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Biocatalysts Based on Immobilized Lipases for the Production of Ethyl Esters of Fatty Acids including Bioactive Gamma-Linolenic Acid from Borage Oil

Gonzalo Berzal [1], Martín García-González [1], Natalia Castejón [1], Paz García-García [1,*], Gloria Fernández-Lorente [2] and Francisco J. Señoráns [1]

[1] Healthy-Lipids Group, Food Science Department, Faculty of Sciences, Universidad Autónoma de Madrid, Francisco Tomás y Valiente, 7, 28049 Madrid, Spain; gonzalo.berzal@inv.uam.es (G.B.); diegom.garcia@estudiante.uam.es (M.G.-G.); natalia.castejon@univie.ac.at (N.C.); javier.senorans@uam.es (F.J.S.)

[2] Laboratory of Microbiology and Food Biocatalysis, Institute of Food Science Research (CIAL, CSIC-UAM), Nicolás Cabrera, 9, UAM Campus, 28049 Madrid, Spain; g.f.lorente@csic.es

* Correspondence: mariap.garcia@uam.es

Abstract: In the present work, borage oil (*Borago officinalis*) was used as the main source of gamma linolenic acid (GLA) to obtain ethyl esters by enzymatic ethanolysis using immobilized enzymes for its application in the food industry. Commercial *Thermomyces lanuginosus* lipase (TLL) was compared to chemical ethanolysis in alkaline medium. In addition, TLL was immobilized by adsorption on hydrophobic porous support (Octadecyl-Sepabeads®) to compare the results. Fatty acid ethyl ester (FAEE) yields of both reactions were compared under the same conditions (25 °C and 200 rpm) and analyzed by GC-MS. Moreover, the conversion yield for borage oil ethanolysis catalyzed by TLL immobilized on C18-Sepabeads® supports was similar to the chemical pathway (93.4% and 99.5%, respectively). When this biocatalyst was used in a solvent-free system (at 40 °C and 200 rpm), it was possible to obtain a high FAEE yield of 84.3% in the first 24 h of reaction. Furthermore, it was possible to re-use the immobilized biocatalyst for the performance of five reaction cycles maintaining 68% of its initial activity. Thus, the use of immobilized enzymes in solvent-free systems is an eco-friendly alternative to obtain GLA ethyl esters for its possible application in cosmetics and food.

Keywords: borage oil; gamma-linolenic acid; *Thermomyces lanuginosus* lipase (TLL); immobilization; ethanolysis; solvent-free system

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1. Introduction

Edible fats and oils are a group of fundamental macromolecules in the human diet. These lipids provide essential fatty acids such as linoleic acid (LA, 18:2 n-6) and alpha-linolenic acid (ALA 18:3 n-3), which play important roles in embryonic development, transport, metabolism and the maintenance of cell membranes. They are also structural components of functionally relevant molecules (steroid hormones and bile acids) and they serve as a vehicle for fat-soluble vitamins (A, D, E and K) [1]. The fundamental role played by w-3 and w-6 polyunsaturated fatty acids (PUFA) in the body is widely documented in the literature, as their absence from a normal diet is associated with the development of some cardiovascular, inflammatory and infectious diseases, certain types of cancer and autoimmune disorders [2,3].

Gamma-linolenic acid (GLA) is the desaturation product Δ6 of LA in the n-6 essential fatty acid metabolic pathway. Although the typical Western diet is rich in LA, the activity of this enzyme is often reduced by different factors (age, stress, alcohol consumption, smoking or vitamin deficiencies), which results in insufficient production of GLA. Its deficiency can represent a problem for the balance of metabolites derived from PUFA w-6. Moreover, there

is also much interest on the beneficial effects of GLA on dermatitis, rheumatoid arthritis, blood pressure regulation and the improvement of the plasma lipid profile [4]. The most representative sources of GLA are certain families of vegetables, including *Boraginaceae*, *Ranunculaceae* or *Primulaceae*, with borage oil (*Borago officinalis*) and evening primrose oil (*Oenothera biennis*) [5].

The growing need to look for processes at an industrial level that provide higher yields and are more selective, while allowing for a lower expenditure of energy, less organic solvents and reducing the disposal of waste has encouraged the use of enzymes and their immobilized biocatalysts to develop processes under the principles of Green Chemistry [6,7]. Thus, lipases stand out to develop transesterification reactions in which lipid composition could be modified. Alcohols are the most commonly used acyl acceptors in these reactions [8], mainly ethanol because of its low toxicity, could be obtained from renewable sources being considered as GRAS (Generally Recognized As Safe) and produce fatty acid ethyl esters (FAEE) [9]. However, ethanolysis is a reversible reaction and, therefore, an excess of ethanol must be added to shift the balance towards the formation of FAEE. Unlike catalysts of an alkaline nature, enzymes do not form soaps. Moreover, immobilized enzymes have a series of advantages such as reutilization under several cycles, mild conditions, and ease recovery of the product [10]. Different lipases are described in literature because of their high efficiencies related to ethanolysis, being *Candida antarctica* lipase (CAL), *Rhizomucor miehei* lipase (RML) and *Thermomyces lanuginosus* lipase (TLL) as the most cited [10–12]. Immobilization of lipases via adsorption onto hydrophobic supports, such as octadecyl-Sepabeads, provokes a conformational change towards the open form of the enzyme (with its active site exposed) in a phenomenon called interfacial activation or hyperactivation [6,13,14].

Green chemistry is a term related to the creation of products whose chemical processes tend to reduce or eliminate both the use and the production of harmful substances, such as organic solvents. To be called "green", each reaction must have three green components: solvent, catalyst and energy consumption. In 1998, Paul Anastas and John Warner developed twelve principles of green chemistry and in their book, *Green Chemistry Theory and Practice* [15], they explained their meaning in practice.

Therefore, the aim of this research is to develop an integrated and eco-friendly method using immobilized lipases coupled to solvent-free systems via ethanolysis to obtain GLA ethyl esters for its possible application in food.

2. Results

2.1. Characterization of Borage Oil

Borage oil was characterized by GC-MS in order to determine the fatty acid profile of the sample. The composition of each peak related to the total is illustrated in Table 1. Linoleic acid represents the 36.83% of the total composition of fatty acids, followed by a 20.56% of gamma-linolenic (GLA). Moreover, long-chained fatty acids were represented in low percentage compared to the total. It can also be seen that α-linolenic was not present in the sample. This profile, experimentally determined by GC-MS, corresponds to other values represented in bibliography [3,16]. Thus, borage oil is an interesting source of GLA that could be used to obtain fatty acid ethyl esters for further development of structured lipids.

Table 1. Fatty acid profile from borage oil determined by GC-MS.

PEAK	Fatty Acid	Composition *
1	Palmitic—16:0	10.75 ± 0.21
2	Stearic—18:0	5.09 ± 0.07
3	Oleic—18:1 n-9	20.08 ± 0.06
4	Linoleic—18:2 n-6	36.83 ± 0.27
5	Gamma-linolenic—18:3 n-6	20.56 ± 0.16
6	Gondoic/eicosenoic—20:1 n-9	3.89 ± 0.06
7	Erucic—22:1 n-9	2.02 ± 0.04
8	Nervonic—24:1 n-9	1 ± 0.1

* Composition is determined as % total ± standard deviation (SD).

2.2. Optimization of Conditions for Borage Oil Ethanolysis

Different molar ratios between ethanol and borage oil were studied in order to improve the conditions for ethanolysis. According to literature, it was shown that decreasing the amount of ethanol in the reaction resulted in a lower FAEE yield due to consumption of hydroxide ions [17]. Thus, the amount of ethanol was increased to study the amount of FAEE. The ratio ethanol:oil studied were 9:1, 12:1 and 24:1. The reaction was developed at 60 °C and 300 rpm for 1 h using 0.5% KOH as chemical catalyst. After removal of glycerin, different aliquots were analyzed by HPLC-ELSD to determine the composition of the reaction products.

The results, represented in Figure 1, corroborate that alcoholysis was favored when the amount of ethanol was higher than the stoichiometric ratio, avoiding secondary reactions such as saponification. According to literature, FAEE yield increases when increasing the amount of ethanol used in the reaction. The best result was achieved using 24 mols of ethanol per mol of borage oil, obtaining 99.5% of FAEE. Under these conditions, the presence of intermediates, such as diacylglycerols (DAG) and monoacylglycerols (MAG), were quite low (results not shown). Moreover, separation of FAEE from the glycerin phase was facilitated, as previously described in the literature [18].

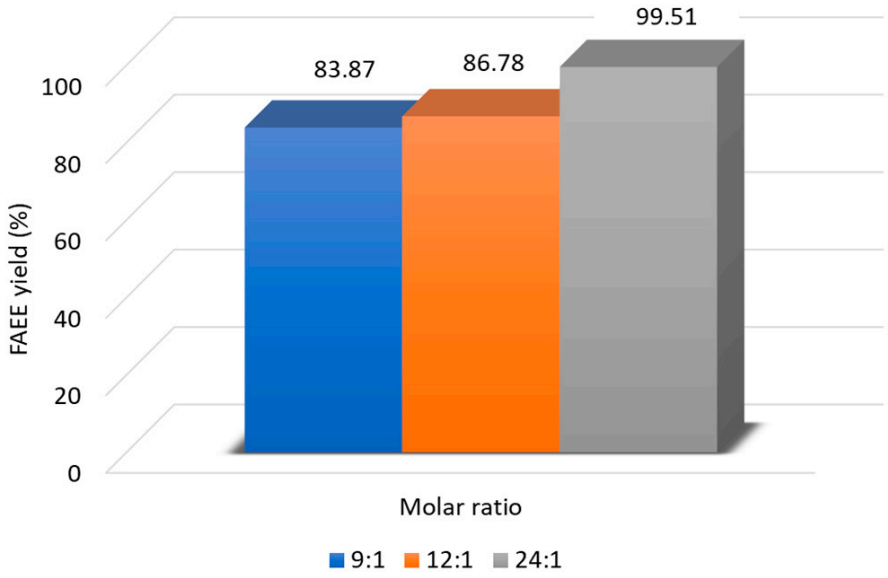

Figure 1. FAEE yield using different molar ratio between ethanol and borage oil for ethanolysis reaction.

2.3. Immobilization of TLL on Sepabeads-C18 Resines

In this work, the development of an enzymatic protocol for borage oil ethanolysis instead of the chemical reaction was a key factor. Thus, highly active enzymatic biocatalysts were developed and immobilization of TLL by hydrophobic adsorption on Sepabeads-C18 resins was studied [12,19]. Immobilization kinetics were studied for 24 h and the results are expressed in Figure 2. As shown, 50% of the protein was immobilized on the support in the first 30 min of incubation. Nevertheless, it lasts 24 h to achieve 89% of the immobilization yield, which corresponds to 35.62 mg of enzymatic load per gram of support. Thus, the recovered activity of the TLL-Sepabeads biocatalyst was 1500 U per gram of catalyst. The activity of the native enzyme (soluble TLL) remains near 100% under the same immobilization conditions for 24 h. In this case, the soluble enzyme had an specific activity of 0.104 U per mg of protein. This represents that the enzyme maintained its integrity and activity during the process and the loss of activity was due to immobilization of the enzyme on the support.

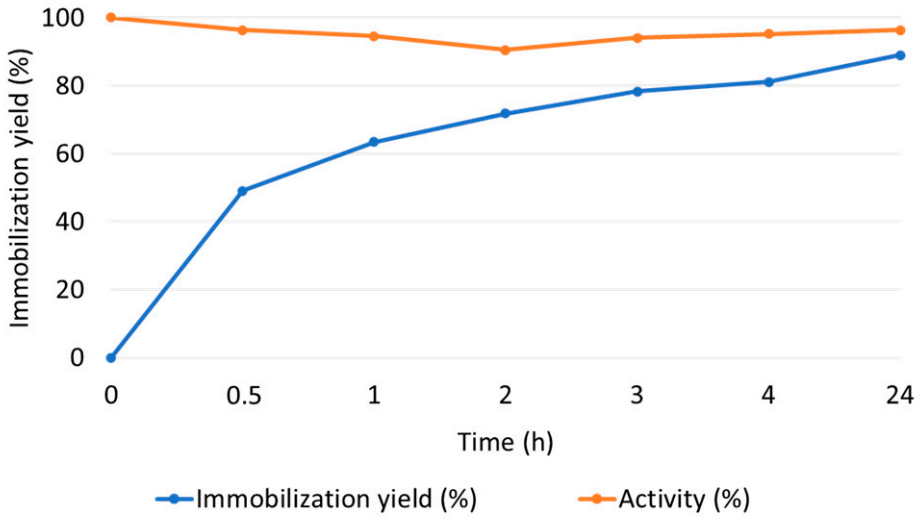

Figure 2. Immobilization yield of TLL on Sepabeads-C18 resines. Blue line represents the kinetics of immobilization at different times under immobilization conditions, and orange line represents the activity of the soluble enzyme under the same conditions.

Immobilization of lipases by adsorption allows the enzyme to be fixed to the solid surface in its open conformation [20]. In this respect, it is pointed out that the surrounding area of the active site of the lipases, rich in hydrophobic residues, is the main area responsible for the interactions that stabilize them on the hydrophobic surface.

2.4. Enzymatic Ethanolisis of Borage Oil Catalyzed by TLL Biocatalysts

Highly active TLL-Sepabeads biocatalysts were tested for borage oil ethanolysis and compared with commercial TLL biocatalysts. The amount of EE, TAG, DAG and MAG was evaluated for 24 h by HPLC-ELSD. Ethanolysis kinetics using a commercial TLL biocatalyst (TLL-Immobead 150) and a TLL-Sepabeads biocatalyst developed in the laboratory are represented in Figure 3. The recovered activity for TLL-Sepabeads was 1500 U per gram of catalyst, whereas the TLL-Immobead 150 had a recovered activity of 3000 U/g.

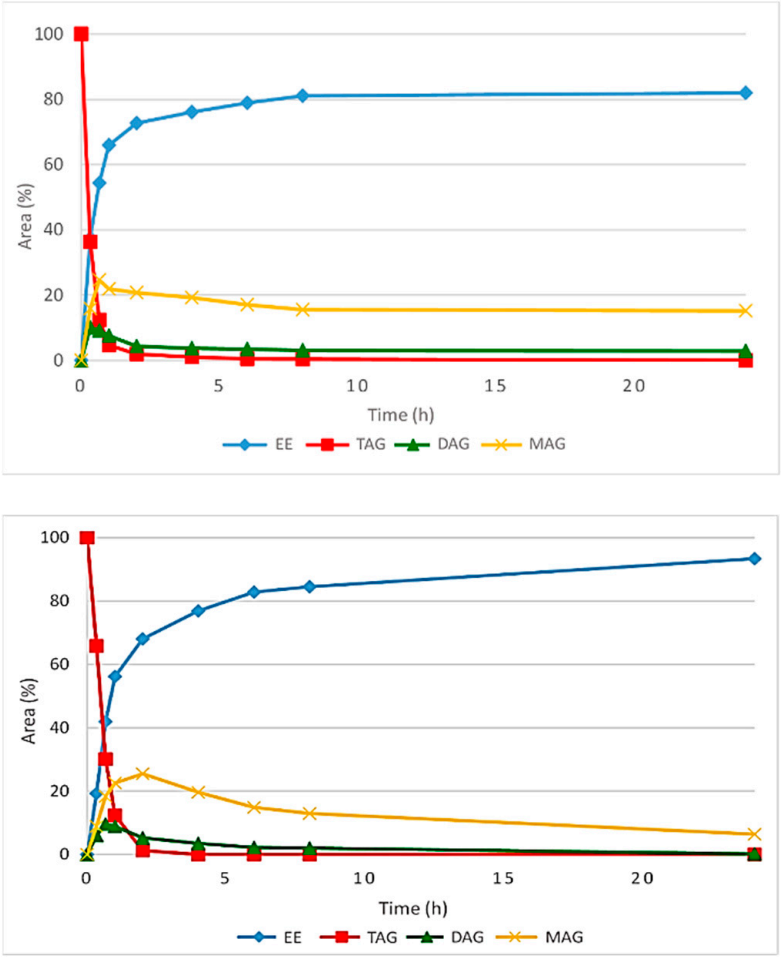

Figure 3. Borage oil ethanolysis catalyzed by commercial TLL-Immobead 150 (**up**) and TLL-Sepabeads (**down**).

Kinetics were remarkably similar using enzymatic biocatalysts with some exceptions. Even though in the first 40 min of reaction the commercial TLL-Immobead 150 had produced 57% EE with a TAG conversion of 63.7%, TAG conversion after this time dramatically slowed down. Furthermore, the percentage of EE produced were maintained around 80% during the experiment (24 h). On the other hand, the TLL-Sepabeads biocatalyst had a slower kinetic of conversion than the commercial biocatalyst as it only produced 42% EE at the same time (40 min), but had a faster conversion of TAG with 70% of TAG. Thus, comparing both biocatalysts, kinetics were similar but better results were obtained with TLL-Sepabeads mainly in EE formation. Using this biocatalyst, the percentage of EE increased progressively for 24 h, obtaining 93.4% EE at 24 h.

Considering the EE yield, results using the TLL-Sepabeads biocatalyst were slightly better than those for the commercial biocatalyst. Thus, the nature of the support in which the enzyme was immobilized is essential for its activity. When TLL was immobilized to hydrophobic supports such as Sepabeads-C18, interfacial activation is developed, and its active conformation is achieved. Moreover, considering the recovered activity of the biocatalysts, results are even better when using TLL-Sepabeads. Even if TLL-Sepabeads presented a lower recovered activity that was almost half of the commercial one, it achieved higher EE yields. Again, those results are highly linked to the immobilization technique used and highlight the importance of a meticulous procedure, taking into account the nature of the enzyme and the support. Additionally, using highly active TLL biocatalysts, which enable greener procedures, the FAEE yield was similar to that obtained in chemical ethanolysis.

The presence of intermediates (DAG and MAG) at 24 h of reaction was also characteristic for each biocatalyst. TLL, immobilized on Sepabeads support, obtained lower quantities of intermediates than commercial TLL biocatalysts. This result was due to the higher activity that TLL presents when it is immobilized on hydrophobic supports. Moreover, the slowdown of MAG conversion in glycerol on account of the regioselectivity of lipases was also represented.

2.5. Re-Use of Immobilized TLL Biocatalysts for Borage Oil Ethanolysis

Five reaction cycles of borage oil ethanolysis (four hour per cycle) were performed using TLL-immobead 150 and TLL-Sepabeads biocatalysts to probe their stability for their possible application in industry. Results are expressed as relative activity (%) according to production of FAEE obtained in the first cycle.

Figure 4 shows that the TLL-Sepabeads biocatalyst is much more stable, as it retained 70% of its activity after being re-used for five cycles of reaction. At the end of the second cycle of application, it retained 92% of its initial activity, and it was kept constant at 70% for the next three cycles. On the contrary, the commercial TLL biocatalyst only retains 10% of its activity after five cycles. After the second re-use of the biocatalyst, it lost 85% of its initial activity and it continuously decreased in every cycle to 10%. Thus, the TLL-Sepabeads was considered the optimum biocatalyst as it was not only responsible for high TAG conversion yields but also it was able to be re-used for five cycles with a minimal loss of activity. Lipases, which are immobilized on hydrophobic supports such as Sepabeads by its enzymatic lid, maintained their open conformation, so they are protected from the reaction media, enabling higher activity and stability compared to commercial biocatalysts.

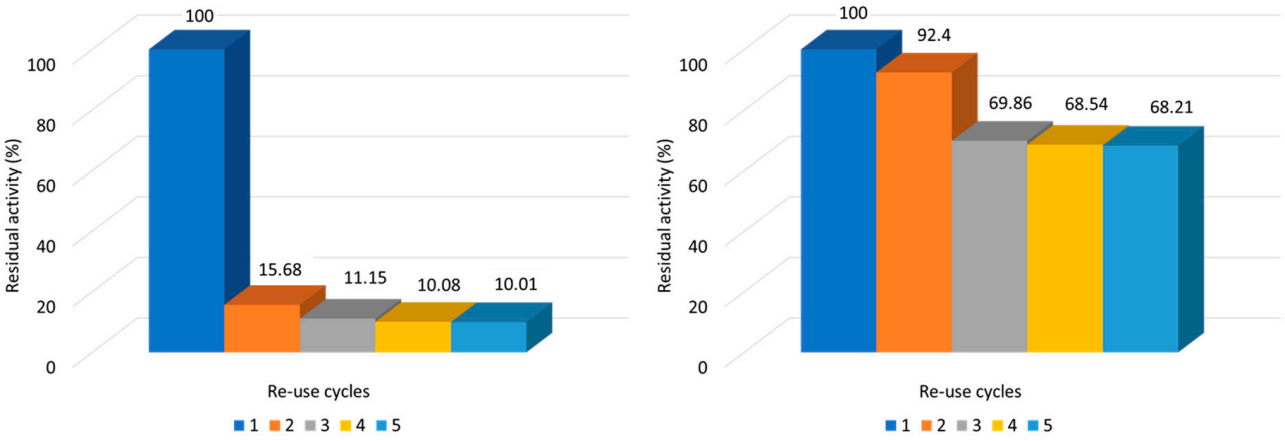

Figure 4. Cycles of reaction for enzymatic ethanolysis of borage oil. On the (**left**), re-use with commercial biocatalyst (TLL-Immobead 150); on the (**right**) side, re-use with TLL immobilized on hydrophobic support (TLL-Sepabeads).

The possibility of biocatalyst re-utilization using immobilized lipases with high catalytic activity favors its industrial application. In this way, the separation of product (FAEE) and biocatalyst is eased, and the catalyst could be re-used for several cycles, enabling an eco-friendly and economical process.

2.6. Characterization of FAEE Obtained via Enzymatic and Chemical Ethanolysis

Fatty acid ethyl esters obtained after enzymatic and chemical ethanolysis of borage oil were further characterized by GC-MS. Results are shown in Table 2.

Table 2. FAEE profile of borage oil after enzymatic and chemical ethanolysis.

PEAK	Rt (min) [1]	Fatty Acid	Composition Chemical Ethanolysis [2]	Composition Enzymatic Ethanolisis [2]
1	13.356	Palmitic—16:00	10.56 ± 0.16	13.56 ± 0.97
2	16.974	Stearic—18:00	4.57 ± 0.16	4 ± 1
3	18.058	Oleic—18:1 n-9	20.13 ± 0.09	22.96 ± 0.22
4	19.752	Linoleic—18:2 n-6	37.65 ± 0.27	47.85 ± 3.92
5	20.913	Gamma-linolenic—18:3 n-6	21.16 ± 0.21	9.70 ± 0.63
6	22.086	Gondoic/eicosenoic—20:1 n-9	3.63 ± 0.01	1.5 ± 0.2
7	26.065	Erucic—22:1 n-9	1.75 ± 0.02	0.5 ± 0.1
8	30.479	Nervonic—24:1 n-9	0.55 ± 0.03	-

[1] Rt refers to retention time of each peak analyzed by GC-MS. [2] Composition is expressed as % total ± standard deviation (SD).

Composition of different FAEE produced by borage oil ethanolysis via chemical reaction using KOH was almost identical to fatty acid profile characterized analyzed in Table 1. This result shows that in chemical ethanolysis, every ester bond reacts with ethanol due to its reactivity of ethoxide anion and tough conditions of reaction media. However, ethanolysis catalyzed by TLL immobilized on Sepabeads-C18 supports showed a different FAEE profile compared to fatty acids from original borage oil. According to Table 2, FAEE obtained linoleic acid, oleic acid and palmitic acid in higher proportions. The last two appeared in higher proportion than in original oil due to regioselectivity for positions sn-1 and sn-3 of TLL enzymes. In addition, ethyl esters of GLA appeared in around 10% related to total composition of fatty acids. Compared to 20% of GLA found in initial borage oil, this loss was due to sn-2 preferred position of GLA. Moreover, there are evidences of acyl migration of sn-2 position to sn-1 and sn-3 positions in the literature [21] that were confirmed in this research.

2.7. Solvent-Free Enzymatic Ethanolysis of Borage Oil

Once enzymatic reaction was preferred, due to the kinetics obtained as well as for the green procedure developed compared to chemical reaction, and TLL-Sepabeads biocatalyst was selected because of its high activity and stability that enabled the development of seven reaction cycles, the development of a solvent-free enzymatic reaction at different temperature conditions (25 and 40 °C) catalyzed by the TLL-Sepabeads was studied.

After 1 h of reaction (Figure 5), when the reaction was developed at 25 °C, there were 39.1% TAG, 14.0% DAG, 12.8% MAG and 34.1% FAEE. In contrast, when the reaction was developed at 40 °C, there were 27.8% TAG, 16.9% DAG, 14.5% MAG and 40.8% FAEE at the same time of reaction. Therefore, in the first hour of solvent-free reaction, the increase in temperature favored the course of the enzymatic reaction. Moreover, when the aliquot was taken at 2 h, the conversion of TAG products was quite similar in the two experimental conditions. From this point on, the reactions became equal and there was no difference due to the temperature change. At 24 h, the conversion to FAEE was higher in the case of conditions at 40 °C (84.3%) than at 25 °C (80.6%), and neither TAG nor DAG was found, which also corroborates the stereospecificity of the lipase used.

Therefore, with the application of a solvent-free reaction at 25 °C coupled to enzymatic ethanolysis, we were able to obtain an eco-friendly and energy and solvent saving process with great results enabling the easy purification and extraction of the product of interest, which was GLA ethyl esters with potential application in food industry.

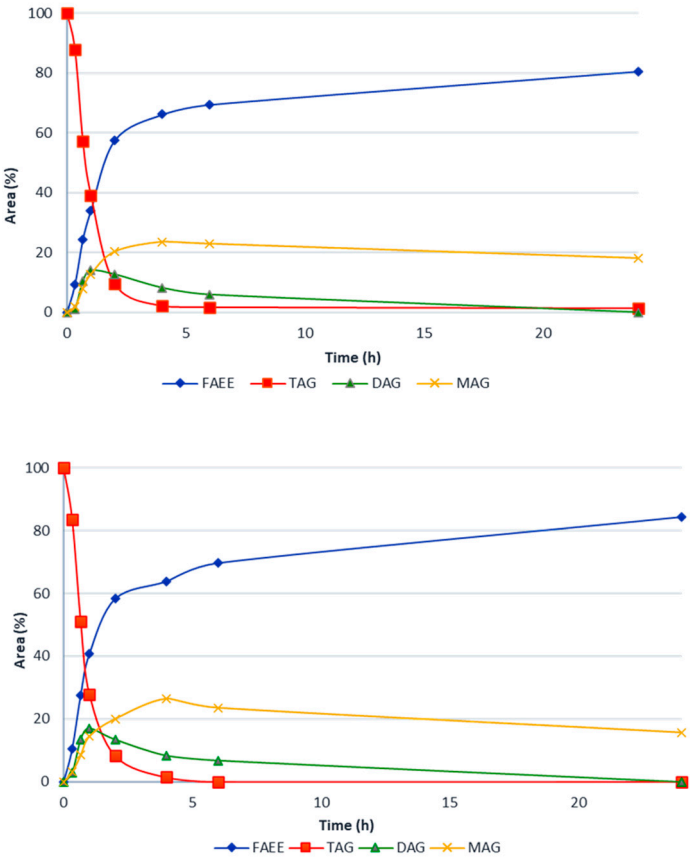

Figure 5. Borage oil solvent-free ethanolysis catalyzed by TLL-Sepabeads at 25 °C (**up**) and 40 °C (**down**).

3. Materials and Methods

3.1. Materials

Borage oil (*Borago officinalis*) was purchased from Pranarôm International (Ghislenghien, Belgium) in 50 mL containers. It was kept under a nitrogen atmosphere at cooling temperature and protected from light to prevent oxidation. Ethanol (C_2H_6O) and potassium hydroxide (KOH) were provided by Panreac Química S.A.U. (Barcelona, Spain). Both p-nitrophenyl butyrate (p-NPB), phosphate buffer and the immobilized commercial biocatalyst of *Thermomyces lanuginosus* (Immobead 150) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). The Sepabeads-C18 was provided by Resindion S.R.L. (Milan, Italy). The commercial immobilized lipases TL IM (*Thermomyces lanuginosus* lipase, TLL) was provided by Novozymes A/S (Bagsvaerd, Denmark). Hexane and methyl tert-butyl ether (both HPLC quality) were acquired from Avantor Performance Materials (Gliwice, Poland). Methanol, acetone, isopropanol (all three HPLC quality) and anhydrous sodium sulphate were provided by Lab-Scan Analytical Science, Poch S.A. (Gliwice, Poland). Finally, the 3 angström (Å) molecular sieve was purchased from Scharlab S.L. (Sentmenat, Spain).

3.2. Methods

3.2.1. Characterization of Borage Oil by GC-MS

The FA content of borage oil was determined in triplicate by derivatization in an alkaline medium, and the FAMEs obtained were analyzed by gas chromatography coupled to a mass spectrometer (GC-MS). To obtain FAME in basic medium, the ISO TC34/SC 5 Standard was followed: 25 mg of oil were weighed and mixed with 200 µL of hexane. In total, 50 µL of a KOH solution in 2 N methanol was added to this mixture and vortexed (Vortex ZX3, Velp Scientifica, Usmate Velate, Italy) for 1 min (prepared daily). After an additional reaction time of 5 min, the reaction was stopped by the addition of 125 mg of

sodium hydrogen sulfate monohydrate ($NaHSO_4 \cdot H_2O$). It was then centrifuged (Mikro 120 centrifuge, Hettich Lab Technology, Tuttlingen, Germany) for 5 min at 5000 rpm. Finally, 100 µL of supernatant (containing the FAME) was collected and dissolved in 400 µL of hexane (final solution) for later analysis.

The fatty acid analysis was performed on an Agilent Technologies (Palo Alto, CA, USA) 5975 MSD Series gas-mass chromatograph with an automatic injector, and He as the carrier gas. An Agilent HP-88 capillary column (Agilent Technologies, Palo Alto, CA, USA) was used, measuring 100 m × 0.25 mm × 0.20 µm. The injection temperature was 250 °C. The oven was kept at 175 °C for 8 min. The temperature was then raised at a rate of 3 °C/min to 230 °C, which was maintained for an additional 10 min. The temperature of the detector was 230 °C. The amount of sample injected was 1 µL with a 1:20 split. The mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) used an ionization potential of 70 eV and an atomic mass range of 30 to 400 µ (atomic mass units).

The fatty acids (FA) in the sample were identified using the NIST Mass-Spectral Library 2.0, expressing the amounts as percentages of the total FA content. The results obtained were compared with updated bibliographic references.

3.2.2. Bradford Method for Protein Quantification

The protein concentration was determined by the method of Bradford [22]. The samples were diluted to obtain different enzymatic solutions. To perform the measurements, 20 µL of the sample was added to 1 mL of Bradford's solution and allowed to react for 30 min. The absorbance was measured at 595 nm on a model UV-Vis UV-1280 spectrophotometer (Shimadzu, Kyoto, Japan). The absorbance range of the samples must be between 0.1 and 1 for measurements to be reliable. Different concentrations were obtained from a known standard curve for bovine serum albumin (BSA). Protein determinations were performed at least in duplicate in all cases.

3.2.3. Immobilization of TLLs in Sepabeads-C18 Resins

In total, 6.66 mL of TLL solution (with concentration of 18 mg/mL TLL according to Bradford determination) was diluted in 23.3 mL of phosphate buffer 5 mM pH 7.0. Then, 3 g of resin (octadecyl-Sepabeads) was added, and the immobilization mixture was kept in a rotary shaker (J.P. Selecta, Barcelona, Spain) at room temperature for 24 h. Periodically, aliquots (20 µL) of supernatant were taken to measure the activity using the p-NPB test. At the same time, the activity of the reference (soluble enzyme TLL diluted in 5 mM phosphate buffer pH 7.0) was measured. The immobilized biocatalyst was dried with vacuum filtration, carrying out consecutive washings with water/acetone mixtures in the following order (volume/volume ratio): 80:20, 50:50, 20:80 and 0:100.

The immobilization yield is defined as the proportion of lipase immobilized in the support, a parameter determined from the fall in the activity of the supernatant over time. The immobilization yield (%) was calculated as $(100 \times (\alpha_0 - \alpha \times 100)/\alpha_0)$, where $\alpha 0$ is the initial enzyme activity, and α is the enzyme activity after immobilization. For this, a mass of 0.1 g of biocatalyst was resuspended in 1 mL of 50 mM phosphate buffer at pH 7.0. The recovered activity (%) of each biocatalyst was calculated as the ratio between the activity of the biocatalyst and the initial enzyme activity multiplied by a hundred. To determine the recovered activity, their dilution factors must be considered.

3.2.4. Measurement of Enzyme Activity (p-NPB Test)

The activity was quantified by the increase in absorbance at 348 nm due to p-nitrophenol (or p-NP) (ϵ = 5.150 $M^{-1}cm^{-1}$) released in the hydrolysis of p-NPB. The spontaneous hydrolysis of the substrate is less than 2% of the total enzymatic activity [23]. The reaction was carried out in a Jasco V-730 spectrophotometer thermostatized at 25 °C and with magnetic agitation. For this purpose, 20 µL of p-NPB 50 mM was added in 2.5 mL of sodium phosphate buffer 25 mM of pH 7.0 in a quartz cuvette and allowed to incubate in agitation for 1 min. Then, 20 µL of supernatant was added. When the activity value

stabilized, the reading was taken. The enzymatic activity (IU) was calculated as the amount of p-NPB (μmoles) hydrolyzed per minute and per mg of the enzyme under the described reaction conditions.

3.2.5. Borage Oil Ethanolysis by Chemical Pathway

The ethanolysis of borage oil was carried out in a 100 mL round-bottomed flask equipped with mechanical agitation (AREX, Velp Scientifica, Usmate Velate, Italy). 5 mL of oil (4.625 g) were weighed and the volume of a freshly prepared solution of KOH in ethanol was added at 0.5% by weight to the weight of oil. The mixture was kept in a water bath at 60 °C for 1 h with constant agitation (300 rpm). After this time, the contents of the flask were transferred to an Eppendorf tube and subjected to a series of washes to purify the FAEE obtained and the remaining acylglycerols. In a first step, 1.85 mL of a 0.5 M sodium chloride aqueous solution was added to the reaction mixture, vortexed for 1 min and centrifuged at 4500 rpm for 5 min with a Digicen 21 centrifuge (Ortoalresa, Madrid, Spain). Then, the upper organic phase was separated with a Pasteur pipette, and it underwent a second wash with 1.85 mL of distilled water. After vortexing for 1 min, the resulting mixture was centrifuged again for 5 min and the organic phase was separated with a Pasteur pipette.

Anhydrous sodium sulphate was added to this phase to eliminate humidity and was filtered into an 8 mL vial to evaporate in a nitrogen stream to a constant weight. To determine the percentage of FAEE, TAG, DAG and MAG by high-performance liquid chromatography (HPLC) coupled to evaporative light scattering detector (ELSD), solutions of the extract were prepared in hexane with a concentration of 5 mg/mL. For GC-MS analysis, 25 mg of the resulting extract was dissolved in 200 μL of hexane. Subsequently, 100 μL was taken from the solution obtained, which was taken to a final volume of 500 μL with hexane. In all cases, measurements were made in duplicate.

3.2.6. Enzymatic Ethanolysis of Borage Oil

In a first step, 200 mg of 3 Å molecular sieve were weighed and used to remove water to prevent hydrolysis reactions, and 100 mg of the immobilized biocatalyst (TLL) was placed in a 25 mL capacity glass vial. Then, 2.05 mL of hexane, 150 μL of absolute ethanol and 300 μL of borage oil were added. The reaction was carried out in a Heidolph incubator equipped with a platform agitator (Unimax 1010, Heidolph, Germany) and a heating unit. The reaction conditions were at constant agitation (200 rpm) at 25 °C. To study the kinetics of the reaction, 50 μL of suspension (reaction medium) was taken at different times (0, 0.3, 0.6, 1, 2, 4, 6, 8 and 24 h), of which 25 μL was diluted 50 times in hexane and analyzed by HPLC-ELSD. The FAEE content was also characterized by GC-MS as explained in Section 3.2.1 (Agilent Technologies, Palo Alto, CA, USA). In all cases, measurements were made in duplicate.

3.2.7. Analysis by HPLC-ELSD

HPLC-ELSD analysis was performed using an Agilent 1260 Infinity Auto-injector Chromatograph (G1329B) with a quaternary pump (G1311B/C), equipped with an Agilent 385 Evaporative Scattered Light Detector (all of them from Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation of the different reaction products obtained by enzymatic ethanolysis was carried out with a silica normal phase column (250 mm × 4.6 mm i.d., 5 μm) maintained at 30 °C [24], using a ternary gradient as follows: 0–2 min, 99.5% A and 0.5% B; at t = 6.5 min, 70% A and 30% B; at t = 11 min, 63% A, 27% B and 10% C; at t = 18 min, 99.5% A and 0.5% B; and at t = 20 min, 99.5% A and 0.5% B. Eluent A consisted of 2,2,4- trimetilpentane, eluent B consisted of methyl tert-butyl ether, and eluent C consisted of 2-propanol. The optimal signal and resolution of the ELSD detector were achieved with the following conditions: evaporator and nebulizer temperature of 30 °C, and evaporator gas (N_2) at 1.6 standard liter per minute (SLM). To identify different lipids represented in the sample such, standards were injected individually and compared

with standards already analyzed in previous studies. In all cases, measurements were made in duplicate.

3.2.8. Reuse of the Immobilized Biocatalyst in Reaction Cycles

Five reaction cycles were carried out with the different immobilized enzymatic biocatalysts. The reactions were carried out in 10 mL syringes equipped with a filter to separate the reaction medium from the Sepabeads and the molecular sieves. At the end of each cycle, the products were collected in an 8 mL vial (amber colour) and the biocatalyst was washed with hexane to remove the substances that were retained in the pores and, at the same time, recover them. After evaporating the solvent, the reaction products were kept in a nitrogen atmosphere and at cooling temperature until the time of the HPLC-ELSD analysis.

3.2.9. Solvent-Free Enzymatic Ethanolysis of Borage Oil

To perform the solvent-free enzymatic ethanolysis, 400 mg of the 3 Å molecular sieve and 200 mg of TLL-Sepabeads were used and placed in a 30 mL capacity glass vial (same quantities as for the solvent reaction). Then, 0.6 mL of dehydrated absolute ethanol and, finally, 1.2 mL of borage oil were added. The reaction was carried out in a Heidolph incubator equipped with a platform agitator (Unimax 1010) and a heating unit. Two different reaction conditions at constant agitation (200 rpm) were used to appreciate the effect of temperature at 25 °C and 40 °C. To study the kinetics of the reaction, 50 µL of suspension (reaction medium) was taken at different times (0, 0.3, 0.6, 1, 2, 4, 6, 8 and 24 h), of which 25 µL was diluted 25 times in hexane and analyzed by HPLC-ELSD. In all cases, reactions were made in duplicate.

3.2.10. Derivatization of Fatty Acids from Reaction Cycles

To determine the fatty acid content of our oil, the sample was previously derivatized into a basic medium (described above in the characterization of borage oil), forming fatty acid methyl esters (FAMEs). In the case of reaction cycles, as there are fatty acid ethyl esters (FAEEs), these will not be methylated, and when we carry out the analysis in the GC-MS, both the FAMEs and the corresponding FAEEs will be appreciated.

4. Conclusions

Borage oil is a rich source of GLA, contains about 20% of total FAs and can be used in ethanolysis to obtain ethyl esters of this FA. The immobilization of lipases on hydrophobic supports is a simple process to obtain biocatalysts with high-catalytic activity. Moreover, it is a relatively fast process since 89% of the enzyme (TLL) present at the beginning of the incubation was fixed in 4 h, resulting in a biocatalyst with an enzyme load of 32.56 mg TLL/g support and an IU of 1477.9 U/g support. After ethanolysis of borage oil with TLL immobilized on octadecyl-Sepabeads, reaction yields (defined as a function of the degree of TAG conversion) were very close to those produced by chemical ethanolysis with KOH (93.4% and 99.5%, respectively). The results obtained were much better when comparing ethanolysis catalyzed with TLL in Sepabeads-C18 (93.4% conversion) with ethanolysis catalyzed with the commercial TLL biocatalyst Immobead 150P (84% conversion). It is corroborated that the immobilization of lipases on hydrophobic supports provides an additional benefit in the transformation of oil modification, the hyperactivation of the enzymes due to the interaction of the enzymes with the apolar support. Regarding the composition of the FAEE formed in the enzymatic ethanolysis, 9.7% were GLA. This represents almost 50% of the GLA residues present in the starting oil (20.56%). Since the TLL-catalysed formation of GLA EE depends on the migration of GLA FA from the sn-2 position to the terminal positions, it would be necessary to reduce the risk of acyl-migration so a solvent-free system with the lowest possible temperature, 25 °C, could be used. It was shown that enzyme immobilization is a process that allows maintaining or improving the initial activity of the enzyme and, at the same time, significantly increasing its stability to be able to reuse it in several reaction cycles and industrial processes. In this work, the TLL

biocatalyst immobilized on octadecyl-Sepabeads was subjected to five cycles of ethanolysis, maintaining about 70% of its initial catalytic activity.

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