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ORIGINAL PAPER

# Production of MLM-Type Structured Lipids Catalyzed by Immobilized Heterologous *Rhizopus oryzae* Lipase

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**Abstract** This work aims to produce triacylglycerols (TAG) containing a medium-chain fatty acid (M) at positions *sn*-1,3 and a long-chain fatty acid (L) at *sn*-2 position, i.e. TAG of MLM type, by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids, catalyzed by 1,3-selective *Rhizopus oryzae* heterologous lipase (rROL) immobilized in Eupergit® C and modified sepiolite. This lipase was produced by the methylotrophic yeast *Pichia pastoris*. Reactions were performed at 25 and 40 °C, for 24 h, either in solvent-free or in *n*-hexane media, at a molar ratio 1:2 (olive oil:free fatty acid). Higher incorporations of C8:0 (21.6 mol%) and C10:0 (34.8 mol%) into the TAG were attained in solvent-free media, at 40 °C, when rROL immobilized in Eupergit® C was used. In organic media, at 40 °C, only 15.9 and 14.1 mol%, incorporation of C8:0 or C10:0 were, respectively observed. Lower incorporations were attained for both acids (3.4–7.0 mol%) when native ROL (nROL) in both supports and rROL in modified sepiolite were used. rROL in Eupergit® C maintained its activity during the first four or three 23-h batches, respectively when C8:0 (half-life time, $t_{1/2} = 159$ h) or C10:0 ($t_{1/2} = 136$ h) were used, decreasing thereafter following a time delay model.

**Keywords** Acidolysis · Capric acid · Caprylic acid · Olive oil · *Rhizopus oryzae* lipase · Structured lipids

**Abbreviations**

| | |
|---|---|
| FFA | Free fatty acid(s) |
| L | Long-chain fatty acid(s) |
| M | Medium-chain fatty acid(s) |
| MAG | Monoacylglycerol(s) |
| MLM | Triacylglycerol(s) containing medium-chain fatty acid at *sn*-1 and *sn*-3 positions (M) and a long-chain fatty acid (L) at position *sn*-2 |
| nROL | Native ROL |
| rROL | Heterologous ROL |
| ROL | *Rhizopus oryzae* lipase |
| SL | Structured lipid(s) |
| TAG | Triacylglycerol(s) |
| $t_{1/2}$ | Half-life time |

## Introduction

The production of triacylglycerols (TAG) with medium chain fatty acids, M, in positions *sn*-1 and *sn*-3, and a long-chain fatty acid, L, in the internal position, i.e. of MLM type, has recently increased due to its nutritional interest. These are low calorie and dietetic structured lipids (SL) adequate to control obesity and also for persons

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with fat malabsorption and other metabolism problems [1, 2]. The presence of medium-chain fatty acids and long-chain fatty acids in the same TAG molecule will contribute to the unique nutritional and metabolic properties of these speciality lipids. Medium-chain fatty acids are saturated fatty acids with 6–12 carbon atoms; long-chain fatty acids are saturated or unsaturated fatty acids with 14–24 carbons [1]. Enzymatic catalysis has been the main process used to obtain SL because of the advantages over the chemical process. Lipases (EC 3.1.1.3, triacylglycerol acylhydrolase) belong to the family of hydrolases that act on carboxylic ester bonds. In nature, lipases catalyze the hydrolysis of triacylglycerols into diacylglycerols (DAG), monoacylglycerols (MAG), free fatty acids (FFA) and glycerol. However, when in non-aqueous media, they can also catalyze esterification, alcoholysis and trans-esterification reactions [3]. The major benefit of using lipases comes from the region-specificity shown by many lipases, which are described as being *sn*-1,3-specific. Therefore, the changes can be made at *sn*-1 and *sn*-3 glycerol positions but not at *sn*-2 position, where the ester group remains unchanged [4]. The *sn*-1,3-specific lipases have been used to promote the incorporation of medium-chain fatty acids, such as caprylic (C8:0) and capric (C10:0) acids, into TAG molecule of several vegetable and fish oils to obtain SL with important clinical and nutritional properties [5–11].

In the majority of these studies, high-cost commercial immobilized lipases have been used. Olive oil enriched with caprylic acid (43 mol%) was obtained by acidolysis catalyzed by a commercial immobilized lipase from *Rhizomucor miehei*, Lipozyme RM IM [12]. Also, Lipozyme TL IM, a commercial immobilized lipase from *Thermomyces lanuginosa,* was used for the acidolysis of corn oil with caprylic acid in *n*-hexane: under optimized conditions, 21.5 mol% of caprylic acid was incorporated [13]. Jennings and Akoh [14] modified rice bran oil with capric acid, using Lipozyme IM as the biocatalyst: after 24 h of the reaction, 26.5 and 24.5 mol% incorporation of capric acid were obtained in hexane and in solvent-free media, respectively.

The limitations of the industrial use of lipases have been mainly due to their high production costs, which may be overcome by molecular technologies to enable the large-scale production of enzymes, as well as in a more purified form [15–17]. Immobilization in solid carriers is perhaps the most used strategy to improve the operational stability of biocatalysts [18]. As alternative to commercial immobilized lipases, Hamam and Shahidi [19] successfully incorporated capric acid into docosahexaenoic acid single cell oil (DHASCO) using lipase PS-30 from *Pseudomonas* sp. as biocatalyst. After a 24 h reaction time and using a mole ratio of 1:3 (DHASCO/capric acid), the incorporation of capric acid was 31.4 mol%. In addition, a novel lipase from *Pichia lynferdii* NRRL Y-7723 was successfully used as catalyst for the acidolysis of borage oil with caprylic acid: after 24 h reaction, 47.5 mol% incorporation of caprylic acid was attained, in *n*-hexane and at an enzyme load of 10 wt% [20].

This work aims to produce TAG of MLM type by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acid, in *n*-hexane and in solvent-free media, catalyzed by native *R. oryzae* lipase (nROL) and heterologous *R. oryzae* lipase (rROL) expressed in *P. pastoris* [21, 22], both immobilized in modified sepiolite and Eupergit® C. Eupergit® C has been successfully used for lipase immobilization by several authors [23, 24]. Modified sepiolite is another solid support that has been used in the immobilization of lipases: the immobilized enzymes showed high stability and great re-usability without a significant loss of their initial catalytic activity [17, 25, 26]. Therefore, the framework of this study is the search for biocatalysts as an alternative to the commercial immobilized lipases used in the majority of acidolysis studies.

## Materials and Methods

### Materials

Portuguese extra virgin olive oil (an acidity of 0.2% expressed as free oleic acid) was purchased from a local supermarket. The fatty acid profile (mol%) of this olive oil is: 18.0% C16:0; 1.5% C16:1; 3.0% C18:0; 72.6% C18:1 and 6.1% C18:2. The native 1,3-selective lipase from *R. oryzae* was kindly given by Amano Enzyme Europe Ltd., UK and the rROL was produced in *P. pastoris* (cf. "Production of Heterologous *R. oryzae* Lipase and Characterization of Native and Recombinant *R. oryzae* Lipase"). Eupergit® C was kindly donated by Röhm GmbH & Co., Degussa, Darmstadt, Germany. Eupergit® C is made by co-polymerization of *N,N′*-methylene-bis(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide. Sepiolite is a natural low-cost hydrated magnesium silicate that presents a fibrous structure and was obtained from Tolsa S.A (Spain). The theoretical formula of the unit cell is $Si_{12}O_{30}Mg_8(OH)_6(–H_2O)_4{\cdot}8H_2O$, where $Si^{4+}$ and $Mg^{2+}$ can be partially replaced by $Al^{3+}$, $Fe^{2+}$ and alkaline ions. Each atom of Mg completes their coordination with two molecules of water.

Porcine pancreatic lipase (30.1 U/mg) was purchased from Applichem. The Bradford protein assay kit was purchased from Bio-Rad. Caprylic acid, capric acid, and fatty acid methyl esters were purchased from Fluka. All solvents and reagents for analyses were chromatographic or analytical grade and obtained from different sources.

## Methods

### *Production of Heterologous R. oryzae Lipase and Characterization of Native and Recombinant R. oryzae Lipase*

The rROL was produced in a *P. pastoris* Mut$^s$ phenotype strain, in a fed-batch cultivation selecting a mixed substrate strategy using sorbitol as co-substrate and methanol as inductor. Culture medium, fed-batch cultivation set up and operational conditions are described in Arnau et al. [27]. The biomass was removed from the culture broth by centrifugation. The supernatant was then concentrated by ultrafiltration with a Centracasette® Pall Filtron system equipped with an Omega membrane of 10 kDa cut-off, and subsequently dialyzed against 10 mM Tris–HCl buffer at pH 7.5 and finally lyophilized.

Native (nROL) and heterologous ROL (rROL) have been previously characterized. Molecular weight of ROL lipase is cited between 29 and 35 kDa [28]. nROL showed three bands recognized by a western blot analysis and the predominant band has a molecular weight slightly higher than 34 kDa. rROL only presented two bands showing a molecular weight slightly lower than nROL. This difference should be due to different post-translational modification between native and recombinant lipase. Interestingly, western blot and zymogram of rROL showed a third band of 45.5 kDa with low activity, probably a native protein from *P. pastoris* linked with the lipase. On the other hand, zymogram of nROL also revealed a positive of a protein of molecular weight of 40 kDa identified as an esterase. These lyophilized powders also showed important differences in terms of substrate specificity against *p*-nitrophenol esters. Similar substrate specificity towards triacylglycerols was observed for nROL and rROL. They preferred middle-chain triacylglycerols (Tri-C8 and Tri-C10). When substrate specificity towards *p*-nitrophenol was studied, rROL specificity increased as carbon chain number was increased, opposite to nROL behavior. This data was corroborated with other commercial extracts from Sigma (nROL). The presence of an esterase on nROL could justify this fact (Guillén et al. 2010, Biochemical Engineering Journal, submitted).

### *Lipase Immobilization in Eupergit® C*

The immobilization procedure of lipases from *R. oryzae* was via oxirane groups as previously described [24]. This conventional method for enzyme immobilization on Eupergit® C support involves the direct enzyme binding on polymers via oxirane groups. Unmodified Eupergit® C (1 g) was incubated with native or recombinant lipase (200 mg) in 5 $cm^3$ of 0.1 M sodium phosphate buffer (pH 7.0) solution at room temperature (25 °C) with no agitation for 96 h. Then, the beads were recovered by vacuum filtration with a glass filter and washed with 0.1 M sodium phosphate buffer solution, pH 7.0 (2 × 25 $cm^3$).

### *Lipase Immobilization in Modified Sepiolite*

Both nROL and rROL were covalently immobilized in modified sepiolite ($AlPO_4$-sepiolite) as described by Bautista et al. [25]. In the present case, covalent immobilization of the enzyme was carried out through the ε-amino group of lysine residues of lipases. The immobilization was carried out at 25 °C by thoroughly mixing the lipase powder (0.5 g) and the modified sepiolite (4 g) in 10 $cm^3$ of 0.1 M phosphate buffer solution (0.1 M $KH_2PO_4$, pH 6.5), for 24 h. After this period, 40 $cm^3$ of the same phosphate buffer solution were added to the immobilization mixture and the immobilized lipase was recovered by centrifugation at 8,720*g* for 5 min.

### *Determination of Lipase Load in Supports*

After immobilization in Eupergit® C and in modified sepiolite, lipase loads (nROL and rROL) were determined according to Bradford method [29]. For each lipase, a standard curve was obtained. The amount of bound enzyme was determined from the difference between the amount of enzyme present in the initial buffer solution ($P_0$), before immobilization support (Eupergit® C and modified sepiolite) was added, and the residual amount of enzyme in this solution and in the washing solutions ($P_1$), after immobilization.

The efficiency of immobilization was estimated as follows:

$$\eta = \frac{P_0 - P_1}{P_0} \times 100 \quad (1)$$

### *Acidolysis Reaction*

The substrate mixture consisted of 3 g of olive oil and 0.98 g of caprylic (C8:0) or 1.17 g of capric acid (C10:0), corresponding to a mole ratio olive oil to free fatty acid (FFA) of 1:2. This molar ratio corresponds to the stoichiometric value needed for the esterification of the free fatty acids at *sn*-1 and *sn*-3 positions because the biocatalysts used are *sn*-1,3-selective. A fixed amount of immobilized lipase (5% or 10 wt% of total substrates) was used. Reactions were carried out in solvent-free media or in *n*-hexane media, at 40 °C, in thermostated-capped cylindrical glass vessels under magnetic stirring at 400 rpm. In *n*-hexane media, a ratio of total substrates/solvent of 1:1.5 (v/v) was used. To select the best biocatalyst and reaction conditions (solvent vs. solvent-free media, reaction temperature); 24 h acidolysis reactions were performed. In time-course

experiments, 0.5 mL samples were withdrawn along 48 h reaction time and stored at −20 °C until analysis. All reactions were performed in triplicate and average values of caprylic or capric acid incorporation were reported.

In order to investigate the presence of eventual significant differences among biocatalysts and system conditions (solvent, solvent-free system, reaction temperature), for each medium-chain fatty acid, ANOVA of molar incorporation values was carried out using LSD post-hoc multiple comparison tests by running the program Statistica™, version 6, from Statsoft, Tulsa, USA.

### *Batch Operational Stability Tests*

The operational stability of the immobilized biocatalyst presenting the highest acidolysis activity in screening experiments was assayed in repeated batches carried out in solvent-free media at 40 °C. A load of 5% of immobilized lipase and a molar ratio olive oil: FFA of 1:2 was used. At the end of each batch (23 h), the immobilized lipase was removed from the reaction medium by centrifugation at 8,720*g* for 5 min and was added into fresh medium and reused in the subsequent batch. Biocatalyst activities were estimated at the end of each batch as the molar incorporation of caprylic or capric acid into the TAG of virgin olive oil. The first batch was used as the reference. The residual activity of the biocatalyst at the end of each batch $n$ ($n = 1,\ldots,13$) was thus estimated as the ratio between the activity observed at the end of batch $n$ and the activity observed at the end of batch one.

The fit of enzyme deactivation kinetics models to experimental data (time, residual activity) was carried out using "Solver" add-in from Excel for Windows, version 8.0 SR2, by minimizing the residual sum-of-squares between the experimental data points and those estimated by the respective model and considering the following options: Newton method; $10^4$ iterations, precision of $10^{-7}$; 2% of tolerance and $10^{-4}$ of convergence. The kinetic constants were obtained by this non-linear regression analysis for the tested models.

### *Analysis of Products*

The product mixture was separated by thin-layer chromatography (TLC) on silica gel plates and developed with *n*-hexane/ethyl ether/acetic acid (70:30:1.5, v/v/v). After, TLC plates were air-dried and sprayed with 0.2% (w/v) 2′,7′-dichlorofluorescein in 95% ethanol and the bands were visualized under ultraviolet light at 366 nm. The various groups of compounds (triacylglycerols, free fatty acids, diacylglycerols and monoacylglycerols) were identified by comparison with standards. The bands corresponding to TAG were scraped from TLC plates and methylated, as follows: the silica gel containing TAG was mixed with 5 mL of methylation reagent (anhydrous methanol/*n*-hexane/concentrated sulfuric acid; 75/25/1, v/v/v), in a conical flask equipped with a Liebig condenser. This mixture was allowed to boil under reflux for 60 min in a water bath at about 80 °C. Then, 10 mL of distilled water and 10 mL of petroleum ether were added and the mixture was transferred to a separating funnel, vigorously agitated and allowed to settle for phase separation. The organic upper layer was recovered, washed twice with distilled water (2 × 10 mL) and dried with anhydrous sodium sulphate. Sodium sulfate was removed by paper filtration, the solution was transferred to a conical-bottom flask and the solvent was evaporated in a Rotavapor at 30 °C under a pressure lower than 200 mbar.

Fatty acid methyl esters (FAME) were dissolved in 100 μL of 0.1% (w/v) methyl myristate (internal standard) in *n*-hexane solution and 1 μL of this solution was analyzed in a GC 14A gas chromatograph (Shimadzu; Kyoto, Japan) equipped with a FID and a DB-wax capillary column (30 m × 0.32 mm i.d., $df = 0.50$ μm). Injection and detector temperatures were set at 250 and 260 °C, respectively. Helium was used as the carrier gas at a column head pressure of 10 kPa and a flow rate of 1 mL/min and the injector was used in split mode with a ratio of 1:20. The oven temperature program was as follows: 110 °C for 7 min, temperature increase to 170 °C at 30 °C/min, a plateau at 170 °C for 45 min, temperature increase to 230 °C at 7.5 °C/min and a final plateau at 230 °C for 15 min.

The relative content of FAME was calculated as a molar percentage on the basis of the molecular weight of each FAME. All analyses were performed in triplicates and average values were reported.

### *Pancreatic Lipase-Catalyzed sn-2 Positional Analysis*

The identification and quantification of the esterified fatty acids at the *sn*-2 position in the structured triacylglycerols was assayed following the pancreatic lipase hydrolysis procedure, adapted from Jennings and Akoh [14]. Two milliliters of 1 M Tris buffer (adjusted to pH 8.0 with HCl), 0.5 mL of 0.05% (w/v) sodium cholate solution, 0.2 mL of 2.2% (w/v) calcium chloride solution and 20 mg of pancreatic lipase were added to TAG samples. The mixture was incubated at 40 °C for 5 min. Then, 1 mL of ethanol, 1 mL 6N hydrochloric acid and 1 mL of diethyl ether were added and the mixture was vortexed and centrifuged at 2,180*g* for 5 min. The clear phase was spotted onto a silica gel TLC plate for separation and the band corresponding to the *sn*-2 monoacylglycerols was scraped from the TLC plate, methylated and analyzed by GC, as previously described (cf. "Analysis of Products").

## Results and Discussion

### Biocatalyst and Reaction Conditions Selection

TAG of MLM type were produced by acidolysis of olive oil with caprylic or capric acid, catalyzed by the 1,3-selective nROL and rROL immobilized in Eupergit® C or in modified sepiolite. The immobilization yields for both lipases immobilized in Eupergit® C or modified sepiolite are shown in Table 1. The incorporation values of caprylic or capric acids into virgin olive oil, obtained with 5% of biocatalyst, are presented in Table 2. Higher immobilization yields were obtained for both lipases in modified sepiolite (about 96%) than in Eupergit® C (86.0 and 95.7%, for the heterologous and the native ROL, respectively). However, high immobilization yields are not synonymous of high acidolysis activity (Table 2). This may probably be ascribed to an eventual mass transfer limitations and/or to lipase deactivation, after covalent immobilization, due to the irreversible stabilization of several inactive conformation structures of lipases.

For each enzyme preparation, the incorporation is always higher for capric than for caprylic acid (Table 2). The preference for a longer chain fatty acid is a characteristic of lipases, in contrast with the behavior of esterases [3]. For each fatty acid, ANOVA of incorporation values was performed (Table 2). Concerning caprylic acid, the majority of incorporation values were statistically different at a significance level lower than 0.05. However, when nROL immobilized in Eupergit® C and rROL immobilized in modified sepiolite were used, similar incorporation values were attained at 40 °C in solvent-free media. With respect to capric acid incorporation, no significant differences were observed when nROL immobilized in Eupergit® C or nROL and rROL in modified sepiolite were used (Table 2).

Higher incorporation values of 21.6 mol% of C8:0 and 34.8 mol% of C10:0 were attained in solvent-free media at 40 °C, when rROL immobilized in Eupergit® C was used. At 25 °C in solvent-free media, the incorporation levels were lower than those obtained at 40 °C: 12.6 and 19.6 mol% of C8:0 and C10:0, respectively. Reaction temperature is an important factor in lipase-catalyzed acidolysis and generally, higher temperatures will promote higher yields for endothermic reactions.

In addition, in organic media at 40 °C, the incorporation of C8:0 or C10:0 into olive oil TAG, catalyzed by the heterologous ROL in Eupergit® C, was only 16.0 and 14.1 mol%, respectively. The presence of *n*-hexane in the reaction media may have a negative effect on the incorporation of C8:0 or C10:0 into olive oil, probably due to a dilution effect of the substrates in the reaction medium, as previously reported by Xu et al. [30]. When rROL was immobilized in modified sepiolite, lower incorporations of caprylic or capric acids into olive oil at 40 °C were observed (4.4 and 5.5 mol%, respectively). Similar and low incorporation levels were obtained when the nROL immobilized in Eupergit® C or in modified sepiolite was used: the incorporation of caprylic or capric acid into the olive oil was only 3.4 or 5.9 mol% and 6.3 or 6.9 mol%, respectively when native ROL was immobilized in Eupergit® C or in modified sepiolite. Therefore, the rROL immobilized in Eupergit® C was the biocatalyst selected for subsequent experiments.

In previous experiments carried out by our group, higher values of incorporation of caprylic and capric acids into virgin olive oil were also observed in solvent-free media than in solvent media, when acidolysis was catalyzed by

**Table 1** Immobilization yield of heterologous and native ROL immobilized in Eupergit® C or in modified sepiolite

| Biocatalyst | Immobilization yield (%) |
|---|---|
| Native ROL in Eupergit® C | 90.2 |
| Native ROL in modified sepiolite | 95.7 |
| Heterologous ROL in Eupergit® C | 86.0 |
| Heterologous ROL in modified sepiolite | 95.7 |

**Table 2** Incorporation of caprylic or capric acid (mol%) into TAG of olive oil, catalyzed by immobilized heterologous and native ROL immobilized in Eupergit® C or in modified sepiolite

| Biocatalyst | Temperature (°C) | Modified olive oil | | | |
|---|---|---|---|---|---|
| | | With C8:0 | | With C10:0 | |
| | | *n*-Hexane | Solvent-free | *n*-hexane | Solvent-free |
| Native ROL in Eupergit® C | 40 | | 3.39 ± 0.50[b] | | 6.27 ± 0.33[b] |
| Native ROL in modified sepiolite | 40 | | 5.86 ± 0.03[c] | | 6.98 ± 1.51[b] |
| Heterologous ROL in Eupergit® C | 25 | | 12.55 ± 1.1[d] | | 19.56 ± 0.26[c] |
| | 40 | 15.98 ± 0.01[a] | 21.60 ± 1.4[e] | 14.09 ± 0.69[a] | 34.82 ± 0.97[d] |
| Heterologous ROL in modified sepiolite | 40 | | 4.38 ± 0.13[b] | | 5.48 ± 0.12[b] |

Mean ± SD, $n = 3$. For each acid, different letters mean that the incorporation values are significantly different at a $p$ value $\leq 0.05$; conversely, results indicated by the same letter mean that they are not significantly different at this $p$ value

the commercial immobilized lipases Lipozyme TL IM, Lipozyme RM IM and Novozym 435 (data not shown).

## The Effect of the Amount of Immobilized rROL in Eupergit® C

Figure 1 shows the time–course of the acidolysis of virgin olive oil with C8:0 or C10:0 in solvent-free media, at 40 °C and for 48 h, using 5 and 10% (weight of immobilized lipase per weight of substrates) of immobilized rROL in Eupergit® C, as biocatalyst. Incorporation of C8:0 or C10:0 into olive oil increased with time, with an initial reaction rate of 0.941 and 1.28 mol%/h of incorporation, respectively, and reached a maximum of incorporation (22.4 or 34.8 mol%) between 10 and 24 h reaction time, when 5% biocatalyst was used. When the biocatalyst load was 10%, the incorporation rate of C10:0 was higher (3.14 mol%/h incorporation) but a similar maximum incorporation level (34.1 mol%) was reached. Therefore, the choice of lipase load will depend on enzyme cost and on the reaction time required. Prolonging the incubation time for more than 24 h did not improve the incorporation levels for both enzyme loads. Similar results were reported by others [14, 31].

For subsequent experiments, the heterologous ROL immobilized in Eupergit® C was used at a load of 5%.

## Operational Stability of Heterologous *R. Oryzae* Lipase

The operational stability of immobilized rROL in Eupergit® C was evaluated in repeated use along 13 consecutive 23-h batches, carried out at 40 °C in solvent-free media. The residual activity data and the deactivation models fitted to these data points are presented in Fig. 2. The biocatalyst showed a time delay ($t_d$) where the enzyme maintains its activity, decreasing thereafter [24]. To describe the

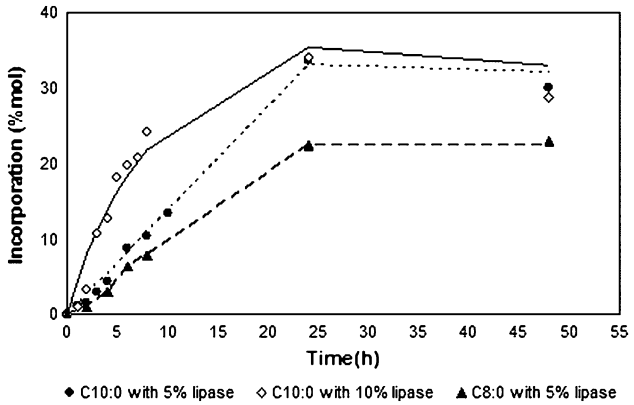


**Fig. 1** Effect of reaction time on the incorporation of caprylic acid (C8:0) or capric acid (C10:0) into olive oil (molar ratio olive oil: free fatty acid of 1:2) catalyzed by 5 and 10% of rROL immobilized in Eupergit® C, at 40 °C, in solvent-free media

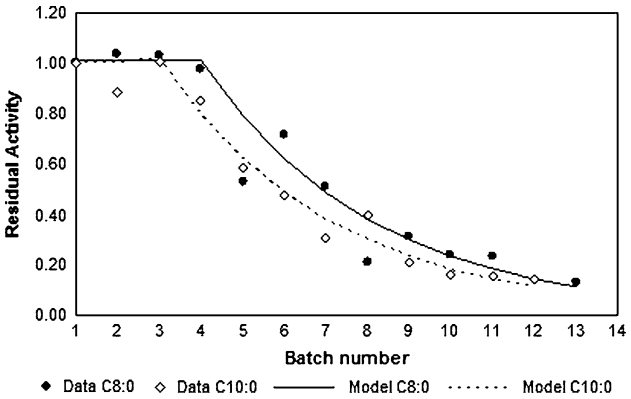


**Fig. 2** Residual activity of rROL immobilized in Eupergit® C at the end of each consecutive 23-h batch, when acidolysis of olive oil with caprylic acid (C8:0) or capric acid (C10:0) was performed

deactivation kinetics, the first-order deactivation model, given by the following equation, was tested:

$$A_n = \mathrm{A}e^{-k_d n} \quad (2)$$

where $A_n$ is the biocatalyst residual activity at batch $n$, A is a constant, $k_d$ is the deactivation rate constant in (batch number)$^{-1}$ and $n$ is the 23-h batch number.

A time delay ($t_d$) model for enzyme inactivation can be formulated as:

$$\mathrm{A} = 1 \quad \text{for } t \leq t_d \quad (3)$$

Then, Eq. 2 is used for $t > t_d$.

The best-fit values of time delay, deactivation constants ($k_d$) and half-life time ($t_{1/2}$) are listed in Table 3. The half-life time of the biocatalyst was higher in the presence of caprylic (159 h) than in the presence of capric acid (136 h). The decrease in the operational stability with the increase in chain length might be due to modifications in the protonation state of the enzyme under the influence of the free fatty acids used: the $p\mathrm{K_a}$ of caprylic acid (4.85) is lower than the $p\mathrm{K_a}$ of capric acid (4.93) [32].

Similar deactivation model was reported for *Candida rugosa* lipase immobilized in Eupergit® C [24].

## Positional Analysis of Structured Lipids

The *sn*-2 positional analysis of native and modified olive oil was determined (Table 4). The modified olive oil was obtained by acidolysis of olive oil and caprylic or capric acids with a molar ratio of 1:2 (oil:free fatty acid), at 40 °C and 5% enzyme load, after 24 h. Of the total fatty acids at *sn*-2 position, C8:0 or C10:0 represent only 15.4 or 17.1%, respectively. Therefore, 82.3 or 80.4% of modified olive oil is of MLM type where long-chain fatty acids, placed at *sn*-2 position, are mainly oleic or linoleic acids. The presence of C8:0 and C10:0 at the *sn*-2 position, even though a 1,3 specific lipase was used, demonstrates some acyl migration.

**Table 3** Deactivation model equations, time delay and half-life for the heterologous *Rhizopus oryzae* lipase immobilized in Eupergit® C

| Reaction system | Model equation | Time delay (h) | Half-life time (h) |
|---|---|---|---|
| Acidolysis of olive oil with C8:0 | $A = 1$ for $t \leq 92.3$ h $A_n = 2.62e^{-0.24n}$ for $t > 92.3$ h | 92.3 | 159 |
| Acidolysis of olive oil with C10:0 | $A = 1$ for $t \leq 70.8$ h $A_n = 2.13e^{-0.25n}$ for $t > 70.8$ h | 70.8 | 136 |

**Table 4** The *sn*-2 positional fatty acid composition (mol%) of olive oil and modified olive oil, obtained by 24 h acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids in solvent-free media, at 40 °C, catalyzed by the heterologous ROL immobilized in Eupergit® C

| Fatty acid | Olive oil | Modified olive oil | |
|---|---|---|---|
| | | With C8:0 | With C10:0 |
| C8:0 or C10:0 | – | 15.40 ± 1.13 | 17.08 ± 0.18 |
| C16:0 | 0.9 ± 0.8 | 2.31 ± 0.13 | 2.56 ± 0.06 |
| C18:1 | 91.1 ± 1.73 | 76.63 ± 1.94 | 75.63 ± 0.28 |
| C18:2 | 8.0 ± 0.87 | 5.65 ± 0.20 | 4.73 ± 0.05 |

Mean ± SD, $n = 2$

Also, Jennings and Akoh [33] obtained 11.5 mol% of C10:0 at sn-2 position after the modification of fish oil in hexane, for 24 h reaction, using Lipozyme RM IM as biocatalyst. Also, no significant differences in acyl migration were observed in structured lipids synthesized by acidolysis of perilla oil and caprylic acid, in solvent or in solvent-free systems, using two commercial immobilized lipases, Lipozyme RM IM and Lipozyme TL IM [5].

## Conclusions

The rROL immobilized in Eupergit® C was able to catalyze the incorporation of 21.6 and 34.8 mol% of caprylic or capric acid into virgin olive oil, upon 24 h at 40 °C in solvent-free media, showing better performance than native ROL. Lower incorporation values (3.4–7.0 mol%) were obtained when acidolysis was catalyzed by rROL immobilized in modified sepiolite and by native ROL immobilized in Eupergit® C or in modified sepiolite.

The selected biocatalyst, rROL immobilized in Eupergit® C, presented high stability for the 4 or 3 first consecutive reuses of 23 h, when C8:0 or C10 was incorporated into olive oil, respectively. After this time delay, its activity decreased rapidly following a first-order deactivation model. Some acyl migration occurred although a 1,3 selective lipase was used for the acidolysis reactions.

The results obtained are rather promising concerning the search for alternative biocatalysts to the high-cost commercial immobilized lipases. The use of a minimal, cheap and defined medium fermentation in *P. pastoris* processes, the high activity of the enzyme produced and the minimum and cheap downstream processing applied to recover ROL suggests that this biocatalyst could be interesting for industrial application.

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