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Immobilization of β-glucosidase from almonds on MANAE- agarose supports by using the chemistry of glutaraldehyde

Inmovilización de β-glucosidasa de almendras sobre soportes de MANAE-agarosa mediante el uso de la química del glutaraldehído

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Abstract

The β-glucosidase from almond was immobilized using different strategies: ionic adsorption on aminated MANAE-agarose beads at pH 5, 7 and 9, followed by glutaraldehyde pre-activated supports. The pH of the immobilization was altered to allow different enzyme molecule orientations on the support surface. The concentration of the enzyme exhibited an effect on the stability of the free enzyme. Immobilization by ion exchange maintained up to 80% of the activity of β-glucosidase, however the stabilization was lower than the immobilization by covalent binding on pre-activated supports. The enzyme immobilized on supports pre-activated at pH 5 showed a higher activity of 78% after 24 h. The immobilized enzymes were inactivated at pH 5 and 7, the enzyme immobilized at pH 7 and inactivated at pH 5 maintained greater stability than the immobilized at the other pH values. Considering the enzymatic activity, the stability and the kinetic parameters Km, Vmax and the Km/Ki ratio, the β-glucosidase immobilized on supports pre-activated with glutaraldehyde at pH 5 and 7 are the best option to use β-glucosidase from almonds.

Keywords: β-glucosidase, enzyme immobilization, glutaraldehyde, amino-agarose supports, enzyme stabilization.

Resumen

La β-glucosidasa de almendras se inmovilizó mediante diferentes estrategias: adsorción iónica en perlas aminadas MANAE-agarosa a pH 5, 7 y 9, seguida de soportes preactivados con glutaraldehído. El pH de inmovilización se modificó para permitir diferentes orientaciones de las moléculas de enzima en la superficie del soporte. La concentración de la enzima mostró un efecto sobre la estabilidad de la enzima libre. La inmovilización por intercambio iónico mantuvo hasta el 80% de la actividad de la β-glucosidasa, sin embargo, la estabilización fue menor que la inmovilización por unión covalente sobre soportes preactivados. La enzima inmovilizada sobre soportes preactivados a pH 5 mostró una mayor actividad de 78% a las 24 h. Las enzimas inmovilizadas se inactivaron a pH 5 y 7, la enzima inmovilizada a pH 7 e inactivada a pH 5 mantuvo una estabilidad mayor que la inmovilizada a los otros valores de pH. Considerando la actividad enzimática, la estabilidad y los parámetros cinéticos Km, Vmax y la relación Km/Ki, la β-glucosidasa inmovilizada sobre soportes preactivados con glutaraldehído a pH 5 y 7 son la mejor opción para utilizar β-glucosidasa de almendras.

Palabras clave: β-glucosidasa, inmovilización de enzimas, glutaraldehído, soportes de amino-agarosa, estabilización enzimática.

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1 Introduction

Nowadays the use of lignocellulosic materials to obtain bioethanol has been in great demand, due to these materials are quite abundant polymers in nature (Mohd Azhar *et al*., 2017; Rastogi and Shrivastava, 2017; Singh *et al*., 2017; Zabed *et al*., 2017). The enzymatic hydrolysis of cellulose starts with enzymes cellulases that attack the polymer; the final product is the disaccharide cellobiose. This disaccharide may be hydrolyzed via acid catalysis (but this has not sense if that has not been used in hydrolysis of cellulose) or enzymatically using β-glucosidases. This last step will release two glucose molecules and then, the fermentation to produce ethanol may proceed. The β-glucosidase may be also used to release some flavors (e.g., glycosylated terpenes in wine) (Barbagallo *et al*., 2004; Krammer *et al*., 1991; Palmeri *et al*., 2017; Palmeri and Spagna, 2007; Todaro *et al*., 2008) or produce some glycosylic bonds (Yang *et al*., 2018).

However, the moderate stability and price of the enzymes are avoiding their industrial implementation (Reetz, 2013). Enzyme immobilization eliminates these problems, as the immobilized enzyme can be reused; this strategy has also been applied to immobilize cells (Trujillo *et al*., 2021), finding that organic residues can be ideal for cell adsorption, in addition a reduction in the production costs of biocatalysts is also achieved. Immobilization allows enzymes to be easily reused, and multipoint immobilization tends to improve enzyme stability as well as create favorable enzyme environments. Therefore, this process improves the properties of enzymes such as activity, selectivity, specificity and even their purity (Cantone *et al*., 2013; Liese and Hilterhaus, 2013; Rodrigues *et al*., 2013; Secundo, 2013; Sheldon and Van Pelt, 2013).

One of the most used methods for the immobilization of enzymes is through a covalent bonding (Castro *et al*., 2013) using activated supports such as glutaraldehyde (Barbosa *et al*., 2014), glyoxyl (Mateo *et al*., 2006), epoxy (Mateo *et al*., 2007), from the latter, the glutaraldehyde stands out due its versatility (Betancor *et al*., 2006; Salazar-Leyva *et al*., 2014; Barbosa *et al*., 2015; Zaak *et al*., 2017). Through the application of this method the activation of primary amino groups is achieved (Barbosa *et al*., 2014). In this way, it may be considered a heterofunctional support (Barbosa *et al*., 2013). This may present some advantages if properly used. Therefore, using the properties of glutaraldehyde, three different routes can be applied to immobilize any enzyme of interest (Betancor *et al*., 2006; Barbosa *et al*., 2012). The first is to immobilize the enzyme by ion exchange and then add glutaraldehyde (Betancor *et al*., 2006). This treatment must assure the modification of each amino group of the protein and the support with only one glutaraldehyde molecule; this is achieved with mild immobilization conditions. (Monsan,1978). This method has the disadvantage that it modifies the entire surface of the protein and this in turn modifies the properties of the enzyme.

The others immobilization strategies consist of in using supports pre-activated with glutaraldehyde (Betancor *et al*., 2006; Barbosa *et al*., 2014). Contrary to the previous method the conditions must be drastic to ensure the modification with two glutaraldehyde molecules; it must be also taken care to avoid the uncontrolled polymerization of glutaraldehyde. (Monsan,1978). Immobilization occurs randomly; if it is carried out at a low ionic strength, the first step is through ion exchange and subsequently covalent bonding is achieved. Otherwise, by using high ionic strength, the covalent immobilization occurs immediately through the most reactive amino group. The appropriate pH for immobilization using glutaraldehyde must be very close to pH 7.0, because in alkaline environments it loses stability. After the first immobilization, the proximity between the glutaraldehyde and the amino of the proteins increases the effective concentration, and thus more enzyme-support bonds can be established. However, at pH 7 the reactivity of amino groups Lys will be very low, while amino glutaraldehyde will maintain reactivity over a wide pH range. (Barbosa *et al*., 2014; Dos Santos *et al*., 2015; Zaak *et al*., 2017).

The alteration of the immobilization conditions can increase the options for the use of glutaraldehyde (Wine *et al*., 2007). Previous studies have shown that the immobilization of enzymes by the adsorption method results in different stabilities (Salazar-Leyva *et al*., 2014). In addition, when using glutaraldehyde (Zaak *et al*., 2017) pH influences the properties of the immobilized enzymes (Barbosa *et al*., 2014).

The β-glucosidase from almonds through biocatalysis is used in the industry to synthesize compounds such as isoflavones used in the formulation of drugs against hypertension due to low production costs (Hati *et al*., 2020), however the obtained glucose can be applied on other studies. Therefore, this research aimed to immobilize the dimeric β-glucosidase enzyme obtained from almonds in amino-

agarose supports preactivated with glutaraldehyde and study the stability under different immobilization conditions.

2 Materials and methods

2.1 Materials

β-glucosidase from almonds (β-glucosidase), ethylenediamine and 4-nitrophenyl-β-D- glucopyranoside (p-NPG) and sepharose CL-4B were purchased from Sigma-Aldrich (St. Louis MO, USA). All other compounds and solvents were of analytical degree. Protein concentration was evaluated using Bradford´s method using bovine serum albumin as standard (Bradford, 1976). The supports of aminated MANAE and MANAE-glutaraldehyde agarose beads were prepared as previously described (Zaak *et al*., 2017).

2.2 Determination of β-glucosidase activity

β-glucosidase activity was determined following the increment of absorbance at 380 nm produced by the release of p-nitrophenol in the hydrolysis of 5 mM p-NPG in 50 mM sodium phosphate at pH 7 and 25ºC (ε was calculated to be 3,459.3 M-1 cm-1 under these conditions) using an spectrum (DR 6000 of HACH, Loveland, Colorado, USA) with stirring and thermostatization. The reaction was initialized by adding 200 μL of enzyme solution or suspension in 2.5 mL of substrate solution, for 5 minutes. The initial activity was taken as reference, which corresponded to 100%. One unit of β-glucosidase activity was defined as the amount of enzyme required to release 1 μmol pNP/min under the stated conditions. The activity was determined for both free and immobilized enzymes. To determine the kinetic parameters, the concentration of p-NPG was varied from 0.1 mM to 10 mM and glucose was added at concentrations from 0 mM to 100 mM.

2.3 β-glucosidase immobilization

10 g of the corresponding support (MANAE or MANAE-glutaraldehyde) was added to 100 mL of β-glucosidase commercial extract solution (containing 1 mg/mL) in 25 mM sodium acetate at pH 5, 25 mM sodium phosphate at pH 7, 25 mM carbonate-bicarbonate at pH 9, and 25ºC. As a reference, an identical suspension was prepared using inert agarose, as a support. Periodically, samples of the supernatant and suspension were taken. After immobilization, the biocatalyst was washed with an excess of distilled water. Immobilization was also carried out in 200 mM sodium phosphate buffer at pH 7 on supports pre-activated with glutaraldehyde. All immobilized enzymes were washed with distilled water and stored at 4 ° C.

2.4 Thermal stability of the different immobilized β-glucosidase

The thermal stability was carried out using free at 52°C and immobilized enzyme at 55°C and a concentration of 1 mg/mL in 25 mM sodium phosphate buffer pH 7 and in 25 mM sodium acetate buffer pH 5. Samples were taken at certain times to measure activity enzymatic using p-NPG as a substrate.

2.5 Effect of pH on immobilized β-glucosidase activity

Activity was determined at pH 3-9, using 50 mM acetate at pH 3-6 or phosphate at pH 6-8. The immobilized enzyme (50 μl) was added to 1.25 mL of the corresponding 100 mM buffer and stirred for 10 minutes to ensure that the internal and external pH were the same, due to the characteristics of the support such as the density of amino groups. Finally, 1.25 mL of 10 mM pNPG was added to initialize the reaction. Enzyme activity was determined at each pH value.

2.6 Desorption of the immobilized enzyme from the support

The immobilized enzymes were incubated in increasing concentrations of NaCl at pH 7 and 25 °C, for 30 minutes. Enzymatic activity of supernatant and suspension were determined. A suspension with free enzyme was used as a reference under the same conditions.

3 Results

3.1 Effect of pH and enzyme concentration in enzyme activity

The Figure 1 shows the stabilities of 1 and 0.1 mg/mL β-glucosidase solutions at pH 5, 6 and 7 and 52° C. The major stability was presented at

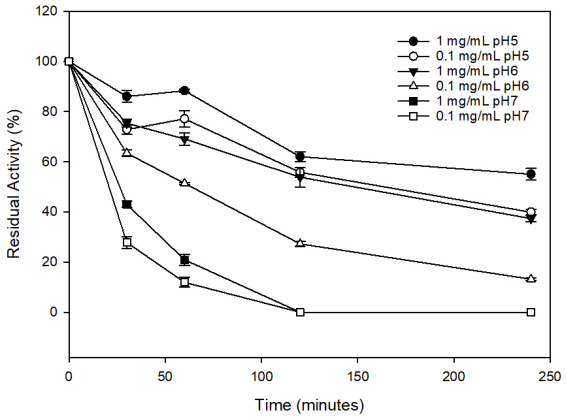

Figure 1. Effect of the concentration on the stability of free β-glucosidase at different pH values at 52°C. Concentrated enzyme solution had 1 mg protein/mL; diluted enzymes solutions had 0.5 mg protein/mL.

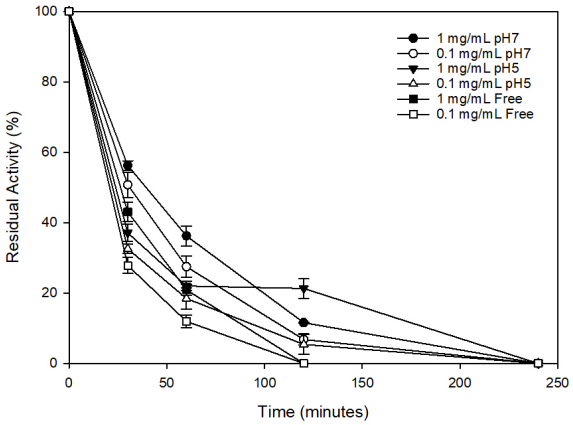

Figure 2. Effect of the enzyme concentration and immobilization pH value in the stability of β-glucosidase at pH 7 and 55°C.

pH 5, decreasing whereas pH increased; it was also observed that the diluted enzyme was less stable than the concentrated one. The inactivation of the diluted enzyme was faster at pH 7; however, all the diluted enzymes were less stable than the concentrated ones. The latter was observed, because the subunits of the enzyme dissociate during the inactivation, generates a dependence with the protein concentration.

3.2 Immobilization of β-glucosidase on aminated supports

Ion exchange immobilization of β-glucosidase was carried out on amino-agarose beads. Table 1 shows the immobilization courses at pH 5 and 7 the supernatant sample was taken after centrifugation and the suspension sample was taken under agitation, that is, the β-glucosidase bound to the support. As observed, immobilization was successful at both pH, however, immobilization was faster at pH 5, no loss of its enzymatic activity was observed. The used support presents two ionizable groups, with pK values of 6.8 and 10 corresponding to the primary and secondary amino groups respectively (Fernandez-Lafuente *et al*., 1993). This faster immobilization at pH 5 is related to the ionizable groups of the enzyme which has more cationic groups at pH 5 than at pH 7, because its isoelectric point is 8.7. An important result was the increase in enzymatic activity after immobilization, at pH 5 reaching up to 147%, while in the one immobilized at pH 7 up to 117%. This increase in the activity of the immobilized enzyme at pH 5 may be due to a change in the assembly of both subunits

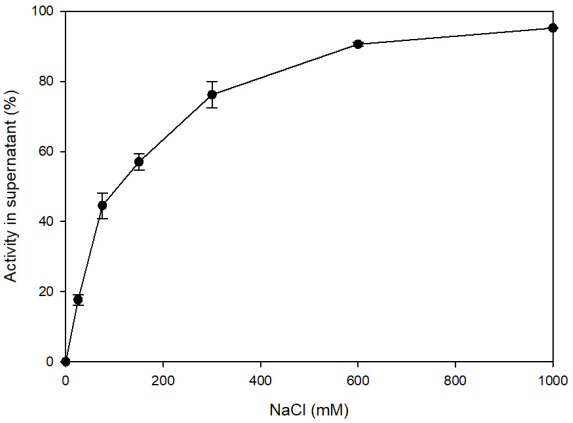

Figure 3. Desorption of the β-glucosidase from the MANAE agarose beads at pH 7 and 25 °C in different NaCl concentrations. Experiments were performed as described in Methods section.

of the protein, thus, a structure with higher enzymatic activity is conformed. However the environment also presented a positive effect, due to enzyme-support interactions (Rodrigues *et al*., 2013).

The β-glucosidase immobilized for adsorption was incubated at 55° C. Figure 2 shows the thermal stability of the immobilized enzyme by ionic adsorption. As observed, an effect of concentration on stability can be noticed, which suggests that the enzyme could bind only by some of its subunits, Otherwise, the enzyme immobilized at pH 7 showed greater stability than that immobilized at pH 5, the concentration effect is more significant in the enzyme which was immobilized at pH 5.

Table 1. Immobilization of β-glucosidase on aminated supports at pH 5 and 7 and 25°C.

	Relative activity (%)*			
	pH 5		pH 7	
Time (hours)	Suspension	Supernatant	Suspension	Supernatant
0	100.00	100.00	100.00	100.00
0.5	147.07±4.342	6.18±0.274	87.68±3.142	10.26±2.313
1	137.23±2.456	7.13±1.854	85.65±4.564	9.48±1.756
2	135.64±5.435	4.32±0.546	117.67±5.435	12.92±3.012
24	129.53±2.945	3.24±1.023	90.22±4.122	8.01±1.465
48	125.95±3.761	1.19±0.734	85.8±2.452	7.62±0.985
52	97.31±3.975	0.89±0.012	81.49±1.386	6.32±0.657
72	70.37±1.245	0.56±0.091	80.91±2.760	5.12±0.734

* Average values ± standard deviation of test performed in duplicate.

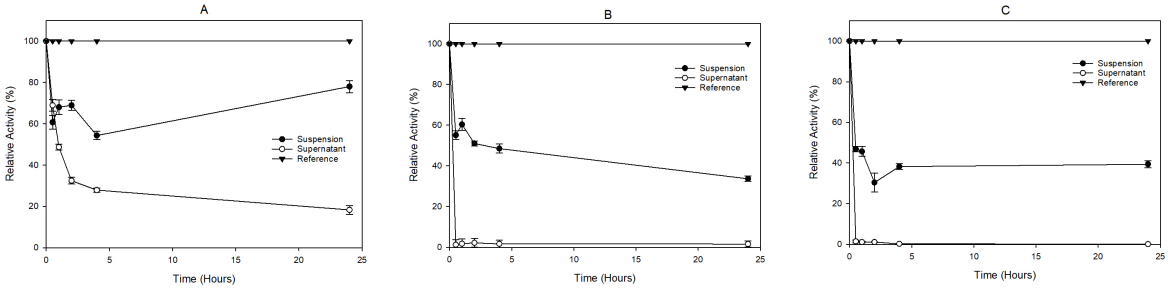

Figure 4. Immobilization courses of β-glucosidase on MANAE-glutaraldehyde pre-activated agarose beads under different conditions. A: Immobilization in 25mM sodium acetate at pH 5 and after 1 h, washed and incubated in 25mM sodium phosphate at pH 7; B: immobilization in 25mM sodium phosphate at pH 7, C: immobilization in 25mM sodium phosphate at pH 9. Other specifications are described in Methods section.

Researchers explain that the enzyme may present different orientations with the support, depending on the immobilization pH (De Albuquerque *et al*., 2016). It is important to notice that all the immobilized enzymes were more stable than the free enzyme. All the immobilized enzymes were incubated in increasing concentrations of NaCl at pH 7 (Figure 3) to check the binding of the enzyme with the support. It can be observed that approximately 60% of the enzyme molecules are released at a concentration of 75 mM. Nevertheless, 24% of the enzymes remained immobilized at 300 mM NaCl. This corroborates that some enzyme molecules were binded by only one subunit, while others were binded by both subunits, due the contact surface with the support was greater (Fernandez-Lafuente, 2009; Lopes *et al*., 2021).

3.3 Immobilization of β-glucosidase on pre-activated supports with glutaraldehyde

Immobilization by ion exchange allowed to obtain enzymes with good activity; even though some stabilization was achieved, it was decided to immobilize β-glucosidase using the versatility of glutaraldehyde. Thus, a reduction in the risk of desorption of the enzyme from the support and dissociation of its subunits may be presented. β-glucosidase was immobilized on MANAE-glutaraldehyde agarose beads at pH 5 (Figure 4A), 7 (Figure 4B) and 9 (Figure 4C), and at pH 7 using 200 mM sodium phosphate to form a covalent bond from the beginning of immobilization (Figure 6) (Barbosa *et al*., 2012). Figures 4B, C and 5 show that the immobilization rate was similar at pH 7, 9 and at high ionic strength (pH 7). The latter may be explained because more than 90% of the enzyme

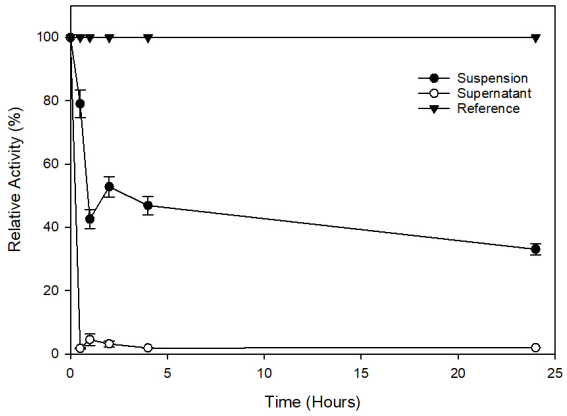

Figure 5. Immobilization in 200 mM sodium phosphate at pH 7.

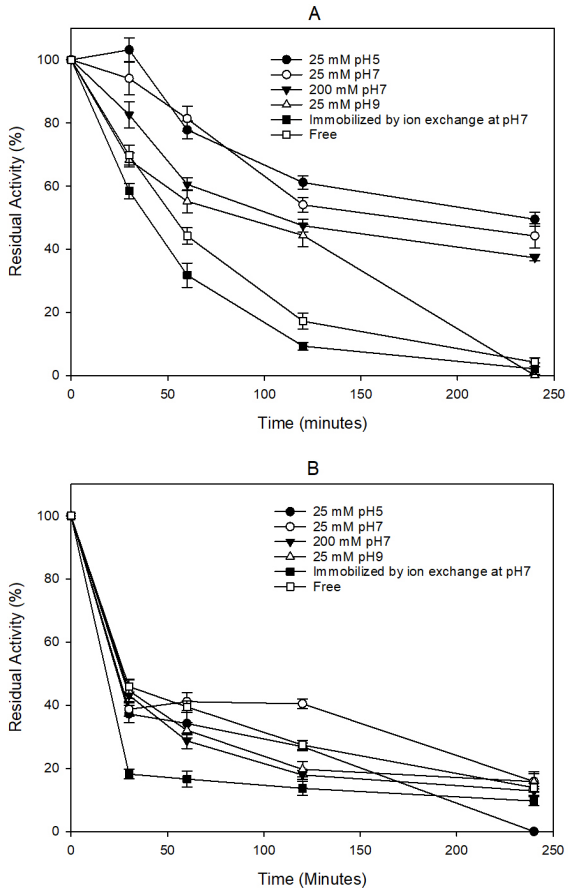

Figure 6. Inactivations courses at pH 5 (A) or pH 7 (B) and 55 °C of different preparations of β-glucosidase immobilized on MANAE-glutaraldehyde pre-activated agarose beads.

was immobilized from the first measurement, while at pH 5 the immobilization rate was slower (Fig 4A). This result may indicate that an ionic exchange of the enzyme occurred in the support pre-activated with glutaraldehyde as the first immobilization step (Andrades *et al*., 2019). In all cases, a decrease in enzyme activity was observed after immobilization. This effect may be generated by steric hindrances (Morellon-Sterling *et al*., 2021) and it can also be related to a distortion of the enzyme caused by the immobilization process or due to diffusion limitation of the substrate (Boudrant *et al*., 2019). Nevertheless, the immobilized enzyme at pH 5 maintains up to 80% of its initial activity, during 24 h. The immobilized enzyme was incubated in NaCl (600mM) pH 7, and no significant amount of the enzyme was released to the supernatant, confirming covalent binding with the support.

3.4 Thermal stability

The thermal stability of the biocatalysts was verified at pH 5 and 7 and 55° C (Figures 6A y B) where in all cases the immobilized enzyme in pre-activated supports was more stable than that immobilized by ion exchange, and this was already more stable than the free enzyme. During the inactivation at pH 5 and after 2h, the immobilized enzymes at pH 5, 7 and at high ionic strength (pH 7), maintained 49, 44 and 37% of its initial activity, respectively. However, the stability at pH 7 did not show a good behavior. These results are comparable with the β-glucosidase from *Malbranchea pulchella* immobilized on MANAE-agarose supports, which maintained 60% of its activity at 50° C and retained only 23% at 60° C (Maria *et al*., 2019). The treatment with glutaraldehyde of the enzymes previously immobilized on amino supports only via ion exchange represents an alternative to improve the stability of immobilized enzymes is (López-Gallego *et al*., 2005), stability can be further increased, by achieving some inter or intramolecular protein crosslinking bonds (Rodrigues *et al*., 2021). This has been previously verified by immobilizing β-glucosidase from *Aspergillus niger* in different amino-agarose beads with glutaraldehyde, obtaining greater stability in the immobilized enzyme and treated with glutaraldehyde, than in the immobilized one on pre-activated supports. (Vazquez-Ortega *et al*., 2018). The enzyme concentration did not show a significant difference in the stability of the enzyme, confirming that both subunits were bound to the support. It is important to notice that the immobilization process also improves the stability of enzymes at low temperatures (Nawaz *et al*., 2021), these researchers

Table 2. Kinetic parameters of different β- glucosidase biocatalyst at pH 7 and 25 °C.

Sample	K_m (mM)	V_{max} (μmol/min/mg protein)	K_i (mM)	K_i/K_m
Free enzyme	12.53±2.481	9.01±0.619	54.19±6.121	4
Pre-activated support - β-glucosidase - pH 5	1.42±0.054	7.46±0.077	46.00±7.527	32
Pre-activated support - β-glucosidase - pH 7	1.20±0.033	17.22±0.012	33.82±23.193	28
Pre-activated support - β-glucosidase - pH 9	10.36±1.817	4.38±0.0329	66.93±12.781	6
Pre-activated support-β-glucosidase - 200 mM/pH 7	5.33±0.861	6.61±0.011	38.09±19.742	7

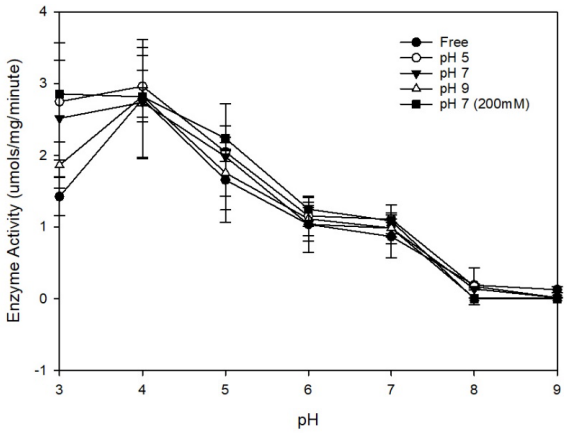

Figure 7. Effect of pH on the activity versus p-NPG of the different β- glucosidase immobilized by ion exchange and covalent bonding. Further details are described in Methods section. Activity is given in μmols/mg/min.

concluded that the immobilized enzyme was more stable than the free one at temperatures of -20 °C, 4 °C and 25 °C; the effect is due to the protective microenvironment provided by the support.

3.5 Effect of pH on the activity immobilized β-glucosidases

The effect of pH in determining the activity of immobilized β-glucosidase under the different conditions was determined (Figure 7). The behavior was similar for all immobilized enzymes; the maximum activity was observed at pH 4 and the activity decreased when the pH moved away from this value. At pH 3, all the immobilized enzymes were more active than the free enzyme. At pH 5, 6 and 7, all immobilized enzymes were 2 - 3-fold more active than the free enzyme, this results contrast with those obtained for other cellulases (Junqueira *et al*., 2019), where greater activity was presented at pH 5 and 6, depending on the type of support used to immobilize. Therefore, immobilization had a positive effect on enzyme activity, depending on the pH at which the activity is measured and the immobilization pH.

3.6 Kinetic parameters of immobilized β-glucosidases at pH 7

Table 2 shows the kinetic parameters of the immobilized enzymes on pre-activated supports. The immobilized enzymes have a lower value of Km, compared with the free enzyme, even obtaining values approximately 10 times lower as in the case of the enzyme immobilized at pH 7. These results showed that the substrate had better accessibility to the immobilized enzyme than the free enzyme. Otherwise, the highest Km value of the immobilized enzymes was given for the immobilized at pH 9, in general it is due to ionic strength, steric effects and diffusion limitations (Da Silva *et al*., 2014). On the other hand, Vmax showed higher values for immobilized β-glucosidases at pH 7. The latter suggests that it was the most active when compared to the others. Previous research affirmed that β-glucosidase is an enzyme which presents some issues for its use, since it suffers a competitive inhibition by glucose (Kuusk and Väljamäe, 2017). Due to this, all the immobilized enzymes on pre-activated supports and the free enzyme were incubated with p-NPG and glucose concentrations, to determine the degree of glucose inhibition. Regarding to this, analogously the inhibition constant (Ki) shows had a similar behavior to Km, except for the enzyme immobilized at pH 9; the latter showed greater tolerance to glucose, with a Ki of 66.93 mM (Table 2). Moreover, this value presented a lower tolerance when compared to the β-glucosidase obtained from *Aspergillus fumigatus* (Ki=543 mM) (Ratuchne and Knob, 2021). However, this enzyme its very tolerant of glucose because it is an unusual enzyme. Ki/Km ratio maintained a similar range for the enzyme immobilized at pH 9 and at pH 7 at high ionic strength (200 mM) when compared to the free enzyme, however the ratio was better with a higher value for the enzyme immobilized at pH 5 and 7.

Considering the enzymatic activity, the stability and the kinetic parameters Km, Vmax and the Km/Ki ratio, the β-glucosidase immobilized on supports pre-activated with glutaraldehyde at pH 5 and 7 are the best option to use β-glucosidase from almonds.

Conclusions

The present study demonstrated the effective immobilization of β-glucosidase from almond by different methods, as ion exchange and covalent immobilization using the versatility of glutaraldehyde; in all cases it was observed that the enzyme was rapidly immobilized to the support. The pH influenced the activity of the immobilized β-glucosidase in supports pre-activated with glutaraldehyde, while in the ionic exchanged it was not so relevant; its activity was maintained above the at pH 5 and 7. Both immobilizations by ion exchange and covalent attachment showed greater stability than the presented by the free enzyme. On the other hand, the role of the inactivation pH was critical to maintain the activity/stability of the immobilized enzymes. Immobilization produces a positive effect on the activity at different values of pH. In addition, the enzyme immobilized on supports pre-activated with glutaraldehyde were more active than the free enzyme. Otherwise, all the enzymes immobilized at the different pH conditions presented lower values of Km and Ki when compared to the free. Thus, the immobilization of β-glucosidase from almonds at pH 5 or 7 in supports pre-activated with glutaraldehyde presents the best results based on the activity, stability and kinetic parameters.

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