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REVIEW ARTICLE

Support engineering: relation between development of new supports for immobilization of lipases and their applications

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Abstract The growing interest in processes with the use of immobilized lipases guides to the development of new supports. In that way, the design and characterization of new supports for lipase immobilization have been increasingly popular in literature. Efforts to obtain ''the perfect support'' (a not accomplished yet) are described in this paper. Obviously, the choice and development of a support is directly related to the process in which it will be used, considering different factors as the media where the immobilzed enzyme will be used (whether aqueous, free or with solvents), potency of agitation, reactor configuration or substrates/products that will be involved. The present work discusses the use of some techniques of support synthesis in the case of core-shell particles, such as: miniemulsion, microemulsion, suspension, dispersion, the use of heterofunctional supports, whole-cell and processes of coimobilization. Some analytical tools for the investigation of enzyme immobilization are also presented, such as fourier transform infrared spectroscopy, as well as support characteristics that may be relevant for its final performance (e.g., specific surface area, particle diameter and particle size distribution and confocal laser scanning microscope).

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Introduction

The increasing and notable interest in enzyme immobilization is based on already widely discussed and proven factors, such as the possibility of improving the stability of the biocatalyst under different environmental conditions, improving mass transfer (case of the use of nanoparticles) and reuse in reactions of interest (Cipolatti, Silva, et al., 2014; Fernandez-Lafuente, Armisén, Sabuquillo, Fernández-Lorente, & Guisán, 1998; Guisan & Blanco, 1987; Rodrigues et al., 2010). A search made in a scientific research platform about enzyme immobilization publications shows the growing interest in the area, that is reflected through the increasing number of studies conducted and published in international journals on this matter (Fig. 1). The graph shows the number of publications since 1974, with a notable increase from 2004. The plotted data indicate that this area is still rising.

In some studies about enzyme immobilization, the focus is in the development of the support. The supports may be rigid or flexible, porous or non porous, macroporous particles, nanoparticles or membranes, among others (Cipolatti et al., 2016; Gumí, Paolucci-Jeanjean, Belleville, & Rios, 2007; Huckel, Wirth, & Hearn, 1996; Sato, Kawakami, & Tokuyama, 2014; Zang et al., 2014).

Some commercial supports can be too expensive for large-scale application, or did not offer the improvement in enzyme properties demanded for a immobilized biocatalyst (Barbosa et al., 2015; Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007; Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013) and this is one of the main reasons why researchers are looking for cheaper and more efficient alternatives. There is also an interest in the new, for discovering new materials, nanocomposites, which provide an efficient immobilization, but also help in the better understanding of the enzyme-support interactions and the ability of the materials to act as supports.

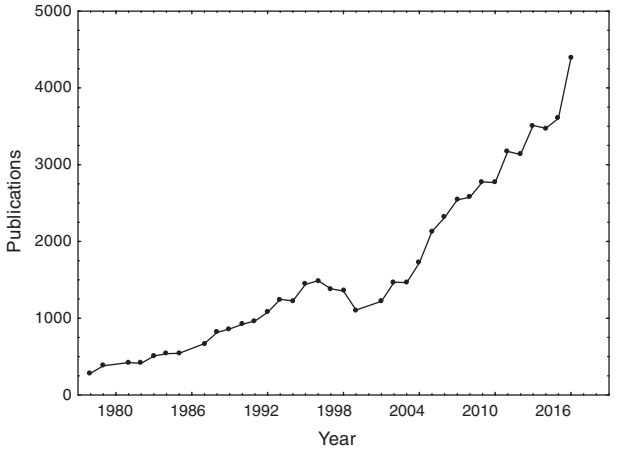

Figure 1 Publications on enzyme immobilization over the years (ScienceDirect, accessed in November, 2016). Keywords: enzyme, immobilization.

Methodologies for the development of supports

Miniemulsion

The miniemulsion technique for the synthesis of supports for enzyme immobilization is still scarcely used; few papers are cited in the literature using this technique (Cipolatti et al., 2014, 2015; Fritzen-Garcia et al., 2013; Valério et al., 2015). Fig. 2 shows an example, CALB enzyme immobilization in poly(methyl methacrylate) (PMMA) nanoparticles obtained by miniemulsion polymerization.

Considering an easy manipulation of the conditions and monomers used in the miniemulsion process, besides the low cost of the polymers, this may be a promising method for the synthesis of supports for immobilization of enzymes. Miniemulsion is classically defined as a relatively stable aqueous dispersion of oil droplets within the 50–500 nm size range prepared by a system containing oil, water, surfactant and a ''cosurfactant'' agent (Landfester, Bechthold, Tiarks, & Antonietti, 1999). The miniemulsion polymerization aims to initiate polymerization when the droplets are already stable to avoid secondary nucleation and minimizing the mass transport (Antonietti & Landfester, 2002). This technique allows drugs, oils or other substances can be incorporated into drops, maintaining its characteristics from the dispersion to obtain the nanoparticles (Landfester, 2009; Valério, Araújo, & Sayer, 2013a; Valério, da Rocha, Araújo, & Sayer, 2014). Typically, the preparation of nanoparticles in miniemulsion systems includes three stages: pre-emulsion of two heterogeneous phases to prepare (macro)emulsions, homogenization of gross emulsions for the miniemulsions and reaction to yield nanoparticles (Qi, Cao, & Ziener, 2014). The nanoparticles can be formed with the use of high pressure homogenizer or ultrasound. This method can be used for encapsulating materials in polymeric nanoparticles (Landfester, 2009).

Furthermore, the miniemulsion has the advantage that the final product can be obtained in one reaction step. The synthesis of PU (polyurethane) nanoparticles in one step consists in to add the monomers (diisocyanate and polyols), chain extender and other reaction components to the reactor simultaneously to form the final product (Cipolatti et al., 2014; Valério, Araújo, & Sayer, 2013b). The miniemulsions can still be classified as direct or reverse, depending on the polarity of the dispersed and continuous phases. In direct miniemulsion, the polarity of the continuous phase is greater than in the dispersed phase, whereas in the inverse miniemulsion the polarity of the continuous phase is lower than in the dispersed phase. In direct miniemulsion, an aqueous solution of surfactant is commonly used as a continuous phase. In reverse miniemulsions, a hydrophobic surfactant solution is used as a continuous phase. Most commonly hydrophobic solvents used are cyclohexane, toluene, hexadecane and isopar M (a hydrocarbons mixture of C12–C14). Systems with direct miniemulsions are used to prepare hydrophobic nanoparticles, whereas the inverse produces hydrophilic particles (Qi et al., 2014).

The polymerization in miniemulsion also has advantages such as the non-excessive use of surfactant, sufficient colloidal stability and incorporation of hydrophobic

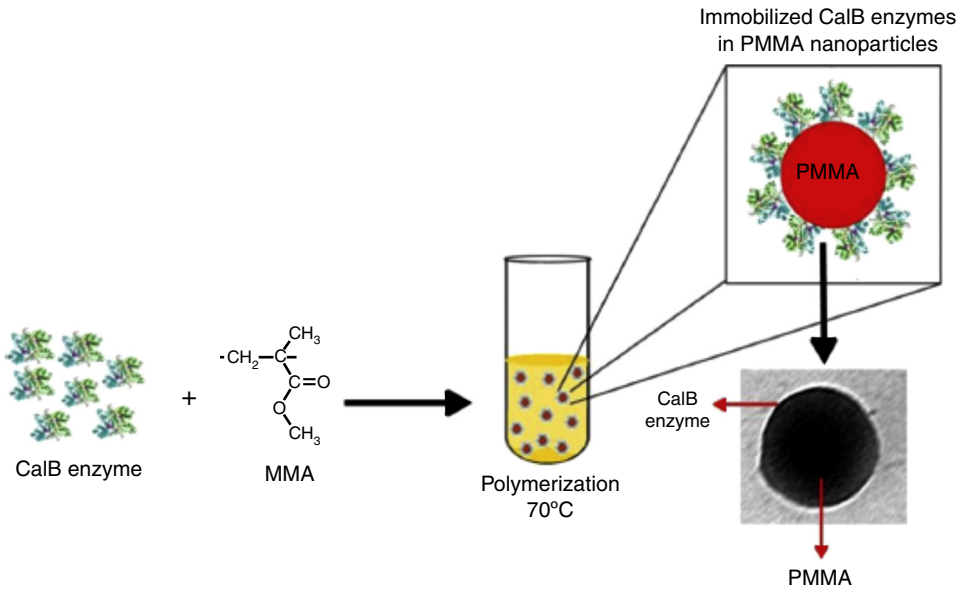

Figure 2 Schematic representation of immobilization of CalB enzyme in PMMA particles using miniemulsion process (Valério et al., 2015). This figure has been reproduced from Ref.: Valério et al. (2015) with permission from Pan Stanford Publishing.

compounds, being still considered a low cost technology (Landfester et al., 1999; Romio, Bernardy, Lemos Senna, Araújo, & Sayer, 2009). Processes that use emulsions for the preparation of core-shell polymers are more easily found (Cunha et al., 2014a), such as that performed by JENJOB et al. (Jenjob, Sunintaboon, Inprakhon, Anantachoke, & Reutrakul, 2012). The authors prepared an emulsion from water and MMA in reactor at 80 °C with nitrogen, then KPS was added and the polymerization process was conducted at the constant temperature for 3 h. Chitosan was used to cover PMMA, forming a core-shell structure, which was used in the immobilization of *Candida rugosa* lipase.

Core–shell supports

The core–shell supports can be synthetized since different polymerization techniquesas heterocoagulation, suspension, emulsion, miniemulsion, dispersion and combinations of one or more of them (Antonietti & Landfester, 2002; Cunha et al., 2014b; Debnath & Khatua, 2011; Ferguson, Russell, & Gilbert, 2002; Lenzi, Lima, & Pinto, 2004; Okubo & Lu, 1996; Waters, 1997; Zang et al., 2014). However the use of emulsion-suspension simultaneously technique has been used efficiently in the immobilization of lipases for different applications, as on the resolution of pharmaceutical derivatives (Cunha et al., 2014a, 2014b; Besteti et al., 2014; Pinto, Freire, & Pinto, 2014; Manoel et al., 2016).

Basically, the particle having a rigid core surrounded by a porous matrix, which can or cannot be of the same core material, is known as core–shell particle. In recent works, the core was formed by polymeric particles nucleated by the suspension of polymerization process, and the shell was formed by polymeric particles nucleated by the emulsion of the polymerization process and coagulated over much bigger suspension particles (Cunha et al., 2014a, 2014b; Lenzi et al., 2004; Pinto et al., 2014).

Although relatively few investigations explains the synthesis of supports with this morphology, specially used for enzymes immobilization, since great results reported by these authors suggest the importance of this type of support for this purpose. The use of polymeric core–shell particles is still little reported.

The use of rigid, inert and inexpensive supports is of great value and interest for a possible application in immobilized lipases in industrial reaction scale. As the main advantage of immobilizing an enzyme, is in enabling its reuse, and maintain a greater stability through different environmental conditions (Cipolatti, Silva, et al., 2014; Fernandez-Lafuente et al., 1998; Mateo et al., 2007; Suescun et al., 2015). Other polymers can be used, as polymethylmethacrylate (PMMA). PMMA has biotechnological and biomedical applications due to its biocompatible character and strength, as well as its low cost, which makes this interesting to use it in the immobilization of lipases (Cerqueira, Santos, Matos, Gutz, & Angnes, 2015; Li, Hu, & Liu, 2004; Valério et al., 2015). Also, PMMA has great commercial importance because of its good transparency optics and high impact resistance. The presence of the methyl group on the α carbon which gives it a greater thermal stability, hardness and stiffness compared to other polyacrylates (Pérez, López-Cabarcos, & López-Ruiz, 2006).

Polystyrene is also used as support for immobilization due to its ideal mechanical strength, adjustable particle size, and favorable chemical stability (Hou et al., 2014; Li et al., 2010). Divinylbenzene is widely used in copolymerization process, Aybastıer and Demir (Aybastıer & Demir, 2010) studied that the styrene–divinylbenzene linking has a peculiar physicochemical and hydrophobic characteristic. For that, it has a good potential to be used as support material for lipase immobilization. Additionally, is a cheap hydrophobic matrix, greatly used in chromatographic processes (Hernandez, Garcia-Galan, & Fernandez-Lafuente, 2011).

Table 1 Use of core–shell particles in the immobilization of lipases.

Lipase/origin	Core	Shell	Application	Reference
Thermomyces lanuginosa	Fe_3O_4	ZnO	Michael addition of active methylene compounds to chalcones	Ghasemi et al. (2014)
Candida rugosa	Fe_3O_4 nanoparticles	Polydopamine	–	Hou, Qi, and Zhu, 2015
Candida rugosa	Fe_3O_4 nanoparticles	MCM-41 silica	Interesterification of soybean oil and lard	Xie and Zang, 2016
Thermomyces lanuginosus (TLL)	Fe_3O_4	Silica	Biodiesel production	
Rhizomucor miehei (RML), *Thermomyces lanuginosus* (TLL), *Candida antarctica* (CALB), Lecitase Ultra (LU)	PS-co-DVB, PS*	PS-co-DVB, PS	Hydrolysis of methyl mandelate, hydrolysis of triacetin.	Manoel et al. (2016)
Burkholderia cepacia	Fe_3O_4 nanoparticles	Chitosan	Biodiesel production	Ghadi et al. (2015)
Lipase B from *Candida antarctica*	PS PS PMMA	PMMA *PS-co-PC PS	Hydrolysis of *p*-Phenil laurate	Besteti et al. (2014)
Lipase B from *Candida antarctica*	PS-co-DVB	PS-co-DVB	Alcoholysis reactions of Pharmaceutical compounds	Cunha et al. (2014a, 2014b)
Lipase B from *Candida antarctica* express *Pichia pastoris*	PS-co-DVB PMMA PMMA-co-DVB	PS-co-DVB PMMA PMMA-co-DVB	Alcoholysis reactions of Pharmaceutical compounds	Manoel et al. (2016)
Lipase B from *Candida antarctica*	Fe_3O_4	Flower-like organosilica	Esterification levulinic acid and alcohols	Gao et al. (2017)

* PS, poly(styrene; PS-co-DVB, poly(styrene-co-divinylbenzene); PS-co-PC, poly(styrene-co-cardanol).

The use of efficient homemade supports, synthesized in a simple and inexpensive way, appears as a viable alternative by making the support readily available for immobilization of several enzymes. Table 1 shows an overview of the use of core–shell particles in the immobilization of lipases.

Heterofunctional

Heterofunctional supports are a trend in the immobilization of lipases, the idea is to use the ability of these enzymes to activate interfacially on hydrophobic surfaces. This immobilization process permitted that the lipase express a greater activity than the soluble enzyme (Fernandez-Lafuente et al., 1998; Manoel, Dos Santos, Freire, Rueda, & Fernandez-Lafuente, 2015; Verger, 1997). The enzyme adsorbed in its open conformation is more stable than the free enzyme and more stable also compared to many derivatives immobilized lipaseby covalent multipoint binding (Mateo et al., 2007).

After this activation, the enzyme is strongly attached to the support by the covalent bond. This way of immobilization involves the chemical modification of an amino acid residue by forming the covalent bond between the biocatalyst and the support, or by cross-linking with the matrix, and several bifunctional agents may be used (Dalla-Vecchia, Nascimento, & Soldi, 2004). If a multi-point covalent union is performed, a strong enzyme-support interaction is possible, conferring greater stability to the derivative obtained (Filho et al., 2008; Rodrigues et al., 2009).

Bernal and coauthors (Bernal, Illanes, & Wilson, 2014) developed a hydrophilic–hydrophobic porous silica for immobilization of lipases from *Pseudomonas stutzeri* and *Alcaligenes sp.* They studied different chemical surfaces with octyl and glyoxyl groups. According to the authors, the derivative exhibited the best behavior as support for lipases immobilization.

The multifunctionality of the supports is a direct consequence of the way they are prepared, and this is the case for glutaraldehyde activated supports. Activation of a support with glutaraldehyde will depend on the pH, time and concentration of the compound, may give three different kinds of interactions with an enzyme: hydrophobic, anionic exchange, and covalent (Barbosa et al., 2013)

Whole-cell

The yeast surface display (YSD) is a strategy that allows the produced enzyme is attached to the outer side of yeast cell wall which acts as a support. The excreted enzyme is attached to the cell by a protein called anchor. The main advantage of using this technique is to obtain an enzyme

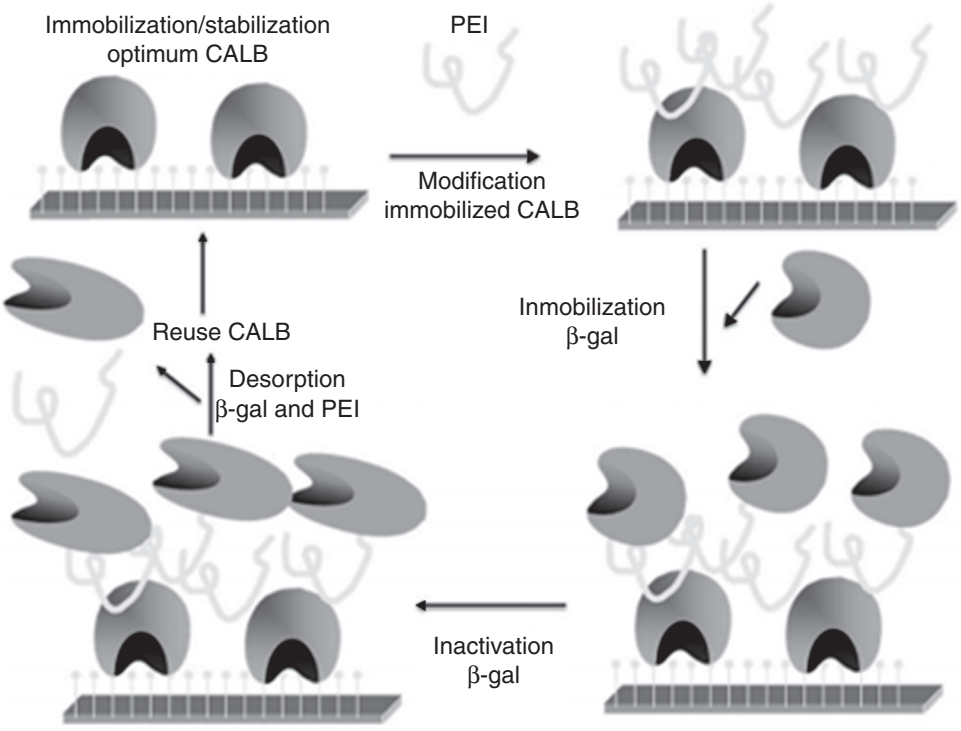

Figure 3 Strategy of coimmobilization of β-gal and CALB on octyl-agarose (Peirce et al., 2016). Reproduced from Ref. Peirce et al. (2016) with permission from The Royal Society of Chemistry. License number: 3984040607683.

immobilized in only one step, with possibility of reuse and improvements in stability (Moura et al., 2015), once free cells in the reaction medium are difficult to reuse.

This type of biocatalyst must have a lower cost of production since it eliminates downstream steps such as enzyme purification. Lipases immobilized on whole cells have been studied for the production of biodiesel (Du, Li, Sun, Chen, & Liu, 2008). Therefore, Ban et al. (Ban, Kaieda, Matsumoto, Kondo, & Fukuda, 2001) immobilized whole cells of *Rhizopus oryzae* on a cross-linked polyurethane backing. The immobilization of the fungal biomass on the support occurred spontaneously during fermentation. A high conversion rate, 90%, was achieved using immobilized cells, with methanol fed batch and 15% of water in the reaction medium. In a later work, Ban et al. (Ban et al., 2002) demonstrated that this same biocatalyst treated with glutaraldehyde solution (crosslinks) had the increased stability of intracellular lipase. Ying and Chen (Ying & Chen, 2007) studied whole cells of *Bacillus subtilis* encapsulated within the network of hydrophobic support with magnetic particles and obtained conversion of about 90% in 72 h of reaction without solvent. This biocatalyst was easily recovered using magnetic separation.

Lipase B from *Candida antarctica* (LipB) was immobilized on the cell surface of the methylotrophic yeast *Pichia pastoris* using the YSD approach. The authors were tested two anchors: Flo9, was identified after a prospection of the *P. pastoris* genome being related to the family of flocculins similar to Flo1 but significantly smaller, and Pir1, protein with internal repeats from *P. pastoris*. Both constructions showed hydrolytic activity toward tributyrin (>100 U/mg_{dcw} and >80 U/mg_{dcw}, respectively), optimal hydrolytic activity around 45 °C and pH 7.0. The biocatalysts were able to maintain more than 80% of its stability after 3 h incubation at 40 °C also showing stability in organic solvents (Moura et al., 2015).

Coimmobilization

The immobilization of more than one enzyme in the same support is appearing as a trend, there are many works that can be found in the literature with the use of different enzymes, whether they are from the same class of enzyme or not. However, we have to be careful when working with this type of catalyst. First, it must be considered whether it is worthy to built this support. Cases where coimmobilization is interesting, and sometimes necessary, include when the product released by enzyme 1 is unstable and serves as a substrate for enzyme 2, thus have a reduction in induction time of production of product 2. The coimmobilization is also interesting if this product 1 can inactivate the enzyme.

In a recent work, was done the coimmobilization of b-galactosidase from *Aspergillus oryzae* (β-gal) and lipase B from *C. antarctica* (CALB). CALB was immobilized on octyl-agarose, and after β-gal was immobilized by ion exchange on the PEI (polyethyleneimine) coated support, like shows the scheme (Fig. 3) developed by authors (Peirce et al., 2016). In this work, the derivative with the two enzymes was compared to the individual ones. The authors affirmed that the requirement for this strategy is that the

immobilization of the first enzyme is cannot only based on ion exchange, otherwise we can desorb the enzyme when desorbing the other enzyme.

Due to the complexity of coimmobilization process and the future application, this type of immobilization is still little explored. Additionally, this process has a number of limitations, since the general stability of the biocatalyst is conditioned to that of the less stable enzyme (Peirce et al., 2016).

Alternatives analytical tools for investigation of enzyme immobilization

Although the classical analytical tools for investigation of enzyme immobilization is still widely used, others techniques allow to understanding the behavior of the structure of proteins, inside or outside the porous of a support, it can also be mentioned.

Fourier transform infrared spectroscopy (FTIR) is a classical method used to determine small molecules, and the ability to obtain many information from biological systems using this technique allows it to be applied to analyze of proteins (Barth, 2007). It is a powerful technique for the determination of the secondary structure of proteins in solution. Quantitative information on the secondary-structure elements of the protein can be obtained by the analysis of the amide I absorption in the 1700–1600 cm^{-1} region (Natalello, Ami, Brocca, Lotti, & Doglia, 2005).

Through this technique is possible to estimate, from simple analyzes to verify if the enzyme is present in the support to more complex analyzes, like as quantification and more refined interactions proteins-carrier (Cipolatti et al., 2014; Foresti, Valle, Bonetto, Ferreira, & Briand, 2010). Therefore, the use of FTIR can be a tool in the immobilization of enzymes. As mentioned, some authors use these spectra to confirm and estimate enzyme immobilization in synthetized supports (Cipolatti et al., 2014; Nicoletti et al., 2015). In Fig. 4, the FTIR spectra confirmed the urethane formation through the absorption band with peak location between 1680 and 1650 cm^{-1} for urea and NH absorption band between 1740 and 1700 cm^{-1} due to stretching vibration of C=O group.

Foresti and coauthors (Foresti et al., 2010) studied *C. antarctica* lipase B, where was immobilized onto titanium dioxide (TiO_2) in a buffer-free, bidistilled aqueous medium. In this interesting work, the authors obtained quantitative information on the relative contribution of the structural elements that constitute the secondary structure of lipase (α-helices, β-sheets, turns and unordered structures) during the immobilization course. A lot of papers with immobilization of enzymes use this technique; and the tendency is that more and more researchers will use and explore it.

Commonly characterization techniques are also used as a way of choosing the future application. One of the factors to consider when immobilizing an enzyme is its load capacity, which is related to its surface area. The specific surface area is a technique used for support characterization and can be done through the method of BET (Brunauer, Emmett and Teller) (Khoobi et al., 2014). Other characterization techniques are also used, such as: particle diameter and particle size distribution and Confocal Laser Scanning Microscope (LSCM).

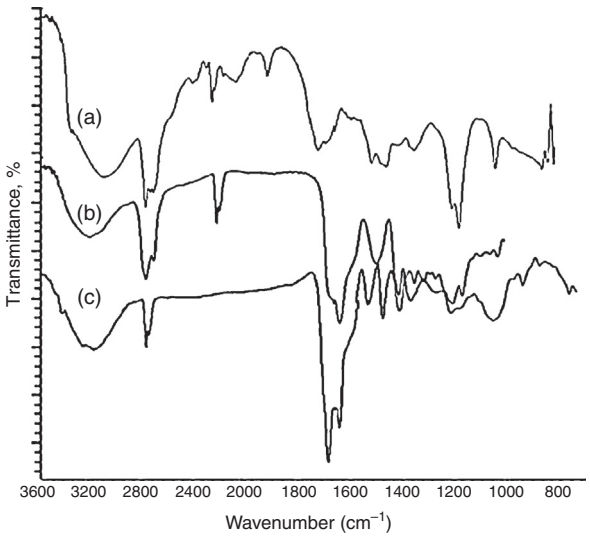

Figure 4 Fourier transform infrared spectra (FTIR) of PEG–PUU nanoparticle synthesized by step miniemulsion polymerization. (a) Free enzyme, (b) immobilized CalB PEGylated poly(urea–urethane) nanoparticle, and (c) PEGylatedpoly(urea–urethane) nanoparticle (Cipolatti et al., 2014). This figure has been reproduced from Ref. Cipolatti et al. (2014) with permission from Pan Stanford Publishing.

Conclusions

The lipases properly immobilized may improve the results obtained during the catalytic reactions. Additionally, the possibility of reuse is directly related to the cost of the final product. The possibility of using an enzyme for several cycles can significantly reduce process costs. Interesting processes with the use of immobilized lipases can be cited: biodiesel (Aguieiras et al., 2014, 2015; de Sousa et al., 2010), Resolution of enantiomers (Machado et al., 2011; Manoel et al., 2012), biolubricants, biosurfactants (Damasceno et al., 2012), production of fatty acid esters (Cipolatti et al., 2015). The efficiency of the cited processes can be significantly improved with the use of adequate immobilized lipases.

Immobilized enzymes, either in laboratory or industrial scale, are the most efficient way to use these biocatalysts, so due to the great importance of this area, it is natural and understandable the researchers' efforts in the development of new supports and new immobilization techniques. However, one should always keep in mind in what reaction system the immobilized enzyme will be used and the costs of the process.

For the development of efficient immobilization protocols, the use of suitable chemical surfaces, which promote good enzyme-substrate interaction, increase of stability, recovery and reuse of the catalyst can be considered (Bernal et al., 2014; Fernandez-Lorente, Palomo, Guisan, & Fernandez-Lafuente, 2007; Wilson et al., 2006). An option to obtain efficient immobilized lipases are the heterofunctional supports, which use hydrophobic adsorption to open the lid, and covalent to the stabilization (Barbosa et al., 2013; Bernal et al., 2014).

Conflicts of interest

The authors declare no conflicts of interest.

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