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Production of human milk fat substitutes enriched in omega-3 polyunsaturated fatty acids using immobilized commercial lipases and *Candida parapsilosis* lipase/acyltransferase

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ABSTRACT

In human milk fat (HMF), palmitic acid (20–30%), the major saturated fatty acid, is mostly esterified at the *sn*-2 position of triacylglycerols, while unsaturated fatty acids are at the *sn*-1,3 positions, conversely to that occurring in vegetable oils.

This study aims at the production of HMF substitutes by enzyme-catalyzed interesterification of tripalmitin with (i) oleic acid (system I) or (ii) omega-3 polyunsaturated fatty acids (omega-3 PUFA) (system II) in solvent-free media. Interesterification activity and batch operational stability of commercial immobilized lipases from *Rhizomucor miehei* (Lipozyme RM IM), *Thermomyces lanuginosa* (Lipozyme TL IM) and *Candida antarctica* (Novozym 435) from Novozymes, DK, and *Candida parapsilosis* lipase/acyltransferase immobilized on Accurel MP 1000 were evaluated. After 24-h reaction at 60 °C, molar incorporation of oleic acid was about 27% for all the commercial lipases tested and 9% with *C. parapsilosis* enzyme. Concerning omega-3 PUFA, the highest incorporations were observed with Novozym 435 (21.6%) and Lipozyme RM IM (20%), in contrast with *C. parapsilosis* enzyme (8.5%) and Lipozyme TL IM (8.2%). In system I, Lipozyme RM IM maintained its activity for 10 repeated 23-h batches while for Lipozyme TL IM, Novozym 435 and *C. parapsilosis* enzyme, linear (half-life time, $t_{1/2}$ = 154 h), series-type ($t_{1/2}$ = 253 h) and first-order ($t_{1/2}$ = 34.5 h) deactivations were respectively observed. In system II, Lipozyme RM IM showed linear deactivation ($t_{1/2}$ = 276 h), while Novozym 435 ($t_{1/2}$ = 322 h) and *C. parapsilosis* enzyme ($t_{1/2}$ = 127 h), presented series-type deactivation. Both activity and stability of the biocatalysts depended on the acyl donor used.

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1. Introduction

In the field of edible oils and fats, research on lipase-catalyzed production of structured lipids (SL) presenting specific functional properties has greatly increased due to the potential benefits of the enzymatic route relatively to chemical processes [1]. Lipases (triacylglycerol acylhydrolases, EC. 3.1.1.3) catalyze ester hydrolysis in aqueous media, but when in organic media at low water activity, they can also catalyze esterification and transesterification reactions [2]. Lipase-catalyzed reactions are carried out under milder conditions (temperature lower than 70 °C, atmospheric pressure) and with a higher selectivity than chemically catalyzed reactions. In addition, the use of 1,3-selective lipases allows to maintain the fatty acids in the *sn*-2 position of the acylglycerols. This is nutritionally desirable and not possible to attain by chemical catalysis.

The synthesis of triacylglycerols (TAG) modified in their fatty acid composition is usually carried out by 1,3-specific lipase-catalyzed acidolysis of an ester (a single TAG, oil or fat) with a free fatty acid (FFA). The term "acidolysis" is classically used to describe a reaction where a fatty acyl group is exchanged through a combination of hydrolysis of a donor ester and esterification of the released alcohol moiety with another free fatty acid.

The human milk fat substitutes obtained by the enzymatic route are among the most important structured lipids, for the food industry. Human milk fat (HMF) contains long-chain fatty acids, namely oleic (30–35%), palmitic (20–30%), linoleic (7–14%) and stearic acids

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(5.7–8%). Unlike in vegetable oils and in cow milk fat, in HMF, palmitic acid, the major saturated fatty acid, is mostly esterified at the *sn*-2 position of the triacylglycerols, while unsaturated fatty acids are at the external positions. The use of vegetable oils and cow milk fat as a substitute of HMF in infant formulas may cause a deficient calcium and fatty acid absorption due to the formation of insoluble calcium soaps with saturated fatty acids released by the action of the 1,3-specific pancreatic lipase [3].

Nowadays, "Betapol™", a commercial structured lipid made of vegetable oils by position-specific enzymatic interesterification, is used as HMF substitute (HMFS) in both premature and term infant formula. This product is manufactured by Lipid Nutrition, Wormerveer, in the Netherlands [4].

In order to produce HMFS that mimic human milk fat, several studies have been carried out using immobilized lipases as catalysts. In the majority of these studies, HMFS were obtained by acidolysis of tripalmitin or lard (rich in palmitic acid) with free fatty acids from different sources.

Thus, HMFS containing palmitic, oleic, stearic and linoleic acid [3], long-chain polyunsaturated and essential fatty acids [5,6], gamma-linolenic acid [7], and omega-3 polyunsaturated fatty acids (omega-3 PUFA) [8], were obtained. The incorporation of omega-3 PUFA in infant formulas, and docosahexaenoic acids (DHA, 22:6n-3) in particular, has known benefits in the development of brain and nervous system of infants [9,10]. HMFS were also obtained by interesterification of (i) tripalmitin with blends of vegetable oils [11,12] or of (ii) lard with soybean oil [13].

Nowadays, the high cost of the commercial non-immobilized and immobilized lipases, together with a low operational stability of the latter, have been recognized as the major constraints to their use in the food industry. To make the enzymatic process competitive, lipases should be reusable and stable.

The high operational stability exhibited by several immobilized lipases in different reaction systems is a key factor in terms of the economical and technological competitiveness with chemical catalysts. The industrial implementation of an enzymatic process depends not only on the catalytic activity of the biocatalyst but also and principally on its operational stability.

The majority of the studies on the production of structured lipids use high cost commercial immobilized lipases. In this study, in order to search for biocatalysts with eventual novel properties as alternative to these commercial biocatalysts, the performance of immobilized *Candida parapsilosis* lipase/acyltransferase, as catalyst for the acidolysis aimed at the production of HMFS, was investigated. Although the free form of this enzyme presents only a slight 1,3-regioselectivity during hydrolysis and transesterification in aqueous media [14], it was included in this study in order to evaluate its potential in acidolysis in organic systems. The lipase/acyltransferase, when in aqueous or in biphasic aqueous/organic media, preferentially catalyzes alcoholysis over hydrolysis [14,15]. We have previously shown that this enzyme was able to catalyze the interesterification of fat blends containing omega-3 PUFA, in solvent-free media, batchwise and in a continuous fluidized-bed reactor. The lipase/acyltransferase presented, at a water activity (a_w) of 0.97, an interesterification activity similar to that exhibited by commercial immobilized lipases at a_w values lower than 0.5 [16,17].

In the present study, the production of HMFS was performed by enzyme-catalyzed acidolysis of tripalmitin with (i) oleic acid (system I) or (ii) omega-3 PUFA (system II), in solvent-free media at 60 °C. Four immobilized enzymes were tested in the same systems: *C. parapsilosis* lipase/acyltransferase and the immobilized commercial lipases from *Rhizomucor miehei* (Lipozyme RM IM), *Thermomyces lanuginosa* (Lipozyme TL IM) and *Candida antarctica* lipase B (Novozym 435). In both reaction systems, the activity and batch operational stability of these enzymes were evaluated in order to select the best biocatalyst.

2. Materials and methods

2.1. Materials

Tripalmitin (95% purity; MW = 807.35), 2′,7′-dichlorofluorescein and methyl myristate standard (>99%) were obtained from Fluka; extra pure oleic acid was from Merck and sodium cholate 99% was from Acros Organics. The commercial concentrate of triacylglycerols rich in omega-3 PUFA, "EPAX 1050TG" (10% eicosapentaenoic acid, EPA, and 50% docosahexaenoic acid, DHA), was a gift from EPAX AS, Lysaker, Norway. Silica-Gel 60 (0.25 mm width, 20 cm × 20 cm) thin layer chromatography (TLC) plates were purchased from Merck. The standards of triolein, diolein (mixed isomers) and monoolein were from Sigma–Aldrich. The other reagents used were *p.a.* and obtained from various sources.

The immobilized thermostable 1,3-selective lipases from *T. lanuginosa* ("Lipozyme™ TL IM") and from *R. miehei* ("Lipozyme™ RM IM"), and the immobilized thermostable preparation of the lipase B from *C. antarctica* ("Novozym™ 435") were kindly donated by Novozymes™, A/S, Bagsvaerd, Denmark. The lipase/acyltransferase from *C. parapsilosis* was produced by overexpression of the corresponding gene in *Pichia pastoris* according to Brunel et al. [18] and immobilized on Accurel MP 1000 (Membrana GmbH, Obernburg, Germany) as previously described [16]. Hog pancreas lipase (30.1 U/mg) was purchased from Fluka.

2.2. Methods

2.2.1. Preparation of free omega-3 PUFA

The preparation of free omega-3 PUFA from "EPAX 1050TG" was carried out according to the method described by Sahín et al. [3]: 25 g of "EPAX 1050TG" were saponified using a mixture of potassium hydroxide (5.75 g), 11 mL of water and 66 mL of 95% (v/v) aqueous ethanol, by refluxing at 100 °C for 60 min, in a flask equipped with a Liebig condenser. Distilled water (50 mL) was added to the saponified mixture and transferred to a separating funnel, where the unsaponifiable matter was extracted by *n*-hexane (2× 100 mL) and discarded. The aqueous layer containing the saponified matter was acidified to pH 1.0 with 3N HCl. The released free fatty acids were extracted with *n*-hexane (50 mL) and dried with anhydrous sodium sulphate. Sodium sulphate was removed by paper filtration and the *n*-hexane was evaporated in a rotavapor at 40 °C and a pressure lower than 200 mbar. A TLC of the obtained FFA was performed (*cf.* 2.2.4.1.) in order to confirm the efficiency of the process. The obtained FFA were stored at −18 °C under nitrogen until use.

2.2.2. Interesterification reaction

Interesterification reactions were performed for 24 h in 20 mL closed thermostated cylindrical batch reactors at 60 °C under magnetic stirring. Two different reaction media were used: (i) 3.90 g tripalmitin and 2.76 g oleic acid (system I) and (ii) 3.90 g tripalmitin and 3.17 g omega-3 PUFA (system II). The amounts of tripalmitin and free fatty acids (FFA) correspond to a molar ratio FFA:tripalmitin of 2:1, calculated on the basis of molecular weights of oleic acid (282.5) and of DHA (328.5), respectively.

A load of 8.9% (w/w tripalmitin) of the immobilized enzyme was added to the reaction medium, after complete melting.

Prior to and after 24 h reaction time, 1 mL samples were taken and the biocatalyst was removed by paper filtration at approximately 70 °C. All samples were stored at −18 °C for subsequent analysis. All the experiments were carried out in triplicate.

For each system, molar incorporation degree (%) was calculated on the basis of molecular weight of oleic acid (system I) and of DHA (system II), respectively.

2.2.3. Batch operational stability tests

In each system, batch operational stability of the biocatalysts was evaluated in consecutive 23 h batches. Interesterification was carried out as previously described (*cf.* 2.2.2.). After each batch, the biocatalyst was removed from the reaction medium by paper filtration and reused in the next batch with fresh medium, under the same reaction conditions. A total of up to 10 batches were performed using the same biocatalyst sample.

The activity of the biocatalyst corresponds to the molar incorporation degree of oleic acid (system I) or omega-3 PUFA (system II) in tripalmitin, observed at the end of each batch. The first batch was used as the reference (100% activity). The residual activity (a_n, %) of the biocatalyst at the end of each batch n ($n = 1, \ldots, 10$) was thus estimated as follows:

$$a_n = \left(\frac{\text{Incorporation}_{\text{Batch-}n}}{\text{Incorporation}_{\text{Batch_1}}}\right) \times 100 \quad (1)$$

The operational half-life time of the biocatalyst, i.e., the operation time needed to reduce its original activity to 50%, was estimated by the models fitted to the observed deactivation profiles.

The fit of the deactivation models to experimental data 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, using the following options: Newton method; 100 iterations, precision of 10^{-5}; 5% of tolerance and 0.001 convergence.

2.2.4. Analysis of reaction products

2.2.4.1. Evaluation of incorporation degree. To determine the amount of oleic acid or omega-3 PUFA incorporated in TAG, the following procedure was followed for each sample: 0.15 g of the reaction medium was dissolved in 25 mL of chloroform *p.a.* and 200 μL of this solution was spotted on a continuous layer on a silica gel TLC plate. Elution was carried out in *n*-hexane/diethyl ether/acetic acid (70/30/1.5, v/v/v) as the mobile phase. Plates were sprayed with 0.2% (w/v) 2′,7′-dichlorofluorescein in 95% ethanol and observed under UV at 366 nm. The various groups of compounds (triacylglycerols, free fatty acids, diacylglycerols and monoacylglycerols) were identified by comparison with standards.

The TAG band was scrapped off and methylated, in order to be assayed as fatty acid methyl esters (FAME) by gas chromatography (GC). For methylation, the silica gel containing TAG was mixed with 5 mL of methylation reagent (anhydrous methanol/*n*-hexane/concentrated sulphuric 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 sulphate 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.

The 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 GC analyzed. A Finnigan TRACE GC Ultra gas chromatograph (Thermo Electron Corporation) equipped with a Thermo TR-FAME capillary column (30 m × 0.25 mm ID × 0.25 μm film), an auto sampler AS 3000 from Thermo Electron Corporation and a flame ionization detector, was used for FAME analysis. Injector (in splitless mode) and detector temperatures were set at 250 °C and 260 °C, respectively. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Air and hydrogen were supplied to the detector at flow rates of 350 mL/min and 35 mL/min, respectively.

For the analysis of samples of system I, the oven temperature program was as follows: 60 °C for 1 min, a temperature increase to 150 °C at 15 °C/min, a plateau at 150 °C for 1 min, followed by temperature increase to 180 °C at 5 °C/min, a plateau at 180 °C for 3 min, an increase in temperature until 220 °C, at a rate of 10 °C/min and a final plateau at 220 °C for 1 min.

For the analysis of samples of system II, the oven temperature program was as follows: 60 °C for 1 min, a temperature increase to 150 °C at 15 °C/min, a plateau at 150 °C for 1 min, followed by temperature increase to 220 °C, at a rate of 5 °C/min and a final plateau at 220 °C for 10 min.

2.2.4.2. Fatty acid composition at the sn-2 position. The fatty acid composition at the *sn*-2 position of the modified triacylglycerols was determined according to the following protocol, adapted from Jennings and Akoh [19]: each sample (1 g of fat) obtained after 24-h enzymatic acidolysis was dissolved in chloroform (5 mL) and 300 μL of this solution was spotted in a continuous layer on a silica gel TLC plate and developed as previously described (*cf.* 2.2.4.1.).

The band corresponding to the TAG fraction was scrapped off, the TAG fraction was extracted by diethyl ether (3× 5 mL) and the solvent evaporated in a rotavapor. TAG were re-suspended in 2 mL 0.1 M Tris–HCl aqueous buffer (pH 8.0) with 0.5 mL of 0.1% (w/v) sodium cholate aqueous solution and 0.2 mL of 22% (w/v) calcium chloride aqueous solution. Pancreatic lipase (50 mg) was added to this mixture and the hydrolysis was carried out at 40 °C. After 5 min, the reaction was stopped by the addition of 1 mL 6N HCl aqueous solution; 3 mL of ethyl ether was added and the mixture was centrifuged for 5 min at 1200 × g. The upper organic layer was recovered, the solvent evaporated, the extract was re-suspended in 300 μL of diethyl ether and spotted in a continuous layer on a silica gel TLC plate and developed. The band corresponding to the *sn*-2 monoacylglycerols was scrapped off, methylated and GC analyzed as previously described (*cf.* 2.2.4.1.).

3. Results and discussion

3.1. Acidolysis activity

All the enzymes were tested as catalysts for both the incorporation of oleic acid or omega-3 PUFA in tripalmitin. Since the hydrolytic activity is not always correlated with interesterification activity [20], the biocatalysts were used at the same weight ratio, in order to give some hints for industrial applications, in terms of process costs estimation [21].

For each system, the molar incorporation degree is presented in Table 1. After 24-h reaction, the incorporation levels of oleic acid were about 27–28%, for all the commercial lipases tested, and only 9% with *C. parapsilosis* enzyme. When omega-3 PUFA were incorporated in tripalmitin, the highest incorporation levels were observed for Novozym 435 (21.6%) and Lipozyme RM IM

Table 1
Average molar incorporation values (%) of oleic acid (system I) or omega-3 PUFA (system II) in tripalmitin, upon 24-h acidolysis of tripalmitin, catalyzed by different biocatalysts (±standard deviation; 3 repetitions).

Biocatalyst	System I	System II
Lipozyme RM IM	27.10 ± 0.35	20.80 ± 1.33
Lipozyme TL IM	27.10 ± 0.07	8.20 ± 0.57
Novozym 435	28.20 ± 0.07	21.60 ± 1.60
Lipase/acyltransferase	9.00 ± 2.13	8.50 ± 2.26

(20.8%); with *C. parapsilosis* lipase/acyltransferase and Lipozyme TL IM, only 8.5 and 8.2% incorporation was detected, respectively. Thus, the immobilized *C. parapsilosis* lipase/acyltransferase was not affected by the replacement of oleic acid with omega-3 PUFA in the reaction medium. Conversely, the immobilized *T. lanuginosa* lipase (Lipozyme TL IM) seemed to present higher affinity for oleic acid than for omega-3 PUFA.

However, in previous studies, Lipozyme TL IM showed to be adequate to catalyze the interesterification of blends of palm stearin, palm kernel oil and concentrates of triacylglycerols rich in omega-3 PUFA ("EPAX 2050TG", rich in DHA, and "EPAX 4510TG", rich in EPA) in solvent-free media [22,23]. Also, a higher selectivity of this biocatalyst for DHA as compared to EPA was previously observed [22]. The commercial immobilized *C. antarctica* lipase B ("Novozym 435") was successfully used as catalyst for the interesterification of palm stearin with a concentrate of triglycerides enriched in omega-3 PUFA and soybean oil ("EPAX 2050TG") in solvent-free media [24]. The different behaviors observed in the present study may be explained by the presence of free oleic acid and free omega-3 PUFA, while in the previous studies these fatty acids were esterified in the triacylglycerol molecules.

Molar incorporation values ranging from 10% to 47% of oleic acid were observed after 24-h acidolysis of tripalmitin with hazelnut fatty acids and stearic acid, in *n*-hexane, catalyzed by Lipozyme RM IM [3]. The highest incorporation value was achieved at 65 °C and at the highest substrate molar ratio tested (1:12:1.5, tripalmitin:hazelnut FA:stearic acid).

HMFS containing gamma-linolenic acid (GLA) was also produced by interesterification of tripalmitin with hazelnut fatty acids and GLA, in *n*-hexane, catalyzed by Lipozyme RM IM and Lipozyme TL IM [7]. Similar results were obtained with both enzymes: to attain both 10% GLA and 45% oleic acid incorporation, the optimal reaction conditions, predicted by response surface methodology, were 55 °C, 24 h reaction time and molar substrate ratio of about 14:1 (moles of total FA/moles of tripalmitin).

When the incorporation of omega-3 PUFA was carried out in a similar system (tripalmitin, hazelnut fatty acids and omega-3 PUFA, in n-hexane), catalyzed by Lipozyme RM IM, the highest incorporation levels of EPA and DHA (5%) and of oleic acid (40%) were predicted to be attained upon 24 h reaction time, at 55 °C, using a substrate ratio (moles of total FFA/moles of tripalmitin) of 12.4:1 [8].

An isoform of *Candida rugosa* lipase (LIP1) and Lipozyme RM IM were also used as catalysts for the interesterification of tripalmitin with oleic acid or methyl oleate in *n*-hexane [25]. In this study, higher oleic acid incorporations were obtained, in general, with methyl oleate as acyl donor, increasing with increasing substrate molar ratio up to 1:3. When oleic acid was used, 26.3% incorporation was obtained with LIP1, at 45 °C, and about 45% with Lipozyme RM IM, at 65 °C, after 24-h reaction and with a substrate ratio of 1:3 (tripalmitin:fatty acid).

It is worthy to notice that the high incorporation values reported in the literature, were obtained using high FFA/tripalmitin molar ratios. These reaction conditions will increase operation costs, namely unconverted substrates recycling and product recovery.

3.2. Fatty acid composition at the sn-2 position

The fatty acids at the *sn*-2 position of the structured TAG obtained after 24-h reaction of tripalmitin with oleic acid, are presented in Table 2.

As observed, the *sn*-2 positions of the structured lipids were predominantly occupied by palmitic acid (61.2–87.3 mol%). When the 1,3-specific lipases Lipozyme RM IM and Lipozyme TL IM were used, despite their region-specificity, the incorporation of oleic acid (16 and 20.6 mol%, respectively) in the *sn*-2 positions occurred due to acyl migration during acidolysis. The acyl migration levels were similar to those reported by others in analogous systems and under similar temperature values [3,7,8,25]. The lowest *sn*-2 incorporation level was observed with *C. parapsilosis* lipase/acyltransferase, probably explained by a low acidolysis rate, which results in a low acyl migration. In general, an increase of acyl migration with temperature has been reported [25,26].

Table 2
Fatty acid (mole percent) at *sn*-2 position of the TAG obtained upon 24-h interesterification of tripalmitin with oleic acid.

Fatty acid	Lipozyme RM IM	Lipozyme TL IM	Novozym 435	Lipase/acyltransferase
C16:0	79.90	75.60	61.20	87.30
C16:1	0.30	0.70	1.70	0.00
C18:0	3.10	2.80	2.70	6.10
C18:1	16.00	20.60	33.20	6.60
C18:2	0.70	0.30	1.20	0.00

The highest incorporation of oleic acid in *sn*-2 position (33.2 mol%) was observed with Novozym 435. In fact, this enzyme is usually not positionally specific towards fatty acid residues in TAG. As reported by the manufacturer, Novozym 435 is a highly versatile catalyst with activity towards a great variety of different substrates and it has been primarily used in the synthesis of optically active alcohols, amines and carboxylic acids, due to its highly enantioselectivity.

3.3. Operational stability tests

In order to select the best biocatalyst for HMFS production, not only the catalytic activity but also batch operational stability was evaluated.

For system I, batch operational stability tests were carried out for the three commercial immobilized biocatalysts, since all of them presented similar interesterification activity. For system II, the operational stability was evaluated for the commercial biocatalysts presenting the highest interesterification activity (Lipozyme RM IM and Novozym 435). In addition, even though the activity of the *C. parapsilosis* lipase/acyltransferase immobilized sample was not high, its operational stability was investigated in both systems, since it had not been tested before.

The residual acidolysis activities of the biocatalysts, at the end of each of the repeated 23-h batches, in systems I and II, are presented in Figs. 1 and 2, respectively. The deactivation models fitted to these results, as well as the respective estimated half-life times, are shown in Table 3.

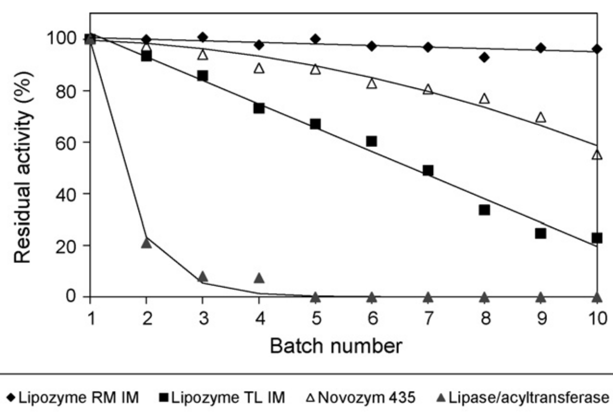

Fig. 1. Batch operational stability test: residual activity of the biocatalysts at the end of each 23-h batch reutilization in the acidolysis of tripalmitin with oleic acid (system I).

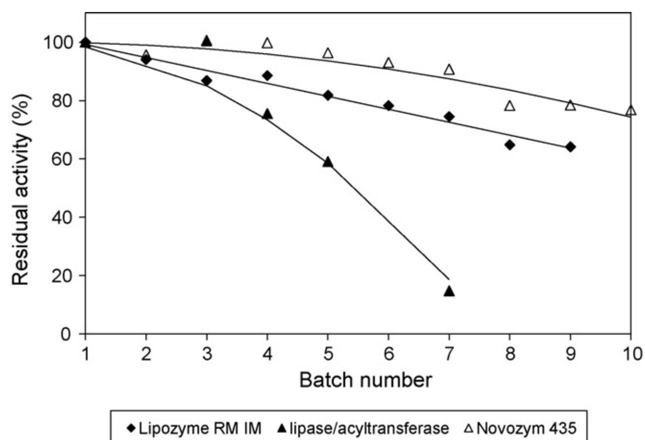

Fig. 2. Batch operational stability test: residual activity of the biocatalysts at the end of each 23-h batch reutilization in the acidolysis of tripalmitin with omega-3 PUFA (system II).

In system I, Lipozyme RM IM maintained its activity for 10 repeated 23-h batches while a linear decrease in Lipozyme TL IM activity was observed (Fig. 1). In analogy with the models fitted to biocatalyst deactivation in continuous bioreactors, the following linear equation could be fitted to the residual activity (a_n) at the end of each consecutive batch, n, for Lipozyme TL IM:

$$a_n = -9.19n + 111.55 \quad (2)$$

(determination coefficient: $R^2 = 0.9886$)

The estimated half-life time of this biocatalyst, $t_{1/2}$, i.e., the operation time required for half the enzyme activity to be lost as a result of deactivation, is about 6.7 batches, corresponding to 154 h operation.

Concerning the immobilized lipase B from *C. antarctica* (Novozym 435), an inactivation parabolic profile was observed for this biocatalyst in system I (Fig. 1). This behavior could be well described by the model of series-type inactivation kinetics proposed by Sadana [27]. The fraction of the original activity (%) of the biocatalyst, a_n, at the end of each batch n, can be given by the following simplified model equation:

$$a_n = 100 - 50k_d n^2 \quad (3)$$

where k_d (batch^{-2}) is the deactivation coefficient. Thus, the deactivation model fitted to the experimental data is given by the following equation where k_d is equal to 0.0082 batch$^{(-2)}$:

$$a_n = 100 - 0.41n^2 \quad (4)$$

The $t_{1/2}$ is given by

$$t_{1/2} = k_d^{(-1/2)} \quad (5)$$

Thus, a half-life time of 11 batches (253 h) was estimated from Eq. (5) for Novozym 435, when used in system I.

In system I, the observed deactivation profile of *C. parapsilosis* lipase/acyltransferase follows a first-order deactivation kinetics model:

$$a_n = A * \ e^{-k_d n} \quad (6)$$

where A is a constant. Therefore, from this model, a half-life time of 1.5 batches (34.5 h) was estimated for this biocatalyst (Eq. (7)):

$$a_n = 428.58 * \ e^{-1.46n} \quad (7)$$

In system II, the replacement of oleic acid (system I) by omega-3 PUFA in reaction media, conducted to changes in operational stability profiles of the biocatalysts (Fig. 2). Lipozyme RM IM, which was very stable in system I, showed a linear activity decrease when reused in system II, in 10 cycles of 23-h each, as follows:

$$a_n = -4.44n + 103.6 \quad (8)$$

($R^2 = 0.9696$)

An estimated half-life time of about 12 batches (276 h) was found for this biocatalyst.

In system II, Novozym 435 presented the highest stability with an estimated half-life time of 14 batches (322 h). As in system I, the inactivation profile of this biocatalyst can be described by Sadana's series-type model [27] with a k_d of 0.00515 batch$^{(-2)}$:

$$a_n = 100 - 0.257n^2 \quad (9)$$

The activity decay observed for the immobilized *C. parapsilosis* lipase/acyltransferase during the consecutive 23-h batches, in system II, could also be described by Sadana's series-type inactivation kinetic model (Eq. (3)) [27], with a k_d of 0.0332 batch$^{(-2)}$:

$$a_n = 100 - 1.66n^2 \quad (10)$$

A half-life time of 5.5 batches (127 h) was estimated for this biocatalyst. The inactivation profile was similar to that observed for Novozym 435 in both systems (Eqs. (4) and (9)).

C. parapsilosis lipase/acyltransferase displayed lower operational stability in the presence of oleic acid (more than 80% loss along the first reutilization) than in the presence of omega-3 PUFA. A difference in operational stability according to the acid used was also observed with the commercial immobilized lipases: Lipozyme RM IM was more stable in the presence of oleic acid (no significant deactivation in 10 batches) than in the presence of omega-3 PUFA (half-life 12 batches), conversely to that observed with Novozym 435. The different behaviors exhibited by the biocatalysts might be due to differences in (i) enzyme sensitivity to by-products accumulating on the immobilization support, including fatty acids oxidation products, or (ii) to enzyme dehydration during the reaction and medium renewal between batches. In fact, the presence of omega-3 PUFA, which are rather prone to oxidation, may explain the lower activity exhibited by Lipozyme RM IM in system II. The negative effect of the presence of hydroperoxides, final oxidation products, phospholipids, chlorophyll and carotenoids and lipid polymers, on lipase stability was also previously observed [23,28–30]. A loss of activity of *C. parapsilosis* lipase/acyltransferase in Accurel MP 1000 was observed in continuous fluidized-bed reactor and during the reutilizations in consecutive batches, due to biocatalyst dehydration [17].

Table 3
Deactivation models and respective half-life times estimated for the biocatalysts used in 10 consecutive 23-h batches in System I and System II (n.d.—not determined).

Biocatalyst	System I (tripalmitin + oleic acid)			System II (tripalmitin + omega-3 PUFA)		
	Deactivation model	Half-life time		Deactivation model	Half-life time	
		Batch no.	h		Batch no.	h
Lipozyme RM IM	No deactivation	No deactivation	No deactivation	Linear	12	276
Lipozyme TL IM	Linear	6.7	154	n.d.	n.d.	n.d.
Novozym 435	Series-type	11	253	Series-type	14	322
Lipase/acyltransferase	First-order	1.5	34.5	Series-type	5.5	127

The decrease in operational stability might also be due to modifications in the protonation state of the enzymes under the influence of the free fatty acids used: pK_a of free fatty acids are known to decrease with unsaturation degree [31–33]. This may explain the higher operational stability of Novozym 435 and *C. parapsilosis* enzyme in the presence of omega-3 PUFA than in the presence of oleic acid. This hypothesis will be further investigated.

4. Conclusions

All the biocatalysts tested presented acidolysis activity aimed at the production of structured lipids adequate to be used as human milk fat substitutes. In system I (tripalmitin and oleic acid), all the immobilized commercial lipases presented similar activities (ca. 27 mol% incorporation) but only Lipozyme RM IM was stable along 10 consecutive 23-h cycles. When oleic acid was replaced by omega-3 PUFA (system II), a decrease in incorporation levels was observed with the commercial lipases tested. The highest incorporation values were obtained with Novozym 435 (21.6 mol%) and Lipozyme RM IM (20.8 mol%). In this system, Novozym 435 presented higher operational stability than when in system I and than Lipozyme RM IM, in system II.

C. parapsilosis lipase/acyltransferase showed similar catalytic activities in both systems, though low incorporation values were obtained (about 9 mol%) in the conditions tested. This enzyme seems to be an adequate catalyst for the acidolysis of tripalmitin with blends of oleic acid and omega-3 PUFA to prepare SL similar to human milk fat. The search for more adequate supports and/or immobilization techniques will be attempted to improve both activity and operational stability of this enzyme.

Concerning the commercial immobilized lipases, only Lipozyme RM IM seems to be adequate for using in reaction media containing blends of oleic acid and omega-3 PUFA.

The results obtained in this study show that the activity and operational stability of the biocatalysts used depend on the acyl donor used.

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