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Immobilization of pectinase on chitosan-magnetic particles: Influence of particle preparation protocol on enzyme properties for fruit juice clarification

Lucas Dal Magro[a,e], Kelly Silva de Moura[a], Betina Elys Backes[a], Eliana Weber de Menezes[b], Edilson Valmir Benvenutti[b], Sabrina Nicolodi[c], Manuela P. Klein[d], Roberto Fernandez-Lafuente[e,*], Rafael C. Rodrigues[a,*]

[a] Biotechnology, Bioprocess and Biocatalysis Group, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil
[b] Laboratory of Solids and Surfaces, Institute of Chemistry, UFRGS, P.O. Box 15003, ZC 91501-970, Porto Alegre, RS, Brazil
[c] Magnetism Laboratory, Institute of Physics, Federal University of Rio Grande do Sul, P.O. Box 15051, ZC 91501-970, Porto Alegre, RS, Brazil
[d] Department of Nutrition, Federal University of Health Sciences of Porto Alegre (UFCSPA), ZC 90050-170, Porto Alegre, RS, Brazil
[e] Department of Biocatalysis, ICP-CSIC, Campus UAM-CSIC, Cantoblanco, ZC 28049, Madrid, Spain

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ABSTRACT

Magnetic-chitosan particles were prepared following three different protocols enabling the preparation of particles with different sizes – nano (Nano-CMag, Micro (Micro-CMag) and Macro (Macro-CMag) – and used for pectinase immobilization and clarification of grape, apple and orange juices. The particle size had a great effect in the kinetic parameters, Nano-CMag biocatalyst presented the highest V_{max} value (78.95 mg. min^{-1}), followed by Micro-CMag and Macro-CMag, with V_{max} of 57.20 mg.min^{-1} and 46.03 mg.min^{-1}, respectively. However, the highest thermal stability was achieved using Macro-CMag, that was 8 and 3-times more stable than Nano-CMag and Micro-CMag biocatalysts, respectively. Pectinase immobilized on Macro-CMag kept 85% of its initial activity after 25 batch cycles in orange juice clarification. These results suggested that the chitosan magnetic biocatalysts presented great potential application as clarifying catalysts for the fruit juice industry and the great importance of the chitosan particles preparation on the final biocatalyst properties.

1. Introduction

There is a growing demand for more selective and clean processes every day, in which the enzymes have been attracting the attention of many researchers. Enzymes are biocatalysts with high potentiality in diverse industries thanks to their high activity under mild conditions, specificity and selectivity, [1,2]. Enzymes are one of the most important tools in modern food processing industry because many intermediate processes are simplified by their use [3].

Nevertheless, their industrial implementation are impaired because of some factors, such as their usual low stability under operational conditions, and the difficulties to their recovery and reuse [4–6]. In this situation, where the reaction conditions will directly define the enzyme activity, the immobilization appears as a way to achieve a suitable biocatalyst for each specific application [7,8]. The scientific advances in enzyme immobilization allow obtaining biocatalysts for industrial applications, improving catalytic properties even in adverse reaction conditions, facilitating the recovering of the enzymes from the reaction medium and their reuse, turning them economically feasible [9–13].

In the juice industry, the application of commercial enzyme preparations, composed by different enzyme, such as hemicellulases, cellulases and pectinases, is usual for obtaining stable and clarified fruit juices [14–17]. These enzymes are responsible for the breakdown of the structural polysaccharides of fruit pulp, which caused juice turbidity [18–20]. The addition of pectinases to the juice reduces its viscosity, improving the press ability of the pulp, disintegrating the jelly structure and increasing the fruit juice yields [3]. Some academic studies have utilized immobilized enzymes for the clarification of orange, apple, pineapple, grape, and carrot juices [21–28], but to the best of our knowledge there is not an industrial process using immobilized enzymes for juice

* Corresponding authors.
E-mail addresses: rfl@icp.csic.es (R. Fernandez-Lafuente), rafaelcrodrigues@ufrgs.br (R.C. Rodrigues).

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clarification, which makes the development of stable and active biocatalysts useful for this task an attractive challenge.

Support nature and immobilization protocols suitable for enzyme immobilization are the key factors for the preparation of an enzyme biocatalysts with high enzymatic activity and operationally stable [29,30]. Chitin is an abundant natural polymer obtained from the shells of shellfish and wastes from the seafood processing industry, that by N-deacetylation produces chitosan [4,31]. The combination of chitosan and magnetic particles could produce an ideal support for the immobilization of enzymes, with high affinity for proteins, easy functionalization with reactive groups for reactions with enzymes, and at same time, providing magnetic separability, allowing easy reuse. Moreover, its hydrophilicity, non-toxicity, biocompatibility, high resistance to chemical degradation and antibacterial properties increase its interest [4,32,33]. Moreover, the magnetic core allows easy and efficient separation of enzyme from the reaction medium by using external magnetic fields [25,34,35], which is important if the product presents some solids in suspension (making filtering useless). Additionally, chitosan particles with different characteristics, especially particle size, can be produced by diverse synthesis methods, such as spray drying, emulsion cross-linking, ionotropic gelation, emulsion-droplet coalescence, reverse micellar and precipitation, methods, providing supports with diverse porosity and different surface/volume ratio for enzyme immobilization [31,33,36–39]. The differences in each method for preparation of chitosan particles may alter the final performance of the biocatalysts. Each synthetic protocol can alter the superficial density of amino groups or the rigidity of the chitosan chains, affecting the final biocatalyst features.

Based on these aspects, the objective of the present work was to prepare chitosan-magnetic biocatalysts with different characteristics by varying the method of synthesis of the support, assessing the effect of the support preparation protocol on the final particle size of the particles and on the immobilized enzyme properties. The biocatalysts were applied to the clarification of apple, orange, and grape juices. The supports were prepared by mixing magnetic particles with chitosan by using three different methods: emulsion cross-linking, precipitation and ionotropic gelation [33,40]. For the immobilization of the enzymes, we used glutaraldehyde as coupling agent, which has been considered an efficient procedure to attain stable covalently attached enzymes [30,41–44]. All biocatalysts were characterized in relation to their magnetic, morphological and textural properties, kinetic parameters (V_{max} and K_M) and thermal stability. Finally, the operational stability of the biocatalysts was study in the clarification of several juices (apple, orange and grape).

2. Materials and methods

2.1. Materials

Freshly pressed apple, orange and grape juices without any treatment were kindly provided by Vitivinícola Jolimont (Canela, RS, Brazil). Rohapect 10 L was from Amazon group (Brazil). Pectin from apple, polygalacturonic acid, galacturonic acid and chitosan were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents were of analytical grade.

2.2. Enzymatic preparation

The enzymatic preparation used in this work was Rohapect 10 L. It is a cocktail of pectinase and cellulase enzymes. The protein concentration was 5.4 mg.mL^{-1}, and the main specific activities (U. mg^{-1}) present in the preparation were: total pectinase, 233.29; polygalacturonase, 537.57; pectin lyase, 13.53; pectin methyl esterase, 168.33; cellulase, 31.99 [45].

2.3. Determination of enzyme activities

Enzymatic activities were measured as previously described by Dal Magro et al. [46]. The polygalacturonase (PG) and total pectinase (PE) activities were determined by the hydrolysis of polygalacturonic acid and pectin (1 g.L^{-1}), respectively. The enzyme was added to substrate prepared in sodium citrate buffer (50 mM, pH 4.8), and incubated at 37 °C, for 1 min for PE activity and 2 min for PG activity, under agitation.The amount of reducing groups was quantified by the 3,5-dinitrosalicyclic acid (DNS) method [47]. The pectin lyase (PL) activity was measured spectrophotometrically by determining the increase in absorbance at 235 nm, through the formation of unsaturated uronide (ε = 5500 M^{-1}. cm^{-1}) [48]. Briefly, the enzyme was added to pectin solution (4 g.L^{-1}) prepared in sodium citrate buffer (50 mM, pH 4.8) and incubated at 37 °C for 1 min, under agitation. The reaction was stopped by adding HCl, 0.5 M. The pectin methyl esterase (PME) activity was measured by titration of carboxylic groups released through the de-esterification of pectin [49]. The enzyme was added to pectin solution (5 g.L^{-1}) prepared in NaCl buffer (0.15 M, pH 4.5). The reaction was conducted at 30 °C for 10 min, under agitation. Total cellulase (CE) activity was determined using Whatman no. 1 filter paper as substrate [50]. The enzyme was added to sodium citrate buffer (50 mM, pH 4.8) containing 50 mg. mL^{-1} of filter paper. The reaction was carried out at 50 °C for 5 min, under agitation, and the amount of released reducing groups was quantified by the 3,5-dinitrosalicyclic acid (DNS) method.

2.4. Synthesis and functionalization of the magnetic particles (MP)

Synthesis of magnetic particles was carried out through solvothermal method [51], using iron (III) chloride as iron precursor and ethylene glycol as high boiling point solvent and reducing reagent. Briefly, $FeCl_3.6H_2O$ (16 mmol, 4.32 g) was dissolved in 100 mL of ethylene glycol, under stirring. Afterward, sodium acetate trihydrate (80 mmol, 10.89 g) was added and the mixture was stirred at room temperature for 1 h, then transferred to a Teflon lined stainless steel autoclave and heated up to 180 °C for 12 h, with heating rate of 3.0 °C.min^{-1}. The system was cooled to room temperature and the precipitate was captured using an external magnet. The magnetic particles, named as MP, were washed thoroughly with ethanol and vacuum-dried for 2 h. In sequence, MP (1.00 g) were dispersed in toluene (50 mL) with 3-aminopropyltrimethoxysilane (APTMS) (1 mmol, 0.174 mL) and stirred under argon atmosphere for 12 h at 70 °C. Subsequently, the functionalized MP with APTMS, named as MP-APTMS, was magnetically separated, washed several times with non-polar and polar solvents to extract any impurity or residue from the product. The sequence of used solvents starts with toluene, which is the solvent used in the reaction, ethanol, water, and finishing with ethanol that is easier to evaporate. Finally the solid was dried under vacuum at 50 °C for 2 h.

2.5. Preparation and activation of chitosan magnetic particles

The chitosan magnetic particles were prepared by three different methodologies to obtain supports with different properties.

Method 1: Chitosan magnetic nano particles (Nano-CMag) were prepared by ionotropic gelation using sodium sulfate as the gelation agent [33]. For this, 0.5 mL of 1.4 mol.L^{-1} sodium sulfate solution was dropped, under sonication, in 9.5 mL of 0.25% w/v chitosan solution in 0.35 mol L^{-1} acetic acid containing 1% v/v

Tween 80 and 0.25% w/v magnetic particles. Then, it was mechanically stirred for 2 h. The Nano-CMag obtained were magnetically separated and washed with distilled water.

Method 2: Chitosan magnetic micro particles (Micro-CMag) were prepared using the cross-linking emulsion technique [40]. 2% w/v chitosan suspension and 2% w/v magnetic particles in 0.35 mol·L^{-1}acetic acid were mixed into a dispersion medium composed of mineral oil:petroleum ether (25:35 v/v) with 0.5% v/v Tween 80. 0.5% (v/v) Glutaraldehyde (was added twice: after 10 min and after 1 h, totaling 1% (v/v) in the final mixture. After the addition of glutaraldehyde, the mixture was stirred for 2 h. Finally, the Micro-CMag particles were separated from the oil phase by centrifugation followed by successive washes with acetone, ethanol and distilled water.

Method 3: Chitosan magnetic macro particles (Macro-CMag) were prepared by the precipitation method [33]. A suspension of 2% w/v chitosan and 2% w/v magnetic particles in 0.35 mol. L^{-1}acetic acid was sonicated for 30 min to remove air bubbles. Then, the mixture was added dropwise in the coagulation solution (1 mol.L^{-1} NaOH) under slow stirring. The formed Macro-CMag particles were magnetically separated and washed with distilled water until neutrality was reached.

2.6. Enzyme immobilization

For immobilization of Rohapect 10 L, all supports were previously activated with glutaraldehyde [26,51]. The activation was performed by incubating 50 mg of each support in 1 mL of phosphate-potassium 0.1 mol.L^{-1} buffer (pH 7.0) with 1% (v/v) glutaraldehyde at 37 °C under stirring for different times (from 30 min to 5 h). After, the supports were magnetically separated and washed 3 times with 50 mmol.L^{-1}sodium citrate at pH 4.8 to remove the excess of glutaraldehyde.

Subsequently, 50 mg of the activated supports were added to 1 mL of the enzyme solution with different protein concentrations (0.2–1.6 mg.mL^{-1}) for different times (5–30 h), at room temperature under gently stirring in a roller mixer. Finally, the biocatalysts were magnetically separated and washed 3 times with 5 mL of 50 mmol sodium citrate at pH 4.8 to remove the non-bound enzymes. To store the biocatalysts, they were suspended in 50 mmol.L^{-1}sodium citrate buffer at pH 4.8 and 4 °C. The immobilization yield (IY), immobilization efficiency (IE) and recovered activity (RA) were determined by the following equations according to Sheldon and Van Pelt [6]:

$$IY\ (\%) = \frac{Immobilized\ activity\ (U)}{Initial\ activity\ (U)} x100 \quad (1)$$

$$IE\ (\%) = \frac{Biocatalyst\ activity\ (U)}{Immobilized\ activity\ (U)} x100 \quad (2)$$

$$RA\ (\%) = \frac{Biocatalyst\ activity\ (U)}{Initial\ activity\ (U)} x100 \quad (3)$$

Immobilized activity is the difference between the initial activity and the activity determined in final supernatant. Initial activity is the activity of the enzyme offered to the support. Biocatalyst activity is the activity measured on the particles after immobilization.

2.7. Kinetic parameters

Kinetic parameters (K_M and V_{max}) of the soluble enzyme, Nano-CMag, Micro-CMag and Macro-CMag were calculated by using different substrate concentrations, varying between 1.39 mmol.L^{-1} and 55.5 mmol.L^{-1} the concentration of pectin at pH 4.8 employing 50 mmol.L^{-1} sodium citrate at 37 °C. The K_M and V_{max} were calculated by non-linear regression of the initial reaction rates corresponding to different substrate concentrations using Solver extension (Microsoft Office Excel 2016).

2.8. Effect of pH and temperature on the enzyme activity

The total pectinase activity was measured for soluble and immobilized enzymes (Nano-CMag, Micro-CMag and Macro-CMag) at different values of pH and temperature. The temperature varied from 30 °C to 90 °C, at pH 4.8 whereas the pH ranged from pH 3.0 to pH 6.0 at 60 °C.

2.9. Thermal and storage stability

Thermal stabilities of the soluble and immobilized enzymes (Nano-CMag, Micro-CMag and Macro-CMag) were analyzed by incubating the samples in 50 mmol.L^{-1}sodium citrate buffer at pH 4.8 and 60 °C. The storage stabilities were performed by incubating the samples in 50 mmol.L^{-1} sodium citrate buffer at pH 3.5 and 4 °C. Periodically, the samples were taken to analyze total pectinase activity. The thermal inactivation was described by a first order reaction:

$$\frac{A}{A_0} = \exp(-k*t) \quad (4)$$

where A is the enzyme activity at time t, A_0 is the initial enzyme activity, t is the inactivation time, and k is the inactivation rate constant at the studied temperature. The half-life time ($t_{1/2}$) was calculated using the k values, according to the equation:

$$t_{1/2} = \frac{\ln(2)}{k} \quad (5)$$

2.10. Operational stability

Operational stability of the biocatalysts was evaluated through reusing them in several consecutive batch cycles for clarification of apple, orange and grape juices. For this, 5 U of total pectinase activity of each biocatalyst were added to 1.0 mL of fresh juice and incubated at 40 °C for 2 h. Between each clarification cycle, the immobilized enzymes were magnetically recovered from the juices and washed with 50 mmol.L^{-1} sodium citrate buffer at pH 4.8. Then, they were added to a new batch of fresh juice to start a new cycle. The decrease in juice turbidity was determined for each cycle, considering the value of the first cycle as 100%. Turbidity was measured spectrophotometrically by detection of the scattered light at 860 nm [52].

2.11. Materials characterization

Scanning Electron Microscopy (SEM) images were acquired using Zeiss microscope model Auriga microscope. The materials were dispersed on double side conduction tape on an aluminum support. The images were obtained with an accelerating voltage of 5 kV and different magnifications. X-ray diffractograms were obtained in a Siemens diffractometer model D500 using CuKα as X-ray source (λ = 0.154056 nm) at a generator voltage of 40 kV and a generator current of 17.5 mA. Magnetic properties were investigated by using an EZ9 MicroSense vibrating sample magnetometer (VSM) at room temperature with a magnetic field (H) cycled between −22 kOe and +22 kOe. N_2 adsorption-desorption isotherms were acquired at liquid nitrogen boiling point, using a Tristar II 3020 Kr Micromeritics equipment. The samples were previously degassed at 60 °C, under vacuum, for 20 h. The specific surface areas were determined by the

BET (Brunauer, Emmett and Teller) multipoint technique and the pore volume and pore size distribution were obtained by using the BJH (Barret, Joyner and Halenda) method [53]. Infrared spectra were acquired by using KBr supported disks (1%). The equipment used was a Shimadzu FTIR Prestigie 21. The spectra were obtained at room temperature with 4 cm^{-1} of resolution and 120 cumulative scans.

3. Results and discussion

3.1. Morphological, structural, magnetic and textural properties

Fig. 1 presents SEM images for Nano-CMag and Micro-CMag and a picture of the Macro-CMag. It is clearly seen that the materials presented size in the nano, micro and macro levels, respectively. The average of particle size (φ) and the standard deviation (σ) were estimated by using Quantikov software as φ =273 nm (σ =58 nm) for Nano-CMag, as φ =82 μm (σ =32 μm) for Micro-CMag and as φ =2.0 mm (σ = 0.2 mm) for Macro-CMag.

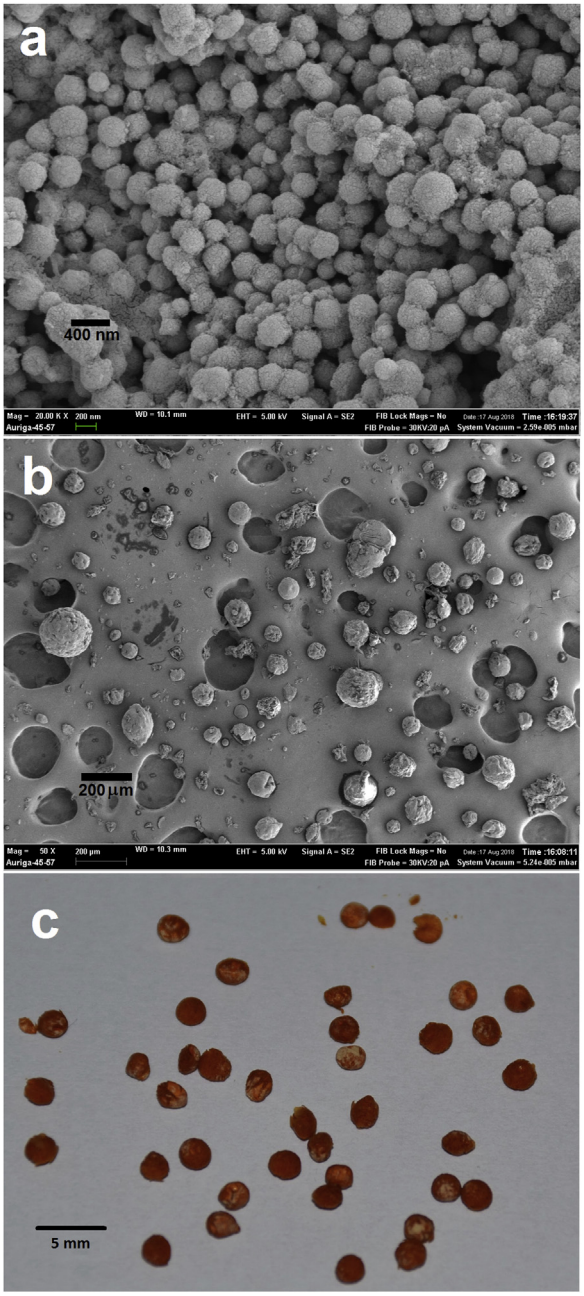

Fig. 1. Images of magnetic chitosan particles: a) Nano-CMag (SEM image, magnification of 20,000x); b) Micro-CMag (SEM image, magnification of 50x) and c) Macro-CMag (picture obtained using Nikon D3100 equipment).

Additional SEM images obtained with higher magnification are presented in Supplementary Material as Figure S1. In these images it is possible to see the spherical shape of the magnetic particle (MP) (Fig. S1a) that is still observed after the functionalization with aminopropyl groups (MP-APTMS) (Fig. S1b). Moreover, it is also possible to see that the functionalization produced a coating on MP surface, making the MP particles more aggregated (Fig. S1b). The images of materials Nano-CMag, Micro-CMag and Macro-CMag are depicted in Fig. S1c, S1d and S1e, respectively, where it is possible to identify the organic moiety, assigned to chitosan, along with the spherical magnetic particles (MP). Roughness on surface of Nano-CMag and Macro-CMag materials can be seen, along with the presence of some macroporosity, i.e. pores with diameter higher than 50 nm [53]. The material Micro-CMag showed a more compacted surface, and no macroporosity can be identified. These analysis confirmed our hypothesis that using different methods for chitosan preparations we are able to obtain particles with different size (nano, micro and macro size), as well as different properties such as particle surface texture and porosity.

Infrared analysis of the materials is presented in Supplementary Material, as Figure S2. The spectra of Nano-CMag, Macro-CMag are very similar to the chitosan spectrum. The broad band with maximum around 3400 cm^{-1} presents contribution of N—H and O—H stretching from amine and hydroxyl groups of chitosan and also adsorbed water [54]. The band around 2900 cm^{-1} is due to C–H stretching of chitosan [54].The broad band around 1620 cm^{-1} has contribution of N-H_2 bending (1653 cm^{-1}) and N—H deformation of amide (1558 cm^{-1}) typical of chitosan [55]. The broad band with maximum at 1070 cm^{-1} was assigned to skeletal vibration of C–O stretching of chitosan [55]. Therefore, the infrared results confirm the presence of chitosan in the materials Nano-CMag and Macro-CMag. Unfortunately, the spectrum of the Micro-CMag sample was not obtained, because we were not able to disperse this sample in the KBr infrared support, as a matter of fact, this sample was not a powder, it presented characteristic such as a soft organic polymer.

The X-ray diffractograms of all materials are presented in Supplementary Material as Figure S3. The diffraction pattern (Fig. S3f) shows Bragg's reflections of typical face-centered cubic (fcc) inverse spinel structure of magnetite (JCPDS N° 19-0629). These reflections are present in all diffractograms (Fig. S3a-e), indicating that the functionalization with APTMS, as well as, the subsequent experimental procedures, used to obtain Nano-CMag, Micro-CMag and Macro-CMag materials, did not affect the crystallinity of magnetite particles. The average crystallite size of magnetite for MP material was calculated by Scherrer's equation using the full width at half maximum for the (311) peak and the obtained value was 39 nm. This result indicates that MP particles are polycrystalline, because from the SEM images (Fig S1a) it was observed that they present diameter in around 200 nm.

The magnetization of the materials was measured as a function of the magnetic field and the non-normalized magnetization curves of all materials are shown in Supplementary Material (Fig. S4). It is clearly observed high magnetic saturation (MS) values for MP and MP-APTMS. The values for MP, MP-APTMS, Nano-CMag, Micro-CMag and Macro-CMag materials were 69, 60, 5.9, 5.8 and 11 $emu \cdot g^{-1}$, respectively. The decreasing in MS for Nano-CMag, Micro-CMag and Macro-CMag materials can be interpreted as a consequence of the incorporation of non-magnetic chitosan moiety to the total mass of the materials. This result is in accordance with the SEM images of the Figure S1, where it was possible to identify both, spherical magnetite particles and organic moieties, which was assigned as chitosan. It is important to point

out that, even presenting lower MS values, when compared to MP and MP-APTMS, the Nano-CMag, Micro-CMag and Macro-CMag materials can be easily and rapidly separated by applying the external magnetic field.

The nitrogen adsorption-desorption isotherms are shown in Supplementary material as Figure S5, along with the pore size distribution curves (inset Fig. S5). The surface area and pore volume values are presented on Table 1. Figure S5a shows the curves for MP and MP-APTMS materials. From the isotherms, it is notice a decreasing in the amount of adsorbed nitrogen, in high relative pressures ($P/P_0 > 0.6$), after the MP functionalization with APTMS. This behavior suggested a decrease in both, surface area and pore volume (Table 1) that was interpreted as a consequence of partial closing of the mesopores, which are pores with diameter between 2 and 50 nm [53], produced by the APTMS coating on the MP material surface, in agreement with the SEM images (Fig. S1b). The isotherm curves for Nano-CMag, Micro-CMag and Macro-CMag materials are shown in Figure S5b, S5c and S5d, respectively. After the enzyme immobilization, there is a decreasing in the nitrogen adsorbed amount in all relative pressures that is markedly in $P/P_0 < 0.2$ for Micro-CMag and Macro-CMag materials (Fig. S5c and S5d, respectively) which corresponds to small mesopores and micropores [53]. In fact, the pore distribution curves confirm the closing of pores with diameter lower than 10 nm for these materials, probably produced by the enzyme coverage on the surface (inset Fig. S5c and S5d). Regarding the Nano-CMag material (Fig 5Sb), its textural characteristics seem not be markedly affected by the enzyme immobilization. All textural characteristics as isotherm profile, pore size distribution, surface area and pore volume remain almost constant.

3.2. Effect of the activation time of the supports with glutaraldehyde on enzyme immobilization

The most common bi-functional cross-linking reagent used is glutaraldehyde because it is inexpensive, easily handled and capable to bind to different enzymes [41]. Thus, firstly, the supports were activated with glutaraldehyde and we evaluated the effect of the activation time on the support performance for pectinase immobilization. The glutaraldehyde concentration was set at 1% (v/v), while the activation time ranged from 30 min to 5 h.

The recovered activity of the biocatalysts increased with the support activation time up to 1 h for Nano-CMag and 3 h for Micro-CMag and Macro-CMag, after these times the recovered pectinase activity decreased (Fig. 2). Similar results were found for the immobilization yield of the biocatalysts (Table S1). In very short activation times, the activation degree of the carrier is low, probably generating fewer covalent attachment points, which resulted in lower immobilization yields, while longer activation times can favored the cross-linking between chitosan activated groups and reduce the possibilities of enzyme-support reaction. Srivatava and Anand [56] verified that higher glutaraldehyde concentrations generated multiple cross-linking points on the chitosan surface causing spatial hindrance among enzyme molecules that decreased the enzyme activity, thus decreasing immobilization yield. Nevertheless, according to our results, this will depend on the form that chitosan is in the support. Thus, the optimal activation time is different for each support.

Table 1
Textural analysis of the materials.

Material	Before enzyme immobilization		After enzyme immobilization	
	S_{BET} [a]	PV_{BJH} [b]	S_{BET} [a]	PV_{BJH} [b]
MP	15.7 ± 0.6	0.038 ± 0.001		
MP-APTMS	12.6 ± 0.5	0.032 ± 0.001	–	–
Nano-CMag	18 ± 0.7	0.051 ± 0.001	18 ± 0.7	0.061 ± 0.001
Micro-CMag	1.7 ± 0.3	< 0.0005	0.46 ± 0.07	< 0.0005
Macro-CMag	11.5 ± 0.5	0.023 ± 0.001	5.4 ± 0.4	0.016 ± 0.001

[a] = BET specific surface area ($m^2\ g^{-1}$).
[b] = BJH pore volume ($cm^3\ g^{-1}$).

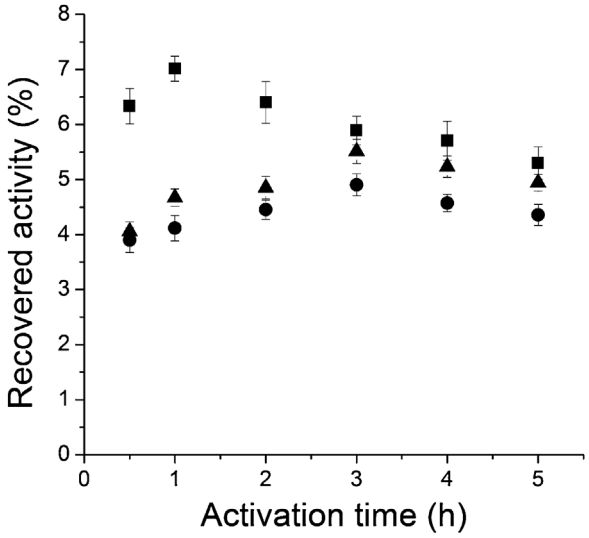

Fig. 2. Influence of the activation time on the recovered activity of the (■) Nano-CMag, (▲) Micro-CMag and (●) Macro-CMag biocatalysts, under the conditions 1% of glutaraldehyde and 0.4 $mg.mL^{-1}$ of protein concentration. The immobilization was carried out during 15 h.

3.3. Enzyme concentration

Different enzyme concentrations were tested for immobilization, ranging from 0.2 $mg.mL^{-1}$ to 1.6 $mg.mL^{-1}$ of protein (4 mg to 32 mg of protein per g of support) and the results are presented in Fig. 3.

The immobilization in all materials presented a similar behavior, where the highest recovered activity was obtained when the lowest enzyme concentration was used. The decrease in the recovered activity could be explained by diffusional limitations of the substrate into the pores, that will be mainly hydrolyzed by the enzymes located in the outer part of the pores and the external particle surface [42,57,58].

On the other hand, the activities of the biocatalysts increased when the enzyme concentration increased. Biocatalysts activity rapidly increased up to 0.8 mg protein.mL^{-1}, while enzyme concentrations higher than 0.8 $mg.mL^{-1}$ only promoted slight increments in the activity for Nano-CMag and Micro-CMag. These results may be related to the lower particle size, that will reduce the enzyme diffusional problems to the inner areas of the supports, allowing a more rapid immobilization of the enzyme. Using Macro-CMag, that has larger particle size, the enzyme diffusion problems increased and immobilization in the core of the particle may become very slow. Moreover, also the substrate diffusion limitations will be higher in these larger particles, producing a decrement of the expressed activity [33]. Thus, to compare all biocatalysts, and in order to achieve high enzyme activity and satisfactory RA%, an enzyme concentration of 0.8 $mg.mL^{-1}$ was chosen for the next experiments.

3.4. Immobilization time

Effects of immobilization time on the biocatalysts recovered activities were evaluated from 5 h to 30 h. (Fig. 4). Regarding the recovered activity, a large increase was observed up to 15 h for all

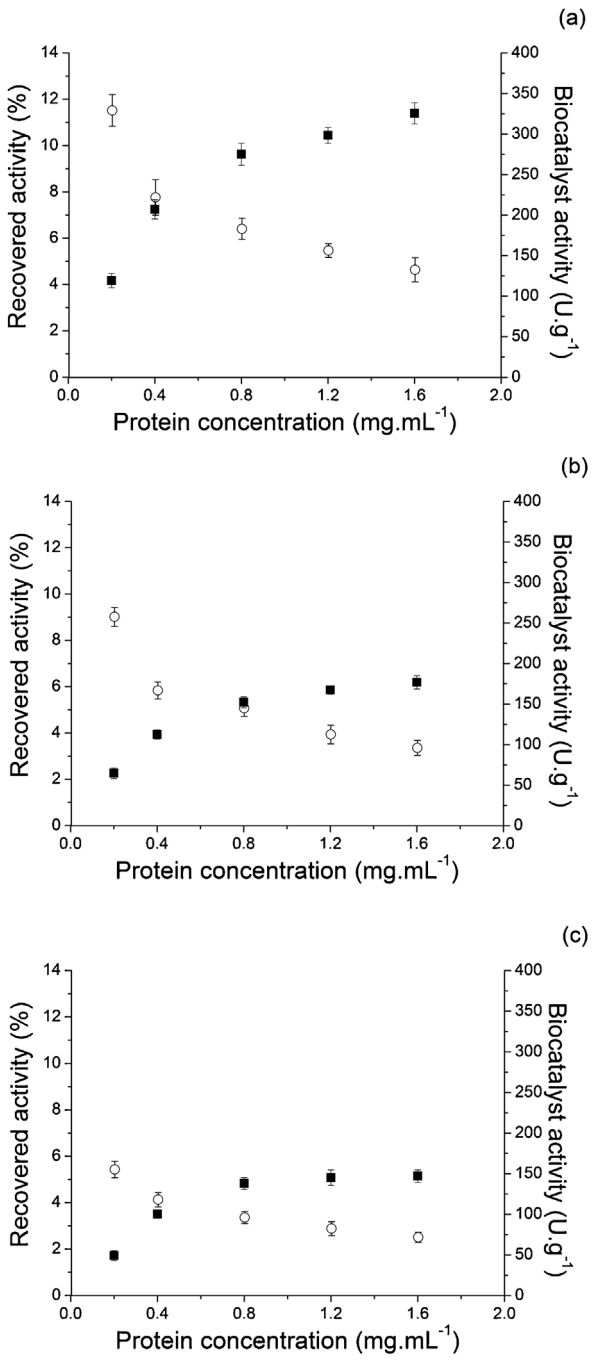

Fig. 3. Influence of enzyme concentration on the (○) RA% and (■) biocatalyst activities of the (a) Nano-CMag, (b) Micro-CMag and (c) Macro-CMag. The immobilization was carried out during 15 h.

biocatalysts, however, Micro-CMag and Macro-CMag biocatalysts showed a slight increase in activity up to 20 h. It could again be related to the diffusional problems of the enzyme to be immobilized on these biocatalysts, whereas in Nano-CMag, the enzymes were immobilized more rapidly in the shorter pores, in the Macro-CMag, the enzymes needed a longer time to reach the inner part of the long pores.

Similar results were observed by Biró et al. [31]. The authors reported that the steric hindrance caused by the enzyme molecules on the macroparticles surface avoided immobilization inside the whole biocatalyst, whereas for the immobilization in smaller particles there was better distribution of the enzyme in whole particle. Thus, considering the highest recovered activities and immobilization yield, an immobilization time of 20 h for all biocatalysts was selected as optimal value for subsequent experiments.

Although the recovered activities were very low, they are similar to other immobilization strategies using the same enzymatic preparation [26,51,59,60]. This is mainly because the diffusion limitations presented by the large substrate on immobilized particles. Moreover, changing the immobilization support and the immobilization protocol it was possible to vary the enzyme orientation to the support, which leads to different activities and stabilities.

In the next steps, we characterized the immobilized preparations on chitosan-magnetic particles regarding activities, kinetic parameters, thermal and operational stabilities.

3.5. Kinetic parameters

The maximum reaction rate (V_{max}), Michaelis constant (K_M) and catalytic efficiency were determined by measuring initial reaction rates, varying the amount of substrate.

Regarding V_{max} and K_M values, all immobilized enzymes showed slower V_{max} and higher K_M values than the soluble enzyme (K_M, 0.96 mg.mL^{-1} and V_{max}, 143.40 mg.min^{-1}). The enzyme kinetic parameters exhibited different results as a function of the type a support, with the expected best properties for the smallest support particle. Thus, the enzyme immobilized on Nano-CMag had the lowest value of K_M (1.70 mg.mL^{-1}) and the highest value of V_{max} (78.95 mg.min^{-1}), followed by Micro-CMag (K_M, 2.23 mg.mL^{-1} and V_{max}, 57.20 mg.min^{-1}), and Macro-CMag (K_M, 2.35 mg.mL^{-1} and V_{max}, 46.03 mg.min^{-1}). Differences were significant but not decisive to discard some of the biocatalysts.

The increase in K_M when increasing the particle size can be attributed, again, to the diffusional limitations of the reacting species, although some structural variations of the immobilized enzymes on supports prepared by the different protocols may be not discarded, such as some enzyme distortions [61]. In fact, the decrease in V_{max} when the particle size increased should not be related to diffusional limitations but to a different structure of the enzyme that can drive to a lower catalytic capacity or some enzyme molecules whose active center is blocked by the support surface [62].

3.6. Enzyme activities of the soluble enzyme and biocatalysts

Total pectinase (PE), polygalacturonase (PG), pectin lyase (PL), pectin methyl esterase (PME) and cellulase (CE) activities were analyzed for each biocatalyst and compared to those of the soluble enzyme.

As can be seen in Table 2, the biocatalyst Nano-CMag presented the highest values for all the enzymatic activities analyzed. These results can be related to lower diffusion limitations on these small particles, facilitating the enzyme access to the macromolecular substrates. On the other hand, Macro-CMag biocatalysts presented the lowest activity recovery for all substrates, possibly because the higher diffusion limitations. Similar results were also found by Klein et al. [33] and Biró et al. [31], for immobilization of β-galactosidase, observing that the smaller the particle size, the higher the recovered activity.

It has been reported that the immobilization of a mixture of enzymes with different sizes on porous supports may be a problem [13]. The protein with the largest size should determine the minimum support pore diameter, because if the support pore is not enough to allow the diffusion of the largest protein, this enzyme can be immobilized on the pore entrance, blocking the access to lower proteins [13]. Various crowding effects and pores blocking effects, related to changes in diffusion rate, protein unfolding, self-

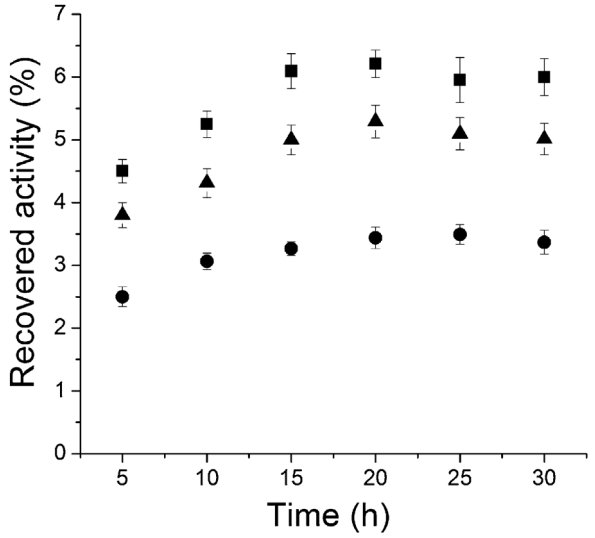

Fig. 4. Influence of immobilization time on the recovered activity of the (■) Nano-CMag, (▲) Micro-CMag and (●) Macro-CMag biocatalysts with of 0.8 mg.mL^{-1} of protein concentration.

Table 2
Enzymatic activities of the soluble enzyme, Nano-CMag, Micro-CMag and Macro-CMag biocatalysts.

Biocatalysts	Recovered activities (%)				
	PE	PG	PL	PME	CE
Soluble enzyme	100	100	100	100	100
Nano-CMag	6.15	5.82	5.73	12.21	9.60
Micro-CMag	5.16	2.92	3.64	2.99	8.93
Macro-CMag	3.29	2.25	2.07	2.90	4.53

association and protein binding enhancement, enzymatic activity alteration and reaction kinetics modification have been reported [63–65].

Moreover, the spatial arrangement and orientation of multiple enzymes in solid supports are very important, facilitating the cooperative catalytic mechanisms, where the reactive intermediates can be transported rapidly from one active site to the next to avoid diffusion losses, as in cascade reactions [66,67].

3.7. Effect of pH and temperature on enzyme activity

The reaction conditions are directly associated to the enzyme catalytic efficiency. Thus, the effect of different temperatures (30 °C–90 °C) and pH values (3–6) were analyzed for the soluble enzyme and immobilized biocatalysts activities. Regarding the temperature, the soluble enzyme presented maximum activity at 50 °C, while the biocatalysts Nano-CMag, Micro-CMag and Macro-CMag showed maximum activity at 60 °C (Fig. 5). This should be consequence of an improvement on enzyme stability after immobilization, where a more stable enzyme exhibits superior activity at higher temperatures [62]. Immobilized/stabilized biocatalysts presented higher activities at elevated temperatures compared to the soluble enzyme. Effect of temperature on enzyme activity is the result of several factors: enzyme activity should increase, but enzyme inactivation (negative conformational changes) may compensate this, moreover, viscosity of the medium may change and that way diffusion of substrate may be greatly altered.

The immobilized enzymes presented higher activities in a wider range of pH values than the free enzyme. The maximum activity was found at the same pH for all forms of enzymes (pH 4),

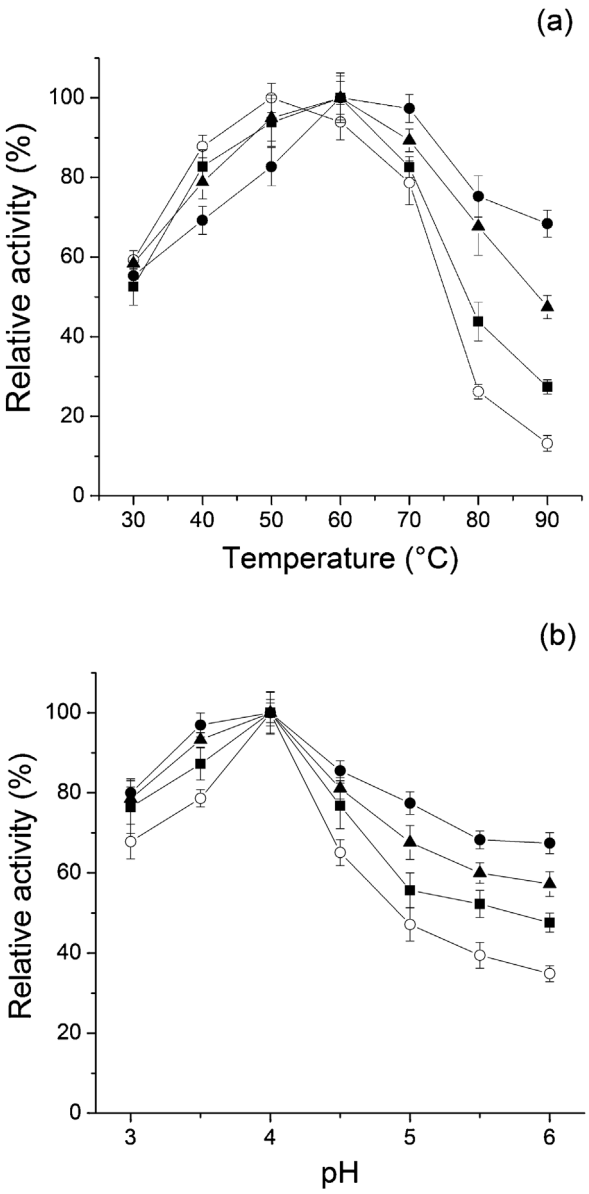

Fig. 5. Relative activity of (○) soluble enzyme, (■) Nano-CMag, (▲) Micro-CMag and (●) Macro-CMag under different conditions of (a) temperature and (b) pH.

however, the immobilized enzymes retained higher activities under extreme conditions. The biocatalysts prepared on the different particles presented higher activity at pH 3 and pH 6 than the soluble enzyme. Additionally, at pH 6, the activity of Macro-CMag was higher than Nano-CMag and Micro-CMag, as occurred at high temperatures. This suggests that the properties of the prepared particles by different methods affected not only the immobilization rate and yield, as presented above, but also the enzyme activity at different conditions.

3.8. Thermal and storage stability

A stable biocatalyst is essential for industrial application, so thermal and storage stabilities of the immobilized biocatalysts and the free enzyme were evaluated. At 60 °C, the soluble enzyme presented the shortest half-life ($t_{1/2}$ = 0.257 min^{-1}) and the highest thermal inactivation rate (k =2.69 min). For Nano-CMag, Micro-CMag and Macro-CMag, an improvement in the thermal

stability was observed, since *k* decreased (0.117, 0.039 and 0.014 min, respectively) and $t_{1/2}$ increased (5.92, 17.77 and 49.51 min, respectively), providing stabilization factors of 2.2, 6.6 and 18.4, respectively, when compared to soluble enzyme (Table 3). These stabilization results explain, at least partially, the wider range of temperatures and pH where the immobilized enzyme may be utilized.

Enzyme immobilization is one way to improve the enzyme stability [6,12,13]. If several linkages between the support and the enzyme are established, they will restrict conformational changes of the enzyme induced by heat and stabilizes the tertiary structure of the enzyme, which is essential to catalytic activity [41,68].

Moreover, a significant improvement in enzyme stability was once again observed for the Macro-CMag biocatalyst, presenting 8-times more thermal stability than the Nano-CMag biocatalyst. These results suggested that the three different support may have conferred different structures to the chitosan coating, developing different electrostatic interactions between the chitosan chains which may to favor the development of more open support, such as Nano-CMag, providing a lower possibility of getting an intense multipoint covalent attachment. Moreover, the rigidification achieved via multipoint covalent attachment also depends on the support rigidity (only a rigid support may produce enzyme rigidification), and this rigidification is likely different for each method of chitosan preparation. Results suggested that using Macro-CMag biocatalyst, the enzyme–support reaction may be more intense or the chitosan polymer may have a lower mobility, favoring the preparation of more rigid and compact support which helped to stabilize the enzymes.

A high thermal stability was also presented when the polygalacturonase from Rohapect 10 L was immobilized on MANAE-agarose using the glutaraldehyde chemistry [60]. Thermal stability was measured in four different pH and temperature conditions and in all of them immobilized enzyme was much more stable than free enzyme. Additionally, stabilization data suggested changing the immobilization pH from 5 to 8 the enzyme was probably attached by a different orientation on the support [60].

Related to the storage stability, the soluble enzyme and the three immobilized biocatalysts were fully stable when stored at 4 °C, in sodium citrate buffer (50 mM), pH 3.5 (near to pH of fruit juices) during 1 month.

3.9. Operational stability

Operational stability is another important parameter for identifying a suitable immobilized enzyme and it is one of the main reasons for immobilization. To evaluate the operational stability, several batch cycles of clarification of grape, orange and apple juices were performed.

As can be observed in Fig. 6, all the biocatalysts were able to perform the clarification of the juices by several cycles of reuses. Due to the juices pH (around 3), the initial activity in the first cycle was lower than the maximal activity presented in Fig. 5b (at pH 4). Thus, the initial activity was around 80% of the maximal one. Nevertheless, the decrease in each juice turbidity in the first cycle was considered as 100%, and the subsequent cycles were compared to that, as this experiment was performed to check the biocatalyst stability during successive reaction cycles. However, the Macro-CMag biocatalyst presented the best results after 25 reuses, presenting in the last reuse a residual activity of 22%, 85% and 25%

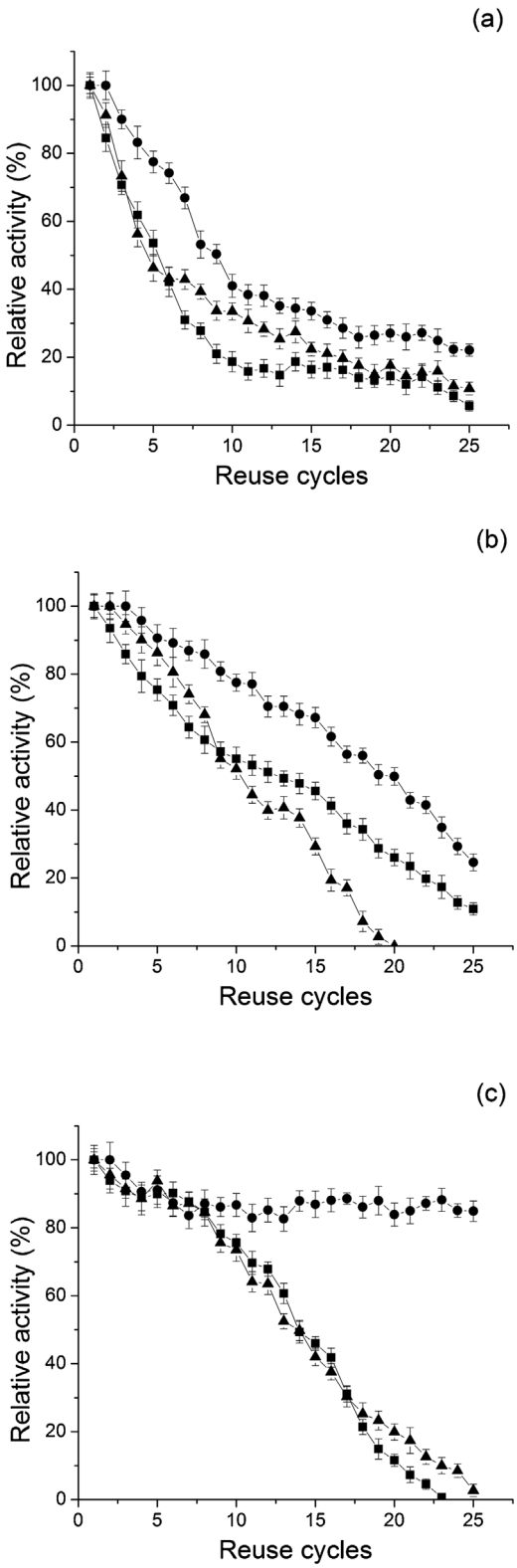

Fig. 6. Reusability of (■) Nano-CMag, (▲) Micro-CMag and (●) Macro-CMag biocatalysts assayed in (a) grape, (b) apple and (c) orange juices.

Table 3
Kinetic parameter of thermal deactivation for the soluble enzyme, Nano-CMag, Micro-CMag and Macro-CMag biocatalysts.

Treatments	k (min^{-1})	$t_{1/2}$ (min)	Stabilization factors
Soluble enzyme	0.257	2.69	1.0
Nano-CMag	0.117	5.92	2.2
Micro-CMag	0.039	17.77	6.6
Macro-CMag	0.014	49.51	18.4

for grape, orange and apple juices, respectively. On the other hand, the Nano-CMag and Micro-CMag biocatalysts presented a residual activity lower than 15% for all the juices in the last reuse.

The more rapid loss of activity over the reaction cycles for the Nano-CMag and Micro-CMag biocatalysts may be related to the lower stability of the enzyme molecules immobilized in these supports when compared to the enzyme molecules immobilized into the pores of Macro-CMag.

Considering that the biocatalysts could be satisfactorily reused until the loss of 50% of its initial activity, each biocatalyst will be used for different number of cycles until its replacement by a new immobilized enzyme. Thus, for grape and apple juices Nano-CMag should be replaced after 5 and 12 cycles, Micro-CMag after 4 and 10 cycles, and Macro-CMag after 9 and 19 cycles, respectively. In each case, to obtain the initial 5 U, it was used 18.93 mg, 29.43 mg and 37.30 mg of Nano-CMag, Micro-CMag and Macro-CMag, respectively, for 1 mL of juice. Then for each biocatalyst the relation mass/cycle was 3.79 mg and 1.58 mg for Nano-CMag, 7.36 mg and 2.94 mg for Micro-CMag, and 4.14 mg and 1.96 mg for Macro-CMag, respectively for grape and apple juices. Then, it can be concluded that, despite the lower stability of Nano-CMag, its higher activity per gram allows the use of a smaller amount of the biocatalyst, reducing the overall cost.

For orange juice the result is even more expressive. The relation mass/cycle for Nano-Cmag and Micro-CMag was 1.26 mg and 1.96 mg, respectively. However, Macro-CMag biocatalyst can be used for at least 25 cycles in orange juice without significant losses of activity (over 80% of the initial activity), that way, for this juice the advantages of this biocatalyst over the other two ones are very great. A proper calculation cannot be performed as the biocatalyst retained 85% of the activity after 25 cycles and very likely inactivation did not follow first order kinetic.

The difference in the operational stability using different juices could be explained by the different composition of each juice. The ionic exchange adsorption of some compounds in chitosan, such as polyphenols, proteins, starch, among others, may have contributed to the increase of diffusion problems, reducing enzyme activity in grape and apple juices. These problems seem to be minimized using orange juice.

Pectin lyase from Rohapect 10 L presented high operational stability when immobilized on glutaraldehyde-activated agarose [59]. After 5 cycles of 72 h each, the immobilized enzymes maintained more than 90% of its initial activity, being a suitable strategy for the preparation of a biocatalyst with high potential for application in pectin hydrolysis, such as Macro-CMag.

4. Conclusions

This paper presents for first time the importance of the method of chitosan particles preparation on the properties of the final biocatalyst when used for enzyme immobilization. Moreover, the use of chitosan and magnetic particles as supports for enzyme immobilization showed to be an interesting strategy for fruit juice clarification. The coating with chitosan allowed the improvement of superficial area compared with the magnetic particles alone, while the magnetic particles facilitated the biocatalysts separation. We compared three different methods for preparation of chitosan particles, providing different particle sizes (nano, micro and macro particles), and also having different textural properties. The Macro-CMag biocatalyst presented the highest thermal stabilities among the biocatalysts prepared. In addition, Macro-CMag presented the highest retained activity after 25 cycles of reuse for all fruit juices, especially orange juice, where the final residual activity was 85%. On the other hand, the Nano-CMag biocatalyst presented the best kinetic parameters and higher enzyme activity recoveries.

From a practical point of view, each biocatalyst presents its own advantages and disadvantages. For fruit juice clarification, it is necessary a smaller amount of Nano-CMag when compared to Macro-CMag, due to its higher activities. On the other hand, the separation of Macro-CMag is easier when compared to Nano-CMag, due to its larger size. Thus, our results bring new lights for immobilization of clarifying enzymes and to the development of an industrial process for juice clarification.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00373.

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