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Optimization of the Enzymatic Synthesis of Pentyl Oleate with Lipase Immobilized onto Novel Structured Support

Valeria Cavallaro [1,2,*], Gabriela Tonetto [2,3] and María Luján Ferreira [1,2]

[1] Departamento de Química, Universidad Nacional del Sur (UNS), Avenida Alem 1253, 8000 Bahía Blanca, Argentina; mlferreira@plapiqui.edu.ar
[2] Planta Piloto de Ingeniería Química—PLAPIQUI (UNS-CONICET), Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina
[3] Departamento de Ingeniería Química, Universidad Nacional del Sur (UNS), Avenida Alem 1253, 8000 Bahía Blanca, Argentina; gtonetto@plapiqui.edu.ar
* Correspondence: valeria.cavallaro@uns.edu.ar; Tel.: +54-291-4861700

Received: 10 April 2019; Accepted: 4 June 2019; Published: 14 June 2019

Abstract: The term biorefinery is related to the sustainable production of value-added bioproducts and bioenergy from biomass. Esters from fatty acids are important compounds synthesized from by-products of the oleochemical industry. In agreement with the biorefinery concept, it is important to search for catalysts that reduce the consumption of energy and water, using moderate operation conditions and low reaction times. In this work, response surface methodology (RSM) was used to optimize the enzymatic synthesis of pentyl oleate using *Candida antarctica* lipase B (CALB) immobilized on a polyethylene-aluminum structured support. A factorial design was employed to evaluate the effects of several parameters on the ester yield. To obtain a model with a good fit, an approach to reaction mechanism and enzyme kinetics was taken into consideration. Experimental findings were correlated and explained using equations of a ping-pong bi-bi kinetic model and considering the inhibitory effects of both substrates. The developed model was consistent with the experimental data predicting an increase in pentyl oleate production with increasing temperature and a decrease with higher oleic acid amounts and alcohol to acid molar ratios. This model could be useful in a future industrial application of CALB/LLDPE/Al to minimize the costs in oleochemical biorefineries.

Keywords: biorefinery; RSM; CALB; esterification; enzyme inhibition; ping-pong bi-bi mechanism

1. Introduction

Nowadays, concern about the impact of industrial processes has led to the search for environment-friendly alternatives. One of them is the concept of a biorefinery, that is, the use of the available biomass with the lowest environmental impact, energy consumption, and manufacturing costs to produce marketable products and energy [1].

An oleochemical biorefinery processes naturally occurring oils and fats that are classically extracted from vegetable biomasses, often farmed for oil production or from animal-based biomasses, resulting as by-products of animal processing in the food and feed industries [2,3]. Biorefinery products are important for a variety of industries and applications. In particular, esters derived from fatty acids are of great commercial importance. Long-chain esters (22–26 carbon atoms) have applications in pharmaceuticals, cosmetics, perfumes, detergents [4], and also as water-resistant agents, plasticizers, and fuel additives [5,6]. Therefore, obtaining these high-added value esters is a promising route to enhance the economics of oil-based biorefineries.

Enzymatic esterification offers significant superiorities over chemical catalysis because of milder reaction conditions, higher selectivity and specificity, and lower energy requirements. Several lipases have been reported in literature to effectively catalyze esterification and transesterification reactions [7–10]. Nevertheless, despite the benefits of this technology, replacement of chemical catalysts with enzymes is not widespread in industrial processes because of several factors such as enzyme low stability, final product contamination, difficulty of biocatalyst recuperation and reutilization, and high costs. Enzyme immobilization is a way of solving these difficulties, allowing enzyme recovery and reuse with low toxicity and favorable mechanical properties and stability (thermal, operational, and storage), making the process economically viable [11–13].

Response surface methodology (RSM) is an efficient statistical procedure used to investigate the consequence of various reaction parameters on the ester yield in esterification reactions to predict the best performance conditions using a minimum number of experiments, thus reducing research time and costs [14]. RSM has successfully been applied to study and optimize the enzymatic synthesis of various esters [10,15,16].

We have recently reported the successful *Candida antarctica* lipase B immobilization on a new polyethylene-aluminum structured support (50CAT) and its use in an esterification reaction. 50CAT demonstrated important advantages such as easy separation from reaction products, reuse, and low cost [17]. The main purpose of this investigation is to find the best experimental conditions in order to maximize ester yield in the lipase-catalyzed synthesis of pentyl oleate (PO) using 50CAT. Applying RSM, a parametric study was carried out and a model was obtained to optimize the process. Furthermore, the effects of reaction temperature, initial concentration of oleic acid, and substrates initial molar ratio on the ester yield were analyzed. This optimization study could contribute to the exploitation of by-products of the oleochemical industry, such as fatty acids, maximizing product obtention. In addition, these results are of relevance for a possible further utilization of 50CAT in a continuous monolithic reaction tubular system. The advantages of this approach are related to the low cost of the support, its ease of use in continuous monolithic reactors, and the avoidance of particulate high-cost commercial biocatalysts, with the well-known problems of enzymatic packed-bed reactor disadvantages (such as clogging or formation of channels).

2. Materials and Methods

2.1. Materials

Candida antarctica lipase B (batch LCN02103) was obtained from Novo Nordisk (Brazil). Potassium hydroxide, oleic acid (OA), and phenolphthalein were purchased from Anedra. Ethylic ether, absolute ethanol, and *n*-heptane were acquired from Dorwill (Argentina). 1-penthanol was supplied by Sigma. All products used were of analytical grade.

Linear low-density polyethylene (LLDPE-IP20) was obtained from Dow Chemical. Support films were produced in a hydraulic press.

2.2. Enzyme Immobilization

Biocatalyst support was a commercial laminated pure aluminum sheet of 50 mm × 50 mm covered with LLDPE films. Support preparation and enzyme immobilization was previously reported in Reference [17]. Briefly, LLDPE/Al supports were pretreated with ethanol and then contacted with an aqueous enzyme solution for 2 h at room temperature. Finally, the catalyst was water washed and dried.

2.3. Lipase Quantification

Sulfur content was measured using inductively coupled plasma atomic emission spectroscopy (AE-ICP Shimadzu 9000, Bahía Blanca, Argentina) to determine biocatalyst enzyme loading as reported

earlier [17]. CALB content (1.94–2.50 mg per catalyst) was calculated based on initial and final immobilization sulfur concentration.

2.4. Biocatalyzed PO Synthesis

Enzymatic ester synthesis was performed as follows: The reactants (OA and 1-penthanol), along with the solvent (40 mL *n*-heptane), were placed in a glass flask with hermetic seal and kept in a thermostatic bath at 1400 rpm stirring. Later, the biocatalyst was added to the mixture and reaction was conducted for 6 h. Reaction samples (0.5 mL) were withdrawn from the system at the beginning and at the end of the reaction, dissolved in 5 mL of ether:ethanol (1:1) and titrated with KOH solution in the presence of phenolphthalein. Fatty acid (FA) conversion percentage was defined as follows:

$$\text{OA Conversion } \% = 100 - \left(\frac{titrated\ acid\ after\ reaction}{initial\ amount\ of\ acid} \right) \times 100$$

Reaction temperature, initial amount of oleic acid, and initial molar substrate ratio were established as stated in the following experimental design section.

2.5. Experimental Factorial Design and Statistical Analysis

In this work, a 2^3 factorial design was used. A total of ten experiments with two central points were performed (Table 1). The variables and their levels were: amount of oleic acid (75–225 mg), reaction temperature (25–65 °C), and molar ratio (1-penthanol: OA) (1–3). The study response was converted OA (mg). To perform the factorial design and the statistical analysis STATGRAPHICS Centurion version XV.2 software was used. The order of the experiments was fully randomized. Multiple regression was used to fit the response. Coefficient of determination (R^2) was calculated to assess the goodness of fit. The statistically significant effect of the variables was tested using ANOVA. Coefficients with p-value > 0.05 were considered with no significance and therefore they were eliminated from the model.

Table 1. 2^3 Experimental design for the synthesis of pentyl oleate (PO) catalyzed by 50CAT (reaction time: 6 h, solvent: *n*-heptane).

Run Number	Experimental Factors			Response
	T (°C)	iOA (mg)	MR	Converted OA (mg)
1	25	75	1	7.4
2	65	225	1	47.9
3	65	75	3	29.2
4	65	75	1	18.6
5	45	150	2	28.8
6	25	225	3	24.8
7	65	225	3	61.9
8	25	225	1	24.1
9	45	150	2	30.0
10	25	75	3	20.7
11 *	25	150	3	30.0
12 *	25	150	1	39.9

* Experimental conditions added by the authors.

3. Results and Discussion

In this work, RSM was used for modeling the effect of initial OA mass (iOA), substrate molar ratio (MR), and reaction temperature (T) on the enzymatic esterification of OA with 1-penthanol. The experimental matrix for the factorial design is shown in Table 1 along with the data for the response factor.

Entries 11–12 did not belong to the original factorial design, instead they were added later by the authors in order to evaluate the possible inhibition of the lipase by substrates.

Table 2 presents the results of the analysis of variance for "Converted OA" response. In this case, four effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. They are reaction temperature (T), initial amount of oleic acid (iOA), molar ratio (MR), and the product of temperature and initial amount of oleic acid (T*iOA).

Table 2. Analysis of variance for "Converted oleic acid (OA)".

Source	Sum of Squares	Df	Mean Square	F-Ratio	*p*-Value
A: T	812.045	1	812.045	74.43	0.0033
B: iOA	856.98	1	856.98	78.55	0.0030
C: MR	186.245	1	186.245	17.07	0.0257
AB	212.18	1	212.18	19.45	0.0216
AC	14.045	1	14.045	1.29	0.3390
BC	10.58	1	10.58	0.97	0.3973
Total error	32.729	3	10.9027		
Total (corr.)	2124.8	9			

The same results are visualized in the standardized pareto chart for response variable (Figure 1), where the length of each bar is proportional to the absolute value of its associated regression coefficient estimate. From the figure it is clear that the initial amount of OA is the most important parameter influencing the ester yield followed by reaction temperature.

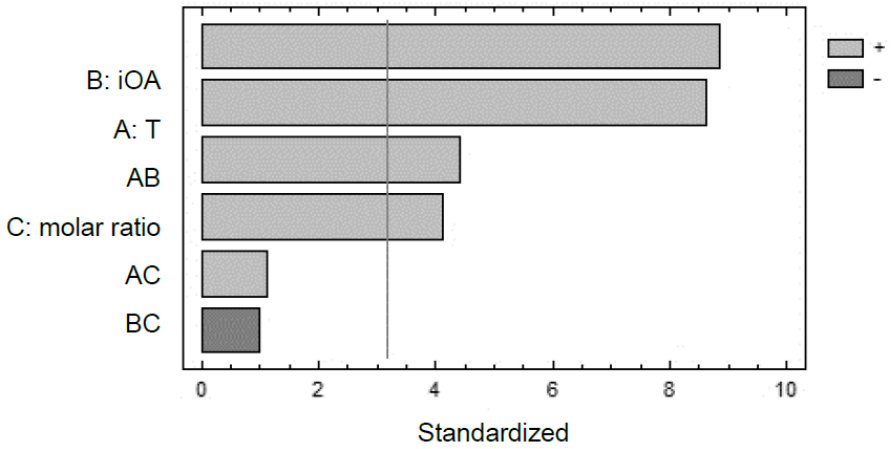


Figure 1. Standardized pareto chart for "Converted OA".

In the experimental range, no simple relationship among variables (or experimental factors) and product yield was found. Consequently, we explored other correlations between variables to determine a mathematical model able to fit the data and to explain the conversion of the reactants (OA and 1-penthanol) into PO.

It is well known that a way to enhance or improve the yield of enzyme catalyzed reactions is to consider their mechanism. Enzyme kinetics can facilitate the way we understand those mechanisms. There are several reported models to elucidate the kinetics of a bio catalyzed esterification [18]. Michaelis-Menten mechanism is generally accepted for reactions with only one substrate but for reactions where the process involves two substrates and two products the bi-bi scheme must be considered. Within this model, the ping-pong and the ternary complex mechanisms are included [19,20]. The ping-pong bi-bi mechanism is the most commonly accepted scheme for lipase catalyzed esterification and transesterification reactions in organic media [4,21]. A general expression for ping-pong bi-bi reaction rate kinetics is shown next, where [A] and [B] are the substrates concentrations,

K^A_M and K^B_M are the [A] and [B] required for half maximal activity, and V_{max} is maximal velocity when both A and B are saturating the enzyme.

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_M^A}{V_{max}[A]} + \frac{K_M^B}{V_{max}[B]} \quad (1)$$

Taking advantage of the accepted kinetic model for CALB catalyzed esterification, Equation (1) was used as a basis for analyzing experimental results. To obtain a better data fit, residual OA amount was used as the response.

Equation (2) shows the obtained expression from the RSM design.

$$\frac{1}{OA_{res}} = 0.00314184 + 1.79018\,\frac{1}{iOA} - 104.869\frac{1}{iOA\ x\ iOH} - 1.19178\frac{1}{T} \quad (2)$$

where OA_{res} is the residual amount of OA after reaction, iOA is the initial acid amount, iOH is the initial 1-penthanol amount, and T is the reaction temperature.

Positive and negative signs in the terms of Equation (2) express synergistic and antagonistic effects, showing the impact of independent variables on ester production. The quality of the model was evaluated using the coefficient of determination (R^2). A high R^2 value (0.982) was obtained, suggesting that the model can explain approximately 98% of variability. The model showed a *p*-value smaller than 0.001 at 95% confidence level indicating the important statistical relationship among variables and response.

As shown in Figure 2, the model predictions and actual values showed good consistency, signifying that the established model provided accurate and satisfactory results. The linear distribution is indicative of a well-fitted model.

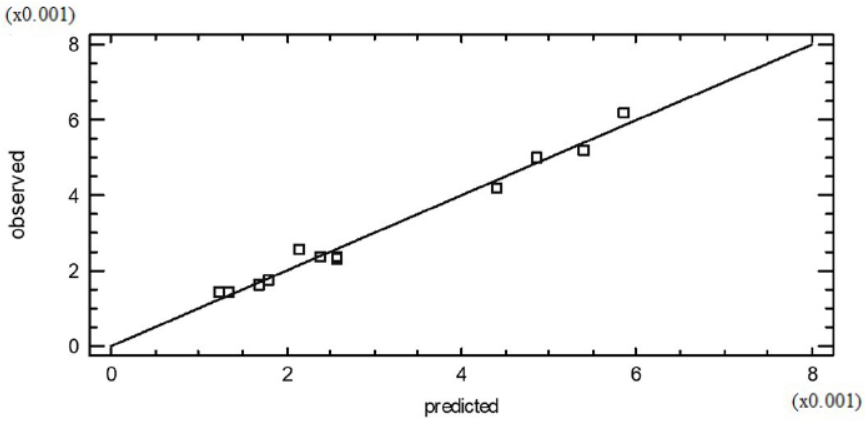


Figure 2. Comparison between the predicted and actual values for the esterification of oleic acid and 1-pentanol.

The performed analysis determined that both initial OA and 1-penthanol amounts played a critical role in PO synthesis. Also, it could be concluded from Equation (2) that while temperature had a positive effect, oleic acid concentration exerted a significant negative effect on ester yield. In summary, we can state that ester yield increases with increasing temperature and decreases with higher acid amounts.

3.1. Reaction Parameters Effect on Pentyl Oleate Conversion

3.1.1. Temperature Effect

Temperature usually plays a significant role in catalytic reactions. An increase in temperature may reduce the viscosity of the reaction mixture, enhance mutual solubility, improve the diffusion of

substrates, and increase the interactions between the catalyst and substrates. However, in biocatalytic processes, high temperature may disrupt the active conformation of the enzyme, causing loss of activity and selectivity. Therefore, it is important to determine the desired temperature in order to obtain optimum enzyme activity. Several studies have indicated that lipases are sensitive to temperature and their activity is significantly decreased at elevated temperatures [22,23]. Equation (2) shows that when reaction temperature increases from 25 °C to 65 °C, ester formation improves.

Several authors have also found that commercial formulations of CALB (Novozym 435®, Bagsvaerd, Denmark) exhibited their optimal activity at high temperatures. Bouaid et.al. optimized the synthesis of Kojic monooleate ester at 83.69 °C while butyl esters from coconut oil were obtained with maximum yield at 65 °C [3,24].

3.1.2. Initial Oleic Acid Concentration Effect

Figure 3 displays the effect of the initial OA concentration on the synthesis of pentyl oleate catalyzed by 50CAT. The results show an increase in ester production with higher oleic acid concentration up to an optimum value at 150 mg of OA (0.532 mmol OA) and a decrease thereafter. This increase was more marked at lower alcohol concentrations. The maximum, optimum OA relative amount to CALB in 50CAT is 0.266 mmol OA/mg CALB. Thus, high acid concentrations appear to inhibit the catalytic activity of lipase. Next, we made initial reaction rate considerations to prove oleic acid inhibition (Figure 4).

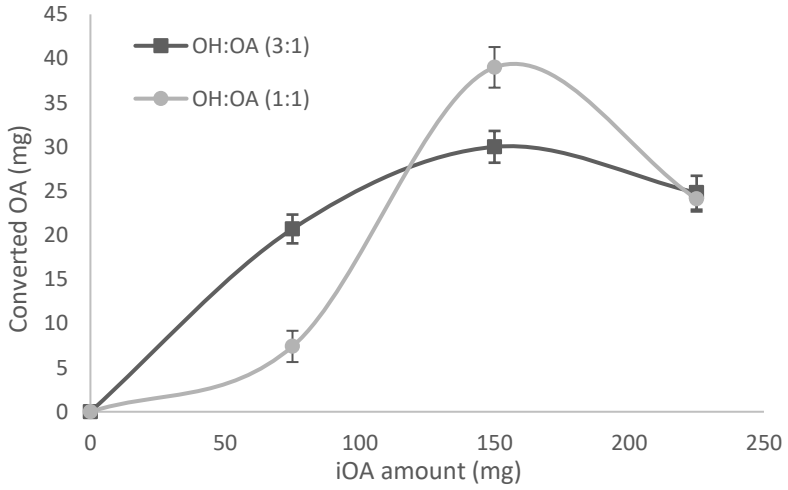


Figure 3. Effect of the initial OA amount on the synthesis of pentyl oleate catalyzed by 50CAT at different molar ratios (reaction time 6 h, T: 25 °C).

Initial reaction rates were determined for two different OA concentrations using fresh biocatalyst and a substrate molar ratio of 1. The results in Figure 4 indicate that the reaction rate increased with higher OA concentration but not as expected, as can be depicted from the equation slopes, confirming lipase inhibition. With lower OA to CALB the initial portion of the kinetics was basically linear, therefore, 0.5 to 2.5 h were selected as reaction times. Furthermore, there is an induction period that is evident with high OA concentration.

Enzyme inhibition by one or more substrates is a very common phenomenon when using enzymatic systems. This effect is the consequence of the formation of an inactive complex among the enzyme and other substrates competing with the main substrate in the reaction medium, or secondary reactions with the protein. In this system, we have found CALB inhibition by OA. There are diverse probable hypotheses that can be proposed for the acid inhibition at higher concentrations. Initially, at high acid concentration, the lower reported conversions could be a consequence of an accumulation of water in the catalytic triad pocket of the lipase as the reaction progresses, causing ester hydrolysis. On the other hand, the excess of acid may cause acidification of the micro-aqueous layer interface

leading to enzyme inactivation and/or high local concentration of OA near the active site with further hindrance for the diffusion of the alcohol to the acyl enzyme [25,26].

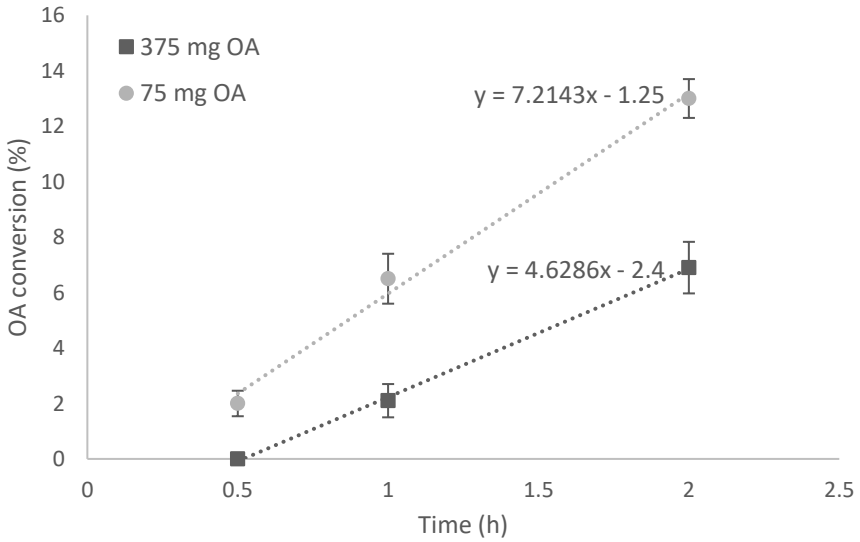


Figure 4. Initial OA conversion in the synthesis of pentyl oleate catalyzed by 50CAT when two different iOA concentrations are used (T: 25 °C, MR: 1).

3.1.3. Substrate Molar Ratio Effect

In this work, diverse molar ratios of pentyl alcohol and oleic acid were explored varying from 1:3 to 3:1 and its influence on ester synthesis was investigated at 25 °C (Figure 5).

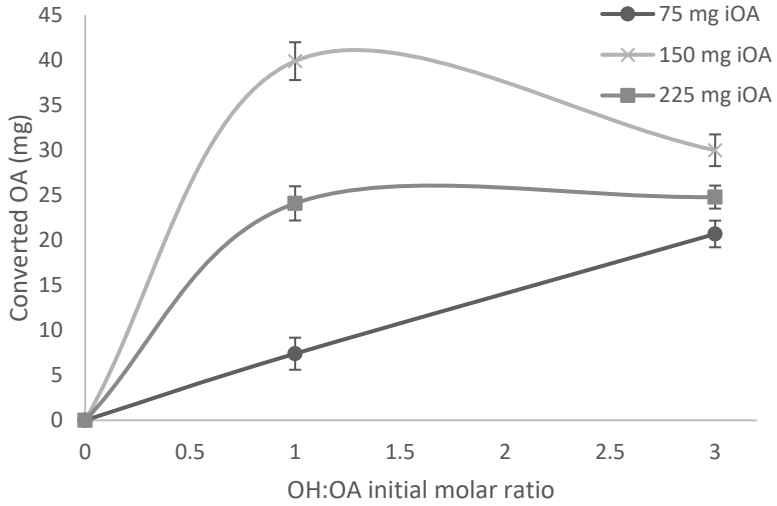


Figure 5. Effect of the 1-pentanol:OA initial molar ratio on the synthesis of pentyl oleate catalyzed by 50CAT (reaction time: 6 h, T: 25 °C).

Experimental results in Figure 5 show that when the initial amount of OA is low (75 mg), an increment in alcohol concentration produces an increase in conversion. Using 150 mg iOA, maximum conversion of 39.9% was obtained at 1:1 molar ratio of 1-penthanol to oleic acid. A higher alcohol concentration (alcohol to acid molar ratio 3:1) reduced the final conversion to 30%, probably due to the blocking of the active site of lipase with alcohol and leading to enzyme inhibition. This effect was also seen in entries 11 and 12 of Table 1, where an increase in alcohol initial concentration, while other variables remained constant, led to a smaller OA conversion.

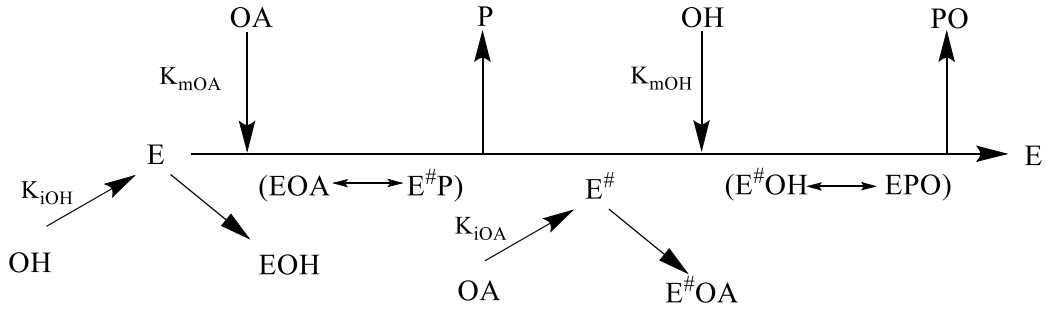
When the amount of iOA increases up to 225 mg, the acid inhibitory effect becomes more evident and alcohol concentration seems to have no effect on ester formation. Therefore, molar ratio 1:1 was chosen as the most appropriate to maximize the ester yield. It is important to highlight that both OA and 1-penthanol increase in Figure 5 when going from 75 mg to 225 mg.

Many reports on alcohol inhibition during lipase catalyzed esterification reactions are available. The presence of an excess of ethanol has been shown to drastically affect the yield of ethyl butyrate [27]. Duan et al. reported the inhibition of *Candida antarctica* lipase by propanol [28]. Methanol has also shown to exert inhibitory effects on the synthesis of dimethyl adipate and lauryl alcohol on lauryl laurate synthesis [29], among others.

While most studies have reported inhibition by alcohol or by acid, only few works have considered inhibition by both substrates as we found in this paper. This effect has been found in reactions among butyric, isovaleric and lactic acid with ethanol [21,30–32], octanoic acid and hexanol [33], and using oleic acid with different alcohols such as methyl, butyryl, furfuryl, and oleyl alcohols [4,34,35].

It is important to highlight that the main goal of this work was not to make a kinetic study. Instead, we aim to find a mathematical model for the prediction of optimal experimental conditions for biocatalyst potential industrial applications. For this reason, we did not obtain kinetic parameters. In this particular case, a heterogeneous system was presented, and substrate molar ratios were used instead of concentrations, which would be difficult for the Michaelis-Menten kinetics application. In addition, Michaelis-Menten does not take enzyme aggregation into consideration, an important effect when dealing with water-soluble enzymes.

In this study, the experimental data was explained by applying a ping-pong bi-bi kinetic model with inhibition by both substrates (Scheme 1). We emphasize that this is not a kinetic study, but an approach to explain the results looking at the mechanistic details to find useful mathematical correlations for practical application.



Scheme 1. General schematic representation of the ping-pong bi-bi mechanism with inhibition of oleic acid and 1-pentanol (E: CALB, OA: oleic acid, P: water, OH: 1-pentanol, PO: pentyl oleate).

The steps of the proposed mechanism considered are: First, the oleic acid [OA] binds to the CALB surface [E] forming the oleic acid–lipase complex [EOA]. This complex then isomerizes to form the acyl–enzyme intermediate [E#] with the release of a water [P] molecule. Then, the 1-penthanol [OH] reacts to the acyl–lipase binary complex to form a second complex [E#OH], which later forms the enzyme–pentyl oleate complex [EPO]. Ultimately, the [EPO] complex produces pentyl oleate [PO] and free lipase [E]. When the enzyme reacts with oleic acid and 1-penthanol respectively, [E#OA] and [EPO] complexes are formed and a dead-end inhibition occurs [36].

4. Conclusions

A comprehensive study to optimize the reaction conditions for the synthesis of pentyl oleate using CALB immobilized onto polyethylene-aluminum support was achieved using RSM. Experimental data was related and explained using an expression of the ping-pong bi-bi mechanistic model, with inhibitory effects of both oleic acid and 1-penthanol substrates. The model generated by the experimental design was statistically significant and allowed us to find the best conditions to maximize pentyl oleate

yield. The statistical analysis showed, within the considered experimental range, an increase in ester production with increasing temperature and a decrease with higher oleic acid amounts and alcohol to acid molar ratios. The optimized values of the parameters, namely initial OA concentration, reaction temperature, and substrates initial molar ratio, were: 150 mg, 65 °C, and 1, respectively. These results may provide important tools for the industrial utilization of CALB/LLDPE/Al as a biocatalyst in the economical and efficient synthesis of pentyl oleate and other similar esters from by-products of the oleochemical industry. Furthermore, this work represents a starting point in the way of process intensification using this biocatalyst in a continuous monolithic tubular reactor.

Author Contributions: G.T. and M.L.F. planned the experiments, V.C. performed the experiments and wrote the manuscript, V.C., G.T. and M.L.F. analyze the data, G.T. and M.L.F. revised the manuscript and acquire the funds for the research.

Funding: This research was funded by the National Agency of Scientific and Technological Promotion, Argentina (ANPCYT—PICT 2015-932/1583 and PICT CABBIO 2016-4687) and the Universidad Nacional del Sur (UNS—PGI 24/M141).

Acknowledgments: The authors would like to thank Universidad Nacional del Sur (UNS), the National Council for Scientific and Technological Research (CONICET) and the National Agency of Scientific and Technological Promotion (ANPCYT) from Argentina. V.C. is postdoctoral fellow of CONICET. G.T. and M.L.F. belong to the CONICET Researcher Career.

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

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