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**Application of commercial and non-commercial immobilized lipases  
for biocatalytic production of ethyl lactate in organic solvents**

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## Abstract

This study explores the potential for enhancing the production of ethyl lactate (EL), a green solvent, through enzymatic esterification. Different solvents were compared as organic media for conversion of lactate and ethanol into EL, catalyzed by Novozym 435. Chloroform and hexane were the most effective in low acid concentrations (0.01-0.1 M) exhibiting maximum EL yields of 88% and 75% respectively. The yield of EL improved as the solvent's Log P increased up to a value of 2. Non-commercial immobilized biocatalysts consisting heterologous *Rhizopus oryzae* (rROL) and *Candida rugosa* (CRL) lipases immobilized on hydrophobic supports were compared to commercial biocatalysts clarifying that Novozym 435 and Lipozyme RM IM could be efficiently applied. Operational stability tests were conducted using Novozym 435, which retained higher activity in chloroform as compared to hexane. Although non-commercial biocatalysts were not competitive in esterification, they exhibited significant activity towards hydrolysis constituting a valuable alternative to higher-cost options.

**Keywords:** Ethyl lactate; Esterification; Lipase; Novozym 435; Operational stability

**Abbreviations:** Ethyl lactate (EL); heterologous *sn*-1,3-regioselective *Rhizopus oryzae* lipase (rROL); *Candida rugosa* lipase (CRL); Gas chromatography (GC); Free fatty acids (FFA); *Candida antarctica* lipase B (CALB).

## 1. Introduction

The replacement of conventional solvents with environmental friendly alternatives is one of the main pillars of Green Chemistry concentrating great technological and economic interest (Dandia et al., 2013). However, the industrial application of green solvents depends on the characteristics of the whole production cycle and it could be accelerated if green solvent properties were similar to those of traditional media, promoting industrial transition for these organic molecules (Aparicio and Alcalde, 2008). The esters of lactic acid with lower alcohols (especially methanol, ethanol and butanol) are considered as green solvents with excellent properties that could be used instead of toxic alternatives in various industrial applications attracting significant interest as commodity chemicals (Corma et al., 2007). EL constitutes the main member in this family of compounds aligned with major Green Chemistry principles, such as: i) production from renewable raw materials, ii) fully biodegradable, iii) recyclable, iv) non-corrosive, v) non-ozone depleting, vi) non-carcinogenic, vii) production via heterogenous catalysis avoiding excessive use of reactants, and viii) production through hybrid technologies which eliminate the use of solvents and involve lower capital cost and energy use (Pereira et al., 2011). Although the United States Environmental Protection Agency considers EL as a Class4A inert compound (Ahmadkalaei et al., 2016), the conventional production method involves the use of chemical catalysts for esterification, integrating energy intensive distillation steps and high investment costs in a range of reaction and separation units (Pereira and Rodrigues, 2014). However, EL can be produced from cellulosic feedstocks (Yang et al., 2016) in processes requiring lower energy consumption, such as membrane bioreactors (Nigiz and Hilmioglou, 2017,

2016a) and synthesis through lipase catalyzed esterification (Hasegawa et al., 2008a, 2008b; Jain and Mishra, 2015).

Lipases present numerous applications in the food, fuel and pharmaceutical sectors as biocatalysts, holding the capacity to catalyze diverse reactions including esterification, interesterification, transesterification, alcoholysis, acidolysis and aminolysis (Stergiou et al., 2013). Nevertheless, the majority of the aforementioned reactions take place in non-aqueous systems resulting in significant activity loss, which considering the elevated cost of commercial lipases constrains their industrial application (Jain and Mishra, 2015). The enzymatic process could become competitive with the use of immobilized lipases on hydrophobic supports through improved operation control, enhanced product recovery, flexible bioprocess design and better enzyme stability. Various water insoluble carriers, such as polyurethane-based supports (e.g. Accurel MP 1000) and resins (e.g. Lewatit) have been widely applied owing to their high porosity and surface area (Nunes et al., 2011; Tufvesson et al., 2011). Moreover, the utilization of non-commercial lipases could serve as a lower process-cost alternative. Thus, the extracellular *sn*-1,3-regioselective lipase from *Rhizopus oryzae* (rROL) has been cloned in *Pichia pastoris* demonstrating 40-fold higher specific activity as compared to the commercial ROL (Guillén et al., 2011) and it has been successfully used for lipid restructuring and biodiesel production (Nunes et al., 2012; Rodrigues et al., 2016a; Simões et al., 2014; Tecelão et al., 2012).

Koutinas et al. (2014) have previously demonstrated the capacity of a hybrid fermentation-enzymatic process to produce EL from dairy waste. Herein, we investigate the potential of increasing the productivity of the enzymatic step in this process. To this end, the loading of lactic acid as well as the effect of different polar and non-polar

solvents was explored relevant to the conversion of the ester using the commercial immobilized lipase from *Candida antarctica*, Novozym 435. Different commercial (Novozym 435, Lipozyme RM IM, Lipozyme TL IM) and non-commercial biocatalyst (rROL immobilized on hydrophobic supports, namely Lewatit VP OC 1600, Accurel MP 1000, Lifetech<sup>TM</sup> ECR1030M and Lifetech<sup>TM</sup> AP1090M) as well as CRL immobilized on Lewatit VP OC 1600, were tested for the production of EL in the most effective solvents. Moreover, batch operational stability tests were performed using the enzymes selected. The results obtained demonstrate that commercial lipases are more applicable to the specific reaction, offering the operational parameters that may enhance the production of the green solvent in the esterification step of the hybrid process.

## 2. Materials and Methods

### 2.1 Reagents

Lemon peel oil was kindly provided by a local citrus juice factory (Kean Soft Drinks Ltd., Limassol, Cyprus), while all chemicals employed were procured from Sigma-Aldrich Company Ltd (UK) and were of ANALAR grade.

### 2.2 Lipases and immobilization supports

The commercial immobilized lipases used for esterification included Novozym 435 (*Candida antarctica* lipase B (CALB) immobilized on acrylic resin), Lipozyme TL IM (*sn*-1,3-regioselective lipase from *Thermomyces lanuginosus*) and Lipozyme RM IM (*sn*-1,3-regioselective lipase from *Rhizomucor miehei*), kindly donated by Novozymes<sup>TM</sup>, A/S, Bagsvaerd, Denmark. Lyophilized CRL was a gift from Amano Enzyme Europe Ltd., UK. Moreover, the non-commercial rROL was used. rROL was

produced by over-expression of the corresponding gene in a mutant strain of *Pichia pastoris* (Barrigón et al., 2013). The rROL used in this study presented a hydrolytic activity of 100 U/mg of total protein according to the methodology previously developed (Resina et al., 2004). rROL was immobilized on different synthetic supports: (i) Lifetech™ ECR1030M, and (ii) Lifetech™ AP1090M, kindly donated by Purolite (Lifetech™, Wales, UK); (iii) Lewatit VP OC 1600, a gift from Lanxess (Germany); and (iv) Accurel MP 1000, from Membrana GmbH (Germany). CRL was only tested immobilized on Lewatit VP OC 1600. According to the literature, Lewatit VP OC 1600 constitutes most probably the same support used by Novozymes™ in Novozym 435 (Cabrera et al., 2009). The main physical and chemical properties of these carriers, as well as the immobilization methods employed are presented in Table 1.

### 2.3 Characterization of supports

All the supports used constitute synthetic resins derived from different polymeric hydrophobic structures (Table 1). In spite of the hydrophobicity of these carriers, they may exhibit some affinity for water molecules. To evaluate the ability of these supports to adsorb water, the aquaphilicity (Reslow et al., 1988) and partition coefficient for water between the support and water-saturated diisopropyl ether were estimated.

#### 2.3.1 Aquaphilicity assessment

Aquaphilicity constitutes a parameter proposed by Reslow et al. (1988), which is defined as the ratio between the amount of water on the support and the amount of water in diisopropyl ether, following establishment of partition equilibrium, under fixed conditions. Thus, the aquaphilicity of each immobilization support was estimated using the protocol developed by Reslow et al. (1988) after modification. The carriers were

completely dried by silica-gel in a desiccator for 24 h. Subsequently, 0.5 g of dried supports was placed in 7.2 g of water-saturated diisopropyl ether for 24 h, to allow migration of water from the solvent to the carrier until partition equilibrium. Solvent aliquots (100  $\mu$ L) were withdrawn and the residual water content was determined by a Metrohm 684 Karl Fischer Coulometer. Given that the quotient (amount of dried carrier/amount of saturated diisopropyl ether) proposed by Reslow et al. (1988) is 0.2 and the value acquired here is 0.069, a conversion factor of 2.88 (i.e.  $0.2/0.069$ ) must be used to convert the results obtained in aquaphilicity values. Thus, the aquaphilicity ( $A_q$ ) of each support was calculated as follows:

$$A_q = 2.88 \frac{W_{support}}{W_{solvent}} \quad (1)$$

$W_{support}$  corresponds to the total amount of water migrating to the support, while  $W_{solvent}$  constitutes the total amount of water in the solvent, following establishment of partition equilibrium. It is worth noticing that aquaphilicity does not express a partition coefficient, since it depends on the ratio of support/solvent applied. Experiments were performed in duplicate for all supports, while at least three aliquots were analyzed for each experiment. The corresponding average values and standard deviations were calculated.

### 2.3.2 Partition coefficient estimation

The partition coefficients (carrier/external diisopropyl ether) for water ( $P_w$ ) were estimated as follows:

$$P_w = \frac{W_{support} \times M_{solvent}}{W_{solvent} \times M_{support}} = 14.4 \frac{W_{support}}{W_{solvent}} \quad (2)$$

$M_{solvent}$  and  $M_{support}$  correspond to the mass of solvent and dry support applied, 7.2 g and 0.5 g, respectively.



#### 2.4 Lipase immobilization

Immobilization of CRL or rROL by adsorption on Lifetech™ AP1090M and Lifetech™ ECR1030M resins was performed as previously described (Rodrigues et al., 2016a). The immobilization of both lipases on Lewatit VP OC 1600 or Accurel MP 1000, using glutaraldehyde as reticulation agent to prevent leaching of the enzyme to the reaction medium during esterification, was derived from Rodrigues et al. (2016b). All enzyme preparations incorporated an enzyme:support ratio of 1:4 (0.25 g enzyme in the original solution per 1 g support).

#### 2.5 Protein assay

The immobilization yield was calculated by using the amount of immobilized protein on the supports. Protein was assayed by the method described by Bradford (1976), while the calibration was established employing bovine serum albumin (Sigma-Aldrich, Saint Louis, USA) as standard. The content of immobilized protein was calculated as the difference between the amount of protein in the initial enzyme solution (prior to the addition of immobilization support) and the residual protein present in the supernatant following immobilization (as well as in subsequent washing solutions). The immobilization yield (%) was calculated as the ratio between the amount of immobilized protein and the initial protein content in the enzymatic solution.

#### 2.6 Assessment of lipases' hydrolytic activity

The hydrolytic activities of the commercial and non-commercial immobilized lipases employed were estimated using the protocol presented in Tecelão et al. (2012), following modification: 1.75 g of gum arabic was added to 25 g of distilled water and the solution was stirred for 15 min. Extra virgin olive oil (25 g) were added to the

viscous mixture, which was further stirred for 1 h. Subsequently, 9 mL of phosphate buffer (0.1 M, pH 7.0) was supplemented and the emulsion was left overnight to homogenize in a magnetic stirrer, while 100  $\mu$ L of Tween 80 were added and the mixture was placed again under stirring overnight. A volume of 1.5 mL of the emulsion was transferred into a conical flask and placed at a shaking incubator at 30  $^{\circ}$ C and 100 rpm for 15 min. The immobilized enzyme (50 mg) was added to the substrate and the mixture was left for 5 min to react. Following hydrolysis, 5 mL of an ethanol:acetone (1:1, v:v) solution were added to the mixture for enzyme inactivation. The content of free fatty acids (FFA) released was measured through direct titration using a 0.1 M sodium hydroxide solution and phenolphthalein as indicator. Blank experiments were conducted without enzyme addition and each reaction was performed in triplicate. The unit of hydrolytic activity (U) was defined as the amount of enzyme catalyzing the hydrolysis of olive oil that released 1  $\mu$ mole of FFA  $\text{min}^{-1}$ .

### 2.7 Batch enzymatic experiments

Enzymatic reactions were conducted in 2 ml screw-capped closed vials, which were stirred at 100 rpm in an incubator operated at 30  $^{\circ}$ C. Immobilized lipases were applied in all esterification experiments at a concentration of 6.7  $\text{mg ml}^{-1}$ , while ethanol and lactic acid were added directly into the solvent used in each reaction. The concentration of ethanol was maintained constant at 0.5 M through the experiments and the concentration of lactic acid ranged between 0.01-1 M according to the requirements of each experiment. Both polar (chloroform, dichloroethane and ethyl acetate), and non-polar (toluene, hexane and decane) solvents and lemon peel oil were tested. Solvent-free systems, where ethanol was used as solvent and reagent, were also tested.

### 2.8 Lipase inactivation experiments

In order to evaluate eventual catalytic effects by the lipase carriers used in commercial preparations, inactivation of the biocatalyst was performed through addition of 8 g of immobilized enzyme in 40 ml of an acetone-methanol solution (1:1, v/v). Lipase inactivation in the mixture was allowed to proceed for 16 h under magnetic stirring as previously described (Kawase and Tanaka, 1989). 6.7 mg ml<sup>-1</sup> of inactivated Novozym 435 and Lipozyme RM IM was added to chloroform or hexane supplemented with 0.01 M of lactic acid and 0.5 M of ethanol. The reaction was allowed to proceed for 24 h at 30 °C and 100 rpm, while the capacity of the carrier as catalyst for the esterification reaction was assessed through determination of the EL yield.

### 2.9 Batch operational stability tests

The operational stability of Novozym 435 was determined in sequential 24 h batches conducted under selected reaction conditions. Esterification was performed as described in section 2.7 using as solvents chloroform and hexane. Ethanol concentration was maintained at 0.5 M and 0.01 M of lactic acid was added. Following each batch, the enzyme was removed through filtration and applied directly in the subsequent reaction using fresh media under the same conditions. A total of 5 batch experiments were conducted with the use of the same biocatalyst. The EL yield ( $Y_{EL}$ ) achieved following completion of each batch (Eq. 3) was used as a measure of enzyme activity, while the activity of the first batch was employed as a reference point (100% activity). Therefore, the enzymatic residual activity ( $a_n$ ) following each batch  $n$  ( $n = 1, \dots, 5$ ) was determined with the use of Eq. 4.

$$Y_{EL} = \frac{\text{EL concentration}}{\text{Maximum theoretical EL concentration}} \times 100\% \quad (3)$$

$$a_n = \frac{Y_{EL\_Batch\_n}}{Y_{EL\_Batch\_1}} \times 100\% \quad (4)$$

## 2.10 Determination of Log P values

The Log P values of the reaction medium (Log  $P_{system}$ ) were estimated for the systems in chloroform and hexane, using the Log P values of substrates and the respective molar fractions as follows:

$$Log P_{system} = x_{solv} Log P_{solv} + x_{Eth} Log P_{eth} + x_{LA} Log P_{LA} \quad (5)$$

$x_{solv}$ ,  $x_{eth}$  and  $x_{LA}$  correspond to the molar fractions of solvent, ethanol and lactic acid in the reaction medium respectively.

## 2.11 GC analysis

Gas Chromatography (GC) was employed for the determination of EL concentration in samples obtained from esterification reactions. A Shimadzu GC-2014 (Shimadzu, UK) was applied, which comprised a flame ionisation detector and a 30 m Zebron ZB-5 capillary column (Phenomenex, UK) with 0.25 mm internal diameter. Nitrogen was used as the mobile phase, while the stationary phase consisted of 5%-phenyl and 95% dimethylpolysiloxane. The closed vials utilized in the enzymatic reactions were transferred into the autosampler of the GC and one  $\mu$ L was injected. The following column temperature programmes were utilized for analysis of EL concentration in each solvent: i) hexane – increase of temperature from 50 °C to 165 °C with a rate of 30 °C  $min^{-1}$ , constant temperature of 165 °C for 1 min and further increase with a rate of 150 °C  $min^{-1}$  up to 250 °C (maintained for 3 min); ii) decane – temperature increase from 100 °C to 200 °C with a rate of 30 °C  $min^{-1}$  (maintained at 200 °C for 1 min) and further increase with a rate of 50 °C  $min^{-1}$  up to 250 °C (maintained for 3 min); iii) chloroform – temperature increase from 50 °C to 160 °C with a rate of 50 °C  $min^{-1}$  (maintained at

160 °C for 1 min) and subsequent increase with a rate of 25 °C min<sup>-1</sup> up to 250 °C; iv) 1,2-dichloroethane and ethyl acetate – temperature increase from 65 °C to 170 °C with a rate of 30 °C min<sup>-1</sup> (maintained at 170 °C for 1 min) and further increase with a rate of 35 °C min<sup>-1</sup> up to 250 °C; v) ethanol – increase of temperature from 70 °C to 165 °C with a rate of 40 °C min<sup>-1</sup>, constant temperature of 165 °C for 1 min and further increase with a rate of 150 °C min<sup>-1</sup> up to 250 °C (maintained for 3 min); vi) citrus peel oil – initial temperature of 120 °C for 2 min followed by an increase of 40 °C min<sup>-1</sup> up to 180 °C (maintained for 1 min); vii) toluene – initial temperature of 90 °C for 1 min followed by an increase of 30 °C min<sup>-1</sup> to 160 °C (maintained for 1 min) and a subsequent increase of 25 °C min<sup>-1</sup> to 250 °C. EL concentration was estimated interpolating from previously established calibration curves and the coefficient of variation for five samples was 2.4% at a concentration level of 0.1 M EL.

## 2.12 Statistical analysis

One-way analysis of variance (ANOVA) was performed on data that concerned the characterization of supports (aquaphilicity and partition coefficients) aiming to evaluate significant differences among supports. The programme Statistica (version 6, Statsoft, Tulsa, USA) was used, while post-hoc comparisons were carried out at a p value of 0.05 and by using the Tukey HSD test.

## 3. Results and Discussion

### 3.1 Solvent selection

The development of the bioprocess envisaged aimed at converting biowaste into EL (Koutinas et al., 2014), requires the esterification of fermentation products with the use

of lipases. Although biocatalysis of the reaction can proceed in a range of organic media with diverse properties, including polar and hydrophobic solvents as well as solvent-free media (Hasegawa et al., 2008a), the nature of the solvent applied should be carefully considered. The choice of the solvent constitutes a crucial process parameter given that highly hydrophilic solvents are toxic for the enzymes removing the water monolayer required for maintaining the active conformation of the enzyme. According to Laane et al. (1985), Log P octanol/water can be applied to measure the hydrophobicity of the solvent. Organic media with Log P values lower than 2 are harmful to the enzymes removing the water layer of the enzyme drastically. Moreover, solvents that include Log P values higher than 4 are non-toxic, while those with values between 2-4 may impose an unpredictable effect on enzyme activity. In the system explored here, hydrophilic substrates, immiscible to hydrophobic solvents, are employed placing a constraint to the use of hydrophobic media. However, the high concentrations of lactic acid often applied in polar solvents cause enzyme inactivation due to high acidity (Reetz, 2002), highlighting that both the concentration of lactic acid and the use of different organic solvents should be tested to ensure high EL productivity.

Several polar and non-polar organic solvents have been previously employed for enzymatic esterification of ethanol and lactic acid (Hasegawa et al., 2008b; Major et al., 2010). Thus, both options were explored including media with different Log P values and the polar solvents tested included ethanol (Log P = -0.31), ethyl acetate (Log P = 0.68), 1,2-dichloroethane (Log P = 1.48) and chloroform (Log P = 2), whereas the non-polar solvents examined were toluene (Log P = 2.5), hexane (Log P = 3.5), and decane (Log P = 5.802). Particular interest was placed on the use of hexane, given its use as a

solvent for food applications (Pires-Cabral et al., 2010), and on lemon peel oil, a natural oil holding the capacity for application as biosolvent (Fidalgo et al., 2016). In all experiments, lactic acid was added in four different concentrations (0.01 M, 0.1 M, 0.5 M and 1 M) to access the acidity effect imposed by the substrate. Ethanol concentration was maintained at 0.5 M, apart from the solvent-free system where it served both as reactant and solvent (Fig. 1).

Enzyme-free experiments were first performed to evaluate the non-enzymatic production of EL in each media (Fig. 1A). The EL yield remained extremely low (< 5%) in all reactions demonstrating that non-enzymatic esterification was suppressed under the conditions tested. These experiments were repeated with the addition of 6.7 mg ml<sup>-1</sup> of Novozym 435 (Fig. 1B). EL yields of 16% and 11% were obtained in solvent-free experiments (performed in ethanol) through application of 0.5 M and 1 M of lactic acid, respectively. However, the productivity of EL was substantially enhanced for a range of solvents at lower acid contents. The use of 0.1 M of lactic acid resulted in 59% and 56% of EL yields in chloroform and 1,2-dichloroethane respectively. When the concentration of lactic acid was reduced to 0.01 M, the most efficient organic media comprised chloroform and hexane generating EL yields of 88% and 75% respectively. Moreover, 1,2-dichloroethane and toluene produced lower EL yields that reached 61% and 60%. Although lemon peel oil holds favourable technological properties as bio-solvent, only 56% of the maximum theoretical yield was achieved and only at the lowest acid concentration employed, while the use of ethyl acetate generated even lower EL production. Therefore, both solvents were disregarded from further tests. The highest yields were achieved in chloroform (using 0.01-0.1 M of lactic acid) and hexane (using 0.01 M of lactic acid). Thus, these two solvents were selected for further

investigation in the system, given their capacity to promote the highest concentrations of EL and due to the applicability of hexane in the food sector.

Fig. 1C presents the ester yield as a function of the Log P values of solvents, demonstrating that the production of EL was enhanced as the Log P value of the solvent increased up to a value of 2 (chloroform). The reduced ester yields achieved when solvents with higher Log P values were applied could be due to the low solubility of substrates in these solvents. A steep decrease in ester yield was observed in esterification employing 0.5 M of ethanol and 0.1 M of lactic acid when solvents with Log P values higher than 2 were applied. Thus, the solubility of substrates constitutes a limiting factor in solvent choice and productivity enhancement.

The presence of 0.5 M of ethanol and 0.1 M of lactic acid in the reaction medium promoted a decrease of Log P as compared to the values of pure solvents (from values of 2 for pure chloroform and 3.5 for pure hexane to 1.89 and 3.21 respectively). Moreover, the reduction of lactic acid's concentration in these systems to 0.01 M slightly increased the Log P values to 1.91 (in chloroform) and 3.25 (in hexane), demonstrating that substrate concentrations affect the hydrophobicity character of the reaction medium, which is subsequently expected to influence enzyme activity.

The strong acidity of lactic acid is known to hinder the esterification activity of lipases (Hasegawa et al., 2008b). Acids with  $pK_a$  values lower than 4.8 may inhibit Novozym 435 irreversibly (lactic acid comprises a  $pK_a$  of 3.86) demonstrating that the acidity imposed by the substrate could be a crucial parameter for the reaction (Hollmann et al., 2009). Moreover, non-polar solvents solubilize only limited amounts of lactic acid further reducing the efficiency of the esterification reaction. Esterification yields in hexane remained at low levels when acid concentrations ranged between 0.1-3.5 M



(Hasegawa et al., 2008a). However, the results of the present study demonstrate that EL formation in non-polar solvents could be substantially enhanced with the use of lower substrate concentrations (0.01 M, Fig. 1B).

### *3.2 Evaluation of immobilized biocatalysts for EL production*

The capacity of various lipases to catalyze a wide range of esterification reactions and the development of a competitive low-cost process require the comparison of commercial and non-commercial biocatalysts. The commercial enzymes employed constituted Novozym 435, Lipozyme RM IM and Lipozyme TL IM, while non-commercial alternatives included CRL using Lewatit VP OC 1600 as the support and rROL immobilized on Accurel MP 1000, Lifetech™ ECR1030M, Lifetech™ AP1090M and Lewatit VP OC 1600.

#### *3.2.1 Support characterization*

Aquaphilicity values and partition coefficients (support/solvent) for water are presented on Table 1 for the supports tested. Aquaphilicity values varied from 0.436, (Accurel MP 1000) to 1.43 (Lewatit VP OC 1600). The lowest partition coefficient (2.18) was observed for Accurel MP 1000 and the highest value (7.13) was achieved for Lewatit VP OC 1600 constituting the most hydrophobic and the less hydrophobic carriers respectively. Although the two Lifetech (Purolite) resins entailed different compositions, they exhibited similar hydrophobicity forming a homogeneous group. Immobilization in these resins was performed by adsorption in AP 1090M and ECR 1030M. Similar aquaphilicity values were obtained for hydrophobic supports such as celite, bonopore, dimethyldichlorosilane-controlled porous glass (CPG), surfasil-CPG and other CPG derivatives as well as for the resin Amberlite XAD-4 (Reslow et al.,

1988). Moreover, hydrophilic materials, such as LH-20 Sephadex, Sephadex G-25 and hydrophilic polyurethane foams (Hypol FHP 2002™ and Hypol FHP 5000™ foams), include higher aquaphilicity values (Ferreira-Dias et al. 1999; Reslow et al., 1988). Regarding the partition coefficients for water between the support and diisopropyl ether, the water molecules always tend to migrate from the water-saturated solvent towards the carriers ( $P_w > 1$ ).

### 3.2.2 Lipase immobilization

Immobilization yields were calculated based on the amount of protein immobilized as shown on Table 1. The yields varied from 34.7% in Accurel MP 1000 (for rROL) to 66 % in Lewatit VP OC 1600 (for rROL). However, the immobilization yields were not significantly different in each support for both enzymes. rROL immobilized on Lewatit VP OC 1600 and on the same Purolite resins have previously exhibited higher immobilization yields (Rodrigues et al., 2016a). This could be ascribed to the use of rROL derived from different fermentation batches, which could also affect the percentage of rROL in the total protein content.

### 3.2.3 Evaluation of immobilized biocatalysts for EL production

In line with the above presented results, the efficiency of these immobilized enzymes was tested for EL production from lactic acid and ethanol employing the two organic solvents selected in Section 3.1 as well as the two lowest lactic acid concentrations applied (Figs. 2A-B). The use of hexane (Fig. 2A) demonstrated that when 0.1 M of lactic acid was used, similarly to the performance of Novozym 435, the production of EL was suppressed with all biocatalysts tested. However, the decrease in lactic acid concentration to 0.01 M resulted in 31% and 75% of EL yield for Lipozyme RM IM and

Novozym 435 respectively. Moreover, the latter biocatalysts were the only enzymes that functioned in the presence of chloroform (Fig. 2B). Specifically, Novozym 435 produced 59% and 88% of EL yield with the use of 0.1 M and 0.01 M of lactic acid, while the production of EL catalyzed by Lipozyme RM IM in the lowest acid concentration employed was 57%. These results demonstrate that apart from Lipozyme TL IM, the commercial immobilized enzymes could efficiently catalyze the reaction in both solvents at the lowest lactic acid concentration. However, at 0.1 M of acid content, Lipozyme RM IM was repressed in both solvents and Novozym 435 functioned only in chloroform. Nevertheless, although rROL and CRL preparations have demonstrated great potential for application in similar esterification reactions, as well as in the production of biodiesel and human milk fat substitutes by interesterification reactions (Rodrigues et al., 2016a; Simões et al., 2014; Tecelão et al., 2012; Pires-Cabral et al., 2010), these enzymes could not esterify lactic acid under the conditions tested.

The capacity of immobilization carriers to directly drive the esterification reaction without the action of lipases was tested through enzyme inactivation for Novozym 435 and Lipozyme RM IM. The application of the carriers following enzyme inactivation in the esterification of ethanol and lactic acid resulted in complete repression of the reaction demonstrating that the capacity of the two commercial preparations to catalyze ester synthesis in chloroform and hexane was due to the action of the enzymatic content.

Novozym 435, Novozym 388, Lipozyme RM IM and CALB displaying yeast cells, as well as non-immobilized lipases (e.g. *Bacillus* lipase, Lipase A and Lipase D) have been previously used as biocatalysts for EL production (Findrik et al., 2012; Hasegawa et al., 2008a; Hasegawa et al., 2008b; Inaba et al., 2009; Jain and Mishra, 2015; Koutinas et al., 2014; Major et al., 2010; Sun et al., 2010). A yield of 56% was achieved

in Cypros 104 (Findrik et al., 2012) and methyl isobutyl ketone (Hasegawa et al., 2008b) with the use of Novozy 435, while the application of the same preparation in acetonitrile (Hasegawa et al., 2008a) and Cyphos 202 (Major et al., 2010) produced ester yields of 53% and 104% respectively. Overall, Novozym 435 was the most efficient enzyme exhibiting in the present study one of the highest EL yields. Lipozyme RM IM was also successfully applied in polar solvents, reaching a yield of 54% in methyl isobutyl ketone (Hasegawa et al., 2008b). However, similarly to the results obtained with the use of hexane, lactic acid concentrations of 0.01 M substantially enhanced the ester yield in non-polar solvents (e.g. heptane) that reached 74% when lyophilized CALB-displaying yeast cells were used (Inaba et al., 2009). Moreover, non-immobilized lipases resulted in substantially lower yields that ranged between 5-28% (Hasegawa et al., 2008a; Hasegawa et al., 2008b; Jain and Mishra, 2015).

### *3.3 Hydrolytic activities of immobilized biocatalysts*

The complex nature of lipase function is strongly affected by the composition of reaction media (Vargas et al., 2008). This fact results in enzymatic action that often demonstrates clear preference to either hydrolysis or esterification depending on the process conditions used (Romero et al., 2012). Moreover, determining the activities of enzymes for different reactions enables identification of potential biotechnological applications. Thus, the hydrolytic activity of the enzymes employed was measured to define alternative industrial applications and to investigate the potential relation between hydrolytic and esterification capacity for each biocatalyst. The assessment of hydrolytic activities for each enzyme preparation (Table 2) demonstrates that similarly to the capacities of Novozym 435 and Lipozyme RM IM for effective synthesis of ester bonds, the same enzymes were the most efficient for the hydrolysis reaction tested,

exhibiting a hydrolytic activity of 260 U and 220 U per g of biocatalyst respectively. However, although the rest of the biocatalysts explored were unable to catalyze the synthesis of EL, Lipozyme TL IM presented hydrolytic activity of 190 U g<sup>-1</sup> and rROL immobilized on Lifetech<sup>TM</sup> AP1090M reached 150 U g<sup>-1</sup> during olive oil hydrolysis. These values are slightly lower as compared to the activity values obtained for Novozym 435 and Lipozyme RM IM. Although hydrolysis and synthesis can be strongly correlated reactions for a wide range of lipases (Otero et al., 2005), it has been previously demonstrated that there is no correlation between esterification and hydrolytic activities for *Penicillium brevicompactum* lipase (Silva et al., 2011) or between interesterification and hydrolysis activities for rROL immobilized on Eupergit C, Accurel MP 1000 or Lewatit VP OC 1600 (Nunes et al., 2012; Tecelão et al., 2012). In line with the literature, the present study presented contradictory results given that the most active enzymes in esterification also exhibited the highest hydrolytic activity. Despite the inability of non-commercial preparations to catalyze the synthesis of EL, these enzymes were active in the hydrolysis reaction of olive oil in emulsion system.

### 3.4 Operational stability assays

The operational stability of Novozym 435 was evaluated in 5 consecutive 24 h batch experiments using 0.01 M of lactic acid and 0.5 M of ethanol in chloroform and hexane at 30 °C. Following the first batch, 80% and 65% of conversion into EL was observed in chloroform or hexane respectively. The results obtained have been expressed as residual activity (Fig. 3). A rapid deactivation was observed in hexane during the second batch reaching a residual activity of 22%, which was maintained for subsequent batches. However, slower deactivation was observed in chloroform and only after 72 h of

operation the residual activity was 52%. The activity remained constant in the last two batches, exhibiting comparable performance to that observed in hexane.

Similar behavior was previously observed for Novozym 435, which was completely inactivated in hexane and exhibited significantly higher stability in polar solvents that included acetonitrile, acetone and tetrahydrofuran (Hasegawa et al., 2008a). Although polar solvents with Log P < 2 are generally regarded as toxic, the enzyme could be more stable in such solvents due to the presence of more negatively charged amino acids on the surface as compared to basic ones, solubilizing the protein, either through generation of a hydrated ion network with cations or via electrostatic repulsive charges avoiding protein aggregation (Doukyu and Ogino, 2010; Jain and Mishra, 2015). Also, considering that Lewatit VP OC 1600 is the carrier used in Novozym 435, it is more hydrophilic than the other supports tested in the present study (Table 1). Due to the hydrophobicity of hexane, ethanol and lactic acid tend to migrate to the microenvironment of the lipase reaching higher and inhibitory concentrations than when chloroform is used. This effect was also observed in the synthesis of ethyl butyrate in *n*-hexane, with CRL immobilized in polyurethane foams with different hydrophilicities, (Pires-Cabral et al., 2010). The deactivation was faster for the lipase in the less hydrophilic foam when higher substrate concentrations were applied.

#### 4. Conclusions

The potential of enhancing the productivity of EL via enzymatic esterification has been explored. It was clarified that although high lactic acid contents suppress the reaction, acid concentrations lower than 0.1 M could substantially enhance the product

yield in solvents such as chloroform and hexane. Moreover, Novozym 435 and Lipozyme RM IM can be efficiently applied as biocatalysts for esterification of lactic acid and ethanol, while non-commercial preparations of rROL and CRL were not effective under the conditions tested. The study presents important operational parameters that could enhance the production of the green solvent through enzymatic esterification.

## 5. Acknowledgements

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## 7. Figure Captions

**Fig. 1.** Ethyl lactate yield achieved in different solvents from esterification of lactic acid with ethanol. Experiments were performed for 24 h using different concentrations of lactic acid (0.01, 0.1, 0.5, 1 M) and 0.5 M of ethanol at 30 °C and 100 rpm. (A) Control experiments performed without the addition of enzyme. (B) Enzymatic reactions catalyzed by Novozym 435 at a concentration of 6.7 mg mL<sup>-1</sup>. (C) Ester yield (%) as a function of the Log P values of solvents applied in esterification catalyzed by Novozym 435 using 0.1 M (open circles) and 0.01 M (closed circles) of lactic acid.

**Fig. 2.** The effect of different commercial and non-commercial immobilized lipases on the production of ethyl lactate. Lactic acid was used in concentrations of 0.01 M and 0.1 M, while ethanol and enzyme concentrations were maintained constant at 0.5 M and 6.7 mg mL<sup>-1</sup>, respectively. The esterification reaction was performed in (A) hexane and (B) chloroform, for 24 h at 30 °C and 100 rpm. Control: experiments performed without the addition of enzyme; CR: *Candida rugosa* lipase; RO: *Rhizopus oryzae* lipase.

**Fig. 3.** Batch operational stability tests for Novozym 435 using 0.01 M of lactic acid and 0.5 M of ethanol. Reactions were performed at 30 °C and 100 rpm using 6.7 mg mL<sup>-1</sup> of lipases. Residual activity of Novozym 435 in: i)  : chloroform, and ii)  : hexane.

## 8. Tables and Figures

**Table 1.** Main physical and chemical properties as well as average aquaphilicity and partition coefficient (support/solvent) values for water in the immobilization support tested and average values of immobilization yields (%) for rROL and CRL. Superscript indexes indicate differences based on Tukey HSD test ( $p \leq 0.05$ ). The indexes indicate no differences between samples.

<i>Physical and Chemical properties</i>					
Carrier	Polymer structure	Method of immobilization	Functional Group	Particle size range (mm)	Pore Diameter (Å)
Accurel MP 1000	Polypropylene	Adsorption+ reticulation	None	0.05-1.0	200
Lewatit VP OC 1600	DVB-crosslinked methacrylate	Adsorption + reticulation	None	0.315 - 1.0	150
Lifetech AP 1090M	Macroporous styrene	Adsorption	None	0.30 - 0.71	900- 1100
Lifetech ECR 1030M	DVB/ methacrylate	Adsorption	None	0.30 - 0.71	250
<i>Aquaphilicity and partition coefficient (support/solvent)</i>					
Carrier	Aquaphilicity	Std	Partition coefficient support/solvent	Std	
Accurel MP 1000	0.436 <sup>a</sup>	0.051	2.18 <sup>a</sup>	0.257	
Lewatit VP OC 1600	1.430 <sup>b</sup>	0.144	7.13 <sup>b</sup>	0.718	
Lifetech AP 1090M	0.706 <sup>c</sup>	0.081	3.53 <sup>c</sup>	0.405	
Lifetech ECR 1030M	0.671 <sup>c</sup>	0.060	3.36 <sup>c</sup>	0.298	
<i>Immobilization yields (%) for rROL and CRL</i>					
Carrier	rROL	CRL			
Accurel MP 1000	34.71	-			
Lewatit VP OC 1600	66.04	59.54			
Lifetech AP 1090M	47.05	-			
Lifetech ECR 1030M	45.55	-			
Lifetech ECR 8285M	-	-			

**Table 2.** Hydrolytic activity of lipases tested.

<b>Enzyme</b>	<b>Enzyme Activity [U g<sup>-1</sup>] with Standard Deviation</b>
Novozym 435	260 ± 33.8
<i>CRL</i> - Lewatit VP OC 1600	50 ± 4.9
<i>rROL</i> - Accurel MP 1000	106 ± 25.5
<i>rROL</i> - ECR1030M	100 ± 28.4
<i>rROL</i> - AP1090M	150 ± 12.4
<i>rROL</i> - Lewatit VP OC 1600	70 ± 6.7
Lipozyme RM IM	220 ± 14.1
Lipozyme TL IM	190 ± 12.2



Fig. 1.

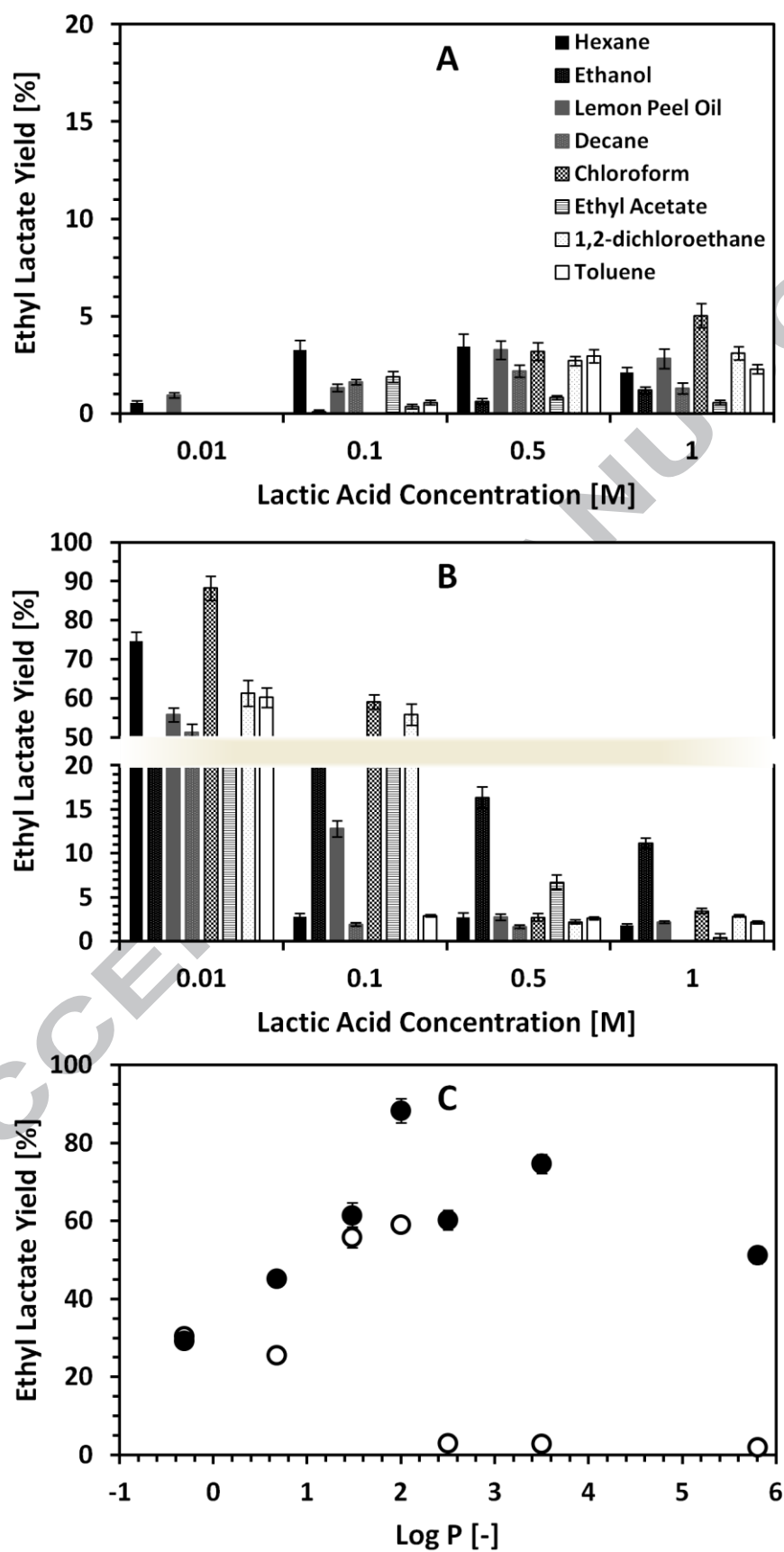


Fig. 2.

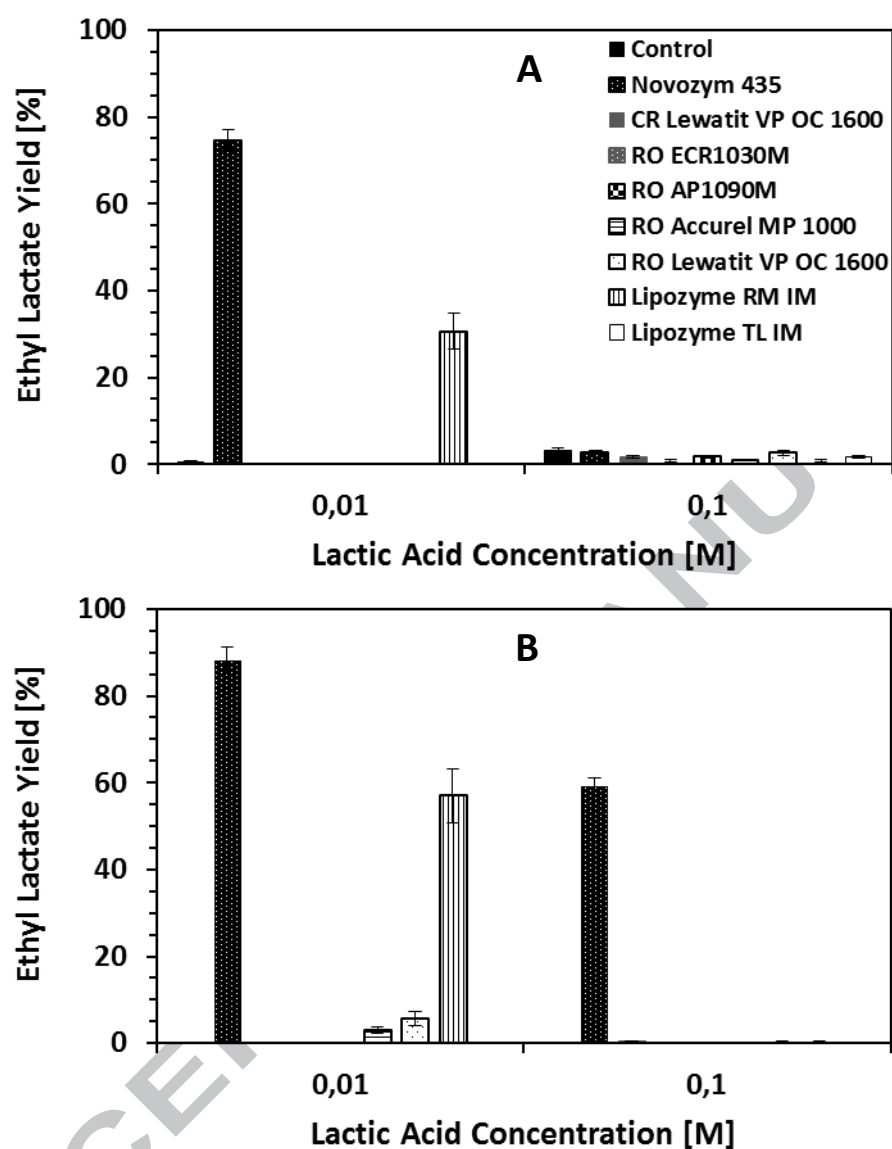
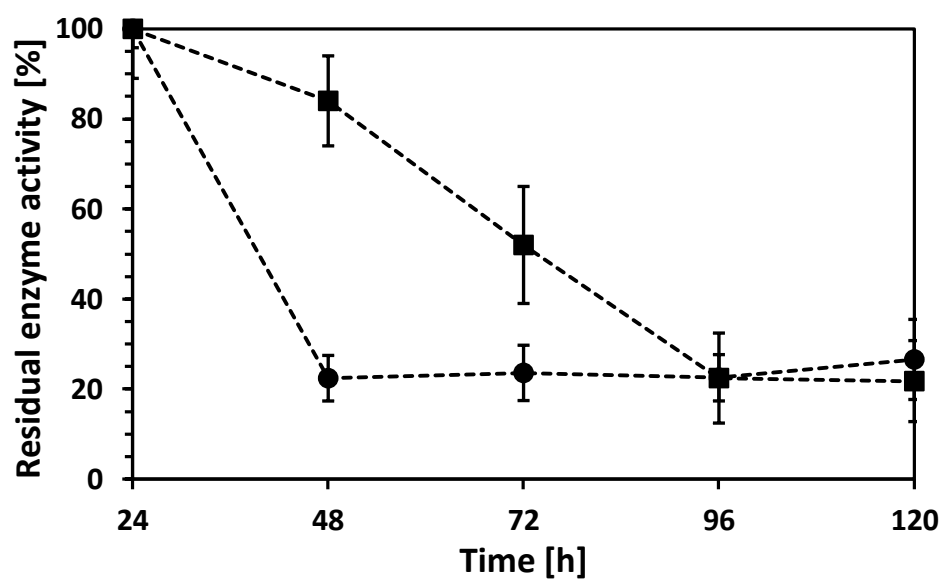


Fig. 3.



### Highlights

- Different solvents were tested for lactate and ethanol esterification
- Chloroform and hexane were the most effective solvents in low acid concentrations
- Novozym 435 and Lipzyme RM IM could be efficiently applied in the reaction
- Novozym 435 retained higher activity in chloroform as compared to hexane
- Non-commercial enzymes exhibited significant activity towards hydrolysis