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Immobilization of naringinase from *Aspergillus niger* CECT 2088 in poly(vinyl alcohol) cryogels for the debittering of juices

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Abstract

Naringinase, induced from *Aspergillus niger* CECT 2088 cultures, was immobilized into a polymeric matrix consisting of poly(vinyl alcohol) (PVA) hydrogel, cryostructured in liquid nitrogen, to obtain biocatalytically active beads. The effects of matrix concentration, enzyme load and pH on immobilization efficiency were studied. Between 95% and 108% of the added naringinase was actively entrapped in PVA cryogel, depending on the conditions of immobilization used. The optimal conditions were: 8% (w/v) PVA at pH 7 and 1.6–3.7 U ml^{-1} of enzyme load. The pH/activity profiles revealed no change in terms of shape or optimum pH (4.5) upon immobilization of naringinase. However, the optimum temperature was shifted from 60 °C to 70 °C and the activation energy of reaction, E_a, was decreased from 8.09 kJ mol^{-1} to 6.36 kJ mol^{-1} by immobilization. The entrapped naringinase could be reused through six cycles (runs of 24 h at 20 °C), retaining 36% efficacy for the hydrolysis of naringin in simulated juice.

Keywords: *Aspergillus niger*; Debittering; Enzyme immobilization; Naringin; Naringinase; Poly(vinyl alcohol) cryogel

1. Introduction

Naringinase, an enzyme complex consisting of rhamnosidase (EC 3.2.1.40) and flavonoid-β-glucosidase (EC 3.2.1.21), catalyzes the hydrolysis of naringin (naringenin 7-rhamnoglucoside) to naringenin, glucose and, rhamnose. This enzyme activity attracts growing biotechnological interest due to its role in debittering of citrus fruit juices (Jimeno, Manjón, Canovas, & Iborra, 1987; Puri & Banerjee, 2000; Tsen & Yu, 1991) and biotransformation of antibiotics and steroids (Thirkettle, 2000). Moreover, its hydrolysis products (rhamnose, prunin and naringenin) show biological activities and can be used as start materials for the synthesis of substances applied in pharmaceutics, cosmetics and food technology (Ellenrieder, Blanco, & Dax, 1998).

In order to minimize naringin content in citrus juices, naringinase has been immobilized on various supports, such as porous glass beads (Manjón, Bastida, Romero, Jimeno, & Iborra, 1985), crab shell chitin (Tsen & Tsai, 1988), sodium alginate (Puri & Marwaha, 1996), cellulose acetate films (Soares & Hotchkiss, 1998), hen egg white (Puri, Seth, Marwaha, & Kothari, 2001) or glutaraldehyde-coated woodchips (Puri, Kaur, & Kennedy, 2005). The use of immobilized enzymes in the food industry requires the ready availability of supports and cheap immobilization procedures. This means that supports must contain readily accessible reactive groups for easy and inexpensive activation, and they must also be totally non-toxic, with adequate stability. Poly(vinyl alcohol) (PVA), which is readily available, fulfills most of these requirements. Nevertheless, although PVA gel has been used as a carrier for immobilized cells and enzymes (Ariga, Kato, Sano, Nakazawa, & Sano, 1993; Giuliano, Schiraldi, Maresca, Esposito, & De Rosa, 2003), its use as an immobilization matrix for naringinase has not been reported and few papers have been concerned with the effect of PVA on the characteristic properties

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of the enzymes (Ariga, Tetsuya, & Sano, 1992). In addition, PVA hydrogel is easily produced by iterative freezing and thawing (IFT) of PVA solution and shows rubber-like elasticity (Lozinsky & Plieva, 1998). This property is advantageous for an immobilizing support used in a bioreactor with vigorous stirring. In fact, one of the substantial advantages of PVA cryotropic carriers is their very good physico-mechanical quality. The matrix is a non-brittle gel material which exhibits little abrasive erosion, even under conditions of intense stirring (Ariga et al., 1993), which contrasts with most other hydrogels commonly employed for enzyme entrapment (alginate, agar, carrageenan or polyacrylamide).

It is worth mentioning that the PVA itself is biologically compatible, non-toxic to organisms and a readily available, low-cost polymer (Chen & Lin, 1994; Ting & Sun, 2000). It is also an advantage that chemicals are not needed to form and stabilize the hydrogel. This gelling agent is reusable in that, after inactivation of an immobilized biocatalyst, it can be melted and reutilized for the preparation of a gel carrier *de novo* (Lozinsky & Plieva, 1998).

In this paper attempts have been made to immobilize naringinase by entrapment in poly(vinyl alcohol) cryogels. Immobilization parameters were determined, kinetic properties of the immobilized enzyme were characterized. These are discussed and its stability during continuous debittering of a simulated fruit juice system is studied.

2. Materials and methods

2.1. Materials

Naringin and diethylene glycol were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(vinyl alcohol), with a number average molecular weight, M_n, of 72,000, a degree of polymeration of 1600 and a saponification value >97.5%, was purchased from Fluka (Sigma Chemical Co., St. Louis, MO). Analytical grade chemicals and reagents were used.

2.2. Cell growth and enzyme production

Aspergillus niger CECT 2088 was obtained from the Spanish culture collection (Burjassot, Valencia) and maintained on Saboraud's dextrose agar (Difco, Becton Dickinsom, Sparks, MD). Fungus was precultured on Martinez-Madrid medium (Martinez-Madrid, Manjon, & Iborra, 1989) with 20% (v/v) orange peel extract (Larrauri, Borroto, & Boys, 1996) and this was used as inoculum. Naringinase was produced by inoculating 1 ml of spore suspension of precultured *A. niger* (10^7 spores ml^{-1}) into modified Martinez-Madrid medium (without fructose and containing 100 μg ml^{-1} of naringin as sole carbon source) with initial pH 7. The flask was incubated at 28 °C for 72 h on a rotary shaker (150 rpm). The cultures were filtered through a Whatman No. 1 filter paper. The cell-free filtrate was used as a source of crude naringinase and stored at 4 °C for further use.

2.3. Assay of naringinase activity

Soluble and immobilized naringinase were estimated by determining the naringin concentration using Davis' method (Davis, 1947). A reaction mixture of 1 ml of 500 μg ml^{-1} of naringin dissolved in 0.2 M acetate buffer (pH 5.0) and 1 ml of crude enzyme extract, or equivalent immobilized enzyme extract, was incubated for 1 h at 50 °C with constant shaking at 150 rpm. After the reaction, aliquots (0.1 ml) were removed and added to 90% diethylene glycol (4.8 ml), followed by the addition of 4 mM NaOH (0.1 ml). The assay mixture was allowed to stand at room temperature for 15 min and the intensity of the yellow colour developed was determined spectrophotometrically at 420 nm. A standard curve of naringin in the range of 0–250 μg ml^{-1} was plotted. One unit (U) of naringinase activity was defined as 1 μg of naringin hydrolyzed per minute under the above assay conditions.

2.4. Immobilization of naringinase in PVA cryogels

Poly(vinyl alcohol) and NaOH (1:0.025 w/w) were dissolved in 16 ml of deionized water by autoclaving for 15 min at 121 °C, cooled to room temperature and neutralized with HCl (35% w/w) before mixing with the crude enzyme extract from *A. niger* (4 ml). The mixture (total volume 20 ml) was dropped into liquid nitrogen, whereupon beads were formed instantly. The beads, frozen and thawed (4 °C) thrice, following the procedure of Giuliano et al. (2003), were shaken with deionized water for 2 h to remove the loosely entrapped enzyme, dried with sterile filter paper and stored at 4 °C until used.

To study the effect of enzyme concentration, four solutions of naringinase with concentrations ranging between 2.0 U ml^{-1} and 5.4 U ml^{-1} were assayed. Increasing (2.0, 2.4, 3.7 and 5.4 U ml^{-1}) concentrations of enzyme were obtained from the fungal crude extract (1.6 U ml^{-1}) by ultrafiltration using an Amicon 8050 cell (Millipore, Bedford, MA), with agitation and gentle pressure, equipped with a 30 kDa molecular weight cutoff polyethersulfone membrane.

2.5. Determination of kinetic parameters

The kinetic parameters for soluble and immobilized naringinase were determined by measuring the reaction rates (under the conditions above-mentioned) at substrate concentrations ranging from 30 μg ml^{-1} to 400 μg ml^{-1}. The Michaelis constants were calculated by analysing the data according to the EnzymePack program (Biosoft®).

2.6. pH and temperature/enzyme activity curves

The effect of pH, of both free and immobilized naringinase, was studied, using buffer solutions with pH values ranging from 5.2 to 12.6. The buffers were 0.1 M disodium

citrate (pH 1.2–5), 0.1 M acetate (pH 3.8–5.6), 0.1 M phosphate (pH 5–8.2) and 0.1 M Tris–HCl (pH 7.2–9.0).

The optimum temperature for hydrolysis of naringin was determined by measuring naringinase activity at seven different incubation temperatures over a range of 30–90 °C under the same experimental conditions as described before. The activation energies (E_a) were calculated from the Arrhenius law.

2.7. Reuse of the immobilized enzyme in simulated juice

The operational stability of immobilized naringinase (0.2 ml free enzyme g^{-1} of support) was determined by quantifying the reduction of naringin in a batch of 50 ml of simulated citrus juice at 20 °C in consecutive cycles of repeated use of the enzyme. The immobilized enzyme concentration in the juice was 10% (w/v) (PVA–naringinase beads). After each cycle of 24 h, immobilized particles were washed with 0.2 M acetate buffer (pH 5.0).

The simulated juice system contained 800 μg ml^{-1} of naringin, 0.48% of sucrose and 0.025% of citric acid. The final pH was adjusted to pH 3.2 with 4 M citric acid.

2.8. Statistical procedure

Multiple analysis of variance (MANOVA) was applied to all data with Statgraphics 4.0 plus for Windows. The results were expressed as level of significance (p) obtained for the effects of each variable studied ($p > 0.05$ was considered as not significant). Values shown in figures and tables represent the averages of at least three triplicate assays.

3. Results and discussion

3.1. Effect of PVA concentration, enzyme load and pH on immobilization process

The effect of poly(vinyl alcohol) concentration used to immobilize the naringinase from *A. niger* is shown in Table 1. The highest activity yield (91.6%) was observed when using a PVA concentration of 8% (w/v). Although a PVA concentration up to 10% could be achieved, production of the beads beyond such a high polymer concentration proved to be very difficult, due to the high viscosity of the mixture. The polymer concentration in the initial solution is of importance: the higher the PVA content in the system to be frozen, the higher is PVA cryogel strength and thermostability (Ariga et al., 1993; Yamura, Karasaka, Tanigami, & Matsuzaea, 1994); however, very concentrated solutions of PVA are excessively viscous for producing small and uniformly distributed beads, especially when the polymer MW exceeds 60–70 kDa (Lozinsky & Plieva, 1998). On the other hand, Lozinsky and Plieva (1998) demonstrated that, upon increasing the PVA concentration, both the amount and size of the macropores decreased and the thickness of the pore walls increased; hence, by regulating the composition of the initial systems, the porosity of the gel matrix can be controlled to a certain extent. Thus, at 4% (w/v) PVA concentration, the pore dimensions are absolutely large enough for the unimpeded diffusion of enzyme molecules, resulting in a negligible activity retention (0.27%); on increasing the concentration to 6% or 8%, this carrier reliably retains enzymes (74% and 91.6% activity retention, respectively) which are entrapped within the matrix during the gel formation.

The efficiencies of immobilization obtained when naringinase was immobilized at different pH values are shown in Table 2. The maximum activity retention of naringinase (92.1%) was obtained at neutral pH. In contrast, significant activity decreases were observed at acid and alkaline pH values. Polyvinyl alcohols are hydrophilic polymers whose concentrated aqueous solutions are capable of gelling *per se* with the formation of a non-covalent spatial networks. The major types of intermolecular links in the junction knots of similar networks are the hydrogen bonds between OH groups of neighboring polymer chains (Fujii, 1971). These junction knots arise only when the OH groups are free to participate in interchain interactions (Lozinsky & Plieva, 1998). The present investigation shows that neutral pH facilitates the gelation process, as well as causes the minimum impact on naringinase activity. With 8% PVA at pH 7, maximal naringinase activity was recorded, indicating an optimal porosity for retention of naringinase, inflow of the substrate, and outflow of the end product.

The effect on enzyme concentration in the PVA solution on the extent of activity retention is presented in Table 3. Activity retention was optimal (95.8%) at 1.6 U ml^{-1} of enzyme load, improved to 108% and 102% at 3.7 and 5.4 U ml^{-1}, respectively, and slightly decreased to 80–90% at 2.0–2.4 U ml^{-1} of enzyme concentration.

Table 1
Effect of PVA concentration on immobilization of naringinase from *A. niger*[a]

PVA concentration (%, w/v)	Activity of immobilized naringinase (U g^{-1} support)	Activity retention (%)[b]
4	0.001 ± 0.007	0.27
6	0.278 ± 0.010	74.0
8	0.344 ± 0.014	91.6

[a] Immobilization conditions: 0.2 ml free enzyme g^{-1} support; pH 7.
[b] It is calculated as activity of immobilized enzyme/activity of free enzyme. Activity of free enzyme = 1.87 ± 0.044 U ml^{-1}.

Table 2
Effect of pH on immobilization of naringinase in PVA beads[a]

Immobilization pH	Activity of immobilized naringinase (U g^{-1} support)	Activity retention (%)[b]
4	0.079 ± 0.011	21.9
5	0.161 ± 0.030	44.8
6	0.282 ± 0.023	78.4
7	0.332 ± 0.018	92.5
8	0.285 ± 0.036	79.3

[a] Immobilization conditions: 0.2 ml free enzyme g^{-1} support; 8% (w/v) PVA.
[b] Activity of free enzyme = 1.80 ± 0.090 U ml^{-1}.

Table 3
Effect of enzyme concentration on naringinase immobilization on PVA cryogels[a]

Enzyme concentration[b]	Activity of immobilized naringinase (U g^{-1} support)	Activity retention (%)[c]
5.4	1.10 ± 0.023	102
3.7	0.804 ± 0.044	109
2.4	0.389 ± 0.017	81.0
2.0	0.360 ± 0.001	90.1
1.6	0.307 ± 0.015	95.8

[a] Immobilization conditions: 0.2 ml free enzyme g^{-1} support; 8% (w/v) PVA; pH 7.
[b] U ml^{-1}.
[c] It is calculated as activity of immobilized enzyme/activity of free enzyme.

Researchers have reported naringinase immobilization using several methods and carriers, with varying results. For instance, naringinase produced by *Penicillium decombens* has been immobilized by entrapping the enzyme in alginate beads, hen egg white cross linked with glutaraldehyde, gelatin open pore matrix and covalently attached to seeds of *Ocimum basilicum* through ethylenediamine arms (Norouzian, Hosseinzadeh, Inanlou, & Moazami, 1999). Immobilization by covalently attachment was found to be more effective than were the other three methods; 57% of the total enzyme activity was attached to the seeds. Puri and Marwaha (1996) found that the efficiency of immobilization of naringinase from *Penicillium* sp. in calcium alginate beads, was around 85% and 140% when binding the enzyme to glutaraldehyde-coated hen egg white (Puri et al., 2001). Recently, the covalent immobilization of naringinase on glutaraldehyde-coated woodchips was reported (Puri et al., 2005). With this treatment, 71% enzyme binding was observed with 1% glutaraldehyde and 120% efficiency of immobilization. Nevertheless, the authors pointed that higher bifunctional reagent concentration inhibited and reduced naringinase activity, increased the cost of immobilization and apparently posed toxic effects.

The preparation obtained from a gel-forming solution at pH 7, containing 8% of PVA and 1.6 U ml^{-1} of enzyme load, was used for comparing the properties of the immobilized with those of the free naringinase in further experiments.

3.2. Determination of kinetic parameters

Michelis constants of naringinase were determined by using a Lineweaver–Burk plot, when naringin concentration was varied from 30 μg ml^{-1} to 400 μg ml^{-1}. Soluble naringinase shows normal kinetic behaviour with K_m = 66.3 μg ml^{-1} and V_{max} = 2.52 U (regression coefficient of Lineweaver–Burk, R^2 = 0.989), whereas the immobilized enzyme has sigmoid behaviour which does not allow kinetic parameters to be calculated from the Lineweaver–Burk graph (Fig. 1). This behaviour may be explained by the diffusional limitations upon the rate of the biocatalyzed reaction (Pifferi & Preziuso, 1987). The substrate concentration decreases from the carrrier surface towards the centre. This is because the substrate can be occupied by enzymes situated further toward the outside and, as a result, enzyme molecules further inside may not come into contact with the substrate (Vieth & Venkatasubramanian, 1974). This would explain the low catalytic activity observed for naringinase at low concentration of substrate and the sigmoidal behaviour.

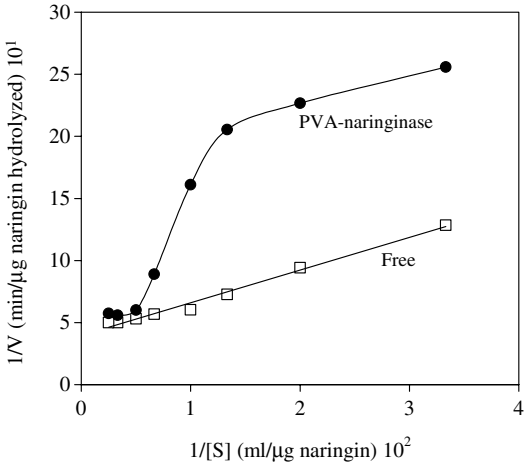

Fig. 1. Lineweaver–Burk graph for soluble and immobilized naringinase in PVA cryogels.

3.3. Effect of pH on enzyme activity

The pH/activity curves for free and immobilized naringinase are shown in Fig. 2. The optimum pH (4.5) for the entrapment enzyme was the same as for the free enzyme, and the behaviours of both enzymes were nearly identical. Comparison of the pH-activity profiles of free and immobilized enzyme can show the effect of the microenvironment surrounding the active site of enzyme protein on the hydrogen-ion concentration. In the present study, the immobilization support is a neutral polymer, so the pH of the buffer used was not changed by the addition of PVA and the alteration of the proton distribution, between the bulk phase and the surroundings of the immobilized enzyme, is also negligible. Romero, Manjón, Bastida, and Iborra (1985) also observed a pH optimum between 4.0 and 5.0 for free naringinase from *A. niger* as also did Puri and Marwaha (1996) for alginate-entrapped naringinase from *Penicillium* sp. In contrast, a lowering of pH optima from 4.5 to 3.0 as a result of immobilization of naringinase from *Penicillium* sp. on glycophase-coated porous glass and on glutaraldehyde-coated woodchips was observed by Manjón et al. (1985) and Puri et al. (2005), respectively.

The high response at low pH levels, observed in the present study, is highly interesting in fruit juice processing industries, because the pH of juices is often less than 5. This means that the enzyme preparation will work under optimal pH conditions, thus increasing the operational stability. Additionally, a low operational pH makes microbial contamination of the substrates, products and the enzyme reactor more unlikely (Lozano, Manjón, Romojaro, & Iborra, 1988).

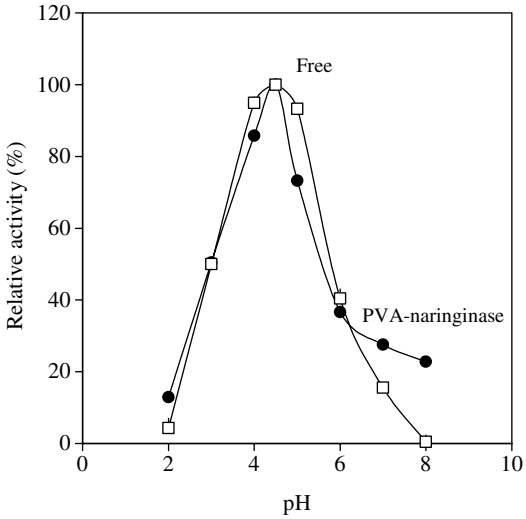

Fig. 2. Effect of pH on naringinase activity.

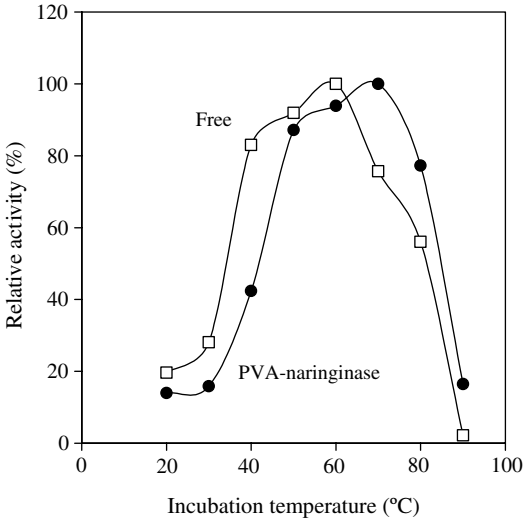

Fig. 3. Relative activity versus temperature, for free and immobilized naringinase in PVA cryogels.

3.4. Influence of temperature on the reaction rate

The temperature-dependence of the rate by the immobilized enzyme was compared with that of the native enzyme, and the results are shown in Fig. 3. The optimum temperature of free enzyme was 60 °C, whereas the activity of immobilized enzyme was highest at 70 °C. Park and Chand (1979) found that commercial naringinase from *A. niger* showed optimum activity at 40 °C, while immobilized enzyme on porous glass beads had an optimum at 55 °C. Similar shifts in optimum temperature of covalently-bound naringinase on hen egg white and alginate-entrapped naringinase were observed by Puri and Marwaha (1996) and Puri et al. (2001), respectively.

Activity of the immobilized enzyme was less affected by temperature than was that of the free enzyme, because immobilization lowered activation energy (from 8.09 kJ mol^{-1} to 6.36 kJ mol^{-1}), resulting in a higher catalytic efficiency for immobilized naringinase. Manjón et al. (1985) and Soares and Hotchkiss (1998) reported a similar decrease in activation energies with naringinase immobilized in glycophase-coated porous glass and cellulose acetate films, respectively.

The optimum temperature is determined by the balance between the effect of temperature on the rate of the enzyme reaction and its effect on the rate of destruction of the enzyme (Cornish Bowden, 1995). In this case, the PVA hydrogels seem to significantly protect the entrapment enzyme, favouring an optimum temperature shift toward higher temperatures. This stabilization is not significant with respect to the use of the immobilized derivative for juice debittering because the industrial process will run below the optimum temperature of the preparation in order to avoid changes in nutritional properties of the juice. However, it is important that immobilization stabilizes the enzymes because this will be reflected in operational stability.

3.5. Operational and storage stability

Taking into account that one of the most important advantages of an immobilized enzyme system is the possibility of its reutilization, the operational stability of the entrapped naringinase was determined by quantifying naringin hydrolysis in consecutive cycles of repeated use of enzymes. As shown in Fig. 4, the PVA–naringinase beads were successively used for hydrolysis of naringin in simulated juice, as described in Section 2. In the first run, the hydrolysis of naringin, with both free and immobilized enzymes after 24 h of treatment at 20 °C, were about 45% and 34%, respectively. After the second use, 23.3% of naringinase reduction was obtained in fresh simulated juice. Furthermore, about 36% efficiency of immobilized enzyme was observed after running six batches through the model system. The observed decrease in hydrolysis of naringin could be the result of the enzyme inhibition by the end-products of reaction (rhamnose and glucose). It has been reported that glucose, fructose and rhamnose

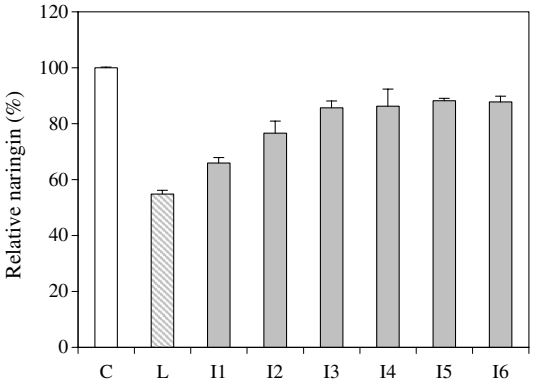

Fig. 4. Operational stability of immobilized naringinase in simulated juice at 20 °C (Cycles of 24 h). C: Control without enzyme; L: free enzyme; In: immobilized enzyme (n = run number).

are non-competitive and competitive inhibitors for both native and immobilized naringinase from *A. niger* and *Penicillium* sp., respectively, during debittering of fruit juice at pH 3 and 4.5 (Tsen, 1984; Tsen & Tsai, 1988). Loss of activity was observed by Ellenrieder et al. (1998) using alginate-entrapped naringinase, from commercial enzyme preparations, to hydrolyse 10% naringin solution. After hydrolysis runs of 4 h and 8 h, the activity of the alginate beads was reduced by about 16% and 30%, respectively. In contrast, Puri et al. (2005) reported no apparent activity loss after seven cycles of operations in kinnow mandarin juice using covalent immobilization of naringinase on glutaraldehyde-coated woodchips.

Finally, storage stability in a cold room (4 °C) for naringinase–PVA beads was also determined. This catalyst could be stored dry, retaining 75% of its initial activity for at least two months. In fact, the immobilized enzyme lost only 22% of its activity during the first 15 days, and then remained almost constant for at least two months (data not shown).

4. Conclusions

Judging from the results reported here we can conclude that crude naringinase extract from *A. niger*, without previous purification and entrapped in PVA cryogels, remains highly active. In addition, the availability and low cost of the carrier (PVA), its biodegradability, the simplicity of the immobilization method chosen and the kinetic behaviour and operational stability of PVA–naringinase beads, render the use of immobilized naringinase in cryogels of PVA of interest for the debittering of citrus fruit juices.

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