Polyethyleneimine coating of magnetic particles increased the stability of an immobilized diglycosidase

Marisol Minig
Laura S. Mazzaferro
Agostina Capecce
Javier D. Breccia*

INCITAP-CONICET (Instituto de Ciencias de la Tierra y Ambientales de La Pampa-Consejo Nacional de Investigaciones Científicas y Técnicas), Departamento de Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Santa Rosa, La Pampa, Argentina

Abstract

The diglycosidase, α-rhamnosyl-β-glucosidase, from *Acremonium* sp. DSM24697 was immobilized by adsorption and cross-linking onto polyaniline–iron (PI) particles. The immobilization yield and the immobilization efficiency were relatively high, 31.2% and 8.9%, respectively. However, the heterogeneous preparation showed lower stability in comparison with the soluble form of the enzyme in operational conditions at 60 °C. One parameter involved in the reduced stability of the heterogeneous preparation was the protein metal–catalyzed oxidation achieved by iron traces supplied from the support. To overcome the harmful effect, iron particles were coated with polyethyleneimine (PEI; 0.84 mg/g) previously for the immobilization of the catalyst. The increased stability of the catalyst was correlated with the amount of iron released from the support. Under operational conditions, the uncoated particles lost between 76% and 52% activity after two cycles of reuse, whereas the PEI-coated preparation reduced 45–28% activity after five cycles of reuse in the range of pH 5.0–10, respectively. Hence, polymer coating of magnetic materials used as enzyme supports might be an interesting approach to improve the performance of biotransformation processes.

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1. Introduction

Acremonium sp. DSM24697, α-rhamnosyl-β-glucosidase (EC 3.2.1.168), is a specific diglycosidase that hydrolyzes 7-*O*-linked flavonoid rutinosides [1]. The enzyme hydrolyzes the flavonoid hesperidin at the heterosidic bond to yield the disaccharide rutinose (α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranose) and the aglycone hesperetin (Fig. 1). The enzyme has transglycosylation activity when using hydroxylated compounds as sugar acceptors instead of water, including acceptors with phenolic OH groups [2]. Because of its substrate specificity and mode of action, the enzyme has potential use for industrial processing of plant-based foods, analytical assaying of flavonoids, and the synthesis of rutinosides [2, 3].

The development of heterogeneous enzyme preparations facilitates kinetic control and allows reutilization, a key requirement for economically relevant biotransformations [4]. Several glycosidases have been stabilized by immobilization onto a number of supports in view of their application in food processing and fine chemistry [5–8]. Polyaniline magnetic particles have received considerable attention due to their high specific surface area, resistance to microbial attack, and the easy removal of a reaction mixture by the application of a magnetic field [9, 10]. In particular, the magnetic removal of the support would reduce the time of catalyst separation from the reaction mixture, allowing an accurate kinetic control of transglycosylation processes. Nevertheless, little is discussed about possible metal leakage from the support, especially at low pH values, with the concomitant destabilization of the immobilized catalyst throughout the metal-catalyzed oxidation. This work describes the immobilization of *Acremonium* sp. DSM24697,

Abbreviations: *PEI, polyethyleneimine; PI, polyaniline-iron support; PI-PEI, polyaniline-iron support coated with polyethyleneimine; SEM, scanning electron microscope; EI, immobilization efficiency; YI, immobilization yield; BET, Brunauer–Emmett–Teller.*

**Address for correspondence: Javier D. Breccia, PhD, INCITAP-CONICET, Departamento de Quimica, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Av. Uruguay 151, (6300) Santa Rosa, La Pampa, Argentina. Tel.: +54 2954 245200/7426; Fax: +54 2954 432535; e-mail: javierbreccia@gmail.com.*

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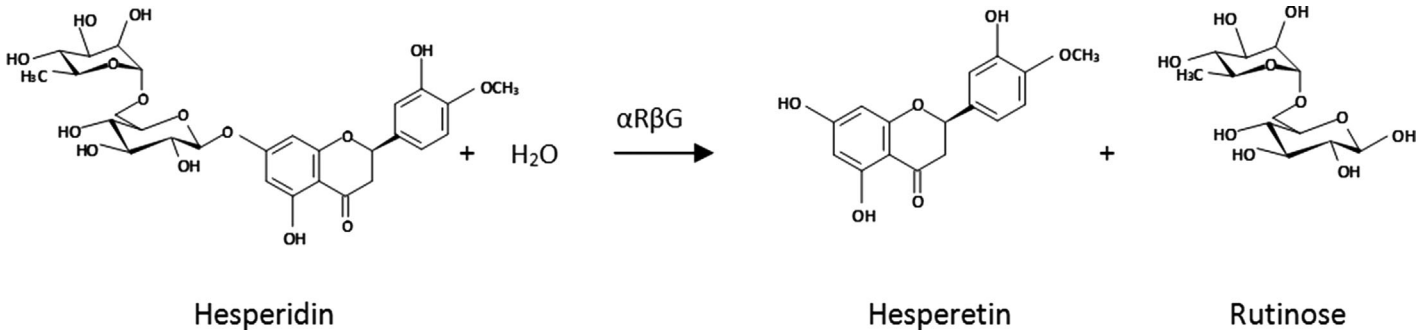

FIG. 1 *Enzymatic reaction catalyzed by α-rhamnosyl-β-glucosidase (EC 3.2.1.168) from Acremonium sp. DSM24697.*

α-rhamnosyl-β-glucosidase—a prone oxidation protein—onto magnetic microparticles and its stabilization by coating the support using a polymer that encompass metal-chelating and -scavenging capacities.

2. Materials and Methods

2.1. Materials

Hesperidin, rutinose, glutaraldehyde (50%), polyethyleneimine (PEI; 50% (w/v); M_W 60 kDa), ammonium peroxydisulfate, and aniline oil were purchased from Sigma (St. Louis, MO, USA), and iron oxide (Fe_3O_4) was purchased from BDH (Poole, Dorset, UK). All the chemicals used were of analytical grade, and the solvents were of the highest quality that is commercially available.

2.2. Production of α-rhamnosyl-β-glucosidase

The α-rhamnosyl-β-glucosidase was obtained from the culture supernatant of *Acremonium* sp. DSM24697, as described elsewhere [1]. The supernatant was freeze-dried and the obtained powder containing 36 U/g was stored at −18 °C.

2.3. Synthesis of polyaniline–iron (PI) support

The magnetic microparticles were prepared by mixing 1.0 g of iron oxide (Fe_3O_4) with 4.5 mL of aniline, 0.5 mL of 10 M HCl, and 50 μL of 10% (w/v) ammonium persulfate at 25 °C for 15 Min [11]. After polymerization, the particles were dispersed and the bundle size was reduced after 2 Min of ultrasonic treatment. The particles were washed four times with distilled water to remove the remaining HCl and stored at 8 °C in Milli-Q water. The morphology was observed by a scanning electron microscope (SEM-Zeiss Supra 40, Gottingen, Germany). Pore size distribution and surface area were estimated by BET analysis of nitrogen adsorption–desorption isotherms performed at 77 K using an Autosorb-1 (Quantachrome Instruments, FL, USA). The particle size distribution was measured by a laser light scattering analyzer (Mastersizer 2000; Malvern Instrument, Malvern, UK).

2.4. Enzyme immobilization

The α-rhamnosyl-β-glucosidase from *Acremonium* sp. DSM24697 was immobilized by adsorption cross-linking using a freeze-dried culture supernatant. The magnetic support (0.5 g) was incubated with 1.0 mL of an enzyme solution containing 25 mg of freeze-dried extract in 50 mM sodium phosphate buffer (pH 8.0). The mixtures were stirred (150 rpm) for 10 Min at 28 °C and then incubated at 10 °C for 14 H. Furthermore, 0.5% (w/v) glutaraldehyde was added and incubated for 30 Min at 5 °C. The insoluble complex was removed by the application of a magnetic field, washed with 50 mM sodium phosphate buffer (pH 6.8), and then incubated for 30 Min in 100 mM Tris–HCl buffer (pH 7.5) at 25 °C to block the unreacted aldehyde groups. The heterogeneous preparation was washed (four times) with 50 mM sodium phosphate buffer (pH 6.5) and stored at 8 °C for further use. The immobilization yield (YI) and the immobilization efficiency (EI) are defined as

$$\mathrm{YI} = \frac{A_{\mathrm{loaded}} - A_{\mathrm{unbound}}}{A_{\mathrm{loaded}}} \times 100$$

$$\mathrm{EI} = \frac{A_{\mathrm{particles}}}{A_{\mathrm{loaded}} - A_{\mathrm{unbound}}} \times 100$$

where A_{loaded} is the enzyme activity loaded, A_{unbound} is the enzyme activity remaining in the supernatant, and the activity bound to the support ($A_{\mathrm{particles}}$) was calculated as the units of enzyme activity per g of wet particles.

2.5. Coating PI particles with PEI

PI–PEI was prepared as described by Pessela et al. [12] with slight modifications. A total of 1.5 g of wet particles was suspended in 10 mL of 1% (w/v) PEI. The suspension was mildly stirred at 25 °C for 3 H. Magnetic particles were removed and washed twice with 20 mL of 100 mM sodium acetate buffer (pH 4.0), twice with 100 mM sodium borate buffer (pH 7.8), and finally with Milli-Q water and stored at 8 °C. The amount of coated PEI was estimated by the difference of bound Cu^{2+} to coated and uncoated support. Briefly, 50 mg of particles was incubated with 1 mM $CuSO_4$ (250 μL) at 25 °C in a rocking table for 15 Min. An aliquot of the supernatant was incubated with 200 μL of 0.1% (w/v) PEI solution. The concentration of Cu^{2+}

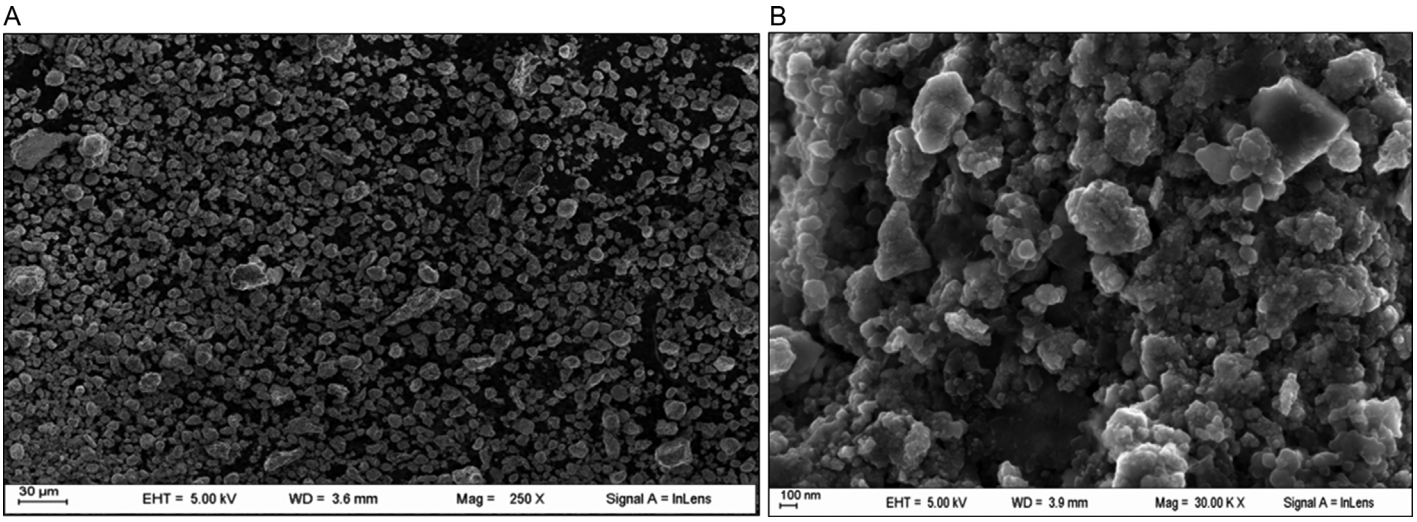

FIG. 2 *SEM digital micrographs of synthesized magnetic PI particles at different magnifications: (A) 250× and (B) 30,000×.*

ions was quantified by measuring the PEI–Cu^{2+} complex (ε = 0.0031 μM^{-1} cm^{-1}) at 273 nm [13].

Iron leakage from magnetic particles was assessed at different pH values. Fifty milligrams of each support (PI and PI–PEI) was suspended in a 0.5 mL of 50 mM buffer solution: Sodium citrate (pH 4.0 and 5.0), sodium phosphate (pH 6.0–8.0), and universal buffer (pH 9.0 and 10) were incubated for 30 Min at 60 °C. The particles were recovered by applying a magnetic field, and the amount of iron in the supernatant was determined by the thiocyanate colorimetric method [14]. The effect of the iron released from the magnetic particles on soluble α-rhamnosyl-β-glucosidase was evaluated. For this purpose supernatant from 10% (w/v) magnetic particles (PI and PI–PEI) in 50 mM sodium citrate buffer (pH 5.0) was incubated for 1 H at 60 °C. The supernatant (50 µL) mixed with 25 µL free α-rhamnosyl-β-glucosidase (1.49 ± 0.074 U/mL) in 50 mM sodium citrate (pH 5.0) was incubated for 30 Min at 70 °C and then the enzymatic activity at 70 °C was measured. The control was performed by incubating the enzyme in 50 mM sodium citrate buffer (pH 5.0).

2.6. Enzyme assays

For quantification of α-rhamnosyl-β-glucosidase activity, each reaction contained 450 µL of substrate, 0.11% (w/v) of hesperidin in 50 mM sodium citrate buffer (pH 5.0), and 50 µL of suitably diluted enzyme solution. The reaction was performed for 30 Min at 60 °C and stopped by adding 500 µL of 3,5-dinitrosalicylic acid (DNS) [15]. For immobilized enzyme activity, each reaction contained 50 mg of particles in 500 µL of the substrate; the catalyst was magnetically separated before adding DNS. The tubes were placed in a boiling water bath for 10 Min and cooled before measuring the absorbance at 540 nm. Another method used for enzyme activity was the measurement of released hesperetin at 323 nm [16]. One unit of α-rhamnosyl-β-glucosidase activity was defined as the amount of enzyme required to release 1 µmol of rutinose per Min. The effect of pH and reuse of the immobilized enzyme were studied in cycles of 30 Min at 60 °C in different 50 mM buffer solutions: sodium citrate (pH 5.0), sodium phosphate (pH 6.0–8.0), and universal buffer (pH 9.0 and 10).

3. Results and Discussion

3.1. PI particles

Microparticles of PI were synthesized according to the method of Lee et al., rendering an amorphous material with a porous surface and high size dispersion. In contrast to the nanoscale particles reported for this bundle preparation, the SEM images of the support showed particles with diameters ranging between 3 and 20 µm exhibiting a wrinkled and porous surface (Fig. 2) [11]. The specific surface of PI particles determined by N_2 adsorption–desorption isotherms was 15.8 ± 8 m^2/g, a pore volume average of 0.0056 cm^3/g, and 2 nm width. Regarding size dispersion, the material showed two microparticle populations, one dispersible lower size (5.3 µm average diameter) responsible for the colloidal behavior that represents <1.0% of the total weight and the major fraction of larger particles (34.4 µm average diameter) that remain settled in a static reactor (Fig. 3A). The PI material was used to produce a heterogeneous preparation of the fungal glycoside hydrolase, α-rhamnosyl-β-glucosidase [1].

3.2. Immobilization of α-rhamnosyl-β-glucosidase on PI particles

The supernatant of the submerged culture of *Acremonium* sp. DSM24697 was used as a diglycosidase source for immobilization onto PI particles by adsorption and cross-linking with glutaraldehyde. The simple adsorption was significant, but the

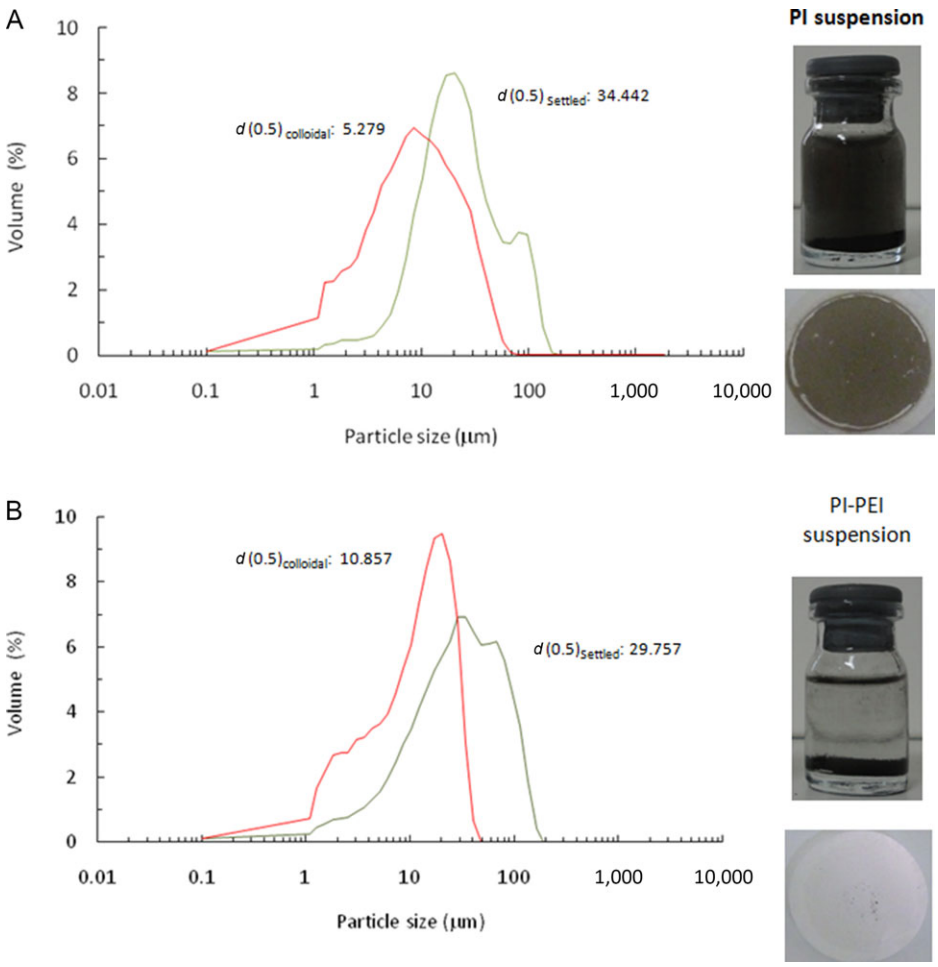

FIG. 3 *Size distribution of (A) PI and (B) PI–PEI measured by laser light scattering: (▁) settled particles and (▁) colloidal fraction. (Right) Flacks containing (A) PI and (B) PI-PEI and below supernatant filtered using a 0.22 µm pore size nylon filter for PI and PI-PEI, respectively.*

enzyme leaked (65%) after the first use. The covalent binding with glutaraldehyde was essential to reach a stable preparation, although the EI was significantly reduced, as it had been demonstrated previously for the immobilization of related enzymes such as β-glucosidase and α-arabinofuranosidase [5, 7, 17]. The final YI of the process was 31.2%, with an EI of 8.9%. The temperature profile of the immobilized biocatalyst exhibited an optimal temperature of 10 °C below compared to the soluble enzyme (Fig. 4A). This slightly reduced stability in conjunction with the prone oxidation nature of α-rhamnosyl-β-glucosidase suggests that metal ions from the support could be involved in the preparation instability [8].

3.3. PI composites coated with PEI

Pessela et al. [6] reported that the protein adsorption strength was higher using PEI supports, where desorption of β-galactosidase could be prevented even at high ionic strength using this polymer (0.5 M NaCl at pH 7.0). On the other hand, PEI was reported as a protein stabilizer against metal-catalyzed oxidation [8, 13]. Therefore, the hydrosoluble polymer was considered an interesting additive for the immobilized preparation to improve catalyst stability. It is known that agglomeration/aggregation of colloidal particles in water can be favored by multivalent cations through physical collision and particle bridging [18]. In this case, the PI coated with PEI (PI–PEI) rendered approximately 0.84 mg/g PEI adsorbed to the magnetic support. Although the preparation contained a lower ratio of PEI (<0.1% w/w), the physical properties of the coated material changed radically. The colloidal fraction was significantly reduced (<0.5% w/w), and the average diameter of particle size rose up to 10.9 µm for the colloidal fraction, whereas the average of the settled fraction was 29.8 µm (Fig. 3B).

Considering the pKa values of branched PEI (4.5 for primary, 6.7 for secondary, and 11.6 for tertiary amine groups), it is reasonable to guess that at pH values ≈5 and below, the polymer PEI will exist in its protonated form [19]. It is known that PEI forms complexes with acidic proteins due to electrostatic forces and the binding of enzymes onto composites

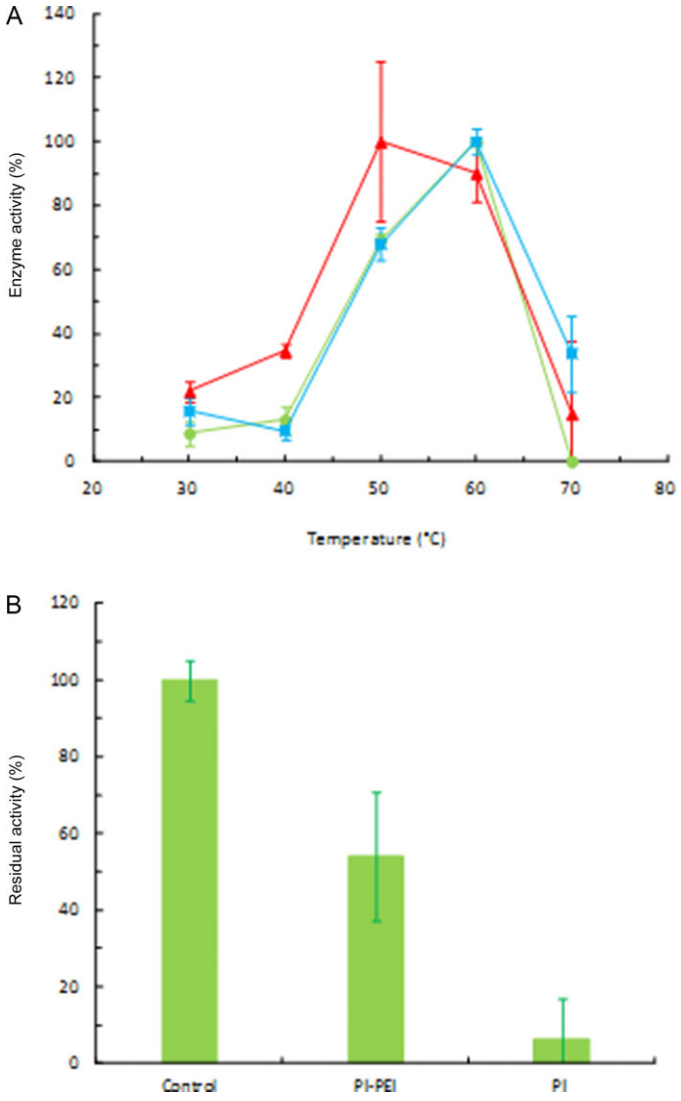

FIG. 4

(A) Temperature profile of the enzyme activity. Free α-rhamnosyl-β-glucosidase (●) and immobilized α-rhamnosyl-β-glucosidase: (▲) PI; (■) PI-PEI. One hundred percent for the free enzyme was 0.89 U/mL and for the immobilized catalyst was PI: 0.019 U/g and PI–PEI: 0.031 U/g. (B) Effect of PI and PI–PEI supernatant on the stability of free α-rhamnosyl-β-glucosidase was assessed through incubation and further hesperidin hydrolysis at 70 °C. Control corresponds to the enzyme incubated in 50 mM sodium citrate buffer (pH 5.0). Each value represents the means of three independent assays ±standard deviation (SD).

with flexible polymers containing a high density of ion exchange moieties would promote a strong adsorption to the detriment of protein stability [6, 20]. Therefore, it was expected that α-rhamnosyl-β-glucosidase (isoelectric point, 5.7) would interact with PEI in the operational conditions of hesperidin hydrolysis. In this way, PI–PEI support showed a slightly reduced yield of immobilization (27%) and the efficiency was half (4.8%) that of the uncoated material. However, the protein stability of PI–PEI preparation recovered the optimal temperature of the soluble form of the enzyme and the residual activity at 70 °C that was null for the soluble enzyme, increased to 34% for the PEI-coated support (Fig. 4A). To demonstrate whether destabilization of the enzyme was due to metal ions leaked from the support, free α-rhamnosyl-β-glucosidase was incubated with a supernatant of the PI particles previously incubated in sodium citrate (pH 5.0) at 70 °C for 30 Min, and the residual activity was also measured at 70 °C (Fig. 4B). The reduction in the activity strongly suggested that traces of iron released from the support promoted protein destabilization, and the increment in thermal stability gained with the PEI-coated support might be associated with the reduction of metal-catalyzed oxidation promoted by the chelating and free radical scavenging properties of the polycation [13].

3.4. Operational stability of the PI and PI–PEI supports

The recycling of the biocatalyst was evaluated during five cycles of reuse (30 Min, 60 °C) at different pH values (Fig. 5). Interestingly, both heterogeneous preparations of the catalyst (PI and PI–PEI) showed the highest activity in alkaline conditions, exhibiting 80–100% activity between pH 8.0 and 10, respectively. In contrast, the soluble form of α-rhamnosyl-β-glucosidase whose optimum pH was reported at 5.0 presented 50–0% activities in the range of pH 8.0–10, respectively [1]. The uncoated support showed a clear loss of activity after two cycles of reuse in the entire range of pH values and presented only 24–48% residual activity in the third cycle, whereas PEI-coated preparation did not exhibit such a drastic reduction in activity, achieving 55–72% residual activity after five cycles of reuse in the entire range of pH values assayed. The stability in operational conditions was correlated with the measurement of released iron from the support. Although iron leakage was undetected in the range of pH between 7.0 and 10 at acidic pH, both preparations presented free iron after exposure at 60 °C. At pH 5.0, the supernatant of uncoated material contained 15.6 times more soluble iron than the PI–PEI preparation (Fig. 6).

Functionalized magnetic particles have rendered highly stable and reduced leakage of immobilized biocatalysts [21, 22]. Nevertheless, the results presented here strongly linked protein stability with metal-catalyzed oxidation promoted by traces of iron leaked from the support. Shaw et al. [23] reported an immobilized esterase on magnetic nanoparticles obtaining similar thermal and pH stability than the free form of the enzyme; in addition, an immobilized cellulase onto iron oxide nanoparticles lost 48% activity after the first use [24]. These data pointed out that immobilization on metal-containing particles might be deleterious to enzyme stability. However, this phenomenon of instability was attributed to other reasons, such as protein unfolding, product inhibition, or loss of one or more individual components of enzyme complexes, whereas protein oxidation was not suggested [23, 24]. Enzyme stabilization promoted by PEI-coated particles was achieved in a wide range

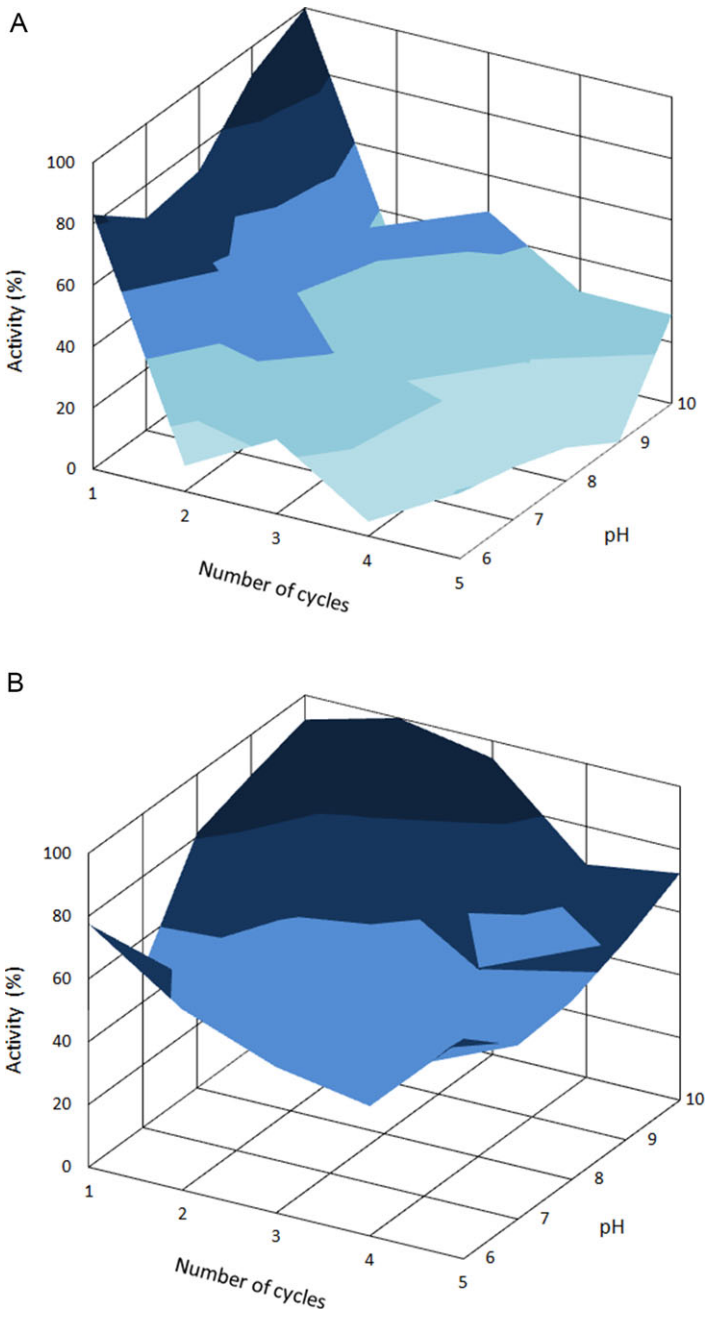

FIG. 5 *Reusability of the immobilized enzyme at different pH values. (A) PI, (B) PI-PEI; the percentage of activity is represented as 100–80, 60–80, 60–40, 40–20, 20–0. The reactions were carried out at 60 °C. One hundred percent of activity corresponds to 0.052 U/g and 0.053 U/g for PI and PI–PEI, respectively. Each value represents the means of three independent assays.*

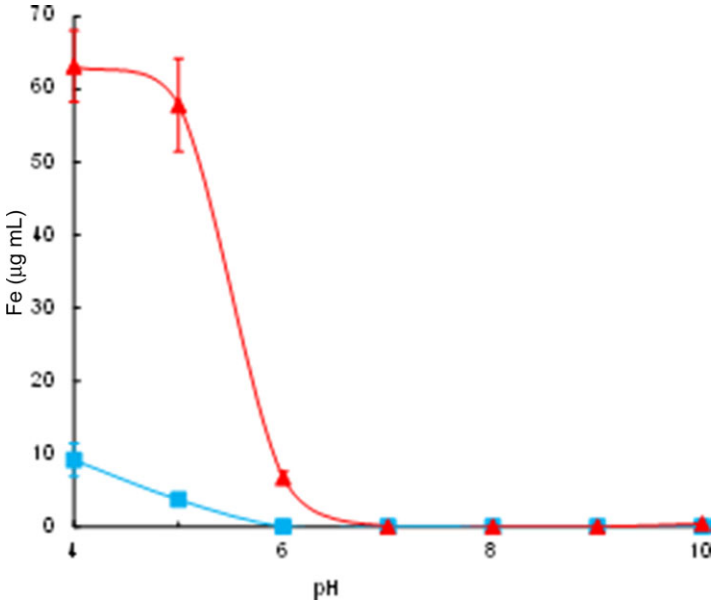

FIG. 6 *Iron released from the synthesized magnetic particles at different pH values. ▲, PI; , PI-PEI. Each value represents the means of three independent assays ±SD.*

of pH (5.0–10), suggesting that metal ions could exert protein deleterious effects in a wide range of pH, whereas chelating as well as scavenger properties of PEI reduced protein damage [13].

4. Conclusions

Hesperidin hydrolysis as well as the synthesis of rutinosylated compounds can be achieved with immobilized α-rhamnosyl-β-glucosidase onto PI. The system will facilitate kinetic control of the process by easily removing the catalyst from the reaction mixture by applying a magnetic field. The reduced stability of the immobilized enzyme onto PI was associated with metal-catalyzed oxidation promoted by free iron released from the support. The stability of the heterogeneous enzyme was improved by coating the iron particles with PEI previous to the immobilization. The PEI-coated preparation of the diglycosidase allowed a more efficient recycling of the catalyst by increasing the stability in a wide range of pH values. Therefore, polymer coating could be an interesting approach to overcome enzyme instability, reach safer heterogeneous biocatalysts, and extend applications of magnetic materials for large-scale biotransformations and food processing.

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6. References

[1] Mazzaferro, L., Piñuel, L., Minig, M., Breccia, J. D. (2010) Arch. Microbiol. 192, 383–393. (2011). Erratum, Arch. Microbiol. 193, 461
[2] Mazzaferro, L. S., Piñuel, L., Erra-Balsells, R., Giudicessi, S. L., Breccia, J. D. (2011) Carbohydr. Res. 347, 69–75.
[3] Minig, M., Mazzaferro, L. S., Erra-Balsells, R., Petroselli, G., Breccia, J. D. (2011) J. Agric. Food Chem. 59, 11238–11243.
[4] Gavrilescu, M., Chisti, Y. (2005) Biotechnol. Adv. 23, 471–499.
[5] Martino, A., Pifferi, P. G., Spagna, G. (1995) Process Biochem. 31, 287–293.
[6] Pessela, B. C. C., Fernández-Lafuente, R., Fuentes, M., Vián, A., García, J. L., Carrascosaa, A., Mateo, C., Guisán, J. M. (2003) Enzyme Microb. Technol. 32, 369–374.
[7] Spagna, G., Andreani, F., Salatelli, I., Romagnoli, D., Pifferi, P. G. (1998) Process Biochem. 33, 57–62.
[8] Piñuel, L., Mazzaferro, L. S., Breccia, J. D. (2011) Process Biochem. 46, 2330–2335.
[9] Bai, S., Guo, Z., Liu, W., Sun, Y. Food Chem. 96, 1–7.
[10] Andrade, L. H., Rebelo, L. P., Netto, C. G. C. M., Toma, H. E. (2010) J. Mol. Catal. B Enzym. 66, 55–62.
[11] Lee, G., Kim, J., Lee, J. (2008) Enzyme Microb. Technol. 42, 466–472.
[12] Pessela, B. C. C., Betancor, L., López-Gallego, F., Torres, R., Dellamora-Ortiz, G. M., Alonso-Morales, N., Fuentes, M., Fernández-Lafuente, R., Guisán, J. M., Mateo, C. (2005) Enzyme Microb. Technol. 37, 295–299.
[13] Breccia, J. D., Andersson, M., Hatti-Kaul, R. (2002) Biochim. Biophys. Acta 1570, 165–173.
[14] Woods, J. T., Mellon, M. G. (1941) Ind. Eng. Chem. 13, 551–554.
[15] Miller, G. L. (1959) Anal. Chem. 31, 426–428.
[16] Mazzaferro, L. S., Breccia, J. D. (2012) Food Chem. 134, 2338–2344.
[17] Spagna, G., Barbagallo, R., Greco, E., Manenti, I., Pifferi, P. G. (2002) Enzyme Microb. Technol. 30, 80–89.
[18] Le Correa, K. S., Valsami-Jonesb, E., Hobbsc, P., Jeffersona, B., Parsonsa, S. A. (2007) Water Res. 41, 419–425.
[19] Demadis, K. D., Paspalaki, M., Theodorou, J. (2011) Ind. Eng. Chem. Res. 50, 5873–5876.
[20] Mazzaferro, L. S., Breccia, J. D., Andersson, M. M., Hitzmann, B., Hatti-Kaul, R. (2010) Int. J. Biol. Macromol. 47, 15–20.
[21] Lei, H., Wang, W., Chen, L., Li, X., Yi, B., Deng, L. (2004) Enzyme Microb. Technol. 35, 15–21.
[22] Kuroiwa, T., Noguchi, Y., Nakajima, M., Sato, S., Mukataka, S., Ichikawa, S. (2008) Process Biochem. 43, 62–69.
[23] Shaw, S. Y., Chen, Y. J., Ou, J. J., Ho, L. (2006) Enzyme Microb. Technol. 39, 1089–1095.
[24] Caoa, M., Lia, Z., Wanga, J., Gea, W., Yuea, T., Lib, R., Colvinc, V. L., Yuc, W. W. (2012) Trends Food Sci. Technol. 27, 47–56.