

Production of Dietetic Triacylglycerols from Olive Oil Catalyzed by Immobilized Heterologous *Rhizopus oryzae* Lipase

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In this study, low calorie triacylglycerols (TAGs) of MLM type (containing a medium-chain fatty acid, M, at positions *sn*-1,3 and a long-chain fatty acid at position *sn*-2) were produced by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids, in solvent-free media. The heterologous *sn*-1,3 regioselective *Rhizopus oryzae* lipase produced by the methylotrophic yeast *Pichia pastoris* (rROL) was immobilized in Amberlite IRA 96® (rROL-IRA) and used as biocatalyst. Acidolysis was optimized as a function of temperature and molar ratio (C:8/TAG or C10:0/TAG), by response surface methodology. The production of new TAGs was followed by the consumption of C8:0 or C10:0, and of initial TAGs from olive oil. From the response-surfaces fitted to the experimental data points, no optimal points were observed inside the experimental region. The highest consumption of initial TAGs, free fatty acids and triolein (the major TAG of olive oil) were achieved at 29 °C and molar ratio (MR) of 2:1 at 24 h reaction time. For the system with virgin olive oil and caprylic acid (C8:0), the highest consumption of TAGs was 76.9% while for the system with virgin olive oil and C10:0, 85.6 % of TAGs consumption was observed. The production of structured TAG from olive oil and medium chain fatty acids (caprylic and capric acids) catalyzed by the heterologous *Rhizopus oryzae* lipase immobilized in Amberlite IRA 96 is a promising alternative to the high-cost immobilized commercial lipases for MLM production.

1. Introduction

The structured lipids (SLs) of MLM-type are defined as triacylglycerols (TAGs) containing medium chain (6–12 carbon atoms) and saturated fatty acids (M) in the *sn*-1 and *sn*-3 positions and long-chain (14–24 carbon atoms) saturated or unsaturated fatty acids (L) in the *sn*-2 position. The interest on these SLs has sharply increased due to their unique nutritional properties (Arifin *et al.*, 2010). For clinical nutritional purposes, MLM structured lipids are of interest since they are hypocaloric (5-7 kcal/g). Furthermore, mono or polyunsaturated long-chain fatty acids are more efficiently absorbed when located at the *sn*-2 bond (Morales-Medina *et al.*, 2017). Triacylglycerols of MLM type can be produced by acidolysis between TAGs (vegetable or fish oils) and medium chain fatty acids (caprylic, C8:0, or capric acids, C10:0) as acyl donors, catalyzed *sn*-1,3 regioselective lipases, mostly from microbial sources, either in solvent or in solvent-free media (Esteban *et al.*, 2011, Nunes *et al.*, 2011a, Nunes *et al.*, 2011b, Nunes *et al.*, 2012, Terada *et al.*, 2015, Sanchez *et al.*, 2017). The main constraint of this methodology has been the price of commercial enzymes currently used in most of the studies on oils and fats modification. However, the use of immobilized and lower-cost non-commercial lipases has made this method potentially viable (Palla *et al.*, 2012, Caballero *et al.*, 2014, Kim and Akoh, 2015). The immobilization process of the enzyme may increase its operational stability and enables enzyme reutilization in repeated batches and the implementation of continuous processes, improving the cost efficiency. The search for non-commercial *sn*-1,3 regioselective lipases capable to catalyze reactions to

produce SLs with specific functional properties, greatly increased during the last decades (Nunes *et al.*, 2011b, Nunes *et al.*, 2012, Casas-Godoy *et al.*, 2013; Faustino *et al.* 2016, Costa *et al.*, 2017). In this trend, the heterologous *Rhizopus oryzae* lipase (rROL) has been produced by our group by over-expression of the corresponding gene in a mutant strain of *Pichia pastoris* (Guillén *et al.*, 2011). rROL immobilized in Eupergit® C was tested as catalyst for the production of MLM by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids, in solvent-free medium (Nunes *et al.* 2011) and the reaction was optimized by response surface methodology (Nunes *et al.*, 2012). At 40 °C, 21.6 mol% of C8:0 and 34.8 mol% of C10:0 incorporation in TAGs of virgin olive oil were attained, after 24 h reaction time. This enzyme was also immobilized in Amberlite® IRA96 resin and used as catalyst for the acidolysis of grapeseed oil with caprylic or capric acids in solvent-free media (Costa *et al.*, 2017). After 24 h acidolysis, 68.5% and 52.4 % yield of new TAGs containing C8:0 or C10:0 were obtained, respectively. These results obtained with rROL are rather promising since they are similar to those attained with high-cost commercial immobilized lipase preparations (Nunes *et al.*, 2011a).

The aim of the present study is to optimize the production of low calorie TAGs by acidolysis of virgin olive oil with C8:0 or C10:0, in solvent-free media, as a function of temperature and molar ratio caprylic acid/olive oil or capric acid/olive oil, by response surface methodology (RSM), using rROL immobilized in Amberlite IRA96 as catalyst. The choice of a solvent-free system will maximize volume productivity, simplify the downstream processing, decrease costs and is a clean and environmentally friendly process, adequate for the food industry.

2. Materials

Extra virgin olive oil (acidity of 0.7% expressed as free oleic acid) was purchased from a Portuguese local supermarket. Caprylic acid (C8:0, octanoic acid) and capric acid (C10:0, n-decanoic acid) were purchased from TCI Europe N.V., Belgium, Amberlit IRA96® resin was obtained from Rohm and Haas, Lenntech, Philadelphia, U.S.A. The standard of monononadecanoin (minimum 99% pure) was obtained from Larodan Fine Chemicals AB, Sweden; N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (minimum 90% pure) was purchased from TCI Europe N.V., Belgium; glutaraldehyde (25 % aqueous solution) was purchased from Merck, Germany. All solvents and reagents for analyses were chromatographic or analytical grade and obtained from different sources. *Rhizopus oryzae* lipase (rROL) was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB), Spain. The rROL was obtained by a fed-batch cultivation of a recombinant *Pichia pastoris* strain using methanol as inductor (Barrigón *et al.*, 2013).

The biomass was separated from the culture broth by centrifugation and microfiltration. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system equipped with an Omega membrane of 10 kDa cut-off, and subsequently dialyzed against 10mM Tris-HCl buffer pH 7.5 and finally lyophilized (Palla *et al.*, 2012).

2.1. Methods

2.1.1. Preparation of immobilized rROL

The procedure followed for rROL immobilization in Amberlit IRA96 was adapted from the methodology described by Wang *et al.*, (2010), according to Costa *et al.* (2017). Anion resins (0.5 g of dry resins) were treated with 50 °C deionized water for 30 min. Then, the resins were recovered by vacuum filtration and washed with 1 M NaOH and 1 M HCl solutions alternately for three times and equilibrated with sodium phosphate buffer (0.2 M, pH 7.5, 2 x 50 mL) in the end. The resins and the lipase solution (5 mL of sodium phosphate buffer 0.2 M, pH 7.5, containing 0.125 g of rROL) were mixed together at 28 °C for 4 h. After this preliminary adsorption, the particles were filtered under reduced pressure and put in a solution of glutaraldehyde (0.5% v/v, 25 mL solution of glutaraldehyde/g resin), at 28 °C during 20 min under agitation. The resin was, once again, recovered by vacuum filtration and washed twice with 50 mL of phosphate buffer solution (0.2 mol/L, pH 7.5) to remove the free enzyme. The immobilized lipase was dried under vacuum for approximately 24 h and stored at 4 °C until use.

2.1.2. Acidolysis Reaction

The acidolysis reactions were carried out in solvent-free media, in thermostated-capped cylindrical glass vessels under magnetic stirring at 400 rpm. Reaction media consisted of 3.0 g of extra virgin olive oil and caprylic (C8:0) or capric acid (C10:0) in amounts dictated by the experimental design corresponding to different molar ratios (MR) of free fatty acid to olive oil. For MR calculations, TAGs of olive oil are assumed to be triolein. The temperature (T) varied according to the experimental design (Table 1). In each experiment, 0.15 g of immobilized enzyme, corresponding to 5 % (w/w) of the amount of TAGs (olive oil), were added to

the reaction medium, after complete melting. After 24 and 48 h of reaction, 0.5 mL of the reaction medium was withdrawn, and the reaction medium was stored at -20 °C until analysis. The acidolysis reaction was monitored by the consumption of caprylic or capric acids, and TAGs of olive oil (consumed TAGs/initial TAGs, w/w- %) including triolein, which is the major TAG of olive oil (Faustino *et al.* 2016, Costa *et al.*, 2017).

2.1.3. Experimental Design and Statistical Analysis

The best reaction conditions for the acidolysis reaction were established via RSM. The statistical optimization experiments were carried out according to a Central Composite Rotatable Design (CCRD) with 11 experiments (3 center points, 4 factorial points and 4 star points), as a function of molar ratio (MR fatty acid/olive oil; 1.6:1-4.4:1) and reaction temperature (T; 29.4-55 °C) (Table 1). For every experiment of the CCRD, the percentage (w/w) of consumed C8:0 or C10:0, TAGs of olive oil were analyzed using the software "Statistica", version 6, from Statsoft, Tulsa, USA.

2.1.4. Quantification and analysis of reaction products

The quantification of substrates and products was achieved by high temperature gas chromatography after derivatization of the samples with pyridine and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), according to the European Standard (EN 14105:2011), after modification (Faustino *et al.* 2016, Costa *et al.*, 2017). A gas chromatograph (Agilent Technologies 7820A) equipped with a flame ionization detector (FID) and capillary column DB5-HT (15 m x 0.32 mm ID x 0.10 µm film), an auto sampler Agilent Technologies 7820 and an on-column mode injector. Injector and detector temperatures were set at 83 °C and 380 °C, respectively. Helium was used as carrier gas at a flow rate of 2.2 mL/min. Air and hydrogen were supplied to the detector at flow rates of 300 mL/min and 30 mL/min, respectively. The oven temperature program was as follows: 50 °C for 1 min, a temperature increase to 180 °C at 15 °C/min, followed by temperature increase at a rate of 7 °C /min to 230 °C, a final ramp at a rate of 10 °C /min to 370 °C and a final plateau at 370 °C for 12 min (total running time of 43 min). Calibration curves for C8:0 and C10:0 were used to quantify the mass percentage of consumed medium-chain fatty acids and the calibration curve for triolein was used to quantify the consumed TAGs (% Consumed TAG/Initial TAG).

3. Results and Discussion

3.1. Modeling MLM Production

The production of dietetic triacylglycerols of MLM type was evaluated through the consumption of capric or caprylic acid, consumption of initial TAG and triolein, after 24-h acidolysis in solvent-free media, catalyzed by rROL immobilized on Amberlite IRA96®, under the conditions dictated by the experimental design. The obtained results concerning the percentage of consumed TAGs are presented in Table 1.

Table 1: Experimental design matrix used (CCRD) as a function of molar ratio of medium-chain fatty acid/TAG (MR) and temperature (T) and the experimental results: percentage of consumed TAGs in the acidolysis of olive oil with C8:0 or C10:0 after 24 h reaction catalyzed by rROL immobilized in Amberlite IRA96®.

Experiment	Coded Matrix		Experimental Design Decoded Matrix		Experiments results Consumed TAGs (%)	
	(X ₁)	(X ₂)	Temperature (X ₁ , °C)	Molar Ratio (X ₂ , MR)	System C8:0	System C10:0
1	-1	-1	29.4	2:1	76.9	85.6
2	-1	+1	29.4	4:1	8.7	39.5
3	+1	-1	50.6	2:1	21.5	29.4
4	+1	+1	50.6	4:1	25.9	40.4
5	-√2	0	25	3:1	52.7	48.8
6	+√2	0	55	3:1	27.9	33.9
7	0	-√2	40	1.6:1	6.9	70.7
8	0	+√2	40	4.4:1	29.9	42.7
9	0	0	40	3:1	46.2	34.1
10	0	0	40	3:1	54.9	39.0
11	0	0	40	3:1	41.1	31.9

For both systems, the highest percentages of consumption of TAGs were achieved in experiment 1 at 29.4 °C and molar ratio of 2:1 after 24 h reaction. Under these conditions, 76.9 and 85.6 % of the initial TAGs were consumed in the system with C8:0 and in the system with C10:0, respectively. Also, for the system with capric acid, a high consumption of initial TAGs (70.7%) was observed in the experiment 7 (MR=1.6:1; T=40 °C; star point). However, under the same reaction conditions, much lower TAGs consumption values (6.9 %) were observed when caprylic acid was used. The preference towards capric acid was also observed for rROL immobilized on Eupergit C or on modified sepiolite in the acidolysis of olive oil, in solvent-free system (Nunes *et al.*, 2011). On the contrary, when rROL immobilized in Amberlite IRA96 was used as catalyst for the acidolysis of grapeseed oil in solvent-free medium, the highest yield of new TAG (68.5 %) was observed with caprylic acid (Costa *et al.*, 2017). In fact, oleic acid is the major fatty acid of olive oil while linoleic acid is the major fatty acid of grapeseed oil. The oil composition and the immobilization carrier seem to affect rROL lipase selectivity and activity.

The main effects of T and MR and of the interaction between MR and T on the acidolysis reaction were calculated (Table 2). A positive or a negative linear effect of a particular factor (MR or temperature), on the response means that an increase in the value of that factor results in an increase or reduction in the response, respectively. A negative (or positive) quadratic effect indicates that the response is described by a convex (or concave) response surface.

Table 2: Linear and quadratic effects of factors and of interaction, and respective p-levels (values between brackets), of MR medium chain fatty acids/triacylglycerols and temperature (T) on the TAGs consumption in the acidolysis of virgin olive oil with C8:0 or C10:0, catalyzed by rROL immobilized in Amberlite IRA96®.

Factor	TAG consumption	
	System with C8:0	System with C10:0
MR (linear term)	-7.8 (0.520)	-18.6 (0.007)
MR (quadratic term)	-27.1 (0.101)	21.6 (0.007)
T (linear term)	-18.3 (0.167)	-19.1 (0.006)
T (quadratic term)	-5.2 (0.718)	6.2 (0.269)
MR x T (interaction)	36.3 (0.0731)	28.5 (0.0048)

For the system with caprylic acid (C8:0), the interaction MR x T has a positive effect on the percentage of consumed TAG indicating that a simultaneous increase in these factors promotes TAG consumption. However, an increase in temperature alone promotes a decrease in TAG consumption, *i.e.* in acidolysis reaction. This can be ascribed to a thermal deactivation of rROL. No significant effects ($p \gg 0.05$) of MR (linear term) and T (quadratic term) were observed. The negative quadratic effect of MR indicates a convex response surface as a function of MR.

With respect to the system with capric acid, an increase in either MR or temperature promotes a decrease in TAGs consumption, while a positive effect of the interaction is observed. Again, high amounts of capric acid or higher temperature values may cause rROL deactivation. It would be expected that an increase in the molar ratio and therefore in available medium-chain fatty acids for the reaction would lead to an increased consumption of TAGs. However, the experimentally observed trend was precisely the inverse: increasing the MR resulted in a decrease in consumption of TAGs. This may be related to an enzyme inhibition by the free fatty acids or a loss of lipase activity. High levels of free fatty acids produce high levels of carboxylic acids, which may acidify the aqueous phase in the microenvironment of the lipase, or cause water adsorption in the interphase where the lipase operates, limiting its activity.

Three-dimensional response surfaces described by second-order polynomial model as a function of T and MR were fitted to the experimental data-points of TAGs consumption in both systems (Table 3, Figure 1).

Table 3: Model equations (decoded values) for the response surfaces fitted to the consumption of TAG during the acidolysis of olive oil with caprylic (C8:0) or capric acid (C10:0) catalyzed by rROL immobilized in Amberlite IRA96 and respective R^2 and R^2_{adj} .

System	Model equations	R^2	R^2_{adj}
Olive oil + C8:0	TAG consumed/TAG initial = 190.36 - 6.12 T - 12.35 MR ² + 1.75 (MR x T)	0.71	0.57
Olive oil + C10:0	TAG consumed/TAG initial = 401.67 - 127.87 MR + 10.77 MR ² - 7.14 T + 0.028 T ² + 1.35 (MR x T)	0.94	0.89

In these equations, only the factors with significant effects or those whose removal would lead to a lack of fit of the models, were retained (Haaland, 1989). The coefficients of determination (R^2) and the adjusted coefficients of determination (R^2_{adj}) of these polynomials are also shown in Table 3. High values of both symbols R^2 and R^2_{adj} these models show a good fit for the consumption of TAGs in the system with caprylic acid ($R^2 = 0.71$), as well as an excellent fit for consumption TAGs ($R^2 = 0.94$) in the system with capric acid.

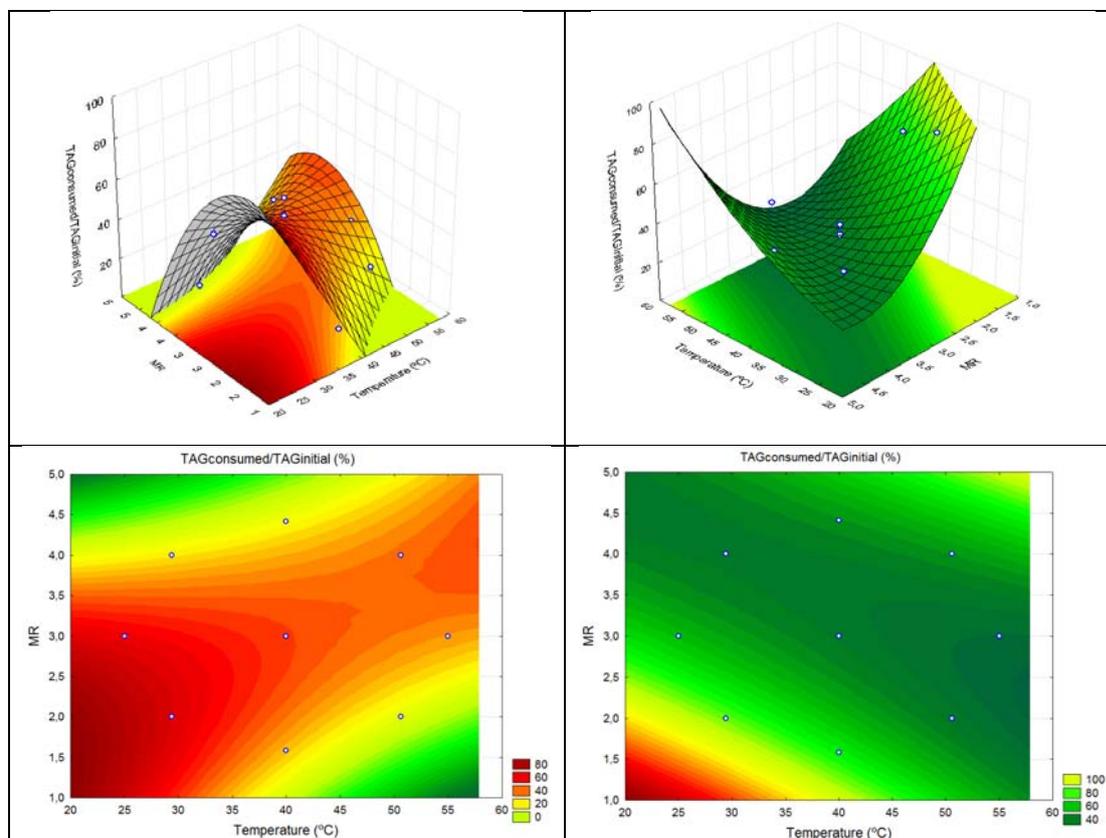


Figure 1: Response surfaces fitted to the experimental data (% Consumed TAGs), and respective contour plots: a) system with C8:0; b) system with C10:0, as a function of the molar ratio (MR) and temperature.

The response surfaces fitted to the percentage of consumed TAGs are saddle-like and concave for the systems with caprylic or capric acid, respectively. From these response-surfaces fitted, no optimal points (maximum TAG consumption) were observed inside the considered experimental region. Thus, only the identification of the regions corresponding to higher TAG consumption could be achieved. For both systems, the best results are expected at low molar ratios (≤ 3 for caprylic acid; ≤ 2 for capric acid) and low temperatures (lower than 30°C- 35 °C).

These values were similar to the optima values predicted by RSM for the production of MLM by acidolysis of virgin olive oil with caprylic or capric acids using the lipase Lip2 from *Yarrowia lipolytica*, immobilized in Accurel MP 1000 (Godoy *et al.*, 2013). Under optimized conditions for Lip2 (48 h reaction at 40 °C, molar ratio of 2:1 medium chain fatty acid/TAG), the highest incorporation yield was 25.51 mol % for C8:0 and 17.9 mol % for C10:0.

In the production of human milk fat substitutes, (i) rROL immobilized in Accurel® MP 1000 was used as catalyst for the acidolysis of lard with omega-3 polyunsaturated fatty acids (Simões *et al.*, 2014) or (ii) immobilized in Lewatit VP OC 1600 and used in the acidolysis of tripalmitin with polyunsaturated fatty acids from camelina oil (Faustino *et al.*, 2016). Again, the best results were obtained for lower values of temperature and molar ratios FFA:TAGs .

4. Conclusions

The lipase-catalyzed production of low-calorie structured triacylglycerols from virgin olive oil and medium chain fatty acids (caprylic and capric acids) in a batch reactor was modelled by RSM. The non-commercial *sn*-

1,3 regioselective rROL immobilized in Amberlite IRA96 was able to catalyze the incorporation of caprylic acid and capric into olive oil, in solvent-free media This biocatalyst showed to be a feasible option to the high-cost immobilized lipases used for structured lipids production.

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