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Omega-3 production by fish oil hydrolysis using a lipase from *Burkholderia gladioli* BRM58833 immobilized and stabilized by post-immobilization techniques

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ABSTRACT

Immobilization of lipase from *Burkholderia gladioli* BRM58833 on octyl sepharose (OCT) resulted in catalysts with higher activity and stability. Following, strategies were studied to further stabilize and secure the enzyme to the support using functionalized polymers, like polyethylenimine (PEI) and aldehyde-dextran (DEXa), to cover the catalyst with layers at different combinations. Alternatively, the construction of a bifunctional layer was studied using methoxypolyethylene glycol amine (NH 2 -PEG) and glycine. The catalyst OCT-PEI-DEXa was the most thermostable, with a 263.8-fold increase in stability when compared to the control condition. When evaluated under alkaline conditions, OCT-DEXa-PEG 10 /Gly was the most stable, reaching stability 70.1 times greater than the control condition. Proportionally, the stabilization obtained for *B. gladioli* BRM58833 lipase was superior to that obtained for the commercial *B. cepacia* lipase. Preliminary results in the hydrolysis of fish oil demonstrated the potential of the coating technique with bifunctional polymers, resulting in a stable catalyst with greater catalytic capacity for the production of omega-3 PUFAs. According to the results obtained, it is possible to modulate *B. gladioli* BRM58833 lipase properties like stability and catalytic activity for enrichment of omega-3 fatty acids.

1. Introduction

Lipases are considered the biocatalysts of greatest industrial interest, exhibiting a wide spectrum of biotechnological applications. Depending on the process in which they are used, these enzymes may suffer under adverse conditions of temperature, pH or even exposition to organic solvents for prolonged periods of time. Exposure to these conditions usually leads to denaturation of the three-dimensional structure and a consequent loss of catalytic capacity, affecting not only productivity, but also the final cost of the products. Obtaining immobilized catalysts is a viable solution to ensure that characteristics like the catalytic potential of the lipase are maintained. Some advantages of enzyme immobilization are the increase in enzyme stability and their possibility of reuse, reducing production costs in industry [1,2].

Stabilizing an enzyme is one of the main challenges of enzymatic engineering and can be achieved by several techniques of immobilization and post-immobilization. For lipases, immobilization on hydrophobic supports via interfacial adsorption has been widely used. However, when immobilized by this method, only a small part of the enzyme surface remains in contact with the support, allowing the application of other combined stabilization strategies (post-immobilization techniques), such as coating immobilized catalysts with polymers [3].

Burkholderia gladioli BRM58833 has been previously reported to produce high levels of lipolytic activity when cultivated by solid-state fermentation in wheat bran [4]. The lipase was resistant against solvents and exhibited an expressive thermal stability when compared to other lipases, revealing the potential of this enzyme in both hydrolysis

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and ester synthesis reactions. Therefore, the immobilization of lipases from *B. gladioli* BRM58833 by interfacial adsorption on hydrophobic supports was studied, as well as their stabilization by post-immobilization techniques. Thus, the objective of this work was to immobilize and stabilize these lipases, generating catalysts with high resistance to temperatures and pH values as well as applying them for the hydrolysis of fish oil for enrichment of omega-3 polyunsaturated fatty acids.

2. Methodology

2.1. Materials

Agarose 10BCL was purchased from Agarose Beads Technologies (Madrid, Spain). Sulfopropyl sepharose, Butyl Sepharose, Octyl Sepharose and Phenyl Sepharose were purchased from GE Healthcare (Uppsala, Sweden). Immobeads-150 Octadecyl, Immobeads-300 Methyl-Estyrene, Lewatit 1600, Polyethyleneimine, Polyallylamine, *p*-nitrophenyl palmitate, docosahexaenoic acid, *Burkholderia cepacia* lipase (Amano), *Thermomyces lanuginosus* lipase (Novozymes), bovine serum albumin and dextran (*Leuconostoc mesenteroides*) were obtained from Sigma-Aldrich Co. (St. Louis, IL, USA). Methoxypolyethylene glycol amine (NH_2-PEG) was purchased from Rapp Polymere GmbH (Tubingen, Germany). All other reagents were of analytical grade.

2.2. Lipase production

Burkholderia gladioli BRM58833 was previously isolated from oil-palm fruits and is preserved in the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries, Embrapa Agroenergia, Brasılia, Brazil. The lipase-containing crude enzymatic extract was obtained through solid-state fermentation of *B. gladioli* BRM58833 in wheat bran as described by Martins et al. [4].

The commercial lipases from *Burkholdeira cepacia* (Amano) and *Thermomyces lanuginosus* (Novozymes) were used to produce catalysts as a way of comparison with *B. gladioli* BRM58833 catalysts.

2.3. Structural modelling

The structural conformation of *Burkholderia gladioli* BRM58833 lipase was predicted by comparative modelling. The lipase sequence (NCBI access: NHH84158) was used to build a model using *B. cepacia* lipase (PDB access: 1ys2.1.A) as a template (sequence identity = 82.7%). The model was constructed by identification of rigid bodies and domain superposition using the automated Swiss-Model server (https://swissmodel.expasy.org/). Annotation of the constituent residues of the catalytic triad was based on Dalal [5].

2.4. Lipase assay

Lipolytic activity assays were performed based on the method by Winkler and Stuckmann [6] with modifications, as described by Martins et al. [4]. Activities were measured according to the increase in absorbance at 410 nm of *p*-nitrophenol (*p*NP) due to the hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) at 37 °C for 1 min in a thermostated cuvette (path length = 1 cm) with constant magnetic stirring (Jasco V-630 Spectrophotometer). One international unit of activity (U) was defined as the amount of enzyme required to release 1 μmol of *p*NP per min under test conditions.

2.5. Purification/immobilization on sulfopropyl sepharose

A purification step by immobilization on sulfopropyl sepharose (SP) was performed to remove Triton X-100 from the lipase-containing crude enzymatic extract. Firstly, the extract was dialysed overnight (membrane cut-off of 12 kDa) against 10 mmol L^{-1} acetate buffer at pH 5.0 and, then, two-fold diluted in the same buffer. The resulting solution was combined with SP in a 1:10 support:solution proportion. This suspension was incubated at 25 °C for 3 h under mild stirring. In order to follow the immobilization process, both suspension and supernatant were periodically evaluated for lipase activity. Finally, the immobilized preparation was vacuum filtered and thoroughly washed with 10 mmol L^{-1} acetate buffer at pH 5.0. The lipase was desorbed from SP with NaCl 0.4 mol L^{-1} in 25 mmol L^{-1} phosphate buffer at pH 8.5 and mild stirring for 1 h. After that, the obtained supernatant was dialysed overnight (membrane cut-off of 12 kDa) against 10 mmol L^{-1} phosphate buffer at pH 7.0 to remove salts and adjust the pH. This resulting lipase solution (henceforth denominated BGLip) was Triton X-100 free and could be applied to other immobilization methods.

2.6. Immobilization on glyoxyl agarose

Agarose 10BCL was esterified with glycidol and then oxidized with sodium periodate to obtain agarose activated with glyoxyl groups (GLA) [7]. The obtained support was used for the immobilization of *B. gladioli* lipases in seven different conditions: (1) incubation with BGLip at pH 8.5 and addition of Triton X-100 and DTT; (2) incubation with SP supernatant at pH 8.5 and addition of DTT; (3) incubation with BGLip at pH 8.5; (4) incubation with BGLip at pH 8.5 and addition of DTT; (5) incubation with BGLip at pH 10.0 and addition of Triton X-100; (6) incubation with SP supernatant at pH 10.0; and (7) incubation with BGLip at pH 10.0. All tests were performed considering 5 mg of protein per g of support using 10 mmol L^{-1} bicarbonate buffer and GLA in a 1:10 support: solution proportion. When present, a final concentration of Triton X-100 0.1% and DTT 50 mmol L^{-1} were considered. Suspensions were incubated at 25 °C for 5 h under mild stirring and were periodically evaluated for lipase activity. Afterwards, sodium borohydride (1 mg mL^{-1}) was added in order to reduce the formed Schiff bases during 30 min at 25 °C under mild stirring. At the end, immobilized preparations were vacuum filtered and thoroughly washed with 10 mmol L^{-1} phosphate buffer at pH 7.0.

2.7. Immobilization on hydrophobic supports

To immobilize *B. gladioli* BRM58833 lipase, BGLip was offered to different hydrophobic supports: Butyl Sepharose (BUT), Octyl Sepharose (OCT), Phenyl Sepharose (PHE), Immobeads-150 Octadecyl (C18), Immobeads-300 Methyl-Estyrene (MES) and Lewatit 1600 (LEW). Each support was added to BGLip in a 1:10 support:solution proportion and incubated at 25 °C in 10 mmol L^{-1} phosphate buffer at pH 7.0 under mild stirring [8]. All tests were performed considering 5 mg of protein per g of support. Lipase adsorption was monitored both in the suspension and supernatant, which were periodically measured by the lipolytic activity assay. At the end of the process, the catalysts were vacuum filtered and thoroughly washed with 10 mmol L^{-1} phosphate buffer at pH 7.0.

2.8. Immobilization parameters

Immobilization yield and expressed activities were used as parameters to evaluate each immobilization procedure and were calculated as depicted in equations (1) and (2):

$$Immob.Yield\left(\%\right) = \left(1 - \frac{Final\ \ supernatant\ \ activity\left(U/mL\right)}{Initial\ \ activity\left(U/mL\right)}\right).100 \quad \text{(Eq. 1)}$$

$$Exp.Act.(\%) = \left(\frac{Suspension\ \ activity\left(U/mL\right).Yield}{Initial\ \ activity\ \left(U/mL\right)}\right).100 \quad \text{(Eq. 2)}$$

2.9. Determination of kinetic parameters: Km and Vmax

The enzymatic solution (either free enzyme or octyl-immobilized lipase) was incubated with *p*NPP with concentrations ranging from 0.02 to 1.26 mmol L^{-1}. The kinetic parameters Vmax and Km were estimated from the Michaelis-Menten equation without linearization in order to improve the fit, avoid approximations and consider experimental uncertainties. An iterative least squares estimation procedure, based on the Gauss-Newton algorithm, was performed to obtain the parameters that best fit the kinetic model to experimental data. The convergence criterion adopted was 10^{-6} with a maximum number of iterations of 100.

2.10. Adsorption of hydrophilic polymers

To improve catalysts stability, two polycationic polymers were adsorbed to the enzyme surface: polyethyleneimine (PEI, MW of either 10, 25 or 60 kDa) and polyallylamine (PAA, MW 17.5 kDa). One gram of octyl-immobilized lipase was combined with 16 mL of a polymer solution at 25 mg mL^{-1} in 10 mmol L^{-1} bicarbonate buffer at pH 8.0 overnight under mild stirring and refrigerated at 4 °C [9]. Afterwards, catalysts were vacuum filtered and thoroughly washed with 10 mmol L^{-1} phosphate buffer at pH 7.0.

2.11. Covalent cross-linking

Aldehyde-dextran (DEXa) was obtained by the oxidation of dextran (MW 25 kDa) in a solution of sodium periodate as outlined by Fernández-Lafuente et al. [10]. Octyl-immobilized lipase was added to aldehyde-dextran in 10 mmol L^{-1} bicarbonate buffer at pH 8.5 in a 1:10 support:solution proportion and incubated at 25 °C overnight and under mild stirring. Afterwards, sodium borohydride (1 mg mL^{-1}) was added and the mixture was incubated for 30 min at 25 °C under mild stirring. At the end, immobilized preparations were vacuum filtered and thoroughly washed with 10 mmol L^{-1} phosphate buffer at pH 7.0 [11]. Different DEXa concentrations (either 10 or 50 mg/g support) were evaluated overtime to determine the strategy that would promote an increase in stabilization the most.

Similarly, a combined strategy was used to evaluate the stabilization effect of DEXa as a second polymeric layer. Hence, using the conditions described above, OCT-PEI and OCT-PAA catalysts were combined with DEXa instead.

2.12. Building of a polymeric bilayer of DEXa and NH_2-PEG

Catalysts containing a first layer of DEXa were further modified with methoxypolyethylene glycol amine (PEG-NH_2) prior to reduction. For that, OCT-DEXa catalysts were incubated with NH_2-PEG (MW of either 2 or 10 kDa) at different concentrations (either 25 or 250 mg g^{-1} of support) in 10 mmol L^{-1} bicarbonate buffer at pH 8.5 in a 1:10 support: solution proportion and incubated at 25 °C overnight and under mild stirring. Furthermore, the combination of NH_2-PEG and glycine (GLY) was evaluated to obtain bifunctional polymers. In this case, incubation with NH_2-PEG was followed by a second incubation with GLY always at 250 mg g^{-1} of support in 10 mmol L^{-1} bicarbonate buffer at pH 8.5. An inverted strategy of incubation (GLY followed by NH_2-PEG) was also studied. For all cases, after coating with polymers, sodium borohydride (1 mg mL^{-1}) was added and the mixture was incubated for 30 min at 25 °C under mild stirring. At the end, immobilized preparations were vacuum filtered and thoroughly washed with 10 mmol L^{-1} phosphate buffer at pH 7.0.

2.13. Stabilization analysis

In order to compare the effects of each stabilization strategy, all catalysts were inactivated at 60°C in 10 mmol L^{-1} sodium phosphate buffer at pH 7.0 and the residual activities were measured periodically along time. Those with greater thermal stability were also evaluated under alkaline conditions, being incubated in 10 mmol L^{-1} bicarbonate buffer at pH 10 and 30 °C. Likewise, residual activities were measured periodically to monitor the inactivation of each catalyst. The half-life was estimated considering residual activities (A) in function of time as depicted in Equation (3), where k_d corresponds to the inactivation rate constants and t corresponds to time.

$$A = A_0.e^{-kd.t} \quad \text{(Eq. 3)}$$

In the end, a stabilization index was calculated dividing each catalyst half-life by the control half-life to rank them according to their stability.

2.14. Fish oil hydrolysis

The hydrolysis of fish oil was carried out in a biphasic system according to the procedure described by Fernández-Lorente et al. [12] with modifications. After combining 5 mL of *n*-heptane with 5 mL of 10 mmol L^{-1} sodium phosphate buffer at pH 7.0 and 0.1 mL of fish oil (NuaDHA 500), the system was left under vigorous stirring for 30 min at 30 °C. The reaction was started with addition of 300 mg of immobilized catalysts. The system was left at 30 °C with constant stirring. Samples of 100 μL of the organic phase were collected for periodic analysis. The production of DHA was evaluated by RP-chromatography on an HPLC system (Spectra Physic SP 100 coupled to a UV detector Spectra Physic SP 8450). Fatty acids were separated using a Kromasil C8 column (15 × 0.4 cm) with isocratic elution at a flow of 1 mL min^{-1} in acetonitrile and 10 mmol L^{-1} Tris buffer pH 3 (70:30 v/v). DHA was detected at 215 nm and quantified by comparison with the pure commercial standard. The retention time for DHA was 14.32 min.

3. Results and discussion

3.1. Immobilization of *B. gladioli* BRM58833 lipase

For an enzyme to be successfully implemented in an industrial process, it must not only have a high activity against its substrate, but it must also be stable. Thus, the lipase obtained from the cultivation of *B. gladioli* BRM58833 in wheat bran was subjected to immobilization on different supports. Initially, the crude enzyme extract was ionically immobilized onto sulfopropyl sepharose (Table 1, catalyst #1) to separate Triton X-100 and other impurities from the enzymes. Although this step had a yield of only 10.5%, it was necessary to adsorb part of the lipase activity to the support, allowing to obtain a Triton X100-free lipase solution after desorption. After this process, two lipase solutions were obtained: BGLip (desorbed from SP, Triton X100-free) and SP-S (which represented the lipase fraction of the crude enzyme extract that could not be adsorbed on SP during the immobilization process). Both lipase solutions were then applied to subsequent immobilization processes to obtain different catalysts.

BGLip and SP-S were covalently immobilized on activated agarose with glyoxyl groups producing GLA-1 and GLA-2 (Table 1, catalysts #2 and #3, respectively). According to López-Gallego et al. [13], under low alkaline pH conditions, the N-terminal portion of the protein (pK ~ 7.5) is much more reactive than the amino groups present in the side chains of the amino acid residues on the surface of the protein (pK Lys ~ 10.5). Thus, under conditions of pH 8.5 as used for BGLip and SP-supernatant, the binding of the lipase to the support is favoured via a single-point bond.

As the structural model for *B. gladioli* lipase BRM58833 demonstrated the location of the N-terminal portion on the surface of the enzyme and at the opposite end of the active site, the binding of the enzyme to the support is sterically favoured, exposing the cavity of the active site to the reaction medium (Fig. 1). To the preparation of GLA-1

Table 1
Immobilization and thermal stability of *B. gladioli* BRM58833 and *B. cepacia* lipases on different supports. Control condition: GLA-1.

#	Catalyst	Used Fraction	Immobilization pH	Yield (%)	Expressed Activity (%)	$t_{1/2}$ (h)	Thermal Stabilization Index
B. gladioli BRM58833 Lipase							
1	SP	Crude Extract	5.0	10.5	11.9	0.7	2.8
2	GLA-1	BGLip[a,b]	8.5	60.4	37.1	0.2	1.0
3	GLA-2	SP-S[b]	8.5	77.0	39.9	0.8	3.5
4	GLA-3	BGLip	8.5	43.0	77.7	3.0	12.4
5	GLA-4	BGLip[b]	8.5	25.2	33.3	1.9	7.9
6	GLA-5	BGLip[a]	10.0	93.9	66.5	0.2	0.9
7	GLA-6	SP-S	10.0	80.8	78.2	0.3	1.4
8	GLA-7	BGLip	10.0	93.4	58.2	1.2	4.9
9	BUT	BGLip	7.0	100.0	99.2	0.8	3.3
10	OCT	BGLip	7.0	100.0	136.8	3.9	16.3
11	PHE	BGLip	7.0	100.0	107.5	0.6	2.6
12	C18	BGLip	7.0	83.2	27.5	0.1	0.6
13	MES	BGLip	7.0	90.2	30.3	0.2	1.0
14	LEW	BGLip	7.0	95.0	35.5	0.3	1.1
***B. cepacia* Lipase**							
15	GLA-cepacia	Commercial	8.5	70.8	47.1	2.9	12.3/1.0[c]
16	OCT-cepacia	Commercial	7.0	100.0	114.1	14.7	62.1/4.8[c]

[a] Addition of Triton X-100 0.1%.
[b] Addition of DTT 50 mmol L^{-1}.
[c] Thermal stabilization index calculated considering GLA-cepacia as control.

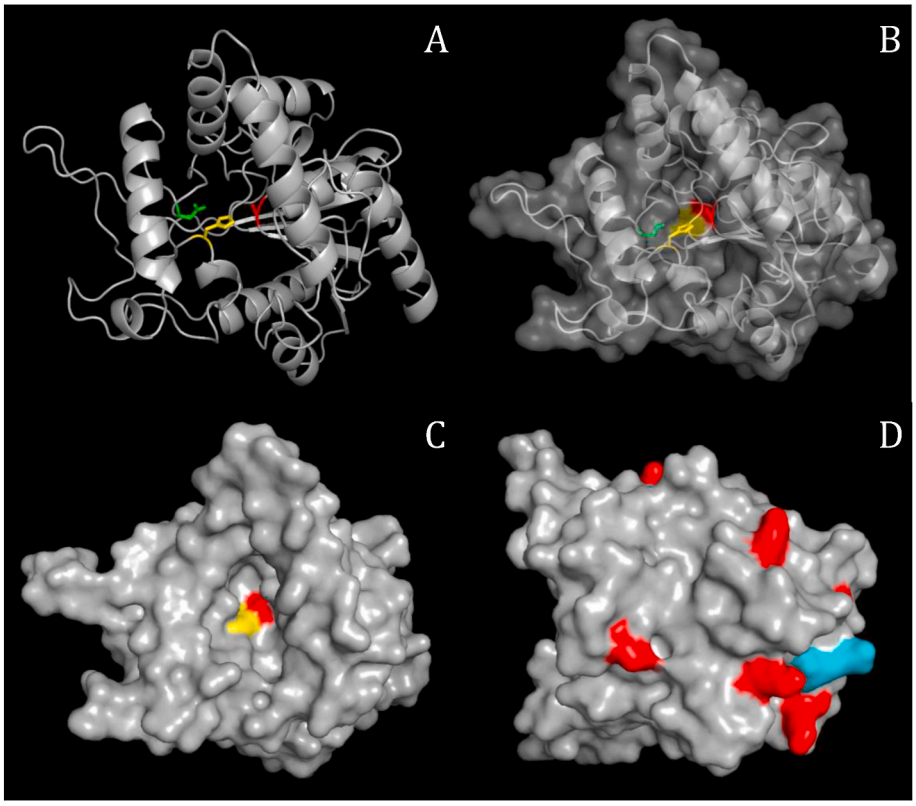

Fig. 1. Model of *B. gladioli* BRM58833 lipase. (A) Cartoon model with highlighted active site (red: S-126; green: D-302; yellow: H-324); (B) Cartoon model with surface overlap; (C) Cavity of the active site; (D) Surface opposite to the cavity of the active site with emphasis on Lys residues (red) and the N-terminal portion (blue); Model obtained using *B. cepacia* lipase (PDB access: 1ys2.1.A) as template. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and 2, DTT and Triton X-100 or only DTT were added, respectively. According to Ortiz et al. [14], lipases can be adsorbed to any hydrophobic surface, causing a tendency to form dimer aggregates resulting from the interaction between two open forms of the lipase. The use of surfactants during immobilization is responsible for breaking up these aggregates, favouring the immobilization of enzymes in their monomeric form. The addition of DTT, on the other hand, provides the stabilization of Schiff bases, preserving the enzyme-support interaction until reduction and formation of a proper covalent bond [15]. This effect is especially desirable in situations of low alkaline pH, in which the formation of a single-point bond is favoured, as in the case presented.

The GLA-1 catalyst represented the most similar variation with the free enzyme with no aggregate formation and with the enzyme attached to the support via a single-point covalent bond without steric impediment of the active site. Even more, when covalently attached via a single bond to the support, the lipase behave as in solution, alternating between its closed and inactive (higher percentage) and its open and active conformation (lower percentage). Additionally, at pH 7.0 the thermal stability of GLA-1 was similar to the stability obtained for the soluble and diluted enzyme. Therefore, GLA-1 was chosen as a reference for comparison with all obtained catalysts.

Similarly to GLA-1, for the construction of catalyst GLA-3 (Table 1,

catalyst #4), BGLip was covalently immobilized on agarose activated with glyoxyl groups, but in the absence of Triton X-100 and DTT to investigate the formation of enzyme aggregates. For GLA-4 (Table 1, catalyst #5) the same conditions as GLA-3 were considered, but DTT was added to assess the influence of this thiolated agent.

The difference in immobilization yields between GLA-3 and 4 reveals that DTT did not fulfil its Schiff base stabilizing role as expected. Bolívar et al. [15] demonstrated that in the presence of DTT, or other thiolated compounds, the imines formed between the aldehyde groups of the support and the reactive portions of the enzyme are transiently reduced, stabilizing the maintenance of Schiff bases. The same effect was not observed for *B. gladioli* BRM58833 lipase. The lower immobilization yield under conditions without Triton X-100 confirms that the lipase in its monomeric form results in more stable interactions with the support, while the resulting conformation of the dimeric aggregate does not present an ideal position that allows greater stabilization of the complex.

For the construction of catalysts GLA-5, GLA-6 and GLA-7 (Table 1, catalysts #6, #7 and #8, respectively), a pH of 10.0 was considered for immobilization. This means that the formation of Schiff bases occurred not only to the N-terminal portion, but also to lysine-rich regions that could be exposed on the surface of the enzyme, providing a multi-punctual covalent attachment. Fig. 1 highlights a lysine-rich region on the enzymatic surface close to the N-terminal portion. Thus, immobilization with a similar orientation to that obtained for GLA-1 is assumed. The formation of a multi-point bond to the support, however, promotes the development of a more rigid structure, which may favour the stability of the catalysts but not necessarily their catalytic activity.

GLA-5 and GLA-7 were obtained from the immobilization of BGLip and GLA-6 to the immobilization of SP-S. GLA-7 differed from GLA-5 for the absence of Triton X-100, which also allowed the attachment of aggregates. It was observed that the conditions of multi-punctual immobilization to the support resulted in higher yields as expected, since the increase in the amount of Schiff bases would better stabilize the catalyst prior to the reduction step.

BGLip was also used to immobilize the lipase of *B. gladioli* BRM58833 on hydrophobic supports by interfacial adsorption, producing catalysts BUT, OCT, PHE, C18, MES and LEW (Table 1, catalysts #9 to #14, respectively). In this approach, lipases are immobilized by affinity with their open forms adsorbed to the hydrophobic support. This process is responsible not only for the stabilization of its structures, but also for favouring the protection of the active sites, since they become less accessible to the physical-chemical modification agents that can be applied [8]. As expected, the use of hydrophobic supports resulted in greater immobilization yields when compared to GLA catalysts. Octyl sepharose was the best support for the immobilization of *B. gladioli* BRM58833 lipase with not only a yield of 100%, but also an expressed activity of 136.8%, indicating a hyperactivation profile (Fig. 2). Hyperactivation of lipases when adsorbed to hydrophobic supports is a phenomenon widely discussed in literature and is one of the reasons why these supports have been widely used to immobilize lipases from different sources.

The lipase hyperactivation profile after immobilization in octyl sepharose can also be confirmed by the altered kinetic parameters of the enzyme. The apparent Vmax was determined at $2.4.10^{-1}$ mol $L^{-1}.min^{-1}.mg^{-1}$ for BGLip and $2.1.10^{-1}$ mol $L^{-1}.min^{-1}.mg^{-1}$ for the enzyme immobilized in octyl sepharose. The Km values obtained were $3.28.10^{-2}$ mol L^{-1} and $1.59.10^{-2}$ mol L^{-1} for BGLip and for the OCT catalyst with coefficients of determination (R^2) of 0.99 and 0.97, respectively. The expressive decrease in the Km value reflects an increase of more than twice the affinity to the substrate by the immobilized lipase when compared to its free form.

For immobilization on hydrophobic supports, although the enzyme's active site is positioned towards the surface of the support, the enzyme pocket formed by the contact with the support still allows the entry of large substrates, enabling high activities to be achieved and, in some cases, even higher than those obtained for free lipase [16]. Furthermore,

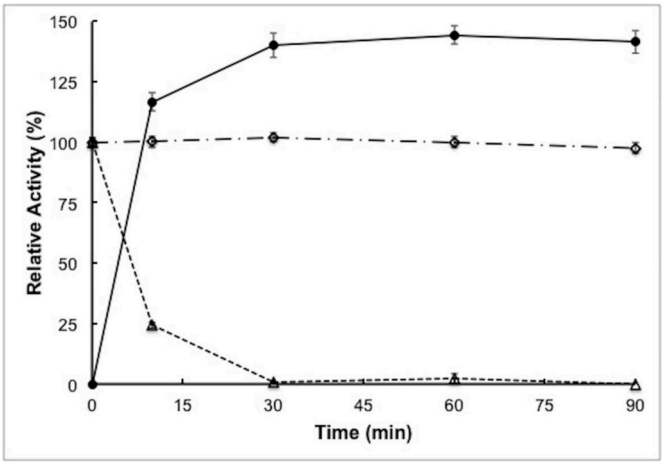

Fig. 2. Immobilization of *B. gladioli* BRM58833 lipase on octyl sepharose. Free enzyme solution, no support added (open circles, dashed line); Immobilization suspension (circles, solid black line); and Immobilization supernatant (triangles, dotted line).

it has been shown that catalysts immobilized by adsorption of the lipases in their open conformation are very stable, being able to achieve even greater stabilities than that of catalysts obtained by multi-punctual covalent bonds [17]. Indeed, OCT showed a thermal stabilization index 16.3 times higher than the control condition and 3.25 times higher when compared to the immobilization condition by the most stable multi-punctual covalent bond catalyst.

3.2. Post-immobilization stabilization

One limitation of the immobilization of lipases by interfacial activation on hydrophobic supports is the possibility of enzyme desorption under high temperature conditions, the presence of organic solvents or even moderate concentrations of surfactants. To circumvent this problem, one of the strategies that can be used is the application of post-immobilization techniques, aiming at establishing more enzyme-support interactions and, thus, providing greater stability to immobilized catalysts.

According to Romero-Fernández et al. [3], among the post-immobilization modification strategies, the use of polymers to coat the enzymatic surface through ionic interactions or covalent bonds stands out as a good complement to immobilization. In this perspective, Guisán et al. [18] proposed the first work involving the coating of lipases immobilized on hydrophobic supports with polyethyleneimine (PEI). PEI is a polycationic polymer displaying a large amount of primary (25%), secondary (50%) and tertiary (25%) amines in its structure, thus functioning as a strong anion exchanger [19]. Another polycationic polymer with interesting properties is polyalylamine (PAA), which has a slightly lower charge density than PEI, displaying all its amines as primary amines. Coating the lipase surface with these polymers results in a protective layer formed from the intense crosslinking between different units of the enzyme and the polymer chain. When adsorbed to hydrophobic supports, the active site cavity is protected from any modification due to its orientation towards the support surface. Another advantage conferred by the process is the hydrophilicity of the formed polymeric layer, which not only reduces enzyme mobility, but also ends up creating microenvironments that function as a protective barrier to the damage of solvents and surfactants and help stabilize the enzyme. According to Rodrigues et al. [16], the intermolecular crosslinking of several lipases with the polymer promotes a multi-point stabilization of the resulting aggregate, requiring the simultaneous desorption of several bonds for the release of the aggregate. Hence, the loss of enzyme to the reaction medium is reduced, allowing the use of the catalyst over more

reaction cycles. Peirce et al. [20] demonstrated this concept when the incubation of CALB immobilized on octyl sepharose in a Triton X-100 solution exhibited a drastically reduced enzymatic loss after coating with PEI.

Since the immobilization of *B. gladioli* BRM58833 lipase in octyl sepharose was the best method to obtain high yields and activities, OCT was chosen as the basis for the development of new catalysts. In this way, OCT was coated with a layer of PEI 25 kDa or PAA 17.5 kDa (Table 2, catalysts #18 and #22, respectively). The PEI coating resulted in a 77.7-fold increase in thermostability when compared to the control and was 3.8 times more stable than the PAA-coated analogue catalyst. Thus, different sizes of PEI, 10 and 60 kDa, were evaluated to produce more catalysts (Table 2, catalysts #17 and #19, respectively). As noted by Romero-Fernández et al. [3], higher molecular weights or coating degrees do not necessarily result in greater stabilities. Different molecular weights will have different effects on the generated microenvironment and on structural changes, resulting in different extensions of stabilization. Thus, after assessing the stability of the constructed catalysts, it was observed that OCT-PEI coated with PEI 25 kDa was the most stable among its 10 and 60 kDa analogues.

A second strategy for coating with polymers was the use of dextran-aldehyde (DEXa). Unlike ionic coating observed for PEI and PAA, coating with DEXa is done through the formation of covalent bonds (mostly two-point bonds with neighbouring amino groups), preventing the reversibility of the process and the regeneration of the supports after its use. Thus, the addition of DEXa is capable of forming small intersections, which reduce the mobility of the immobilized enzyme and enable a stability increase of the developed conjugate [11].

The combination of stabilization strategies with the construction of layers of polycationic polymers and DEXa produces a viscous polymer with interesting stabilizing effects on immobilized lipases. The PAA-coated catalyst was especially expected to improve its stability with the addition of a second layer of DEXa, as PAA contains two close amino groups capable of reacting with DEXa aldehydes. Indeed, the increase in stability for OCT-PAA-DEXa (Table 2, catalyst #23) was 5.4 times greater than its single-layer counterpart. However, although the increase observed for catalyst # 23 was greater than the 3.4-fold increase achieved for OCT-PEI-DEXa (Table 2, catalyst #20) when compared each with its one-layer counterpart, catalyst #20 was the one that obtained the highest thermal stabilization index, exhibiting a stability 263.8 times greater than the control.

When not used freshly prepared, DEXa aldehyde groups tend to react with each other, producing a polymer with different characteristics. This aged (oxidized) DEXa polymer is also capable of reacting with amino groups, but in a way that one-point bonds are favoured. Therefore, the same combination used to produce catalysts #20 and #23 was applied to the construction of catalysts with aged DEXa (Table 2, catalysts #21 and #24). The combination of the PAA-covered catalyst with aged DEXa produced a derivative 1.8 times more stable than its counterpart made with freshly prepared DEXa. For the catalyst covered with PEI as a first layer, however, the preparation with fresh DEXa resulted in a more stable catalyst than with the use of aged DEXa.

Another strategy in the construction of stable catalysts of *B. gladioli* BRM58833 lipase was the use of DEXa as a first layer. Thus, OCT was coated either 10 mg g^{-1} or 50 mg g^{-1} of DEXa (Table 3, catalysts #29 and #30, respectively). As discussed earlier, higher coating degrees do not necessarily result in greater stabilities. In fact, treatments with different DEXa concentrations resulted in 2.6-fold improvements in the stabilization index for the catalyst obtained with less DEXa.

Alternatively, the enzyme immobilized in OCT was first coated with DEXa and, before the reduction step, methoxypolyethylene glycol amine (NH_2-PEG) was offered to the preparation. NH_2-PEG is a hydrophobic polymer capable of reacting with remaining aldehyde groups from the initial OCT incubation in DEXa. In this way, a second protective layer is added to the catalyst, assisting in the reduction of adverse effects of high temperatures or solvents present in the reaction medium [21]. Thus, NH_2-PEG (2 kDa) was added at a concentration of either 25 mg g^{-1} or 250 mg g^{-1} to the DEXa layer of catalyst #29, resulting in catalysts #31 and #32, respectively (Table 3). Indeed, the addition of NH_2-PEG induced the formation of more stable catalysts, with a 1.5-fold increase in the stability of the derivative coated with a single layer of DEXa.

The use of less aggressive reducing agents was also assessed. Therefore, the use of 2-picoline borane instead of sodium borohydride was evaluated. Since the reduction with this reducing agent takes longer, this reaction step was extended for 16 h. Thus, catalyst #34 was produced with 2-picoline borane as a reducing agent and catalyst #33 with the standard method using sodium borohydride after being exposed to NH_2-PEG for 16 h. After treatment with 2-picoline borane, a 7.9% increase in half-life was observed. However, despite the prolonged

Table 2
Thermal stabilization of *B. gladioli* BRM58833 and *B. cepacia* lipases immobilized in octyl sepharose.

#	Support	Coating with Polymers		$t_{1/2}$ (h)	Thermal Stabilization Index
		First Layer	Second Layer		
B. gladioli BRM58833 Lipase					
17	OCT	PEI 10 kDa	–	11.6	49.1
18	OCT	PEI 25 kDa	–	18.4	77.7
19	OCT	PEI 60 kDa	–	8.0	34.0
20	OCT	PEI 25 kDa	DEXa	62.5	263.8
21	OCT	PEI 25 kDa	DEXa (aged)	53.5	226.1
22	OCT	PAA 17.5 kDa	–	4.8	20.2
23	OCT	PAA 17.5 kDa	DEXa	25.9	109.5
24	OCT	PAA 17.5 kDa	DEXa (aged)	46.7	197.2
***B. cepacia* Lipase**					
25	OCT-cepacia	PEI 25 kDa	–	42.5	179.4/12.3[a]
26	OCT-cepacia	PEI 25 kDa	DEXa	74.8	315.8/24.2[a]
27	OCT-cepacia	PAA 17.5 kDa	–	25.6	108.0/7.2[a]
28	OCT-cepacia	PAA 17.5 kDa	DEXa	84.9	358.7/27.8[a]

[a] Thermal stabilization index calculated considering GLA-cepacia as control.

Table 3
Thermal stabilization of *B. gladioli* BRM58833 lipase immobilized in octyl sepharose with dextran-aldehyde and bifunctional polymers.

#	Support	Coating with polymers		$t_{1/2}$ (h)	Thermal Stabilization Index
		First Layer	Second Layer		
B. gladioli BRM58833 Lipase					
29	OCT	DEXa 10 mg g^{-1}	–	18.3	77.2
30	OCT	DEXa 50 mg g^{-1}	–	6.9	29.1
31	OCT	DEXa 10 mg g^{-1}	$PEG_{2\ kDa}$ 25 mg g^{-1}	26.7	112.9
32	OCT	DEXa 10 mg g^{-1}	$PEG_{2\ kDa}$ 250 mg g^{-1}	27.9	117.7
33	OCT	DEXa 10 mg g^{-1}	$PEG_{2\ kDa}$ 250 mg g^{-1} (overnight)	31.5	133.1
34	OCT	DEXa 10 mg g^{-1}	$PEG_{2\ kDa}$ 250 mg g^{-1} (overnight) + 2-picolino	34.0	143.8
35	OCT	DEXa 10 mg g^{-1}	$PEG_{10\ kDa}$ 250 mg g^{-1}	47.6	201.2
36	OCT	DEXa 10 mg g^{-1}	PEG_{10}/Gly	38.2	161.2
37	OCT	DEXa 10 mg g^{-1}	Gly/PEG_{10}	28.5	120.4

exposure to sodium borohydride, this method was still considered more interesting, since it involved a simpler methodology and less preparation time.

When added to catalysts, NH_2-PEG is able to increase viscosity and stability at the cost of also increasing hydrophobicity and the chance of exposing enzyme's hydrophobic pockets, which could, in turn, reduce its activity. However, the construction of a second layer with NH_2-PEG resulted in a more stable catalyst than the coating with a single layer of DEXa. Still, to evaluate whether the construction of a second layer formed by a bifunctional polymer (with hydrophilic and hydrophobic characteristics) would be advantageous, the addition of glycine to the NH_2-PEG layer was studied (Fig. 3). This is the first report of an attempt to build a bifunctional layer combining NH_2-PEG and glycine. Along these lines, catalyst #29 was first offered to NH_2-PEG and then to glycine to obtain catalyst #36. Alternatively, catalyst #37 was obtained by reversing the incubation order, adding glycine first. Contrary to expectations, the construction of a second layer with bifunctional polymers did not result in more stable catalysts than when using only 10 kDa NH_2-PEG.

The catalysts that stood out for their thermal stability were selected to stability evaluation under alkaline conditions, being incubated at pH 10 (Table 4). In general, all catalysts showed higher half-lives when subjected to an alkaline treatment, showing greater susceptibility to thermal inactivation than by the alkaline effect of the medium. As observed for thermal inactivation, the construction of polymeric layers covering the lipase immobilized on octyl sepharose resulted in stability gains. Among the catalysts built in this work, OCT-PEI-DEXa (#20) stands out, being the catalyst most resistant to thermal inactivation and the third most resistant to the effect of an alkaline pH. However, the catalyst that showed greater stability at pH 10 was OCT-DEXa-PEG_{10}/Gly, exhibiting a half-life of 184.3 h and a stabilization index of 70.1 in relation to the control condition. This result highlights a better effect of the bifunctional layer on the protection against alkaline conditions for the studied lipase.

3.3. Comparison with B. cepacia lipase

The lipase from *B. cepacia* was used as a standard for comparison with the results obtained for the *B. gladioli* lipase BRM58833. Thus, the same conditions of immobilization and stabilization that resulted in greater stabilities for the lipase of *B. gladioli* BRM58833 were reproduced with its commercially available counterpart. Firstly, *B. cepacia* lipase was immobilized on activated agarose with glyoxyl groups to obtain GLA-cepacia (Table 1, catalyst #15), which, like GLA-1, represented the preparation most similar to the free enzyme and could be used as a reference for comparisons with other catalysts. GLA-cepacia exhibited not only a higher yield and expressed activity than GLA-1, but was also 12.3 times more stable (Fig. 4a).

Subsequently, the *B. cepacia* lipase was immobilized on octyl sepharose to obtain OCT-cepacia (Table 1, catalyst #16), as this support resulted in the best immobilization yield and activity expressed for the *B. gladioli* BRM58833 lipase. As observed for OCT, OCT-cepacia also indicated a hyperactivation profile with an expressed final activity of 114.3%. In terms of stabilization, OCT-cepacia was 15.9-fold more stable than OCT (Fig. 4b). Proportionately, however, the *B. gladioli* BRM58833 lipase exhibited a better improvement in activity and stability than its counterpart when each was compared to its initial conditions.

Finally, the conditions used to coat OCT with polycationic polymers and DEXa were replicated for OCT-cepacia, since these catalysts were the ones that resulted in the highest stabilization indexes for *B. gladioli* BRM58833 lipase. Therefore, catalysts #25 to #28 were obtained in the same way as their counterparts (Table 2). As observed for OCT, OCT-cepacia was also better stabilized when coated in two layers. Differently, however, PAA served as a better coating polymer than PEI in this case, resulting in a stabilization index of 27.8-fold greater when compared to GLA-cepacia and 358.7-fold greater when compared to GLA-1. Despite presenting a high stabilization index when compared to GLA-1, proportionally the stabilization obtained for the lipase of *B. gladioli* BRM58833 was superior to that obtained for *B. cepacia* when each one was compared with its control condition (Fig. 4c).

Table 4
Stabilization of immobilized catalysts of *B. gladioli* BRM58833 and *B. cepacia* lipases at alkaline pH.

#	Support	Coating with Polymers		$t_{1/2}$ (h)	Alkaline Stabilization Index
		First Layer	Second Layer		
B. gladioli BRM58833 Lipase					
2	GLA-1	–	–	2.6	1.0
10	OCT	–	–	47.5	18.1
20	OCT	PEI 25 kDa	DEXa	85.8	32.6
21	OCT	PEI 25 kDa	DEXa (aged)	49.4	18.8
23	OCT	PAA 17.5 kDa	DEXa	53.0	20.2
24	OCT	PAA 17.5 kDa	DEXa (aged)	93.8	35.7
32	OCT	DEXa 10 mg g^{-1}	$PEG_{2\ kDa}$ 250 mg g^{-1}	70.9	27.0
35	OCT	DEXa 10 mg g^{-1}	$PEG_{10\ kDa}$ 250 mg g^{-1}	74.9	28.5
36	OCT	DEXa 10 mg g^{-1}	PEG_{10}/Gly	184.3	70.1
***B. cepacia* Lipase**					
15	GLA-cepacia	–	–	5.7	2.2/1.0[a]
16	OCT-cepacia	–	–	6.3	2.4/1.1[a]
26	OCT-cepacia	PEI 25 kDa	DEXa	18.6	7.1/3.3[a]
28	OCT-cepacia	PAA 17.5 kDa	DEXa	27.9	10.6/4.9[a]

[a] Alkaline stabilization index calculated considering GLA-cepacia as control.

3.4. Hydrolysis of fish oil

After immobilization and stabilization of *B. gladioli* BRM58833

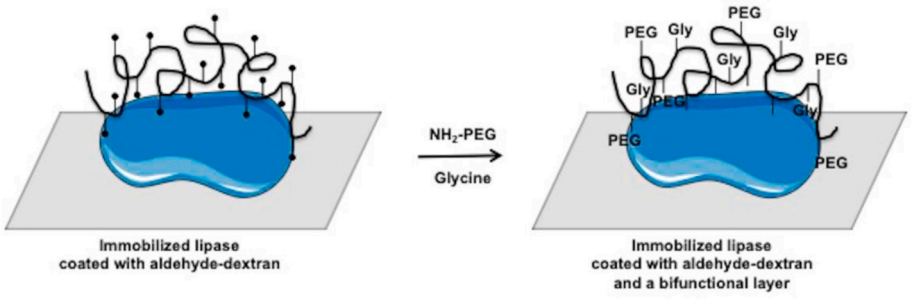

Fig. 3. Dextran-aldehyde as a basis for the construction of a bifunctional layer of methoxypolyethylene glycol amine and glycine around the surface of the enzyme.

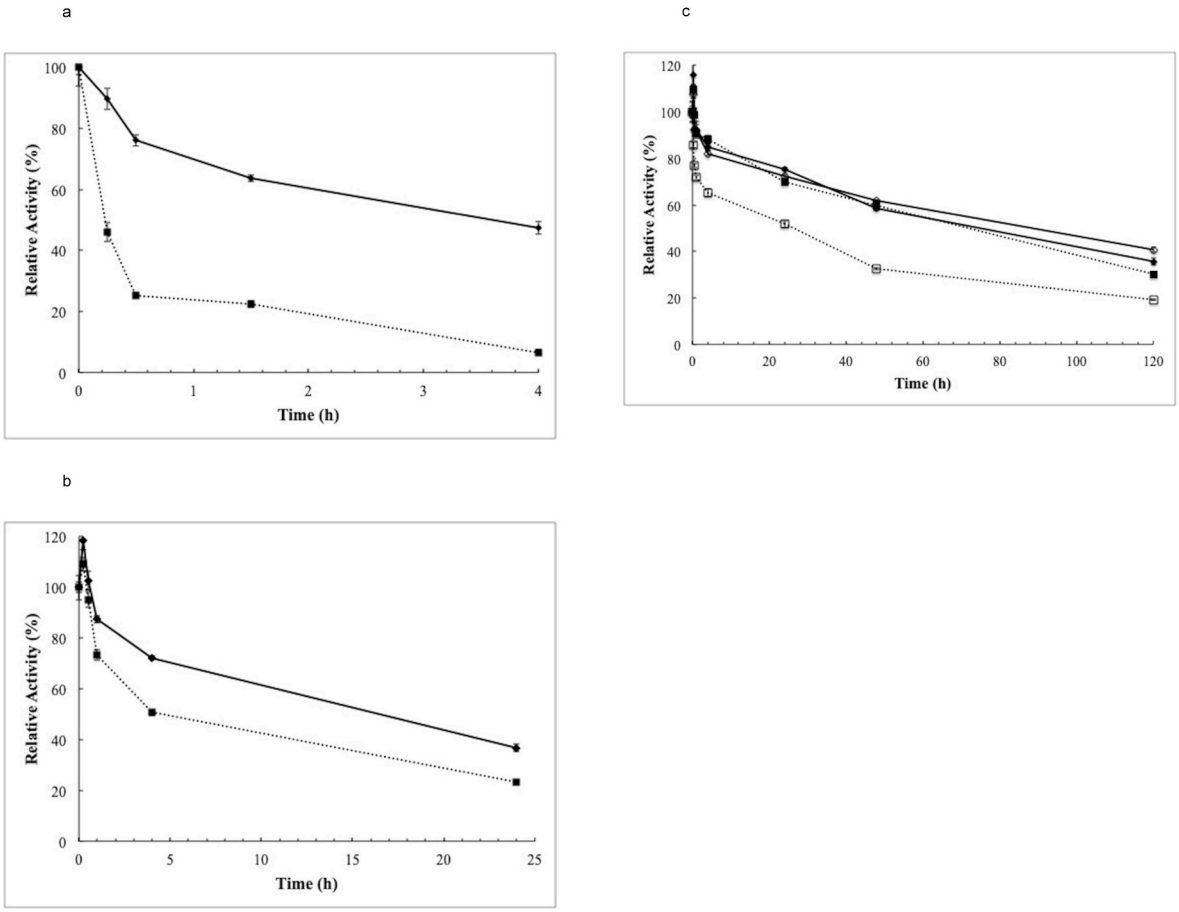

Fig. 4. Thermal inactivation course of immobilized catalysts of *B. cepacia* (solid lines) and *B. gladioli* BRM58833 (dotted lines) lipases. (A) Lipases immobilized on agarose activated with glyoxyl groups; (B) Lipases immobilized on octyl sepharose; (C) Two-layer coated catalysts: *B. cepacia* OCT-PEI-DEXa (filled diamond, solid line); *B. cepacia* OCT-PAA-DEXa (empty diamond, continuous line); *B. gladioli* BRM58833 OCT-PEI-DEXa (filled square, dotted line); *B. gladioli* BRM58833 OCT-PAA-DEXa (empty square, dotted line).

lipase, the most stable catalysts were applied for the hydrolysis of fish oil for the production of docosahexaenoic acid (DHA).

Initially, the action of the lipase of *B. gladioli* BRM58833 immobilized in octyl sepharose was studied in comparison to the lipases of *B. cepacia* and *Thermomyces lanuginosus* immobilized in the same conditions (Fig. 5). *T. lanuginosus* lipase is a well studied enzyme in the hydrolysis of PUFAs, having been extensively applied to fish oil hydrolysis reactions [12,22] and, for this reason was selected for comparison.

When the immobilized catalysts were obtained considering the same low enzymatic load on octyl sepharose (1 mg g^{-1}), a higher DHA content was observed for the reactions with *B. cepacia* lipase, reaching a DHA concentration of 23.6 ± 0.1 mmol L^{-1} in 48 h of reaction, which corresponded to 55.6% of the calculated maximum content. As for the catalysts of *T. lanuginosus* and *B. gladioli* BRM58833, 28.4 and 8.4% of the maximum content were found, respectively (Fig. 5a). On the other hand, when the immobilization was standardized by lipolytic activity offered to the support (60 U g^{-1}), a higher DHA content was observed in the reactions prepared with the *T. lanuginosus* lipase, reaching 64.3% of the maximum content in 48 reaction h (Fig. 5b). Obtaining a *T. lanuginosus* lipase catalyst by standardizing lipolytic activity against *p*NPP resulted in a much higher hydrolysis when evaluated against fish oil. *T. lanuginosus* lipase exhibit a low activity against *p*NPP under the test conditions used, requiring a higher protein load to match *Burkholderia* lipases. Thus, the discrepancy resulting from the extrapolation of lipolytic activity between different substrates is evident.

The lipase of *B. gladioli* BRM58833 immobilized in octyl sepharose did not reached a competitive performance for the hydrolysis of fish oil when compared to the commercial enzymes. The highest concentration of DHA obtained was 3.6 mmol L^{-1} after 48 h of reaction, equivalent to 8.4% of the calculated maximum content. In order to evaluate the influence of the stabilization techniques used, the hydrolysis of fish oil was carried out with the two catalysts that stood out the most for their stability. Thus, OCT-PEI-DEXa was selected for its thermal stability and OCT-DEXa-PEG_{10}/Gly was selected for its stability against alkaline environments (Fig. 6).

This study showed that hydrolysis catalysed by *B. gladioli* BRM58833 lipase resulted in successful concentrations of DHA with different efficiencies depending on the immobilized catalyst used. After 72 h of reaction, the levels of DHA obtained for OCT, OCT-PEI-DEXa and OCT-DEXa-PEG_{10}/Gly were 12.7%, 5.2% and 19.5%, respectively. There was a significant increase in the level of DHA with the use of the OCT-DEXa-PEG_{10}/Gly, demonstrating the potential of the coating technique with bifunctional polymers to obtain more stable catalysts with greater catalytic capacity.

If taken into consideration the initial release rates obtained for all catalysts applied to fish hydrolysis, however, the potential of *B. gladioli* BRM58833 lipase catalysts and the stabilization techniques used is revealed (Table 5). OCT initial DHA release rates obtained are comparable to those obtained for CALB and RML immobilized in Octyl sepharose [23]. Even more, *B. gladioli* BRM58833 lipase catalysts coated with polymers resulted in faster initial rates than its solely immobilized counterpart. Considering assay conditions, OCT-DEXa-PEG_{10}/Gly stands out once more both as a faster and more stable catalyst than OCT.

In general, however, it can be said that in the long run there is still

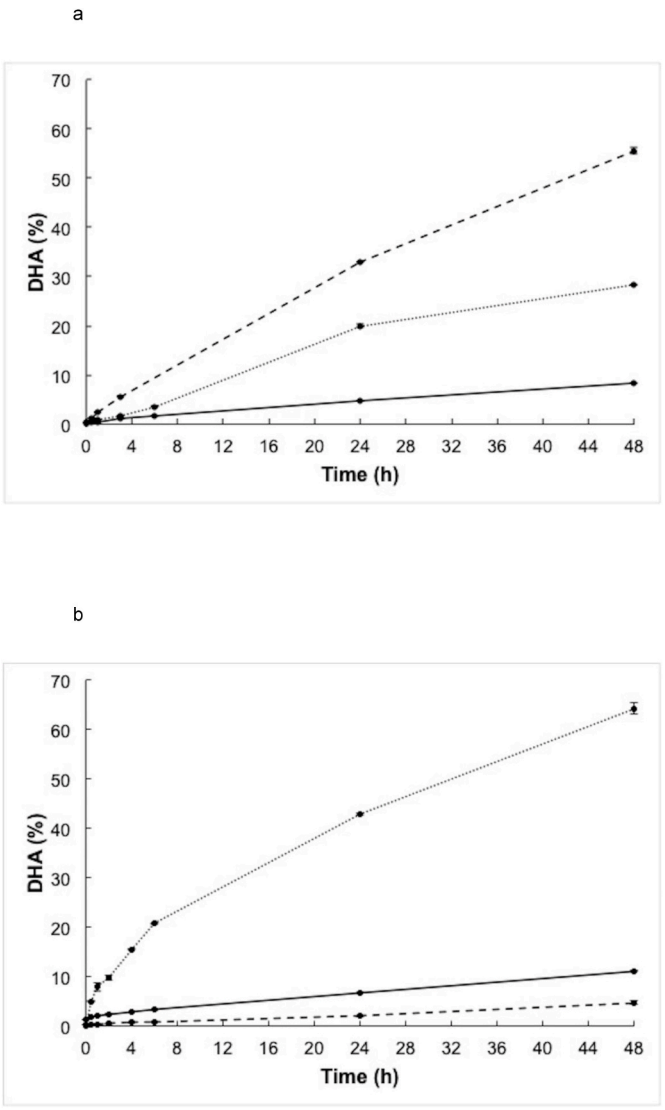

Fig. 5. Hydrolysis of fish oil to obtain docosahexaenoic acid. Comparison between the lipases of *Thermomyces lanuginosus* (dotted line), *Burkholderia cepacia* (dashed line) and *B. gladioli* BRM58833 (solid line) immobilized in octy sepharose. (A) Standardized immobilization by enzymatic load of 1 mg of protein per g of support; (B) Standardized immobilization with 60 U per g of support.

much to be developed until the *B. gladioli* BRM58833 lipase becomes competitive with the commercial enzymes concomitantly evaluated. According to Pizarro et al. [24], the dynamics of hydrolysis behaves differently for saturated and monounsaturated and for PUFAs. Whereas for the first group hydrolysis behaves as a saturation curve, for PUFAs the hydrolysis increases over time in a proportional manner. This circumstance reveals a restriction catalytic effect. Indeed, the structural conformation of PUFAs, characterized by long chains curved by the presence of consecutive unsaturations, may represent a steric impediment to the catalytic activity of some lipases [25]. The curvature of the chains brings the methyl terminal groups of PUFAs closer to the ester bonds of the triacylglycerol molecule, preventing the active site to reach the bond and protecting these compounds from hydrolysis catalysed by lipases. As for the relatively straight chains of saturated and monounsaturated fatty acids, the same effect is not observed. Thus, it has been suggested that triacylglycerols with PUFAs in their composition should be hydrolysed afterwards to triacylglycerols without these compounds in their composition due to the recognition of the molecular structure of the ester. This phenomenon could explain the low activity of the

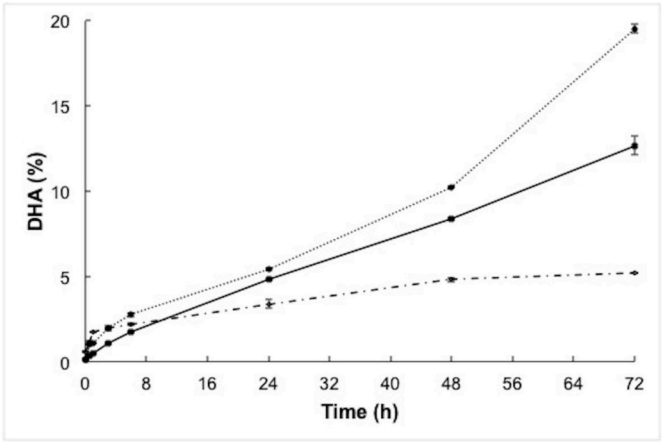

Fig. 6. Use of stabilized lipase catalysts of *B. gladioli* BRM58833 for the hydrolysis of fish oil. OCT: solid line; OCT-PEI-DEXa: dashed line; OCT-DEXa-PEG10/Gly: dotted line.

Table 5
Hydrolysis of fish oil. Activity is expressed as micromoles of DHA released per min and per milligram of immobilized lipase within 1 h of reaction.

Catalyst	Activity ($U.mg^{-1}$)
OCT-TLL	0.096 ± 0.011
OCT-cepacia	0.284 ± 0.001
OCT	0.057 ± 0.003
OCT-PEI-DEXa	0.207 ± 0.002
OCT-DEXa-PEG_{10}/GLY	0.127 ± 0.001

immobilized lipase catalysts of *B. gladioli* BRM58833 against these substrates. Nevertheless, further studies with higher enzyme loads and different reaction conditions are needed to demonstrate the potential of these lipases in the hydrolysis of fish oil.

4. Conclusion

The lipase obtained from *Burkholderia gladioli* BRM58833 was successfully immobilized and, in accordance to other literature reports, hydrophobic supports resulted in higher yields of immobilization and activity. Coating the surface of octyl sepharose immobilized enzymes with polymers resulted in catalysts with increased stabilities against high temperatures and alkaline conditions. OCT-PEI-DEXa and OCT-DEXa-PEG_{10}/Gly were the most stable regarding thermal and alkali inactivation. According to the results obtained, the combination of immobilization and post-immobilization techniques can modulate not only the lipase stability but also its catalytic properties. The strategies proposed and evaluated throughout this study could effectively stabilize *B. gladioli* BRM58833 lipase, resulting in stabilization indexes proportionally higher than those obtained for *B. cepacia* lipase. In terms of fish oil hydrolysis, high initial rates of hydrolysis were obtained for the stabilized catalysts that are comparable to other studies reported in literature. Preliminary results demonstrate the potential of the coating technique with bifunctional polymers formed by NH_2-PEG and glycine, resulting in a stable catalyst with greater catalytic capacity for the production of omega-3 PUFAs. This was the first study that reported the construction of a bifunctional layer in this manner. Yet, further studies are necessary to improve reaction conditions so higher contents of omega-3 PUFAs may be obtained. Finally, it is believed that the stabilization of *B. gladioli* lipase BRM58833 can improve its resistance and, therefore, allow its reuse for more reaction cycles, preserving its activity and selectivity for longer periods of time, reducing the associated costs to the use of these enzymes and enabling their industrial application.

Author statement

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Declaration of competing interest

The authors declare no conflicts of interest.

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