International Journal of Biological Macromolecules 134 (2019) 936–945

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Comparison of the immobilization of lipase from *Pseudomonas fluorescens* on divinylsulfone or *p*-benzoquinone activated support

Nathalia Saraiva Rios [a,b], Davino M. Andrade Neto [c], José Cleiton Sousa dos Santos [d], Pierre Basílio Almeida Fechine [c], Roberto Fernández-Lafuente [b,*], Luciana Rocha Barros Gonçalves [a,*]

[a] Departamento de Engenharia Química, Universidade Federal do Ceará, Campus do Pici, Bloco 709, 60455-760 Fortaleza, CE, Brazil
[b] Departamento de Biocatálisis, ICP-CSIC, Campus UAM-CSIC, Madrid, Spain
[c] Departamento de Química Analítica e Físico-Química, Centro de Ciências, Universidade Federal do Ceará, Av. Mister Hull s/n, Pici, 60455-760, Fortaleza, CE CP 12200, Brazil
[d] Instituto de Engenharias e Desenvolvimento Sustentável, Universidade da Integração Internacional da Lusofonia Afro-Brasileira, 62785000 Acarape, CE, Brazil

ARTICLE INFO

Article history:
Received 6 March 2019
Received in revised form 14 May 2019
Accepted 18 May 2019
Available online 20 May 2019

Keywords:
Lipase
Enzyme immobilization
Adsorption
Covalent binding
Enzyme stabilization

ABSTRACT

$NiZnFe_2O_4$ superparamagnetic nanoparticles were coated with silica by impregnation with tetraethoxysilane (TEOS) and further activated with divinylsulfone (DVS) and *p*-benzoquinone (BQ) for covalent immobilization lipase from *Pseudomonas fluorescens* (PFL), producing the biocatalysts TEOS-NANO-DVS-PFL and TEOS-NANO-BQ-PFL. The optimal conditions for enzyme immobilization were found to be pH 7 and 0.1 M of both activating reagents. PFL was also immobilized on TEOS nanoparticles without any activation as a reference (TEOS-NANO-PFL). Results indicated that TEOS could be released from the nanoparticles at alkaline pH value. Optimal TEOS-NANO-PFL exhibited a recovered activity of 55% and a $t_{1/2(60°C)}$ of just over 150 min; while TEOS-NANO-DVS-PFL showed 82% of activity recovered and $t_{1/2(60°C)}$ of 225 min; being the TEOS-NANO-BQ-PFL the biocatalyst offering the best results (89% of recovered activity and a half-life over 1440 min), the maximum enzyme load was ≈300 U/g.

1. Introduction

Biocatalysis permits to perform the most complex processed under very mild conditions [1–3]. Lipases (triacylglycerol hydrolases EC 3.1.1.3) are hydrolytic enzymes and are the most used enzymes in industry, catalyzing many reactions, namely hydrolysis, esterification, transesterification, acidolysis, aminolysis, among others [4,5]. Although lipases have a wide range of applications in reactions of industrial interest, their soluble form has poor stability and their reuse becomes problematic [3]. To overcome these drawbacks, lipase immobilization has been extensively studied in order to produce active, stable and reusable biocatalysts [2,6]. A proper immobilization process may simplify the separation of the biocatalyst from the reaction media and permit a more effective control of the reaction, enabling the application of biocatalysts in continuous processes [3,7]. In addition, immobilization techniques can improve most enzyme properties, such as activity, stability, specificity or selectivity [8,9]. Therefore, enzyme immobilization has become an important tool to produce economically viable industrial biocatalysts with suitable performance [10].

The selection of a proper immobilization protocol is critical to fully benefit from it [3,11]. Therefore, in this paper we have compared two different support activation reagents (divinylsulfone and *p*-benzoquinone) to analyze their possibilities to produce enzyme immobilization and stabilization via multipoint covalent attachment [11–13]. Both bifunctional reagents may react with hydroxyl groups of a support to produce its activation. Divinylsulfone (DVS) is a bifunctional molecule that has been used to modify supports for enzyme immobilization [13–16] and they have been recently proposed as almost ideal immobilization supports to get very intense multipoint covalent attachment [13]. Vinyl sulfone groups can react with different moieties of enzymes, such as amine, hydroxyl, phenyl, thiol, among others, producing highly stable biocatalysts [13,14]. Therefore, some homo and heterofunctional supports have been raised using vinyl groups for enzyme immobilization, such as divinylsulfone-activated agarose, octyl agarose activated with divinylsulfone, amino functionalized SBA-15 activated with divinylsulfone, Fe_3O_4 magnetic nanoparticles coated with oleic acid and activated with divinylsulfone, among others [16–19].

p-Benzoquinone (BQ) is another bifunctional molecule that has been used for support activation and enzyme immobilization [15,20,21]. Similar to DVS, BQ can react with amine, hydroxyl or thiol moieties among other groups of the proteins. Despite its high potential as an activating agent, *p*-Benzoquinone has not been properly studied to stabilize enzymes via multipoint covalent attachment. Thus, in this paper we intend to compare DVS as the most promising reported support activator to produce an intense multipoint covalent attachment with BQ.

* Corresponding authors.
E-mail addresses: rfl@icp.csic.es (R. Fernández-Lafuente), lrg@ufc.br (L.R.B. Gonçalves).

https://doi.org/10.1016/j.ijbiomac.2019.05.106
0141-8130/

To the best of our knowledge, silica coated $NiZnFe_2O_4$ nanoparticles have never been activated with DVS or BQ to produce TEOS-NANO-DVS or BQ support for enzyme immobilization. In this context, the activation protocol of TEOS nanoparticles with DVS and BQ should be investigated to produce active and stable biocatalysts after lipase immobilization.

Another critical point in the design of immobilized biocatalysts is the support. In this regard, superparamagnetic nanoparticles are considered very suitable supports for enzyme immobilization due to their high surface area, large surface-volume ratio and minimized diffusional limitations [22–29]. Moreover, superparamagnetic nanoparticles enable the separation of biocatalyst from the reaction medium by applying an external magnetic field [22,30]. This allows the use of the immobilized enzyme even in a medium containing solids, as it does not require the use of filters [31]. The immobilization of the enzyme in the external surface of the support enables the use of the immobilized enzyme to modify even solid substrates [32], although this also causes the enzyme to be unprotected from the environment (e.g., from interaction with hydrophobic interfaces [33]).

Lipases from *Pseudomonas* genus are extracellular enzymes that play an important role in biotechnological process, due to their application in biofuels, food and pharmaceutical products [34,35]. For this reason, as model enzyme to check the possibilities of BQ as support activating reagent, we have selected the lipase from *Pseudomonas fluorescens* (PFL) [34,35].

Thus, in this work, mixed nanoparticles ($NiZnFe_2O_4$) were synthetized. This material has remarkable chemical stability, good mechanical resistance, moderate saturation magnetization and anticorrosion properties [36,37]. In this approach, $NiZnFe_2O_4$ nanoparticles were coated with silica (tetraethoxysilane) to enhance its chemical stability, preventing the aggregation of the superparamagnetic core in liquid media and improving its biocompatibility [38,39]. Supports coated with silica have some very adequate physical and chemical properties for enzyme immobilization, such as nontoxicity, stability to redox reactions and facility to chemical derivatization, enabling the easy activation [40]. The silica-coated nanoparticle was activated with DVS and BQ, producing TEOS-NANO-DVS and TEOS-NANO-BQ supports in order to covalently bind the lipase on them. Fig. 1 shows a scheme of the immobilization strategies used in this paper. Therefore, PFL was immobilized on superparamagnetic $NiZnFe_2O_4$ coated with silica and activated or not with DVS or BQ and the biocatalysts performances were compared.

2. Materials and methods

2.1. Materials

PFL (Lot: MKBW 5216 V), tetraethoxysilane (TEOS) (purity 98% *v*/v), *p*-nitrophenyl butyrate (*p*NPB) ≥ 98%, divinylsulfone (97% purity), *p*-benzoquinone (≥98% purity), Triton X-100, cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The chemical reagents for the synthesis of magnetic nanoparticles were $FeCl_3 \cdot 6H_2O$ (97%), $NiCl_2 \cdot 6H_2O$ (97%) and $ZnCl_2$ (99%) and they were purchased from Dinâmica Química Contemporânea LTDA. All other reagents and solvents were of analytical grade.

2.2. Methods

2.2.1. Synthesis of superparamagnetic $NiZnFe_2O_4$ nanoparticles

The hydrothermal synthesis of superparamagnetic $NiZnFe_2O_4$ nanoparticles was performed according to the procedure reported by Freire et al. [41], with some modifications. Solutions of chloride salts of the different metals were prepared (molar ratio was (0.25):Ni^{2+} (0.25):Zn^{2+}(1):Fe^{3+}) and mixed under vigorous stirring (6000 rpm) for 10 min. After that, 10 mL of 35 mM sodium hydroxide was added dropwise to precipitate the desired hydroxides. The mixture was transferred to a Teflon-lined autoclave for 30 min reaction at 250 °C. The precipitate was recovered with a magnet and washed several times with distilled water until neutral pH. Finally, the $NiZnFe_2O_4$ nanoparticles were dried in a vacuum desiccator (360 mmHg) at 25 °C.

2.2.2. Functionalization of superparamagnetic $NiZnFe_2O_4$ nanoparticles with tetraethoxysilane (TEOS)

The functionalization of $NiZnFe_2O_4$ nanoparticles was performed according to Thangaraj et al. [39], with some modifications. A mass of 100 mg of nanoparticles was dispersed in 50 mL of a solution formed

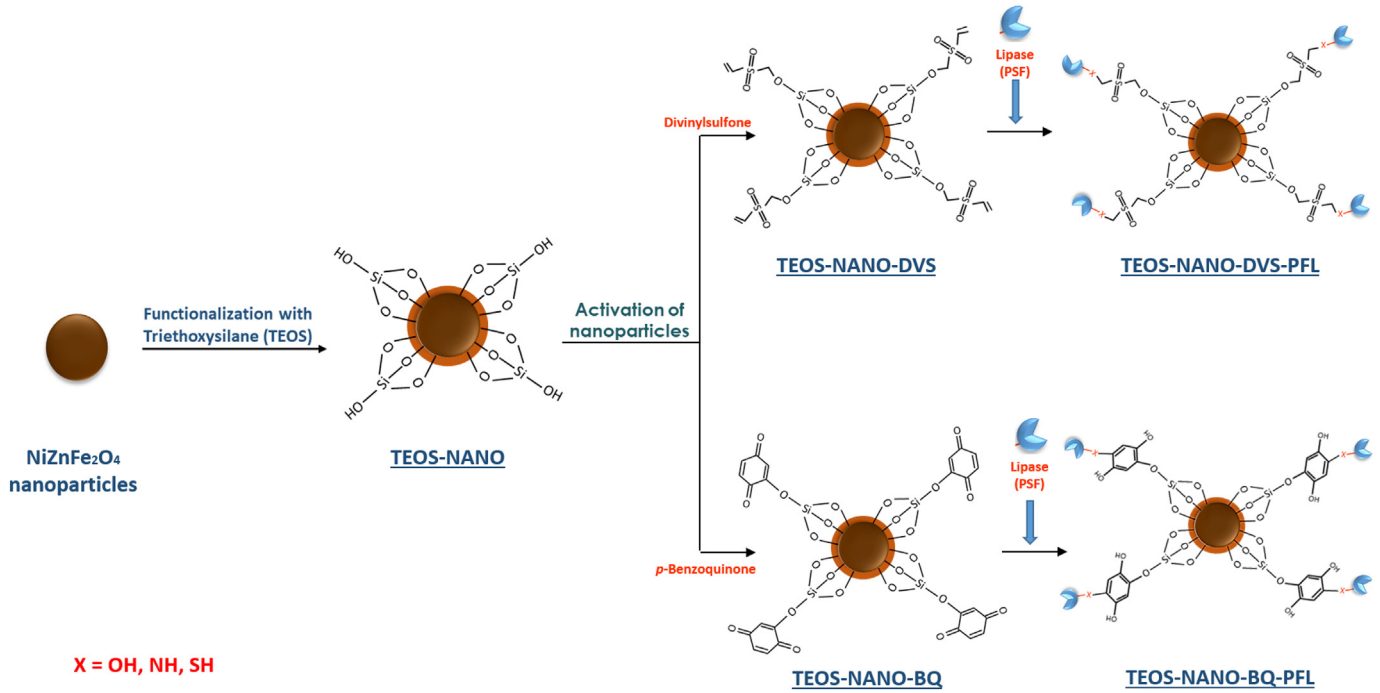

Fig. 1. Schematic representation of the preparation of TEOS-NANO-DVS-PFL and TEOS-NANO-BQ-PFL.

by 90% (*v*/v) ethanol and 10% (v/v) distilled water by sonication. Then, TEOS was added to the $NiZnFe_2O_4$ nanoparticles suspension (nanoparticle:TEOS ratio: 1:5 *w*/*v*). Subsequently, 10 mL of 25% (v/v) aqueous solution of ammonium hydroxide was added slowly dropwise and the mixture was maintained under stirring (750 rpm) for 6 h at 25 °C. The silica functionalized nanoparticles (TEOS nanoparticles) were collected with a magnetic bar and washed with distilled water and ethanol. Following that, the material was dried in a vacuum desiccator (360 mmHg) at 25 °C for 48 h.

2.2.3. Activation of TEOS functionalized nanoparticles with divinylsulfone (DVS) or p-benzoquinone (BQ)

1 g of TEOS nanoparticles was suspended in 10 mL of DVS or BQ solutions at the indicated concentrations at 25 °C. The pH (5, 7, 10 and 12.5) and reagents concentrations (0.05, 0.1, 0.25, 0.5 M) were altered in the activation process. TEOS nanoparticles were activated with DVS for 35 min. The procedure for TEOS nanoparticles activation with *p*-benzoquinone was conducted according to Brandt et al. [42]. 1 g of TEOS nanoparticles was suspended in 10 mL of *p*-benzoquinone solutions, containing 20% (*v*/v) of ethanol, during 1 h at room temperature. After that, the *TEOS* nanoparticles activated with DVS (TEOS-NANO-DVS) or BQ (TEOS-NANO-BQ) were extensively washed with immobilization buffer to be used for subsequent lipase immobilization.

2.2.4. Characterization of superparamagnetic modified and non-modified $NiZnFe_2O_4$ nanoparticles

Fourier Transform Infrared spectroscopy (FTIR) analysis was conducted in order to identify the vibration binding profile of the non-modified and modified $NiZnFe_2O_4$ nanoparticles. The typical vibration mode of the $NiZnFe_2O_4$ nanoparticles was identified on a Perkin Elmer spectrometer. For these measurements, the samples were previously diluted in KBr and then the data were collected in the range of 400–4000 cm^{-1}. In addition, some studies were carried out on an ATR spectrometer (Agilent) and the data were collected in the range of 600–4000 cm^{-1}.

2.2.5. Immobilization of PFL on TEOS functionalized nanoparticles

PFL was immobilized by two different strategies, namely: physical adsorption and covalent attachment. The PFL immobilization processes were carried out under mechanical stirring, at 25 °C. The enzyme load of immobilization was 20 U/g of support to prevent diffusion limitations or enzyme-enzyme interactions that could alter the observed properties of the immobilized enzymes [43,44].

2.2.5.1. Immobilization of PFL on TEOS-NANO-DVS or TEOS-NANO-BQ. Lyophilized PFL was dissolved in 5 mM sodium phosphate buffer pH 7 (20% *w*/*v*). Afterwards, 100 mg of TEOS-NANO-DVS or TEOS-NANO-BQ support was added into 1 mL of 5 mM buffer solutions (sodium acetate buffer pH 5, sodium phosphate buffer pH 7 and sodium bicarbonate buffer pH 10), containing <5% of the lipase solution The pH was rechecked after mixing enzyme and immobilization buffer solutions. The process of immobilization was conducted in a batch reactor and the effect of immobilization time was investigated. The biocatalysts were named TEOS-NANO-DVS-PFL and TEOS-NANO-BQ-PFL.

2.2.5.2. Immobilization of PFL on no treated TEOS-NANO. A PFL solution was prepared as in the previous point, but only in 5 mM sodium phosphate at pH 7. Afterwards, 100 mg of TEOS nanoparticles was added into 1 mL of this enzyme solution. The process of immobilization was conducted in a batch reactor for 24 h.

2.2.6. Enzyme activity

PFL activity was determined by hydrolysis of *p*NPB at pH 7 and 25 °C, according to the methodology described by Garcia-Galan et al. [45]. 50 μL of 50 mM *p*NPB in acetonitrile were added to 2.5 mL of 25 mM sodium phosphate at pH 7 and 50 μL of the sample at 25 °C. The product, *p*-nitrophenol, released during the hydrolysis of *p*NPB, was measured at 348 nm ($\varepsilon_c = 5.236\ mol^{-1} \cdot cm^{-1}$). One unit (U) is defined as the amount of enzyme capable of hydrolyzing 1 μmol of substrate per minute under the described conditions.

2.2.7. Immobilization parameters

The immobilization parameters were calculated in order to evaluate the efficiency of the produced biocatalysts. The immobilization yield (*IY*) was defined as initial activity (At_i) minus the supernatant activity (At_s) divided by the initial activity (At_i) [46]. The theoretical activity (At_T) was defined using the immobilization yield and the amount of lipase offered per gram of support [46]. The recovered activity (At_R) was defined as the ratio between the lipase activity observed in the biocatalysts (At_D U g − 1) and the theoretic activity (At_T U g − 1) [46].

2.2.8. Thermal inactivation of immobilized enzymes

10 mg of immobilized preparations were suspended in 0.5 mL of 25 mM sodium phosphate buffer at pH 7 and 60 °C. Periodically, samples were withdrawn, and their activities were determined employing the *p*NPB assay. The enzyme deactivation profile was built using the initial measure before incubation at 60 °C as 100%. The half-life time ($t_{1/2}$)

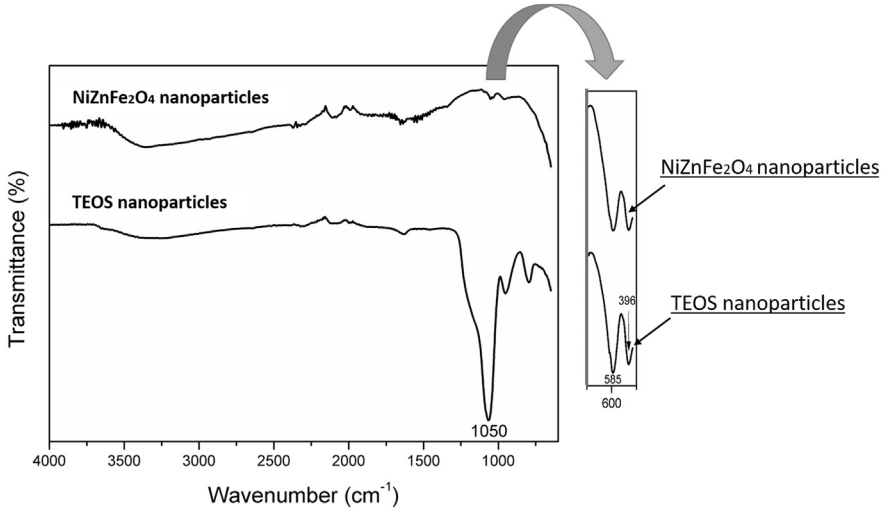

Fig. 2. FTIR spectra of samples of unmodified $NiZnFe_2O_4$ nanoparticles and TEOS nanoparticles. Experiments were performed as described in Methods. On the left: measures conducted at FTIR Agilent technologies ATR spectrometer (4000–600 cm^{-1}). On the right: measures conducted at Perkin Elmer spectrometer (760–400 cm^{-1}).

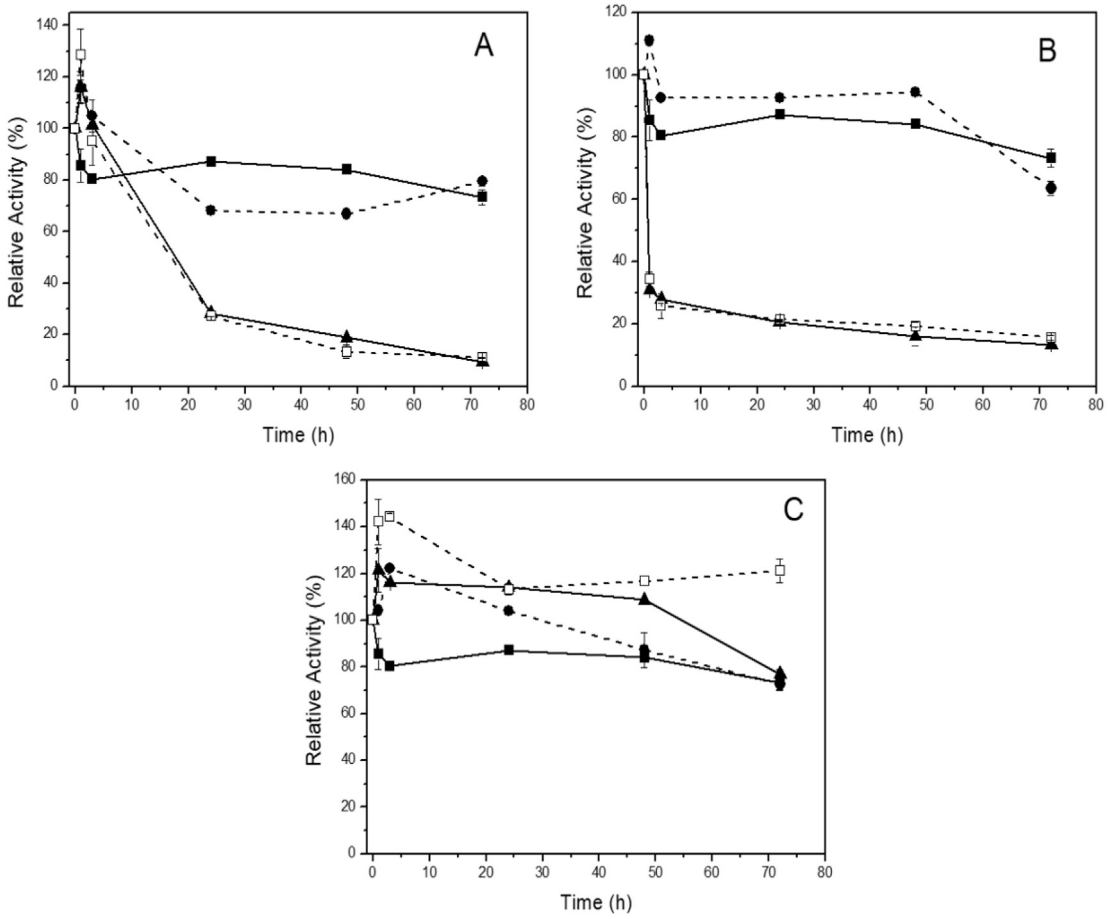

Fig. 3. Effect of different surfactants on the stability of free *PFL*. A: Anionic surfactant – SDS; B: Cationic surfactant – CTAB; C: Non-ionic surfactant - Triton X-100. Experiments were performed at pH 7 and 25 °C, other specifications are described in methods. Concentration of surfactants: Control – 0%: Solid line (■); 0.01%: Dashed line (●); 0.05%: Solid line (▲); 0.1%: Dashed line (□). The relative activity was calculated considering as 100% the lipase activity in initial time of assay. The lines represent the tendency of the experimental data.

for each immobilized preparation was calculated according to Sadana and Henley deactivation model [47], using a Microcal Origin program, version 8.1.

2.2.9. Support loading capacity

The support loading capacity was determined by increasing the amount of enzyme offered per gram of support (from 20 to 750 U/g). These assays were performed using optimal activated supports and protocols.

2.2.10. Operational stability of immobilized biocatalysts

The feasibility of the biocatalysts capture and the operational stabilities of the biocatalysts in aqueous media were evaluated in the hydrolysis of 0.962 mM *p*NPB in 25 mM sodium phosphate buffer pH 7.0, at 25 °C for 5 min. Consecutive cycles of hydrolysis reaction were performed using 10 mg of biocatalyst. Between the cycles, the biocatalyst was captured with a magnet bar and washed with sodium phosphate buffer (pH 7.0, 25 mM). Afterwards, the relative activity of the biocatalyst was determined, taking as 100% the activity in the first cycle of *p*NPB hydrolysis.

3. Results and discussion

3.1. Fourier transform infrared spectroscopy (FTIR) of TEOS-$NiZnFe_2O_4$ superparamagnetic nanoparticles

In order to study the incorporation of *TEOS* groups on the surface of $NiZnFe_2O_4$ superparamagnetic nanoparticles, infrared spectroscopy of the naked samples of $NiZnFe_2O_4$ nanoparticles and *TEOS* modified nanoparticles was conducted (Fig. 2). The broad band between 1000 and 1250 cm^{-1}, absent in the case of the naked particles, in the *TEOS* modified nanoparticles spectrum represents the asymmetric vibrations of Si—O bonds [39]. In addition, the new bands at 950 and 800 cm^{-1} are assigned to the presence of silanol groups (Si—OH) and the symmetrical tension of Si—O bonds, respectively [48]. The appearance of all these bands on the TEOS-$NiZnFe_2O_4$ demonstrates the successful functionalization of the nanoparticles with silica.

In Fig. 2 (right-hand side), all samples present bands at 585 and 360 cm^{-1}, which are attributed to the ion vibrations in the crystal lattice of $NiZnFe_2O_4$ nanoparticles. Therefore, these bands represent the stretching vibrations of Fe—O and Zn—O bonds in tetrahedral sites

Table 1
Values of the parameters of immobilization and thermal stability of TEOS-NANO-DVS-PFL biocatalyst. Immobilization conditions: sodium phosphate buffer, 5 mM, pH 7; Triton X-100 0.1% (*v*/v), during 24 h, at 25 °C. Other specifications are described in methods. Inactivation conditions: 60 °C in presence of sodium phosphate buffer, 25 mM, pH 7.

Immobilization parameters	TEOS-NANO-PFL biocatalyst
Immobilization yield (*IY* -%)	60.89 ± 0.5
Immobilized lipase activity (At_D - U/g)	6.75 ± 0.0
Theoretic activity (At_T - U/g)	12.18 ± 0.1
Recovered activity (At_R - %)	55.46 ± 0.5
Half-life ($t_{1/2}$ – min)	152.2

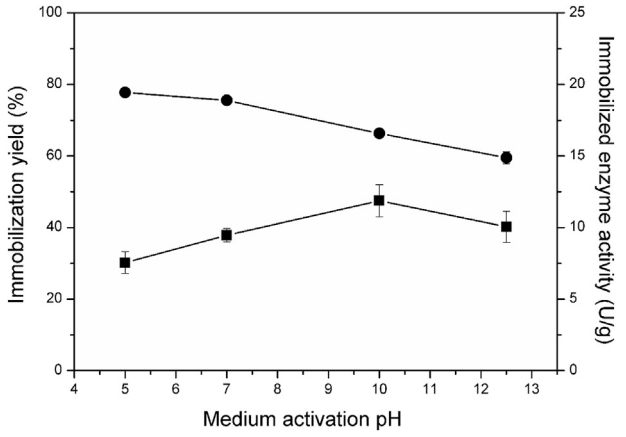

Fig. 4. Immobilization of PFL on TEOS nanoparticles activated with BQ at different pH values and 25 °C. These assays were performed using 0.1 M of BQ in acetate, phosphate, carbonate-bicarbonate or carbonate buffer at pH values 5, 7, 10 and 12.5, respectively. Experiments were carried out as described in methods, Immobilization yield (●); Immobilized enzyme activity (■). The lines represent the tendency of experimental data.

(585 cm^{-1}) and the stretching vibrations of Fe—O and Ni—O bonds in octahedral sites (396 cm^{-1}) [49]. Thus, it is possible to confirm that the functionalization process of $NiZnFe_2O_4$ nanoparticles with silica does not affect the internal structure of the $NiZnFe_2O_4$ nanoparticles.

3.2. Effect of surfactants on the stability of PFL

Several studies have shown that lipases can be immobilized in the presence of surfactants to improve the activity of the immobilized enzyme active biocatalysts [50–53]. The presence of surfactants has several effects on the lipase behavior. PFL is typically known for forming bimolecular aggregates, changing some of its properties due to the existence of an open but partially blocked active center of the lipase in the intersection between the two lipase molecules [54,55]. That is, it is possible that in a covalent immobilization, dimer and monomer mixtures may become immobilized, making the results hardly reproducible and having its activity/stability properties fully altered. The presence of surfactants has been shown to break these aggregates, allowing the individual enzyme molecules to bind to the support. If detergents are used in an immobilization process, this may produce biocatalysts with improved activities and easier to reproduce, as only monomeric enzyme molecules will be immobilized. Moreover, surfactants shift the open-closed equilibrium of lipases towards the open and more active form, in some instances, the immobilization can freeze this open form of the lipase [50–52,56]. Additionally, surfactants avoid enzyme immobilization on fairly hydrophobic supports via interfacial activation, leaving the covalent reaction of the enzyme with the support as the only reason for enzyme immobilization [12,16]. However, the use of detergents may also have negative effects on enzyme properties, e.g., reducing enzyme stability or producing the enzyme inhibition [53].

Therefore, before using a detergent in the immobilization of a lipase, it is worth investigating their effects on the lipase stability. Fig. 3 presents the effect of three distinct surfactants (Sodium dodecyl sulfate – SDS/anionic surfactant, cetyltrimethylammonium bromide – CTAB/cationic surfactant and Triton X-100/non-ionic surfactant) at various concentrations (0.01, 0.05 and 0.1% *v*/v) on the evolution of the activity of free PFL along time.

In the first hours of incubation with 0.01% SDS, the free PFL maintained 100% of its activity. However, after 24 h of incubation, free PFL presented slightly lower activities (See Fig. 3 - A). At high concentrations of SDS (0.05 and 0.1%), lipase progressively lost activity, maintaining only 10% of its relative activity after 72 h of incubation in both concentrations of SDS. Fig. 3 – B shows the effect of CTAB on enzyme stability. It is possible to observe that 0.01% (*v*/v) CTAB (during 48 h) did not significantly decrease the PFL activity. However, using 0.05 or 0.1% the activity progressively decreased (residual activity around 20% after 24 h of incubation). This way, both detergents did not seem appropriate for their use in PFL immobilization.

Fig. 3-C shows that Triton X-100 even increased the activity of the enzyme when compared with the enzyme in absence of detergent (although the samples were much diluted during the activity assay (maximal concentration was 0.0192% (*v*/v)). In the presence of 0.1% (v/v) Triton X-100, the relative activity of free PFL remained over 100% during 72 h of incubation. This hyperactivation can be explained by the stabilization of the open and monomeric form of lipase molecules [51,53,57,58].

Taking into account these results, the PFL immobilizations were performed in the presence of 0.1% (v/v) Triton X-100.

3.3. Immobilization of PFL on TEOS-NANO support

PFL was incubated in the presence of TEOS-NANO support in the presence of 0.1% (v/v) detergent, producing the biocatalyst TEOS-NANO-PFL and Table 1 shows the results. PFL was adsorbed on TEOS-NANO support, presenting an immobilization yield about 60% and recovered activity about 55%, after 24 h of immobilization. In addition, the biocatalyst TEOS-NANO-PFL showed a significant thermal stabilization at 60 °C ($t_{1/2}$ about 150 min). The increase of the detergent concentration (to 0.5%) or the increase of the ion strength (using 200 mM NaCl) do not avoid enzyme adsorption, suggesting that the enzyme was immobilized in a mixed way, including ion exchange and interfacial activation versus the support. This way, the influence of enzyme adsorption (or at least the enzyme/support interactions later one) on the nanoparticle surface cannot be discarded when the particle was activated with BQ or DVS. As these effects were positive for enzyme stability, they do not look a problem.

Table 2
Values of the parameters of immobilization and thermal stabilities of TEOS-NANO-BQ-PFL biocatalysts produced at different concentrations of activating agent. Immobilization conditions: sodium phosphate buffer, 5 mM, pH 7; Triton X-100 0.1% (v/v), during 24 h, at 25 °C. Other specifications are described in methods. Inactivation conditions: 60 °C in presence of sodium phosphate buffer, 25 mM, pH 7.

Immobilization parameters	BQ concentration			
	0.05 M	0.1 M	0.25 M	0.5 M
Immobilization yield (*IY* -%)	66.68 ± 1.2	66.31 ± 0.2	62.88 ± 4.3	68.45 ± 2.7
Immobilized lipase activity (At_D - U/g)	6.11 ± 0.0	11.87 ± 1.1	10.38 ± 2.7	3.75 ± 0.4
Theoretic activity (At_T -U/g)	13.34 ± 0.2	13.26 ± 0.0	12.57 ± 0.9	13.69 ± 0.2
Recovered activity (At_R - %)	45.81 ± 0.8	89.50 ± 8.7	81.98 ± 16.2	27.37 ± 2.5
Half-life ($t_{1/2}$ – h)	>24	>24	18.3	17

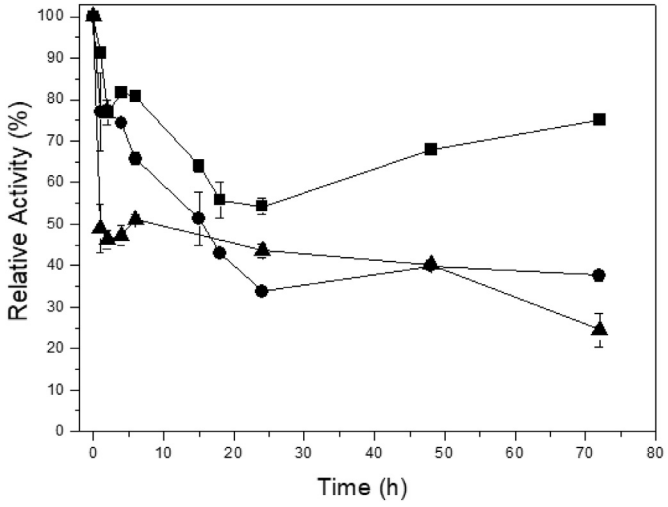

Fig. 5. PFL Immobilization course on TEOS-NANO-BQ. Other specifications are described in Methods. Relative activities of supernatant during immobilization at pH 5 (▲); pH 7 (●) and pH 10 (■). The lines represent the tendency of experimental data.

3.4. Immobilization of PFL on TEOS-NANO-BQ support: optimization of the support activation

Fig. 4 shows that the immobilization yield decreased when increasing the support activation pH, while the immobilized enzyme activity was improved, achieving maximum activity values when the support activation was performed at pH 10, shortly followed by the support activated at pH 12.5. The lower immobilization yield at high pH value is not easy to explain, as it may be expected that the activation of the hydroxyl groups in the support is more efficient at alkaline pH value, and the number of active groups in the support should determine the immobilization rate. This may be explained considering the stability of the silanol bonds of TEOS with the nanoparticle, silica become solubilized at alkaline or acid pH values and that way the number of active groups in the support decreased at alkaline pH values even though very likely the hydroxyl activation is more efficient. The lower amount of reactive groups in the support will give a lower enzyme-support reaction, permitting to maintain a higher activity of the immobilized enzymes, even though the amount of immobilized enzyme is lower.

Table 3
Half-lives of different TEOS-NANO-BQ-PFL immobilized at pH 5, 7 or 10 for 24 of contact (enzyme-support). Other specifications are described in methods. Inactivation conditions: 60 °C in presence of sodium phosphate buffer, 25 mM, pH 7.

Immobilization condition	Half-life (min)
pH 5, 24 h	243.42
pH 7, 24 h	>1440
pH 10, 24 h	350.11

Table 2 shows the results of immobilization parameters and thermal stabilities of PFL immobilized on supports activated at various BQ concentrations. The best results were achieved using 0.1 M of BQ, since high recovered activity (about 89.5%) and high thermal stability ($t_{1/2}$ > 24 h, at 60 °C) were obtained. This was much higher than that observed using the TEOS nanoparticles and shows the importance of the BQ activation in the biocatalyst performance. The use of higher concentrations of BQ produced a decrease of these parameters, perhaps due to some BQ polymerization that can generate non homogenous and longer spacer arms and generate some steric hindrances for the reaction of the enzyme with the non-polymerized BQ moieties (that is, the longer spacer arms will produce both steric hindrances for the reaction with the BQ groups below this "long pacer arms" and a lower enzyme rigidity for each of the enzyme-support bonds formed).

3.4.1. Immobilization of Pseudomonas fluorescens lipase on TEOS-NANO-BQ support: optimization of immobilization conditions

The immobilization pH value plays many roles. In the case of DVS and very likely BQ, enzymes may become immobilized in a wide range of pH values, but in each pH the most reactive group may be different. Therefore, the enzyme can be immobilized following different orientations, only varying the immobilization pH [16,18]. This may become more important considering that BQ has a certain anionic character [20]. In this context, the influence of the immobilization pH of PFL on BQ activated support was studied, and results are shown in Fig. 5.

Fig. 5 shows the PFL immobilization course at pH 5, 7 and 10 on TEOS-NANO-BQ that has been activated under optimal conditions. At pH 5, 50% PFL is immobilized in the first hours of immobilization and, at longer incubation times (48 h), 60% of enzyme activity was immobilized. At pH 7 and 10, PFL was also immobilized, achieving the maximum immobilization yield after 24 h of contact (relative activities about 35 and 55% for pH 7 and 10, respectively). Therefore, immobilization rates at pH 7 and 10 were lower in relation to immobilization rates

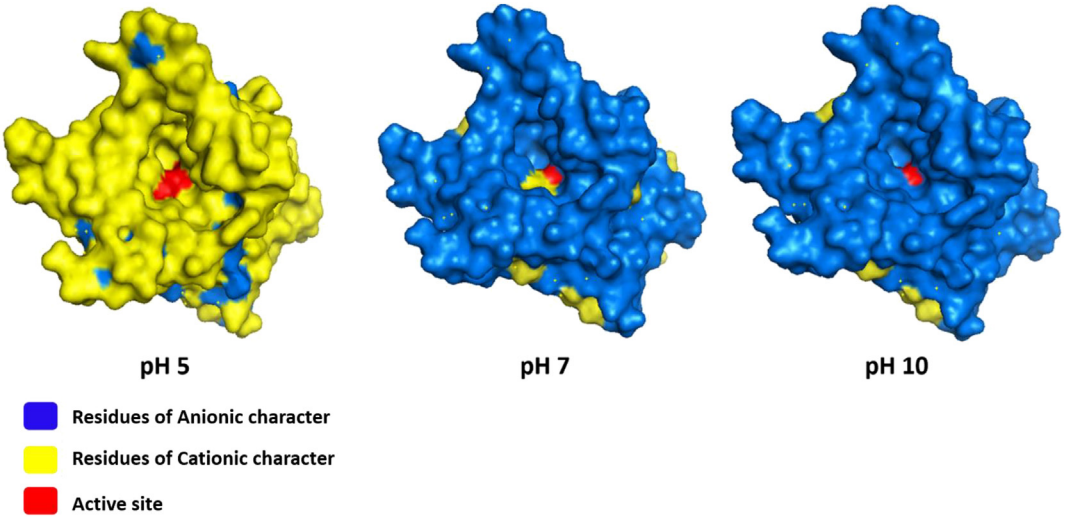

Fig. 6. Schematic representation of PFL immobilization at pHs 5, 7 and 10. This figure was made using pymol educational version (PDB lipase code: 2LIP).

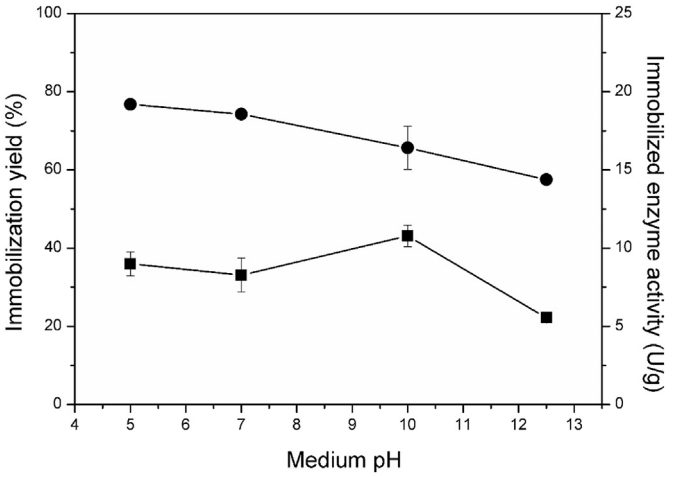

Fig. 7. Immobilization of PFL on TEOS nanoparticles activated with DVS at different pH values. These assays were performed with 0.1 M of DVS in acetate, phosphate, carbonate-bicarbonate or carbonate buffer, 200 mM, at pHs 5, 7, 10 and 12.5, respectively, at 25 °C. Other specifications are described in methods. Immobilization yield (●); Expressed activity in the biocatalyst (■). The lines represent the tendency of experimental data.

at pH 5. This is against the expected results considering just the reactivity of the enzyme groups with BQ, which should increase with pH. Even more important, at pH 10 it is possible to observe how the enzyme started to be released from the support after 24 h of immobilization. Considering that the enzyme-support covalent bonds should be irreversible, the likeliest explanation for this result is that the TEOS moiety where the enzyme molecules are immobilized become released to the medium at pH 10, taking the immobilized enzyme molecules with them. This agrees with the results on support activation. To confirm this point, an enzyme immobilized at pH 7 was incubated at pH 10 for 48 h. It was again detected that a significant percentage of enzyme activity (around 30%) was released to the medium (not shown results). However, at pH 7 no enzyme leakage was detected after one week of incubation at 4 or 25 °C.

The faster immobilization rate at pH 5 compared to pH 7 could be explained by the anionic character of *p*-benzoquinone groups, that can produce an ion exchange with the cationic groups of the lipase, whose isoelectric point is around 5.9. Fig. 6 presents a scheme of possible ionic character (anionic or cationic) of PFL at immobilization pHs 5, 7 and 10. At pH 5, PFL has a cationic character, enabling a fast first adsorption on the anionic BQ activated support. At pH 7 and pH 10, PFL has an anionic character, avoiding the first adsorption via ion exchange and yielding a low immobilization rate.

After this first adsorption, the enzyme molecules can become covalently immobilized via an intramolecular reaction, as in other multifunctional supports [12,14]. At pH 7, the enzyme immobilization must be directly via covalent bonds. Other authors also reported low immobilization rates at pH 10, when bovine serum albumin was immobilized on agarose activated with *p*-benzoquinone at various pHs (3 to 10). They observed that the highest immobilization rate of the proteins was achieved when immobilization was conducted at pH around 3 and, on the other hand, the lowest amount of immobilized proteins was obtained at pH 10 [42]. Again, a likely explanation may be reached by the different cationic/anionic state of the enzyme at different pH values. Similar results are described for aminated supports activated with glutaraldehyde, but, in this case, the result is the opposite, as the enzyme adsorption is via anion exchange [59].

The incubation of some of the immobilized enzyme preparations at 1 M NaCl showed the release of a certain percentage of enzyme (around 20%), suggesting that some of the enzyme molecules could be just immobilized on the support via ion exchange. Table 3 presents the half-lives of biocatalysts immobilized at pH 5, 7 and 10. The results show that the highest stability was obtained when PFL was immobilized on TEOS-NANO-BQ at pH 7 for 24 h.

3.5. Immobilization of PFL on TEOS-NANO-DVS support: optimization of support activation

The optimal activation of supports such as agarose with DVS are described at pH 12.5, but the supports used here have very different properties, as previously commented in this paper. Thus, the effect of the pH during DVS activation of the TEOS-NANO support was studied. The immobilization of PFL was performed at pH 7, optimal conditions found using BQ. Fig. 7 shows that the increase in the pH (in the pH range from 5 to 12.5) of support activation causes a slight decrease in immobilization yield, as using BQ.

At alkaline pH, the reaction of DVS with the hydroxyl groups in the supports occurs faster than at neutral or acid pHs, introducing larger amounts of DVS groups on the support. Therefore, the expected result when increasing the activation pH is that more DVS groups are introduced on the support as long as the active group is stable. If we have more groups on the support, enzyme molecules are immobilized on it in faster, increasing the immobilization speed. However, an opposite trend occurred in this paper (see Fig. 7), that makes results fully different to the ones obtained using agarose [18]. DVS molecules may undergo inactivation or polymerization at very alkaline pH values, but that will occur also when using agarose and activation time is relatively short.

The distinctive property of the new material is the fact that the TEOS bound to the nanoparticles is unstable at drastic pH values [60,61], that is, silica is dissolved in the medium at drastic pH values and the number of groups in the support decreased instead of increasing as it may be expected, when using this drastic alkaline pH value. Fig. 7 also shows the final enzyme activities of PFL immobilized on supports activated under different pH values. The most active immobilized preparation was obtained when activation was conducted at pH 10. Explanations may be like in the case of BQ activation, the lower activation at high

Table 4
Data of parameters of immobilization and thermal stabilities of TEOS-NANO-DVS-PFL biocatalysts produced at different concentrations of activating agent. Other specifications are described in methods. Immobilization conditions: sodium phosphate buffer, 5 mM, pH 7; Triton X-100 0.1% (v/v), during 24 h, at 25 °C. Inactivation conditions: 60 °C in presence of sodium phosphate buffer, 25 mM, pH 7.

Immobilization parameters	DVS concentration			
	0.05 M	0.1 M	0.25 M	0.5 M
Immobilization yield (*IY*-%)	62.30 ± 3.5	65.61 ± 5.5	60.07 ± 4.6	61.29 ± 2.1
Immobilized lipase activity (At_D - U/g)	3.38 ± 0.4	10.78 ± 0.7	5.46 ± 0.0	2.81 ± 0.0
Theoretic activity (At_T - U/g)	12.46 ± 0.7	13.12 ± 1.1	12.01 ± 0.9	12.26 ± 0.4
Recovered activity (At_R - %)	27.24 ± 4.9	82.18 ± 1.6	45.60 ± 3.5	22.90 ± 0.5
Half-life ($t_{1/2}$ – min)	108.5	225	138.5	95

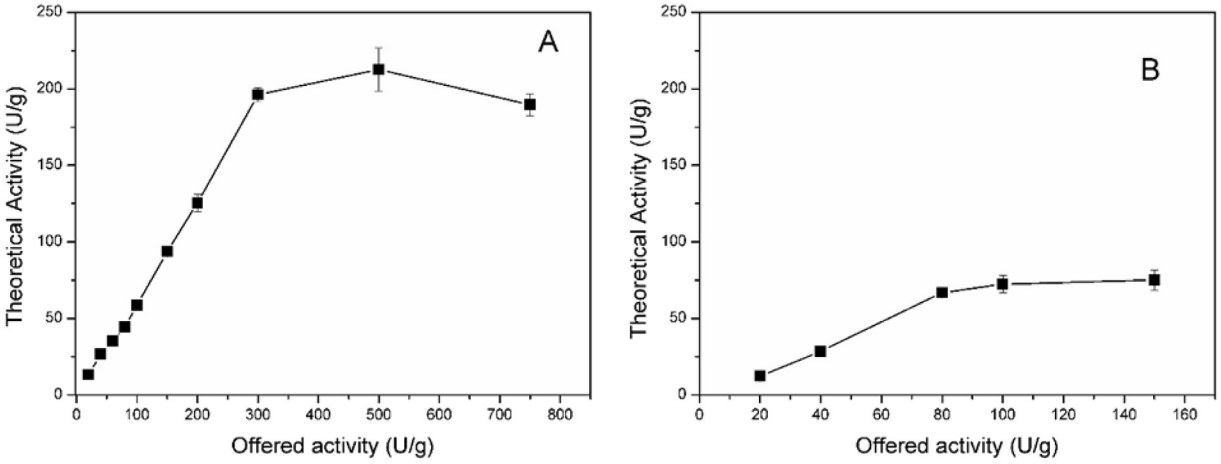

Fig. 8. Theoretic activity versus offered activity of TEOS-NANO-BQ-PFL (A) and TEOS-NANO-PFL (B). The lines represent the tendency of experimental data. Other specifications are described in methods.

pH values produce a slower immobilization, but also a lower enzyme distortion.

Next, the effect of concentration of DVS in the activation step on immobilized enzyme properties was analyzed. Table 4 presents some parameters of immobilization and thermal stabilities of PFL immobilized on supports activated at different DVS concentrations.

It is possible to highlight that the best results were obtained using 0.1 M of DVS and support activation at pH 10. In this condition, the biocatalyst was more active and stable ($At_D = 10.8$ U/g and $t_{1/2} = 225$ min). Moreover, this biocatalyst was able to retain a large percentage of its initial activity after the process of immobilization, yielding 82% of recovered activity. When 0.25 and 0.5 M of DVS were used, recovered activities (45.6 and 22.9%) and enzyme stabilities (half-lives of 138.5 and 95 min) decreased quickly, for 0.25 and 0.5 M of DVS preparations, respectively. This could be due to the polymerization of DVS, that gives a heterogeneous surface to immobilize the enzyme on and even the growing of the length spacer arm will reduce the positive effects of each enzyme-support bond on enzyme stability.

The best biocatalysts TEOS-NANO-BQ-PFL and TEOS-NANO-DVS-PFL were obtained under similar support activation conditions. This demonstrates that the bifunctional agent *p*-benzoquinone and divinylsulfone act similarly during the process of support activation, as mentioned by other authors [15]. However, the use of *p*-benzoquinone allows producing a more robust biocatalyst in this specific case, with higher thermal stabilities than the NANO-DVS-PFL preparations. For instance, at optimized conditions, the half-life of TEOS-NANO-BQ-PFL was higher than 24 h, while TEOS-NANO-DVS-PFL half-life was lower than 4 h. Thus, further work was performed using TEOS-NANO-BQ-PFL, comparing it with the enzyme immobilized in the non-activated TEOS-NiZnFe_2O_4 nanoparticle.

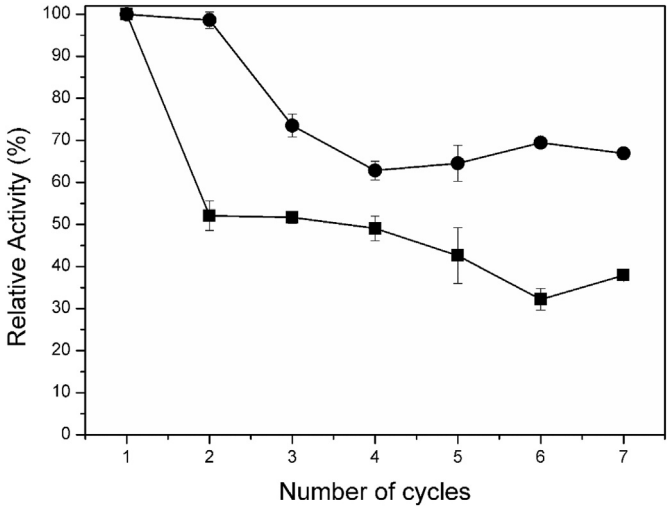

Fig. 9. Operational stability of TEOS-NANO-BQ-PFL (●) and TEOS-NANO-PFL (■) preparations. Hydrolysis of *p*NPB was carried out at 25 °C, aqueous medium and pH 7. Other specifications are described in methods. The lines represent the tendency of experimental data.

3.6. Enzyme load of TEOS-NANO-BQ-PFL and TEOS-NANO-PFL biocatalysts

The loading capacity is an important feature of the support, mainly for industrial applications in biocatalysts. In this context, the enzyme load capacity of TEOS-NANO-BQ and TEOS-NANO was investigated and results are presented in Fig. 8.

Initially, the theoretic activity increases with the offered amount of PFL and after 300 and 100 U/g for TEOS-NANO-BQ-PFL and TEOS-NANO-PFL, respectively, the values of theoretic activity remained almost constant. Considering that the surface should be similar for both supports, this suggested that the physical immobilization on non-activated TEOS nanoparticles may be more complex that using the BQ activated ones, with perhaps a competition between free enzymes and the support (e.g., this has been described for immobilization via interfacial activation on hydrophobic supports) [58].

3.7. Operational stability of immobilized PFL

TEOS-NANO-BQ-PFL and TEOS-NANO-PFL were utilized in several cycles of *p*NPB hydrolysis and the results are shown in Fig. 9. TEOS-NANO-BQ-PFL maintained higher residual activity (70%) compared to the TEOS-NANO-PFL (35%) activity after 7 cycles of reaction. A similar difference in the operational stability was also observed by Bezerra et al. [50], when PEI coated Fe_3O_4 nanoparticles were used to immobilize lipase from *Thermomyces lanuginosus* by covalent attachment (glutaraldehyde coupling) and adsorption. The covalent and adsorbed biocatalysts showed about 40 and 20% of their initial activities after 8 cycles of in *p*NPB hydrolysis, respectively. It is worth pointing out that the biocatalysts produced in this work are operationally more stable than the biocatalysts produced by Bezerra et al. [50]. This shows that the *p*-benzoquinone coupling produces robust biocatalysts with high stabilities than the biocatalysts produced with the standard glutaraldehyde coupling.

4. Conclusion

The results obtained in this study show that experimental changes in the activation and immobilization conditions may alter the final properties of the biocatalyst. Although bifunctional agents (divinylsulfone and *p*-benzoquinone) with similar groups involved in the process of activation and immobilization were used, changes in the enzyme-support reactivity were observed. This can be attributed to the fact that quite different biocatalysts, mainly in relation to thermal stability, were prepared.

One of the main conclusions is that using these TEOS coated supports, the changes in the pH of activation and immobilization must consider the risks of TEOS release to the medium, when using drastic pH, and that can make the understanding of the results more complex. This also limits the range of conditions where these biocatalysts may be used to those where TEOS release to the medium may be not relevant (e.g., pH near to neutrality).

BQ activation permits a rapid immobilization via cation exchange at moderately acid pH, that later is followed by covalent bonds. The preparation of TEOS-NANO-BQ-PFL under optimized conditions allowed obtaining a robust biocatalyst that maintains almost 90% of its activity after immobilization. Also, remarkable parameters of thermal stability were obtained in one-step immobilization of PFL on TEOS-NANO-BQ at optimized conditions ($t_{1/2}$ > 24 h at 60 °C).

Acknowledgements

The authors thank to CAPES, CNPq, FUNCAP and Ministerio de Ciencia, Innovación y Universidades from Spanish Government (project number CTQ2017-86170-R) for financial support. We thank Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP), project number BP3-0139-00005.01.00/18. Nathalia S. Rios thanks CNPq for a predoctoral fellowship (CNPq scholarship – Brazil). We thank to Professor Hosiberto Batista de Sant'Ana for kindly supplying the FTIR ATR analyses. The help and suggestions of Dr. Ángel Berenguer (Departamento de Química Inorgánica and Instituto Universitario de Materiales, Universidad de Alicante) are gratefully recognized.

References

[1] Y. Gu, P. Xue, *Pseudomonas* sp. lipase immobilized on magnetic porous polymer microspheres as an effective and recyclable biocatalyst for resolution of allylic alcohols, J. Chin. Chem. Soc. 65 (2018) 696–705, https://doi.org/10.1002/jccs.201700424.
[2] M.P. Pinheiro, N.S. Rios, T.D.S. Fonseca, F.D.A. Bezerra, E. Rodríguez-Castellón, R. Fernandez-Lafuente, M. Carlos de Mattos, J.C.S. dos Santos, L.R.B. Gonçalves, Kinetic resolution of drug intermediates catalyzed by lipase B from *Candida antarctica* immobilized on immobead-350, Biotechnol. Prog. (2018) 1–12, https://doi.org/10.1002/btpr.2630.
[3] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, Chem. Soc. Rev. 42 (2013) 6223–6235, https://doi.org/10.1039/c3cs60075k.
[4] N.N. Gandhi, Applications of lipase, J. Am. Oil Chem. Soc. 74 (1997) 621–634, https://doi.org/10.1007/s11746-997-0194-x.
[5] A. Salihu, M.Z. Alam, Solvent tolerant lipases: a review, Process Biochem. 50 (2015) 86–96, https://doi.org/10.1016/j.procbio.2014.10.019.
[6] J.C.S. Dos Santos, N. Rueda, R. Torres, O. Barbosa, L.R.B. Gonçalves, R. Fernandez-Lafuente, Evaluation of divinylsulfone activated agarose to immobilize lipases and to tune their catalytic properties, Process Biochem. 50 (2015) 918–927, https://doi.org/10.1016/j.procbio.2015.03.018.
[7] C.S. Bezerra, C.M.G. De Farias Lemos, M. De Sousa, L.R.B. Gonçalves, Enzyme immobilization onto renewable polymeric matrixes: past, present, and future trends, J. Appl. Polym. Sci. 132 (2015) 1–15, https://doi.org/10.1002/app.42125.
[8] O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Strategies for the one-step immobilization-purification of enzymes as industrial biocatalysts, Biotechnol. Adv. 33 (2015) 435–456, https://doi.org/10.1016/j.biotechadv.2015.03.006.
[9] J.C.S. dos Santos, N. Rueda, L.R.B. Gonçalves, R. Fernandez-Lafuente, Tuning the catalytic properties of lipases immobilized on divinylsulfone activated agarose by altering its nanoenvironment, Enzym. Microb. Technol. 77 (2015) 1–7, https://doi.org/10.1016/j.enzmictec.2015.05.001.
[10] R.A. Sheldon, Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs), Appl. Microbiol. Biotechnol. 92 (2011) 467–477, https://doi.org/10.1007/s00253-011-3554-2.
[11] J.C.S. dos Santos, O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Importance of the support properties for immobilization or purification of enzymes, ChemCatChem 7 (2015) 2413–2432, https://doi.org/10.1002/cctc.201500310.
[12] O. Barbosa, R. Torres, C. Ortiz, Á. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Heterofunctional supports in enzyme immobilization: from traditional immobilization protocols to opportunities in tuning enzyme properties, Biomacromolecules 14 (2013) 2433–2462, https://doi.org/10.1021/bm400762h.
[13] J.C.S. dos Santos, N. Rueda, O. Barbosa, J.F. Fernández-Sánchez, A.L. Medina-Castillo, T. Ramón-Márquez, M.C. Arias-Martos, M.C. Millán-Linares, J. Pedroche, M. del M. Yust, L.R.B. Gonçalves, R. Fernandez-Lafuente, Characterization of supports activated with divinyl sulfone as a tool to immobilize and stabilize enzymes via multipoint covalent attachment. Application to chymotrypsin, RSC Adv. 5 (2015) 20639–20649, https://doi.org/10.1039/C4RA16926C.
[14] H. Zaak, M. Sassi, R. Fernandez-Lafuente, A new heterofunctional amino-vinyl sulfone support to immobilize enzymes: application to the stabilization of β-galactosidase from *Aspergillus oryzae*, Process Biochem. 64 (2018) 200–205, https://doi.org/10.1016/j.procbio.2017.09.020.
[15] P. Zucca, E. Sanjust, Inorganic materials as supports for covalent enzyme immobilization: methods and mechanisms, Molecules 19 (2014) 14139–14194, https://doi.org/10.3390/molecules190914139.
[16] N.S. Rios, M.P. Pinheiro, J.C.S. dos Santos, T. de S. Fonseca, L.D. Lima, M.C. de Mattos, D.M.G. Freire, I.J. da Silva, E. Rodríguez-Aguado, L.R.B. Gonçalves, Strategies of covalent immobilization of a recombinant *Candida antarctica* lipase B on pore-expanded SBA-15 and its application in the kinetic resolution of (*R,S*)-Phenylethyl acetate, J. Mol. Catal. B Enzym. 133 (2016) 246–258, https://doi.org/10.1016/j.molcatb.2016.08.009.
[17] A.L. Medina-Castillo, J. Morales-Sanfrutos, A. Megia-Fernandez, J.F. Fernandez-Sanchez, F. Santoyo-Gonzalez, A. Fernandez-Gutierrez, Novel synthetic route for covalent coupling of biomolecules on super-paramagnetic hybrid nanoparticles, J. Polym. Sci. Part A Polym. Chem. 50 (2012) 3944–3953, https://doi.org/10.1002/pola.26203.
[18] J.C.S. Santos, N. Rueda, A. Sanchez, R. Villalonga, L.R.B. Gonçalves, R. Fernandez-Lafuente, Versatility of divinylsulfone supports permits the tuning of CALB properties during its immobilization, Process Biochem. 5 (2015) 35801–35810, https://doi.org/10.1039/C5RA03798K.
[19] T.L.d. Albuquerque, N. Rueda, J.C.S. dos Santos, O. Barbosa, C. Ortiz, B. Binay, E. Özdemir, L.R.B. Gonçalves, R. Fernandez-Lafuente, Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment, Process Biochem. 51 (2016) 865–874, https://doi.org/10.1016/j.procbio.2016.04.002.
[20] M.S. Eldin Mohy, E.I. Seuror, M.A. Nasr, H.A. Tieama, Affinity covalent immobilization of glucoamylase onto ρ-benzoquinone- activated alginate beads: II.Enzyme immobilization and characterization, Appl. Biochem. Biotechnol. 164 (2011) 45–57, https://doi.org/10.1007/s12010-010-9113-y.
[21] M.S.M. Eldin, Beta-galactosidase covalent immobilization on the surface of alginate beads and its application in lactose hydrolysis - effect of introducing ethylene diamine spacer, Dtsch. Leb. 101 (2005) 309–314.
[22] H. Vaghari, H. Jafarizadeh-Malmiri, M. Mohammadlou, A. Berenjian, N. Anarjan, N. Jafari, S. Nasiri, Application of magnetic nanoparticles in smart enzyme immobilization, Biotechnol. Lett. (2015) 223–233, https://doi.org/10.1007/s10529-015-1977-z.
[23] E. Aslani, A. Abri, M. Pazhang, Immobilization of trypsin onto Fe3O4@SiO2–NH2 and study of its activity and stability, Colloids Surfaces B Biointerfaces 170 (2018) 553–562, https://doi.org/10.1016/j.colsurfb.2018.06.022.
[24] W. Xie, J. Wang, Enzymatic production of biodiesel from soybean oil by using immobilized lipase on Fe3O4/poly(styrene-methacrylic acid) magnetic microsphere as a biocatalyst, Energy and Fuels 28 (2014) 2624–2631, https://doi.org/10.1021/ef500131s.
[25] W. Xie, X. Zang, Lipase immobilized on ionic liquid-functionalized magnetic silica composites as a magnetic biocatalyst for production of trans-free plastic fats, Food Chem. 257 (2018) 15–22, https://doi.org/10.1016/j.foodchem.2018.03.010.
[26] W. Xie, X. Zang, Covalent immobilization of lipase onto aminopropyl-functionalized hydroxyapatite-encapsulated-γ-Fe2O3 nanoparticles: a magnetic biocatalyst for interesterification of soybean oil, Food Chem. 227 (2017) 397–403, https://doi.org/10.1016/j.foodchem.2017.01.082.
[27] W. Xie, Y. Han, H. Wang, Magnetic Fe3O4/MCM-41 composite-supported sodium silicate as heterogeneous catalysts for biodiesel production, Renew. Energy 125 (2018) 675–681, https://doi.org/10.1016/j.renene.2018.03.010.
[28] W. Xie, M. Huang, Immobilization of *Candida rugosa* lipase onto graphene oxide Fe3O4 nanocomposite: characterization and application for biodiesel production, Energy Convers. Manag. 159 (2018) 42–53, https://doi.org/10.1016/j.enconman.2018.01.021.
[29] W. Xie, X. Zang, Immobilized lipase on core-shell structured Fe3O4-MCM-41 nanocomposites as a magnetically recyclable biocatalyst for interesterification of soybean oil and lard, Food Chem. 194 (2016) 1283–1292, https://doi.org/10.1016/j.foodchem.2015.09.009.
[30] D. Zheng, S. Wang, S. Qiu, J. Lin, X. Diao, Synthesis of butyl oleate catalyzed by cross-linked enzyme aggregates with magnetic nanoparticles in rotating magneto-microreactor, J. Biotechnol. 281 (2018) 123–129, https://doi.org/10.1016/j.jbiotec.2018.07.011.
[31] E.P. Cipolatti, R.O. Henriques, D.E. Moritz, J.L. Ninow, D.M.G. Freire, E.A. Manoel, R. Fernandez, Nanomaterials for biocatalyst immobilization – state of the art and future trends, RSC Adv. 6 (2016) 104675–104692, https://doi.org/10.1039/c6ra22047a.

[32] C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente, R.C. Rodrigues, Potential of different enzyme immobilization strategies to improve enzyme performance, Adv. Synth. Catal. 353 (2011) 2885–2904, https://doi.org/10.1002/adsc.201100534.
[33] L. Betancor, M. Fuentes, G. Dellamora-ortiz, L. Fernando, A. Hidalgo, N. Alonso-morales, C. Mateo, M. Guis, R. Fern, Dextran aldehyde coating of glucose oxidase immobilized on magnetic nanoparticles prevents its inactivation by gas bubbles, J. Mol. Catal. - B Enzym. 32 (2005) 97–101, https://doi.org/10.1016/j.molcatb.2004.11.003.
[34] E.J. Gilbert, *Pseudomonas* lipases: biochemical properties and molecular cloning, Enzym. Microb. Technol. 15 (1993) 634–645, https://doi.org/10.1016/0141-0229(93)90062-7.
[35] N.S. Rios, B.B. Pinheiro, M.P. Pinheiro, R.M. Bezerra, J.C.S. dos Santos, L.R.B. Gonçalves, Biotechnological potential of lipases from *Pseudomonas*: sources, properties and applications, Process Biochem. 75 (2018) 99–120, https://doi.org/10.1016/j.procbio.2018.09.003.
[36] A. Miszczyk, K. Darowicki, Study of anticorrosion and microwave absorption properties of NiZn ferrite pigments, Microwaves 1 (2011) 13–21, https://doi.org/10.1108/00035591111097657.
[37] C. Wang, Y. Niu, P. Pei, Y. Shen, H. Zhang, A. Xie, Synthesis, characterization and dielectric properties of polyaniline@Ni0.5Zn0.5Fe2O4composite nanofibers, Mater. Sci. Semicond. Process. 40 (2015) 140–144, https://doi.org/10.1016/j.mssp.2015.06.067.
[38] A. Kunzmann, B. Andersson, C. Vogt, N. Feliu, F. Ye, S. Gabrielsson, M.S. Toprak, T. Buerki-Thurnherr, S. Laurent, M. Vahter, H. Krug, M. Muhammed, A. Scheynius, B. Fadeel, Efficient internalization of silica-coated iron oxide nanoparticles of different sizes by primary human macrophages and dendritic cells, Toxicol. Appl. Pharmacol. 253 (2011) 81–93, https://doi.org/10.1016/j.taap.2011.03.011.
[39] B. Thangaraj, Z. Jia, L. Dai, D. Liu, W. Du, Effect of silica coating on Fe3O4 magnetic nanoparticles for lipase immobilization and their application for biodiesel production, Arab. J. Chem. (2016)https://doi.org/10.1016/j.arabjc.2016.09.004.
[40] E. Aghaei, R. Alorro, A. Encila, K. Yoo, Magnetic adsorbents for the recovery of precious metals from leach solutions and wastewater, Metals (Basel) 7 (2017) 529, https://doi.org/10.3390/met7120529.
[41] R.M. Freire, T.S. Ribeiro, I.F. Vasconcelos, J.C. Denardin, E.B. Barros, G. Mele, L. Carbone, S.E. Mazzetto, P.B.A. Fechine, MZnFe2O4 (M = Ni, Mn) cubic superparamagnetic nanoparticles obtained by hydrothermal synthesis, J. Nanopart. Res. 15 (2013)https://doi.org/10.1007/s11051-013-1616-3.
[42] J. Brandt, L. Andersson, J. Porath, Covalent attachment of proteins to polysaccharide carriers by means of benzoquinone, Biochim. Biophys. Acta 386 (1975) 196–202.
[43] L. Fernandez-Lopez, S.G. Pedrero, N. Lopez-Carrobles, B.C. Gorines, J.J. Virgen-Ortíz, R. Fernandez-Lafuente, Effect of protein load on stability of immobilized enzymes, Enzyme Microb. Technol. 98 (2017) 18–25, https://doi.org/10.1016/j.enzmictec.2016.12.002.
[44] H. Zaak, E.-H. Siar, J.F. Kornecki, L. Fernandez-Lopez, S.G. Pedrero, J.J. Virgen-Ortíz, R. Fernandez-Lafuente, Effect of immobilization rate and enzyme crowding on enzyme stability under different conditions. The case of lipase from *Thermomyces lanuginosus* immobilized on octyl agarose beads, Process Biochem. 56 (2017) 117–123, https://doi.org/10.1016/j.procbio.2017.02.024.
[45] C. Garcia-Galan, O. Barbosa, K. Hernandez, J.C.S. Dos Santos, R.C. Rodrigues, R. Fernandez-Lafuente, Evaluation of styrene-divinylbenzene beads as a support to immobilize lipases, Molecules 19 (2014) 7629–7645, https://doi.org/10.3390/molecules19067629.
[46] J.A. Silva, G.P. Macedo, D.S. Rodrigues, R.L.C. Giordano, L.R.B. Gonçalves, Immobilization of *Candida antarctica* lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies, Biochem. Eng. J. 60 (2012) 16–24, https://doi.org/10.1016/j.bej.2011.09.011.
[47] J.P. Henley, A. Sadana, Categorization of enzyme deactivations using a series-type mechanism, Enzyme Microb. Technol. 7 (1985) 50–60, https://doi.org/10.1016/0141-0229(85)90013-4.
[48] P. Arévalo-Cid, J. Isasi, F. Martín-Hernández, Comparative study of core-shell nanostructures based on amino-functionalized Fe3O4@SiO2and CoFe2O4@SiO2nanocomposites, J. Alloys Compd. 766 (2018) 609–618, https://doi.org/10.1016/j.jallcom.2018.06.246.
[49] A.T. Raghavender, N. Biliškov, Ž. Skoko, XRD and IR analysis of nanocrystalline Ni-Zn ferrite synthesized by the sol-gel method, Mater. Lett. 65 (2011) 677–680, https://doi.org/10.1016/j.matlet.2010.11.071.
[50] R.M. Bezerra, D.M.A. Neto, W.S. Galvão, N.S. Rios, A.C.L.d.M. Carvalho, M.A. Correa, F. Bohn, R. Fernandez-Lafuente, P.B.A. Fechine, M.C. de Mattos, J.C.S. dos Santos, L.R.B. Gonçalves, Design of a lipase-nano particle biocatalysts and its use in the kinetic resolution of medicament precursors, Biochem. Eng. J. 125 (2017) 104–115, https://doi.org/10.1016/j.bej.2017.05.024.
[51] U.M.F. de Oliveira, L.J.B. Lima de Matos, M.C.M. de Souza, B.B. Pinheiro, J.C.S. dos Santos, L.R.B. Gonçalves, Effect of the presence of surfactants and immobilization conditions on catalysts' properties of *Rhizomucor miehei* lipase onto chitosan, Appl. Biochem. Biotechnol. 184 (2018) 1263–1285, https://doi.org/10.1007/s12010-017-2622-1.
[52] G. Fernández-Lorente, J.M. Palomo, C. Mateo, R. Munilla, C. Ortiz, Z. Cabrera, J.M. Guisán, R. Fernández-Lafuente, Glutaraldehyde cross-linking of lipases adsorbed on aminated supports in the presence of detergents leads to improved performance, Biomacromolecules (7) (2006) 2610–2615, https://doi.org/10.1021/bm060408+.
[53] K. Holmberg, Interactions between surfactants and hydrolytic enzymes, Colloids Surfaces B Biointerfaces 168 (2018) 169–177, https://doi.org/10.1016/j.colsurfb.2017.12.002.
[54] J.M. Palomo, M. Fuentes, G. Fernández-Lorente, C. Mateo, J.M. Guisan, R. Fernández-Lafuente, General trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the enzyme functionality, Biomacromolecules 4 (2003) 1–6, https://doi.org/10.1021/bm025729+.
[55] L.N. De Lima, C.C. Aragon, C. Mateo, J.M. Palomo, R.L.C. Giordano, P.W. Tardioli, J.M. Guisan, G. Fernandez-Lorente, Immobilization and stabilization of a bimolecular aggregate of the lipase from *Pseudomonas fluorescens* by multipoint covalent attachment, Process Biochem. 48 (2013) 118–123, https://doi.org/10.1016/j.procbio.2012.11.008.
[56] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (2007) 1451–1463, https://doi.org/10.1016/j.enzmictec.2007.01.018.
[57] C. Mateo, V. Grazú, B.C.C. Pessela, T. Montes, J.M. Palomo, R. Torres, F. López-Gallego, R. Fernández-Lafuente, J.M. Guisán, Advances in the design of new epoxy supports for enzyme immobilization-stabilization, Biochem. Soc. Trans. 35 (2007) 1593–1601, https://doi.org/10.1042/BST0351593.
[58] E.A. Manoel, J.C.S. dos Santos, D.M.G. Freire, N. Rueda, R. Fernandez-Lafuente, Immobilization of lipases on hydrophobic supports involves the open form of the enzyme, Enzyme Microb. Technol. 71 (2015) 53–57, https://doi.org/10.1016/j.enzmictec.2015.02.001.
[59] O. Barbosa, R. Torres, C. Ortiz, R. Fernandez-Lafuente, Versatility of glutaraldehyde to immobilize lipases: effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*, Process Biochem. 47 (2012) 1220–1227, https://doi.org/10.1016/j.procbio.2012.04.019.
[60] A.L. Pham, D.L. Sedlak, F.M. Doyle, Dissolution of mesoporous silica supports in aqueous solutions: implications for mesoporous silica-based water treatment processes, Applied Catal. B, Environ. 126 (2012) 258–264, https://doi.org/10.1016/j.apcatb.2012.07.018.
[61] Y. Niibori, M. Kunita, O. Tochiyama, T. Chida, Y. Niibori, M. Kunita, O. Tochiyama, T. Chida, Dissolution rates of amorphous silica in highly alkaline solution dissolution rates of amorphous silica in highly alkaline solution, J. Nucl. Sci. Technol. 37 (2012) 349–357, https://doi.org/10.1080/18811248.2000.9714905.