catalysts

MDPI

Article

Novel *Combi-lipase* Systems for Fatty Acid Ethyl Esters Production

Esteban C. Toro [1], Diego F. Rodríguez [1,2], Nelson Morales [1], Lina M. García [1] and César A. Godoy [1,*]

[1] Departamento de Química (LIBB), Grupo de Investigación en Ingeniería de los Procesos Agroalimentarios y Biotecnológicos (GIPAB), Universidad del Valle, C.P. 76001 Cali, Colombia; esteban.toro@correounivalle.edu.co (E.C.T.); dfrodriguez1@uc.cl (D.F.R.); nelson.morales@correounivalle.edu.co (N.M.); lina.mayerly.garcia@correounivalle.edu.co (L.M.G.)

[2] Facultad de Química y de Farmacia, Pontificia Universidad Católica de Chile, Vicuña Mackenna 4860, C.P. 7820436 Macul, Santiago, Chile

* Correspondence: cesar.godoy@correounivalle.edu.co; Tel.: +57-2-3212100 (ext. 756)

Received: 1 May 2019; Accepted: 20 May 2019; Published: 18 June 2019

Abstract: Most Combi-lipases (CL) are based on mixtures of different lipases immobilized on different supports. The increased CL efficiency has been attributed solely to the complementary selectivity of lipases. However, the role of the immobilization support in CL or in co-immobilized systems (*co*CL) and the application of kinetic models to account CL composition effects, have not been assessed. In this work, commercial lipases from *Thermomyces lunuginosus* (TLL), *Candida antarctica* (CALB) and *Rhizomocur miehei* (RML) and supports as Lewatit®VPOC1600 (LW) and Purolite®ECR1604 (PU), were combined to produce new CL systems for the production of fatty acid ethyl esters (EE) which are the main component of ethylic biodiesel: *Co*-immobilization slightly altered palm olein EE yields with regard to that of equivalent CL systems, e.g., the best *co*CL of TLL and CALB in LW (89.5%) and the respective CL (81.8%). The support did affect CL behavior: (i) The best *co*CL of TLL and RML on LW produced 80.0% EE while on PU 76.4%; (ii) CL based on mixtures of the same enzyme, but immobilized on different supports (*semi*CL) show complementarity: The best TLL *semi*CL produced 86.1% EE while its constituents (LW) and (PU) produced individually 78.2 and 70.3%, respectively. The proposed model accounts adequately the EE production properties for CL systems based on TLL, CALB and LW. This work expands the tools to obtain new CL systems for EE production.

Keywords: immobilization; Combi-lipases; support; biodiesel; lipases; transesterification

1. Introduction

Optimization of mixtures of immobilized lipases (Combi-lipases, CL) has been applied in the production of fatty acid alkyl esters (the main biodiesel component) or vegetable oil hydrolysates as a strategy to increase rate and yield with regard to single-lipase catalysts [1–5]. In most cases, those increments have been attributed to the complementary selectivity of the lipases involved, e.g., one lipase is not-specific and the other *sn*-1 and -3 specific [1]. In such cases, CL consist in mixtures of Novozyme®435 Lipozyme TL IM® and/or Lipozyme RM IM® [1–4], that are industrial derivatives of immobilized lipases from *Candida antarctica* (CAL B), *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei* (RML), respectively; the supports which each lipase is immobilized on are non-compressible silica gel, Lewatit®-VP-OC-1600 (LW) and Duolite®ES-562 (a weak-anion exchanger phenol-formaldehyde copolymer resin [6]), respectively. Since the immobilization support nature often plays a key role in tuning the immobilized enzyme properties [7–9], it is very likely that in those CL

catalyzed reactions the presence of different supports (in addition to the different lipases) contributes to the effects observed. In the case of applications of *co*CL for fatty acid alkyl esters production, only a few reports are found: One research group has dealt with *Rhizopus oryzae* lipase (ROL) and *Candida rugose* lipase (CRL) mixtures co-immobilized covalently on non-commercial support [10,11]. However, well-defined immobilization parameters for each lipase in the obtained *co*CL, as the immobilized activity (or protein) or the effect of the support nature, were not reported. The latter is important, since in other enzymatic systems support nature may also affect the overall biocatalyst behavior for co-immobilized enzymes [12,13].

Conversely, even when there are several kinetic models for fatty acid alkyl ester production catalyzed by lipases [11,14,15], as far as we know, these have not yet been applied to explain one of the key parameters when dealing with CL: The composition effect.

The main goal of this work was to assess both the contribution of support and lipase nature in CL systems based on commercial components in the production of fatty acid ethyl esters (EE) as the main components of ethylic biodiesel. To this end, CL based on the combination of derivatives of the same enzyme (*semi*CL), conventional CL (where different enzymes are present) and co-immobilized lipases in different supports (*co*CL), were produced and compared with regard to its constituents. Thus, commercial enzymes from Novozyme® (CALB, RML and TLL) and supports of different nature from Lewatit® VP OC 1600 (LW) and Purolite® ECR 1604 (PU) were used to obtain mono-enzymatic derivatives for the production of fatty acid ethyl esters (EE) from palm olein under very mild reaction conditions [16]. The effect of the enzyme loading in EE production for the most active derivatives was determined. The influence of lipase composition in binary *semi*CL, CL and *co*CL systems were measured and compared to that obtained from a modified and adjusted kinetic model [14,15]. Finally, catalytic and operational properties of selected CL were compared to those of the industrial derivative in biodiesel production using Novozyme®435 [17].

2. Results and Discussion

2.1. Immobilization of Lipases on Selected Supports and EE Production Activity

The activity immobilization yield was above 80% for the lipases assayed except for CALB on the strong anionic exchanger PU were immobilization was negligible (Table 1). Interestingly, the immobilization yield in terms of protein was below to that of the activity. This means that these enzymes result purified once immobilized on the support surface: This was previously observed for lipases immobilized on supports where immobilization is driven by the hydrophobic effect as on LW [18,19].

Table 1. Immobilization yield on different screened supports in highly loaded biocatalysts.

Enzyme	Support	Immobilized Protein (mg/g)	Protein Immobilization Yield (%) [a]	Immobilized Activity (IU *p*-NPB/g)	Activity Immobilization Yield (%) [a]
Thermomyces lanuginosus lipase (TLL)	Lewatit® VP OC 1600 (LW)	70.40 ± 9.29	77.20 ± 7.66	0.814 ± 0.045	98.15 ± 4.84
	Purolite® ECR 1604 (PU)	19.40 ± 2.36	19.55 ± 2.37	0.157 ± 0.008	89.34 ± 3.19
Candida antarctica lipase B (CALB)	LW	69.12 ± 2.74	69.34 ± 0.99	0.866 ± 0.026	94.42 ± 4.58
	PU	3.76 ± 2.74	3.80 ± 2.76	0.011 ± 0.026	1.20 ± 0.27
Rhizomucor miehei lipase (RML)	LW	34.80 ± 1.09	34.80 ± 1.10	0.257 ± 0.010	92.90 ± 3.62
	PU	23.66 ± 1.43	23.66 ± 1.43	0.317 ± 0.068	87.99 ± 2.65
Novozyme® 435	LW	87.22 ± 15.86	-	-	-

[a] Immobilization yield under defined immobilization conditions (Section 3.4) was calculated as 100% ($1-X_{SI} \times X_{CS}^{-1}$), where X_{SI} and X_{CS} indicate the total protein or activity content after 24 h in the supernatants that were in contact before the measures with and without support, respectively [16].

On hydrophobic supports as LW (with a polymethacrylate/divinylbenzene copolymer matrix) it is expected that lipases transit from the close and more hydrophilic form in aqueous media to the open and more lipophilic form once immobilized on the support [20]. By means of the hydrophobic patches detection tool (Swiss PDV Viewer http://www.expasy.org/spdbv/) and the proper PDB numbers, it was possible to stablish that in the expected immobilized form, RML (4TGL), CALB (5A71) and TLL (1DTE), have a major hydrophobic patch area (open lid domain) of 2287, 2205 and 1789 Å^2, respectively.

However, even when the initial rate of immobilization was different (Figure 1a), the extension of the immobilized activity at 24 h was significantly the same, which means that the hydrophobicity of the support was high enough to make those differences irrelevant between lipases under the experimental conditions (Section 3.4).

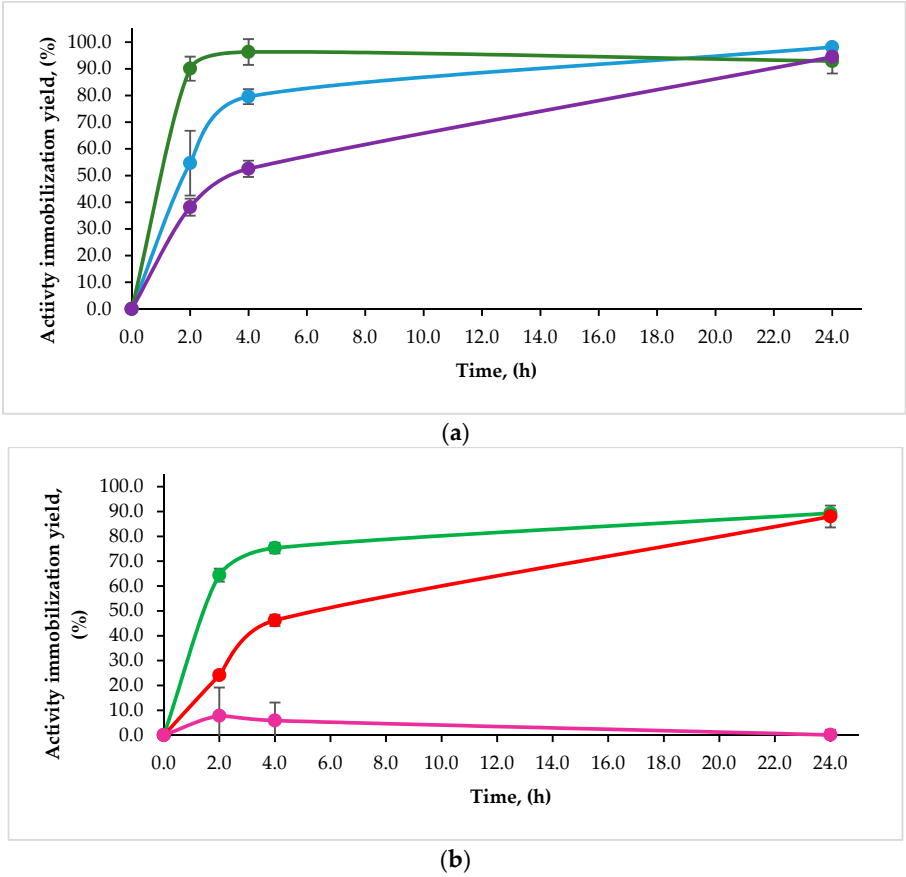


Figure 1. (**a**) Hydrolytic activity (*p*-NPB) immobilization time-course for lipases on LW: TLL (blue), RML (orange) and CALB (violet); (**b**) on PU: TLL (green), RML (red) and CALB (orchid). Immobilization conditions according to Section 3.4.

High values (>85%) of activity immobilization were achieved for TLL and RML on PU after 24 h (Figure 1b). For these enzymes, the difference between the immobilized activity and protein where more marked than that on LW (Table 1). This implies a higher degree of purification of the lipases on the PU support surface probably due to its low hydrophobicity (and thus, higher selectivity) compared to LW. For CALB, immobilization of activity or protein on PU was negligible probably due to the fact that CALB has the higher pI among the lipases assayed (6.0 [21] vs. 3.8 [22] and 4.4 [21] for RML and TLL, respectively). In fact CALB was also unable to be immobilized (even at pH 10) on the strong anionic exchanger Q-Sepharose® [23] and with low activity immobilization yields (below 20%) on other anionic exchanger supports assayed in our laboratory as Nekrolith®RAM-1 or Lewatit® MP800 (Table S1). Using the tool H^{++}3.0 [24] it is possible to establish that at the immobilization pH, CALB has a net charge of ~0 while RML-7 and TLL-9, which may explain the low interaction of CALB

with anionic exchangers and the relative rates of immobilization of RML and TLL in both PU and LW supports (Figure 1).

2.2. *Effect of Protein Loading in Selected Derivatives*

Palm olein fatty acid ethyl esters yield (%EE) under mild reaction conditions (Section 3.3), was assessed for the derivatives obtained. The EE production activity order of the lipase preparations was TLL ≥ RML > CALB (Table 2, fourth column); this pattern was previously seen under similar reaction conditions using waste cooking oil or fats [25]. The lower EE yield observed for the reference derivative Novozyme®435 (and for the closely related derivative CALB-LW) has been attributed to the fact that CALB has low catalytic activity against triglycerides [14].

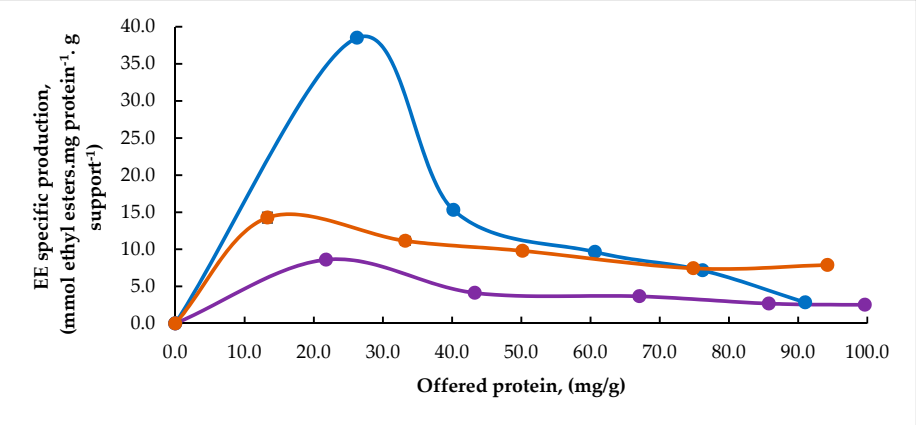
Table 2. Specific palm olein fatty acid ethyl esters production for the obtained derivatives.

Enzyme	Support	Specific Production at 6 h (mmol EE/g Derivative-mg Protein) [a]	EE After 24 h (%)
TLL	LW	2.79 ± 0.11 38.0 ± 0.35 [b]	92.5 ± 3.32 78.2 ± 3.80 [b]
	PU	9.68 ± 0.07 21.7 ± 0.08 [b]	68.1 ± 3.52 70.3 ± 3.41 [b]
CALB	LW	2.48 ± 0.72	47.4 ± 3.60
RML	LW	7.79 ± 0.11 9.67 ± 0.61 [b]	70.7 ± 4.34 68.6 ± 4.05 [b]
	PU	3.90 ± 0.07 6.42 ± 0.61 [b]	69.7 ± 4.34 63.0 ± 3.26 [b]
Novozyme®435	LW	3.62 ± 1.53	52.7 ± 3.50

[a] For the calculation it was assumed the average molecular weight of 301.2 g.mol^{-1} for palm olein fatty acid ethyl esters according to the results obtained by GC-MS (Table S2). [b] Values after the minimization of the protein offered to the support.

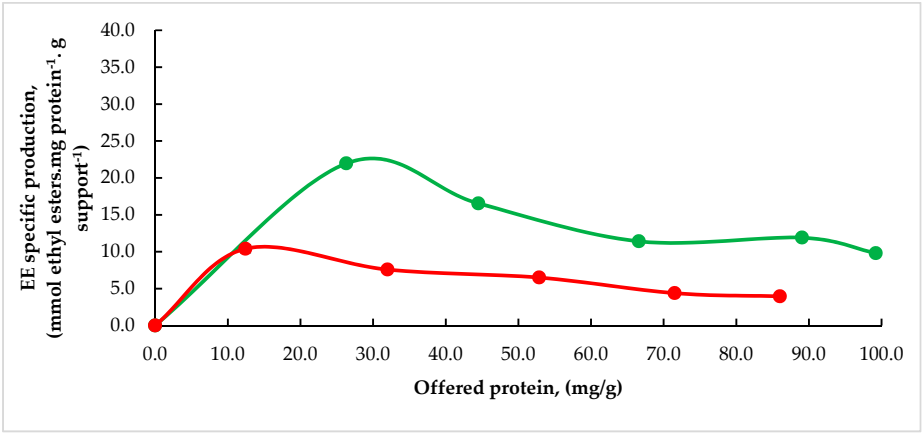
Since the enzyme accounts for most of the price of the biocatalysts [26], it is important to minimize the spent amount during derivative production, however, this important aspect has been omitted for most of the CL reported so far [1–3,5].

In this work, highly loaded biocatalysts were initially produced offering around 100 mg of protein per g of support (Table 1); the specific EE production (mmol EE. mg protein^{-1}. g support^{-1}) was determined at 6 h (upper rows, Table 1), time when the EE production rate was relatively constant (Figure S2). Then, lower amounts of protein were offered to produce lowly loaded derivatives and the same catalytic properties were determined (Figure 2).



(**a**)

Figure 2. *Cont.*



(**b**)

Figure 2. Effect of the TLL protein offered to the supports on the specific EE production at 6 h. (**a**) In LW for TLL (blue), RML (orange) and CALB (purple); (**b**) in PU (lower panel) for TLL (green), RML (red).

The highest specific activity value for TLL-LW was obtained when 26 mg of protein per g of support was offered (Figure 2a): This derivative produces 78.2% EE from palm olein at 24 h, which is lower than that of the highly loaded biocatalyst (92.5% EE), but reduces protein expending by 70%. For the best TLL-PU derivative, the protein saved was 73% with a 70% EE yield at 24 h (Table 2). Lower values in the protein saved were found for RML derivatives (~37% in both LW and PU) and none for CALB-LW. In these cases, the highest specific activity was found from 10–20 mg per g of support, however, the %EE at 24 h for these derivatives were below 40%. In further experiments, this implied to choose derivatives obtained with higher amounts of offered protein (~50 mg for RML and ~100 mg for CALB-LW) to obtain a %EE closer to those of the highly loaded biocatalysts (Table 2). The fact that TLL allowed a higher protein saving may be related to its higher activity in transesterification compared to CALB or RML [16,25,27]. This makes the reaction to be more dominant on the external TLL-derivative surface, which implies that a higher proportion of the enzyme immobilized within the particle cannot process substrate (higher k_{cat}, higher Thiele modulus and lower enzyme utilization [28]). Thus, this seems to reduce more markedly the specific activity of the TLL-biocatalyst especially at higher values ofprotein offered or to increase it at lower values of protein offered with lower sacrifices in EE yields than for the other enzymes (Figure 2).

2.3. Composition Effects on EE Production with CL, semiCL and coCL

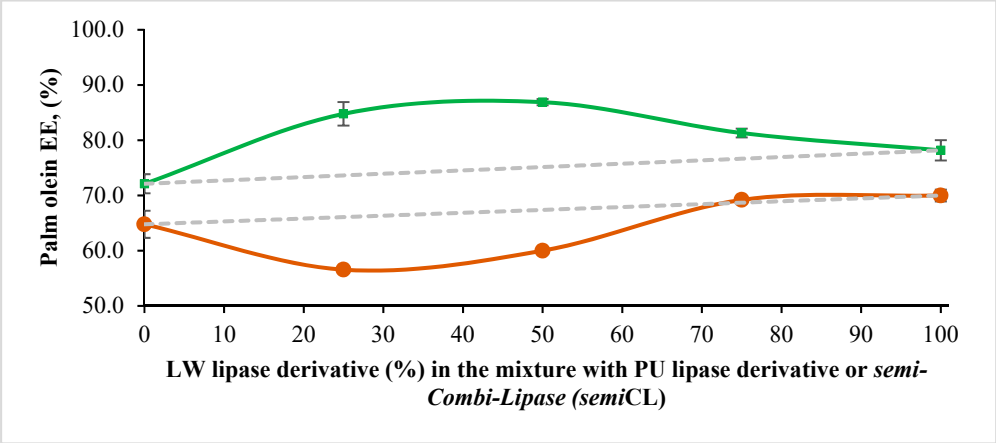
To quantify the effect in EE production of the combination of the biocatalysts with regard to the EE production obtained assuming a simple additive effect, it is defined the complementarity factor, CF, of a binary Combi-lipase as:

$$CF_{C_1,C_2} = \frac{\%EE_{Combi}\ (C_1, C_{2)}}{\%\ EE_{calc}\ (C_1/100 * \%EE_1 + C_2/100 * \%EE_2)}, \quad (1)$$

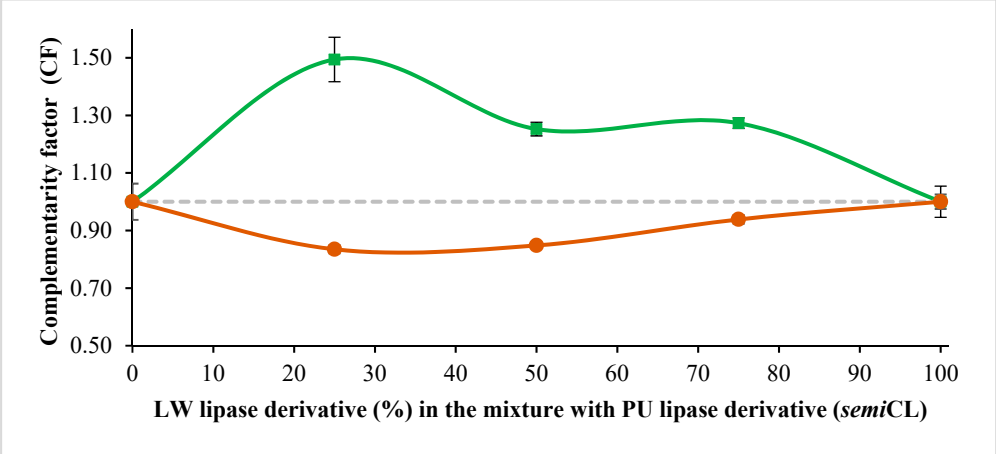
where $\%EE_{Combi}$ is the yield measured at 24 h for a binary Combi-lipase catalyst (CL, *semi*CL or *co*CL) with a percentage composition C_1 and C_2; the $\%EE_{calc}$ results from the weighted sum of the %EE at 24 h for each component at 100% under the same reaction conditions (Section 3.3).

2.3.1. Semi Combi-lipases (*semi*CL): TLL-LW/TLL-PU and RML-LW/RML-PU

Different combinations of derivatives of the same lipase (TLL or RML) on the supports PU and LW were made and the values of %EE and CF vs. composition were determined, as seen in Figure 3.



(**a**)



(**b**)

Figure 3. (**a**) Effect of the composition of the *semi*CL on %EE yield and in (**b**) CF at 24 h. Combination of TLL (green) or RML (orange) LW and PU preparations. The discontinuous lines represent a calculated weighted additive behavior.

Both RML and TLL has been characterized as *sn*-1,3 specific lipases [29,30]. It has been reported that this specificity can be altered depending on the immobilization support or the solvent used [29,31]. Other reports establish that silica support could catalyze by itself acyl migration, known as the rate-limiting step in EE production for these specific lipases [32]. Therefore, there is evidence that the support can alter biocatalyst properties in transesterifications, however, this has been not evaluated in the Combi-lipases context.

In this work, it is shown that the immobilization support alone plays also a key role in the CL properties assessed: The CF values obtained for the biocatalysts obtained by the combination of derivatives of the same lipase on LW and PU supports (*semi*CL) differ from a merely additive effect (Figure 3). In general, it is expected a synergistic effect in CL ($CF_S > 1$) when the component biocatalysts complement each other in their respective rate-limiting steps, e.g., one is faster acting on triglycerides, but slower than the other on partial glycerides [33]. Negative effects ($CF_S < 1$), when inhibitors and/or inactivators (e.g., glycerol or ethanol [34]) for each biocatalyst are accumulated along the reaction time in a higher degree for the presence of the other component with regard to the situation when each biocatalyst was alone. In the case of the *semi*CL studied here, that type of differences must emerge from the different orientation on the surface and microenvironment that the lipase acquires after immobilization on the support [7,16]: It is expected that in LW the lipase adsorbs through the open lid surface [20] while in PU through the surface with the higher content of anionic residues. As the CFs for RML and TLL in their respective *semi*CL shown an opposite behavior with regard to additivity

(Figure 3), it is clear that even when the orientation on each support type may be similar for these lipases, the exact effect in catalytic complementarity is lipase-specific [25,35], and since the composition media changes during the reaction course, also dynamic.

2.3.2. Combi-lipases Based on Mono-lipasic Derivatives Mixtures (CL)

As commented, the Combi-lipases reported in the literature are made of the combination of biocatalyst based on different enzymes and supports [1–4]. In this work, novel Combi-lipases systems were obtained and their properties in EE production are summarized in Table 3. Interestingly, all these biocatalysts were more active than the commercial biocatalyst Novozyme®435 and with a reduced amount in the enzyme content up to 70%.

Table 3. Complementarity factors and maximum biodiesel yield for different Combi-lipase systems.

Type		Component C1	Component C2	Min. CF [a] and Composition (%C1)	Max. CF [a] and Composition (%C1)	Max. EE Yield (%) and Composition (% C1)
*semi*CL		TLL-LW	TLL-PU	1.06 (75)	1.19 (25)	86.1 (50)
		RML-LW	RML-PU	0.83 (25)	0.94 (75)	70.7 (100)
CL		TLL-LW	CALB-LW	1.16 (75)	1.41 (25)	81.8 (75)
		TLL-LW	RML-LW	1.01 (75)	1.15 (25)	82.0 (25)
		TLL-LW	RML-PU	1.00 (75)	1.10 (25)	78.2 (100)
		TLL-PU	RML-PU	0.80 (75)	1.18 (50)	78.5 (50)
		TLL-PU	RML-LW	0.95 (75)	1.07 (25)	73.7(25)
		TLL-PU	CALB-LW	1.05 (25)	1.23 (50)	81.4 (75)
		RML-LW	CALB-LW	0.94 (25)	1.07 (50)	70.7 (100)
*co*CL (co-immobilized)	in LW	TLL	CALB	1.25 (50)	1.49 (25)	89.5 (75)
		TLL	RML	1.06 (75)	1.15 (25)	80.1 (75)
	in PU	TLL	RML	1.05 (75)	1.13 (50)	75.3 (50)

[a] Values of CF calculated accordingt to Equation (1) at the respective C1 compostion.

Values of CFs from 0.8–1.5 for the CL assayed were obtained. This means, that even when the catalytic properties for most CL were not additive, the positive or negative synergistic effect was moderate as previously seen for CL of the studied enzymes, e.g., the CFs calculated for binary mixtures of TLL, RML or CALB in Reference [2] were from 1.05–1.8. In this work, the highest CFs values (1.41 and 1.49) were achieved for combinations of TLL and CALB and the highest %EE (81.8) were found at 75% TLL-LW. This is the same proportion found for the optimization of a CL composed of Lipozyme TL IM and Novozyme®435 (based on TLL and CALB, respectively) for the production of rapeseed oil EE [36]. This seems to reflect their documented complementary catalytic properties in transesterification: TLL is *sn*-1,3 specific [37], faster processing triglycerides [15] and inhibited by free fatty acids [15,38], while CALB is unspecific [37], faster processing partial glycerides [33] and can esterify free fatty acids [39]. Values of CF that range from 1.0–1.2 were obtained from combinations of TLL and RML: This lower values with regard to those of TLL and CALB were expected, since RML [40] has catalytic properties closer to TLL in transesterification [15].

Conversely, RML did not complement in the same extent with CALB as TLL did (CFs 0.94–1.07). This also has been seen in CL of covalently immobilized RML and CALB that, depending on the reaction conditions, showed low complementarity or even antagonist effects in waste cooking oil EE production [41].

2.3.3. Co-Immobilized Lipases (coCL)

Relatively low increments (+9%) in CFs (Figure 4) or EE yield (Table 3) were found for the *co*CL of CALB and TLL with regard to the respective CL; for the *co*CL of RML and TLL the effect of co-immobilization was slightly negative (−3%) in EE yield (Table 3). The composition values for maximum CF were the same in CL and *co*CL.

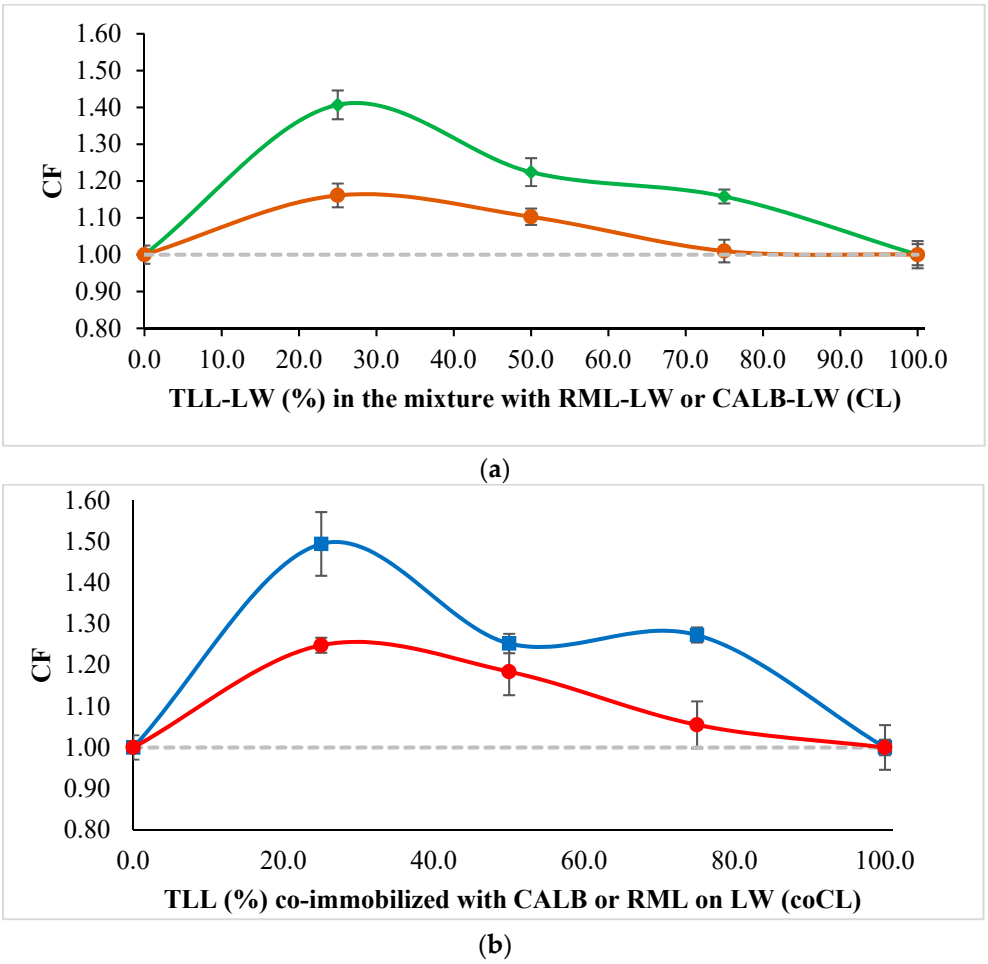


Figure 4. Effect of the composition of the biocatalyst on the CFs. (**a**) Combi-lipases based on mixtures of TLL-LW with CALB-LW (green) or with RML-LW (orange); (**b**) co-immobilized lipases on LW (lower panel) of TLL with CALB (blue) or with RML (red).

The moderate increment caused for lipase co-immobilization found in this work, agrees with the results for covalently co-immobilized lipases in silica gel (+6%) in the transesterification of soybean oil and methanol in terms of initial rate (EE yield was not reported) [11]. Conversely, the support effect was manifested on the *co*CL of TLL and RML in the value of the maximum %EE and the respective composition (Table 3): The *co*CL in LW produces 80.1% EE (TLL/RML 3:1) while in PU 75.3% (TLL/RML 1:1). This may be a reflection of the fact that in LW (Section 2.1) the open (and more active) conformation of the lipases is favored [20].

2.3.4. Application of an Adjusted Kinetic Model

As for most reactions catalyzed for lipases, transesterification for EE production follows the Ping-Pong Bi-bi mechanism [11,14,15]. However, only a few reports contain information about the kinetic forward and reverse constants involved in each step [14,15]. From the information in Reference [15], it is possible to estimate values of equilibrium constants ($K = k_{forward}/k_{reverse}$) for each transesterification step. For the model assayed here, it was assumed independence of temperature, type of alcohol and oil for the values of K. Thus, values of K for each reversible step in the Ping-Pong Bi-bi mechanism [15] were used as a constraint to adjust the kinetic constants in accordance to the catalytic properties in transesterification for each lipase (Section 2.3.2). Other processes, as the transfer of reactants and products from the bulk media to the reactive surface of the heterogeneous biocatalyst, ethanol inactivation, inhibition by free fatty acids or glycerol and free fatty acid esterification (for

CALB [39] and RML [42]), were modeled in a simplified way [14,15]. The parameters involved in these processes were adjusted to fit the experimental time-course of EE production for each mono-lipasic derivative (Table S3). The program Kinetiscope (version 1.1.8) was used to run simulations of the time-course of the EE production.

In Figure S2 (discontinuous lines) it is shown the adjustment to the time-course in EE production for each active mono-lipasic derivative. In all cases, the values achieved for R^2 were above 0.99 (%EE experiment vs. %EE adjusted model). As an attempt to evaluate the CL composition effect on CFs and %EE at constant biocatalyst content (Table S4), simulations were running maintaining the adjusted values for each mono-lipasic component. The comparison of the model outcomes vs. experimental results for the CL assayed is summarized in Table 4.

Table 4. Comparison of model and experimental for some Combi-lipase properties.

Type		Component C1	Component C2	Max. CF and Composition (%C1) [a]		Max. %EE Yield and Composition (%C1) [b]	
				Model	Experimental	Model	Experimental
*semi*CL		TLL-LW	TLL-PU	**1.05 (25)**	**1.19 (25)**	79.5 (75)	86.1 (50)
		RML-LW	RML-PU	0.92 (50)	0.94 (75)	**68.0 (75)**	**69.2 (75)**
CL		TLL-LW	CALB-LW	**1.50 (25)**	**1.41 (25)**	**89.6 (75)**	**81.8 (75)**
		TLL-LW	RML-LW	1.02 (75)	1.16 (25)	76.2 (75)	82.0 (25)
		TLL-LW	RML-PU	1.08 50)	1.10 (25)	75.0 (75)	76.2 (50)
		TLL-PU	RML-PU	1.09 (25)	1.18 (50)	69.1 (75)	78.5 (50)
		TLL-PU	RML-LW	1.04 (75)	1.07 (25)	72.6 (75)	73.7(25)
		TLL-PU	CALB-LW	1.23 (50)	1.19 (75)	**76.9 (75)**	**81.4 (75)**
		RML-LW	CALB-LW	**1.28 (50)**	**1.07 (50)**	74.4 (50)	67.3(75)
*co*CL	in LW	TLL	CALB	**1.50 (25)**	**1.49 (25)**	**89.6 (75)**	**89.5 (75)**
		TLL	RML	1.02 (75)	1.15 (25)	**76.2 (75)**	**80.1 (75)**
	in PU	TLL	RML	1.09 (25)	1.13 (50)	69.1 (75)	75.3 (50)
RSMD in model value %C1 for CF_{max} or $\%EE_{max}$				29.8		26.0	
RSMD in model value for CF_{max}				0.022		-	
RSMD in values for $\%EE_{max}$				-		2.28	

[a] Maximum and different from 1.0. [b] Maximum for the best CL.

In most cases, the model fails to find the exact values of %C1 where the maximum CF or %EE is found (RSMD 26–30). The reasons for this are that it may rely on the model assumptions, as the invariability of equilibrium constants with regard to temperature and the type of alcohol. Other reason, is that for each component of the system at the beginning and during the reaction course, the assumed unitary (an invariable) value for the activity coefficients (implicitly in Table S4) does differ from one and change along the reaction process [43].

Beyond that, the model found correctly the % C1 for $\%EE_{max}$ for the CL of TLL-PU and CALB-LW, TLL-PU and CALB-LW, the *co*CL of TLL and RML on LW, the *semi*CL of RML and both the % C1 for CF_{max} (25% TLL) and $\%EE_{max}$ (75% TLL) for the CL and *co*CL of TLL and CALB on LW (Figure 5) which presented the highest conversions (Table 4 in bold). In general, parameters as the model maximum values of CFs and %EE had a relatively lower RSMD. Other information that can be derived from the model (data not shown), is that a degree of complementarity higher than that found experimentally here (CF > 2) could only be reached if the ratios in the values of kinetic constants for the respective rate-limiting steps are much higher than those proposed here, e.g., above a thousand and with a % EE for each component below 50% at 24 h.

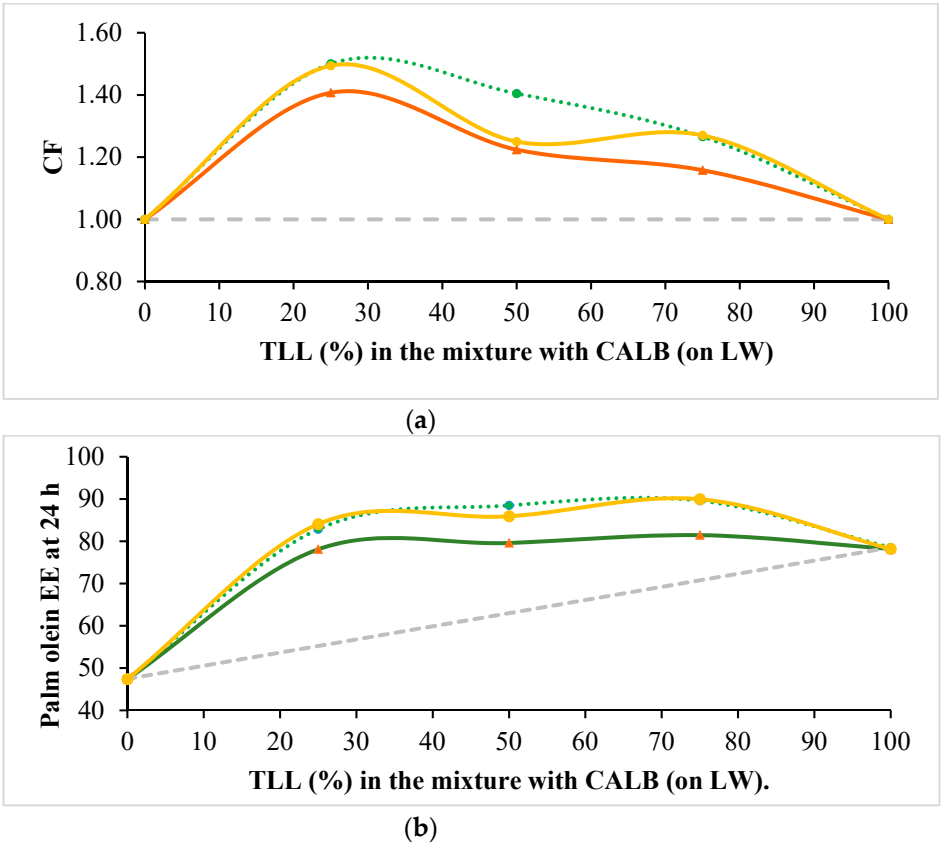


Figure 5. Experimental and model values of (**a**) CF and (**b**) %EE with regard to the composition of the CL based on TLL, CALB and LW. Values according to the model (green); experimental values for *co*CL (purple) and CL (orange). As references, the discontinuous lines (grey) represent a merely weighed additive effect between the CL constituents.

Thus, developing new CL exploring different enzymes and supports may allow increasing even more the rates and yields of EE production found here. This CL may include *sn*-2 lipases as CALA [44], catalytically active supports [45] and different approaches as the dynamic optimization of the CL composition (and other reactions conditions) during the reaction course.

2.4. Reuse of Selected CL

The *co*CL of TLL (75%), CALB (25%) and on LW were selected for reuse experiments given their higher EE production yield (Table 4). The mono-lipasic related derivatives TLL-LW and CALB-LW and Novozyme®435, were chosen as references. The operational stability of the *co*CL is high until the fourth cycle and seems to reflect the stability of TLL on LW; for CALB on LW and the closely related industrial biocatalyst Novozyme®435 the operational stability is very similar (Figure 6) [29].

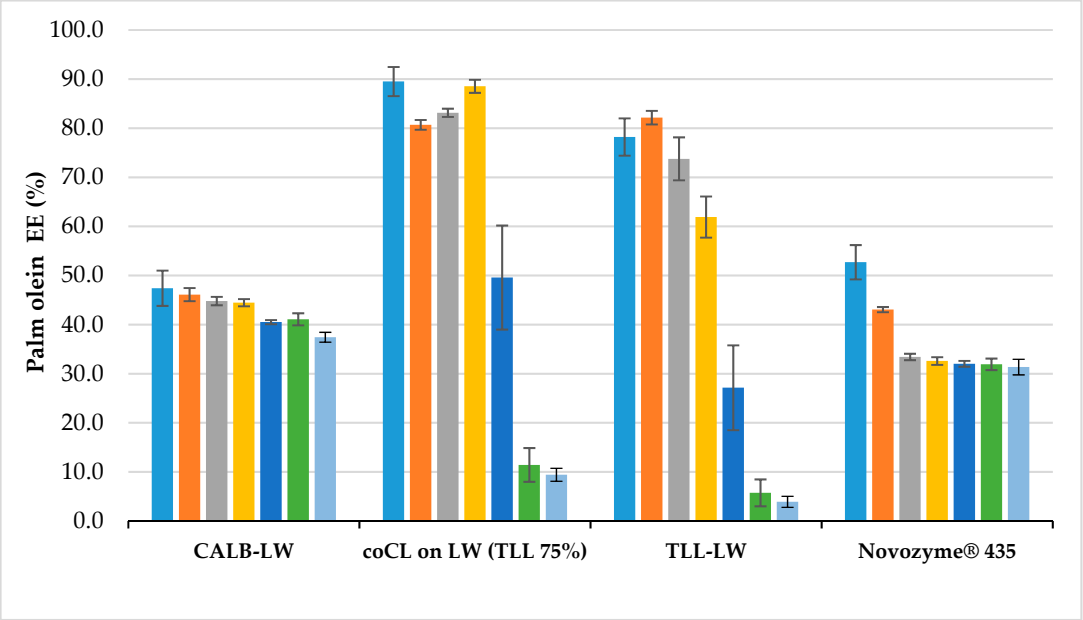


Figure 6. Palm olein EE (%) after successive cycles for selected biocatalysts. Experimental conditions according to Sections 3.3 and 3.8.

Thus, it seems that the weakest component of the *co*CL is TLL. The reason of the relatively low stability of this component may not be related with ethanol inactivation alone, as seen in other transesterification reactions [14,15]: After 2 h of incubation in absolute ethanol (Section 3.8), TLL-LW preserves 83.6% of the initial EE production (CALB-LW 76.5% and RML-LW 5.30%). This high stability has been seen also for TLL immobilized on other hydrophobic supports [29]. Another reason seems to rely on enzyme desorption from the biocatalyst particle: The protein content on the support after the last reaction cycle was reduced in 74.8%. This aspect can be potentially improved with strategies, such as physical or chemical derivative modification, as those applied in analog biocatalysts based on TLL [29].

3. Materials and Methods

3.1. Materials

Candida antarctica lipase B (CAL B), *Thermomyces lanuginosus* lipase (TLL), *Rhizomocur miehei* (RML), *p*-nitrophenyl butyrate (*p*-NPB), Cetyltrimethylammonium bromide (CTAB), Ethylenediaminetetraacetic acid (EDTA), Bicinchoninic Acid Kit (BCA), Bovine serum albumin (BSA), ethanol (96%) and salts for buffering solutions were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Palm olein was purchased in a local store; Novozyme® 435 (a commercial biocatalyst based on immobilized CALB) was a gift from Novozymes (Bagsværd, Denmark). Other reagents and solvents were of analytical or HPLC grade. Supports from Lewatite® (VP OC 1600 (LW) based on polymethacrylate/divinylbenzene copolymer and MP 800 (MP), based on cross-linked polystyrene functionalized with Type I quaternary ammonium groups) were kindly donated by Lanxess® (Cologne, Germany) and Purolite® ECR1604 (PU), based on based on polymethacrylate/divinylbenzene copolymer functionalized with Type I quaternary ammonium groups, was donated by Purolite Ltd. (Llantrisant, UK).

3.2. Esterase Activity and Protein Determination

Esterase activity of soluble or immobilized enzymes against *p*-NPB (*p*-nitrophenyl butyrate) was assayed at pH 7.0 (25 mM sodium phosphate buffer) and 25 °C as described before [18] with the following modifications: Presence of Triton® X-100 (TX) 0.01% for CALB or RML and CTAB 0.001%

and TX 0.01% for TLL. One international unit (IU) is defined as the amount of enzyme required to hydrolyze one μmol of *p*-NPB. min^{-1} under the conditions described above.

Protein determination was performed according to the Protein Assay Kit protocol at 37 °C for 30 min using BSA (bovine serum albumin) as a standard (Pierce® BCA). The quantity of protein was measured in proper dilutions of filtered aliquots of control and immobilization supernatants after the decantation of the support. The protein loading on the different immobilization supports was calculated from the difference in protein content measured between the control and the respective immobilization supernatant after 24 h [16].

3.3. One-Step Solvent-Free Ethyl Biodiesel Production (EE)

Both the procedure to the determination of fatty acid ethyl esters (EE) by FTIR-ATR and to perform the biocatalyzed reaction and was described before [16] with these modifications: The biocatalytic EE production temperature was reduced to 37 °C and the overall mass of the reaction mixture proportionally reduced 60%.

The composition of the vegetable oil used was determined by GC-MS (Table S2 and Figure S1). Initially, the vegetable oil was converted to fatty acid ethyl esters (FAEEs): 0.08 g NaOH was initially dissolved with ethanol (4 mL) at 1% (with regard to the oil mass). The ethanol: Oil molar ratio was six. The NaOH/Ethanol solution was added to the oil and mixed at 200 rpm with magnetic stirring and the temperature set to 65 °C under reflux for 5 h. The reaction mixture was centrifuged at 5000 rpm at 40 °C. An aliquot of the upper organic phase (5.0 mg) was diluted in 1 mL of hexane. Then, 1.0 μL of the mixture was injected into the injector port (split 1:200); injector temperature was 280 °C. The column used was a 30.0 m length SH-Rxi^{TM}-5MS (Crossbond™ 5% diphenyl / 95% dimethyl polysiloxane) with 0.25 μm of thickness (helium flow 1.0 mL/min). The program of the column oven temperature was 5 min at 100 °C, then increased to 290 °C using a ramp of 7 °C/min and maintained 3 min. The conditions for the MS were: 230 °C for the ion source, 280 °C interface temperature and solvent cut time of 2.5 min. The total time of analysis was 37 min (Figure S1).

3.4. Production of Lipase Derivatives

Production of lipase derivatives was performed mixing 1.00 g of support and 22.2 mL of a solution with 2 mM EDTA, 10% glycerol and different protein concentration (4.50, 3.38, 2.25 or 1.12 mg/mL) and 10.0 mM buffer (sodium phosphate or citrate) for 24 h at 28 °C and pH 7.0. Subsequently, the derivatives were concomitantly washed with immobilization solution without enzyme and then with distilled water. Finally, the derivatives were dried at 37 °C for 24 h and stored at 4 °C until use.

3.5. Production of Co-Immobilized Lipases (coCL)

It was used a two-step procedure using the immobilization conditions described above for each enzyme: In the first step, the enzyme with lower support affinity (lower immobilization rate and/or yield, Figure 1) was immobilized at the desired enzyme loading (Section 3.4). After three washes with the properly buffered solutions, an immobilization solution containing the second enzyme was added and once reached the desired enzyme loading, the solid containing the co-immobilized lipases, was washed thrice with immobilization solution (without enzyme) and then with water. Finally, the *co*CL was dried and stored as described before (Section 3.4).

3.6. Combi-Lipases (CL) and Combi-Catalysts of the Same Enzyme (semiCL)

The CL and *semi*CL were obtained by weighing and mixing the proper constituent dried derivatives at proportions 25:75; 50:50 and 75:25 and then used in palm olein EE production as described (Section 3.3). The total biocatalyst mass proportion (5.75%) with regard to the oil mass was maintained in all the experiments.

3.7. Application of an Adjusted Kinetic Model

A previous model for enzymatic transesterification [14,15] was modified (Table S3 and Section 2.3.4) and adjusted to fit the time-course of each mono-lipasic derivative constituent of the CL. Simulations of the time-course of the reactions were run using the program Kinetiscope version 1.1.8 (the adjusted values of kinetic constants and other parameters are summarized in Tables S3 and S4).

3.8. Operational Stability of Selected Derivatives in EE Production

Derivatives were collected after each reaction cycle (24 h) from the reaction medium by decantation and filtration, washed with *tert*-butanol, dried, weighed and reused following the procedure described previously [16].

3.9. Stability in Ethanol of Selected Biocatalyst

Prior to EE production, 50 mg of selected biocatalyst were immersed in 490 µL of 96% ethanol during 2 h at 37 °C and 1700 rpm. Then, the derivatives were dried and used as described before (Section 3.3).

3.10. Statistical Analysis

The experiments described were performed in triplicate. An ANOVA procedure ($p < 0.05$) was used to evaluate significant differences among means.

4. Conclusions

Novel Combi-lipases based on commercial components as Purolite®, Lewatit® and lipases from Novozymes® were produced. Their yields in the production of fatty acid ethyl esters (the main ethylic biodiesel component) were higher than that of the commercial biocatalyst under mild reaction conditions: 89.5% for the *co*CL 3:1 of TLL and CALB, respectively, and 52.7% for Novozyme®435. The adjusted model constitutes an initial approach to explain the CL composition effect on fatty acid ethyl ester yield and complementarity, being mainly useful for CL based on TLL, CALB and Lewatit® VP OC 1600 (LW). However, information derived from measures of kinetic and thermodynamic constants in the specific context of these reactions may contribute to improving the model applicability.

Conversely, both the enzyme and the support nature alter the overall properties of the CL: It was possible to observe non-additive effects even in mixtures of derivatives based on the same enzyme, but immobilized on different supports (*semi*CL) as LW and PU (Purolite® ECR 1604). In general, LW was more effective than PU in enhancing CL properties in palm olein transesterification. Therefore, in the design of a given CL it is important to choose a proper combination of lipases and immobilization supports that can enhance complementary properties related to the improvement of rate-limiting chemical or mass transfer steps, and that minimize enzyme inhibition or inactivation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/9/6/546/s1, Table S1: Activity immobilization yield for CALB on anionic exchangers. Table S2: Composition of the palm olein used in this work. Figure S1: Chromatogram of palm olein ethyl esters, Figure S2: Time-course of EE production for the lipase derivatives on LW, Table S3: Values of the adjusted parameters used for the simulation of the reaction course of EE production for the obtained derivatives and Table S4: Initial reaction conditions for the simulations of EE production (using Kinetiscope 1.1.8).

Author Contributions: Conceptualization, C.A.G.; Formal analysis, E.C.T., D.F.R. and C.A.G.; Funding acquisition, C.A.G.; Investigation, E.C.T., D.F.R., N.M. and L.M.G.; Methodology, E.C.T., D.F.R., N.M. and L.M.G. and C.A.G.; Supervision, C.A.G; Writing—original draft, E.C.T. and D.F.R.; Writing—review and editing, E.C.T. and C.A.G.

Funding: Financial and logistic support from Colombian Universidad del Valle (CI 140) and COLCIENCIAS (CI 71083-Grant 745-2016-Project 110671250425) are gratefully recognized.

Acknowledgments: Authors greatly acknowledge Lewatit® and Purolite® supports donations from Lanxess (Phil Fatula) and Purolite® (Alessandra Basso), respectively; José H. Isaza guides for the GC-MS set-up and Claudia Ortiz manuscript proof-reading and suggestions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Alves, J.S.; Vieira, N.S.; Cunha, A.S.; Silva, A.M.; Záchia Ayub, M.A.; Fernandez-Lafuente, R.; Rodrigues, R.C. Combi-lipase for heterogeneous substrates: A new approach for hydrolysis of soybean oil using mixtures of biocatalysts. *RSC Adv.* **2014**, *4*, 6863. [CrossRef]
2. Poppe, J.K.; Matte, C.R.; do Carmo Ruaro Peralba, M.; Fernandez-Lafuente, R.; Rodrigues, R.C.; Ayub, M.A.Z. Optimization of ethyl ester production from olive and palm oils using mixtures of immobilized lipases. *Appl. Catal. A Gen.* **2015**, *490*, 50–56. [CrossRef]
3. Poppe, J.K.; Matte, C.R.; Fernandez-Lafuente, R.; Rodrigues, R.C.; Ayub, M.A.Z. Transesterification of Waste Frying Oil and Soybean Oil by Combi-lipases Under Ultrasound-Assisted Reactions. *Appl. Biochem. Biotechnol.* **2018**, *186*, 576–589. [CrossRef]
4. Poppe, J.K.; Matte, C.R.; de Freitas, V.O.; Fernandez-Lafuente, R.; Rodrigues, R.C.; Záchia Ayub, M.A. Enzymatic synthesis of ethyl esters from waste oil using mixtures of lipases in a plug-flow packed-bed continuous reactor. *Biotechnol. Prog.* **2018**, *34*, 952–959. [CrossRef] [PubMed]
5. Qiao, H.; Zhang, F.; Guan, W.; Zuo, J.; Feng, D. Optimisation of combi-lipases from *Aspergillus niger f* or the synergistic and efficient hydrolysis of soybean oil. *Anim. Sci. J.* **2017**, *88*, 772–780. [CrossRef]
6. Fallavena, L.P.; Antunes, F.H.F.; Alves, J.S.; Paludo, N.; Ayub, M.A.Z.; Fernandez-Lafuente, R.; Rodrigues, R.C. Ultrasound technology and molecular sieves improve the thermodynamically controlled esterification of butyric acid mediated by immobilized lipase from *Rhizomucor miehei*. *RSC Adv.* **2014**, *4*, 8675–8681. [CrossRef]
7. Mateo, C.; Palomo, J.M.; Fernandez-Lorente, G.; Guisan, J.M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzym. Microb. Technol.* **2007**, *40*, 1451–1463. [CrossRef]
8. Rodrigues, R.C.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Fernández-Lafuente, R. Modifying enzyme activity and selectivity by immobilization. *Chem. Soc. Rev.* **2013**, *42*, 6290–6307. [CrossRef]
9. Guisan, J.M. *Immobilization of Enzymes and Cells*; Humana Press: New York, NY, USA, 2006; ISBN 978-1-58829-290-2.
10. Lee, J.H.; Kim, S.B.; Yoo, H.Y.; Lee, J.H.; Han, S.O.; Park, C.; Kim, S.W. Co-immobilization of *Candida rugosa* and *Rhyzopus oryzae* lipases and biodiesel production. *Korean J. Chem. Eng.* **2013**, *30*, 1335–1338. [CrossRef]
11. Lee, J.H.; Kim, S.B.; Yoo, H.Y.; Lee, J.H.; Park, C.; Han, S.O.; Kim, S.W. Kinetic modeling of biodiesel production by mixed immobilized and co-immobilized lipase systems under two pressure conditions. *Korean J. Chem. Eng.* **2013**, *30*, 1272–1276. [CrossRef]
12. Kazenwadel, F.; Franzreb, M.; Rapp, B.E. Synthetic enzyme supercomplexes: Co-immobilization of enzyme cascades. *Anal. Methods* **2015**, *7*, 4030–4037. [CrossRef]
13. Rocha-Martín, J.; de las Rivas, B.; Muñoz, R.; Guisán, J.M.; López-Gallego, F. Rational Co-Immobilization of Bi-Enzyme Cascades on Porous Supports and their Applications in Bio-Redox Reactions with In Situ Recycling of Soluble Cofactors. *ChemCatChem* **2012**, *4*, 1279–1288. [CrossRef]
14. Fedosov, S.N.; Brask, J.; Pedersen, A.K.; Nordblad, M.; Woodley, J.M.; Xu, X. Kinetic model of biodiesel production using immobilized lipase Candida antarctica lipase B. *J. Mol. Catal. B Enzym.* **2013**, *85–86*, 156–168. [CrossRef]
15. Firdaus, M.Y.; Brask, J.; Nielsen, P.M.; Guo, Z.; Fedosov, S. Kinetic model of biodiesel production catalyzed by free liquid lipase from *Thermomyces lanuginosus*. *J. Mol. Catal. B Enzym.* **2016**, *133*, 55–64. [CrossRef]
16. Godoy, C.A. New Strategy for the Immobilization of Lipases on Glyoxyl–Agarose Supports: Production of Robust Biocatalysts for Natural Oil Transformation. *Int. J. Mol. Sci.* **2017**, *18*, 2130. [CrossRef]
17. Norjannah, B.; Ong, H.C.; Masjuki, H.H.; Juan, J.C.; Chong, W.T. Enzymatic transesterification for biodiesel production: A comprehensive review. *RSC Adv.* **2016**, *6*, 60034–60055. [CrossRef]
18. Fernandez-Lorente, G.; Cabrera, Z.; Godoy, C.; Fernandez-Lafuente, R.; Palomo, J.M.; Guisan, J.M. Interfacially activated lipases against hydrophobic supports: Effect of the support nature on the biocatalytic properties. *Process Biochem.* **2008**, *43*, 1061–1067. [CrossRef]
19. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. *Biotechnol. Adv.* **2015**, *33*, 435–456. [CrossRef]

20. Manoel, E.A.; dos Santos, J.C.S.; Freire, D.M.G.; Rueda, N.; Fernandez-Lafuente, R. Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. *Enzym. Microb. Technol.* **2015**, *71*, 53–57. [CrossRef]
21. Melton, L.; Shahidi, F.; Varelis, P. *Encyclopedia of Food Chemistry*; Elsevier: Amsterdam, The Netherlands, 2018; ISBN 978-0-12-814045-1.
22. Rodrigues, R.C.; Fernandez-Lafuente, R. Lipase from *Rhizomucor miehei* as an industrial biocatalyst in chemical process. *J. Mol. Catal. B Enzym.* **2010**, *64*, 1–22. [CrossRef]
23. Marciello, M.; Filice, M.; Palomo, J.M. Different strategies to enhance the activity of lipase catalysts. *Catal. Sci. Technol.* **2012**, *2*, 1531–1543. [CrossRef]
24. Anandakrishnan, R.; Aguilar, B.; Onufriev, A.V. *H*++ 3.0: Automating p*K* prediction and the preparation of biomolecular structures for atomistic molecular modeling and simulations. *Nucleic Acids Res.* **2012**, *40*, W537–W541. [CrossRef] [PubMed]
25. Tacias-Pascacio, V.G.; Virgen-Ortíz, J.J.; Jiménez-Pérez, M.; Yates, M.; Torrestiana-Sanchez, B.; Rosales-Quintero, A.; Fernandez-Lafuente, R. Evaluation of different lipase biocatalysts in the production of biodiesel from used cooking oil: Critical role of the immobilization support. *Fuel* **2017**, *200*, 1–10. [CrossRef]
26. Amini, Z.; Ilham, Z.; Ong, H.C.; Mazaheri, H.; Chen, W.-H. State of the art and prospective of lipase-catalyzed transesterification reaction for biodiesel production. *Energy Convers. Manag.* **2017**, *141*, 339–353. [CrossRef]
27. Mangas-Sánchez, J.; Adlercreutz, P. Highly efficient enzymatic biodiesel production promoted by particle-induced emulsification. *Biotechnol. Biofuels* **2015**, *8*, 58. [CrossRef] [PubMed]
28. AL-Muftah, A.E.; Abu-Reesh, I.M. Effects of internal mass transfer and product inhibition on a simulated immobilized enzyme-catalyzed reactor for lactose hydrolysis. *Biochem. Eng. J.* **2005**, *23*, 139–153. [CrossRef]
29. Abreu Silveira, E.; Moreno-Perez, S.; Basso, A.; Serban, S.; Pestana-Mamede, R.; Tardioli, P.W.; Sanchez-Farinas, C.; Castejon, N.; Fernandez-Lorente, G.; Rocha-Martin, J.; et al. Biocatalyst engineering of *Thermomyces lanuginosus* lipase adsorbed on hydrophobic supports: Modulation of enzyme properties for ethanolysis of oil in solvent-free systems. *J. Biotechnol.* **2019**, *289*, 126–134. [CrossRef]
30. Utsugi, A.; Kanda, A.; Hara, S. Lipase Specificity in the Transacylation of Triacylglycerin. *J. Oleo Sci.* **2009**, *58*, 123–132. [CrossRef]
31. Caballero, E.; Soto, C.; Olivares, A.; Altamirano, C. Potential Use of Avocado Oil on Structured Lipids MLM-Type Production Catalysed by Commercial Immobilised Lipases. *PLoS ONE* **2014**, *9*, e107749. [CrossRef]
32. Du, W.; Xu, Y.-Y.; Liu, D.-H.; Li, Z.-B. Study on acyl migration in immobilized lipozyme TL-catalyzed transesterification of soybean oil for biodiesel production. *J. Mol. Catal. B Enzym.* **2005**, *37*, 68–71. [CrossRef]
33. Šinkūnienė, D.; Adlercreutz, P. Effects of Regioselectivity and Lipid Class Specificity of Lipases on Transesterification, Exemplified by Biodiesel Production. *J. Am. Oil Chem. Soc.* **2014**, *91*, 1283–1290. [CrossRef] [PubMed]
34. Xiao, M.; Mathew, S.; Obbard, J.P. Biodiesel fuel production via transesterification of oils using lipase biocatalyst. *GCB Bioenergy* **2009**, *1*, 115–125. [CrossRef]
35. Tacias-Pascacio, V.G.; Peirce, S.; Torrestiana-Sanchez, B.; Yates, M.; Rosales-Quintero, A.; Virgen-Ortíz, J.J.; Fernandez-Lafuente, R. Evaluation of different commercial hydrophobic supports for the immobilization of lipases: Tuning their stability, activity and specificity. *RSC Adv.* **2016**, *6*, 100281–100294. [CrossRef]
36. Li, L.; Du, W.; Liu, D.; Wang, L.; Li, Z. Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. *J. Mol. Catal. B Enzym.* **2006**, *43*, 58–62. [CrossRef]
37. Huang, Y.; Zheng, H.; Yan, Y. Optimization of Lipase-Catalyzed Transesterification of Lard for Biodiesel Production Using Response Surface Methodology. *Appl. Biochem. Biotechnol.* **2008**, *160*, 504–515. [CrossRef]
38. Liu, W.-H.; Beppu, T.; Arima, K. Effect of Various Inhibitors on Lipase Action of Thermophilic Fungus *Humicola lanuginosa* S-38. *Agric. Biol. Chem.* **1973**, *37*, 2487–2492. [CrossRef]
39. Mata, T.M.; Andrade, S.; Correia, D.; Matos, E.; Martins, A.A.; Caetano, N.S. Acidity reduction of mammalian fat by enzymatic esterification. *Energy Procedia* **2017**, *136*, 290–295. [CrossRef]
40. Piyatheerawong, W.; Yamane, T.; Nakano, H.; Iwasaki, Y. Enzymatic preparation of enantiomerically pure sn-2,3-diacylglycerols: A stereoselective ethanolysis approach. *J. Am. Oil Chem. Soc.* **2006**, *83*, 603–607. [CrossRef]

41. Babaki, M.; Yousefi, M.; Habibi, Z.; Mohammadi, M. Process optimization for biodiesel production from waste cooking oil using multi-enzyme systems through response surface methodology. *Renew. Energy* **2017**, *105*, 465–472. [CrossRef]
42. Von der Haar, D.; Stäbler, A.; Wichmann, R.; Schweiggert-Weisz, U. Enzymatic esterification of free fatty acids in vegetable oils utilizing different immobilized lipases. *Biotechnol. Lett.* **2015**, *37*, 169–174. [CrossRef] [PubMed]
43. Yancy-Caballero, D.M.; Guirardello, R. Modeling and parameters fitting of chemical and phase equilibria in reactive systems for biodiesel production. *Biomass Bioenergy* **2015**, *81*, 544–555. [CrossRef]
44. Mendoza, L.D.; Rodriguez, J.A.; Leclaire, J.; Buono, G.; Fotiadu, F.; Carrière, F.; Abousalham, A. An ultraviolet spectrophotometric assay for the screening of sn-2-specific lipases using 1,3-*O*-dioleoyl-2-*O*-α-eleostearoyl-sn-glycerol as substrate. *J. Lipid Res.* **2012**, *53*, 185–194. [CrossRef] [PubMed]
45. Kim, M.; Salley, S.O.; Ng, K.Y.S. Transesterification of Glycerides Using a Heterogeneous Resin Catalyst Combined with a Homogeneous Catalyst. *Energy Fuels* **2008**, *22*, 3594–3599. [CrossRef]