Grapeseed Oil + C8:0 (C10:0)

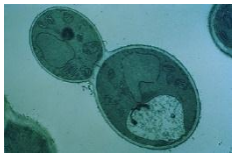
Non-commercial immobilized lipases

Dietetic TAGs

M
L
M

Biocatalysts

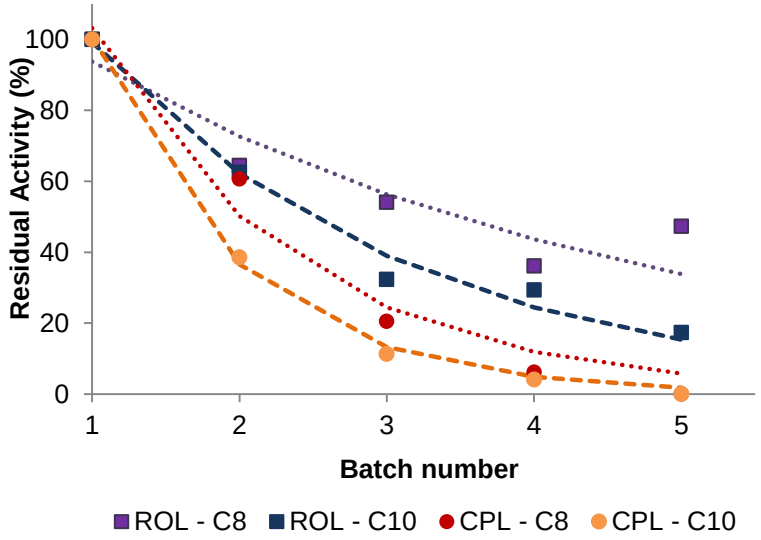
Carica papaya lipase self-immobilized in the latex (CPL)



Recombinant Lipase *Rhizopus oryzae* (rROL)

Biocatalyst	Half-life time (h)
ROL-C8	166
ROL-C10	118
CPL-8	96
CPL-10	81

High Operational Stability



Production of MLM type Structured Lipids from Grapeseed Oil Catalyzed by Non-Commercial Lipases

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Running title: Production of MLM Lipids from Grapeseed Oil

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Abstract

Low calorie triacylglycerols (TAG) presenting medium-chain fatty acids (M) at positions *sn*-1,3 and long-chain fatty acids (L) at position *sn*-2, are known as MLM. This study aimed at the production of MLM by acidolysis of grapeseed oil with medium-chain caprylic (C8:0) or capric (C10:0) acids. Grapeseed oil was used as source of long-chain polyunsaturated fatty acids, especially linoleic acid, at *sn*-2 position in TAG. Reactions were performed in batch, in solvent-free systems, during 48 h. Novel non-commercial *sn*-1,3 regioselective lipases were used as alternative to high cost commercial biocatalysts, namely: the heterologous lipase from *Rhizopus oryzae* (rROL) immobilized in Amberlite™ IRA 96 and *Carica papaya* lipase (CPL) self-immobilized in papaya latex. The highest productions of new TAG were achieved at 40 °C, molar ratio TAG:M of 1:2, after 48 h, with both biocatalysts, with yields varying between 38 and 69 %. rROL immobilized in Amberlite IRA 96 showed a preference towards caprylic acid while CPL showed no preference towards caprylic or capric acid. First-order deactivation kinetics was observed for both biocatalysts. Half-lives at 40 °C were 166 and 118 h for rROL and 96 and 81 h for CPL, in the acidolysis of grapeseed oil with C8:0 or C10:0, respectively.

Practical applications

Grapeseeds of *Vitis vinifera* L. are a by-product of the wine industry. Using grapeseed oil to produce added-value functional oils rich in linoleic acid (essential fatty acid) may be a way of improving the revenues of the enological and oil sectors.

Both lipases, and mainly *Carica papaya* lipase self-immobilized in papaya latex, due to its low-cost production and easy preparation, are promising non-commercial biocatalysts for the synthesis of MLM in solvent-free media. The use of a solvent-free system, in addition of being a green option, is also preferred for economic reasons, avoiding the costs with solvent and solvent recovery. Also, the use of the stoichiometric molar ratio TAG:M of 1:2 will (i) decrease

costs related with the recovery and reutilization of medium chain fatty acids in excess, product recovery and purification, and (ii) avoid biocatalyst deactivation by high amounts of free fatty acids in reaction media.

Key words: *Carica papaya* lipase; grapeseed oil; low calorie lipids; *Rhizopus oryzae* lipase; structured lipids.

1. Introduction

Lipids are essential constituents of food diet mainly because of their high energy value and for being a good source of bioactive compounds such as liposoluble vitamins, antioxidants and essential fatty acids. Triacylglycerol (TAG) structure can be modified, namely by changing the original fatty acids (FA) by other FA with beneficial characteristics and/or by changing their original position in the glycerol skeleton. These modified fats, known as “structured lipids” (SL) or ‘‘taylor-made fats’’, can be produced either chemically or enzymatically. They represent a food industry growing segment in order to achieve natural and healthier fats (when enzymatic catalysts are used) as a response to the current consumer’s demand [1-3]. Dietetic TAG with low caloric value contain medium chain fatty acids (M) esterified at *sn*-1,3 positions and long chain fatty acids (L) esterified at *sn*-2 position of the glycerol backbone, and they are known as MLM. During digestion, the medium chain fatty acids, released from TAG by the action of the *sn*-1,3-regioselective pancreatic lipase, are preferentially transported via the portal vein to the liver, where they are metabolized as rapidly as glucose. Since these FA are not readily re-esterified into new TAG, they are not stored as fat, with weight control benefits [1, 3]. MLM present lower caloric value (5 to 7 kcal/g) than conventional fats and oils (9 kcal/g) [4]. In addition, the production of these SL is also important as a food source for persons with pancreatic insufficiency and other malabsorption problems [5].

Lipases (EC 3.1.1.3; triacylglycerol acylhydrolase) can be used as catalysts for SL production since they can catalyze esterification, acidolysis, alcoholysis and interesterification reactions, when used in non-aqueous media. Also, they are highly selective and some of them are regio-selective, which is not an option for chemical catalysts [2, 6]. They can be used non-immobilized or immobilized in solid supports in order to be reused in batch or used in continuous bioreactors. However, the high prices of commercial lipases, together with their frequently low operational stability, were recognized as the major constraints to the use of lipase-catalyzed processes [2]. Thus, the search for novel lipases with high activity and operational stability is needed for the implementation of enzymatic processes in the food industry.

Rhizopus oryzae lipase (rROL), cloned and expressed in the methylotrophic yeast *Pichia pastoris* [7], was used as a feasible non-commercial catalyst for the synthesis of TAG of MLM type [8-10]. *Carica papaya* lipase (CPL) self-immobilized in papaya latex is an example of a low-cost alternative to the commercial lipases. This lipase is extracted from green or sick papaya plants with high levels of defense enzymes from agro-wastes of papaya plantations and can be used as catalyst for SL production [11-13]. Table 1 shows some examples of using these non-commercial biocatalysts in the synthesis of structured lipids in solvent-free media.

The aim of this work was to produce MLM rich in linoleic acid, by acidolysis in solvent-free media, using non-commercial *sn*-1,3 regioselective recombinant *R. oryzae* lipase and *Carica papaya* lipase self-immobilized in papaya latex as catalysts. MLM were obtained by acidolysis of grapeseed oil with caprylic or capric acids (medium-chain FA). Grapeseed oil was used as source of polyunsaturated fatty acids (PUFA), especially linoleic acid (58.0-78.0 %), which is an essential fatty acid for humans. Grapeseeds of *Vitis vinifera* L. are a by-product of wine industry, which is an important agri-business in the Mediterranean region countries, USA, Argentina, Chile and Australia, among others. The use of grapeseed oil for the production of high added-value SL will be a revenue for the oenology sector.

2. Materials and Methods

2.1 Lipases and immobilization support

Non-commercial heterologous *Rhizopus oryzae* lipase (rROL) was produced by over-expression of the corresponding gene in a mutant strain of *Pichia pastoris* [7]. *Carica papaya* lipase (CPL), a self-immobilized biocatalyst preparation from papaya latex free of proteases, was obtained as previously described [12]. Only papaya latex particles smaller than 10 mesh were used as catalyst. CPL was extracted from unripe or sick fruits, because they contain higher levels of defensive enzymes [14]. Amberlite® IRA96, from Rohm and Haas, Lenntech, Philadelphia, U.S.A, was used for rROL immobilization. It is a macroreticular weak base anion exchange resin (styrene divinylbenzene copolymer with at least 85 % of tertiary amine as functional group).

2.2 Substrates

The substrate used as source of linoleic acid was refined grapeseed oil, produced by Smile Noël, Pont-Saint-Esprit, France. The fatty acid composition of refined grapeseed oil used in this work was evaluated by capillary gas chromatography as fatty acid methyl esters, and is presented in Table 2. The substrates used as source of medium chain fatty acids were caprylic acid (> 98 % purity; MW= 144.21) and capric acid (> 98 % purity; MW= 172.26), both from TCI Europe N.V., Belgium.

2.3 Chemicals

Glutaraldehyde (25 % aqueous solution) was purchased from Merck, Germany. Bovine serum albumin (BSA) (≥ 96 %), Coomassie-Brilliant Blue G (≥ 90 %) with purity *p.a.*, the standards trilinolein (99 %) and linoleic acid (99 %) were obtained from Sigma-Aldrich, Sintra, Portugal. The standard of monononadecanoin (99 %) was obtained from Larodan Fine Chemicals AB, Sweden; N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; > 90 %), was purchased from

TCI Europe N.V., Belgium; pyridine with purity *p.a.*, was obtained from Panreac Química S.L.U., Spain. The other reagents used were *p.a.* and obtained from different sources.

2.4 rROL immobilization in Amberlite® IRA96

The immobilization procedure for rROL in Amberlite® IRA96 was carried out according to the method described by Wang *et al* [14] after modification. Initially, 5 g of resin were added to 50 mL of deionized water, under magnetic stirring, during 30 minutes at 50 °C. After, the support was rinsed with 25 mL of 1 M NaOH aqueous solution followed with 25 mL of 1 M HCl aqueous solution, for three times, and subsequently equilibrated with sodium phosphate buffer (0.2 M, pH 7.5). The resin (1 g) and the lipase solution (10 mL of sodium phosphate buffer 0.2 M, pH 7.5, containing 0.25 g of rROL) were mixed together at 28 °C, for 4 h, under gentle magnetic stirring. These particles were filtered under reduced pressure and then rinsed with glutaraldehyde aqueous solution (0.5 % v/v) (25 mL/1 g resin). The immobilized lipase was washed three times with 20 mL sodium phosphate buffer, dried under vacuum filtration and stored at 4 °C until use.

2.5 Determination of rROL immobilization yield

The immobilization yield was determined in terms of (i) immobilized protein or (ii) estimated hydrolytic activity in the immobilized rROL. The protein content was assayed by the Bradford method using BSA as standard, by quantifying the unbound protein content in the supernatant before and after immobilization [15]. The immobilization protein yield (η_P) was estimated, according to the following equation:

$$\eta_P = \frac{P_0 - P_1}{P_0} \times 100 \qquad (1)$$

where P_0 is the protein amount present in the initial enzymatic solution (mg), before the immobilization support was added, and P_1 is the residual amount of protein (mg) present in the supernatant after immobilization, as well as in the subsequent washing solutions.

The hydrolytic activity of rROL was assayed using the method described by Soares *et al* [16], after modification: 50 g of extra virgin olive oil were mixed with 50 g of distilled water and 3.5 g of gum arabic. This mixture was stirred for 10 min. Then, 80 mL of phosphate buffer solution (pH 7.0, 0.1 M) were added and the final emulsion was put in a 20 mL closed thermostated cylindrical batch reactor, at 40 °C, under magnetic stirring. After, 1mL of initial enzyme solution was added and the hydrolysis was allowed to proceed for 5 min. After this time, 20 mL of ethanol/acetone solution (1:1 v/v) were added in order to inactivate the enzyme. The amount of free fatty acids (FFA) released during hydrolysis was determined by direct titration with a 0.1 N sodium hydroxide aqueous solution, using phenolphthalein as indicator. In parallel, blank experiments were carried out in the absence of enzyme. The same procedure was followed for the supernatants obtained after immobilization and washing the immobilized rROL, using 5 mL of each solution and 10 min reaction time.

The immobilization hydrolytic yield (η_H) was estimated as follows:

$$\eta_H = \frac{H_0 - H_1}{H_0} \times 100 \qquad (2)$$

Where H_0 is the hydrolytic activity (U) of the initial enzymatic solution, before the immobilization support was added, and H_1 is the residual hydrolytic activity (U) in the supernatant after immobilization, as well as in the subsequent washing solutions. One unit of hydrolytic activity (U) was defined as the amount of enzyme that catalyzes the hydrolysis of olive oil releasing one micromole of FFA per minute.

2.6 Assessment of hydrolytic activity of immobilized rROL and CPL preparations

The hydrolytic activity of rROL and CPL preparations was assayed using 0.3 g of immobilized enzyme and the hydrolysis was allowed to proceed for 5 min, as previously described (*cf*.

Determination of rROL immobilization yield). The assessment of hydrolytic activity of CPL preparation was performed directly as papaya latex lipase is a self-immobilized enzyme.

2.7 Acidolysis reactions

Acidolysis reactions were performed batchwise in closed thermostated 20 mL cylindrical batch reactors, under magnetic stirring, in solvent-free medium. A molar ratio TAG:FFA of 1:2 was used. This molar ratio corresponds to the stoichiometric value needed for the esterification of the free fatty acids at *sn*-1 and *sn*-3 positions by *sn*-1,3-selective lipases. The substrate mixture consisted of 3 g of grapeseed oil and 0.98 g of caprylic (C8:0) or 1.17 g of capric acid (C10:0). This reaction occurred at 40 °C, unless otherwise stated. A biocatalyst amount of 5 % (w/w) of the total TAG (grapeseed oil), i.e. 0.15 g of immobilized lipase, was used. Samples (0.05 mL) were taken from the reaction medium at 0, 24 and 48 h of reaction time. All the samples were stored at -18 °C for subsequent analysis. Reactions were carried out in triplicate.

2.8 Acidolysis kinetics and temperature effect

The reaction conditions for the MLM production assays were the same as described for acidolysis reaction, except for the reaction temperatures and the sampling times. These assays were performed at three different temperatures: 30, 40 and 50 °C, and samples were taken at 0, 3, 5, 8, 24 and 48 h of reaction time. Reactions were carried out in triplicate.

2.9 Batch operational stability tests

The operational stability of the biocatalysts was assayed under the same conditions of the acidolysis reactions, at 40 °C. Five consecutive 48 h-batches were carried out for rROL and CPL. At the end of each batch, the lipase was removed from the reaction medium and was added to fresh medium and reused in the next batch. Biocatalyst activities were estimated by the production of new TAG. The first batch was used as the reference (activity equal to 100 %). The

residual activity of the biocatalyst at the end of each batch was calculated relatively to the initial activity of the biocatalyst at the end of the first batch. 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 results was performed using "solver" from Excel for Windows, by minimizing the residual sum-of-squares between the experimental data points and those estimated by the model.

2.10 Analysis of reaction products

For TAG, mono- (MAG) and diacylglycerol (DAG), and FFA quantification, samples were analyzed by GC following the procedure described by the European standard EN 14105 [17], after modification, using monononadecanoin (Mono C19) as internal standard [18]. A solution of 0.5 % (w/v) of reaction medium in *n*-hexane was prepared. A volume of 0.5 mL of this solution was withdrawn and the solvent was evaporated in a rotavapor at 40ºC and 60 mbar. The dry residue was mixed with 400 μL of the internal standard solution (50 ± 0.1 mg of Mono-C19, dissolved in 20 mL of tetrahydrofuran), 200 μL of pyridine and 200 μL of N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). The reagents must be added by the mentioned order and under vigorous stirring, keeping the samples protected from humidity. After 15 minutes at room temperature, 4 mL of *n*-heptane was added to the mixture and 1 μL of this solution was GC analyzed. A gas chromatograph Agilant Tecnologies 7820A, equipped with on-column injector, a DB5-HT capillary column (15 m x 0.32 mm ID x 0.10 μm film) and a flame ionization detector was used. Injector and detector temperatures were set at 83 °C and 380 °C, respectively. Air and hydrogen were supplied to the detector at flow rates of 300 mL/min and 30 mL/min, respectively. Helium was used as carrier gas at a flow rate of 2.2 mL/min. The oven temperature program was as follows: 50 °C for 1 min, a temperature increase to 180 °C at 15 °C /min (ramp 1), followed by temperature increase at a rate of 7 °C /min (ramp 2) to 230 °C, a

final ramp (ramp 3) at a rate of 10 °C /min to 370 °C and a final plateau at 370 °C for 12 min. The total run time is 43 min.

The identification of the various groups of compounds was carried out by comparison with the chromatograms shown in the European Standard EN 14105 [17], and also by the retention times and reference standards. The reference standards used for the calibration curves were: trilinolein, linoleic acid, capric acid and caprylic acid. The conversion degree (%, w/w) of a determined compound was calculated by the ratio between the amount of consumed substrate and the amount of initial substrate. The mass of new TAG formed during the reaction was quantified considering the sum of the areas of all new peaks (retention times between 26 and 31 min) in TAG region of the chromatogram, using the calibration curve for trilinolein. The new TAG were quantified in terms of mass yields (%, w/w) determined by the ratio between the amount of new TAG produced and the amount of initial TAG. The masses of partial acylglycerols (MAG and DAG) were not quantified because they represented a reduced percentage in the samples, as confirmed by thin layer chromatography.

2.11 Statistical analysis

For each system, eventual significant differences between biocatalysts, for the amounts of new TAG produced and/or consumed substrates were investigated by one-way analysis of variance (ANOVA). The software Statistica, version 6, from Statsoft, Tulsa, OK, USA, was used and *post-hoc* comparisons were carried out using the Tukey test at a *p*-value of 0.05.

3. Results and Discussion

3.1 Immobilization yield of rROL in Amberlite IRA96 and biocatalyst hydrolytic activity

The amount of immobilized protein was 0.935 mg/g IRA96, corresponding to an immobilization protein yield of 22.3 %, while the hydrolytic yield was estimated as 64.7 %. This shows a

greater affinity of rROL molecules than of other proteins with no catalytic activity, present in the lyophilized extract, to the immobilization support. However, the hydrolytic activity directly measured for immobilized rROL was 497 U/g (std = 35; 3 replicates), corresponding to a retention of hydrolytic activity of only 35.6%. This yield is lower than the estimated yield in terms of hydrolytic activity, which may be due to some inactivation of rROL molecules during immobilization and/or mass transfer limitations inside the support. The hydrolytic activity for self-immobilized *Carica papaya* lipase was 698.2 U/g (std = 58; 3 replicates).

The hydrolytic activities of lipase preparations used in this study were only determined to evaluate if the enzymes have catalytic activity, but do not indicate which preparation has the highest catalytic activity in acidolysis. In fact, no direct relationships between hydrolytic and interesterification activities of rROL were previously found [9, 19].

3.2 Synthesis of MLM

Lipase-catalyzed acidolysis is a reversible reaction that has been considered as a hydrolysis-esterification two-step mechanism reaction [20]. Therefore, along the reaction catalyzed by *sn*-1,3 regioselective lipases, long-chain fatty acids at positions *sn*-1,3 of TAG of grapeseed oil are released to the reaction medium and C8:0 (or C10:0) molecules are esterified to form new DAG and new TAG (with one or two substitutions in the acylglycerol backbone). At equilibrium, based on the law of probabilities, the theoretical maximum value of fatty acid incorporation is 66.7 mol-%, when a *sn*-1,3 selective lipase is used [20].

The production of TAG of MLM type was evaluated by new TAG yields, and capric or caprylic acid consumptions. New TAG, MAG, DAG and also the release of FFA from grapeseed oil were observed during the reaction time (Fig. 1).

Table 3 shows the results obtained after 24 and 48 h acidolysis of grapeseed oil with caprylic or capric acid, aimed at the production of MLM, catalyzed by rROL or CPL. Concerning new TAG synthesis, no significant differences were observed between the results obtained

with rROL after 24 or 48 h reaction. For the systems where *C. papaya* lipase was used, an increase in new TAG was observed from 24 to 48 h. The yields of new TAG obtained with rROL were always higher than the values obtained with *C. papaya* lipase. In fact, the highest yield of new TAG (68.5 %) was observed with rROL for the incorporation of C8:0 after 24 h reaction. CPL showed no preference towards caprylic or capric acid. For rROL, a preference towards caprylic acid was observed. On the contrary, rROL immobilized on Eupergit C or on modified Sepiolite showed a preference towards capric acid, when used in the acidolysis of olive oil, in solvent-free system [8]. Again, the immobilization carrier seems to affect rROL lipase selectivity.

TAG consumption (Table 3) increased along 48 h-acidolysis for both biocatalysts, either in the presence of caprylic or capric acid. The highest values of TAG consumed after 48 h reaction (81.5 and 81.7 %) were observed for the systems with caprylic acid, catalyzed by rROL or CPL, respectively. These similar values of TAG consumption were not accompanied by similar values of new TAG formed (66.8 and 40.8 %, respectively). However, C8:0 or C10:0 consumptions were similar for both biocatalysts, increasing with reaction time. This may be explained by the high hydrolytic activity of *C. papaya* lipase. It is worth to notice that a 1.4-fold hydrolytic activity of *C. papaya* lipase with respect to rROL was not observed in terms of acidolysis activity. This was also verified with rROL obtained from different fermentation batches, having different hydrolytic activities but similar acidolysis activities [9].

In spite of the lack of preference shown by CPL towards C8:0 or C10:0 in the formation of new TAG, a preference towards C8:0 is observed in TAG consumption. Indeed, a preference for octanoin was observed in hydrolytic characterization of CPL [21].

The synthesis of MLM from virgin olive oil was also carried out in solvent free medium, with a molar ratio olive oil:FFA of 1:2 at 45 °C, using commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) as catalysts [22]. Capric acid incorporation was between 27.1 and 30.4 mol-% for all the biocatalysts, while the highest incorporation values of

C10:0 were observed with Novozym 435 (25.5 mol-%) and Lipozyme RM IM (25.7 mol-%). MLM TAG were also produced by Wang *et al* [23] with canola oil and caprylic acid (MR 1:4 canola oil/C8:0, 10 % lipase) catalyzed by Lipozyme RM IM. After 15 h acidolysis at 50-60 ºC, 45.4 mol-% incorporation of caprylic acid was observed. Öztürk *et al* [24] used Lipozyme TL IM for the acidolysis of corn oil and caprylic acid. Under optimized conditions (MR 1:3.9 corn oil/C8:0, 13.2 % lipase and 3.1 h reaction time), 21.5 mol.% of caprylic acid incorporation was obtained.

Although the higher yields in new TAG rich in caprylic or capric acid, obtained with rROL, both rROL and CPL (due to its lower-cost and easy preparation) were used for subsequent studies on MLM synthesis.

3.3 Temperature effect on the production of MLM

In order to investigate the effect of temperature on the production of new TAG, time-course experiments were carried out at 30, 40 and 50 °C. The initial rates of synthesis of new TAG, as well as the amounts of new TAG obtained after 48 h acidolysis, are presented in Fig. 2. An increase in initial rates with temperature is observed, mainly when *C. papaya* lipase is used. At 50 °C, the initial rates of acidolysis catalyzed by this lipase are similar for C8:0 and higher for C10:0 than those observed with rROL. When rROL was used, the initial rates showed not to depend so strongly from the temperature, especially with capric acid.

With respect to the new TAG production, the results showed a maximum at 40 °C and lower yields at 30 and 50 °C, for both biocatalysts, either in the system with caprylic or capric acids. At 40 °C, the highest yields of new TAG were obtained when rROL was used as catalyst, with preference for caprylic acid. At 30 or 50 °C, the results were similar for all the systems under study. In fact, in spite of high initial rates observed at 50 °C, for *C. papaya* lipase, low yields of new TAG are obtained at this temperature. This may be explained by a lipase thermal deactivation [21].

In the production of MLM using extra virgin olive oil, high acidolysis activity of rROL immobilized in Eupergit C was observed at 40 °C, in solvent-free system [8]. In the optimization of reaction conditions catalyzed by this biocatalyst, 37 and 35 °C were found as the optima for acidolysis with caprylic or capric acid, respectively [9].

3.4 Operational stability assays

The high cost of lipases, together with their low operational stability, were recognized as the main obstacles for the application of enzyme-catalyzed processes in the food industry. The use of reusable and stable immobilized lipases is a way to make enzymatic processes economically feasible. In our study, the use of non-commercial lipases aims at reducing the costs related to the use of expensive commercial lipases. Batch operational stability tests were carried out to evaluate the stability of the biocatalysts used during the acidolysis reaction of grapeseed oil with caprylic or capric acid. Acidolysis activity was evaluated in terms of new TAG yield. The residual acidolysis activity of immobilized rROL and *C. papaya* lipase, at the end of each 48 h-batch, is presented in Fig. 3. For both biocatalysts, the observed behaviour can be described by first-order deactivation kinetics, given by the following model equation:

$$A_n = Ae^{-k_d n} \qquad (3)$$

where A_n is the biocatalyst residual activity (%) at batch n, A is a constant representing the initial activity of the enzyme, before deactivation, n is the batch number and k_d is the deactivation rate constant, expressed in n^{-1}.

The deactivation models fitted to the experimental data, determination coefficients of these models, as well as half-life times ($t_{1/2}$) of each biocatalyst are presented in Table 4. Either in presence of caprylic or capric acids, a good fit of a first order deactivation model is observed for both biocatalysts ($0.845 \leq R^2 \leq 0.998$).

The highest operational stability was observed for immobilized rROL, either in presence of caprylic ($t_{1/2}$ = 166 h) or capric acid ($t_{1/2}$ = 118 h). *C. papaya* lipase also showed a good operational stability both in presence of caprylic ($t_{1/2}$ = 96 h) or capric acid ($t_{1/2}$ = 81 h). For both biocatalysts, higher operational stability was obtained in presence of caprylic acid.

According to Nunes *et al* [8], higher operational stability was also obtained for rROL immobilized in Eupergit C, in presence of caprylic acid ($t_{1/2}$= 159 h) instead of in presence of capric acid ($t_{1/2}$= 136 h).

In previous studies [10, 19], an increase in operational stability was observed when rROL immobilized in different supports was rehydrated between batches. The loss of activity could be explained by a progressive dehydration along the reactions. Faustino *et al* [18] concluded that a high initial water activity of rROL favours the acidolysis reaction. In fact, in all these experiments, rROL was used with its initial water activity after immobilization (a_w = 0.95).

However, the estimated half-life of rROL immobilized in Eupergit C was higher with capric acid ($t_{1/2}$= 54.3 h) than with caprylic acid ($t_{1/2}$= 39.0 h), when rehydrated between consecutive reuses in the acidolysis of olive oil [10]. Simões *et al* [25] obtained an estimated half-life of 112 h also using rROL immobilized in Accurel MP 1000 for the production of human milk fat substitutes (HMFS) by acidolysis of lard and polyunsaturated fatty acids. Tecelão *et al* [13] also used *C. papaya* latex for the HMFS production from tripalmitin and oleic acid. In the experiments with a MR oleic acid/tripalmitin of 2:1, the biocatalyst still retained 71.5 % of its initial activity at the end of the third consecutive 23 h-batch. The operational stability of this enzyme showed to decrease with the amount of free oleic acid in the reaction medium. In addition, a low acidolysis activity was observed in presence of polyunsaturated fatty acids [13].

Also, batch operational stability was assayed for the commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) used as catalysts for the synthesis of MLM from virgin olive oil, in solvent free medium [22]. After 10 consecutive 23 h-batches, the half-life times of these biocatalysts were estimated. The obtained values varied from 47 h for Novozym

435 with capric acid to around 300 h for Lipozyme RM IM with caprylic acid, while no deactivation was observed for Lipozyme RM IM in presence of capric acid [22].

Therefore, the operational stability of both non-commercial biocatalysts used in the present work is comparable to the values observed for the high cost commercial immobilized enzymes.

4. Conclusions

The heterologous *Rhizopus oryzae* lipase immobilized in Amberlite IRA 96 and *Carica papaya* lipase self-immobilized in papaya latex are promising non-commercial biocatalysts for the synthesis of low calorie TAG from grapeseed oil, as alternative to high cost commercial biocatalysts. The yields of new TAG rich in caprylic or capric acid, obtained after 48 h acidolysis in solvent-free media, varied from 38 to 41 % with *Carica papaya* lipase, or from 54 to 69 %, when immobilized rROL in Amberlite IRA 96 was used as catalyst. Both biocatalysts showed high operational stability at 40 °C, with half-life times higher than 81 h. The highest operational stability was observed for rROL in presence of caprylic acid ($t_{1/2}$ = 166 h). However, even presenting lower acidolysis activity than immobilized rROL, CPL did not show any preference towards caprylic or capric acid to produce new TAG and exhibited a high operational stability. In addition, the low cost of this biocatalyst obtained from agro-residues of papaya plantations may compensate its lower acidolysis activity in comparison with rROL immobilized in the resin Amberlite IRA 96. This is particularly important for industrial implementation of the enzymatic process. This study also showed that grapeseed oil is a good source of unsaturated fatty acids (namely linoleic acid) to incorporate in dietary TAG of MLM type. The use of this oil to produce added-value oils with important functional properties may be a way of improving the revenues of the oenological and oil industries.

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Figure captions:

Fig. 1: MLM production- example of a chromatogram after 48 h acidolysis reaction between capric acid and grapeseed oil catalyzed by immobilized rROL (DAG- diacylglycerols; LLL- trilinolein; MAG- monoacylglycerols; Mono C19- mononononadecanoin used as internal standard; TAG- triacylglycerols).

Fig. 2: Initial rates of new TAG production (A) and new TAG yields (B) after 48 h reaction, at 30, 40 and 50 °C, catalyzed by rROL or CPL.

Fig. 3: Residual activities of biocatalysts at the end of each consecutive 48 h batch, when acidolysis of grapeseed oil with capylic or capric acid was performed at 40°C.

Table 1. Examples of production of Structured Lipids (Human milk fat substitutes, HMFS, and MLM) catalyzed by rROL or CPL, in solvent-free media.

Structured Lipid type	Biocatalyst	Support	Reaction system	Fatty acid incorporation in new TAG (mol-%)	Reference
HMFS	rROL	Lewatit VP OC 1600	Tripalmitin + Oleic acid	30	[19]
		Accurel MP 1000	Tripalmitin + Oleic acid	22	[19]
		Eupergit C	Tripalmitin + Oleic acid	4.2	[19]
		Accurel MP 1000	Lard + PUFA	17	[24]
		Lewatit VP OC 1600	Tripalmitin + Fatty Acids from camelina oil	43.6	[18]
		Relizyme OD403/S	Tripalmitin + Fatty Acids from camelina oil	18.3	[18]
	CPL	Self-immobilized in the latex	Tripalmitin + Oleic acid	22.1	[13]
			Tripalmitin + PUFA	8.7	[13]
MLM	rROL	Eupergit C	Olive oil + caprylic acid	21.6	[8]
		Eupergit C	Olive oil + capric acid	34.8	[8]
		Modified sepiolite	Olive oil + caprylic acid	4.4	[8]
		Modified sepiolite	Olive oil + capric acid	5.5	[8]

Table 2. Fatty acid composition (mol.%) of grapeseed oil

Fatty Acid Group	Fatty acid	Concentration (mol.%)
Saturated Fatty Acids	C14:0	< 0.01
	C15:0	0.01
	C16:0	7.73
	C17:0	0.07
	C18:0	4.69
	C20:0	0.23
	C21:0	0.10
	C22:0	0.07
	C24:0	0.09
Monounsaturated Fatty Acids	C16:1	0.12
	C17:1	0.03
	C18:1	1.22
	C20:1	0.24
Polynsaturated Fatty Acids	C18:2	84.96
	C18:3	0.39
	C20:2	0.04

Table 3. New TAG yields, TAG consumed, caprylic (C8:0) or capric (C10:0) acids consumed for the four MLM production systems, at 24 or 48 h reaction time, catalyzed by rROL or CPL. In each type of compound, superscript indexes indicate differences based on Tukey test ($p \leq 0.05$) and with ± equal to the standard deviation.

		System			
Compounds	**Time (h)**	**rROL – C8:0**	**rROL – C10:0**	**CPL – C8:0**	**CPL – C10:0**
TAG new (%, w/w)	24 h	68.5^{c} ±4.1	$52.4^{b,c}$±6.6	28.3^{a}±7.4	32.7^{a}±5.7
	48 h	66.8^{c}±3.8	$54.3^{b,c}$±7.3	40.8^{b}±0.3	38.2^{b}±3.4
TAG consumed (%, w/w)	24 h	$69.0^{b,c}$±0.8	43.7^{a}±8.6	48.5^{a}±0.4	63.7^{b}±1.3
	48 h	81.5^{d}±0.8	64.2^{b}±0.2	81.7^{d}±2.9	73.9^{c}±1.9
C8:0 or C10:0 consumed (%, w/w)	24 h	$35.0^{b,c}$±1.1	$30.8^{a,b}$±9.6	25.9^{a}±0.3	$37.3^{c,d}$±5.0
	48 h	$45.7^{e,f}$±5.6	$42.7^{d,e}$±8.4	$45.4^{e,f}$±8.1	49.5^{f}±2.7

Table 4. Deactivation models fitted to rROL and *Carica papaya* lipase (CPL) in the systems with caprylic (C8:0) or capric (C10:0) acids, respective determination coefficients (R^2) and half-life times ($t_{1/2}$) of the biocatalysts.

System	Model equation	R^2	$t_{1/2}$(h)
rROL + C8:0	$A_n = 120.92e^{-0.25n}$	0.845	166
rROL + C10:0	$A_n = 158.27e^{-0.47n}$	0.984	118
CPL + C8:0	$A_n = 212.22e^{-0.72n}$	0.968	96
CPL + C10:0	$A_n = 276.36e^{-1.01n}$	0.998	81

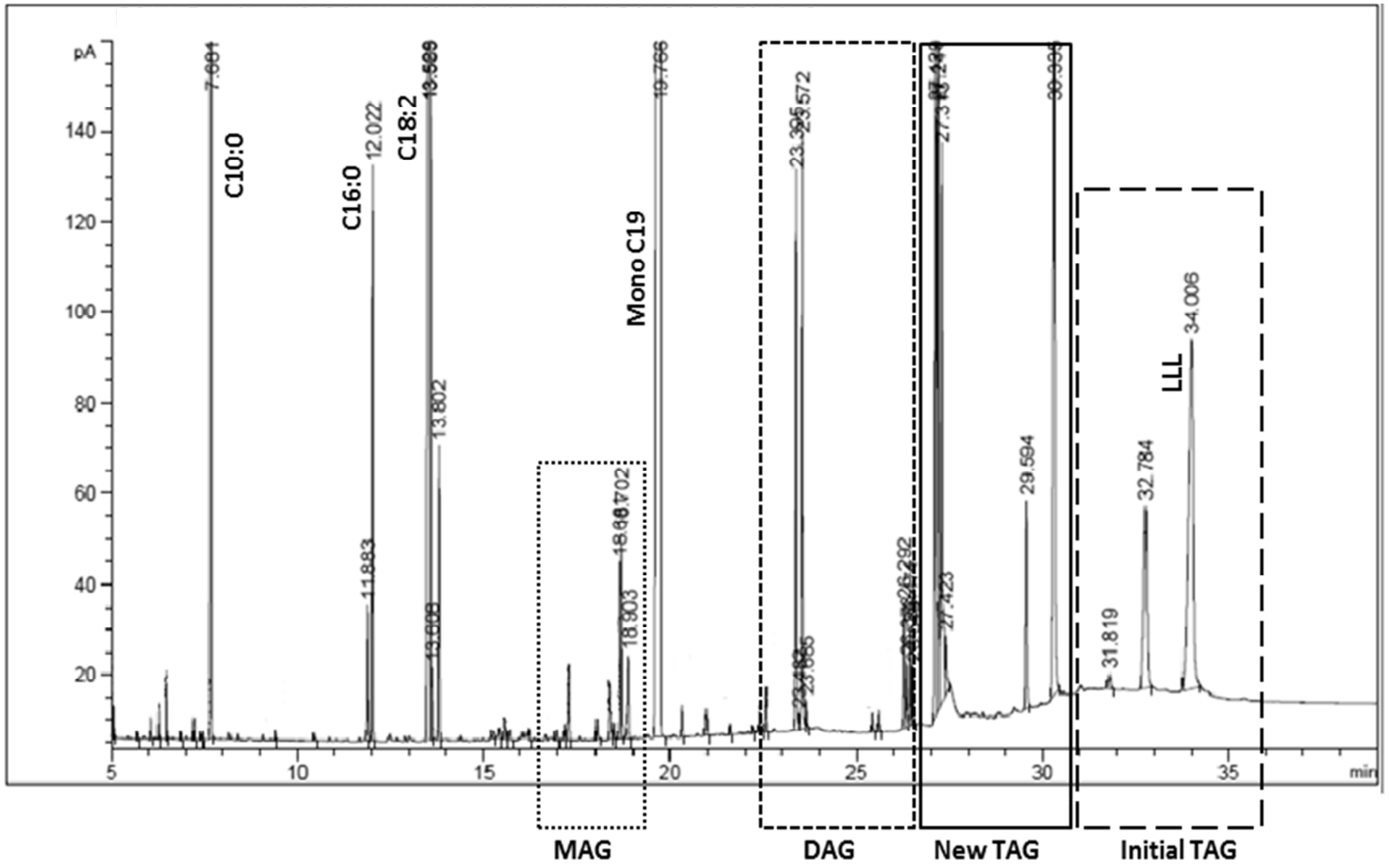
pA
140
120
100
80
60
40
20
C10:0
C16:0
C18:2
Mono C19
LLL
12.022
13.802
11.883
18.903
19.766
23.572
29.594
32.784
34.006
31.819
5
10
15
20
25
30
35
min
MAG
DAG
New TAG
Initial TAG

Fig. 1

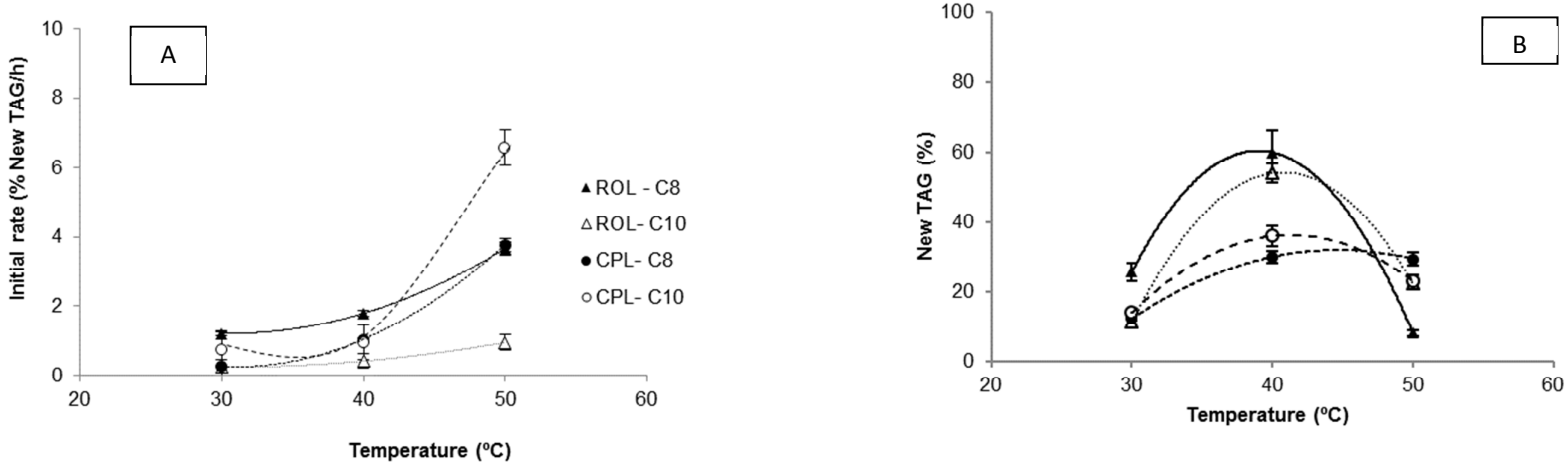
A
Initial rate (% New TAG/h)
Temperature (ºC)
ROL - C8
ROL- C10
CPL- C8
CPL- C10
B
New TAG (%)
Temperature (ºC)

Fig. 2

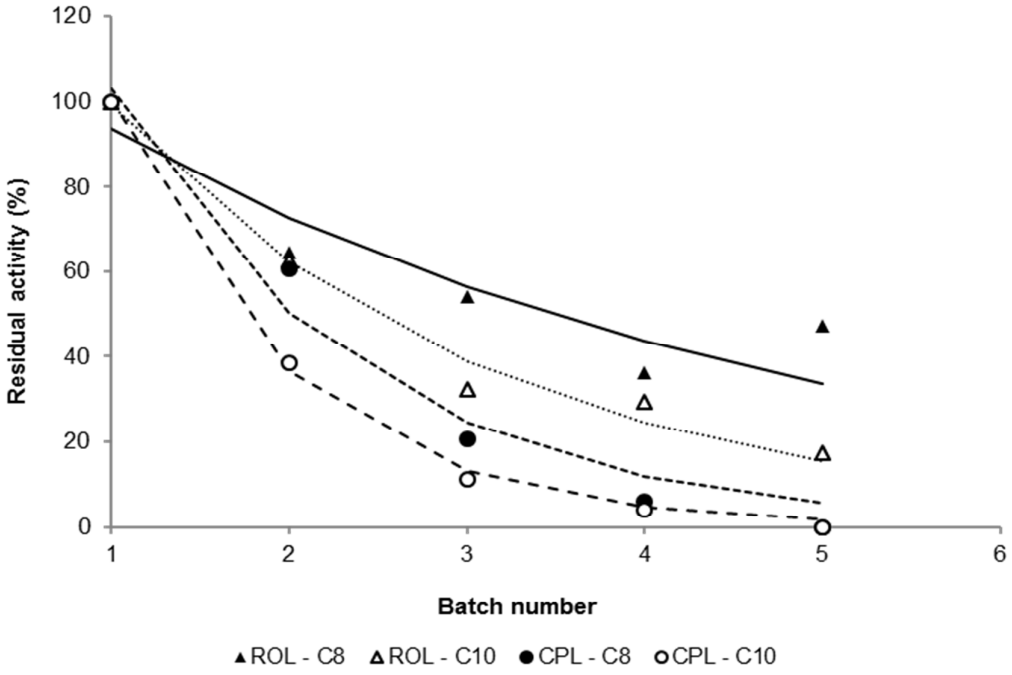
120
100
80
60
40
20
0
Residual activity (%)
1
2
3
4
5
6
Batch number
▲ROL - C8
△ROL - C10
●CPL - C8
○CPL - C10

Fig. 3

Production of MLM type Structured Lipids from Grapeseed Oil Catalyzed by Non-Commercial Lipases

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Running title: Production of MLM Lipids from Grapeseed Oil

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Abstract

Low calorie triacylglycerols (TAG) presenting medium-chain fatty acids (M) at positions *sn*-1,3 and long-chain fatty acids (L) at position *sn*-2, are known as MLM. This study aimed at the production of MLM by acidolysis of grapeseed oil with medium-chain caprylic (C8:0) or capric (C10:0) acids. Grapeseed oil was used as source of long-chain polyunsaturated fatty acids, especially linoleic acid, at *sn*-2 position in TAG. Reactions were performed in batch, in solvent-free systems, during 48 h. Novel non-commercial *sn*-1,3 regioselective lipases were used as alternative to high cost commercial biocatalysts, namely: the heterologous lipase from *Rhizopus oryzae* (rROL) immobilized in Amberlite™ IRA 96 and *Carica papaya* lipase (CPL) self-immobilized in papaya latex. The highest productions of new TAG were achieved at 40 °C, molar ratio TAG:M of 1:2, after 48 h, with both biocatalysts, with yields varying between 38 and 69 %. rROL immobilized in Amberlite IRA 96 showed a preference towards caprylic acid while CPL showed no preference towards caprylic or capric acid. First-order deactivation kinetics was observed for both biocatalysts. Half-lives at 40 °C were 166 and 118 h for rROL and 96 and 81 h for CPL, in the acidolysis of grapeseed oil with C8:0 or C10:0, respectively.

Practical applications

Grapeseeds of *Vitis vinifera* L. are a by-product of the wine industry. Using grapeseed oil to produce added-value functional oils rich in linoleic acid (essential fatty acid) may be a way of improving the revenues of the enological and oil sectors.

Both lipases, and mainly *Carica papaya* lipase self-immobilized in papaya latex, due to its low-cost production and easy preparation, are promising non-commercial biocatalysts for the synthesis of MLM in solvent-free media. The use of a solvent-free system, in addition of being a green option, is also preferred for economic reasons, avoiding the costs with solvent and solvent recovery. Also, the use of the stoichiometric molar ratio TAG:M of 1:2 will (i) decrease

costs related with the recovery and reutilization of medium chain fatty acids in excess, product recovery and purification, and (ii) avoid biocatalyst deactivation by high amounts of free fatty acids in reaction media.

Key words: *Carica papaya* lipase; grapeseed oil; low calorie lipids; *Rhizopus oryzae* lipase; structured lipids.

1. Introduction

Lipids are essential constituents of food diet mainly because of their high energy value and for being a good source of bioactive compounds such as liposoluble vitamins, antioxidants and essential fatty acids. Triacylglycerol (TAG) structure can be modified, namely by changing the original fatty acids (FA) by other FA with beneficial characteristics and/or by changing their original position in the glycerol skeleton. These modified fats, known as “structured lipids” (SL) or ‘‘taylor-made fats’’, can be produced either chemically or enzymatically. They represent a food industry growing segment in order to achieve natural and healthier fats (when enzymatic catalysts are used) as a response to the current consumer’s demand [1-3]. Dietetic TAG with low caloric value contain medium chain fatty acids (M) esterified at *sn*-1,3 positions and long chain fatty acids (L) esterified at *sn*-2 position of the glycerol backbone, and they are known as MLM. During digestion, the medium chain fatty acids, released from TAG by the action of the *sn*-1,3-regioselective pancreatic lipase, are preferentially transported via the portal vein to the liver, where they are metabolized as rapidly as glucose. Since these FA are not readily re-esterified into new TAG, they are not stored as fat, with weight control benefits [1, 3]. MLM present lower caloric value (5 to 7 kcal/g) than conventional fats and oils (9 kcal/g) [4]. In addition, the production of these SL is also important as a food source for persons with pancreatic insufficiency and other malabsorption problems [5].

Lipases (EC 3.1.1.3; triacylglycerol acylhydrolase) can be used as catalysts for SL production since they can catalyze esterification, acidolysis, alcoholysis and interesterification reactions, when used in non-aqueous media. Also, they are highly selective and some of them are regio-selective, which is not an option for chemical catalysts [2, 6]. They can be used non-immobilized or immobilized in solid supports in order to be reused in batch or used in continuous bioreactors. However, the high prices of commercial lipases, together with their frequently low operational stability, were recognized as the major constraints to the use of lipase-catalyzed processes [2]. Thus, the search for novel lipases with high activity and operational stability is needed for the implementation of enzymatic processes in the food industry.

Rhizopus oryzae lipase (rROL), cloned and expressed in the methylotrophic yeast *Pichia pastoris* [7], was used as a feasible non-commercial catalyst for the synthesis of TAG of MLM type [8-10]. *Carica papaya* lipase (CPL) self-immobilized in papaya latex is an example of a low-cost alternative to the commercial lipases. This lipase is extracted from green or sick papaya plants with high levels of defense enzymes from agro-wastes of papaya plantations and can be used as catalyst for SL production [11-13]. Table 1 shows some examples of using these non-commercial biocatalysts in the synthesis of structured lipids in solvent-free media.

The aim of this work was to produce MLM rich in linoleic acid, by acidolysis in solvent-free media, using non-commercial *sn*-1,3 regioselective recombinant *R. oryzae* lipase and *Carica papaya* lipase self-immobilized in papaya latex as catalysts. MLM were obtained by acidolysis of grapeseed oil with caprylic or capric acids (medium-chain FA). Grapeseed oil was used as source of polyunsaturated fatty acids (PUFA), especially linoleic acid (58.0-78.0 %), which is an essential fatty acid for humans. Grapeseeds of *Vitis vinifera* L. are a by-product of wine industry, which is an important agri-business in the Mediterranean region countries, USA, Argentina, Chile and Australia, among others. The use of grapeseed oil for the production of high added-value SL will be a revenue for the oenology sector.

2. Materials and Methods

2.1 Lipases and immobilization support

Non-commercial heterologous *Rhizopus oryzae* lipase (rROL) was produced by over-expression of the corresponding gene in a mutant strain of *Pichia pastoris* [7]. *Carica papaya* lipase (CPL), a self-immobilized biocatalyst preparation from papaya latex free of proteases, was obtained as previously described [12]. Only papaya latex particles smaller than 10 mesh were used as catalyst. CPL was extracted from unripe or sick fruits, because they contain higher levels of defensive enzymes [14]. Amberlite® IRA96, from Rohm and Haas, Lenntech, Philadelphia, U.S.A, was used for rROL immobilization. It is a macroreticular weak base anion exchange resin (styrene divinylbenzene copolymer with at least 85 % of tertiary amine as functional group).

2.2 Substrates

The substrate used as source of linoleic acid was refined grapeseed oil, produced by Smile Noël, Pont-Saint-Esprit, France. The fatty acid composition of refined grapeseed oil used in this work was evaluated by capillary gas chromatography as fatty acid methyl esters, and is presented in Table 2. The substrates used as source of medium chain fatty acids were caprylic acid (> 98 % purity; MW= 144.21) and capric acid (> 98 % purity; MW= 172.26), both from TCI Europe N.V., Belgium.

2.3 Chemicals

Glutaraldehyde (25 % aqueous solution) was purchased from Merck, Germany. Bovine serum albumin (BSA) (≥ 96 %), Coomassie-Brilliant Blue G (≥ 90 %) with purity *p.a.*, the standards trilinolein (99 %) and linoleic acid (99 %) were obtained from Sigma-Aldrich, Sintra, Portugal. The standard of monononadecanoin (99 %) was obtained from Larodan Fine Chemicals AB, Sweden; N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; > 90 %), was purchased from

TCI Europe N.V., Belgium; pyridine with purity *p.a.*, was obtained from Panreac Química S.L.U., Spain. The other reagents used were *p.a.* and obtained from different sources.

2.4 rROL immobilization in Amberlite® IRA96

The immobilization procedure for rROL in Amberlite® IRA96 was carried out according to the method described by Wang *et al* [14] after modification. Initially, 5 g of resin were added to 50 mL of deionized water, under magnetic stirring, during 30 minutes at 50 °C. After, the support was rinsed with 25 mL of 1 M NaOH aqueous solution followed with 25 mL of 1 M HCl aqueous solution, for three times, and subsequently equilibrated with sodium phosphate buffer (0.2 M, pH 7.5). The resin (1 g) and the lipase solution (10 mL of sodium phosphate buffer 0.2 M, pH 7.5, containing 0.25 g of rROL) were mixed together at 28 °C, for 4 h, under gentle magnetic stirring. These particles were filtered under reduced pressure and then rinsed with glutaraldehyde aqueous solution (0.5 % v/v) (25 mL/1 g resin). The immobilized lipase was washed three times with 20 mL sodium phosphate buffer, dried under vacuum filtration and stored at 4 °C until use.

2.5 Determination of rROL immobilization yield

The immobilization yield was determined in terms of (i) immobilized protein or (ii) estimated hydrolytic activity in the immobilized rROL. The protein content was assayed by the Bradford method using BSA as standard, by quantifying the unbound protein content in the supernatant before and after immobilization [15]. The immobilization protein yield (η_P) was estimated, according to the following equation:

$$\eta_P = \frac{P_0 - P_1}{P_0} \times 100 \qquad (1)$$

where P_0 is the protein amount present in the initial enzymatic solution (mg), before the immobilization support was added, and P_1 is the residual amount of protein (mg) present in the supernatant after immobilization, as well as in the subsequent washing solutions.

The hydrolytic activity of rROL was assayed using the method described by Soares *et al* [16], after modification: 50 g of extra virgin olive oil were mixed with 50 g of distilled water and 3.5 g of gum arabic. This mixture was stirred for 10 min. Then, 80 mL of phosphate buffer solution (pH 7.0, 0.1 M) were added and the final emulsion was put in a 20 mL closed thermostated cylindrical batch reactor, at 40 °C, under magnetic stirring. After, 1mL of initial enzyme solution was added and the hydrolysis was allowed to proceed for 5 min. After this time, 20 mL of ethanol/acetone solution (1:1 v/v) were added in order to inactivate the enzyme. The amount of free fatty acids (FFA) released during hydrolysis was determined by direct titration with a 0.1 N sodium hydroxide aqueous solution, using phenolphthalein as indicator. In parallel, blank experiments were carried out in the absence of enzyme. The same procedure was followed for the supernatants obtained after immobilization and washing the immobilized rROL, using 5 mL of each solution and 10 min reaction time.

The immobilization hydrolytic yield (η_H) was estimated as follows:

$$\eta_H = \frac{H_0 - H_1}{H_0} \times 100 \qquad (2)$$

Where H_0 is the hydrolytic activity (U) of the initial enzymatic solution, before the immobilization support was added, and H_1 is the residual hydrolytic activity (U) in the supernatant after immobilization, as well as in the subsequent washing solutions. One unit of hydrolytic activity (U) was defined as the amount of enzyme that catalyzes the hydrolysis of olive oil releasing one micromole of FFA per minute.

2.6 Assessment of hydrolytic activity of immobilized rROL and CPL preparations

The hydrolytic activity of rROL and CPL preparations was assayed using 0.3 g of immobilized enzyme and the hydrolysis was allowed to proceed for 5 min, as previously described (*cf*.

Determination of rROL immobilization yield). The assessment of hydrolytic activity of CPL preparation was performed directly as papaya latex lipase is a self-immobilized enzyme.

2.7 Acidolysis reactions

Acidolysis reactions were performed batchwise in closed thermostated 20 mL cylindrical batch reactors, under magnetic stirring, in solvent-free medium. A molar ratio TAG:FFA of 1:2 was used. This molar ratio corresponds to the stoichiometric value needed for the esterification of the free fatty acids at *sn*-1 and *sn*-3 positions by *sn*-1,3-selective lipases. The substrate mixture consisted of 3 g of grapeseed oil and 0.98 g of caprylic (C8:0) or 1.17 g of capric acid (C10:0). This reaction occurred at 40 °C, unless otherwise stated. A biocatalyst amount of 5 % (w/w) of the total TAG (grapeseed oil), i.e. 0.15 g of immobilized lipase, was used. Samples (0.05 mL) were taken from the reaction medium at 0, 24 and 48 h of reaction time. All the samples were stored at -18 °C for subsequent analysis. Reactions were carried out in triplicate.

2.8 Acidolysis kinetics and temperature effect

The reaction conditions for the MLM production assays were the same as described for acidolysis reaction, except for the reaction temperatures and the sampling times. These assays were performed at three different temperatures: 30, 40 and 50 °C, and samples were taken at 0, 3, 5, 8, 24 and 48 h of reaction time. Reactions were carried out in triplicate.

2.9 Batch operational stability tests

The operational stability of the biocatalysts was assayed under the same conditions of the acidolysis reactions, at 40 °C. Five consecutive 48 h-batches were carried out for rROL and CPL. At the end of each batch, the lipase was removed from the reaction medium and was added to fresh medium and reused in the next batch. Biocatalyst activities were estimated by the production of new TAG. The first batch was used as the reference (activity equal to 100 %). The

residual activity of the biocatalyst at the end of each batch was calculated relatively to the initial activity of the biocatalyst at the end of the first batch. 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 results was performed using "solver" from Excel for Windows, by minimizing the residual sum-of-squares between the experimental data points and those estimated by the model.

2.10 Analysis of reaction products

For TAG, mono- (MAG) and diacylglycerol (DAG), and FFA quantification, samples were analyzed by GC following the procedure described by the European standard EN 14105 [17], after modification, using monononadecanoin (Mono C19) as internal standard [18]. A solution of 0.5 % (w/v) of reaction medium in *n*-hexane was prepared. A volume of 0.5 mL of this solution was withdrawn and the solvent was evaporated in a rotavapor at 40ºC and 60 mbar. The dry residue was mixed with 400 μL of the internal standard solution (50 ± 0.1 mg of Mono-C19, dissolved in 20 mL of tetrahydrofuran), 200 μL of pyridine and 200 μL of N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). The reagents must be added by the mentioned order and under vigorous stirring, keeping the samples protected from humidity. After 15 minutes at room temperature, 4 mL of *n*-heptane was added to the mixture and 1 μL of this solution was GC analyzed. A gas chromatograph Agilant Tecnologies 7820A, equipped with on-column injector, a DB5-HT capillary column (15 m x 0.32 mm ID x 0.10 μm film) and a flame ionization detector was used. Injector and detector temperatures were set at 83 °C and 380 °C, respectively. Air and hydrogen were supplied to the detector at flow rates of 300 mL/min and 30 mL/min, respectively. Helium was used as carrier gas at a flow rate of 2.2 mL/min. The oven temperature program was as follows: 50 °C for 1 min, a temperature increase to 180 °C at 15 °C /min (ramp 1), followed by temperature increase at a rate of 7 °C /min (ramp 2) to 230 °C, a

final ramp (ramp 3) at a rate of 10 °C /min to 370 °C and a final plateau at 370 °C for 12 min. The total run time is 43 min.

The identification of the various groups of compounds was carried out by comparison with the chromatograms shown in the European Standard EN 14105 [17], and also by the retention times and reference standards. The reference standards used for the calibration curves were: trilinolein, linoleic acid, capric acid and caprylic acid. The conversion degree (%, w/w) of a determined compound was calculated by the ratio between the amount of consumed substrate and the amount of initial substrate. The mass of new TAG formed during the reaction was quantified considering the sum of the areas of all new peaks (retention times between 26 and 31 min) in TAG region of the chromatogram, using the calibration curve for trilinolein. The new TAG were quantified in terms of mass yields (%, w/w) determined by the ratio between the amount of new TAG produced and the amount of initial TAG. The masses of partial acylglycerols (MAG and DAG) were not quantified because they represented a reduced percentage in the samples, as confirmed by thin layer chromatography.

2.11 Statistical analysis

For each system, eventual significant differences between biocatalysts, for the amounts of new TAG produced and/or consumed substrates were investigated by one-way analysis of variance (ANOVA). The software Statistica, version 6, from Statsoft, Tulsa, OK, USA, was used and *post-hoc* comparisons were carried out using the Tukey test at a *p*-value of 0.05.

3. Results and Discussion

3.1 Immobilization yield of rROL in Amberlite IRA96 and biocatalyst hydrolytic activity

The amount of immobilized protein was 0.935 mg/g IRA96, corresponding to an immobilization protein yield of 22.3 %, while the hydrolytic yield was estimated as 64.7 %. This shows a

greater affinity of rROL molecules than of other proteins with no catalytic activity, present in the lyophilized extract, to the immobilization support. However, the hydrolytic activity directly measured for immobilized rROL was 497 U/g (std = 35; 3 replicates), corresponding to a retention of hydrolytic activity of only 35.6%. This yield is lower than the estimated yield in terms of hydrolytic activity, which may be due to some inactivation of rROL molecules during immobilization and/or mass transfer limitations inside the support. The hydrolytic activity for self-immobilized *Carica papaya* lipase was 698.2 U/g (std = 58; 3 replicates).

The hydrolytic activities of lipase preparations used in this study were only determined to evaluate if the enzymes have catalytic activity, but do not indicate which preparation has the highest catalytic activity in acidolysis. In fact, no direct relationships between hydrolytic and interesterification activities of rROL were previously found [9, 19].

3.2 Synthesis of MLM

Lipase-catalyzed acidolysis is a reversible reaction that has been considered as a hydrolysis-esterification two-step mechanism reaction [20]. Therefore, along the reaction catalyzed by *sn*-1,3 regioselective lipases, long-chain fatty acids at positions *sn*-1,3 of TAG of grapeseed oil are released to the reaction medium and C8:0 (or C10:0) molecules are esterified to form new DAG and new TAG (with one or two substitutions in the acylglycerol backbone). At equilibrium, based on the law of probabilities, the theoretical maximum value of fatty acid incorporation is 66.7 mol-%, when a *sn*-1,3 selective lipase is used [20].

The production of TAG of MLM type was evaluated by new TAG yields, and capric or caprylic acid consumptions. New TAG, MAG, DAG and also the release of FFA from grapeseed oil were observed during the reaction time (Fig. 1).

Table 3 shows the results obtained after 24 and 48 h acidolysis of grapeseed oil with caprylic or capric acid, aimed at the production of MLM, catalyzed by rROL or CPL. Concerning new TAG synthesis, no significant differences were observed between the results obtained

with rROL after 24 or 48 h reaction. For the systems where *C. papaya* lipase was used, an increase in new TAG was observed from 24 to 48 h. The yields of new TAG obtained with rROL were always higher than the values obtained with *C. papaya* lipase. In fact, the highest yield of new TAG (68.5 %) was observed with rROL for the incorporation of C8:0 after 24 h reaction. CPL showed no preference towards caprylic or capric acid. For rROL, a preference towards caprylic acid was observed. On the contrary, rROL immobilized on Eupergit C or on modified Sepiolite showed a preference towards capric acid, when used in the acidolysis of olive oil, in solvent-free system [8]. Again, the immobilization carrier seems to affect rROL lipase selectivity.

TAG consumption (Table 3) increased along 48 h-acidolysis for both biocatalysts, either in the presence of caprylic or capric acid. The highest values of TAG consumed after 48 h reaction (81.5 and 81.7 %) were observed for the systems with caprylic acid, catalyzed by rROL or CPL, respectively. These similar values of TAG consumption were not accompanied by similar values of new TAG formed (66.8 and 40.8 %, respectively). However, C8:0 or C10:0 consumptions were similar for both biocatalysts, increasing with reaction time. This may be explained by the high hydrolytic activity of *C. papaya* lipase. It is worth to notice that a 1.4-fold hydrolytic activity of *C. papaya* lipase with respect to rROL was not observed in terms of acidolysis activity. This was also verified with rROL obtained from different fermentation batches, having different hydrolytic activities but similar acidolysis activities [9].

In spite of the lack of preference shown by CPL towards C8:0 or C10:0 in the formation of new TAG, a preference towards C8:0 is observed in TAG consumption. Indeed, a preference for octanoin was observed in hydrolytic characterization of CPL [21].

The synthesis of MLM from virgin olive oil was also carried out in solvent free medium, with a molar ratio olive oil:FFA of 1:2 at 45 °C, using commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) as catalysts [22]. Capric acid incorporation was between 27.1 and 30.4 mol-% for all the biocatalysts, while the highest incorporation values of

C10:0 were observed with Novozym 435 (25.5 mol-%) and Lipozyme RM IM (25.7 mol-%). MLM TAG were also produced by Wang *et al* [23] with canola oil and caprylic acid (MR 1:4 canola oil/C8:0, 10 % lipase) catalyzed by Lipozyme RM IM. After 15 h acidolysis at 50-60 ºC, 45.4 mol-% incorporation of caprylic acid was observed. Öztürk *et al* [24] used Lipozyme TL IM for the acidolysis of corn oil and caprylic acid. Under optimized conditions (MR 1:3.9 corn oil/C8:0, 13.2 % lipase and 3.1 h reaction time), 21.5 mol.% of caprylic acid incorporation was obtained.

Although the higher yields in new TAG rich in caprylic or capric acid, obtained with rROL, both rROL and CPL (due to its lower-cost and easy preparation) were used for subsequent studies on MLM synthesis.

3.3 Temperature effect on the production of MLM

In order to investigate the effect of temperature on the production of new TAG, time-course experiments were carried out at 30, 40 and 50 °C. The initial rates of synthesis of new TAG, as well as the amounts of new TAG obtained after 48 h acidolysis, are presented in Fig. 2. An increase in initial rates with temperature is observed, mainly when *C. papaya* lipase is used. At 50 °C, the initial rates of acidolysis catalyzed by this lipase are similar for C8:0 and higher for C10:0 than those observed with rROL. When rROL was used, the initial rates showed not to depend so strongly from the temperature, especially with capric acid.

With respect to the new TAG production, the results showed a maximum at 40 °C and lower yields at 30 and 50 °C, for both biocatalysts, either in the system with caprylic or capric acids. At 40 °C, the highest yields of new TAG were obtained when rROL was used as catalyst, with preference for caprylic acid. At 30 or 50 °C, the results were similar for all the systems under study. In fact, in spite of high initial rates observed at 50 °C, for *C. papaya* lipase, low yields of new TAG are obtained at this temperature. This may be explained by a lipase thermal deactivation [21].

In the production of MLM using extra virgin olive oil, high acidolysis activity of rROL immobilized in Eupergit C was observed at 40 °C, in solvent-free system [8]. In the optimization of reaction conditions catalyzed by this biocatalyst, 37 and 35 °C were found as the optima for acidolysis with caprylic or capric acid, respectively [9].

3.4 Operational stability assays

The high cost of lipases, together with their low operational stability, were recognized as the main obstacles for the application of enzyme-catalyzed processes in the food industry. The use of reusable and stable immobilized lipases is a way to make enzymatic processes economically feasible. In our study, the use of non-commercial lipases aims at reducing the costs related to the use of expensive commercial lipases. Batch operational stability tests were carried out to evaluate the stability of the biocatalysts used during the acidolysis reaction of grapeseed oil with caprylic or capric acid. Acidolysis activity was evaluated in terms of new TAG yield. The residual acidolysis activity of immobilized rROL and *C. papaya* lipase, at the end of each 48 h-batch, is presented in Fig. 3. For both biocatalysts, the observed behaviour can be described by first-order deactivation kinetics, given by the following model equation:

$$A_n = Ae^{-k_d n} \qquad (3)$$

where A_n is the biocatalyst residual activity (%) at batch n, A is a constant representing the initial activity of the enzyme, before deactivation, n is the batch number and k_d is the deactivation rate constant, expressed in n^{-1}.

The deactivation models fitted to the experimental data, determination coefficients of these models, as well as half-life times ($t_{1/2}$) of each biocatalyst are presented in Table 4. Either in presence of caprylic or capric acids, a good fit of a first order deactivation model is observed for both biocatalysts ($0.845 \leq R^2 \leq 0.998$).

The highest operational stability was observed for immobilized rROL, either in presence of caprylic ($t_{1/2}$ = 166 h) or capric acid ($t_{1/2}$ = 118 h). *C. papaya* lipase also showed a good operational stability both in presence of caprylic ($t_{1/2}$ = 96 h) or capric acid ($t_{1/2}$ = 81 h). For both biocatalysts, higher operational stability was obtained in presence of caprylic acid.

According to Nunes *et al* [8], higher operational stability was also obtained for rROL immobilized in Eupergit C, in presence of caprylic acid ($t_{1/2}$= 159 h) instead of in presence of capric acid ($t_{1/2}$= 136 h).

In previous studies [10, 19], an increase in operational stability was observed when rROL immobilized in different supports was rehydrated between batches. The loss of activity could be explained by a progressive dehydration along the reactions. Faustino *et al* [18] concluded that a high initial water activity of rROL favours the acidolysis reaction. In fact, in all these experiments, rROL was used with its initial water activity after immobilization (a_w = 0.95).

However, the estimated half-life of rROL immobilized in Eupergit C was higher with capric acid ($t_{1/2}$= 54.3 h) than with caprylic acid ($t_{1/2}$= 39.0 h), when rehydrated between consecutive reuses in the acidolysis of olive oil [10]. Simões *et al* [25] obtained an estimated half-life of 112 h also using rROL immobilized in Accurel MP 1000 for the production of human milk fat substitutes (HMFS) by acidolysis of lard and polyunsaturated fatty acids. Tecelão *et al* [13] also used *C. papaya* latex for the HMFS production from tripalmitin and oleic acid. In the experiments with a MR oleic acid/tripalmitin of 2:1, the biocatalyst still retained 71.5 % of its initial activity at the end of the third consecutive 23 h-batch. The operational stability of this enzyme showed to decrease with the amount of free oleic acid in the reaction medium. In addition, a low acidolysis activity was observed in presence of polyunsaturated fatty acids [13]. Also, batch operational stability was assayed for the commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM and Novozym 435) used as catalysts for the synthesis of MLM from virgin olive oil, in solvent free medium [22]. After 10 consecutive 23 h-batches, the half-life times of these biocatalysts were estimated. The obtained values varied from 47 h for Novozym

435 with capric acid to around 300 h for Lipozyme RM IM with caprylic acid, while no deactivation was observed for Lipozyme RM IM in presence of capric acid [22].

Therefore, the operational stability of both non-commercial biocatalysts used in the present work is comparable to the values observed for the high cost commercial immobilized enzymes.

4. Conclusions

The heterologous *Rhizopus oryzae* lipase immobilized in Amberlite IRA 96 and *Carica papaya* lipase self-immobilized in papaya latex are promising non-commercial biocatalysts for the synthesis of low calorie TAG from grapeseed oil, as alternative to high cost commercial biocatalysts. The yields of new TAG rich in caprylic or capric acid, obtained after 48 h acidolysis in solvent-free media, varied from 38 to 41 % with *Carica papaya* lipase, or from 54 to 69 %, when immobilized rROL in Amberlite IRA 96 was used as catalyst. Both biocatalysts showed high operational stability at 40 °C, with half-life times higher than 81 h. The highest operational stability was observed for rROL in presence of caprylic acid ($t_{1/2}$ = 166 h). However, even presenting lower acidolysis activity than immobilized rROL, CPL did not show any preference towards caprylic or capric acid to produce new TAG and exhibited a high operational stability. In addition, the low cost of this biocatalyst obtained from agro-residues of papaya plantations may compensate its lower acidolysis activity in comparison with rROL immobilized in the resin Amberlite IRA 96. This is particularly important for industrial implementation of the enzymatic process. This study also showed that grapeseed oil is a good source of unsaturated fatty acids (namely linoleic acid) to incorporate in dietary TAG of MLM type. The use of this oil to produce added-value oils with important functional properties may be a way of improving the revenues of the oenological and oil industries.

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Figure captions:

Fig. 1: MLM production- example of a chromatogram after 48 h acidolysis reaction between capric acid and grapeseed oil catalyzed by immobilized rROL (DAG- diacylglycerols; LLL- trilinolein; MAG- monoacylglycerols; Mono C19- mononononadecanoin used as internal standard; TAG- triacylglycerols).

Fig. 2: Initial rates of new TAG production (A) and new TAG yields (B) after 48 h reaction, at 30, 40 and 50 °C, catalyzed by rROL or CPL.

Fig. 3: Residual activities of biocatalysts at the end of each consecutive 48 h batch, when acidolysis of grapeseed oil with capylic or capric acid was performed at 40°C.

Table 1. Examples of production of Structured Lipids (Human milk fat substitutes, HMFS, and MLM) catalyzed by rROL or CPL, in solvent-free media.

Structured Lipid type	Biocatalyst	Support	Reaction system	Fatty acid incorporation in new TAG (mol-%)	Reference
HMFS	rROL	Lewatit VP OC 1600	Tripalmitin + Oleic acid	30	[19]
		Accurel MP 1000	Tripalmitin + Oleic acid	22	[19]
		Eupergit C	Tripalmitin + Oleic acid	4.2	[19]
		Accurel MP 1000	Lard + PUFA	17	[24]
		Lewatit VP OC 1600	Tripalmitin + Fatty Acids from camelina oil	43.6	[18]
		Relizyme OD403/S	Tripalmitin + Fatty Acids from camelina oil	18.3	[18]
	CPL	Self-immobilized in the latex	Tripalmitin + Oleic acid	22.1	[13]
			Tripalmitin + PUFA	8.7	[13]
MLM	rROL	Eupergit C	Olive oil + caprylic acid	21.6	[8]
		Eupergit C	Olive oil + capric acid	34.8	[8]
		Modified sepiolite	Olive oil + caprylic acid	4.4	[8]
		Modified sepiolite	Olive oil + capric acid	5.5	[8]

Table 2. Fatty acid composition (mol.%) of grapeseed oil

Fatty Acid Group	Fatty acid	Concentration (mol.%)
Saturated Fatty Acids	C14:0	< 0.01
	C15:0	0.01
	C16:0	7.73
	C17:0	0.07
	C18:0	4.69
	C20:0	0.23
	C21:0	0.10
	C22:0	0.07
	C24:0	0.09
Monounsaturated Fatty Acids	C16:1	0.12
	C17:1	0.03
	C18:1	1.22
	C20:1	0.24
Polynsaturated Fatty Acids	C18:2	84.96
	C18:3	0.39
	C20:2	0.04

Table 3. New TAG yields, TAG consumed, caprylic (C8:0) or capric (C10:0) acids consumed for the four MLM production systems, at 24 or 48 h reaction time, catalyzed by rROL or CPL. In each type of compound, superscript indexes indicate differences based on Tukey test ($p \leq 0.05$) and with ± equal to the standard deviation.

		System			
Compounds	**Time (h)**	**rROL – C8:0**	**rROL – C10:0**	**CPL – C8:0**	**CPL – C10:0**
TAG new (%, w/w)	24 h	68.5^{c} ±4.1	$52.4^{b,c}$±6.6	28.3^{a}±7.4	32.7^{a}±5.7
	48 h	66.8^{c}±3.8	$54.3^{b,c}$±7.3	40.8^{b}±0.3	38.2^{b}±3.4
TAG consumed (%, w/w)	24 h	$69.0^{b,c}$±0.8	43.7^{a}±8.6	48.5^{a}±0.4	63.7^{b}±1.3
	48 h	81.5^{d}±0.8	64.2^{b}±0.2	81.7^{d}±2.9	73.9^{c}±1.9
C8:0 or C10:0 consumed (%, w/w)	24 h	$35.0^{b,c}$±1.1	$30.8^{a,b}$±9.6	25.9^{a}±0.3	$37.3^{c,d}$±5.0
	48 h	$45.7^{e,f}$±5.6	$42.7^{d,e}$±8.4	$45.4^{e,f}$±8.1	49.5^{f}±2.7

Table 4. Deactivation models fitted to rROL and *Carica papaya* lipase (CPL) in the systems with caprylic (C8:0) or capric (C10:0) acids, respective determination coefficients (R^2) and half-life times ($t_{1/2}$) of the biocatalysts.

System	Model equation	R^2	$t_{1/2}$(h)
rROL + C8:0	$A_n = 120.92e^{-0.25n}$	0.845	166
rROL + C10:0	$A_n = 158.27e^{-0.47n}$	0.984	118
CPL + C8:0	$A_n = 212.22e^{-0.72n}$	0.968	96
CPL + C10:0	$A_n = 276.36e^{-1.01n}$	0.998	81

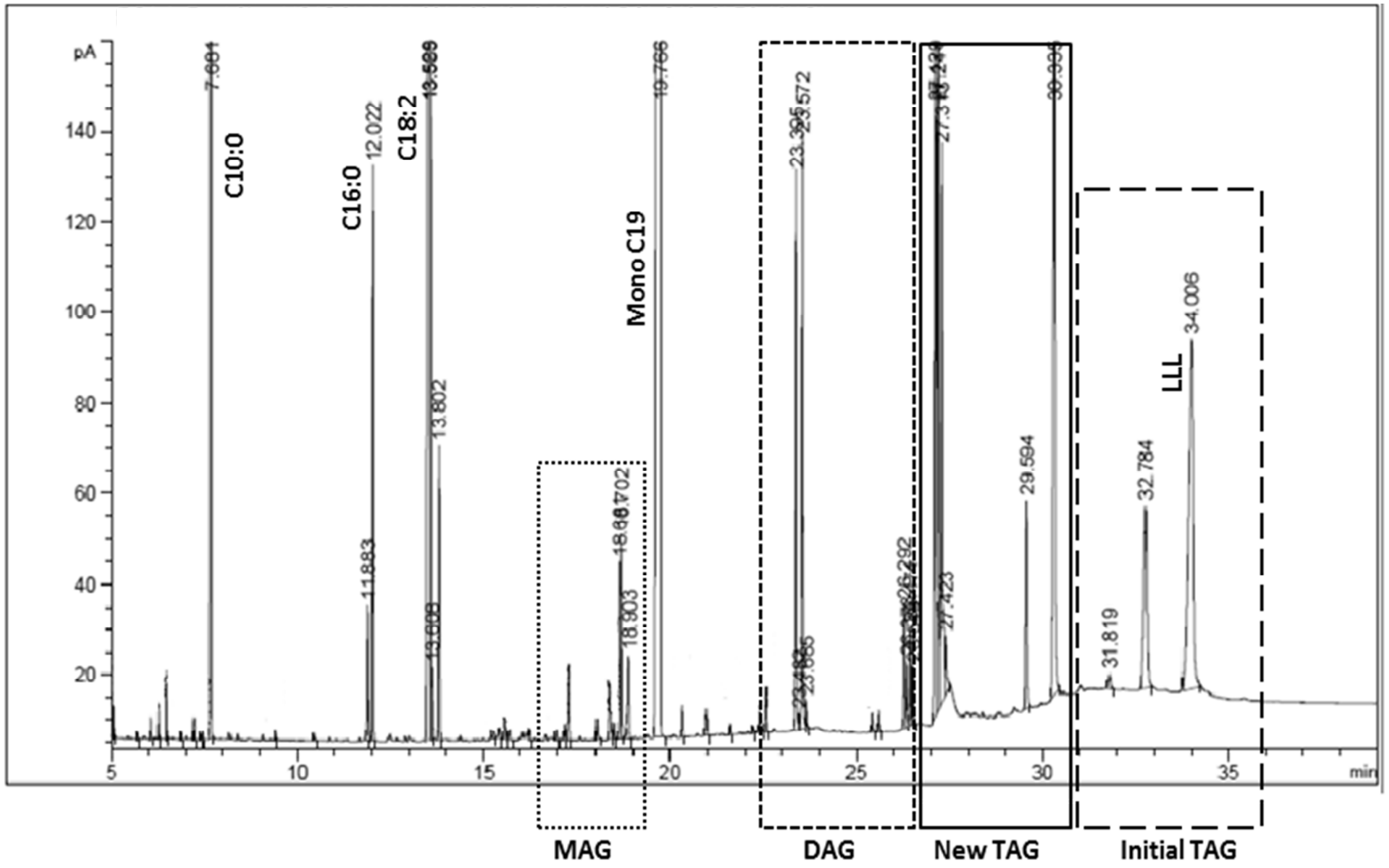
pA
140
120
100
80
60
40
20
C10:0
C16:0
C18:2
Mono C19
LLL
12.022
11.883
13.802
19.766
18.903
23.572
29.594
32.784
34.006
31.819
5
10
15
20
25
30
35
min
MAG
DAG
New TAG
Initial TAG

Fig. 1

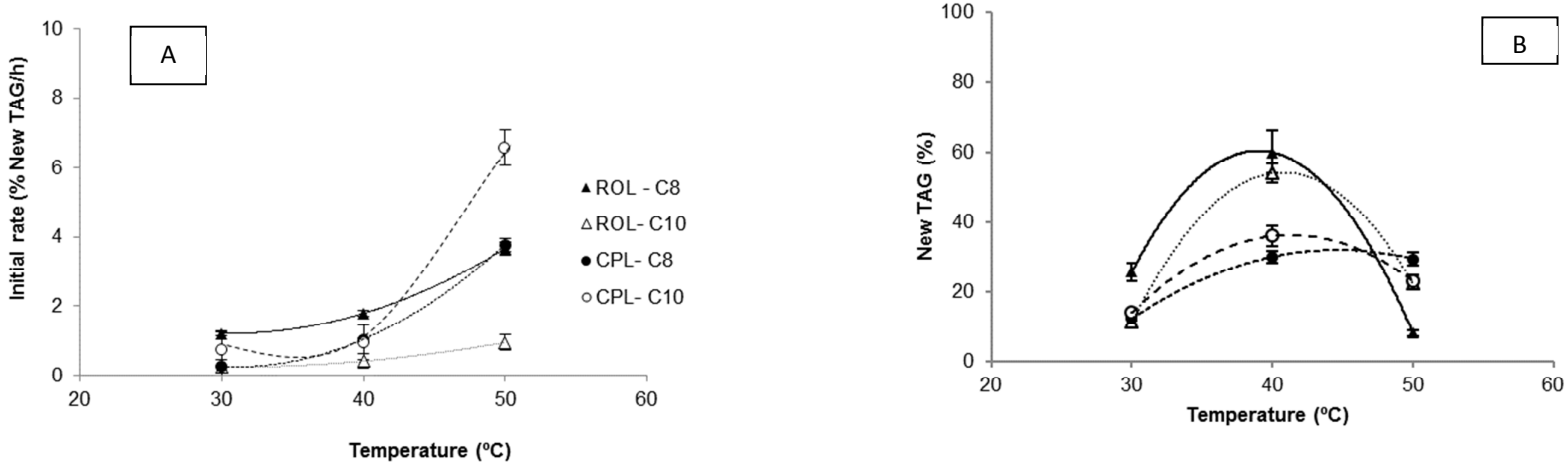
A
Initial rate (% New TAG/h)
Temperature (ºC)
ROL - C8
ROL- C10
CPL- C8
CPL- C10
B
New TAG (%)
Temperature (ºC)

Fig. 2

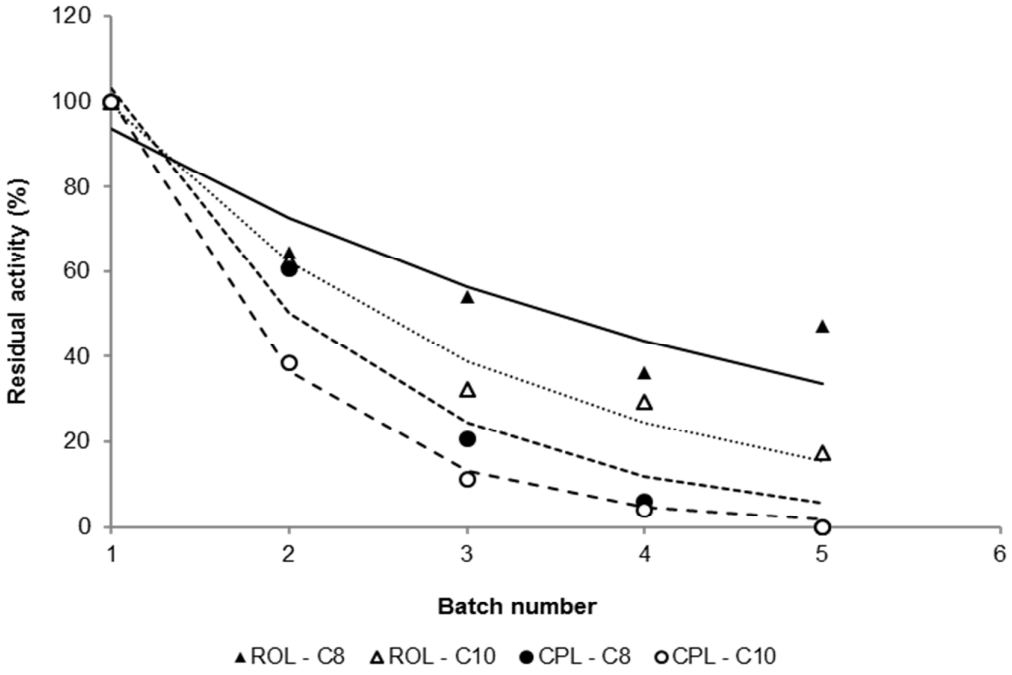
120
100
80
60
40
20
0
Residual activity (%)
1
2
3
4
5
6
Batch number
ROL - C8
ROL - C10
CPL - C8
CPL - C10

Fig. 3