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IMPROVEMENT OF RECOVERED ACTIVITY AND STABILITY OF THE *Aspergillus oryzae* β-GALACTOSIDASE IMMOBILIZED ON DUOLITE® A568 BY COMBINATION OF IMMOBILIZATION METHODS

Article Highlights

- β-Galactosidase from *Aspergillus oryzae* was immobilized on Duolite® A568
- Three steps in the immobilization process allows a higher catalytic activity
- Physical adsorption, covalent multipoint and cross-linking process were investigated
- The biocatalyst obtained was highly stable over the entire pH range studied
- The first-order model described well the kinetics of thermal deactivation

Abstract

The immobilization and stabilization of Aspergillus oryzae β-galactosidase on Duolite® A568 was achieved using a combination of physical adsorption, incubation step in buffer at pH 9.0 and cross-linking with glutaraldehyde and in this sequence promoted a 44% increase in enzymatic activity as compared with the biocatalyst obtained after a two-step immobilization process (adsorption and cross-linking). The stability of the biocatalyst obtained by three-step immobilization process (adsorption, incubation in buffer at pH 9.0 and cross-linking) was higher than that obtained by two-steps (adsorption and cross-linking) and for free enzyme in relation to pH, storage and reusability. The immobilized biocatalyst was characterized with respect to thermal stability in the range 55-65 °C. The kinetics of thermal deactivation was well described by the first-order model, which resulted in the immobilized biocatalyst activation energy of thermal deactivation of 71.03 kcal/mol and 5.48 h half-life at 55.0 °C.

Keywords: β-galactosidase, Duolite® A-568, immobilization, incubation in buffer at pH 9.0.

The β-galactosidases obtained from filamentous fungi have shown to be a promising alternative for use in the lactose hydrolysis of cheese whey, since these enzymes present optimum pH in the acidic region and high thermal stability and thus have potential applications in food processing [1-3].

The β-galactosidase primary biochemical function is associated with lactose hydrolysis, for this reason major fields of its applications include processing of lactose-containing products, allowing the improvement of digestibility and technological and sensorial characteristics of sweetened condensed and frozen dairy products, as well as solving the substantial problem of whey utilization and appropriate disposal [4,5]. However, the use of this enzyme to catalyze the kinetically controlled reaction of transgalactosylation has received great attention recently in the synthesis of physiologically active galactosides, such as the galacto-oligosacharides (GOS) [6-8].

β-Galactosidase has been isolated from different microbial species, plants and animals, however, according to its source, the properties of the enzyme vary markedly. The sources of the enzymes that are used for the hydrolysis of milk and its derivatives are *Aspergillus sp.* and *Lactobacillus bulgaricus* because

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the enzymes obtained from these microorganisms are more stable and active at an acidic pH [3,9].

The β-galactosidase from *A. oryzae* is industrially important. This enzyme is composed of 985 amino acid residues, presents monomeric structure [10], having molecular weight of 105 kDa and maximum activity at pH 4.5 with *o*-NPG (ortho-nitrophenyl-β-galactoside) and at pH 4.8 with lactose as substrate. The optimum temperature is 50 °C and it is stable in the pH range from 4.0 to 9.0 [11,12].

For most applications of enzymes as biocatalysts an immobilization process is required, since it is the most common method for improving enzyme stability [13]. A proper immobilization process, in addition to improving the enzyme stability, may also greatly enhance other enzyme properties, altering of the enzyme environment, or promoting protein rigidification [14]. Besides enhanced stability, immobilization of enzymes permits efficient recovery of biocatalysts from the reaction medium as well as allows their use in continuous operations, consequently reducing the enzyme and product costs significantly [15-17].

Furthermore, the terms immobilization and stability are closely associated; however, if the immobilization procedure is not well designed, permitting uncontrolled enzyme-support interactions, immobilized enzymes can be even less stable than soluble enzymes [18]. On the other hand, if an adequate technique is utilized the stability of the enzyme may be also increased by immobilization process [18].

Various immobilization methods for β-galactosidase, including entrapment, cross-linking, adsorption and covalent binding have already been suggested [8]. Nevertheless, immobilization by physical adsorption onto solid carriers can be a compelling strategy to improve the operational stability and reusability, thus ensuring process cost effectiveness, since that its employment was ensured through a simple protocol, mild immobilization conditions, minimal damage of catalytic activity, and a wide variety of inexpensive materials that can serve as enzyme carriers with the possibility of simple regeneration [8]. Physical adsorption is a standard technique constituted by binding between enzyme and solid support based on van der Waals, ion exchange, and/or hydrophobic interactions. In the case of using this technique to immobilize proteins, those that do not become adsorbed on the support will be discarded.

Enzyme immobilization by ion exchange is a multipoint process, since the enzyme is fixed to the support just when a high sufficiently number of ionic bridges is established between the protein and the support to compensate the ionic strength of the media [19,20]. However, adsorption of the enzyme on a support is not always advantageous. The main disadvantage of these procedures is that the enzyme can be released from the support during the reaction in function of the pH value change or increase of ionic strength, so to minimize the enzyme desorption is recommended a cross-linking step, generally using glutaraldehyde as reticulant agent [16].

Chemical enzyme modification can be performed to further upgrade enzyme stability (*e.g.*, *via* chemical cross-linking), and may be also utilized as another tool to modify enzyme catalytic characteristics [21]. Glutaraldehyde is one of the most employed reagents in enzyme cross-linking and immobilization [22].

In addition to the numerous studies on the immobilization conditions and techniques that are available in the literature, there is great interest in the stabilization of the biocatalyst immobilized through multipoint covalent immobilization (it is not the unique methodology for this aim), which comprises all combinations of support functional groups and amino acid residues that provide formation of multiple covalent bonds. Thus, this multipoint covalent immobilization promotes stiffening of the immobilized enzyme structure and reduces the conformational changes involved in its inactivation [23,24].

According Gürdaş *et al.* [25] the anion exchange resin Duolite® A568 has a highly porous matrix, is granular, weakly basic and based on a crosslinked phenol-formaldehyde polycondensate with tertiary amine functional groups that attach the enzyme through a combination of the ion exchange bonding between the tertiary amine groups presents in the resin and the carboxylic acid groups of the enzyme and the simple adsorption by hydrogen bonds and van der Waals forces. These authors studied a simple adsorption mechanism to immobilize *A. oryzae* β-galactosidase in Duolite® A568. This resin has a hydrophilic structure and controlled pore size distribution that make it suitable for employment as a support for enzyme in a variety of bioprocessing applications.

Abuquerque *et al.* [20] studied the effect of the immobilization pH *via* adsorption on the final performance of the β-galactosidase from *A. oryzae*. These authors concluded that the enzyme immobilization in ion exchangers may be more versatile than supposed. If the protein has specially enriched areas in anion groups, the change in the immobilization pH during ion exchange may alter the immobilized enzyme functional properties, and the simplest explanation for this is that the enzyme orientation regarding the support may be altered by using different pH value.

The present work investigated the immobilization procedure of β-galactosidase from *A. oryzae* on Duolite® A568 combining the immobilization methods: physical adsorption, incubation in buffer pH 9.0 to permit multi interaction and cross-linking process, in order to aggregate the advantages of each method. The influence of the buffer composition used in the incubation step and the effect of the presence of the substrate during the immobilization process were studied. It was evaluated the stability of the biocatalyst obtained in relation to pH, temperature, storage and reusability.

MATERIALS AND METHODS

Materials

The β-galactosidase (E.C. 3.2.1.23) from *A. oryzae* was purchase from Sigma. The enzyme activity determined experimentally was 4 U per mg solid using lactose as a substrate. The unit of enzymatic activity (U) was defined as the amount of β-galactosidase that hydrolyzes 1 μmol of lactose per min at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate. The protein concentration of the commercial enzyme, determined using the method reported by Lowry *et al.* [26], was 79.4 mg per g of solid. The ion exchange resin employed in this work (Duolite® A568) was kindly donated by Dow Chemical Brazil S.A. All of the other reagents used in the study were of analytical purity.

Determination of the activity of the immobilized enzyme

The catalytic activity of the immobilized β-galactosidase for the catalysis of the lactose hydrolysis reaction was measured by the initial rate method, and the glucose produced in the reaction was determined by the glucose oxidase method [27].

The hydrolysis reactions were performed in a reactor containing 100 mL of a 50 g/L lactose solution prepared with 0.1 M acetate buffer pH 4.5, at 35±1 °C under magnetic agitation, according Guidini *et al.* [28]. The unit of activity ($U/g_{support}$) was defined as the amount of β-galactosidase that hydrolyzes 1 μmol of lactose per min per g of biocatalyst immobilized at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate. All of the experiments were conducted in triplicate.

The immobilization efficiency and yield were measured according to Zhang *et al.* [29] and Facin *et al.* [30] by Eqs. (1)-(3), where α_i is the total enzymatic activity of immobilized biocatalyst and α_f is the total activity of the initial enzyme solution; p_f is the total protein content of the initial enzyme preparation and p_w is the total protein content remained in solution after immobilization process; α_{si} is the total specific activity of immobilized biocatalyst and α_{sf} is the total specific activity of the initial enzyme solution.

$$\text{Immobilization efficiency (\%)} = 100\frac{\alpha_i}{\alpha_f} \quad (1)$$

$$\text{Immobilization yield (\%)} = 100\frac{p_f - p_w}{p_f} \quad (2)$$

$$\text{Immobilization specific efficiency (\%)} = 100\frac{\alpha_{si}}{\alpha_{sf}} \quad (3)$$

The specific activity ($U/mg_{protein}$) was determined as the quantity of β-galactosidase that hydrolyzes 1 μmol of lactose per min per mg of protein at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate.

Immobilization procedure

To promote the formation of multipoint links between the enzyme and the support, the immobilization process was composed into three steps: immobilization by adsorption to the resin, incubation at pH 9.0 and cross-linking with glutaraldehyde. The procedures employed for each step are outlined below.

Immobilization. The immobilization procedure involved the adsorption of the enzyme onto the ion exchange resin Duolite® A568 which was previously activated conform described by Guidini *et al.* [28]. Samples of 0.5 g of this resin were mixed with 10 mL of the enzyme solution (at the indicated concentration for each experiment) in 0.1 M acetate buffer, pH 4.5, and incubated at 25±1 °C for 15 h in a shaking incubator with an agitation of 150 rpm. The immobilization conditions used were those employed by Guidini *et al.* [28]. The strength of the buffer used was chosen based on the work of Husain *et al.* [31], Ansari *et al.* [32] and Urrutia *et al.* [33].

Incubation in buffer at pH 9.0. The incubation step was performed by immersion the resin or the enzyme derivative in 10 mL of 0.1 M phosphate buffer, pH 9.0, in a shaking incubator with an agitation of 150 rpm at 25±1 °C for 24 h [34,35].

Cross-linking. In this step, glutaraldehyde was used as the cross-linking agent at a ratio of 1 g of resin to 10 mL of 3.5 g/L glutaraldehyde. A volume of 5 mL of the 3.5 g/L glutaraldehyde solution H 3.86 was added to the biocatalyst immobilized, and the reaction system was maintained at a stirring of 150 rpm at 25±1 °C for 1.5 h. The employed variables (the glutaraldehyde concentration and the cross-linking time) were defined by Guidini *et al.* [28].

Influence of the buffer composition used during the incubation step at pH 9.0 of the immobilized biocatalyst

To evaluate the effect of the buffer composition employed in the incubation step, three samples were prepared using the three steps, immobilization by adsorption, with enzyme concentration of 16 g/L, followed by cross-linking with glutaraldehyde and finally incubation step. Three different buffers (borate, Tris (hydroxymethyl) and phosphate) 0.1 M, pH 9.0, were used. The pH 9.0 phosphate buffer was prepared by 955.0 mL of 0.1 M disodium hydrogen phosphate solution (14.2 g/L) with 45.0 mL of 0.1 M HCl, according to the procedure reported by Farag *et al.* [36].

Influence of the sequence of the steps on the immobilized β-galactosidase

To study the influence of the step sequence in the preparation of the biocatalyst, experiments were performed using different methods as described previously. The first experiment evaluated the influence of the incubation in buffer at pH 9.0 of the resin before the immobilization step, as described by Letca *et al.* [35]. The sample of 0.5 g of Duolite® A568, previously activated, was mixed with 10 mL of 0.1 M phosphate buffer, pH 9.0, in a shaking incubator at 150 rpm at 25±1 °C for 24 h. After this step the equilibrated resin was submitted to the immobilization by adsorption, followed by cross-linking with glutaraldehyde.

In two other experiments, was studied the influence of performing the incubation in buffer pH 9.0 of the immobilized enzyme before and after the cross--linking step. The relative activity (ratio of the activity of the biocatalyst obtained after the three steps to the activity of the biocatalyst obtained only after adsorption step and cross-linking with glutaraldehyde) for each biocatalyst was measured by the method of initial rates. In all of the experiments performed in this part of the study, the concentration of the enzyme solution used in the immobilization was 16 g/L.

Two experiments were conducted using the enzyme obtained after the three steps (immobilization by adsorption, incubation in buffer 9.0 and cross-linking): the resin was washed with acetate buffer pH 4.5 between each step in the first experiment, whereas the resin was not washed in the second experiment, and the cross-linking step carried out at pH 9.0.

To analyze the effect of the washing step, the protein concentration in the supernatant was determined before and after the immobilization step using the method related by Lowry *et al.* [26]. In these experiments, the concentration of the enzyme solution used in the immobilization step was 5 g/L.

Effect of the addition of substrate during the immobilization process

To verify the influence of the substrate (lactose) during the immobilization of the enzyme, six assays were performed using the immobilization by adsorption and the cross-linking steps as described previously. The amount of substrate added in each step used to obtain the biocatalyst was calculated for a lactose concentration of 50 g/L. In these experiments, the concentration of the enzyme solution used was 16 g/L.

pH Stability of the immobilized β-galactosidase

Samples of 0.5 g of the immobilized enzyme obtained after the three-step (immobilization by adsorption using an enzyme solution of 5 g/L, incubation in buffer pH 9.0 and cross-linking) immobilization process, according to the procedure described previously, were incubated in 10 mL of a buffer solution with a pH value in the range of 1.5 to 9.0 at 25±1 °C for 24 h. After the incubation, the biocatalysts were washed with 0.1 M acetate buffer, pH 4.5. The residual enzymatic activity was calculated in relation to the initial activity. The following buffers were used: hydrochloric acid-potassium chloride (0.1 M) at pH 1.5; 0.1 M citrate at pH 3.0, 0.1 M acetate for the pH values between 4.0 to 5.0, and 0.1 M phosphate at the pH range of 6.0 to 9.0. For pH range of 6.0 to 8.0 were used stocks solutions of monobasic sodium phosphate and dibasic sodium phosphate. The pH 9.0 phosphate buffer was prepared as mentioned previously.

Storage stability

The biocatalysts obtained after the immobilization by adsorption, incubation in buffer pH 9.0 and cross-linking steps, using an enzyme solution of 5 g/L, were stored in buffer solution pH 4.5 (0.1 M sodium acetate) at 4 °C in for 98 days. The stability of two different samples was studied: one of the samples was obtained by washing the resin between each immobilization step, and the other was obtained without washing the resin. The residual enzymatic activity in comparison to the initial activity was determined by the method of initial rates.

Reusability of immobilized β-galactosidase

The immobilized biocatalyst obtained by the three-step (immobilization by adsorption, incubation in buffer pH 9.0 and cross-linking) process using an enzyme solution of 5 g/L, was used in 10 consecutive lactose hydrolyses assays of 300 min each. The glucose produced in the reaction was determined by the glucose oxidase method [27]. Between each hydrolysis reaction, the biocatalyst was washed with a 0.1

M sodium acetate buffer, pH 4.5 and stored at 4 °C in the same buffer until the next hydrolysis reaction. The conditions utilized for all hydrolyses experiments were temperature of 35±1.0 °C, 0.1 M sodium acetate buffer, pH 4.5 and initial lactose concentration of 50 g/L.

Thermal stability of the immobilized β-galactosidase

With the purpose of studying the thermal stability of the biocatalyst immobilized obtained after the three-step (immobilization by adsorption, incubation in buffer pH 9.0 and cross-linking) immobilization process, the samples of β-galactosidase immobilized obtained using an enzyme solution of 5 g/L, were incubated into 50 mL of pH 4.5 acetate buffer in a thermostatic bath and the temperature ranged between 55.0 to 65.0 °C. For each studied temperature, the samples of immobilized biocatalyst were removed of the thermostatic bath at specific intervals of time (5 min for 65.0±1 °C, 7 min for 62.5±1 °C, 10 min for 60.0±1 °C, 15 min for 57.5±1 °C, 20 min for 55.0±1 °C) and were cooled fast in an ice bath, and the residual activity was measured by the initial rate of reaction using 50 g/L of initial lactose concentration, pH 4.5 at 35±1 °C. The residual enzymatic activities obtained as a function of incubation time for each temperature were adjusted to first order thermal deactivation model and in series thermal deactivation model in a single step [38] by the numerical method of Levenberg-Marquardt [37] using the Statistica® 7.0 software to obtain the kinetic parameters for the best fit [38].

RESULTS AND DISCUSSION

Influence of the buffer composition used during the incubation step at pH 9.0 of the immobilized biocatalyst

The results of the relative enzymatic activity (ratio of the activity of the biocatalyst obtained using different buffers in relation to the activity of the biocatalyst obtained using phosphate buffer) of the immobilized enzymes obtained using different buffers during the incubation step are presented in Table 1.

Table 1. Results of the influence of the buffer composition used in the incubation step in the immobilization process

Buffer composition	Activity[a], $U/g_{support}$	Relative activity, %
Borate	155.56±6.67	32
Phosphate	486.67±12.78	100
Tris	462.22±11.11	95

[a]The unit of activity ($U/g_{support}$) was defined as the amount of β-galactosidase that hydrolyzes 1 μmol of lactose per min per g of biocatalyst immobilized at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate

The analysis of the results presented in Table 1 show that the choice of buffer significantly affects the enzymatic activity of the biocatalyst. The use of borate decreases the activity almost by 70% when compared with the activity of the derivative obtained employing phosphate buffer.

A blank assay was performed employing enzyme solution 5 g/L prepared with 0.1 M borate buffer pH 9.0 and then determined the activity under the same conditions employed by immobilized enzyme. The enzymatic activity for soluble enzyme achieved using borate buffer was 13.33 U, which corresponds to about 7% of the soluble enzyme activity prepared in acetate buffer (194.44 U).

This result can be explained by the fact of borate buffer contains boric acid in its composition, that behaves like a Lewis acid (electron acceptor), allowing their complexation with the electron pair of the amino groups of the enzyme, causing a decrease in enzyme activity [39,40].

Shubhada and Sundaram [41] have studied the stability of β-galactosidase from *A. oryzae* in water-miscible organic solvents at different pH values in various buffers. These authors noted that in the studied pH values, the loss of stability was greater in sodium borate buffer than in sodium citrate-sodium phosphate or sodium phosphate buffers. These authors state that the greater loss of stability observed when used sodium borate buffer, in comparison with other studied solvents, is related to the fact that borate ions are known to complex with the carbohydrate part of glycoproteins. Since β-galactosidase is a glycoprotein, borate complexation may further assist the access of organic solvents, possibly through a change of conformation.

The use of phosphate and Tris buffers produced immobilized biocatalysts with similar catalytic activities, with a lower cost for the phosphate buffer. Thus, phosphate buffer was chosen to prepare the samples of biocatalyst in the work sequence.

Influence of the sequence of the steps on the immobilized β-galactosidase

The influence of the sequence of the immobilization by adsorption, cross-linking and incubation (in buffer pH 9.0) steps on the immobilized enzyme activity is shown in Table 2, that demonstrate that the incubation step (immersion in 0.1 M phosphate buffer, pH 9.0) increased the enzyme activity, regardless of where in the sequence this step was performed. In addition, an increase in enzyme activity of 44% was achieved when the enzyme was subjected to immobil-

Table 2. Results of the enzymatic activity of the biocatalyst obtained by combination of steps in the immobilization process compared with the enzymatic activity achieved by the immobilized biocatalyst obtained by adsorption and subsequent cross-linking

Steps for obtaining the biocatalyst	Activity[a], $U/g_{support}$	Relative activity, %
Immobilization	401.11 ± 18.33	106
Immobilization + Crosslinking	377.78 ± 10.56	100
Immobilization + Incubation at pH 9.0	462.22 ± 11.11	124
Incubation at pH 9.0 + Immobilization + Crosslinking	451.11 ± 10.00	119
Immobilization + Incubation at pH 9.0 + Crosslinking	543.33 ± 15.00	144
Immobilization + Crosslinking + Incubation at pH 9.0	486.67 ± 12.78	129

[a]The unit of activity ($U/g_{support}$) was defined as the amount of β-galactosidase that hydrolyzes 1 μmol of lactose per min per g of biocatalyst immobilized at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate

ization by adsorption followed by incubation in buffer pH 9.0 and then exposed to the cross-linking agent.

The improved in enzyme activity can be consequence of conformational changes that drive to a final conformation with more activity since in fact, the results shows that the loss of activity caused by the immobilization step was prevented.

One of the experimental conditions that can alter the balance between cationic and anionic groups on a protein surface is the pH. It is well known and accepted that this variable determines if an enzyme is adsorbed or not during anion exchange [19-20]. However, it has shown that the immobilization on an ion exchanger support may involve different areas depending on the immobilization pH value and how this may affect the final enzyme properties, *e.g.*, stability, activity or selectivity [20].

Albuquerque *et al.* [20] affirm that if the protein has specially enriched areas in anion groups, the variation in the immobilization pH during ion exchange may modify the immobilized enzyme functional properties, and the simplest explanation for this is that the enzyme orientation in relation to the support may be altered by using different pH value. According to the authors, their work was the first to probe this statement for the *β*-galactosidase from *A. oryzae* (same enzyme studied in the present work).

The catalytic activities of the enzymes obtained after each step (immobilization by adsorption, stabilization and cross-linking) were determined. As shown in Table 2, a gradual increase in the activity was obtained after each step.

In the first step, immobilization by adsorption, the immobilization of the enzyme occurs through interactions between support and the enzyme. The incubation of the enzyme immobilized by adsorption in a buffer at pH 9.0 can permit to achieve an intense multi interaction enzyme-support. In the last step the addition of glutaraldehyde was responsible for the appearance of new links between the enzyme molecules linked in the support to other molecules still available in the immobilization medium. In this way that glutaraldehyde's contribution could probably be ascribed predominantly to cross-linking between enzyme molecules.

An important aspect of pH influence on immobilization by adsorption can be explained by the fact that enzymes are more susceptible to interact with positively charged functional groups if they are negatively charged, which occurs at pH values above their pI, which is around 4.6 for *β*-galactosidase from *A. oryzae* [6].

Though, overall enzyme charge is not the only factor that impact adsorption via ionic interactions, once distribution of charged residues on the surface of enzyme also influences the efficiency of immobilization by directing the orientation of adsorbed molecules. Since surface of the support is positively charged throughout examined pH range due to primary amino groups, it can be assumed that distribution of negatively charged residues and properties of residues in their proximity cause large discrepancies in efficiency of immobilization at different pH values. The effect of pH on the efficiency of immobilization could be further explained by distribution of amino acid residues relevant for adsorption on the surface of *β*-galactosidase from *A. oryzae* [6].

Banjanac *et al.* [6] studied the immobilization of *β*-galactosidase from *A. oryzae* onto unmodified, amino modified and cyanuric chloride functionalized amino modified nonporous fumed nano-silica particles (FNS, AFNS and CCAFNS, respectively) and it can be concluded that immobilization of *β*-galactosidase is more favorable via carboxyl residues than via amino acid residues present on enzyme surface which is in agreement with previously study reported by Carevic *et al.* [8].

The influence of washing between each step in the process used to obtain the biocatalyst was studied, and the specific activities and yield obtained after the three steps of the process with and without the washing step are presented in Table 3.

As shown in Table 3, the suppression of the washing step between the preparation steps of the

Table 3. Results of the enzymatic activity, specific activity, immobilization yield and efficiency in comparison with free enzyme

Enzyme	Enzyme activity[a], U	Specific activity[b], $U/mg_{protein}$	Immobilization yield, %	Immobilization efficiency, %	Immobilization specific efficiency, %
Soluble (Free)	194.44	32.03±1.71	-	-	-
Immobilized without washing	166.66	31.32±0.96	88	86	98
Immobilized with washing	158.33	28.56±0.65	75	81	89

[a]The unit of activity (U) refers to enzyme activity defined as the amount of β-galactosidase that hydrolyzes 1 μmol of lactose per minute at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate; [b]the unit of activity ($U/mg_{protein}$) refers to specific activity defined as the amount of β-galactosidase that hydrolyzes 1 μmol of lactose per minute per mg of protein at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate

enzymatic derivative increased the immobilization yield by approximately 17.3%, which resulted in an increase in the specific activity of 9.7%.

The analysis of the results of Table 3 shows similar catalytic activities exhibited by the soluble and immobilized enzymes. Moreover, the experiences resulted in a high immobilization specific efficiency (98%). A blank assay with the soluble enzyme was performed, and after incubation in the same immobilization conditions there was no loss of activity. This result indicates that nearly all of the catalytic activity of the soluble enzyme was retained after the immobilization procedure.

Effect of the addition of substrate during the immobilization process

The biocatalyst obtained after three-steps has presented high activity retention, nevertheless in the present work was studied the possibility of increase the activity using the substrate as a protector of the active site. Table 4 shows the enzymatic activities obtained in the study of the effect of the presence of the substrate (lactose) during the immobilization process.

As shown in Table 4, the presence of lactose does not significantly influence the immobilized biocatalyst under the conditions evaluated. The enzymatic activities obtained when the enzyme was immobilized by adsorption process in the presence and in the absence of substrate were similar. The analysis of the cross-linking process showed that the enzymatic activities of the enzymes obtained after the cross-linking step performed in the presence and in the absence of the substrate were not significantly different.

The presented results show that the immobilization of β-galactosidase on anion exchange resin (Duolite® A568) does not affect the active site of the enzyme, even in the absence of lactose. This finding is likely because the site is well protected inside the protein and does not significantly suffer from steric hindrance or obstructions during the immobilization process. Thus, the addition of lactose as a protector of active site did not change the activity of the immobilized enzyme, which indicates that the active site is not directly involved in the immobilization process and that the addition of the substrate during the immobilization process is unnecessary [42].

The results above are consistent with the work of Aguiar-Oliveira [43], who studied the effect of the addition of salts and substrate during the immobilization of fructosyltransferase on niobium and the effect of these compounds on the catalytic properties of the resulting immobilized biocatalyst. The researcher found that the presence of the substrate does not affect the immobilized enzyme activity in the synthesis of FOS (fructooligosaccharide).

Based on the knowledge that the existence of the substrate or a competitive inhibitor can preserve the active site during the cross-linking and immobilization processes, Oosterom *et al.* [44] performed the immobilization of the β-galactosidase from *A. oryzae* on Duolite® S761 in the presence of 0.1 M lactose. The enzymatic activity of this immobilized enzyme increased around 30% in comparison with the immobilized biocatalyst obtained in the absence of lactose. However, the presence of lactose had no effect on the performance of the immobilized biocatalyst when

Table 4. Results of the effect of the addition of substrate during the immobilization process; the unit of activity ($U/g_{support}$) was defined as the amount of β-galactosidase that hydrolyzes 1 μmol of lactose per min per g of biocatalyst immobilized at pH 4.5 and 35 °C, employing lactose 50 g/L as substrate

Sample	Steps used to obtain the biocatalyst	Enzymatic activity ($U/g_{support}$)
1	Immobilization by adsorption without lactose	401.11±18.33
2	Immobilization by adsorption with lactose	400.00±12.78
3	Immobilization by adsorption without lactose + cross-linking without lactose	377.78±10.56
4	Immobilization by adsorption without lactose + cross-linking with lactose	367.78±10.56
5	Immobilization by adsorption with lactose + cross-linking without lactose	326.67±6.11
6	Immobilization by adsorption with lactose + cross-linking with lactose	330.00±9.44

dimethyl adipimidate (DMA) was used as the cross--linking agent.

pH influence on the stability of the immobilized biocatalyst

The reaction medium pH exerts a great influence on the catalytic stability of most enzymes. Figure 1 shows the residual activity, which is determined by the ratio of the activity after a 24-h incubation to the initial activity.

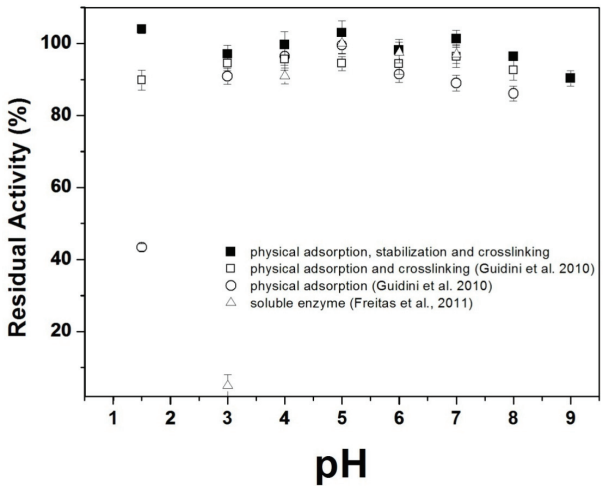

Figure 1. pH influence in the stability of the immobilized β-galactosidase.

The study of the pH stability of the immobilized biocatalyst obtained by three-step immobilization process (adsorptive immobilization, incubation at pH 9.0 and cross-linking with glutaraldehyde) shows that the enzymatic derivative presents high stability at an extensive range of pH values. The residual enzyme activity was approximately 100% in relation to initial activity at all pH values studied, which indicates high stability.

The maintenance of the activity of the biocatalyst after a 24-h incubation at different pH values may be further explained by the additional step of incubation at pH 9.0 realized between the immobilization by adsorption and the cross-linking with glutaraldehyde steps. The results observed by Guidini *et al.* [28], who studied the stabilities of the biocatalysts obtained after a one-step (immobilization by adsorption) and a two-step immobilization procedure (immobilization by adsorption followed cross-linking with glutaraldehyde), presented in Figure 1 for comparison, show less stability compared with that obtained in present work.

According to the work of Guidini *et al.* [28], the enzyme obtained after the one-step immobilization process (immobilization by adsorption) was stable over a narrow pH range (from 4 to 5). However, after the addition of the cross-linking with glutaraldehyde step, these authors obtained the residual activity approximately 95% for a pH range of 2 to 7.

Freitas *et al.* [45] present a study of kinetic properties of β-galactosidase from *A. oryzae* soluble and immobilized in alginate, gelatin and glutaraldehyde. The enzyme maintain its total activity after 18 h of incubation, for a pH extension of 4.0–7.0, in its free and immobilized forms, which coincides with the stability of the soluble enzyme, as recommended by the manufacturer and the literature [1,45,46].

Grazú *et al.* [47] immobilized penicillin G acylase (PGA) at neutral pH for 24 h and then incubated the immobilized enzyme for 48 h at pH 10. These researchers found that more than 90% of the catalytic activity was recovered after the stiffening obtained during the immobilization process.

The results obtained in present work are in accordance with the findings related by Albuquerque *et al.* [20] that explain, in first time, that the immobilization on an ion exchanger support may involve different areas depending on the immobilization pH value and this may affect the final enzyme properties, *e.g.*, stability.

Storage stability

The influence of the storage time on the activity retention of immobilized β-galactosidase was evaluated. The results show that the residual activity obtained for the samples with and without washing maintained their activities after 98 days of storage in 0.1 M sodium acetate buffer, pH 4.5, and at a temperature of 4±2 °C, as shown in Figure 2. These results indicate that the immobilization procedure improved the

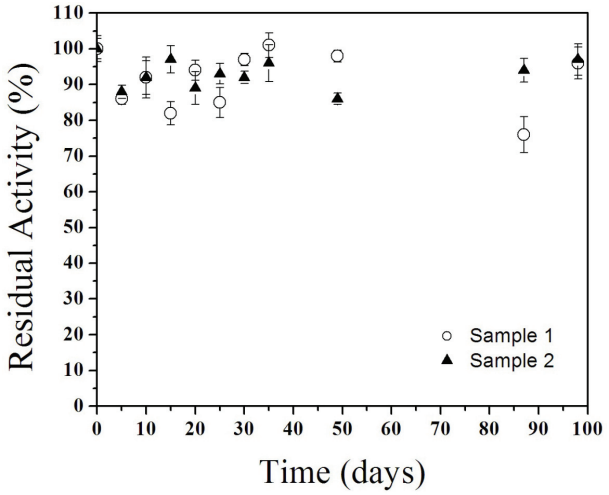

Figure 2. Storage stability of immobilized β-galactosidase (sample 1 – the biocatalyst was washed between each step used to obtain the same; sample 2 – without the washing step).

stability of the biocatalyst in relation to the storage, since, according to Haider and Husain [1], the soluble *A. oryzae* β-galactosidase, in the same storage conditions, has maintained only 40±2.5% of the initial activity after 60 days.

These results are consistent with the work of Guidini *et al*. [28], who reported that the stability of the β-galactosidase enzyme from *A. oryzae* immobilized on Duolite® A568 by adsorption and then crosslinked with glutaraldehyde was maintained after 90 days of stocking in acetate buffer, pH 4.5, at 4±2 °C.

Haider and Husain [1] immobilized the β-galactosidase from *A. oryzae* on calcium alginate and crosslinked with concanavalin A. These researchers found that the resulting immobilized enzyme maintained an activity of 93% compared with the baseline after 2 months of storage at 4 °C.

Ansari and Husain [11] described the storage stability of free and immobilized β-galactosidase from *A. oryzae* at 4 °C in 0.1 M acetate buffer, pH 4.5 for more than 2 months. The crosslinked enzyme presented 78% activity after 2 months of storage while its soluble form retained only 40% activity under similar storage conditions.

Reusability of immobilized β-galactosidase

The immobilization of enzymes is an important tool that improves the utilization of enzymatic catalysts increasing stability and allowing their reuse. The results of reuse of immobilized β-galactosidase have been presented in Figure 3. The biocatalyst was used during 10 consecutive hydrolyses assays of 300 min each and the conversion of lactose into glucose and galactose was the same for all assays. These results indicate the high stability of the biocatalyst in the reaction conditions (35 °C and 0.1 M acetate buffer pH 4.5) allowing their reuse for long and repeated times.

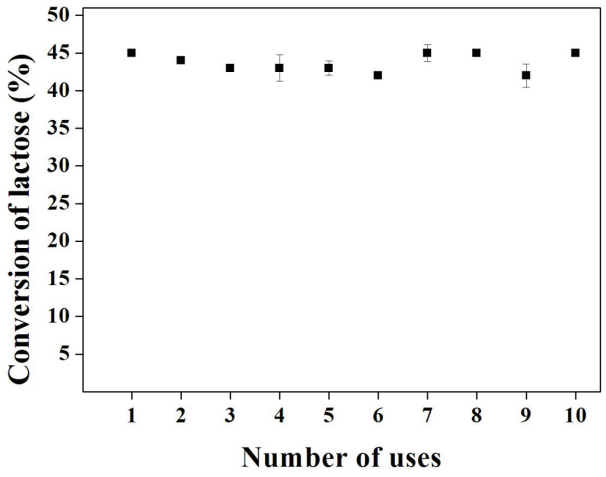

Figure 3. Conversion of lactose using β-galactosidase immobilized in relation to the number of uses.

The results obtained in the present work were better than those obtained by Guidini *et al*. [28], who studied the reuse of β-galactosidase immobilized by adsorption on the same support followed by crosslinking with glutaraldehyde, in which they found a loss of activity of 10% after the fifth 10 min assay in the same conditions.

Ansari and Husain [11] obtained for β-galactosidase adsorbed on Con A-celite and adsorbed and crosslinked on Con A-celite, a residual activity of 64 and 71% after 7th repeated use, respectively.

Thermal stability

Figure 4 shows the strong dependence of the stability of the immobilized biocatalyst on temperature. It is observed that for 65.0 °C in 15 min there was a decrease of 50% of the activity in relation to the initial activity. For the temperatures of 60.0, 57.5 and 55.0 °C the biocatalyst maintained the initial activity until 30-min incubation, thereafter was observed the thermal inactivation process, demonstrating that for the range of 55 to 60 °C, the immobilized enzyme is more resistant to thermal deactivation. For the temperature of 55 °C, the biocatalyst retains almost all of its initial activity after 60-min incubation and after 2.3 h, the biocatalyst maintained 80% of its initial activity.

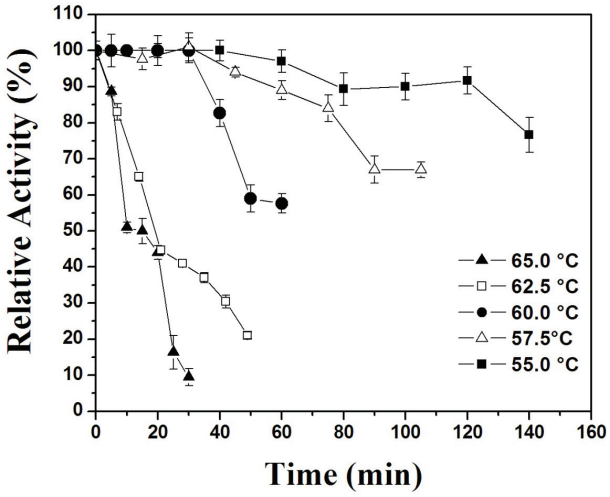

Figure 4. Activity relative as a function of incubation time for the temperatures of 55.0-65.0 °C.

This maintenance of initial activity until 30 min incubation can be related to the insertion of the incubation in buffer pH 9.0 step in obtaining a biocatalyst, since this was not observed by Guidini *et al.* [48] that studied the thermal stability of the β-galactosidase immobilized by two-step immobilization process (immobilization by adsorption followed cross-linking)

and the results obtained showed a fall of 10% activity after 60 min incubation at 55 °C.

Freitas [49] studied the thermal stability of β-galactosidase from *A. oryzae* free and was observed for the temperature of 65 °C a rapid enzyme deactivation, requiring only five minutes for the enzyme lost 80% of its catalytic activity.

The results obtained experimentally for the residual enzymatic activity were adjusted to the model of thermal deactivation in the first order and in series in a single stage [38] by the numerical method of Levenberg-Marquardt [37] employing the Statistica® 7.0 software. The significance analysis was realized using the Student's *t*-test test considering similar significant parameters as those with significance levels smaller than 10%.

Analyzing the results (in supporting information, available from the author upon request) of the modeling of thermal deactivation, it follows that for all temperatures, the best adjust was achieved by applying first order deactivation kinetics. On the other hand, the model of deactivation in series in a single step was inappropriate for the fit in all temperatures.

With the experimental results of residual enzymatic activity on incubation time were calculated the thermal deactivations constants, k_d, for first order model for each incubation temperature, which were used to calculate the half-life, as showed in Table 5. Freitas [49] reported in your work, the half-life measured by the first order thermal deactivation model for soluble form of β-galactosidase from *A. oryzae* at 65, 63, 61, 57 and 55 °C were 2.40, 11.68, 13.59, 57.70 and 177.70 min, respectively.

These results clearly show the biocatalyst in the immobilized form has higher thermal stability in comparison to the soluble enzyme. Haider and Husain [1] obtained for β-galactosidase from *A. oryzae* in the free form a half-life of approximately 30 min for thermal stability at 60 °C and pH 4.6. Husain *et al.* [31] and Ansari *et al.* [32] have studied the thermal denaturation of same enzyme in the soluble form at 60 °C in 0.1 M sodium acetate buffer (pH 4.5) and obtained the half-life of 30 to 40 min under these conditions.

The authors Haider and Husain [1] evaluated the thermal stability of calcium alginate entrapped preparations of *A. oryzae* β-galactosidase and obtained for entrapped soluble and insoluble concanavalin complex half-lifes of 45 and 60 min respectively, at 60 °C in 0.1 M sodium acetate buffer (pH 4.5). An strong dependence on the thermal stability of the enzyme in function of method of immobilization was observed.

The k_d values shown in Table 5, were adjusted to the Arrhenius model utilizing the Origin® 7.0 software. With a determination coefficient of 0.96 the linear fit was obtained. Therefore, the activation energy determined from the process of thermal deactivation was 71.03 kcal/mol, a value less than to the free enzyme obtained by Freitas [49], which was 88.14 kcal/mol for the same enzyme. These results indicate that the immobilized enzyme in the present work is more stable for an increment temperature in the lactose hydrolysis process in comparison to the same enzyme in the soluble form.

CONCLUSIONS

The immobilization process of *A. oryzae* β-galactosidase by adsorption, incubation in buffer pH 9 and cross-linking, in this sequence, employing Duolite® A568 as support, resulted in a biocatalyst with better activity and stability compared with the biocatalyst obtained only by adsorption and cross-linking. The immobilized biocatalyst obtained presented a good thermal stability at temperatures around 50 °C, with a half-life of 5.48 h at 55 °C and also excellent pH stability in the range from 1.5 to 9.0. There was also an increase of stability in comparison to the storage time and in relation to the number of uses of the biocatalyst immobilized. Based on the previously mentioned conclusions, the use of sequence of the steps of adsorption, followed by incubation in buffer pH 9.0 and cross-linking with glutaraldehyde in the immobilization of β-galactosidase of *A. oryzae* in Duolite® A568 as support presents potential for industrial applications of this enzyme in the immobilized form.

Table 5. Half-life for each temperature using the model of thermal deactivation of the first order

Temperature, °C	k_d / min^{-1}	R^2	$t_{1/2}$ / min	$t_{1/2}$ / h
65.0	0.0546	0.92	12.70	0.21
62.5	0.0313	0.98	22.15	0.37
60.0	0.0211	0.95	62.85	1.05
57.5	0.0052	0.92	163.30	2.72
55.0	0.0024	0.94	328.81	5.48

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REFERENCES

[1] T. Haider, Q. Husain, Int. J. Biol. Macromol. *41* (2007) 72–80

[2] R. Panesar, P.S. Panesar, R.S. Singh, J.F. Kennedy, J. Chem. Technol. Biotechnol. **86** (2011) 42-46

[3] I. Van De Voorde, K. Goiris, E. Syryn, C. Van Den Bussche, G. Aerts, Process Biochem. **49** (2014) 2134–2140

[4] A. Nath, S. Mondal, S. Chakraborty, C. Bhattacharjee, R. Chowdhury, Asia-Pac. J. Chem. Eng. **9** (2014) 330-348

[5] M. Khan, Q. Husain, A. H. Naqvi, RSC Adv. **6** (2016) 53493-53503

[6] K. Banjanac, M. Carević, M. Ćorović, A. Milivojević, N.Prlainović, A.Marinkovićc, D.Bezbradica, RSC Adv. **6** (2016) 97216-97225

[7] A. Kamran, Z. Bibi, A. Amana, S.A.U. Qader, Biocatal. Agric. Biotechnol. **5** (2016) 99-103

[8] M. Carević, M. Ćorović, M. Mihailović, K. Banjanac, A. Milisavljević, D. Velicković, D. Bezbradica, Int. Dairy J. **54** (2016) 50-57

[9] V.Gékas, M.Lopez-Leiva, Process Biochem. **20** (1985) 2-12

[10] M.M. Maksimainen, A.Lampio, M. Mertanen, O.Turunen, J. Rouvinen, Int. J. Biol. Macromol. **60** (2013) 109-115

[11] S.A. Ansari, Q. Husain, Food Bioprod. Process **90** (2012) 351-359

[12] Q. Husain, Critical Rev. Biotechnol.**30** (2010) 41-46

[13] N. An, C.H. Zhou, X.Y. Zhuang, D.S. Tong, W.H. Yu, Appl. Clay Sci. **114** (2015) 283-296

[14] F.F. Freitas, L.D.S. Marquez, G.P. Ribeiro, G.C. Brandão, V.L. Cardoso, E.J. Ribeiro,Braz. J. Chem. Eng. **29** (2012) 15-24

[15] L.D.S. Marquez, B.V. Cabral, V.L. Cardoso, E.J. Ribeiro, J. Mol. Catal., B-Enzym. **51** (2008) 86-92

[16] V. Stepankova, S. Bidmanova, T. Koudelakova, Z. Prokop, R. Chaloupkova, J. Damborsky, ACS Catal. **3** (2013) 2823-2836

[17] B. Das, A.P. Roy, S. Bhattacharjee, S. Chakraborty, C. Bhattacharjee, Ecotox. Environ. Safety **121** (2015) 244-252

[18] O. Barbosa, R. Torres, C. Ortiz, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Biomacromolecules **14** (2013) 2433-2462

[19] X.D. Tong, X.Y. Dong, Y. Sun, Biochem. Eng. J. **12** (2002) 117-124

[20] T.L. de Albuquerque, S. Peirce, N. Rueda, A. Marzocchella, L.R.B. Gonçalves, M.V.P. Rocha, R. Fernandez-Lafuente, Process Biochem. **51** (2016) 875-880

[21] A. Díaz-Rodríguez, B.G. Davis, Curr. Opin. Chem. Biol. **15** (2011) 211-219

[22] O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, RSC Adv. **4** (2014) 1583-1600

[23] Y.A.R. Tapias, C.W. Rivero, F. López Gallego, J.M. Guisán, J.A. Trelles, Food Chem. **208** (2016) 252-257

[24] B.C.C. Pessela, R. Torres, M. Fuentes, C. Mateo, M. Filho, A.V. Carrascosa, R. Fernandez-Lafuente, Biotechnol. Progress **20** (2004) 1507-1511

[25] S. Gürdaş, H.A. Güleç, M. Mutlu, Food Bioprocess Technol. **5** (2012) 904-911

[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randal, J. Biol. Chem. **193** (1951), pp. 193, 265-275

[27] J. Raba, H.A. Mottola, Crit. Rev. Anal. Chem. **25** (1995) 1-42

[28] C.Z. Guidini, J. Fischer, L.N.S. Santana, V.L. Cardoso, E.J. Ribeiro, Biochem. Eng. J. **52** (2010) 137-143

[29] Y. Zhang, M. Jeya, J. Lee, Appl. Microbiol. Biotechnol. **89** (2011) 1435-1442

[30] B.R. Facin, B. Moret, D. Baretta, L.A. Belfiore, A.T. Paulino, Food Chem. **179** (2015) 44-51

[31] Q. Husain, S.A. Ansari, F. Alam, A. Azam, Int. J. Biol. Macromol. **49** (2011) 37-43

[32] S.A. Ansari, R. Satar, F. Alam, M.H. Alqahtani, A.G. Chaudhary, M.I. Naseer, S. Karim, I.A. Sheikh, Process Biochem. **47** (2012) 2427-2433

[33] P. Urrutia, C. Mateo, J.M. Guisan, L. Wilson, A. Illanes, Biochem. Eng. J. **77** (2013) 41-48

[34] C. Mateo, O. Abian, R. Fernandez-Lafuente, J.M. Guisan, Enzyme Microb. Technol. **26** (2000) 509-515

[35] D. Letca, C. Hemmerling, M. Walter, D. Wullbrand, K. Buchholz, Roman. Society Biol. Sci. **9** (2004) 1879-1886

[36] R.S. Farag, M.S. Afifi, M.M. Abd-Rabow, Int. J. Pharm. Sci. Res. **2** (2011) 1197-1203

[37] J.J. Moré, G.A. Watson, Lect. Notes Mathematics **630** (1977) 105-116

[38] J.P. Henley, A. Sadana, Enzyme Microb. Technol. **7** (1985) 50-60

[39] A.F.C. Alcântara, H.S. Barroso, D. Piló-Veloso, Quim. Nova **25** (2002) 300-311

[40] J.P. Montalvo-Andia, L.A. Teixeira, PhD Thesis, Pontifical Catholic University of Rio de Janeiro (2009)

[41] S. Shubhada, P. Sundaram, Enzyme Microb. Technol. **15** (1993) 881-886

[42] M. Wiltchek, T.J. Miron, Biochem. Bioph. Methods **55** (2003) 67-70

[43] E. Aguiar-Oliveira, PhD Thesis, Faculty of Food Engineering, State University of Campinas (2012)

[44] M.W. Oosterom, H.J.A. Belle, F. Rantwijl, R.A. Sheldon, J. Mol. Catal., A: Chem. **134** (1998) 267-274

[45] F.F. Freitas, L.D.S. Marquez, G.P. Ribeiro, G.C. Brandão, V.L. Cardoso, E.J. Ribeiro, Biochem. Eng. J. **58-59** (2011) 33-38

[46] A.S. Tanriseven, E. Dogan, Process Biochem. **38** (2002) 27-30

[47] V. Grazú, F. López-Gallego, T. Montes, O. Abian, R. González, J.A. Hermoso, J.L. García,C. Mateo, J.M. Guisán, Process Biochem. **45** (2010) 390-398

[48] C.Z. Guidini,J. Fischer, M.M. Resende, V.L. Cardoso, E.J. Ribeiro, J. Mol. Cat., B-Enzym. **71** (2011) 139-145

[49] F.F. Freitas, PhD Thesis, Faculty of Chemical Engineering, Federal University of Uberlândia (2007).

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NAUČNI RAD

POBOLJŠANJE OBNOVLJENE AKTIVNOSTI I STABILNOSTI β-GALACTOZIDAZE IZ *Aspergillus oryzae* IMOBILISANE NA DUOLITU A568 KOMBINACIJOM IMOBILIZACIONIH METODA

Imobilizacija i stabilizacija β-galaktozidaze iz Aspergillus orizae na Duolitu A568 su izvršene korišćenjem kombinacije fizičke adsorpcije, inkubacije u puferu pri pH 9,0 i umrežavanja sa glutaraldehidom, pri čemu je enzimska aktivnost povećana za 44% u odnosu na biokatalizator dobijen nakon procesa dvostepene imobilizacije (adsorpcija i umrežavanje). Stabilnost biokatalizatora dobijenog u tristepenom procesu imobilizacije (adsorpcija, inkubacija u puferu na pH 9,0 i umrežavanje) je veća od biokatalizatora dobijenog dvostepenim postupkom (adsorpcija i umrežavanje), kao i od slobodnog enzima u odnosu na pH, skladištenje i ponovno korišćenje. Imobilizovani biokatalizator je okarakterisan u odnosu na termičku stabilnost u opsegu 55-65 °C. Kinetika termičke deaktivacije opisana je modelom prvog reda, pri čemu je energija aktivacije termičke deaktivacije imobilizovanog biokatalizatora 297,2 kJ/mol, a poluvremem života 5,48 h na 55,0 °C.

Ključne reči: β-galaktozidaza, Duolit A-568, imobilizacija, inkubacija u puferu pri pH 9,0.