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# Comparison of the performance of commercial immobilized lipases in the synthesis of different flavor esters

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ABSTRACT

In this work, it is compared the performance of three commercial lipase preparations (Novozym 435, Lipozyme TL-IM, and Lipozyme RM-IM) in the synthesis of flavor esters obtained by esterification of acetic, propionic, and butyric acids using ethanol, isopropyl alcohol, butanol, or pentanol. A comprehensive comparison was performed verifying activities of these three enzyme preparations versus the different couples of substrates, checking the obtained yields. In general, the longer the acid chain, the higher the reaction yields. Novozym 435 was the most efficient enzyme in most cases, and only Lipozyme RM-IM offered better results than Novozym 435 in the production of ethyl butyrate. Reactions with butyric acid showed the highest conversion rates using all biocatalysts. Using optimal substrates, the reactions catalyzed by the three enzymes were optimized using the response surface methodology, and the catalytic performance of the biocatalysts in repeated batches was assessed. After optimization, yields higher than 90% were obtained for all three enzymes, but Lipozyme TL-IM needed four-times more biocatalyst content than the other two preparations. Novozym 435 kept over 80% of its activity when reused in 9 successive batches, whereas Lipozyme RM-IM can be reused 5 times and Lipozyme TL-IM only 3 times. In general, Novozym 435 showed to be more suitable for these reactions than the other two enzyme preparations.


## 1. Introduction

Many commercially important fruity notes are esters formed by short chain alcohols and carboxylic acids [1]. The extraction of these notes from natural products is expensive and, as a consequence, their chemical synthesis has become the standard industrial practice, i.e., the reaction using inorganic catalysts and elevated temperatures (200–250 °C) [2], and the obtained esters are classified as "artificial flavors". In contrast, the enzymatic syntheses of flavor esters catalyzed by lipases allow their classification as "natural flavors", with immediate economical advantages [3]. Lipases are well-studied enzymes, showing hydrolytic activities, but also catalyzing reactions of esterification, transesterification, and alcoholysis [3–6]. Several researches reported on esterifications catalyzed by lipases, especially those using a short chain alcohol and a long chain fatty acid [2,7–11]. However, reactions using short carboxylic acids have been the subject of fewer studies [12]. The enzymatic syntheses of flavor esters require very low water contents in order to shift the thermodynamic equilibrium toward synthesis, thus non-aqueous media are required. Solvent-free systems [13,14], ionic liquids [15,16] or supercritical fluids [17] have been used as media for lipase catalyzed reactions. However, still most of the examples that can be found in the literature use the most traditional organic solvents [13,16,18].

Although this is a very common reaction, and much effort has been expended to find an ideal biocatalyst, a comparison of the activity of the most used lipases has not been reported to date. This may be interesting to understand the behavior and specificities on the synthesis of flavor esters, and, for first time, this has been performed in this manuscript. Thus, the main objective of this work was to evaluate the performance of three of the most used commercially available biocatalysts (Novozym 435; Lipozyme TL-IM; and Lipozyme RM-IM) for the synthesis of esters of different short chain acids (acetic, propionic, and butyric acids), and

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alcohols (ethanol, butanol, and pentanol) on organic solvents to comprehend the enzymes activities and specificities against these substrates. A secondary alcohol (2-propanol) has also been tested in order to allow us a better understanding of the enzymes specificities. The resulting esters are among the most interesting fruit flavors, such as pineapple, banana, peach, apricot, mango, among others.

Novozym 435 is possibly the most used biocatalyst [19]. This immobilized preparation of lipase B from *Candida antarctica* (CALB) is obtained by immobilization via interfacial activation of the enzyme on a moderately hydrophobic resin, Lewatit VP OC 1600 [20].

The second used immobilized lipase biocatalyst was that from *Thermomyces lanuginosus* (TLL). It is immobilized on a cationic silicate via anion exchange (Lipozyme TL IM®) [21]. This enzyme has been used in multiple reactions [22] and is produced by a genetically modified strain of *Aspergillus oryzae* [23]. Its structure is also well known and the enzyme has a large lid, able to fully seclude the active center from the reaction medium in the closed form [24].

The third biocatalyst used in this study is the immobilized lipase from *Rhizomucor miehei* (RML), commercially available from Novozymes as immobilized form (Lipozyme RM-IM). The support of this immobilized enzyme is Duolite ES 562, a weak anion-exchange resin based on phenol–formaldehyde copolymers [25]. The enzyme has been used on many processes [26,27] and its structure shows a typical lid that isolates the active center of the enzyme from the medium when closed [28]. Therefore, these three biocatalysts differ not only about the enzyme source, but also in the nature of the matrix and on the mechanism of immobilization of the enzyme and significant differences on enzyme activity, specificity, and stability may be expected [29].

We also studied the enzyme performance on some of the esterification reactions using the response surface methodology, and tested the biocatalysts activities during reuse in several batches.

## 2. Materials and methods

### 2.1. Chemicals

The enzymes used in this work were *Candida antarctica* lipase B immobilized in a macroporous resin (Novozym 435); *T. lanuginosus* lipase immobilized in a silicate support (Lipozyme TL-IM); and *R. miehei* lipase immobilized in an anion-exchange resin (Lipozyme RM-IM), all of them kindly donated by Novozymes (Spain). Acetic, propionic, and butyric acids; ethanol, 2-propanol, 1-butanol, 1-pentanol, and other chemicals were of analytical grade and purchased from Sigma-Aldrich (Sigma, St. Louis, USA).

### 2.2. Esterification reactions

#### 2.2.1. Screening experiments

Esterification reactions were carried out using 0.1 M of each different acid and alcohol (1:1 alcohol:acid molar ratio) in the presence of *n*-hexane as solvent into 50 mL Erlenmeyer flasks (working volume of 10 mL), under agitation in an orbital shaker (200 rpm) for 2 h. Reaction temperature and the amount of each biocatalyst were set according to previous studies [30–32]: 10% (mass fraction of substrate) for Novozym 435 and Lipozyme RM-IM; 30% for Lipozyme TL-IM; 40 °C for Novozym 435 and Lipozyme RM-IM; and 50 °C for Lipozyme TL-IM.

#### 2.2.2. Reaction optimization

From the preliminary screening, one combination of acid and alcohol was chosen for each enzyme. The optimization was performed by central composite design (CCD) and response surface methodology (RSM). The variables studied on the CCD were reaction temperature (30–60 °C), substrate molar ratio (1:1–5:1 alcohol:acid molar ratio), amount of added water (0–1%) and biocatalyst content (% considering in the calculations the mass of both substrates in stoichiometric ratio). The range for biocatalyst content was different for each enzyme according to their activity: 1–10% for Novozym 435 and Lipozyme RM-IM; 5–45% for Lipozyme TL-IM. Reactions were carried out by mixing 0.1 M of acid with different concentrations of alcohol into 50 mL Erlenmeyer flasks (working volume of 10 mL), followed by the addition of various amounts of water, immobilized enzyme (dried as described above), and *n*-hexane as solvent. The mixtures of acids, alcohol, and enzymes were incubated in an orbital shaker (200 rpm) at various reaction temperatures for 2 h for Novozym 435 and Lipozyme RM-IM, and 5 h for Lipozyme TL-IM. The conditions for each reaction were determined by the CCD, which is presented in Table 1.

**Table 1**
Experimental design and results of CCD.

| Run | *X*1 | *X*2 | *X*3 | *X*4 | Novozym 435 (%) | Lipozyme RM-IL (%) | Lipozyme TL-IM (%) |
|---|---|---|---|---|---|---|---|
| 1 | −1 | −1 | −1 | −1 | 25.67 | 30.17 | 43.24 |
| 2 | −1 | −1 | −1 | 1 | 25.19 | 23.48 | 48.38 |
| 3 | −1 | −1 | 1 | −1 | 54.43 | 60.00 | 77.62 |
| 4 | −1 | −1 | 1 | 1 | 59.71 | 62.18 | 81.71 |
| 5 | −1 | 1 | −1 | −1 | 11.51 | 29.17 | 13.14 |
| 6 | −1 | 1 | −1 | 1 | 19.94 | 33.87 | 12.10 |
| 7 | −1 | 1 | 1 | −1 | 53.81 | 51.45 | 44.67 |
| 8 | −1 | 1 | 1 | 1 | 47.58 | 42.06 | 30.29 |
| 9 | 1 | −1 | −1 | −1 | 32.09 | 48.75 | 0.48 |
| 10 | 1 | −1 | −1 | 1 | 50.17 | 53.49 | 24.06 |
| 11 | 1 | −1 | 1 | −1 | 78.17 | 72.29 | 17.14 |
| 12 | 1 | −1 | 1 | 1 | 75.37 | 67.47 | 24.48 |
| 13 | 1 | 1 | −1 | −1 | 43.58 | 29.76 | 28.86 |
| 14 | 1 | 1 | −1 | 1 | 45.13 | 39.32 | 4.19 |
| 15 | 1 | 1 | 1 | −1 | 69.64 | 78.47 | 45.62 |
| 16 | 1 | 1 | 1 | 1 | 67.94 | 74.62 | 37.62 |
| 17 | −2 | 0 | 0 | 0 | 14.92 | 35.92 | 42.52 |
| 18 | 2 | 0 | 0 | 0 | 59.68 | 1.25 | 18.19 |
| 19 | 0 | −2 | 0 | 0 | 45.57 | 44.35 | 68.86 |
| 20 | 0 | 2 | 0 | 0 | 52.47 | 40.17 | 0.76 |
| 21 | 0 | 0 | −2 | 0 | 25.32 | 16.13 | 3.71 |
| 22 | 0 | 0 | 2 | 0 | 73.23 | 81.25 | 87.90 |
| 23 | 0 | 0 | 0 | −2 | 49.57 | 62.77 | 55.52 |
| 24 | 0 | 0 | 0 | 2 | 38.09 | 53.97 | 46.86 |
| 25 | 0 | 0 | 0 | 0 | 48.60 | 43.86 | 60.10 |
| 26 | 0 | 0 | 0 | 0 | 36.44 | 46.67 | 55.00 |
| 27 | 0 | 0 | 0 | 0 | 47.94 | 55.29 | 58.76 |
| 28 | 0 | 0 | 0 | 0 | 44.13 | 48.62 | 48.38 |

*X*1: temperature; *X*2: substrate molar ratio; *X*3: biocatalyst content; *X*4: added water.

### 2.3. Hydrolytic activity

The hydrolytic activity of the immobilized enzymes was measured according to methodology previously developed [33]. A volume of 5 mmol of soybean oil was added into 50 mL Erlenmeyer flasks, followed by addition of 60 mmol of water (12:1 water:oil molar ratio), and 10% of biocatalyst (by oil mass). The mixtures of soybean oil, water, and lipases were stirred in an orbital shaker (200 rpm) for 1 h at 40 °C. The progress of hydrolysis was monitored by determination of the free fatty acid released by titration of 0.3 g samples using 0.01 M NaOH using phenolphthalein as pH indicator and 5 mL of ethanol as quenching agent. One unit (U) was defined as the amount of enzyme that releases 1 μmol of fatty acid per minute at the experimental conditions.

### 2.4. Reaction analysis

The progress of esterification was monitored following the determination of the residual acid content by titration of 0.5 mL

samples using 0.01 N NaOH, phenolphthalein as pH indicator, and 5 mL of ethanol as quenching agent. The amount of ester was calculated as being equivalent to the amount of acid consumed. The molar conversion was determined from the values obtained for the blank and the test samples. The accuracy of this method was also tested by determination of ester concentration on gas chromatograph (SHIMADZU, GC-2010 plus), equipped with a flame ionization detector (FID) and an AT.FFAP column (30 m × 0.32 mm × 0.25 μm). The carrier gas was nitrogen. The temperatures of the injector and detector were both set to 250 °C, and the split ratio was 1:10. The oven temperature program was: start at 60 °C, 10 °C min$^{-1}$ to 90 °C, 30 °C min$^{-1}$ to 240 °C, and then was held at 240 °C for 2.5 min.

### 2.5. SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [34] using a SE 250-Mighty small II electrophoretic unit (Hoefer Co.); 15% (concentration fraction) running gel in a separation zone of 9 × 6 cm, and a stacking gel of 5% (concentration fraction) polyacrylamide. Samples of 100 mg of the commercial preparations of the immobilized enzyme were re-suspended in 1 mL of rupture SDS-buffer 2% (mass fraction), 10% (volume fraction) mercaptoethanol, and 1 M NaCl, boiled for 5 min. A volume of 20 μL aliquots of the supernatant was loaded onto gels. After running, gels were stained with Coomassie brilliant blue. Low molecular weight markers (GE Healthcare) were used as reference (14,000–97,000 Da).

### 2.6. Statistical analysis

The conversions obtained in the esterification reactions were calculated. CCD and analysis of results were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed as analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, $p(t)$, were determined by Student's $t$-test; the second order model equation significance was determined by Fisher's $F$-test. The variance explained by model was given by the multiple determination coefficients, $R^2$. For each variable, the quadratic models were represented as contour plots (2D). The second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

where $Y$ is the response variable, $\beta_0$ the constant, $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and $X_i$ and $X_j$ the coded level of variables $x_i$ and $x_j$. The above quadratic equation was used to plot surfaces for all variables.

### 2.7. Enzyme reuse

After the esterification reaction, the immobilized enzyme was separated from the reaction medium by vacuum filtration using a sintered glass funnel. In some cases, in order to remove any adsorbed substance from the support, the recovered biocatalysts were washed with 10 volumes of $n$-hexane, dried for 24 h at 25 °C and reused in a new fresh reaction [32]. Hexane is highly volatile, providing a dry biocatalyst after 24 h. In other cases, the reuse was performed after vacuum filtration without further treatments.

## 3. Results and discussion

### 3.1. Screening of alcohols and acids

Fig. 1 shows the behavior of the three commercial immobilized enzymes Novozym 435, Lipozyme RM-IM, and Lipozyme TL-IM in the esterification of three short chain acids (acetic, propionic and butyric), and the four selected alcohols (ethanol, 2-propanol, 1-butanol, and 1-pentanol). Novozym 435 showed the highest activity when used with most of the substrates, except for butyric acid (Fig. 1c), which produced good and similar conversions with all lipases. In the production of ethyl butyrate, Lipozyme RM-IM offered better results than Novozym 435. Lipozyme TL-IM was the biocatalyst generally showing the lowest yields. The only secondary alcohol assayed, 2-propanol, showed to be the poorest substrate for esterification, independent of the acids and enzymes used, except for the combination Lipozyme TL-IM and acetic acid, when this enzyme presented highest activity with 2-propanol (Fig. 1a). The selectivity of Novozym 435 versus different fatty acids (C4–C16) and propanol or 2-propanol was studied by Arsan and Parkin [35]. The authors observed that Novozym 435 presented an initial reaction rate higher with propanol than 2-propanol. Moreover, using hexanoic acid, Novozym 435 showed lower activity with other secondary alcohols such as 2-butanol, phenol, cyclohexanol, and (−)menthol [35]. Although lipases can be reactive versus secondary alcohols [36] and secondary esters [37], their reaction rates with primary alcohols and esters are markedly faster.

As a general trend, it can be established that the longer the acid chain, the higher the yield. This may be exemplified by Novozym 435 as biocatalyst and pentanol as alcohol, producing conversions of 56, 72, and 83% for acetic, propionic, and butyric acids, respectively. Using Lipozyme RM-IM, regardless the alcohol used, increasing the acid chain, the yield was improved. When using Novozym 435 and Lipozyme TL-IM as biocatalysts, it produced lower yields using propionic acid, except when using pentanol. Although the observed yield using propionic acid was lower than those using the other two acids for Novozym 435, with butanol and pentanol it was reached yields near to 70% in 2 h. Kuperkar et al. [38] under their optimal conditions obtained 90% in the synthesis of isobutyl propionate catalyzed by Novozym 435 in solvent-free system after 10 h.

In general, concerning the effect of the alcohol used in the reaction, pentanol, and especially butanol, showed to be the best substrates for most enzymes and acids combinations. Ethanol also produced good conversions, especially in combination with butyric acid. Moreover, using acetic acid as substrate and Novozym 435 as biocatalyst, ethanol showed to be a good acyl acceptor.

Results shown in Fig. 1 demonstrate the different behaviors of these three commercial biocatalysts during esterification, with significant interactions between the used acyl donors and the nucleophiles, strongly affecting the enzymes activities.

It is worthwhile noting that these reactions were carried out using fixed conditions (see Section 2.2.1), with further influences in the reaction expected caused by the immobilization protocol used [31,39], and therefore these results must be taken as an indicative trend.

### 3.2. SDS-PAGE of protein loading of the different biocatalysts

Fig. 2 shows the result of SDS-PAGE, which measured (semi-quantitatively) the amount of lipase immobilized in each support. This experiment helps to better understand the relation between the biocatalysts activities and enzyme load. The gel shows that Lipozyme TL-IM has the highest protein load, followed by Novozym 435, and Lipozyme RM-IM, with the lowest load. Because Lipozyme TL-IM produced the lowest esterification activities (Fig. 1) among

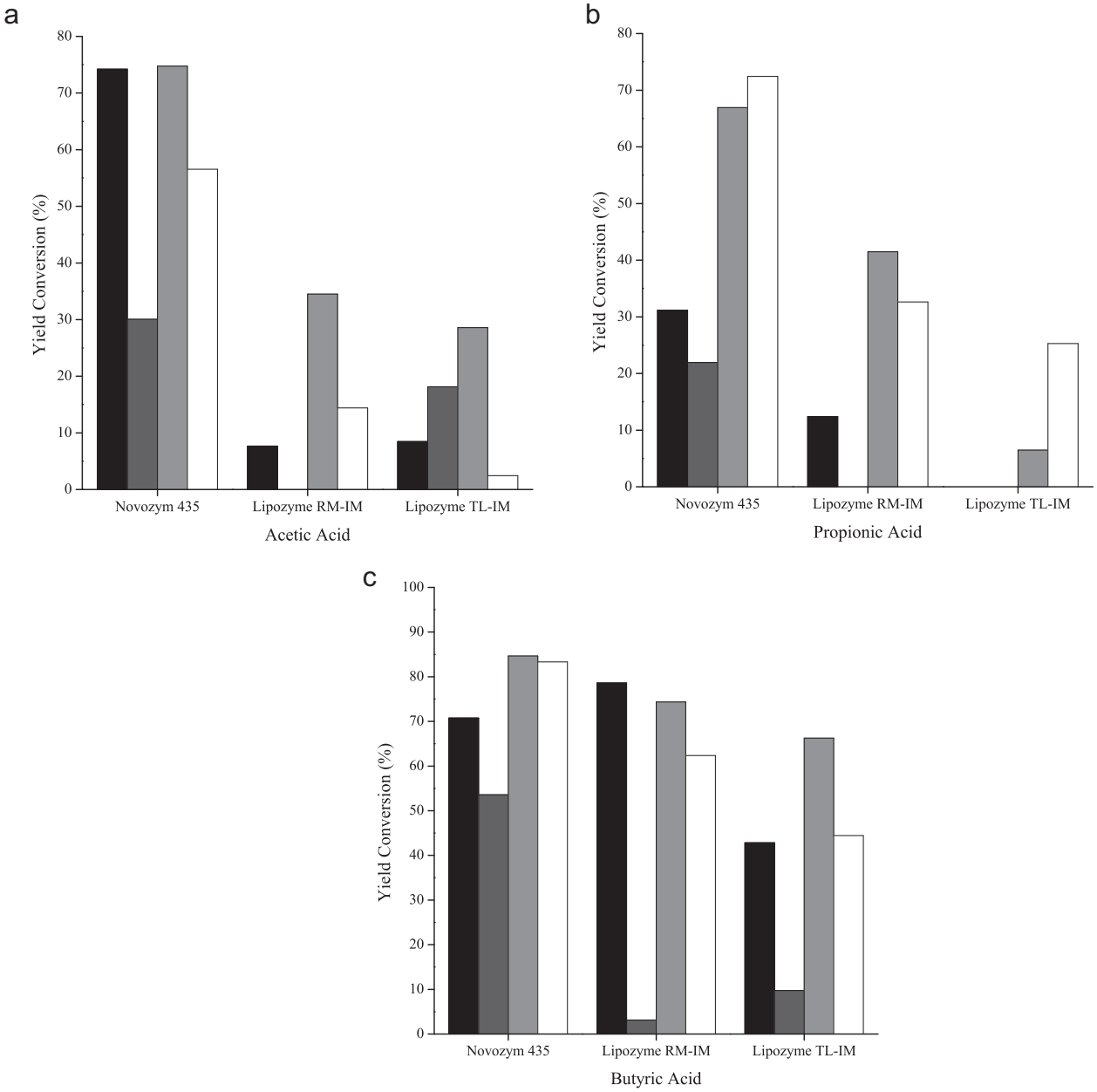


**Fig. 1.** Esterification of short chain acids catalyzed by the three lipases. (a) Acetic acid; (b) propionic acid; (c) butyric acid. Bars: black, ethanol; dark gray, 2-propanol; light gray, butanol; white, pentanol.

all three preparations, its high protein load suggests a significant lower specific activity for these reactions.

Nevertheless, when we compared the hydrolytic activity, the Lipozyme TL-IM showed the highest activity (16.6 U $g^{-1}$ of biocatalyst), followed by Lipozyme RM-IM (13.6 U $g^{-1}$ of biocatalyst) and Novozym 435 (3.6 U $g^{-1}$ of biocatalyst). It is important to note that the best biocatalyst in the esterification reaction is the worst in hydrolysis, confirming the complexity of lipase catalyzed reactions. Novozym 435 is 4-times more active than Lipozyme TL-IM in synthesis, and Lipozyme TL-IM was 5-times more active than Novozym 435 in hydrolysis. Lipozyme RM-IM showed good activity in both reactions.

Several aspects might influence the activities of the biocatalysts. Enzyme sources (microorganism) should naturally be expected to show structural differences, having strong influence on the biocatalysts properties and activities, even in similar reaction systems [40,41]. The nature of the immobilization support has also been reported to alter the properties of the same lipase in one specific reaction [42], as it has been shown for esterification reactions [39,43]. The nature of the support can affect the enzyme conformation, as well as the partition of substrates and products from the enzyme environment, which might difficult the access of the substrate to the enzyme active site. The immobilization protocol is another important aspect that has been shown to alter enzyme properties even when immobilized on the same support [44,45]. Finally, the reaction pH and water content will influence enzyme properties and reaction rates.

The nature of the immobilization supports of the three biocatalysts used in this work was different. Lewatit, which was used to immobilize CALB to produce Novozym 435, has a fairly hydrophobic surface [46,47], while a very hydrophilic silicate was used to immobilize TLL to produce Lipozyme TL-IM [21] and Duolite ES 562, a weak anion-exchange resin based on phenol–formaldehyde copolymers was used to immobilized RML [25]. Concerning the

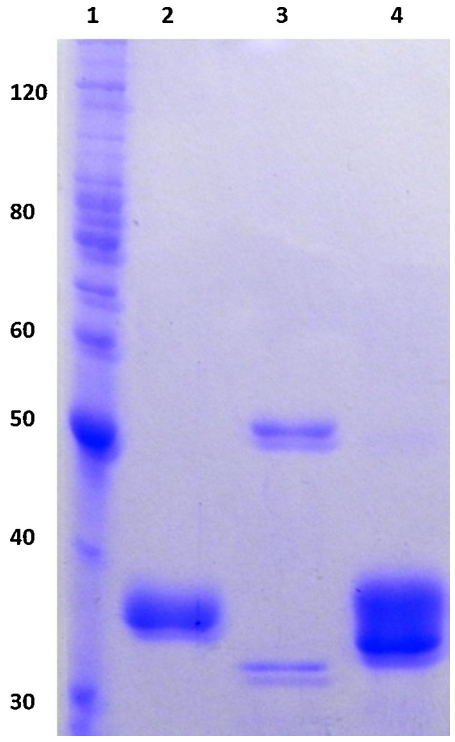


**Fig. 2.** SDS-PAGE of the immobilized lipases. Lane 1: molecular weight markers (kDa); lane 2: Novozym 435; lane 3: Lipozyme RM-IM; lane 4: Lipozyme TL-IM.

immobilization protocols, Novozym 435 is prepared via interfacial activation of the enzyme on the hydrophobic surface of the support [46,47], whereas the other two enzymes are immobilized via anionic exchange [21,25]. All these differences of the biocatalysts make more complex the understanding of the interactions of acids and alcohols on the enzymes activities.

Based on the results presented in Figs. 1 and 2, and considering all discussed complexities that influence enzyme reactions, it was chosen one combination of acid and alcohol for each immobilized enzyme in order to perform the optimization of the reaction parameters using RSM.

### 3.3. Experimental design for flavor esters syntheses

The esters chosen for each enzyme were: pentyl acetate (banana and apple flavors) for Novozym 435; pentyl butyrate (pear and apricot flavors) for Lipozyme RM-IM; and ethyl butyrate (pineapple and mango flavors) for Lipozyme TL-IM. These combinations were chosen because they present some room to improvement and due to their importance as flavor for food industry. Reaction temperature, substrate molar ratio, biocatalyst content and added water were evaluated and the results for flavors esters synthesis are presented in Table 1. The reaction times considered for analyses were 2 h for Novozym 435 and Lipozyme RM-IM, and 5 h for Lipozyme TL-IM, which were chosen in order to obtain relative high yields, but not as high as to make it difficult to detect improvements in activity when changing reaction conditions. The range of the variables studied in the CCD were the same, the only exception being the biocatalyst content for Lipozyme TL-IM that, due to its low activity, was varied from 5 to 45%, while for the other 2 biocatalysts it was from 1 to 10%. The highest yields obtained were around 80% for each enzyme (Table 1). Conversions varied widely, with yields lower than 10% to higher than 80%, clearly showing the importance of reaction optimization.

In order to check the models fitness, Fisher's statistical test for analysis of variance (ANOVA) was performed for the 3 reactions,

**Table 2**
Linear effects of the variables in the reactions catalyzed by the three enzymes used in this work.

| Variable | | Novozym 435 | Lipozyme RM-IM | Lipozyme TL-IM |
|---|---|---|---|---|
| $X1$ | Temperature | 21.14* | 5.07 | −18.03* |
| $X2$ | Substrate molar ratio | −2.32 | −3.95 | −19.73* |
| $X3$ | Biocatalyst content | 29.09* | 29.23* | 29.42* |
| $X4$ | Added water | −0.06 | −1.76 | −2.11 |

* Statistically significant at 95% of confidence level.

presenting $F$-values of 13.06, 2.63, and 6.77 for reactions catalyzed by Novozym 435, Lipozyme RM-IM, and Lipozyme TL-IM, respectively, all being statistically significant ($p$-values: <0.0001, 0.0450, and 0.0007, respectively). The determination coefficients ($R^2$) and the correlation coefficients ($R$) were higher than 0.8 for all models, meaning that more than 80% of the variation for the syntheses are attributed to the independent variables, and could be explained by their respective models, while the high correlation coefficients suggest a satisfactory representation of the process models and good correlations between experimental results and theoretical values predicted by the models equations. Therefore, the second-order polynomial models are given by

$$\begin{aligned} Y1 = {} & 44.27 + 10.57X1 - 1.30X1X1 - 1.16X2 + 1.62X2X2 \\ & + 14.54X3 + 1.69X3X3 - 0.03X4 + 0.32X4X4 \\ & + 1.41X1X2 - 0.81X1X3 - 0.98X1X4 + 0.50X2X3 \\ & - 1.12X2X4 - 2.06X3X4 \end{aligned} \quad (2)$$

$$\begin{aligned} Y2 = {} & 48.60 + 2.53X1 - 6.28X1X1 - 1.97X2 - 0.27X2X2 \\ & + 14.61X3 + 1.33X3X3 - 0.88X4 + 3.75X4X4 \\ & - 0.03X1X2 + 1.40X1X3 + 0.52X1X4 + 0.92X2X3 \\ & + 0.350X2X4 - 1.76X3X4 \end{aligned} \quad (3)$$

$$\begin{aligned} Y3 = {} & 55.55 - 9.01X1 - 7.55X1X1 - 9.86X2 - 6.37X2X2 \\ & + 14.71X3 - 3.62X3X3 - 1.05X4 - 2.28X4X4 \\ & + 12.55X1X2 - 3.13X1X3 + 0.94X1X4 + 0.27X2X3 \\ & - 5.51X2X4 - 0.86X3X4 \end{aligned} \quad (4)$$

where $Y1$, $Y2$, and $Y3$ are the percentage yields for pentyl acetate catalyzed by Novozym 435, pentyl butyrate catalyzed by Lipozyme RM-IM, and ethyl butyrate catalyzed by Lipozyme TL-IM, respectively, and $X1$, $X2$, $X3$, and $X4$ are the coded values of temperature, substrate molar ratio, enzyme content, and added water, respectively.

### 3.4. Effects of process variables

The relation between the process variables can be observed in the contour plots of Fig. 3. For each enzyme, it was plotted the curves for two variables according to their linear effects as presented in Table 2: biocatalyst content and temperature for Novozym 435 and Lipozyme RM-IM (Fig. 3a and b), and substrate molar ratio (alcohol:acid) and temperature for Lipozyme TL-IM (Fig. 3c). Positive values mean that the response is improved when increasing the variable from level (−1) to level (1), and inversely for negative values. The response in this case is the reaction yield. It can be seen in Table 2, that the content of the biocatalyst was the variable presenting the highest reaction effect and that, independently of the enzyme and acid/alcohol combination, the effect

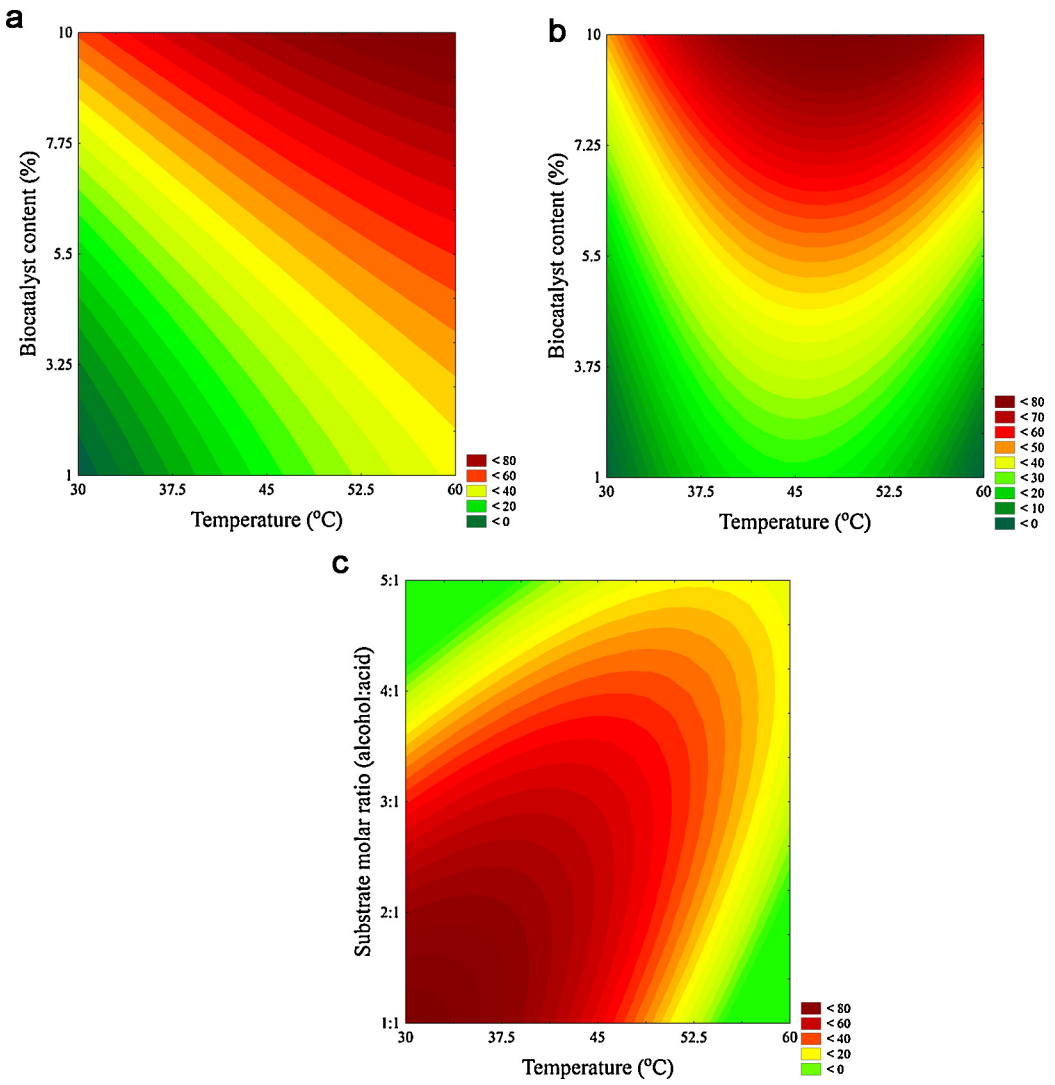


**Fig. 3.** Contour plots for esterification reactions catalyzed by the three biocatalysts used in this work. (a) Pentyl acetate, Novozym 435; (b) pentyl butyrate, Lipozyme RM-IM; (c) ethyl butyrate, Lipozyme TL-IM.

of biocatalyst content was very similar. This high and positive effect was expected because the amount of enzyme should directly relate to the reaction rate. More complex and interesting results were observed for the effects of temperature and substrate molar ratio on the yields of reactions. It is expected that temperature, should positively affect reaction rate up to a point where enzyme inactivates, which was observed for Novozym 435 and Lipozyme RM-IM. Observing the contour plots of Fig. 3a, increasing the reaction temperature and the biocatalyst content, the reaction yield was improved, which was expected because both biocatalyst content and temperature should increase reaction rate. Concerning Lipozyme RM-IM (Fig. 3b), the temperature had a positive effect around the central point, meaning that low and high temperatures will negatively affect the enzyme activity.

Although biocatalyst content presented the highest effect for Lipozyme TL-IM, temperature and substrate molar ratio showed important effects on the reaction. Somewhat surprisingly, temperature showed a significant and negative effect on the reaction catalyzed by Lipozyme TL-IM, since the enzyme in this biocatalyst is known to be thermophilic [22]. One possible explanation could be the combination effect of substrate molar ratio and temperature. This interaction effect was highly significant when using Lipozyme TL-IM ($X1X2 = 25.11$, $p = 0.0024$), and can be best understood analyzing data presented in Table 1 and Fig. 3c. At high substrate molar ratios, low yields were obtained, independent of the temperature, but fixing the substrate molar ratio at the lower level and increasing the temperature, the yield increased.

### 3.5. Optimal reaction conditions and model validation

The optimal conditions for the syntheses of the different esters using the three biocatalysts were determined by the response desirability profile, calculated using the Statistica 7.0 software, where the optimal values for each variable were obtained for the desired response, which in this work was the maximal yields after 2 h or 5 h of reaction, depending on the enzyme. The optimal conditions for each enzyme are shown in the Table 3.

In order to validate the prediction models, experiments were carried out at the optimal conditions and the time-courses are presented in Fig. 4. Novozym 435 was slightly faster in the synthesis of pentyl acetate than the other lipases in the synthesis of their respective esters. Moreover, it is important to remark that Lipozyme TL-IM needs four-times more biocatalyst in order to show similar reaction rates. The synthesis of ethyl butyrate was also studied by Guillén et al. [48], and the authors needed two-times more enzyme for the reaction catalyzed by *Rhizopus oryzae*, immobilized on Accurel

**Table 3**
Optimal conditions for the esterification reactions catalyzed by the three enzymes used in this work.

| Enzyme | Novozym 435 | Lipozyme RM-IM | Lipozyme TL-IM |
|---|---|---|---|
| Temperature (°C) | 50 | 47.5 | 32.5 |
| Substrate molar ratio[a] | 3:1 | 2.4:1 | 1.7:1 |
| Biocatalyst content[b] (%) | 7.75 | 10 | 40 |
| Water[b] (%) | 0.25 | 0.12 | 0.38 |

[a] (alcohol:acid).
[b] (% by mass of substrate).

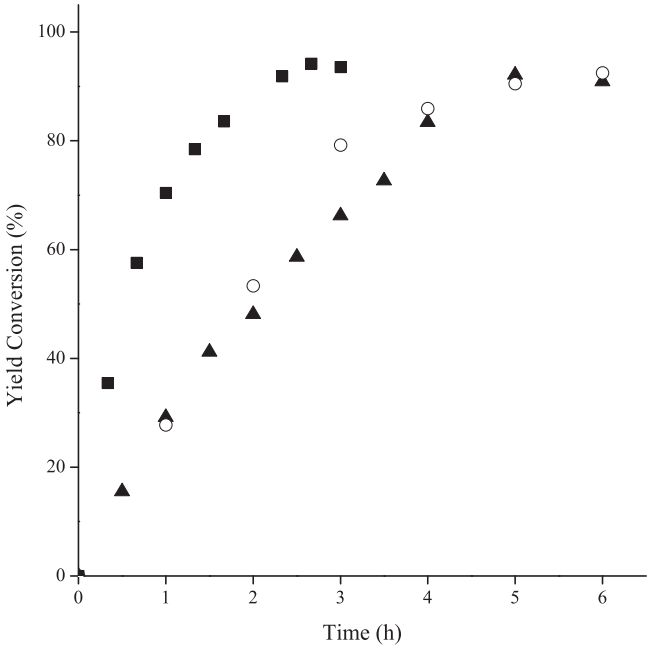


**Fig. 4.** Time course of esterification reactions under optimal conditions catalyzed by (■) Novozym 435, (▲) Lipozyme RM-IM and (◯) Lipozyme TL-IM.

EP100, compared to the results obtained using Lipozyme TL-IM in the present work. Moreover, the authors obtained 90% of conversion in 8 h [48]. Nevertheless, in our study for all 3 biocatalysts, yields over 90% were obtained in less than 6 h of reaction, and the results were better than others found in the literature. Milašinović et al. under their optimized conditions for the synthesis of isobutyrate catalyzed by *Candida rugosa* lipase obtained over 90% of ester yield after 48 h [6]. Our productivity was 10-fold higher (around 50 mmol $L^{-1}$ $h^{-1}$ against 5 mmol $L^{-1}$ $h^{-1}$ [6]). Using the lipase from *Staphylococcus simulans*, Ghamgui et al. achieved 64% of conversion after 8 h for the synthesis of isoamyl acetate in a solvent-free system [13]. In another study, de Paula et al. [40] tested seven lipases in the synthesis of butyl butyrate. The authors found that *C. rugosa* lipase was the most feasible to ester synthesis obtaining around 75% of conversion after 24 h.

### 3.6. Enzyme reuse

One of the most important properties of immobilized enzymes is the possibility of recovering and reusing them, which are important economic and environmental aspects and could define their future industrial applications. As previously described [32], washing the biocatalyst with *n*-hexane between reaction batches helps to remove any excess of substrates or products from inside the porous of the biocatalysts, which is the main cause for decreasing enzyme activity. Therefore, we decided to test the reuse of the three immobilized lipases, performing a wash with *n*-hexane between batches, and the results are presented in Fig. 5. Novozym 435 showed the best performance, being possible to use the biocatalyst for 10 successive batches retaining more than 80% of its initial activity. Similar stability was obtained by Kuperkar et al. [38], who observed that there were marginal changes in percentage conversion after 7 cycles of reuse using Novozym 435 in the synthesis of isobutyl propionate in solvent free system. Lipozyme TL-IM, however, was fully inactivated after five batches, while Lipozyme RM-IM was after seven batches. These differences could be explained by diverse enzymes properties, such as CALB being more stable than the other two lipases under the conditions of reactions. Another hypothesis is the difference in the immobilization protocol and nature of supports, as it has been pointed out before. Finally, the different substrates in each reaction might also have affected the enzyme stability, as they can have a lower or higher partition effect from the enzyme environment.

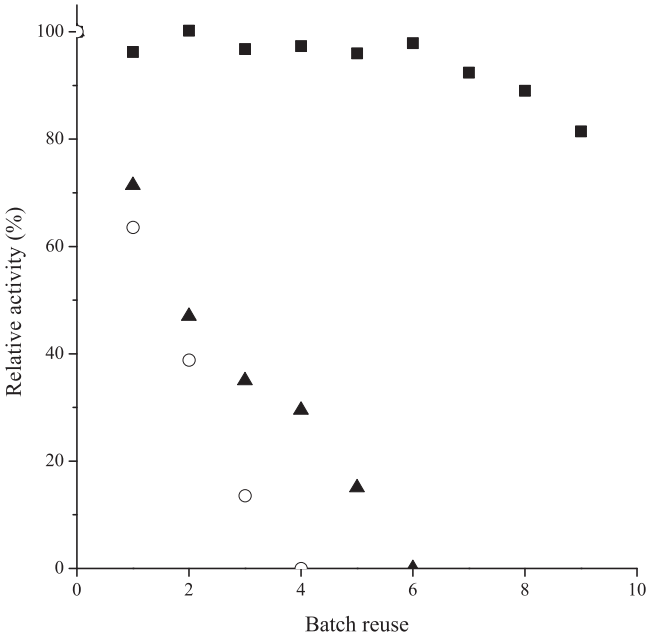


**Fig. 5.** Stability of the biocatalysts over repeated batches under optimal conditions. (■) Novozym 435, (▲) Lipozyme RM-IM and (◯) Lipozyme TL-IM.

## 4. Conclusion

A comprehensive comparison of three of the most commonly used commercial immobilized biocatalysts was performed as biocatalyst of esterification reactions, analyzing the effects of alcohol and acid natures in the reaction rate. The results suggested that Novozym 435 is a better biocatalyst than Lipozyme RM-IM and Lipozyme TL-IM. However, their performances were highly dependent on the alcohols and acids used, and Lipozyme RM-IM was found to be more active for the production of ethyl butyrate. Moreover, although Lipozyme TL-IM presents a higher protein load, it needs four-fold more mass of biocatalyst to give the same reaction rates than the other two biocatalysts. Nevertheless, after optimization, the 3 biocatalysts showed good activity for specific flavor esters, and in less than 6 h of reaction high yields could be obtained.

Results have shown that the enzymatic reactions are dependent upon several factors, varying from the more predictable, such as temperature, to the more complex ones such as substrates combination, and types of biocatalysts. It may be expected that using other immobilization protocols, the performance of all the enzymes may be tuned and the results presented in this paper may be modified.

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