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ORIGINAL PAPER

Immobilization of the recombinant invertase INVB from *Zymomonas mobilis* on Nylon-6

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Abstract The recombinant invertase INVB (re-INVB) from *Zymomonas mobilis* was immobilized on microbeads of Nylon-6, by means of covalent bonding. The enzyme was strongly and successfully bound to the support. The activity of the free and immobilized enzyme was determined, using 10% (w/v) sucrose, at a temperature ranging between 15 and 60 °C and a pH ranging between 3.5 and 7. The optimal pH and temperature for the immobilized enzyme were 5.5 and 25 °C, respectively. Immobilization of re-INVB on Nylon-6 showed no significant change in the optimal pH, but a difference in the optimal temperature was evident, as that for the free enzyme was shown to be 40 °C. The values for kinetic parameters were determined as: 984 and 98 mM for K_m^{app} of immobilized and free re-INVB, respectively. K_{cat}^{app} values for immobilized and free enzymes were 6.1×10^2 and 1.2×10^4 s^{-1}, respectively, and immobilized re-INVB showed V_{max}^{app} of 158.73 μmol h min^{-1} mg^{-1}. Immobilization of re-INVB on Nylon-6 enhanced the thermostability of the enzyme by 50% at 30 °C and 70% at 40 °C, when compared to the free enzyme. The immobilization system reported here may have future biotechnological applications, owing to the simplicity of the immobilization technique, the strong binding of re-INVB to the support and the effective thermostability of the enzyme.

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Introduction

Enzyme immobilization represents an aspect of increasing importance in biocatalyst research, as it contributes significantly to improving enzyme stability, which has many potential applications, including the industrial production of pharmaceuticals, food, and chemical products, as well as the treatment of residues and the diagnosis and treatment of disease. Enzyme immobilization on solid supports, by means of covalent union is one of the most commonly used methods, for which substantial information is available [1, 2].

Invertase catalyzes the hydrolysis of sucrose, producing invert sugar. This product, also termed fructose-rich syrup has principally been used in the beverage and food industries. The immobilization of enzymes in their active state may increase their potential application for industrial purposes [3]. Recombinant enzymes are active in their accurate-folded and soluble forms, but can be totally inactivated, if converted into insoluble aggregates [4]. Thus, for industrial purposes, it is paramount to find a method for immobilizing the enzyme invertase, in its active state [5].

Zymomonas mobilis is a strictly fermentative Gram negative bacterium that has attracted considerable attention as a promising agent for large scale production of ethanol due to its unusual physiological and biochemical properties as well as its high efficiency in ethanol production. *Z. mobilis* produces two invertases, one intracellular (INVA) and other extracellular (INVB) [6]. These enzymes, among the most studied bacterial invertases, are found in family

32 (GH32) and family 68 (GH68), respectively, of the classified glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY) [7].

This study describes the immobilization of the recombinant invertase INVB (re-INVB) from *Z. mobilis* on microbeads of the polymeric support Nylon-6 and the biochemical characterization of both the immobilized and the free form of the enzyme.

Materials and methods

The construction of the plasmid *invb*petK has been described previously [4]. *E. coli* BL21(*DE3*) strain was provided by Invitrogene (US). All chemicals were of analytical grade and supplied by Fluka, J. T. Baker and Sigma. Ultrapure water from Milli-Q system (Millipore Corp.) was used throughout this research.

Recombinant protein production

A fresh clone of *E. coli* BL21 (*DE3*), harboring the *invb*petK plasmid was grown in 500-ml shake flasks, containing 100 ml of 2TY medium [8]. Cultures were incubated at 37 °C for 2 h at 250 rpm, in an orbital shaker (KBLee1001, Daiki) until they reached an optical density of 0.8 at 600 nm (OD_{600}). 25% (v/v) inoculum ratio was used for recombinant protein expression, in order to seed a 1.0-L fermenter, containing 0.4 L of 2TY medium [8]. Dissolved oxygen was manually controlled at 20–30% of saturation by means of flow air (0.2–0.25 vvm) and agitation speed (400–900 rpm) manipulations. Expression of re-INVB was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.

Cells contained in 1 ml of culture broth were harvested by centrifugation (4,000*g* at 4 °C for 20 min) and resuspended in Laemmli sample buffer, prior to the analysis of total protein extract, by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [9]. Soluble protein was prepared from the bacterial pellet of the remaining culture broth as follows. Bacterial pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5) at 20:1 (w/v) ratio. Then cells were lysed with lysozyme (10 mg ml^{-1}), at 4 °C for 30 min and then passed through a French Press AMINCO HA6027 at 16,000 psi. Cell debris was removed by centrifugation (10,000*g* at 4 °C for 15 min) and the supernatant, used as a source of crude invertase, was analyzed in order to reveal invertase activity, as well as its protein profile, using 10% SDS-PAGE.

Enzyme purification

All steps were carried out at 4 °C. Re-INVB was purified from the soluble fraction of the bacterial lysate (crude invertase) by nickel-affinity chromatography on a Ni Sepharose High Performance column (Amersham Pharmacia), according to the manufacturer instructions. Briefly, proteins in the crude invertase preparation were fractionated with a linear gradient of imidazol (10–250 mM) in buffer A (20 mM sodium phosphate, 10 mM imidazol, 300 mM NaCl, pH 7.5). Four milliliter fractions were collected and analyzed, by 10% SDS-PAGE and invertase activity. Then fractions with purified re-INVB were pooled and dialyzed against 10 mM Tris-HCl, 50 mM NaCl, pH 7.5.

Preparation of Nylon-6

Microbeads of Nylon-6 were activated with glutaraldehyde, using polyethylenimine (PEI) as a spacer [5, 10, 11]. The procedure was adapted and optimized for re-INVB as follows: 1.0 g of Nylon-6 microbeads was resuspended in 10 ml of 9.25% (v/v) glutaraldehyde in 200 mM sodium tetraborate, pH 9.3. The mixture was incubated at 70 °C for 20 min, with gentle agitation. Then, the microbeads were filtered, and thoroughly washed with deionized water and resuspended in 5 ml of 5% (w/v) PEI in 200 mM sodium tetraborate buffer, pH 9.3 and incubated at 25 °C, for 3 h. For a second time, the microbeads were filtered and washed with an ample volume of deionized water. The polymeric support was reactivated with 1% (v/v) glutaraldehyde in 50 mM potassium phosphate buffer pH 7.0 at 25 °C, for 15 min. Then once again, the Nylon-6 microbeads were filtered and washed with deionized water.

Enzyme immobilization

Re-INVB was immobilized at pH 5.5, which is the optimum pH for the free enzyme [4]. To immobilize re-INVB, one gram of Nylon-6, previously activated as described above, was suspended in 5 ml of coupling buffer (50 mM sodium acetate, pH 5.5, 50 mM KCl, 10 mM $MgCl_2$, 5 mM L-cysteine and 5% glycerol) containing 20 mg of purified re-INVB. The immobilization was allowed to proceed for 3 h at 4 °C. Then, immobilized re-INVB on Nylon-6 microbeads were rinsed with distilled water and stored at 4 °C in coupling buffer with 10% glycerol.

Invertase activity assay

Invertase activity was determined by measuring the release of reducing sugars using 3′5-dinitrosalicylic acid

(DNS) as described by Miller [12]. The assay was carried out at room temperature, using a 290 mM sucrose solution in 50 mM sodium acetate buffer, pH 5.5. An equimolar mixture of glucose and fructose was used as standard. One unit of invertase activity was defined as the amount of enzyme required for hydrolysis of 1 μmol of sucrose per minute. In many cases, the results were normalized, with 100% enzymatic activity, representing the highest value obtained for each of the series of measurements made.

Protein assay

Protein concentration was estimated as described by Lowry [13] using bovine serum albumin as a standard. The amount of immobilized protein was determined by measuring the amount of protein in the coupling buffer, both before and after the immobilization process.

Effect of pH and temperature on enzyme activity

The effect of pH on the enzymatic activity of the immobilized and free invertase INVB was determined for different pHs, ranging from 3.5–7.5, at room temperature. For the pH range of 3.5–5.5, the sucrose solutions (290 mM) were prepared in both 50 mM sodium acetate buffer as well as in 50 mM sodium phosphate buffer, and for a pH range of 6–7 in 50 mM sodium phosphate buffer. The effect of temperature on the enzymatic activity of the samples was assayed for different temperatures ranging between 15 and 60 °C, at the optimal pH value.

Kinetic parameters

The apparent kinetic parameters *K*m (apparent Michaelis constant) and *V*max (apparent maximum rate) of immobilized and free re-INVB were obtained by measuring in triplicate the initial rates (*V*o) of the reaction with different sucrose concentrations and fitting the data by the Lineweaver-Burk plot [14]. The apparent *K*cat were determined using sucrose solutions ranging from (0.15–2.3 M) in 50 mM sodium acetate buffer, pH 5.5 at 25 °C or 40 °C for the free and immobilized forms of the purified re-INVB, respectively.

Thermal stability

The thermal stability of free and immobilized re-INVB was studied by incubating samples at the desired temperature (15–60 °C) for different lengths of time. Remaining invertase activity was determined using the DNS method [12].

Results and discussion

Expression of the INVB recombinant protein

The recombinant invertase INVB from *Z. mobilis* was expressed as a soluble protein from *E. coli* BL21(*DE3*)/*invb*petK, following induction with 1 mM IPTG. 10% SDS-PAGE analysis indicated the paths followed for expression until re-INVB was purified (Fig. 1). The analysis of protein extracts from bacteria, harboring the construct *inv*BpetK, showed a band of 51 kDa (Fig. 1, lines 7–10), which accords with the expected, theoretical, molecular weight of re-INVB [4].

Immobilization of re-INVB on Nylon-6

The discovery of methods for biocatalyst immobilization is viewed as one of the most important innovations in biotechnology [5]. Kinetics for invertase coupling, using activated Nylon-6 microbeads, indicated that enzyme coupling was completed, after 15 min, when 20 mg of protein per g of Nylon-6 had bonded (Fig. 2). These results indicate that immobilization on Nylon-6 happens more quickly than on other supports [15], and is similar to that for the fusion protein INVB-CBD$_{cex}$, when immobilized on crystalline cellulose [16].

Behavior of immobilized re-INVB on Nylon-6

Several aspects were investigated, in order to study the properties of immobilized re-INVB on Nylon-6 and assess the suitability of this support for immobilizing this enzyme, as well as its potential, future application for industry.

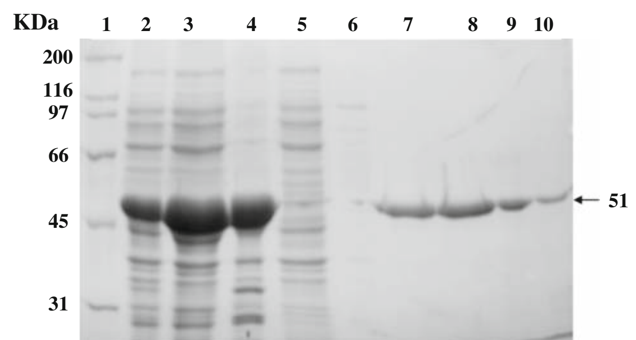

Fig. 1 10% SDS-PAGE analysis of total protein extracts of transformed *E. coli* BL21(*DE3*), induction and purification. *Lanes 1* molecular weight markers; *Lanes 2* non induced cells; *Lanes 3–4* induced cells with IPTG, soluble and insoluble fractions, respectively; *Lanes 5–6* proteins not bound or weakly bound to the nickel column, respectively; *Lanes 7–10* purified re-INVB

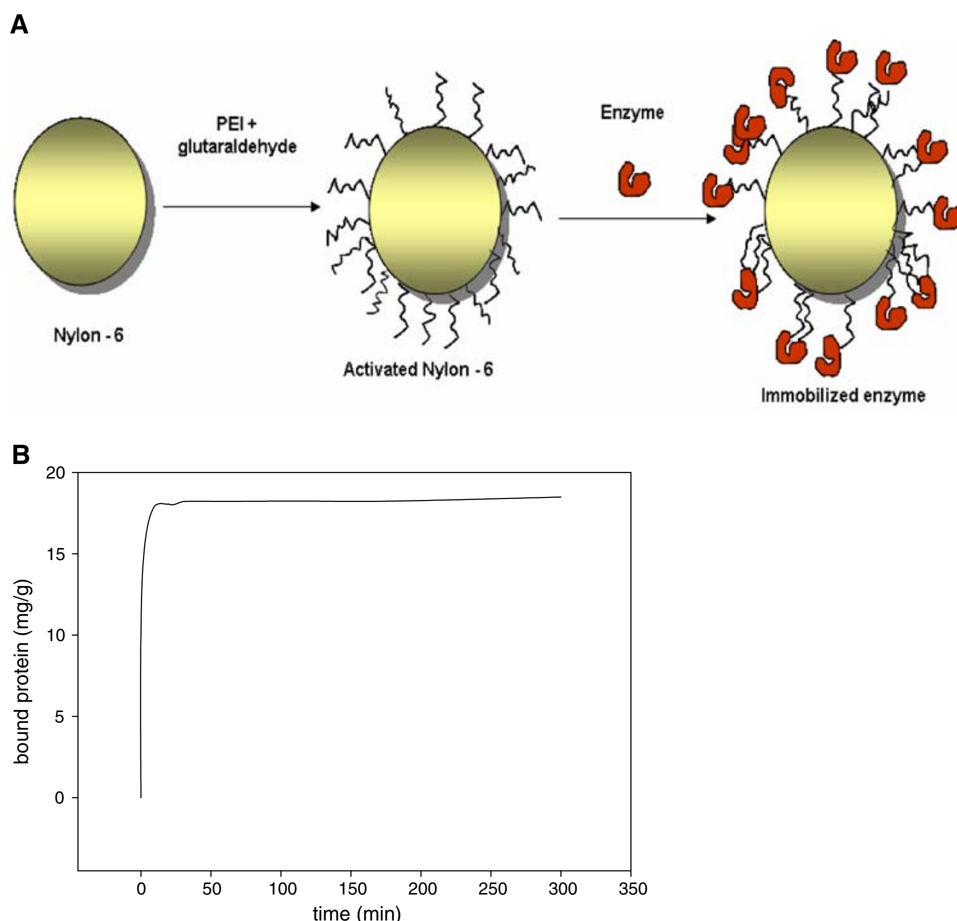

Fig. 2 **a** Schematic illustration of the Nylon-6 activation and the coupling of re-INVB to the support. **b** Kinetics of the coupling of re-INVB from *Z. mobilis* to Nylon-6 microbeads at 4 °C in 50 mM sodium acetate, 50 mM KCl, 10 mM $MgCl_2$, 5 mM L-cysteine and 5% glycerol, pH 5.5

Effect of pH and temperature on catalytic activity

We observed the effect of pH, varying from 3.5–7, on the activity of immobilized and free re-INVB, at room temperature (Fig. 3a). The optimum pH for the hydrolysis of sucrose by immobilized re-INVB on Nylon-6 was pH 5.5; the same as that observed for the free enzyme. The optimum pH was shown to be similar to that reported for other immobilized invertases [5, 16, 17]. Other reports [15, 18–20] show optimal pH for immobilized invertases to be in the range of 3.0–6.1. Previous studies have indicated that both the support and the method used for immobilization are the factors, which most influence the optimum pH [15].

The results presented in Fig. 3b indicate the activity of immobilized and free enzyme at temperatures between 15 and 60 °C. The optimal temperature for re-INVB immobilized on Nylon-6 was 25 °C, while for the free enzyme it was 40 °C [4]. A decrease in optimal temperature, upon enzyme immobilization has been reported for other enzymes. This temperature shift may be due to the covalent bond, which forms between particular functional groups, resulting in a less disordered enzyme and in a greater loss in active sites, than that of the ionically bonded enzyme [21]. Interestingly, at high temperatures, immobilized

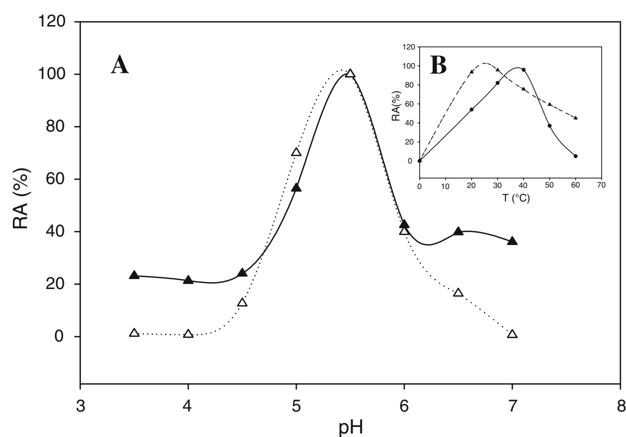

Fig. 3 **a** Effect of pH on re-INVB enzymatic activity. (*filled circle*) Immobilized re-INVB on Nylon-6; (*filled triangle*) free re-INVB. Relative activity, RA (%), versus pH. **b** Effect of temperature on re-INVB enzymatic activity. (*filled circle*) Immobilized re-INVB on Nylon-6; (*filled triangle*) free re-INVB. Relative activity, RA (%), versus temperature, T (°C)

INVB retained more catalytic activity than the free enzyme. Free invertase lost about 90% of its catalytic activity at 55 °C, whereas immobilized invertase lost only 50% (Fig. 3b). This suggests that the immobilization of the

recombinant invertase INVB on Nylon-6 confers stability to the immobilized enzyme, even at temperature values, up to 20 °C higher than the optimal.

Thermal stability

The thermal stability of immobilized enzymes is one of the most important factors, requiring consideration in the case of further applications. In order to study the effect of immobilization on the thermal stability of INVB, samples of immobilized and free enzyme were incubated at 30 °C (Fig. 4) and 40 °C (Fig. 5). When immobilized re-INVB was incubated at 30 and 40 °C, 50% of the initial activity continued after 80 and 60 min, respectively. In contrast, the initial activity of free re-INVB declined by 50% after 40 and 20 min, at 30 and 40 °C, respectively. Thus, immobilized re-INVB was more resistant to thermal inactivation than free enzyme at both temperatures assayed, with this effect being more evident at the higher temperature. An observable decrease in the stability of free and immobilized enzyme, when incubation temperature increased, has been described [3, 5, 17]. Also, an increase in the thermo stability of re-INVB was observed upon immobilization of the enzyme on D-sorbitol cinnamic ester [22].

Kinetic study of re-INV immobilized on Nylon-6

We determined maximum steady-state rate, V_{max}^{app}, Michaelis constant, K_m^{app} and catalytic constant K_{cat}^{app} values, for immobilized and free re-INVB. There was observable Michaelis–Menten type kinetic behavior in both cases. After fitting the data, using Lineweaver–Burker plot, we obtained values of 984 and 98 mM for K_m^{app} for immobilized and free re-INVB, respectively. K_{cat}^{app} values of

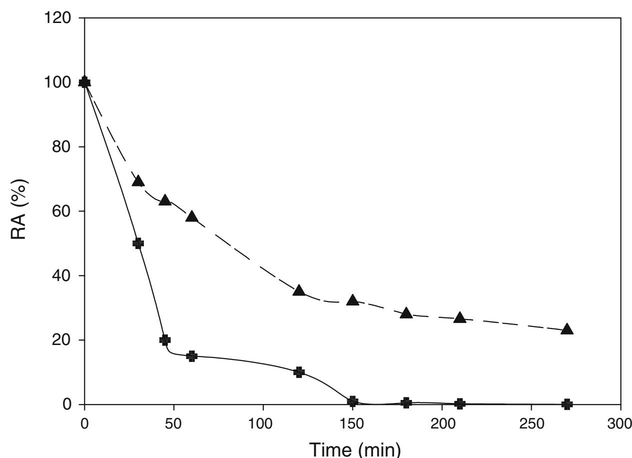

Fig. 4 Thermal stability of re-INVB immobilized on Nylon-6 (*filled circle*) and free re-INVB (*filled triangle*) at 30 °C. Residual enzymatic activity, RA (%), versus time (min)

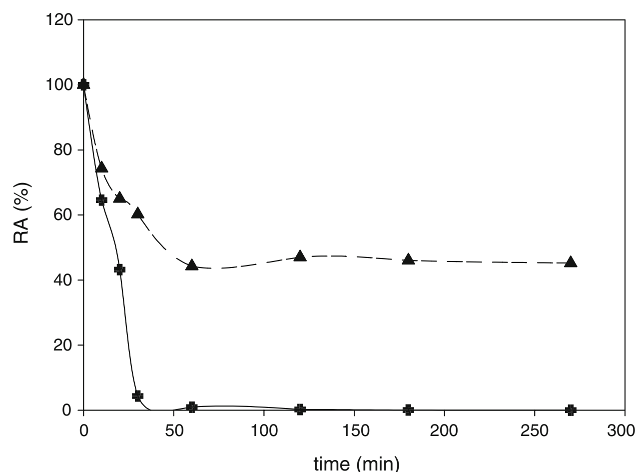

Fig. 5 Thermal stability of re-INVB immobilized on Nylon-6 (*filled circle*) and free re-INVB (*filled triangle*) at 40 °C. Residual enzymatic activity, RA (%), versus time (min)

immobilized and free enzyme were 6.1×10^2 and $1.2 \times 10^4\ s^{-1}$, respectively, and immobilized re-INVB showed V_{max}^{app} of 158.73 µmol h min^{-1} mg^{-1}. These data suggest that the kinetic parameters of re-INVB were affected, when immobilized on Nylon-6, probably due to steric hindrance between the immobilized enzyme and the insoluble support [23]. A decline in catalytic activity for the invertase and other glycoenzymes upon immobilization has been reported [15, 24, 25]. Immobilization of invertase of yeast in poly(maleic anhydride-hexen-1) membrane [26] or in poly(vinyl alcohol) membrane suggested that the formation of the enzyme–substrate complex was more difficult for immobilized invertase, than for native invertase [27]. Where immobilization is induced by the entrapping method, enzymes are confined in the gel, so that the enzyme reaction is mostly governed by the diffusion of substrates into the gel. It is difficult to pass the substrates through the cross-linked gel. Thus, there may be a lesser concentration of substrates within the gel, than there is in the solution. This appears to result in a greater value for K_m, as in the case of immobilized invertase of yeast [28]. A diffusion substrate problem may also be the cause of the increased K_m, manifested when re-INVB is immobilized on Nylon-6.

Stored stability of immobilized enzyme

In many cases, enzymes are not stable when stored in solution and their activities are gradually reduced or lost over time [5]. The storage stability of the immobilized re-INVB was determined at 4 °C in 50 mM acetate buffer pH 5.5 and periodically sampled to determine its residual activity. It was found that the immobilized re-INVB continued to show 80% of its original activity, after 40 days

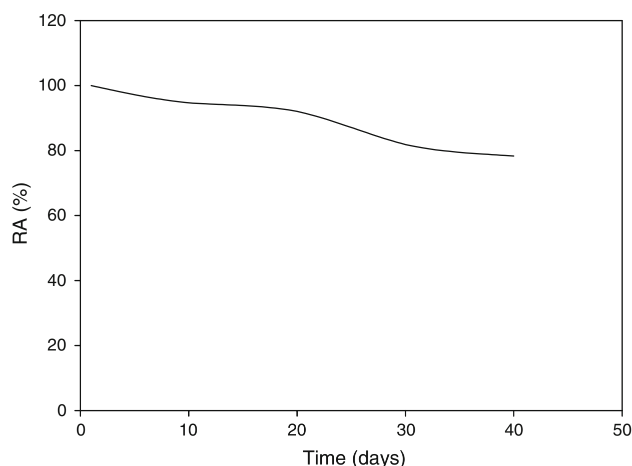

Fig. 6 Storage life of re-INVB immobilized on Nylon-6. Residual enzymatic activity, RA (%), versus time (days)

(Fig. 6). This result indicates that Nylon-6 provides a stable environment for the immobilization of re-INVB and loss of activity was prevented, by storing the enzyme under the tested conditions. Native extracellular invertase from *Saccharomyces cerevisiae* exhibited similar behavior when immobilized on Nylon-6 [5]. Papain, trypsin and pepsin all retained activity for much longer, when immobilized on Nylon-6 than in their soluble forms, probably because of the greatly reduced autohydrolysis and these could be stored for 2 months or more in aqueous suspensions at 4 °C [29].

Conclusions

The re-INVB enzyme immobilized on Nylon-6 by means of covalent union, provided thermostability to the enzyme and the results indicated expedient reproducibility.

The kinetic parameters were affected by the immobilization, probably due to steric hindrance or problems concerning diffusion in the substrate. However, the immobilized enzyme presented better thermostability than free invertase, possibly because of conformational stability. These results suggest that covalent immobilization on Nylon-6 confers additional stability to invertase, probably as a result of its enhanced resistance to unfolding, provided by its multipoint attachment to the support.

The immobilized re-INVB was stable and showed a good storage life, with a number of potential applications that would not be feasible in the case of a soluble enzyme. Further studies are currently underway, concerning storage stability and thermal stability for the immobilized enzyme, under varying conditions.

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