature and thermal stability

The optimum temperature was determined by performing the reaction in the temperature range 20–90 °C (with 10 °C increments). The reaction conditions were as described in Section 2.4.

Thermal stability was ascertained at 50, 60 and 70°C. One milliliter of free CWI solution and 200 beads of immobilized enzyme were mixed with 0.05 M acetate buffer and allowed to incubate for various time intervals (for 14 days at 50°C, for 180 min at 60 °C and for 15 min at 70 °C), during which time enzyme activity was monitored as described in Section 2.4.

2.7. Determination of activation energies

The activation energies of free CWI and the immobilized CWIs were calculated using the Arrhenius equation after measuring enzyme activities at 20, 30 and 40 °C (Hartmeier, 1988).

2.8. Determination of kinetic values

Michaelis–Menten kinetics were established by studying the effect of substrate concentration (1–200 mM) on the reaction rate at 25 °C. K_m and V_{max} values were obtained using non-linear regression (Graph Pad Prism 3.0).

2.9. The batch process

The CWI-157 biocatalyst was tested in a batch reactor. Twelve gram of CWI-157 immobilizate was incubated in 100 ml of 70% sucrose at 50 °C with continuous stirring (100 rpm).

3. Results and discussion

3.1. CWI immobilization

The elaboration of methods for biocatalyst immobilization is conceived as one of the most important innovations in biotechnology (Amaya-Delgado et al., 2006). The amount of CWI that was used for immobilization was chosen after the maximum activity of immobilizate was obtained (Fig. 1). The appropriate amount of CWI was found to be 100 mg CWI in 1 ml of 1% alginate for all three types of alginate. The activities of CWI-S, CWI-155 and CWI-157 were 108, 74 and 71 U/g, respectively. The biocatalyst beads had a regular spherical shape with a diameter of 2.5 ± 0.1 mm. The immobilization of CWI within alginate beads was complete and without loss of enzyme. Our biocatalyst overcame previously described glutaraldehyde–polyethylenimine CWI disadvantages (Hasal et al., 1992). The immobilizate was stable which could have been a consequence of putting the CWI in the protective alginate gel matrix without any aggressive chemical treatment of CWI. Our biocatalyst could be made with a 0.5 mm bead diameter for sucrose concentrations below 1 M in which there are no diffusion barriers (Hasal, Čejková, & Vojtíšek, 1992). For more viscous sucrose solutions, larger alginate beads are more convenient, so the invertase reaction can be stopped at any desired point. Hence there is no enzyme leakage and it is easy to prepare syrups of defined characteristics for the food industry (Arslan, Tümtürk, Çaykara, Şen, & Güven, 2000).

3.2. Determination of optimum pH

The effect of pH on the activity of free and immobilized CWI preparations was studied at various pH values at 25 °C. The results are presented in Fig. 2. Free CWI showed

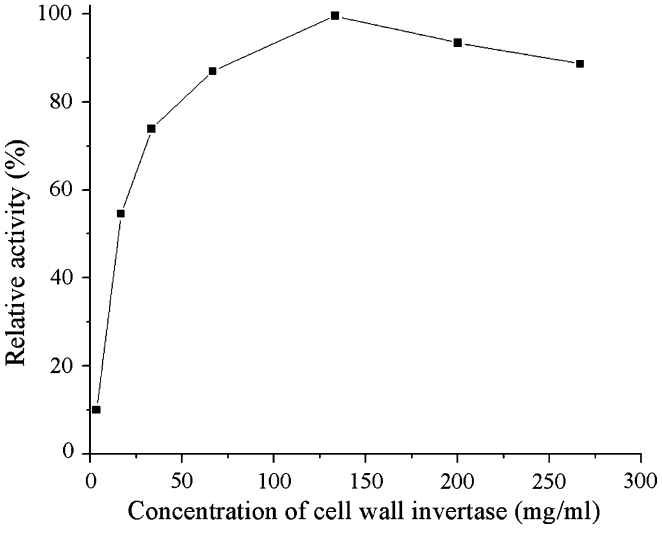

Fig. 1. Optimisation of immobilized CWI activity. CWI concentration is indicated in mg CWI in 1 ml of 1% alginate.

maximum activity at pH 4.5 whereas immobilized CWI was most active at pH 5.0. This shift in optimum pH was most likely due to the secondary interactions between the polyanionic carrier material and CWI. Similar observations concerning pH shifts upon immobilization have been previously reported (Akgöl, Kaçar, Denizli, & Arıca, 2001; Emregül et al., 2006; Tümtürk, Arslan, Disli, & Tufan, 2000).

3.3. Determination of optimum temperature and thermal stability

The effect of temperature on the activity of free and immobilized CWI preparations was studied. The results are presented in Fig. 3. The activities of free CWI and CWI-S were maximal at 60 °C. With increasing temperature, activity was rapidly lost. Due to this result CWI-S was not included in further experiments. CWI-155 had maximum activity at 80 °C whereas the optimum temperature for CWI-157 ranged from 50 °C to 80 °C.

The thermal stabilities of free CWI, CWI-155 and CWI-157 were determined at 50, 60 and 70 °C. At 50 °C the activity of free CWI dropped to 50% after 37 h, whereas no changes in CWI-155 and CWI-157 activities were apparent, even after 14 days (Fig. 4a). At 60 °C, free CWI lost 50% of its activity after 40 min, whereas CWI-155 and CWI-157 retained 60% and 75% of their activities after 180 min, respectively (Fig. 4b). At 70 °C, free CWI retained 20% of its activity after 1 min, whereas CWI-155 retained 20% of its activity after 6 min. CWI-157 was the most stable, as it retained 20% of its activity after 15 min (Fig. 4c). Clearly CWI-155 and CWI-157 were heat-inactivated at a much slower rate than was free CWI. CWI-157 was the most stable at high temperature. It is a generally accepted fact that thermally stable enzymes (enhanced by immobilization) represent valuable tools for a number of biotechnological processes. For this reason, CWI-157 was chosen for further experiments. Enhanced thermal stability

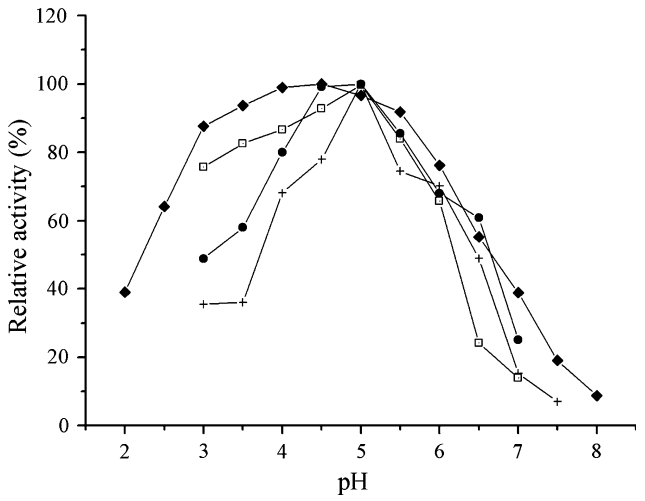

Fig. 2. The influence of pH on the activity of free CWI and immobilized CWIs. -♦-, free CWI; -●-, CWI-S; -+-, CWI-155; and -□- CWI-157.

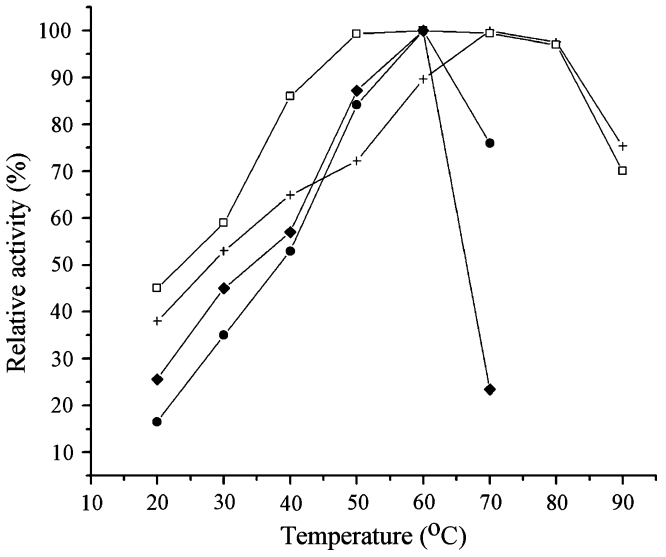

Fig. 3. The influence of temperature on the activity of free CWI and immobilized CWIs. -♦-, free CWI; -●-, CWI-S; -+-, CWI-155; and -□- CWI-157.

of immobilized enzymes has been previously reported (Akgöl et al., 2001; Arıca, Şenel, Alaeddinoğlu, Patir, & Denizli, 2000).

3.4. Activation energies

This is an important parameter within an immobilized enzyme system as it may indicate diffusion limitations (Bahar & Tuncel, 2002). It has been postulated that activation energies decrease as a result of the influence of intraparticle diffusion with increasing particle size (Miyamoto, Fujii, Tamaoki, Okazaki, & Miura, 1973).

Activation energies were found to be 32 kJ/mol for free CWI and 45, 21 and 25 kJ/mol for CWI-S, CWI-155 and CWI-157, respectively. This indicates that the CWI-155 and CWI-157 reaction systems were slightly limited by internal diffusion but were more stable at higher temperatures (Amaya-Delgado et al., 2006).

3.5. Kinetic values

The activities of free CWI and immobilized CWI-157, at various substrate concentrations, were plotted using a computer programme. V_{max} and K_m values were calculated using non-linear regression. The Michaelis constants (K_m) of free and immobilized CWI-157 were 28.4 mM and 72 mM, respectively. The V_{max} values were estimated to be 4.5 mM/min and 0.42 mM/min, respectively. This indicates that the formation of the enzyme–substrate complex is impaired in the case of immobilized CWI. Similar results have been reported previously (Tümtürk et al., 2000; Tanriseven & Doğan, 2001). The lower immobilized CWI V_{max} value than that of the free CWI is due to restricted diffusion of substrate through the beads (Prodanović et al., 2001).

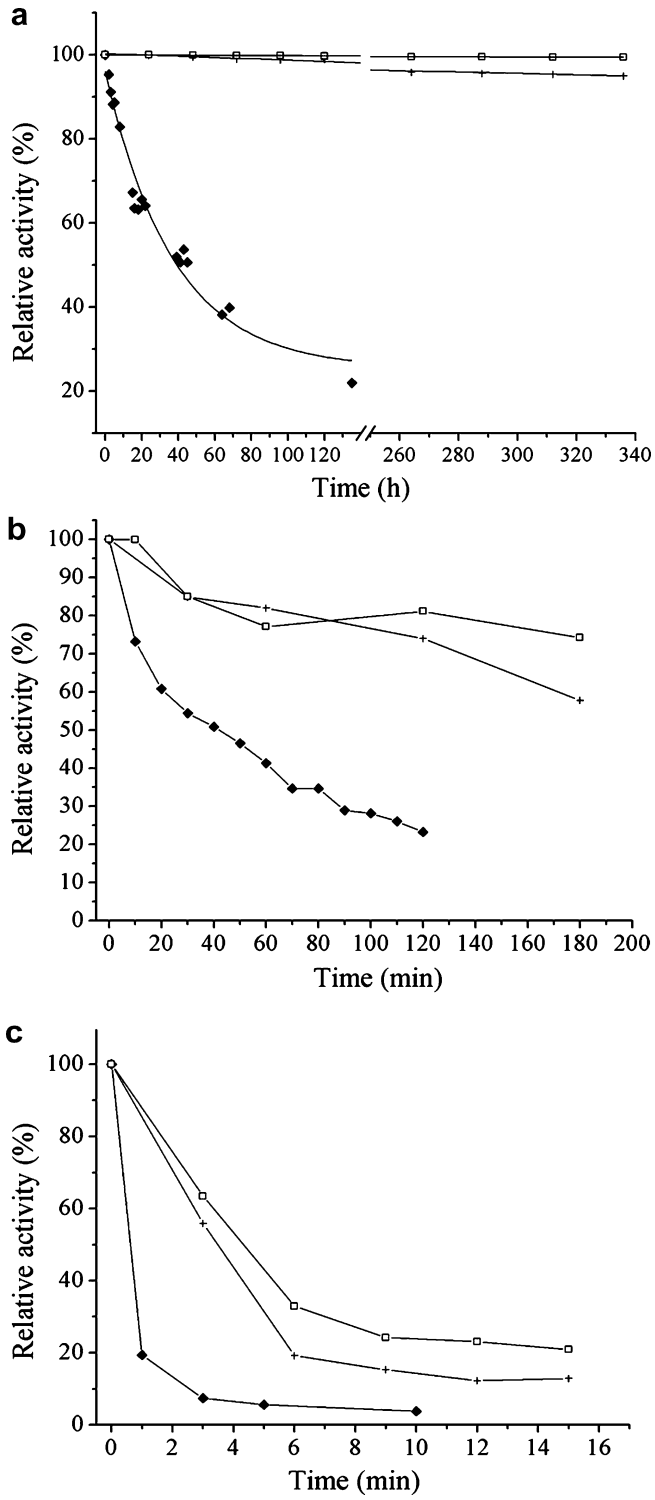

Fig. 4. Thermal stability of free CWI and immobilized CWIs: (a) 50 °C, (b) 60 °C and (c) 70 °C. -♦-, free CWI; -+-, CWI-155; and -□- CWI-157.

3.6. The batch process

For the continuous hydrolysis of sucrose, a batch reactor was set-up, containing CWI-157 in 70% sucrose solution buffered with 50 mM acetate, pH 4.5, with continuous stirring at 50 °C (maintained using a thermoregulated water bath). The degree of sucrose hydrolysis was monitored using DNS reagent at selected time points. Complete sucrose conversion was achieved after 55 h (Fig. 5).

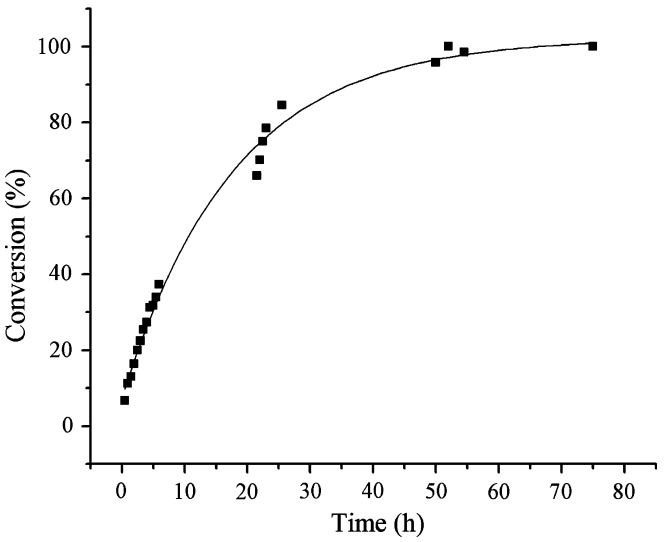

Fig. 5. Sucrose hydrolysis by immobilized CWI-157 in a stirred batch reactor.

In our batch reactor, the amount of reducing sugars produced/minute was at least 10 times higher than that produced by other biocatalysts consisting of immobilized invertase within alginate beads (Arruda & Vitolo, 1999; Tanriseven & Doğan, 2001).

3.7. Repeated use capability

Forty batch processes (each of 1 h duration) were sequentially run within a period of 5 days. The measured CWI activities are indicated in Fig. 6. After the 40th cycle, the immobilized CWI retained 90% of its original activity.

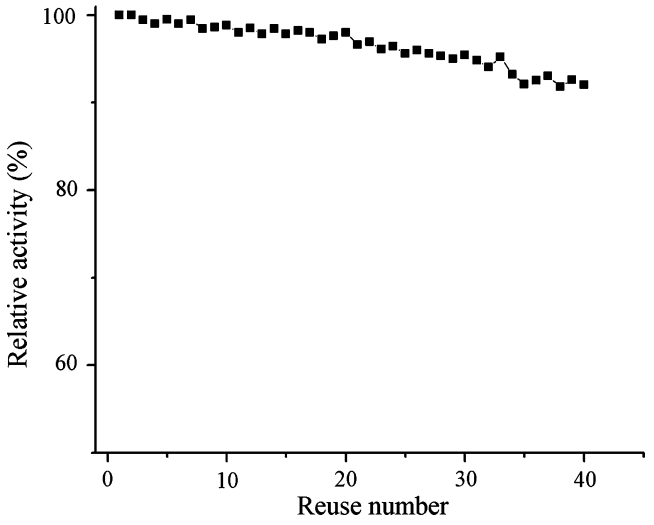

Fig. 6. The effect of repeated use on the activity of immobilized CWI-157.

4. Conclusion

In this study CWI was entrapped within calcium alginate beads. The immobilization of CWI within alginate beads was complete and without loss of enzyme. The total buried activity of CWI was 1400 U/g of biocatalyst. This provided a 'depot' effect (similar to that produced by drugs), thus allowing long operation stability of the biocatalyst (at least 40 batch cycles without considerable loss of activity). This is an attractive feature, as long-term operation and stability of biocatalysts are necessary in the food industry (Emregül et al., 2006).

Our obtained biocatalysts had enhanced thermal stability compared with the free enzyme, therefore extending their potential use. As complete conversion of sucrose was achieved after 55 h of continuous operation in the batch reactor, our biocatalyst may be successfully used for the production of invert sugar.

Another advantage of the bioreactor described in our study is its increased volumetric activity, that allows decreased reactor dimensions and reduced production costs (White & Kennedy, 1985). We estimate that the cost of producing 1 kg of our biocatalyst would be less then 5 $. The properties of our biocatalyst satisfy many of the requirements for continuous operation in the sugar industry.

Acknowledgement

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