Bioprocess and Biosystems Engineering (2019) 42:1625–1634
https://doi.org/10.1007/s00449-019-02159-w

RESEARCH PAPER

Benzyl propionate synthesis by fed-batch esterification using commercial immobilized and lyophilized Cal B lipase

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Received: 21 November 2018 / Accepted: 6 June 2019 / Published online: 2 July 2019

Abstract
In this work, a fed-batch approach was adopted to overcome propionic acid lipase inactivation effects in the benzyl propionate direct esterification mediated by lipases. The ester synthesis was performed using commercial immobilized (Novozym 435) and lyophilized form *Candida antarctica* fraction B lipase (Cal B) as biocatalysts of the esterification between benzyl alcohol and propionic acid in a solvent-free system. The reaction involved the propionic acid-controlled addition during the first 5 h ensuring an excess of alcohol to dilute the media. The biocatalyst Novozym 435 showed a good performance in the first cycle of the fed-batch esterification, ensuring 90 and 99% of conversion at substrates molar ratio of 1:1 and 1:5 (acid:alcohol), respectively. However, the enzyme lost the activity and the conversions were sharply reduced at the second cycle. A novel qualitative protein content analysis by optical microscopy showed that the lipase was desorbed from the support after the esterification, and this behavior was strongly related to the presence of propionic acid in the reaction medium. The lyophilized Cal B was also tested as biocatalyst of the benzyl propionate esterification and showed a similar performance (related to the Novozym 435) in ester conversion and initial reaction rates for all substrates molar ratios tested. Since the substrates affected the performance of the Novozym 435, the lyophilized Cal B is the most suitable catalyst to the benzyl propionate esterification with conversions above 90%, considering a the fed-batch approach in a solvent-free system.

Keywords Acid inactivation · Fed-batch esterification · Lipase · Protein content · Enzyme desorption

Introduction

The natural synthesis of aromatic esters is a very interesting area for the pharmaceutical and food industries [1, 2]. Among the catalysts that may be used in the production, lipases play an important role since the aroma esters produced by microbial or enzymatic methods may be labeled as natural in accordance with the United States and European legislations, thereby satisfying the consumer trend towards natural products in various industries [1, 3–5]. Lipases are an important group in the organic chemistry, biodegradable and responsible to catalyze many organic reactions, operating in mild conditions of pH and temperature in homogeneous or heterogeneous form, and have showing to be promising biocatalysts in the aromatic ester synthesis with potential industrial applications [1, 6–10].

The enzyme-catalyzed systems can be carried out as an equilibrium-controlled process (thermodynamically controlled) or as a kinetically controlled process [11]. In equilibrium-controlled processes, the enzyme only accelerates the rate with which the equilibrium is obtained (e.g., hydrolysis or esterification). In kinetically controlled processes, an activated acyl donor is necessary and the enzyme acts as a transferase. The maximum and transient yields that are over the thermodynamics of the process came from the properties of the catalyst (hydrolysis of activated acyl donor and of the product, versus rate of product production) [11, 12].

Generally, in controlled acyl-transfer esterifications that are diluted in aqueous media, the equilibrium of reaction is largely favored to hydrolysis, while, in nonpolar organic media with low water amount the enzyme remains active and the reaction equilibrium is shifted in favor of synthesis

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[12–14]. Commonly, organic solvents are used to favor esterification reaction over hydrolysis [13–20]; however, many works have been reporting solvent-free systems (SFS) for ester synthesis and molecular sieves showed to be a suitable water absorbent, shifting the reaction equilibrium to synthesis [21–25].

The benzyl propionate is an aromatic ester that possesses a fruit smell and occurs naturally in plums (*prunus* species), passion fruits (*passiflora* species), and melons, and is broadly used as a fragrance ingredient in many decorative cosmetics, fine fragrances, toiletries, and non-cosmetic products such as household cleaners and detergents [26, 27]. In addition, the benzyl propionate is a safe ingredient included by Council of Europe in the list of substances granted A that may be used in foodstuff and considered generally recognized as safe (GRAS) by Food and Drug Administration (FDA) and by Flavor and Extract Manufacturers Association (FEMA) [27].

The natural synthesis of the benzyl propionate is still a poorly explored field and there are only two reports in the literature for its lipase-mediated synthesis. In the first study, an investigation of the benzyl propionate synthesis was performed using three different propanoyl donors (propionic acid, vinyl propionate, and methyl propionate), and a *Pseudomonas cepacia* lipase immobilized in eco-friendly support as biocatalyst [28]. The authors concluded that the low conversions found for the acid (8%) and methyl propionate (17%) were associated to inhibitory effects, and only the transesterification with vinyl propionate was able to reach high conversions (99%). The second and more recent work, investigated the ester synthesis by batch esterification between benzyl alcohol and propionic acid using the Novozym 435 as biocatalyst, but the maximum conversion found was 44% with a molar ratio of 1:1 considering a SFS [29]. The benzyl propionate esterification had shown, until the present moment, low ester conversions, and this behavior has been associated to the use of the short-chain acids as esterification substrate, which leads to enzyme limitations as inhibition and inactivation events [2, 16, 18, 30–33].

The benzyl propionate esterification mediated by lipases is still a challenge as shown in the previous works [28, 29], and more studies are necessary to feasible the esterification reaction that still is the most simple and viable reaction to produce aromatic esters targeting future industrial applications. To overcome these drawbacks related to the use of the short chain acids (like propionic acid) as acyl donors, the present work adopted a fed-batch approach to the benzyl propionate esterification, considering a controlled fed of the acid during the initial hours of reaction.

Here, a maximized condition from previous work [29] was used and adapted to the fed-batch strategy using Novozym 435 (the commercial immobilized prepared of the *Candida antarctica* fraction B lipase—Cal B) and the lyophilized Cal B, as biocatalysts in a SFS using molecular sieves to absorb water. The fed-batch esterification using both biocatalysts was tested in different molar ratios of propionic acid and benzyl alcohol, and the immobilized enzyme was tested in the recycle. The initial reaction rate was also determined, and some characterization around the enzyme activity and protein content of the immobilized enzyme before and after the reaction cycles was conducted to determine the best biocatalyst and to make viable the benzyl propionate synthesis via esterification.

Materials and methods

Enzymes and chemicals

The esterification substrates were propionic acid (Neon) and benzyl alcohol (Neon). The biocatalysts Novozym 435, a commercial immobilized *Candida antarctica* fraction B lipase (Cal B), and Sorbitol solution of free Cal B (Novozymes NZL-102, CALB) were kindly donated by Novozymes®. Molecular sieves (4 Å, beads 8–12 mesh, Sigma-Aldrich) were used as water adsorbent. The solvents acetone (Quimis) and dichloromethane (Quimis) were employed for immobilized lipase wash in recycle and gas chromatography quantification, respectively.

Benzyl propionate quantification

The benzyl propionate obtained after acid and alcohol esterification was analyzed by a calibration curve previously prepared using the ester standard. The analyses were carried out in a gas chromatograph (Shimadzu GC 2010) with auto-injector coupled (Shimadzu AOC 5000), equipped with a DB-5 column (27 m length × 0.25 mm internal diameter × 0.25 µm film thickness). The samples were diluted in dichloromethane and injected into the column with an initial temperature of 100 °C for 2 min, then the temperature was raised from 100 to 230 °C with a rate of 10 °C/min and kept for 10 min; injector and detector were kept at 250 °C [29].

Benzyl propionate fed-batch synthesis using immobilized lipase

Initially, a study of the benzyl propionate fed-batch esterification was made at the same conditions of temperature, enzyme type and amount, and acid:alcohol molar ratio (MR) described in a previous work that maximized the ester synthesis in batch mode [29]. The fed-batch reaction involved the addition of 35 mmol of benzyl alcohol in a glass-jacketed reactor previously heated at 50 °C, containing molecular sieves and 5, 10 or 15 wt% (related to the amount of substrates) of Novozym 435. The propionic acid was added to

the medium during the first 5 h, totalizing 35 mmol after six additions (5.83 mmol/h). The reaction was magnetically stirred at 150 rpm during all reaction time, and aliquots were taken in 0, 1, 2, 3, 4, 5, 6, 8, 10 and 24 h of reaction and adequate diluted for quantification. All experiments were carried out in duplicate.

Effect of substrates molar ratio in the immobilized enzyme reuse

The Novozym 435 performance as biocatalyst in the fed-batch esterification of the benzyl propionate was evaluated using different substrates molar ratios. The esterification reaction involved the addition of the adequate amount of benzyl alcohol, molecular sieves and 10 wt% of Novozym 435 in a glass-jacketed reactor pre-heated (50 °C). The propionic acid was added to the medium during the first 5 h with a fed rate of 5.83, 2.91 and 1.83 mmol/h, ensuring the adequate final MR of 1:1, 1:3 and 1:5 (acid:alcohol), respectively. The reaction was magnetically stirred at 150 rpm during all reaction time, and aliquots were taken in 0, 1, 2, 3, 4, 5, 6, 8, 10 and 24 h of reaction and adequate diluted for quantification. All experiments were carried out in duplicate.

The enzyme reuse study involved the use of the same immobilized enzyme (Novozym 435) over a new reaction cycle using the same reaction parameters. After the first cycle, the enzyme was recuperated from de medium by vacuum filtration using a glass funnel and filter paper. The molecular sieves beads were collected, and the enzyme was washed with acetone (m/v ratio of 1:10) three times to remove the product or substrate residues. In sequence, the immobilized enzyme was dried overnight (50 °C) and reused in a new fresh reaction fed-batch.

Benzyl propionate fed-batch synthesis using lyophilized Cal B lipase

The enzymatic liquid containing the free Cal B lipase was concentrated using the cellulose membrane method described elsewhere [34] and then, the enzyme was recuperated with lyophilization using a LIOTOP Lyophilizer, model L101. The lyophilized Cal B was stored under refrigeration (4 °C) for further analysis.

The benzyl propionate fed-batch esterification using the 50 mg of lyophilized Cal B lipase as biocatalyst involved the use of the same reaction conditions and the procedure described above. The quantity of the lyophilized lipase used for these esterification reactions was based on the amount of Cal B physically immobilized onto the macroporous acrylic polymer resin Lewatit VP OC 1600 from Novozym 435 [10], which remains around 8% [35]. The amount of lyophilized Cal B was 8 wt% related to the total amount of the supported Novozym 435 used in the previous fed-batch reactions.

Initial reaction rate

The initial reaction rate (r) was calculated as follows:

$$r = \left(\frac{dC_{\text{ester}}}{dt}\right)_{t=0}, \quad (1)$$

where r is the initial rate of reaction (h^{-1}); C_{ester} is the ester conversion (wt%) at time t; and t is the reaction time (h) [36].

Immobilized lipase activity

The enzyme activity (U/g) was determined by the lauric acid and *n*-propanol esterification technique described elsewhere with minor modification [37]. The esterification reaction was conducted in a substrate MR of 1:1, enzyme amount of 5 wt% (related to the substrates) during 40 min at 60 °C. The remaining lauric acid content was determined by titration with NaOH 0.01 N. A unit of activity (U) was defined as the amount of enzyme necessary to consume 1 µmol of lauric acid per minute. All enzymatic activity determinations were carried out in triplicate.

Qualitative protein content determination by optical microscopy

The qualitative analysis of the protein immobilized in the Novozym 435 support, and the content of lipase that remained on the support after the fed-batch cycle was realized using optical microscopy. The samples were prepared to analysis in glass microscope slides, the immobilized enzymes were marked using lugol iodine solution, the excess of solution was removed, and immersion oil was dropped directly under the Novozym 435 support beads. In sequence, the samples were taken to analysis in an Olympus BX 41 microscope using bright-field illumination with a magnification of four and ten times and the images were recorded using a digital camera Q-imaging (3.3 mpixel).

Results and discussion

Fed-batch esterification of benzyl propionate ester using Novozym 435 as biocatalyst

The benzyl propionate natural production via lipase-mediated esterification is still a challenge due to the propionic acid short chain characteristics that generally implies in enzyme inhibitory/inactivation effects, as already reported to others short chain acids [2, 16, 18, 30, 31, 33]. Researchers had reported that the main reason for acid inhibition is

associated with the fact that acids may cause acidification of the microaqueous interface leading to enzyme inactivation [30, 31, 33].

In our previous work, the benzyl propionate was synthesized in batch esterification using Novozym 435 as biocatalyst, a commercial and very well-known lipase, and after the experimental design the maximum ester conversion was around 44%, considering the immobilized enzyme highest level (15 wt% related to the total amount of substrates) [29]. The low conversion values were related to enzyme acidic inactivation, and to overcome the drawbacks related to the propionic acid, a fed-batch approach was adopted in this work and the acid was added to the reaction during the initial 5 h.

Figure 1 shows the results of the fed-batch esterification between the benzyl alcohol and propionic acid using three different enzyme amounts of Novozym 435 in presence of molecular sieves and fixed reaction conditions of 50 °C, 150 rpm, and substrates molar ratio of 1:1. The molecular sieves are able to absorb the water present in the reaction media (reaction product) reducing the amount of water in the lipase microaqueous environment and avoiding the protein deactivation [2, 20, 22, 23]. A great increase in the ester conversion was found (~90%) compared with the previous results found in batch mode (~44%) [29], showing that the fed-batch approach is able to overcome the acid inhibitory effects, visualized in batch reactor configuration. The maximum conversion was ensured after 8 h of reaction with 15 wt% of biocatalyst; however, the reaction medium was saturated due to the high amount of enzyme during the first 4 h, making difficult the fed-batch process. The enzyme amount of 5 wt% was not able to increase the ester conversion to the maximum levels. The enzyme amount of 10 wt% was enough to ensure a high conversion (~90%) after 24 h, and good fed-batch performance with lower enzyme amount in the reaction medium. Based on these ester conversions results, the immobilized enzyme amount was fixed in 10 wt% for the next experimental step.

The effect of the substrates molar ratio was investigated in the benzyl propionate fed-batch esterification and Fig. 2 shows the results of the ester conversion versus time for the molar ratio of 1:1, 1:3 and 1:5 (acid:alcohol). All molar ratios tested reached in the first cycle of reaction high benzyl propionate conversions in different periods of reaction. As the alcohol amount was increased the global conversion was also increased, and the MR of 1:5 had the maximum conversion (~99%) after 6 h of reaction, i.e., the maximum benzyl propionate conversion was reached right after the last acid addition, showing a rapid and effective performance. These results may be related to the capacity of high amounts of benzyl alcohol dilute the propionic acid present in the reaction media, together with the slower addition of the acid in the medium, indicating that the enzyme inactivation effects were reduced in the fed-batch conditions, ensuring high conversions. A similar increase in ester conversion was found for a study of oleic acid ethyl ester synthesis using crude rice bran (*Oryza sativa*) lipase in a fed-batch system. The alcohol acted as a lipase inhibitor, and the controlled fed was able to overcome the negative effects and increase the conversion [38].

The recycle of the immobilized enzyme was realized in the same fed-batch reaction conditions as shown in Fig. 2. The ester conversion was sharply reduced in the second cycle of the Novozym 435 for the MR 1:1 and 1:3, remaining around 12 and 16%, respectively. The higher amount of alcohol present in the medium (MR acid to alcohol 1:5) allowed a slight improvement in the enzyme performance, but still, a great reduction of ester conversion was observed after 24 h of reaction (conversion of ~63%). Similar results were found in the benzyl butyrate esterification using Novozym 435 as biocatalyst in a fed-batch configuration with the controlled fed of the butyric acid. The performance of the enzyme was improved in larger amounts of benzyl alcohol [2].

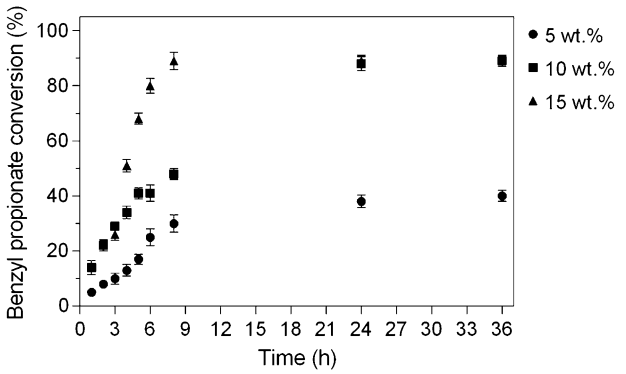

Fig. 1 Fed-batch benzyl propionate conversion versus time at different Novozym 435 amount of 5, 10, and 15 wt% related to the substrates total weight. The reaction conditions were substrates molar ratio of 1:1, 50 °C and 150 rpm

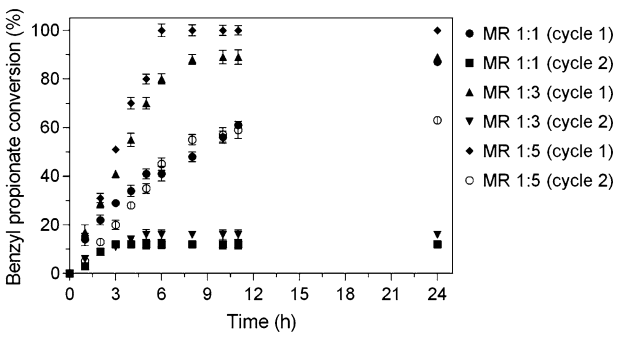

Fig. 2 Reusability of Novozym 435 for benzyl propionate synthesis using the fed-batch approach and different acid:alcohol molar ratios (1:1 to 1:5)

This strong negative effect on enzyme performance was probably accentuated with the use of a SFS configuration, which requires high amounts of alcohol to dilute the propionic acid. Other researchers synthesized many aroma esters using, commonly, organic solvents like *n*-hexane for the enzymatic syntheses with short-chain acids as acyl donors [15–20]. The presence of a great amount of a nonpolar solvent favors the reaction equilibrium to the ester synthesis and increases substrates and products solubility, reducing the acid damaging effects against the enzyme active site [1, 14, 20].

Some investigation around the enzyme activity was also conducted to understand this sharp reduction in the ester conversion even at high amounts of alcohol in the reaction medium in SFS. Table 1 shows the Novozym 435 activity before the reaction, after the second reaction cycle for all acid:alcohol molar ratios tested, and after contact with acid and alcohol separately. Before the reaction, the immobilized enzyme showed an activity of 54.6 U/g, and a similar value was visualized after the alcohol contact, showing that either enzyme support and enzyme active site were not harmed by the alcohol contact. On the other hand, the lipase activity was sharply reduced after the acid contact and the same behavior was visualized after the reactions with molar ratio acid:alcohol 1:1 and 1:3 (with an increased amount of acid), which indicates the propionic acid is responsible for the enzyme loss activity. The enzyme activity after the reaction with molar ratio 1:5 still presented some activity (~ 18 @@U/g) which explains the lower conversion shown in Fig. 2. However, it is worth mentioning the mixtures/reactions visual appearance showed that the Novozym 435 support was not dissolved after the reaction or substrates contact.

To understand the relationship between the propionic acid and the Novozym 435 loss of activity a qualitative investigation about the protein content in the polymeric support of the biocatalyst was also realized using an optical microscopy assay with lugol iodine solution as protein marked. Figure 3 shows the micrographs obtained by optical microscopy for the Novozym 435 before and after the reaction cycles. The protein content (brown filling) in the support of the Novozym 435 was large before the esterification (Fig. 3a), all polymeric support is filled with lipase. Figure 3b shows the support spheres (clear yellow spheres) almost unfilled after the enzyme recycle (molar ratio acid:alcohol 1:3), and a similar behavior is visualized in Fig. 3c that shows the immobilized enzyme used in the reaction with substrate molar ratio of 1:5; however, some protein (brown filling) remains inside the support spheres.

Table 1 Novozym 435 activity before, and after fed-batch esterification (cycle 2) and after substrates contact

Condition	Novozym 435 esterification activity (U/g)
Before reaction	54.6 ± 0.7
After esterification reaction MR 1:1	1.0 ± 0.2
After esterification reaction MR 1:3	1.1 ± 0.4
After esterification reaction MR 1:5	18.5 ± 1.2
After acid contact	1.7 ± 0.7
After alcohol contact	46.2 ± 0.4

Novozym 435 is a commercial immobilized enzyme obtained by immobilization via interfacial activation of the lipase on a moderately hydrophobic macroporous resin [29, 39]. The interfacial activation is very used for lipase immobilization, however, as reported in the literature, the main drawback related to this protocol is the enzyme desorption during high-temperature exposition and in contact with detergents or organic solvents [40–42].

Although Novozym 435 is generally very resistant and used to catalyze successfully many organic reactions [39, 43–47] the micrographs showed that the presence of the lipase inside the support was strongly reduced after the reaction, and the protein leaching was pronounced at higher amounts of propionic acid in the medium (molar ratio of 1:1 and 1:3). These results corroborate with the previous results shown in Fig. 2 and Table 1, in which the Novozym 435 had lost almost completely its activity at the first cycle of use in presence of high amounts of propionic acid (molar ratio of 1:1 and 1:3), but some activity remained after the reaction with substrates molar ratio of 1:5. This behavior is visibly related to the inactivation/desorption of the Cal B immobilized in the Novozym 435 support after the acid contact, leading to the breakdown of the physical bonds between the enzyme and the polymer support. The absence of covalent bonds is the main disadvantage of the interfacial activation immobilization technique. The lipase adsorbed on the macroporous acrylic resin possesses a weak bond and if the enzyme is irreversibly deactivated and/or desorbed, both the enzyme and the support are rendered unusable [10].

A similar result was observed for Cal B lipase immobilized in onoctyl agarose (CALB-OA) using the same immobilization technique (interfacial activation) [40]. The CALB-OA beads used in the first cycle of 100 mM tributyrin hydrolysis exhibited a linear reaction course. However, a drastic drop in the enzyme activity was observed after the second cycle. SDS-PAGE gels of the supernatant and biocatalyst showed significant enzyme desorption after the reaction. Similar results were found using triacetin and sunflower oil, suggesting the substrates and products may act as a lipase detergent causing massive enzyme desorption.

Several works reported that an excess of water affects the reaction conversion but a minimal amount of water is necessary to ensure the enzyme optimal conformation and then to become optimally active [48–50]. The difference

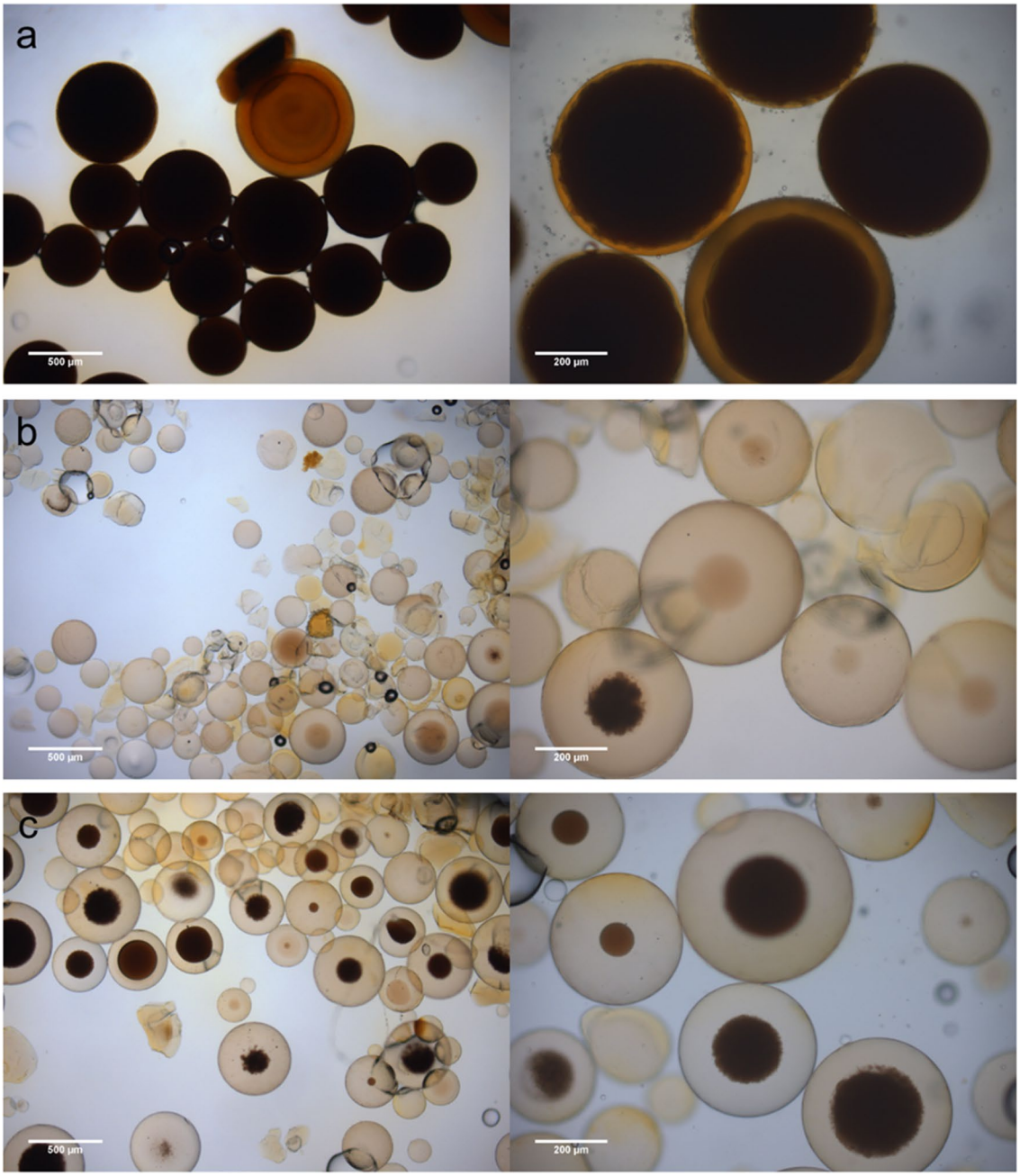

Fig. 3 Optical micrographs of the enzyme support beads showing the protein content (brown filling) into the polymeric support (clear yellow sphere) of the **a** Novozym 435 before use and **b** after recycling with substrates molar ratio of 1:3 and **c** molar ratio of 1:5 in the benzyl propionate fed-batch esterification (colour figure online)

in the nature of the supports might also promote changes in the level of water depending on the biocatalyst. Poppe et al. related that CALB immobilized on styrene–divinylbenzene beads presents a more hydrophobic matrix than Lewatit, which is used in Novozym 435, likely affecting the enzyme activity and conformation [51]. However, the presence of a water layer leads to changes in the enzyme catalytic activity and the present results indicate the desorption phenomena was predominant.

Some researches had already reported that other highly polar aliphatic acids of short-chain may cause enzyme inhibition and inactivation like acetic acid [16, 18, 31] and butyric acid [2, 17]. However, there are not any other work showing, until the present moment, that these acids can act

as a lipase detergent, causing the lipase desorption from the hydrophobic support during the esterification reaction even at increased alcohol molar ratio.

These results suggest that the use of an immobilized enzyme as catalyst of this specific reaction should possess irreversible bonds between the support and enzyme, like in hetero-functional supports, to prevent the lipase desorption [40, 52, 53]. Another alternative is the use of a crosslink agent as glutaraldehyde, polyethylenimine and aldehyde dextran to reinforce the enzyme support bonds, preventing the enzyme release [39, 54, 55].

Fed-batch esterification of benzyl propionate using lyophilized Cal B

Novozym 435 is one of the most used immobilized lipases, extensively employed in different reactions and processes [39]. The immobilized form of Cal B is preferred for laboratory and industrial applications owing to the improved stability besides the traditional advantages of immobilized enzyme systems, as reusability and products high purity related to the easier downstream processing.

However, although high conversions were obtained only in the first reaction cycle of the Novozym 435, the recycle results unfeasible the use of an immobilized enzyme to the present benzyl propionate synthesis, since the recycle is the most important advantage of the immobilized enzyme usage. Based on this, some tests were conducted using the lyophilized Cal B as biocatalyst of the propionic acid and benzyl alcohol esterification. Figure 4 shows the results of conversion for the fed-batch esterification using substrates in molar ratio of 1:1, 1:3, and 1:5. As possible seen, the lyophilized enzyme showed a similar performance to the Novozym 435, ensuring high conversions for all molar ratios tested in the same period of reaction. These results may be related to the lipase-lyophilized form, which did not ensure a free enzyme configuration due to the presence of aggregates in the reaction medium.

The initial fed-batch reaction rate was determined for both lyophilized and immobilized biocatalysts and Fig. 5 displays the results for the three MR tested. As the amount of alcohol was increased, an increase in the reaction rate was found for both Cal B forms, showing that the lyophilized enzyme had the same performance of the immobilized enzyme. It is worth noting that alcohol plays an important role in the reaction rate and was able to dilute the propionic acid present in the medium, reducing the inhibition effects and ensuring a great ester conversion.

Based on these results, the lyophilized Cal B could be used in the lyophilized form to catalyze the benzyl propionate reaction. However, a proper immobilization system may improve enzyme stability and some techniques as crosslinking and hetero-functional supports might prevent the lipase desorption problems and feasible the use of a heterogeneous catalyst for the benzyl propionate esterification. The hetero-functional supports possesses several distinct functionalities on its surface able to interact covalently with the lipase [56] and generally are suitable to the CALB immobilization ensuring enhancement in the results and increase enzyme stability as already reported by some authors [56–62].

Conclusions

The present work showed a good, viable and natural alternative to the esterification of the propionic acid and benzyl alcohol using a biotechnological approach. The use of a fed-batch configuration was able to overcome the inhibitory effects related to the use of propionic acid in the direct esterification, with an increase from 44% of benzyl propionate conversion in batch mode to 99% in fed-batch mode. The

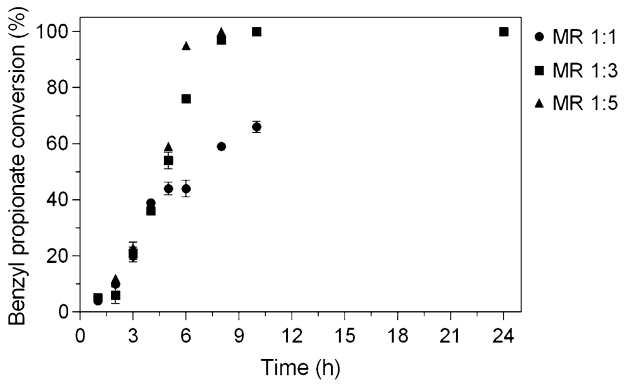

Fig. 4 Benzyl propionate conversion over 24 h of reaction using lyophilized Cal B as biocatalyst in fed-batch system varying substrates molar ratio from 1:1 to 1:5 (acid:alcohol). The reaction conditions were fixed in 50 °C, 8 wt% of lyophilized Cal B (related to the Novozym 435 initial weight), and 150 rpm

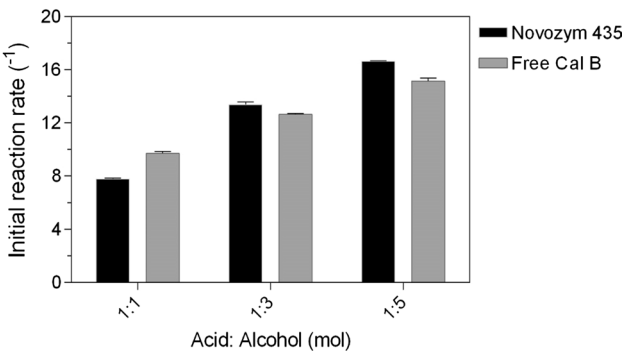

Fig. 5 Initial reaction rate of the fed-batch esterification between benzyl alcohol and propionic acid using Novozym 435 and lyophilized Cal B as biocatalysts with different acid:alcohol molar ratios

Novozym 435 was effective in the first cycle of conversion ensuring high conversion; however, the lipase immobilized was desorbed from the support due to the presence of propionic acid. Although high amounts of alcohol were used to dilute the propionic acid, all substrates molar ratios tested showed enzyme desorption, making unfeasible the use of an immobilized enzyme to the benzyl propionate esterification. The lyophilized Cal B presented similar conversions and initial reaction rates to those found for Novozym 435. These results indicated the possibility of using a free enzyme as biocatalyst to ensure a great conversion and a feasible process for the esterification of benzyl alcohol and propionic acid. However, more studies are needed to find a suitable support material to ensure a good immobilized enzyme process performance, considering an immobilization technique that avoids the enzyme desorption since the propionic acid in solvent-free conditions acted as a lipase detergent.

Acknowledgements The authors thank the financial support from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and Laboratório Multiusuário de Estudos em Biologia from Universidade Federal de Santa Catarina (LAMEB/UFSC) for optical microscopy analyses.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflict of interest.

References

1. Sá AGA, de Meneses AC, de Araújo PHH, de Oliveira D (2017) A review on enzymatic synthesis of aromatic esters used as flavor ingredients for food, cosmetics and pharmaceuticals industries. Trends Food Sci Technol. https://doi.org/10.1016/j.tifs.2017.09.004
2. de Meneses AC, Almeida Sá AG, Lerin LA et al (2019) Benzyl butyrate esterification mediated by immobilized lipases: evaluation of batch and fed-batch reactors to overcome lipase-acid deactivation. Process Biochem 78:50–57
3. Leszczak JP, Tran-Minh C (1998) Synthesis of benzoates by enzymatic catalysis in heterogeneous medium. J Mol Catal B Enzym 5:277–281. https://doi.org/10.1016/S1381-1177(98)00093-9
4. Tomke PD, Rathod VK (2015) Ultrasound assisted lipase catalyzed synthesis of cinnamyl acetate via transesterification reaction in a solvent free medium. Ultrason Sonochem 27:241–246. https://doi.org/10.1016/j.ultsonch.2015.04.022
5. Vanin AB, Orlando T, Piazza SP, Puton BMS (2014) Antimicrobial and antioxidant activities of clove essential oil and eugenyl acetate produced by enzymatic esterification. Appl Biochem Biotechnol 174:1286–1298. https://doi.org/10.1007/s12010-014-1113-x
6. Badgujar KC, Bhanage BM (2015) The combine use of ultrasound and lipase immobilized on co-polymer matrix for efficient biocatalytic application studies. J Mol Catal B Enzym 122:255–264. https://doi.org/10.1016/j.molcatb.2015.09.012
7. Gryglewicz S, Jadownicka E, Czerniak A (2000) Lipase catalysed synthesis of aliphatic, terpene and aromatic esters by alcoholysis in solvent-free medium. Biotechol Lett 22:1379–1382. https://doi.org/10.1023/A:1005600631600
8. Tischer W, Wedekind F (1999) Immobilized enzymes: methods and applications. Top Curr Chem 200:95–126. https://doi.org/10.1007/3-540-68116-7_4
9. Schmid A, Dordick JS, Hauer B et al (2001) Industrial biocatalysis today and tomorrow. Nature 409(6817):258–268
10. Sheldon RA (2007) Enzyme immobilization: the quest for optimum performance. Adv Synth Catal 349:1289–1307. https://doi.org/10.1002/adsc.200700082
11. Kasche V, Haufler U (1987) Equilibrium and kinetically controlled synthesis with enzymes: semisynthesis of penicillins and peptides. Methods Enzymol 136:280–292
12. Kasche V (1986) Mechanism and yields in enzyme catalysed equilibrium and kinetically controlled synthesis of fl-lactam antibiotics, peptides and other condensation products. Enzyme Microb Technol 8:4–16
13. Halling PJ (1990) High-affinity binding of water by proteins is similar in air and in organic solvents. Biochim Biophys Acta 1040:225–228
14. Halling PJ (1984) Effects of water on equilibria catalysed by hydrolytic enzymes in biphasic reaction systems. Enzyme Microb Technol 6:513–516
15. Wu Z, Qi W, Wang M et al (2014) Lipase immobilized on novel ceramic supporter with Ni activation for efficient cinnamyl acetate synthesis. J Mol Catal B Enzym 110:32–38. https://doi.org/10.1016/j.molcatb.2014.09.010
16. Hari Krishna S, Divakar S, Prapulla SG, Karanth NG (2001) Enzymatic synthesis of isoamyl acetate using immobilized lipase from *Rhizomucor miehei*. J Biotechnol 87:193–201. https://doi.org/10.1016/S0168-1656(00)00432-6
17. Hari Krishna S, Prapulla SG, Karanth NG (2000) Enzymatic synthesis of isoamyl butyrate using immobilized *Rhizomucor miehei* lipase in non-aqueous media. J Ind Microbiol Biotechnol 25:147–154. https://doi.org/10.1038/sj.jim.7000045
18. Claon PA, Akoh CC (1994) Effect of reaction parameters on SP435 lipase-catalyzed synthesis of Citronellyl acetate in organic solvent. Enzym Microb Technol 16:835–838. https://doi.org/10.1016/0141-0229(94)90056-6
19. Huang SY, Chang HL, Goto M (1998) Preparation of surfactant-coated lipase for the esterification of geraniol and acetic acid in organic solvents. Enzyme Microb Technol 0229:552–557
20. Marty A, Chulalaksananukul W, Willemot RM, Condoret JS (1991) Kinetics of lipase-catalyzed esterification in supercritical CO_2. Biotechnol Bioeng 39:273–280
21. Khan NR, Rathod VK (2015) Enzyme catalyzed synthesis of cosmetic esters and its intensification: a review. Process Biochem 50:1793–1806. https://doi.org/10.1016/j.procbio.2015.07.014
22. Paludo N, Alves JS, Altmann C et al (2015) The combined use of ultrasound and molecular sieves improves the synthesis of ethyl butyrate catalyzed by immobilized *Thermomyces lanuginosus* lipase. Ultrason Sonochem 22:89–94. https://doi.org/10.1016/j.ultsonch.2014.05.004
23. Bansode SR, Rathod VK (2014) Ultrasound assisted lipase catalysed synthesis of isoamyl butyrate. Process Biochem 49:1297–1303. https://doi.org/10.1016/j.procbio.2014.04.018
24. Sekeroglu G, Fadiloglu S, Ibanoglu E (2002) Production and characterisation of enzymatically produced lauric acid esters of fructose. J Sci Food Agric 1522:1516–1522. https://doi.org/10.1002/jsfa.1222
25. Khan NR, Pratap AP (2013) Green synthesis of isopropyl ricinoleate. J Oleo Sci 158:153–158
26. McGinty D, Letizia CS, Api AM (2012) Fragrance material review on benzyl propionate. Food Chem Toxicol 50:S486–S490. https://doi.org/10.1016/j.fct.2013.08.067

27. VCF (2017) Volatile compounds in food: database. In: Nijssen LM, van Ingen-Visscher CA, Donders JJH (eds) Version 16.4. Triskelion B.V, Zeist, pp 1963–2017
28. Badgujar KC, Bhanage BM (2014) Enhanced biocatalytic activity of lipase immobilized on biodegradable copolymer of chitosan and polyvinyl alcohol support for synthesis of propionate ester: kinetic approach. Ind Eng Chem Res 53:18806–18815. https://doi.org/10.1021/ie501304e
29. Sá AGA, de Meneses AC, Lerin LA et al (2018) Biocatalysis of aromatic benzyl-propionate ester by different immobilized lipases. Bioprocess Biosyst Eng. https://doi.org/10.1007/s00449-018-1893-4
30. Güvenç A, Kapucu N, Mehmetoğlu Ü (2002) The production of isoamyl acetate using immobilized lipases in a solvent-free system. Process Biochem 38:379–386. https://doi.org/10.1016/S0032-9592(02)00099-7
31. Romero MD, Calvo L, Alba C et al (2005) Enzymatic synthesis of isoamyl acetate with immobilized *Candida antarctica* lipase in n-hexane. Enzym Microb Technol 37:42–48. https://doi.org/10.1016/j.enzmictec.2004.12.033
32. Badgujar KC, Pai PA, Bhanage BM (2016) Enhanced biocatalytic activity of immobilized *Pseudomonas cepacia* lipase under sonicated condition. Bioprocess Biosyst Eng 39:211–221. https://doi.org/10.1007/s00449-015-1505-5
33. Zare M, Golmakani MT, Niakousari M (2019) Lipase synthesis of isoamyl acetate using different acyl donors: comparison of novel esterification techniques. Lwt 101:214–219. https://doi.org/10.1016/j.lwt.2018.10.098
34. Chiaradia V, Soares NS, Valério A et al (2016) Immobilization of *Candida antarctica* lipase B on magnetic poly(urea-urethane) nanoparticles. Appl Biochem Biotechnol 180:558–575. https://doi.org/10.1007/s12010-016-2116-6
35. Chen B, Miller ME, Gross RA (2007) Effects of porous polystyrene resin parameters on *Candida antarctica* lipase B adsorption, distribution, and polyester synthesis activity. Langmuir 23:6467–6474. https://doi.org/10.1021/la063515y
36. Zenevicz MCP, Jacques A, Furigo AF et al (2016) Enzymatic hydrolysis of soybean and waste cooking oils under ultrasound system. Ind Crops Prod 80:235–241. https://doi.org/10.1016/j.indcrop.2015.11.031
37. Ceni G, Lerin LA, de Conto JF et al (2010) Optimization of 1-glyceryl benzoate production by enzymatic transesterification in organic solvents. Enzym Microb Technol 46:107–112. https://doi.org/10.1016/j.enzmictec.2009.09.011
38. Hidayat C, Prastowo I, Hastuti P, Restiani R (2014) Effect of ethanol concentrations on rice bran protease activity and ester synthesis during enzymatic synthesis of oleic acid ethyl ester in a fed-batch system using crude rice bran (*Oryza sativa*) lipase. Biocatal Biotransform 32:231–235. https://doi.org/10.3109/10242422.2014.934683
39. Ortiz C, Ferreira ML, Barbosa O et al (2019) Novozym 435: the "perfect" lipase immobilized biocatalyst? Catal Sci Technol 9:2380–2420. https://doi.org/10.1039/c9cy00415g
40. Virgen-ortíz JJ, Tacias-pascacio VG, Hirata DB et al (2017) Relevance of substrates and products on the desorption of lipases physically adsorbed on hydrophobic supports. Enzym Microb Technol 96:30–35. https://doi.org/10.1016/j.enzmictec.2016.09.010
41. Rueda N, Albuquerque TL, Bartolome-cabrero R et al (2016) Reversible immobilization of lipases on heterofunctional octyl-amino agarose beads. Molecules 21:646. https://doi.org/10.3390/molecules21050646
42. Panahi Y, Akhavan A, Sahebkar A et al (2014) Investigation of the effectiveness of *Syzygium aromaticum*, *Lavandula angustifolia* and *Geranium robertianum* essential oils in the treatment of acute external otitis: a comparative trial with ciprofloxacin. J Microbiol Immunol Infect 47:211–216. https://doi.org/10.1016/j.jmii.2012.10.002
43. Rosso Comim SR, Veneral JG, De Oliveira D et al (2015) Enzymatic synthesis of poly(ε-caprolactone) in liquified petroleum gas and carbon dioxide. J Supercrit Fluids 96:334–348. https://doi.org/10.1016/j.supflu.2014.07.004
44. Gumel AM, Annuar SM, Heidelberg T (2013) Single-step lipase-catalyzed functionalization of medium-chain-length polyhydroxyalkanoates. J Chem Technol Biotechnol 88:1328–1335. https://doi.org/10.1002/jctb.3980
45. Polloni AE, Veneral JG, Rebelatto EA et al (2017) Enzymatic ring opening polymerization of ω-pentadecalactone using supercritical carbon dioxide. J Supercrit Fluids 119:221–228. https://doi.org/10.1016/j.supflu.2016.09.019
46. Rathore V, Madras G (2007) Synthesis of biodiesel from edible and non-edible oils in supercritical alcohols and enzymatic synthesis in supercritical carbon dioxide. Fuel 86:2650–2659. https://doi.org/10.1016/j.fuel.2007.03.014
47. Talukder MMR, Wu JC, Lau SK et al (2009) Comparison of Novozym 435 and Amberlyst 15 as heterogeneous catalyst for production of biodiesel from palm fatty acid distillate. Energy Fuels 23:1–4. https://doi.org/10.1021/ef8006245
48. Colombie S, Tweddell RJ, Condoret J-S, Marty A (1998) Water activity control: a way to improve the efficiency of continuous lipase esterification. Biotechnol Bioeng 60:362–368
49. Foresti ML, Pedernera M, Bucal V, Ferreira ML (2007) Multiple effects of water on solvent-free enzymatic esterifications. Enzyme Microb Technol 41:62–70. https://doi.org/10.1016/j.enzmictec.2006.11.023
50. Nie K, Xie F, Wang F, Tan T (2006) Lipase catalyzed methanolysis to produce biodiesel : optimization of the biodiesel production. J Mol Catal B Enzym 43:142–147. https://doi.org/10.1016/j.molcatb.2006.07.016
51. Poppe JK, Garcia-galan C, Matte CR et al (2013) Optimization of synthesis of fatty acid methyl esters catalyzed by lipase B from *Candida antarctica* immobilized on hydrophobic supports. J Mol Catal B Enzym 94:51–56. https://doi.org/10.1016/j.molcatb.2013.05.010
52. Abaházi E, Boros Z, Poppe L (2014) Additives enhancing the catalytic properties of lipase from *Burkholderia cepacia* immobilized on mixed-function-grafted mesoporous silica gel. Molecules 19:9818–9837. https://doi.org/10.3390/molecules19079818
53. Rueda N, Santos JCS, Torres R et al (2015) Improved performance of lipases immobilized on heterofunctional octyl-glyoxyl agarose beads. RSC Adv 5:11212–11222. https://doi.org/10.1039/c4ra13338b
54. Zaak H, Fernandez-lopez L, Otero C et al (2017) Improved stability of immobilized lipases via modification with polyethylenimine and glutaraldehyde. Enzym Microb Technol 106:67–74. https://doi.org/10.1016/j.enzmictec.2017.07.001
55. Filice GFM, Carolina DL, Lorena P et al (2011) Cross-linking of lipases adsorbed on hydrophobic supports: highly selective hydrolysis of fish oil catalyzed by RML. J Am Oil Chem Soc 88:801–807. https://doi.org/10.1007/s11746-010-1727-2
56. Barbosa O, Torres R, Ortiz C et al (2013) Heterofunctional supports in enzyme immobilization : from traditional immobilization protocols to opportunities in tuning enzyme properties. Biomacromolecules 14(8):2433–2462
57. Garcia-galan C, Fernandez-lafuente R, Rodrigues RC (2011) Potential of different enzyme immobilization strategies to improve enzyme performance. Adv Synth Catal 354:2885–2904. https://doi.org/10.1002/adsc.201100534
58. de Melo RR, Alnoch RC, Ferreira A et al (2017) New heterofunctional supports based on glutaraldehyde-activation: a tool for enzyme immobilization at neutral pH. Molecules 22(7):1088. https://doi.org/10.3390/molecules22071088

59. Arana-Peña S, Lokha Y, Fernández-Lafuente R (2018) Immobilization of eversa lipase on octyl agarose beads and preliminary characterization of stability and activity features. Catalysis. https://doi.org/10.3390/catal8110511
60. Hirata DB, Albuquerque TL, Rueda N et al (2016) Evaluation of different immobilized lipases in transesterification reactions using tributyrin: advantages of the heterofunctional octyl agarose beads. J Mol Catal B Enzym. https://doi.org/10.1016/j.molcatb.2016.08.008
61. Peirce S, Tacias-pascacio VG, Russo ME et al (2016) Stabilization of *Candida antarctica* lipase B (CALB) immobilized on octyl agarose by treatment with polyethyleneimine (PEI). Molecules. https://doi.org/10.3390/molecules21060751
62. Rueda N, Santos JCS, Torres R et al (2015) Improved performance of lipases immobilized on heterofunctional octyl-glyoxyl agarose beads. RSC Adv 5:11212–11222. https://doi.org/10.1039/c4ra13338b