Summary of the dissertation for the degree of Master in Biotechnology

Lipase-Catalysed Production of Dietetic Structured Lipids Using Olive Pomace Crude Oils, in Continuous Bioreactor

Joana de S. Gonçalves[1,2], Arsénio Fialho[1] Suzana Ferreira-Dias[2]
1 – Instituto Superior Técnico, Universidade de Lisboa, Portugal
2 – Instituto Superior de Agronomia, Universidade de Lisboa, Portugal

Low-calorie structured lipids, namely MLM-type, are triacylglycerols (TAG) known for their nutrition benefits in our diet. The aim of this work was the lipase-catalysed production of MLM containing caprylic acid (C8:0) or capric acid (C10:0), by acidolysis or interesterification with ethyl esters (C8EE e C10EE), using crude olive pomace oil (OPO) as raw-material. OPO presented high acidity, oxidation products and chlorophylls. Reactions were performed in continuous packed-bed bioreactor (60-122 h), using *sn*-1,3 regioselective commercial immobilized lipases (Lipozyme TLIM or Lipozyme RMIM), in solvent-free media, at 40°C. The results on the production of New TAGs (50-60 % yield) were rather promising, in the range of the assessed acidity. Along with continuous bioreactor operation, both biocatalysts demonstrated high activity and operational stability. First-order model (C8EE + Lipozyme RMIM), linear model (C10:0 + Lipozyme TLIM) and Sadana model (C10EE + Lipozyme TLIM) were fitted to data. Half-lives of 74, 228 and 213 h were estimated, respectively. In the other systems, the biocatalysts did not lose activity along operation. Lipozyme RMIM cost is more than 8-fold the costs of Lipozyme TLIM. Therefore, the use of Lipozyme TLIM and crude OPO will reduce process costs (biocatalyst and oil refining), promoting the sustainability of the process.

Keywords: Structured Lipids, Olive pomace oil, Continuous bioreactor, Interesterification, Acidolysis, Lipases.

1. Introduction

Lipids are a diverse group of molecules, crucial compounds of the population diet providing energy, essential fatty acids, vitamins and antioxidants. Some of them can be considered as functional foods (*e.g.*, virgin olive oil) but others can be modified, being an important subject for different food applications (Zhang *et al.*, 2021).

Natural oils and fats have their functional and nutritional characteristics associated to fatty acid (FA) composition and, in turn, organised in TAG molecules. Since TAG molecules are the biggest source of dietary FAs (up to 97 % of the energy), it is important to take into account their native arrangement, in order to modify them to give better characteristics to the fat products and reduce calories (Kim *et al.*, 2015, Cockcroft, 2021).

Fat substitutes made of carbohydrate and protein cannot be subject to high temperatures. Thus, industries have been trying to produce reduced-calorie fats and improve nutrition properties for a better healthy diet, mimicking all the properties of

natural fat (Abed *et al*., 2016). One of the solutions is modifying lipids, making them functional foods, known as "structured lipids" (SLs) for different applications. Low-calorie TAGs are one of the examples of SLs with enhanced benefits when compared with the ones that exist in nature (Mota *et al*., 2020).

By enzymatic way, the most common reactions are interesterification and acidolysis that modify molecule's architecture through the FAs position using *sn*-1,3 regioselective lipases as biocatalysts (Meynier *et al*., 2017; Zhang *et al*., 2020). Under interesterification, one TAG molecule reacts with methyl or ethyl esters of medium-chain FA or medium-chain TAG, originating new TAGs containing medium-chain FAs. Acidolysis has the same methodology, but TAG molecules react with free fatty acids (FFA). Within their regioselective classification, these enzymes can be classified as *sn*-1,3 regioselective lipases: have preference for the acyl ester bonds at the first and third positions of TAGs so it will give the desired structure for MLM lipids (Kim *et al*., 2015).

For the MLM lipids production, two types of operation mode can be developed for the bioreactors: batch (or discontinuous) and continuous mode. In batch, some factors have a considerable weight in yield of MLM synthesis, such as substrate ratio, enzyme load, stirring rate, temperature and reaction time itself (Utama *et al*., 2019; Jadhav *et al*., 2021). Here, the bioreactor feed is done in the start with substrate and immobilized enzyme. On the contrary, continuous bioreactors have the substrate continually entering in the feed. Within continuous type, it is possible the use of packed-bed reactors (PBR), where the kinetics conditions are more favourable, since it does not have the presence of high tensions created by agitation. The enzymes used in reactions are well positioned and packed in the chamber of the reactor (bed of enzymes), while reaction medium passes through it. Consequently, the medium contacts with the biocatalyst and the reaction occurs and results into a final product.

The essays to produce new TAG molecules with MLM configuration may be developed using low-cost raw-materials, namely olive pomace oil (OPO). The use of crude oils, as raw materials in SLs production, are considered an advantage for the low-cost of enzymatic processes use. However, the use of crude oils can decrease the activity and stability of enzymes of MLM production, although their availability and economical costs (Mota *et al*., 2020).

In this way, the main goal of this work was the production of MLM type lipids by acidolysis of medium-chain FA or interesterification of ethyl esters and crude olive pomace oil (OPO), through enzymatic way, in continuous packed-bed bioreactor.

2. Materials and Methods

2.1. OPO characterization

The olive pomace oil was centrifuged (40ºC, over 30 minute at 10000 rpm) in order to remove the major impurities in the oil.

Both oil acidity and oxidation products were determined according to the Commission Regulation Nr 2568/91 (1991), relative to olive and olive pomace oils characteristics and related analysis methods (Commission Regulation EEC, 1991).

Acidity was determined regarding the quantity of FFA on the sample, in this case, respecting to the oleic acid, since it is the major FA of the olive and olive pomace oils.

Oxidation products were analysed by absorbances at 232 and 270 nm, which are related with quality of oil (K_{232}: presence of initial products of oxidation; K_{270}: presence of final oxidation products).

Colour characterization was assessed by quantification of chlorophyll pigments in the samples, after measurement of absorbances at 630, 670 and 710 nm against air. The pigments content was expressed as pheophytins.

In addition, FA composition was determined using capillary gas chromatography in accordance with European standard (Commission Regulation EEC, 1991).

2.2. Flow rate and residence time selection

The flow rate (*Q*) was measured by the ratio between the recovered volume (*V*, mL) of the reaction medium and respective time (*t*, min). Consequently, the residence time (τ) was calculated for each lipase system, according to the Eq. 1 and regarding the flow rate obtained, volume of enzyme bed ($V_{enzyme\ bed}$) and porosity (ε) of the biocatalyst.

$$\tau = (V_{enzyme\ bed} * \varepsilon)/Q \qquad \text{(Eq. 1)}$$

2.3. Interesterification and acidolysis reactions

Interesterification and acidolysis reactions were developed in continuous bioreactors, with packed-bed column with lipases (10 g of each: Lipozyme TLIM and Lipozyme RMIM) and an external jacket for temperature control at 40ºC by water circulation from a bath. Ethylic ester from capric acid (C10EE) and ethylic ester from caprylic acid (C8EE) were used for interesterification and the acid form (caprylic acid (C8:0) and capric acid (C10:0)) for acidolysis reactions. All of them with >98 % purity. The reaction mixture consisted of OPO after centrifugation, and FA (or Ethyl Ester) in a 1:2 molar ratio of oil/reagent.

2.4. Gas chromatography analysis of reaction products

The reactions were followed by analysis of 1 μL samples by gas chromatography according to the European Standard EN 14105 (2011) with modifications. The gas chromatograph (Agilent Technologies 7820A) has a flame ionization detector. The temperature in the on-column injector was at 53 ºC, while in the detector at 380 ºC. The gases were applied with a flow rate of 30 mL/min (helium, hydrogen and nitrogen) or 300 mL/min (compressed air). In each chromatogram the identification of each peak was made comparing with standards of pure compounds and with the chromatograms shown in the European Standard EN 14105 (2011).

After obtaining the results, the yield of New TAG molecules that were formed, the degree of TAG and FFA/Ethyl Esters conversion were determined.

2.5. Operational stability tests

Operational stability tests were carried out throughout the operation of the continuous reactor.

The activities of the biocatalysts were estimated along the reactions, through the results obtained by the GC analysis, as the molar incorporation of FFA and ethyl esters in the TAG of OPO. Consequently, the deactivation kinetics models (*e.g.* linear, first-order and Sadana models) allowed the evaluation of the effect of crude oil quality on biocatalyst stability and activity. In addition, operational half-life time was also estimated.

3. Results and Discussion

3.1. OPO characterization

The results for the characterization of the three OPO used along this thesis are available in Table 1. The results are presented by the average of three replicas ± SD for each oil sample (centrifuged, OPO-1, 2 and 3).

The values for acidity of OPO varied from 12.05 to 28.75 %, which may be explained by the increasing storage time of olive pomace before oil extraction. During this step, some hydrolysis reactions in the oil catalysed by lipases can occur, and, therefore, increasing its acidity.

For the oxidation products, following the Regulation 2568/91, K value for crude OPO is not defined. However, the obtained values are considered high (K_{232}: 4.92 – 6.06; K_{270}: 1.90 – 2.36), which means that this oil presents a high oxidation state, both for primary and secondary products (232 nm and 270 nm, respectively).

Regarding chlorophyll pigments, high values (366.97 – 447.50 mg pheophytin a/kg) were observed in all OPO samples. Chlorophyll pigments are pro-oxidant molecules. As for the acidity and oxidation products determination, there is no defined limit for the quantification of chlorophyll pigments. However, it is possible to conclude that these parameters are associated with the influence of different factors, such as olive cultivars and olive maturation stage (Ouachab *et al*., 2013).

The analysis of the FA composition of all OPO samples was also performed and demonstrated similarity with olive oil, since oleic acid was the major fatty acid (69 - 71 %).

3.2. Flow rate and residence time selection

The flow rate and residence time are related to each other, *i.e.* the calculated value of residence time is directly dependent on the flow rate obtained during the reactions. For all the reactions, the molar ratio between OPO and ether/acid forms was kept 1:2.

In reactions with Lipozyme TL IM, the bed volume filled by the biocatalyst was 19.16 mL and the results showed a flow rate of 0.6 mL/min. When Lipozyme RM IM was used, the bed volume filled by the biocatalyst was 30.79 mL and the results also showed a flow rate of 0.6 mL/min.

As previously mentioned, the residence time was calculated, using Eq. 1. For Lipozyme TL IM, the value obtained was 0.34 for the void fraction (ε), by the ratio between the enzyme volume and the total volume (enzyme volume + spent substrate volume) (Xu *et al*., 1998). The residence time was 11 minute. For Lipozyme RM IM, ε was 0.39 and the residence time corresponded to 20 minute.

3.3. Low-calorie MLM synthesis

Acidolysis and interesterification reactions of OPO were carried out in the presence of C8:0, C10:0, C8EE or C10EE, in solvent-free media, with Lipozyme TLIM and Lipozyme RMIM.

Since in the presence of an *sn*-1,3 regioselective lipase enzyme, in this type of reactions, the original FA of the TAG molecule in positions 1 and 3 will be changed, while in position 2 they will be kept. The time-course of reactions catalysed by Lipozyme TL IM and RM IM and the respective kinetics models are presented in Figures 1 and 2, respectively.

The yield of new TAGs and the conversion degrees of TAG and FFA/Ethyl Esters were evaluated. The formation of new TAGs was observed, with the consumption of the original TAG molecules of the fat used. The new TAG molecules formed can be of the MLM or MLL type, according to the replacement of FA that was performed with the substrate involved. Given that these reactions produce intermediate molecules (monoacylglycerols, MAGs, and diacylglycerols, DAGs), it is possible to check that the conversion values of TAGs and esters/acids are higher than the yield of new TAGs throughout the reaction operation.

Table 1. Results of chemical characterization (acidity, oxidation products, and chlorophyll pigments) for three samples of olive pomace oil centrifuged (OPO-1, OPO-2 and OPO-3) (it is represented an average value of 3 replicas ± standard deviation for each sample).

Characterization essays	OPO-1	OPO-2	OPO-3
Acidity (%)	12.05 ± 0.45	15.06 ± 0.93	28.75 ± 0.91
K_{232}	4.92 ± 0.09	6.18 ± 0.36	6.06 ± 0.71
K_{270}	1.90 ± 0.08	2.36 ± 0.16	2.28 ± 0.26
Chlorophyll Pigments (Pheophytin a; mg/kg)	366.97 ± 31.35	447.50 ± 15.84	374.35 ± 11.70

Both interesterification and acidolysis reactions of OPO were carried out in a column packed-bed bioreactor at 40ºC, between 60 and 122 hours continuous operation. TAG content was analysed at pre-determined times to study reaction development. Only New TAG data of this work are depicted in Figure 1 and 2. With respect to the results obtained for the yield of New TAGs produced, in acidolysis reactions with the Lipozyme TL IM (Figure 1), a steady state with values of 29 % with C8:0 was reached, while with C10:0, an average yield of 50 % was observed. For the interesterification reaction, when C8EE or C10EE were used, yield values around 40 % and 49 % were attained, respectively. These results suggest that Lipozyme TL IM showed higher affinity for C10:0 and C8EE.

No difference was verified between C10EE and C10:0. With Lipozyme RM IM (Figure 2), in the acidolysis with C8:0, the average yield in New TAGs of 31 % was lower than C10:0 with 54 %. For the esters, C8EE has values of 50 % and C10EE with 56 %, when the equilibrium was attained. As expected, these results propose a higher distinction between acidolysis and interesterification, demonstrating a higher affinity of Lipozyme RM IM for esters and longer molecule main chain length. Previous studies for OPO valorization, in batch bioreactors, revealed that the use of both medium-chain FA showed values for New TAG production of 53-57 % (Ferreira-Dias and Osório, 2020), which did not determine the preference of the lipase for the substrate, contrary to the results obtained in this study.

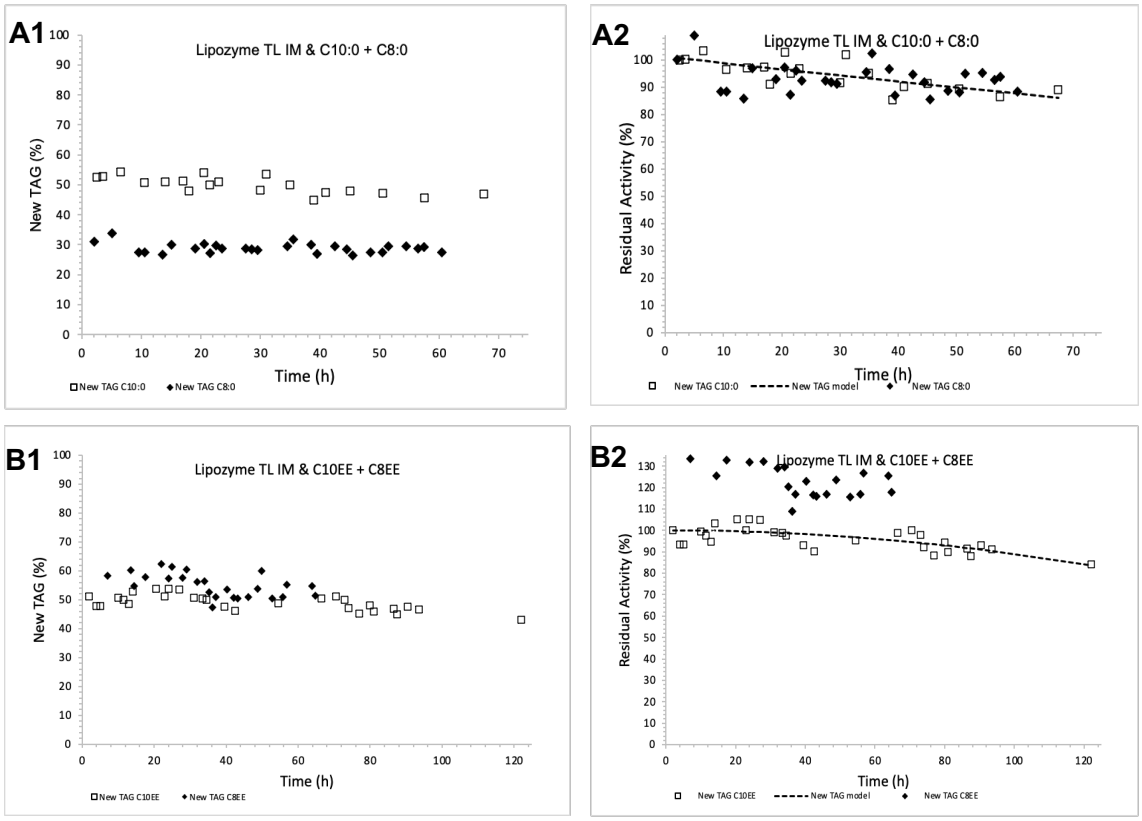

Figure 1. New TAG yield (%) along continuous interesterification and acidolysis reactions of OPO with (A1 and A2) C10:0 + Linear deactivation model and C8:0 and (B1 and B2) C10EE + Sadana deactivation model and C8EE, catalysed by Lipozyme TL IM biocatalyst. OPO samples with 12.05 % (A1, A2, B1 and B2) and 28.75 % (A1, A2, B1 and B2) of acidity.

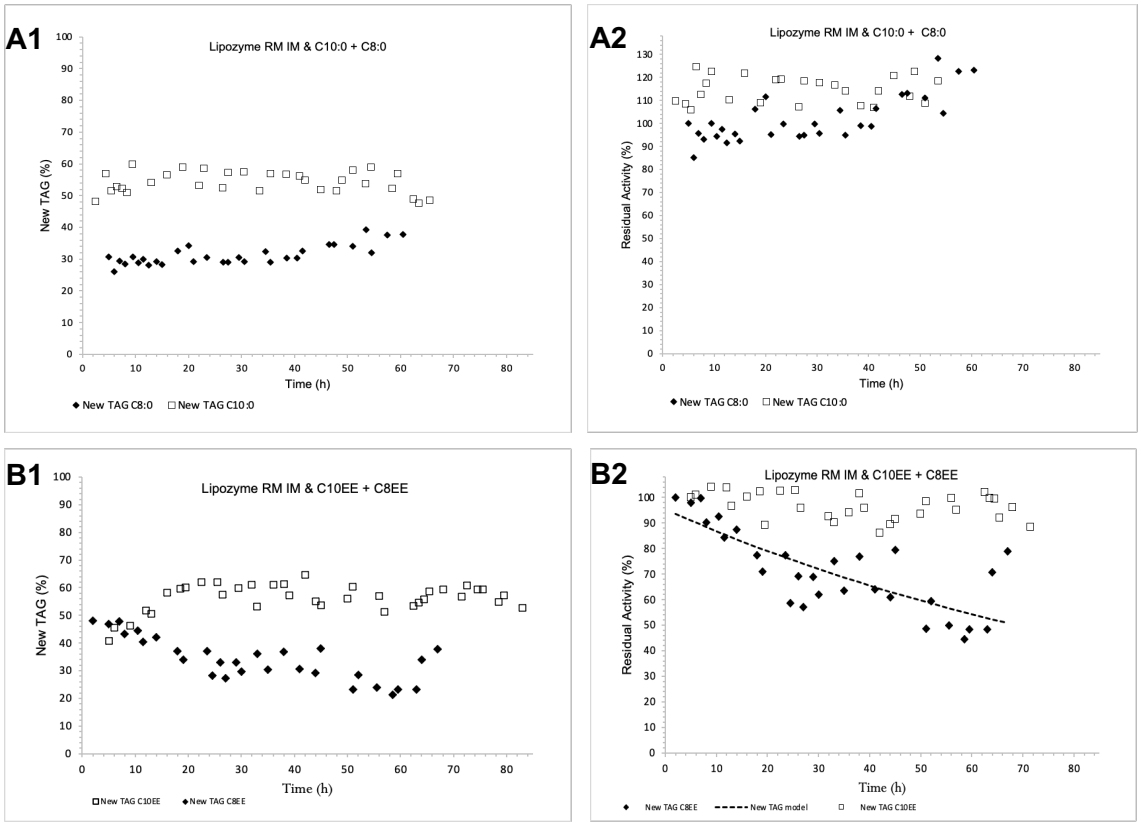

Figure 2. New TAG yield (%) along continuous interesterification and acidolysis reactions of OPO with (A1, A2) C10:0 and C8:0 and (B1, B2) C10EE and C8EE + First-order deactivation model, catalysed by Lipozyme RM IM. OPO samples with 12.05 % (B1 and B2), 15.06 % (A1 and A2) and 28.75 % (A1, A2, B1 and B2) of acidity.

In this study, at a molar ratio of 1:2 (OPO:FFA/Ethyl Ester) for Lipozyme TL IM- and Lipozyme RM IM-catalysed interesterification and acidolysis reactions, a high yield of new TAG (%) was obtained.

3.4. Operational stability tests

The activities of the biocatalysts were estimated along the reactions. The residual activity during the bioreactor operation was calculated as a function of the initial activity, when the steady state was attained (assumed after three residence times), both for yield and conversion degrees.

The data obtained for interesterification and acidolysis reactions were converted into normalised data, calculating the residual activity of the enzymes along the continuous operation of the bioreactor. The ratio between the observed yield for each sample and the initial yield was chosen to measure the residual activity of the biocatalysts. Then it was possible to fit the most adequate deactivation model and estimate half-life time values from the fitted model.

The deactivation profiles for Lipozyme TL IM and Lipozyme RM IM, obtained from New TAG values, are represented in Figures 1 and 2. The parameters for each deactivation model fitted are presented in Table 2.

Table 2. Operational stability parameters of biocatalysts Lipozyme TL IM and Lipozyme RM IM (deactivation model, model equation and half-life, h) for each interesterification and acidolysis reactions, with respective OPO with different acidity and FFA/Ethyl Esters as acyl donor (C10EE, C8EE, C10:0 and C8:0), in continuous bioreactor (n.d.: not determined).

Type of reaction	FFA/Ethyl Esters	Biocatalyst	Deactivation model	Half-life (h)	Oil Acidity (%)
Interesterification	C10EE	Lipozyme TL IM	Sadana	213.3	12.05
	C8EE		n.d.	-	28.75
	C10EE	Lipozyme RM IM	n.d.	-	12.05
	C8EE		First-order	74	28.75
Acidolysis	C10:0	Lipozyme TL IM	Linear	228.3	12.05
	C8:0		n.d.	-	28.75
	C10:0	Lipozyme RM IM	n.d.	-	15.06
	C8:0		n.d.	-	28.75

In Figure 1, in acidolysis with C8:0 and interesterification with C8EE reactions catalysed by Lipozyme TL IM, no loss of activity was observed, where the steady state was readily reached with high residual activity value. In the acidolysis with C10:0, in Lipozyme TL IM profile was fitted the linear model with a high half-life of 228.3 h. In interesterification with C10EE reaction, Lipozyme TL IM was slowly deactivated, with Sadana model applied with also high half-life of 213.3 h. The profiles from the reactions catalysed by Lipozyme RM IM, showed that in the acidolysis reactions with C8:0 and C10:0 and interesterification with C10EE, the biocatalyst kept its activity and operational stability. In interesterification with C8EE, a decrease during 67 h of bioreactor operation, a first-order deactivation model was fitted, although the lipase never loses activity. The results also showed that the operational stability of the biocatalysts seemed not to be affected by the acidity of the OPO used.

In previous studies, in batch bioreactor with OPO as substrate, Heinzl *et al*., (2022), a first-order deactivation model was verified with C8:0 catalysed by Lipozyme RM IM, while in this study no deactivation was registered. In addition, Cozentino *et al*., (2020) in the acidolysis of grape seed oil with C10:0, and using Lipozyme RM IM, a half-life of 209.6 h was obtained, similar to 228.3 h with C10:0 in this work with Lipozyme TL IM.

4. Conclusions

The main objective was the production of new MLM-*type* TAG molecules, through interesterification reactions between the TAGs of OPO and MCFAs (or their esters form). In this work, a natural crude oil (OPO), which is a by-product of olive oil extraction industry, was used as raw material for the production of

structured lipids, providing it a significant high added-value.

OPO acidity, oxidation products and chlorophyll pigments were analysed, in order to provide a better oil characterization. In this study, the results of the three oil batches show that all oils present a high acidity, a high level of oxidation products and green pigments. In addition, all OPO presented a high percentage of oleic acid, similar to the composition of olive oil.

Due to the advantages of enzymatic approaches, regioselective *sn*-1,3 lipases (Lipozyme TL IM and Lipozyme RM IM) were successfully used as biocatalysts, and their operational stability in continuous process was assayed. Both biocatalysts showed high activity with yields in New TAG varying from 30 to 60 %, depending on the biocatalyst itself and on the acyl donor. Moreover, high operational stability was observed for Lipozyme TL IM and Lipozyme RM IM along continuous operation of the bioreactor.

The lower cost of Lipozyme TL IM when compared with Lipozyme RM IM, and similar behavior when acting in continuous packed bed reactors, makes Lipozyme TL IM the best choice as biocatalyst for MLM production from crude OPO.

This study demonstrated that it is possible to produce MLM SLs in continuous bioreactors with crude acidic oils, decreasing the cost of oil refining and raising the value of OPO, while also enhancing the process sustainability and reducing the environmental effect and promoting circular economy in the olive oil industries.

References

[1] Abed, S.M., Ali, A.H., Noman, A., Niazi, S., Ammar, A.-F. & Bakry, A.M. (2016). Structured Lipids: Enzymatic Synthesis, Health Benefits and Nutraceutical Characteristics – A Review, International Journal of Research in Agricultural Sciences, 3, pp: 206-215, ISSN: 2348-3997.

[2] Cockcroft, S. (2021). Mammalian lipids: structure, synthesis and function. *Essays in Biochemistry*, 65(5): 813-845, doi: 10.1042/EBC20200067.

[3] Commission Regulation (EEC) No 2568/91 of 11st July 1991, on the characteristics of olive oil ando live-residue oil and on the relevant methods of analysis. Official Journal of the European Union, L248.

[4] Cozentino, I.S.C., Rodrigues, M.F., Mazziero, V.T., Cavallini, D.C.U. & De Paula, A.V. (2020). Enzymatic synthesis of structured lipids from grape seed (Vitis vinifera L.) oil in associated packed bed reactors. *Biotechnology and Applied Biochemistry*, doi: 10.1002/bab.2085.

[5] Ferreira-Dias, S. & Osório, N. (2020). Valorization of crude olive pomace oil: lipase-catalyzed production of dietetic structured lipids, emulsifiers and biodiesel. *Inform Magazine*, 31(7), pp. 14- 17, doi: 10.21748/inform.07.2020.14.

[6] Heinzl, G.C, Mota, D.A., Martinis, V., Martins, A.S., Soares, C.M.F., Osório, N., Gominho, J., Nampoothiri, K.M., Sukumaran, R.K., Pereira, H. & Ferreira-Dias, S. (2022). Integrated bioprocess for structured lipids, emulsifiers and biodiesel production using crude acidic olive pomace

oils. *Bioresource Technology*, 346, 126646, doi: 10.1016/j.biortech. 2021.126646.

[7] Jadhav, H.B. & Annapure, U. (2021). Designer lipids-synthesis and application – A review. *Trends in Food Science & Technology*, 116:884-902, doi: 10.1016/j.tifs.2021.08.020.

[8] Kadhum, A.A.H., & Shamma, M.N. (2017). Edible lipids modification processes: A review. *Critical Reviews in Food Science and Nutrition*, 57(1): 48-58, doi: 10.1080/10408398.2013.848834.

[9] Kim, B.H. & Akoh, C.C. (2015). Recent Research Trends on the Enzymatic Synthesis of Structured Lipids. *Journal of Food Science*, 80(5): C1713-1724, doi: 10.1111/1750-3841.12953.

[10] Meynier, A. & Genot, C. (2017). Molecular and structural organization of lipids in foods: their fate during digestion and impact in nutrition. *Oliseeds & fats Crops and Lipids*, 24(2): pp. 12, doi: 10.1051/ocl/2017006.

[11] Mota, D.A., Rajan, D., Heinzl, G.C., Osório, N.M., Gominho, J., Krause, L.C., Soares, C.M.F., Nampoothiri, K.M., Sukumaran, R.K. & Ferreira-Dias, S. (2020). Production of low-calorie structured lipids from spent coffee grounds or olive pomace crude oils catalyzed by immobilized lipase in magnetic nanoparticles. *Bioresource Technology*. 307:123-223, doi: 10.1016/j.biortech.2020.123223.

[12] Osório, N.M., Da Fonseca, M.M.R. & Ferreira-Dias, S. (2006). Operational stability of Thermomyces lanuginose lipase during interesterification of fat in continuous packed-bed reactors. *European Journal of Lipids Science and Technology*, 108:545-553, doi: 10.1002/ejlt.200600029.

[13] Ouachab, N., Tsoutsos, T., (2013). Study of the acid pretreatment and biodiesel production from olive pomace oil. *J. Chem. Technol. Biotechnol*. 88, 1175–1181. https://doi. org/10.1002/jctb.3940 (cited in Heinzl *et al*., 2022).

[14] Pokorny, J., Kalinova, L., & Dysseler, P. (1995). Determination of chlorophyll pigments in crude vegetable oils: Results of a collaborative study and the standardized method (Technical Report). *Pure and Applied Chemistry*, 67(10), pp. 1781-1787, doi: 10,1351/pac199567101781.

[15] Utama, Q.D., Sitanggang, A.B., Adawiyah, D.R. & Hariyadi, P. (2019). Lipase-Catalyzed Interesterification for the Synthesis of Medium-Long_Medium (MLM) Structured Lipids– A Review. *Food Technology & Biotechonology*. 57:305-318, doi: 10,17113/ftb.57.03.19.6025.

[16] Xu, X., Balchen, S., HØy, C.-E. & Alder-Nissen, J. (1998). Production of Specific-Structured Lipids by Enzymatic Interesterification in a Pilot Continuous Enzyme Bed Reactor. AOCS Press, 75:1573-79, doi: 10.1007/s11746-998-0096-6.

[17] Zhang, Y., Zhang, T., Liang, Y., Jiang, L. & Sui, X. (2021). Dietary Bioactive Lipids: A Review on Absorption, Metabolism, and Health Properties. *Journal of Agricultural and Food Chemistry*, 69(32): 8929-8943, doi: 10.1021/acs.jafc.1c01369.