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Short communication

Immobilization of a keratinolytic protease from *Purpureocillium lilacinum* on genipin activated-chitosan beads

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ABSTRACT

Keratinase from *Purpureocillium lilacinum* LPSC # 876 was immobilized on chitosan beads using two different cross-linking agents: glutaraldehyde and genipin. For its immobilization certain parameters were optimized such as cross-linker concentration, activation time and activation temperature. Under optimum conditions, enzyme immobilization resulted to be 96 and 92.8% for glutaraldehyde and genipin, respectively, with an activity recovery reaching up to 81% when genipin was used. The immobilized keratinase showed better thermal and pH stabilities compared to the soluble form, retaining more than 85% of its activity at pH 11 and 74% at 50 °C after 1 h of incubation. The residual activity of immobilized keratinase remained more than 60% of its initial value after five hydrolytic cycles. The results in this study support that glutaraldehyde could be replaced by genipin as an alternative cross-linking eco-friendly agent for enzyme immobilization.

1. Introduction

Of the many supports that have been used for enzyme immobilization, organic or inorganic, natural or synthetic, chitosan is of interest in that it offers some attractive characteristics. It is non-toxic, biocompatible and biodegradable, and it can be used in the form of gel, membrane, beads, or powder. In addition, the effectiveness of chitosan as a support can be associated with certain properties such as its elevated porosity, high hydrophilicity, large adhesion area, and thus, small mass transfer resistance to enzymes [1]. It has also been shown to be an efficient support for the immobilization of several enzymes such as glycosidases, proteases, glucose oxidase, and others [2–4].

Overwhelmingly, as cross-linking agent for enzyme immobilization on activated chitosan beads, glutaraldehyde has been used. This cross-linker not only causes damage to the active sites of the enzyme but also – concerning to adverse health effects to humans – it has been shown to induce eye, skin, and upper respiratory tract irritation, nausea, fatigue, and occupational asthma [5–7]. To overcome this health problem, a lesser toxic cross-linking agent than glutaraldehyde is proposed. Genipin is obtained by enzymatic hydrolysis of geniposide, which is extracted from the fruits of *Gardenia jasminoides* [8] and has been proved to be an excellent natural cross-linker for proteins and chitosan. It was reported that it might replace glutaraldehyde with the advantages of stability and biocompatibility of the cross-linked products in biomedical applications such as drug delivery [9], cartilage regeneration [10], tissue fixation [11], etc. been also applied in leather processing [12], fabrication of food dyes [9], and in herbal medicine [13]. Although genipin have been used to immobilize several enzymes such as β-glucosidase, glucoamylase and naringinase [14] and lipases [15,16] there is none report in which this cross-linking agent has been used for keratinase immobilization.

Keratinases constitute a special group of proteases that hydrolyze hard-to-degrade keratin substrates. *Purpureocillium lilacinum* LPSC # 876 produced a 37 kDa serine protease when grown using hair waste as substrate [17]. Despite the growing interest in these enzymes, just few immobilization studies have been reported where chitosan and glutaraldehyde were used [18,19].

In this work, the use of a natural cross-linker agent for the immobilization of *P. lilacinum* keratinase as an alternative to glutaraldehyde is reported. Various properties of the immobilized keratinase, including optimum conditions for immobilization, pH and thermal stability, reusability and storage stability were

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investigated. To our knowledge, although others keratinases has been immobilized on several supports, the use of genipin as cross-linker on chitosan beads has not been attempted in the past.

2. Materials and methods

2.1. Microorganism and keratinase production

P. lilacinum LPSC # 876 belonging to Spegazzini Institute fungal culture collection (La Plata National University, La Plata, Buenos Aires, Argentina) was cultivated in a hair waste medium for 110 h at 28 °C and 200 rpm. After 110 h of cultivation all the contents of each flask was withdrawn and centrifuged at 5000 × *g* and 4 °C for 20 min in order to purify the enzyme.

2.2. Enzyme purification

Keratinase was purified from the culture supernatant as described elsewhere [17]. The purification protocol consisted of sequential enzyme concentration with ammonium sulphate precipitation (0–85% saturation), gel-filtration chromatography in a Sephadex G-25 column, anion-exchange chromatography in a DEAE-Sephadex column followed by cation-exchange chromatography in a SP-Sepharose-FF column and gel-filtration chromatography in a Superdex-75 column. All chromatographic steps were carried out on an Amersham FPLC-U900 system (General Electric, USA).

2.3. Assay of enzyme activity

Proteolytic activity was measured with azocasein as substrate as described elsewhere [20]. An aliquot of 0.1 ml of the enzyme preparation, suitably diluted, was mixed with 0.250 ml of Tris–HCl buffer (100 mM, pH 9.0) containing 1% (w/v) azocasein, and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 1.0 ml of trichloro acetic acid (10%, w/v). After further 15 min at room temperature, the mixture was centrifuged at 5000 × *g* for 10 min. A reaction blank was performed with 0.1 ml of heat-inactivated enzyme. One ml of 1 M NaOH was then added to 1 ml of the supernatant and the absorbance measured at 440 nm. One unit of protease activity was defined as the amount of enzyme that, under the experimental conditions, causes an increase of 0.1 units in the absorbance at 440 nm per minute.

The activity of the immobilized enzyme was analyzed following the same procedures that used for the corresponding free enzyme with minor modifications: 0.06 g of the immobilized enzyme beads and 0.250 ml of azocasein solution in Tris–HCl buffer (100 mM, pH 9.0) were incubated 3 h at 37 °C and after that, the reaction was stopped by the addition of 1.0 ml of trichloro acetic acid (10%, w/v). After a further 15 min at room temperature, the mixture was centrifuged at 5000 × *g* for 10 min. One ml of 1 M NaOH was then added to 1 ml of the supernatant and the absorbance measured at 440 nm. A reaction blank was performed under the same conditions, except that trichloroacetic acid was added before the addition of the enzyme. Azocasein was synthesized as described by Riffel and Brandelli [21].

2.4. Preparation of chitosan beads

Chitosan beads were prepared according to Chiou et al. [15] with some modifications. 3 g of chitosan powder (degree of deacetylation 85%; Sigma Chemical Co.) was dissolved in 100 ml of 0.25 M lactic acid. The resulting viscous solution was ultrasounded for 30 min, and the beads were formed by extruding the solution through a syringe needle into a coagulant bath of 2 M NaOH with constant stirring. After 3 h incubation, beads (2.17 ± 0.22 mm) were removed by filtration and washed with distilled water until neutrality and stored at 4 °C in 20 mM Tris–HCl buffer (pH 7.0) until activation.

2.5. Activation of chitosan beads

An aliquot of chitosan beads (2 g) was incubated with varying concentrations of glutaraldehyde (1.25; 2.50 and 5.00%; Sigma Chemical Co.) at 30 °C and 150 rpm for 12 h. After the activation time, beads were washed with distilled water to remove the excess of glutaraldehyde and then, stored at 4 °C in 20 mM Tris–HCl buffer (pH 7.0) until used [22].

When genipin (Challenge Bioproducts Co., Yun-Lin Hsien, Taiwan) was tested as cross-linking agent, 2 g of beads were incubated varying genipin concentration (0.05% y 0.150%, m/v), activation time (6 and 12 h) and the activation temperature (30–50 °C). At 30 °C the activation time tested was 12 h, while at 40 and 50 °C was 6 h, always at 150 rpm. After activation time, the beads were washed with distilled water to remove the excess of genipin and stored at 4 °C in 20 mM Tris–HCl buffer (pH 7.0) until used. Genipin solutions were prepared dissolving the crystalline powder with one ml of EtOH and letting stand for 10 min in the dark, after that period of time, solutions were taken up to volume (50 ml) with 20 mM Tris–HCl buffer (pH 7.0) [15].

2.6. Immobilization of P. lilacinum keratinase

Fungal keratinase was immobilized on the cross-linked chitosan beads by incubating 40 beads (0.54 ± 0.01 g) with 4 ml (0.3 U/ml) of enzyme at 4 °C overnight at 150 rpm. Thereafter, beads were recovered and washed with distilled water and stored at 4 °C in 20 mM Tris–HCl buffer (pH 7.0) until further use. An aliquot of free enzyme was incubated under the same conditions in order to quantify the enzyme denaturation produced during the immobilization procedure [18].

The enzyme loading on the chitosan beads achieved during the immobilization process was calculated in terms of enzyme immobilization (*EI*):

$$EI\,(\%) : \frac{U_0 - U}{U_0} \times 100$$

while enzyme loading capacity (*LC*) was calculated according to the equation:

$$LC\,(\mathrm{g}^{-1}) : \frac{U_0 - U}{\text{beads weight}} \times 100$$

where U_0 (U/ml) is the enzyme activity in the solution used for immobilization and U (U/ml) is the activity remaining in the supernatant at the end of the immobilization procedure [3,18,23].

2.7. Activity of immobilized keratinase with respect to time

After the selection of the best condition for enzyme immobilization – in terms of the highest enzyme immobilization percentage achieved – using both cross-linking agents, the effect of reaction time was studied. Several tubes containing approximately 0.03 g of beads were incubated in the presence of azocasein at 37 °C and the incubation time was varied from 2 to 8 h. After that period of time, enzyme activity was measured as described above and expressed as ΔAbs 440 nm.

2.8. Stability and reuse of immobilized keratinase

To evaluate the pH and thermal stability of the free and immobilized enzyme, the residual activities were measured after the

Table 1
Optimization of immobilization conditions for keratinase on activated chitosan beads. Results are expressed as mean ± SD.

Conditions	*EI* (%)[b]	*LC* (g^{-1})[c]
Glutaraldehyde (%, v/v)		
1.25	96.0 ± 0.6	26.5 ± 0.9
2.50	91.1 ± 0.8	26.6 ± 0.4
5.00	93.5 ± 0.4	27.5 ± 0.1
Genipin (%, m/v) (T: 30 °C, AT[a]: 12 h)		
0.050	63.5 ± 2.3	58.8 ± 0.2
0.150	92.8 ± 2.4	92.3 ± 1.7
Genipin (%, m/v) (T: 40 °C, AT[a]: 6 h)		
0.050	70.0 ± 1.6	62.4 ± 1.4
0.150	89.9 ± 1.4	82.4 ± 2.8
Genipin (%, m/v) (T: 50 °C, AT[a]: 6 h)		
0.050	56.1 ± 1.8	32.4 ± 1.5
0.150	70.0 ± 2.6	40.4 ± 1.3

[a] AT (activation time).
[b] *EI* (%): $(U_0 - U)/U_0 \times 100$.
[c] *LC* (g^{-1}): $(U_0 - U)$/beads weight × 100.

samples were incubated for 60 min at different temperatures (20, 30, 40, 50 and 60 °C) and in pH range from 3 to 11.

The initial activity of the immobilized enzyme was measured and then compared with the residual activity of the enzyme obtained after its repeated use for five cycles. After of each cycle (duration of each cycle: 6 h), the immobilized enzyme was filtered, washed with 20 mM Tris–HCl buffer (pH 7.0), and reintroduced into a fresh reaction medium [18]. The remaining enzyme activity was measured as describe earlier.

Both stability and reusability were expressed in a normalized form, with the highest values set as 100% activity.

2.9. Storage stability

The storage stability of free and immobilized keratinase was evaluated by determining the enzyme activity after storage at 4 °C for seven days [23].

2.10. Statistical analysis

All analyses were performed at least in triplicate. The data are expressed as means ± standard deviations. Univariate analysis of variance (ANOVA) was employed on each experiment and tested for their significance.

3. Results and discussion

Keratinase produces by *P. lilacinum* LPSC # 876 when it growths on whole hair waste medium was purified as described elsewhere with an overall purification factor of 19.8 fold with a final yield of 1.3% [17]. The molecular mass of the purified keratinase determined by SDS-PAGE was 37 kDa. Its biochemical features such as pH and temperature stability suggest the suitability of this enzyme as a valuable biocatalyst for industrial processes and motivated us the study and characterization its immobilization on chitosan beads [17].

3.1. Optimum conditions for keratinase immobilization

Various conditions for the immobilization of *P. lilacinum* keratinase using two different cross-linking agents were studied (Table 1). Different concentrations of glutaraldehyde in the range of 1.25–5.00% (v/v) were used and it was observed that in all cases, the percentage of immobilization (*EI* %) was higher than 90%, being 1.25% (v/v) the most effective concentration with 96% of

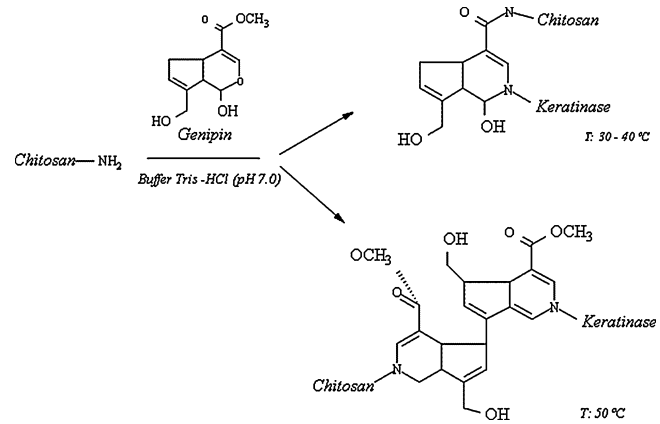

Fig. 1. Hypothetical illustration of the genipin cross-linked chitosan-keratinase network under different activation temperatures.

keratinase immobilization. It can be noticed that when glutaraldehyde concentration increases the immobilization percentage slightly decreased, indicating that as more aldehyde groups are available on the activated chitosan beads, multiple-point attachments of keratinase molecules to the beads will likely occur leading to inactivation of the enzyme [22,24,25]. Concerning to enzyme loading capacity, this parameter did not vary with glutaraldehyde concentration. Even at low concentrations of this cross-linking agent there are enough amount of reactive groups on the surface of chitosan beads to almost immobilize the entire enzyme.

On the other hand, when genipin was used as cross-linker, it was observed that *EI* (%) increases as far as genipin concentration increases, but it decreases with the incrementing of temperature. At 30° and 40 °C, *EI* (%) – using 0.150% (m/v) of genipin – was 92.8 and 89.9%, respectively, while at 50 °C this percentage dropped till 70.0%. When high concentration of genipin is used, a parallel reaction between genipin molecules can take place and cross-link bridges of polymerized genipin can be formed. This parallel reaction can be accelerating by high pH values or high temperatures [11,26,27] explaining why when keratinase was immobilized using 0.150% (m/v) of genipin and 50 °C, the loading enzyme capacity *LC* (g^{-1}) dropped to 40.4. At 50 °C genipin undergone to a self-polymerization reaction prior to cross-link chitosan, giving less reaction groups available on chitosan surface for immobilize the enzyme (Fig. 1).

Enzyme immobilization using genipin is not commonly seen, just a few reports can be found. For example Chiou et al. [15] and Wang et al. [16] reported the use of this cross-linking agent for the immobilization of different lipases onto chitosan beads and mesoporous resins, respectively. While Fujikawa et al. [14] reported β-glucosidase immobilization in calcium alginate gel with a *EI* of 60%.

A glutaraldehyde concentration of 1.25% (v/v) and a genipin concentration of 0.150% (m/v) (activation time and temperature 6 h and 40 °C, respectively) with an immobilization time of 12 h at 4 °C were selected for further work.

3.2. Activity of immobilized keratinase with respect to time

Time course of the product formation using the two immobilized keratinase systems described earlier was studied. As can be seen in Fig. 2, product formation was linear up to 8 h for both systems, while in the case of free enzyme product formation was only linear up to 4 h. This feature was also reported by Bhandari et al. [22] and Ahmed et al. [28], these authors ascribed this feature to diffusional limitations of the substrate to the immobilized enzyme. Although the *EI* (%) obtained using glutaraldehyde was higher than

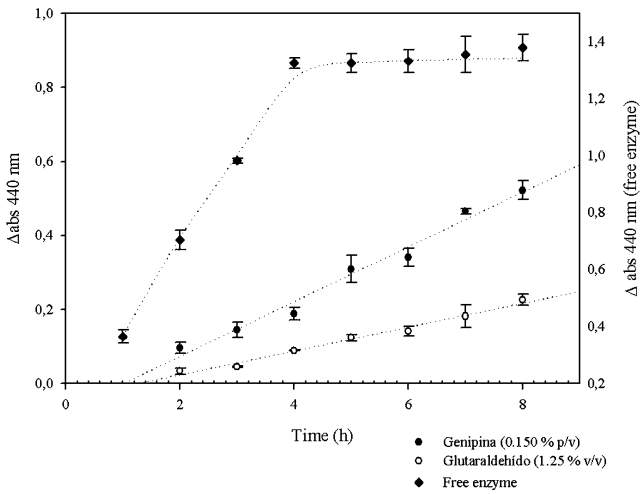

Fig. 2. Time course of the product formation using azocasein as substrate when glutaraldehyde and genipin were tested as cross-linking agent for keratinase immobilization. Experiments were performed in triplicate. The error bars indicate the standard deviation (<10%).

the one obtained using genipin as cross-linking agent (96 and 92.8%, respectively), the highest activity per gram of carrier was reached using genipin (0.39 U/g). The immobilization yield of keratinase on glutaraldehyde–chitosan beads was around 46%, while it increased up to 81% on genipin–chitosan beads, suggesting that the negative effects of glutaraldehyde on keratinase were much greater than genipin. This behaviour may be due to the participation of the catalytic groups of the enzyme in some interactions responsible for the immobilization when glutaraldehyde was used, indicating that genipin is less toxic than glutaraldehyde for the enzyme. Wang et al. [16] reported similar results for the immobilization of lipase from *Candida* sp. 99–125 onto mesoporous resins. The yield of activity of lipase on S-8 resins was less than 30% when glutaraldehyde was used as cross-linking agent increasing to 86% using genipin.

3.3. pH and thermal stability of immobilized keratinase

pH stability of the free and immobilized keratinase (using genipin as cross-linker) was studied in a pH range between 3.0 and 11. It was observed that in a pH range between 4.0 and 9.0 both free and immobilized enzymes retained 100% of its enzyme activity after 1 h of incubation at each pH. At pH 3.0, 10.0 and 11.0 the stability of the immobilized enzyme was greater than that of the free enzyme (Fig. 3A). At pH 3.0 residual activity increased from 42% up to 70% after its immobilization. At pH 11.0 residual activity increased from 68 to 85% after immobilization. The greater stability of the immobilized enzyme at lower pH (3.0) probably reflects the ability of chitosan to form intramolecular salt bridges with anionic groups of the protein, improving the stability of the enzyme [22,29]. Comparing to others keratinases, free and immobilized *P. lilacinum*'s keratinase resulted to be more stable at more harsh acidic conditions. Keratinase from *Chryseobacterium* sp. kr6 immobilized on glutaraldehyde-activated chitosan beads increased its proteolytic residual activity from 90 to 95% at pH 8.0, while at pH 5.0 its increased from 40 to 75% after immobilization [18]. *Aspergillus flavus* keratinase immobilized on calcium alginate showed sensitivity in acidic condition. Comparing with soluble keratinase, at pH 12 the immobilized keratinase retained about 50% of its residual activity; while at acidic pH (pH 4.0 and 5.0) the immobilized enzyme lost all the activity [30]. Similar results were reported by Farag and Hassan [19] in the immobilization of *Aspergillus oryzae* keratinase on sintered glass beads. They reported that high-pH (pH 10.1) did not decrease keratinase activity of both forms but acidic pH did. At

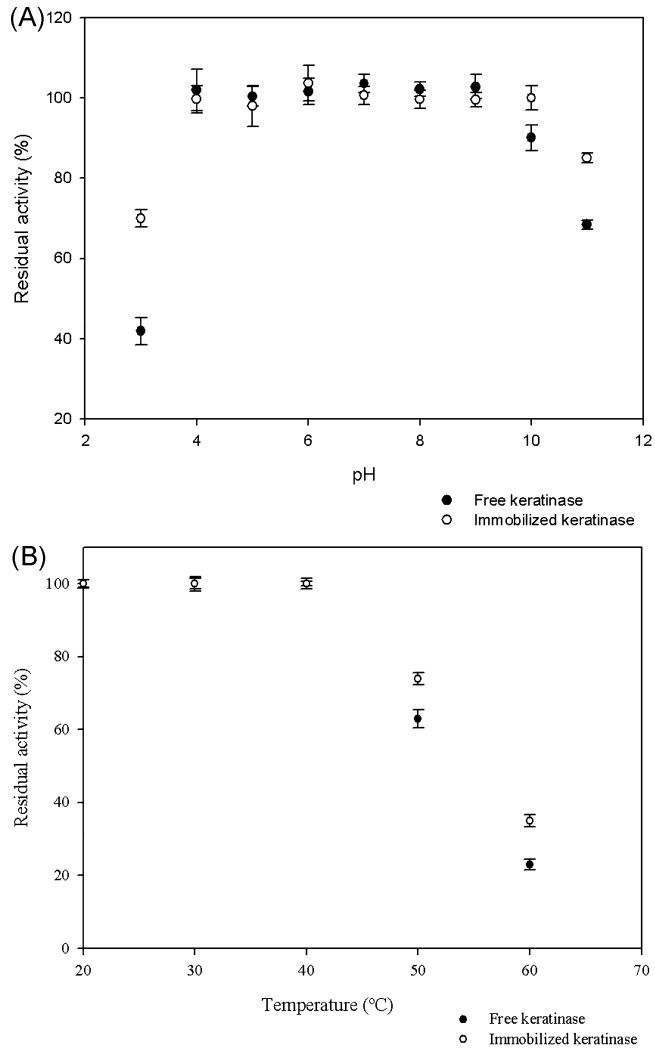

Fig. 3. Effects of pH (A) and temperature (B) on stability of free and immobilized keratinase using genipin as cross-linker. Experiments were performed in triplicate. The error bars indicate the standard deviation (<6%).

pH 3.5 residual activity of free keratinase was about 20% while the immobilized enzyme retained about 35% of the initial activity at the same pH.

The immobilized and free keratinases were incubated at 20, 30, 40, 50 and 60 °C in a water bath for 60 min, and Fig. 3B shows the residual activity at each temperature. Thermal stability of the immobilized enzyme was greatly increased at 50 and 60 °C compared with free enzyme. At 50 °C, residual activity of the free enzyme was 63%, whereas the immobilized enzyme exhibited – at the same temperature – 74% of residual activity. Similar results have previously been reported for other immobilized keratinases [18,19,31].

3.4. Reusability and storage stability of immobilized keratinase

The operational stability of the immobilized keratinase was evaluated in repeated batch process using fresh azocasein in each batch cycle (Fig. 4). *P. lilacinum*'s keratinase can be used repeatedly over an extended period of time. The immobilized keratinase retained 61.37% of its activity after 5 cycles. According to this result, Silveira et al. [18] reported that *Chryseobacterium* sp. kr6's keratinase immobilized on activated glutaraldehyde chitosan beads after 5 cycles of casein hydrolysis showed 63.4% of its residual activity. *Bacillus subtilis* keratinase immobilized on magnetic nanoparticles

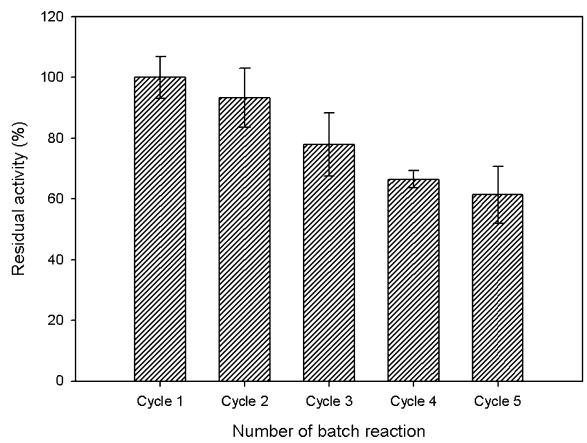

Fig. 4. Reuse of immobilized keratinase. Experiments were performed in triplicate. The error bars indicate the standard deviation (<10%).

showed a structural stabilization with no significant loss in the activity of bound keratinase after being reused five times [31]. The drop tendency in activity may be due because of the leaching of the enzyme during repeated uses, conformational changes and enzyme denaturalization [3,32,33].

Storage stability of free and immobilized keratinase was evaluated determining the proteolytic activity after storage at 4 °C for seven days. Free and immobilized keratinase showed high stability over time. The first day, the free enzyme showed an activity of 0.3 ± 0.007 U/ml and 7 days after the activity was 0.28 ± 0.05, while the immobilized enzyme exhibited an activity of 0.38 ± 0.01 U/g at the beginning of the experience to end with an activity of 0.37 ± 0.04 U/g. In both cases $p > 0.05$, showing that there was no statistical significant difference in activity between day 1 and day 7.

Even though the storage stability of free keratinase was similar to the immobilized keratinase, the immobilized keratinase has the advantage that it could be used repeatedly during an industrial process.

4. Conclusions

In this study, purified keratinase from *P. lilacinum* LPSC # 876 was immobilized on to chitosan beads using glutaraldehyde and genipin as cross-linking agent. Comparing the activity recovery of both systems it was demonstrated that genipin can be effectively used in the immobilization of the keratinase, being this cross-linking agent less toxic to the enzyme than glutaraldehyde. Thermal stability increased after immobilization as well pH stability did. Furthermore, 61.37% of the activity of the immobilized enzyme remained after it reused five times. Therefore, genipin can be considered as an alternative non-toxic and green crosslinking reagent for the immobilization of enzymes.

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