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STABILITY OF IMMOBILIZED *Rhizomucor miehei* LIPASE FOR THE SYNTHESIS OF PENTYL OCTANOATE IN A CONTINUOUS PACKED BED BIOREACTOR

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Abstract - The enzymatic synthesis of organic compounds in continuous bioreactors is an efficient way to obtain industrially important chemicals. However, few works have focused on the study of the operational conditions and the bioprocess performance. In this work, the aliphatic ester pentyl octanoate was obtained by direct esterification using a continuous packed bed bioreactor containing the immobilized enzyme Lipozyme® RM IM as catalyst. Enzymatic deactivation was evaluated under different conditions for the operational parameters substrate/enzyme ratio (5.00, 1.67, 0.83 and 0.55 $mmol_{substrate} \cdot min^{-1} \cdot g^{-1}_{enzyme}$) and temperature (30, 40, 50 and 60 °C). The optimal condition was observed at 30 ºC, which gave the minimum enzymatic deactivation rate and the maximum conversion to the desired product, yielding approximately 60 mmols of ester for an enzyme loading of 0.5 g into the bioreactor. A first-order deactivation model showed good agreement with the experimental data.
Keywords: Continuous bioreactor; Biosynthesis of ester; Biocatalysis; Deactivation.

INTRODUCTION

Esters are important industrial compounds obtained either by organic synthesis (esterification, transesterification or interesterification) or by solid-liquid extraction from natural resources. Among their several applications, esters have gained wide interest as solvents, fragrances (aromatic esters), flavors (aliphatic esters) and precursors for several processes (including the food, drug and cosmetics industries) (Gandhi, 1997; Abbas and Comeau, 2003; Rodrigues and Fernandez-Lafuente, 2010a).

However, the reactions for ester production have a slow rate and require the use of specific catalysts in order to be economically viable. Traditional processes use inorganic acids (e.g., sulfuric acid) and bases (e.g., sodium hydroxide) as catalysts, which often lead to difficulties in operational conditions (e.g., temperature, pressure and pH) and downstream, due to the generation of by-products (Kasche, 1986). Based

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on this scenario, there is strong interest in the use of enzymes as catalysts for esterification reactions, since these biomolecules allow the conduction of the reaction under mild conditions, specificity of substrates (and, consequently, reduced amount of by-products due to side reactions), and decreased cost (Rodrigues and Fernandez-Lafuente, 2010a). In addition, the enzymatic synthesis of esters has economical advantages, since the production by a biological route characterizes them as natural products, which enhances considerably their market value (Gabelman, 1994; Abbas and Comeau, 2003; Chang *et al*., 2007; Rodrigues and Fernandez-Lafuente, 2010a).

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are widely used enzymes due to their ability to recognize several substrates and to catalyze many different reactions (Barros *et al*., 2010; Rodrigues and Fernandez-Lafuente, 2010a). In particular, several flavors (short and long chain aliphatic esters) of industrial interest have been obtained by esterification, transesterification or interesterification reactions using lipases (Gandhi, 1997; Alcantara *et al*., 1998; Abbas and Comeau, 2003; Silva and Jesus, 2003; Hasan *et al*., 2006), with applications in juices, cheeses, baked goods, candies and beverages, for instance (Mahapatra *et al*., 2009). The demand for these chemicals has continuously increased at the rate of 4.3% per year (Dhake *et al*., 2013). Rodrigues and Fernandez-Lafuente (2010b) and Dhake *et al*. (2013) presented relevant reviews concerning the wide application of lipases for the synthesis of flavors.

In particular, extracellular lipase obtained from *Rhizomucor* (formerly *Mucor*) *miehei* (RML) has been successfully applied in these synthesis reactions. A wide range of flavors has been obtained with the use of this catalyst, including farnesyl laurate (Rahman *et al*., 2010), butyl butyrate (Lorenzoni *et al*., 2012) and hexyl laurate (Chang *et al*., 2007). Furthermore, its suitability for ester synthesis has been evaluated under different conditions of temperature and pressure (Noel and Combes, 2003), water content (Valivety *et al*., 1992) and substrates (Somashekar *et al*., 2007).

The RML enzyme has been commercialized in the immobilized form as Lipozyme® RM IM, where the weak anion exchange resin Duolite ES 562, based on phenol-formaldehyde copolymers, has been used as support (Rodrigues and Fernandez-Lafuente, 2010a; Rodrigues and Fernandez-Lafuente, 2010b). This kind of support attends most of the requirements for proper immobilization of the biocatalyst, such as large area for interaction with the biomolecule (Mateo *et al*., 2007).

The immobilization of the enzyme, along with its stabilization, is a key technology for its use in industrial processes and enhances its features, since it can result in higher activity, selectivity and a decrease in inhibition, and allows for the reutilization of the biocatalyst (Rodrigues and Fernandez-Lafuente, 2010a; Garcia-Galan *et al*., 2011). Non-immobilized enzymes are, in general, soluble, inhibited by substrates and products and exhibit low stability, as well as low activity for the catalysis of reactions involving non-physiological compounds (Garcia-Galan *et al*., 2011).

Furthermore, RML is stable and remains active even at low water activity (a_w), which permits its application with organic solvents, an important condition for its use in the direct esterification reaction (with free acids) (Rodrigues and Fernandez-Lafuente, 2010a). In this sense, it should be noted that non-polar solvents have been reported as less harmful to the enzyme than highly polar ones (Rodrigues and Fernandez-Lafuente, 2010a; Dhake *et al*., 2013).

Enzymes in the immobilized form can be used in all types of bioreactors (Mateo *et al*., 2007), such as the packed bed configuration (Brady and Jordaan, 2009; Ju *et al*., 2008), which minimizes labor and overhead costs, as well as allows easy recuperation of the catalyst after the reaction, hence reducing variable costs due to catalyst replacement. Recent works have studied the potential of the use of immobilized enzymes in packed bed bioreactors for a variety of processes, including the synthesis of esters and chemical intermediates (Ju *et al*., 2008), biodiesel (Chen *et al*., 2011a) and polymers (Zhang *et al*., 2012). Also noteworthy is the potential application of immobilized enzymes, including lipases, in clean technologies, particularly in bioreactors designed for environmental protection applications (Demarche *et al*., 2012).

Few works have dealt with the study of the stability of immobilized enzymes, in particular RML, in direct esterifications carried out in continuous bioreactors under a wide range of operational conditions, which may allow an optimization of the process. In this work, direct esterification for the production of an aliphatic ester was carried out. In particular, the reaction (nucleophilic acyl substitution) for production of pentyl octanoate (also known as amyl octanoate or amyl caprylate), an ester of interest in the food industry, where it can be applied as a synthetic flavoring agent for the reproduction of grape taste, was used as a model. A hydrophobic solvent (*n*-hexane) was used, allowing the application of a simple support-based enzyme immobilization protocol (Yahya *et al*., 1998). The scheme of the reaction is represented in Figure 1.

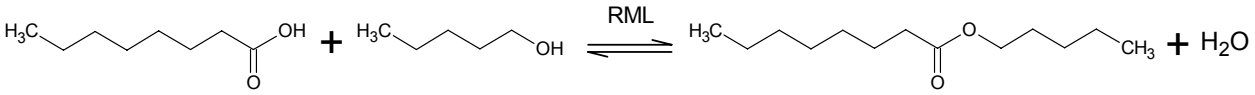

Figure 1: Scheme of the reaction of direct esterification for production of pentyl octanoate from octanoic acid and pentan-1-ol.

This reaction was previously studied by our group in batch bioreactors (Skoronski *et al*., 2010). Thus, the aim of this work was to study the stability of the commercial immobilized lipase Lipozyme® RM IM as catalyst in a packed bed bioreactor for the synthesis of pentyl octanoate under different operational conditions. In particular, the temperature and the substrate/enzyme ratio were evaluated.

EXPERIMENTAL DETAILS

Reagents

Octanoic acid (organic fatty acid with the chemical formula $C_8H_{16}O_2$, also known as caprylic acid) and pentan-1-ol (primary alcohol with the chemical formula $C_5H_{12}O$, also known as normal amyl alcohol), both provided by Merck®, were used as substrate, with purity degrees greater than 97%; n-hexane (chemical formula C_6H_{14}, provided by Vetec®) was used as the (hydrophobic) solvent. Immobilized lipase from *Rhizomucor miehei* (Lipozyme® RM IM), kindly provided by Novozymes® (Araucária-PR, Brazil), was used as biocatalyst. The average diameter of the immobilized enzyme was 1.7 mm and phenolic resin was the material support. It should be noted that the reagents were dried over molecular sieves before each reaction.

Synthesis Conditions

The ester synthesis was carried out in a continuous isothermal packed bed bioreactor (10 mm in diameter), where the temperature was controlled by pumping recirculation water through a jacket around the device. The substrates (pentan-1-ol and octanoic acid) were dissolved in *n*-hexane, with concentrations of 0.5 $mol \cdot L^{-1}$, in a molar ratio of 1:1 (pentan-1-ol:octanoic acid). This solution was fed to the bioreactor in a continuous flow of 0.5 $mL \cdot min^{-1}$. Different amounts of enzyme were used inside the bioreactor (0.05, 0.15, 0.30 and 0.45 g) resulting in feed ratios (substrate/enzyme) of 5.00, 1.67, 0.83 and 0.55 $mmol_{substrate} \cdot min^{-1} \cdot g^{-1}_{enzyme}$. The feed ratio was calculated according to Equation (1):

$$\varphi = C_{A_0} \cdot Q / w, \quad (1)$$

where φ is the feed ratio (dimensionless), C_{Ao} is the concentration of substrate fed to the bioreactor ($mmol \cdot L^{-1}$), Q is the volumetric flow ($L \cdot min^{-1}$) and w is the amount of enzyme (g). The reaction was carried out at four different temperatures: 30, 40, 50 and 60 ºC. No water was fed to the reaction.

The chemical reaction kinetics was considered to be the controlling mechanism and, therefore, the effect of mass transfer limitations was neglected. This assumption is supported by Kasche *et al*. (1987), who claimed that, in general, the biosynthesis of condensation products, e.g., esterification reactions, is kinetically controlled.

Yield Determination

The reaction conversion was determined by the evaluation of octanoic acid concentration in the inlet and outlet streams through acid-base titration, using standard sodium hydroxide solution ($0.01\ mol \cdot L^{-1}$) and phenolphthalein as indicator. Samples of 0.2 mL were collected at time intervals of 10 min and their volume was adjusted to 2 mL using ethanol as solvent.

The production of pentyl octanoate was confirmed by the FTIR (Fourier Transformed Infrared Spectrometry) technique. In particular, the equipment Spectrum One Spectrometer (PerkinElmer®) was used. The cell used had a fixed path length of 1.0 mm, with transparent potassium bromide plate windows held in a stainless-steel mount with a polytetrafluoroethylene spacer providing the fixed separation between the plates. Liquid samples were directly added to the cell in order to fill the free space between the plate windows. The carbonyl groups of octanoic acid and the ester showed peaks in the FTIR spectrum at the wavenumbers of 1709 and 1736 cm^{-1}, respectively, with 10 scans accumulated.

Amount Accumulated of Produced Ester

The amount of ester obtained was computed by Equation (2):

$$m = \int_0^t C_{A_0} \cdot Q \cdot X(t) dt, \quad (2)$$

where m is the accumulated amount of ester (mmol), C_{Ao} is the initial concentration of substrate (mmol·L^{-1}), Q is the volumetric flow (L·min^{-1}), t is time (min) and $X(t)$ is the reaction conversion (dimensionless).

RESULTS AND DISCUSSION

Figure 2 shows the results obtained for the conversion of substrates into product (flavor) and enzyme deactivation along the time for each temperature (30, 40, 50 and 60 ºC) and four substrate/enzyme ratios (5.00, 1.67, 0.83 and 0.55 mmol$_{substrate}$·min^{-1}·g^{-1}$_{enzyme}$).

An explicit relation between enzyme deactivation and substrate/enzyme ratio was observed for each temperature studied. Decreasing the substrate/enzyme ratio resulted in a greater deactivation time. In other words, the total time for enzyme deactivation increased with the amount of enzyme added to the bioreactor, in each temperature condition.

For all the conditions studied, the conversion of substrates into products decreased during the reaction, indicating a strong deactivation of the biocatalyst by the operational conditions, especially the temperature and the produced water.

At the temperature of 30 °C (Figure 1a) longer operations were observed. For a substrate/enzyme ratio of 0.55 mmol$_{substrate}$·min^{-1}·g^{-1}$_{enzyme}$ the substrate was exhausted after 640 min, approximately. This time interval was reduced when the reaction was carried out at warmer temperatures, resulting in a total deactivation time of about 300 min at 60 °C for a substrate/enzyme ratio of 0.55 mmol$_{substrate}$·min^{-1}·g^{-1}$_{enzyme}$. However, the highest initial conversion of 90% was observed at 40 °C (Figure 1b) for the substrate/enzyme ratio of 0.55 mmol$_{substrate}$·min^{-1}·g^{-1}$_{enzyme}$. The operation at 60 °C resulted in the lowest initial conversion for all substrate/enzyme ratios, not reaching 70% any case.

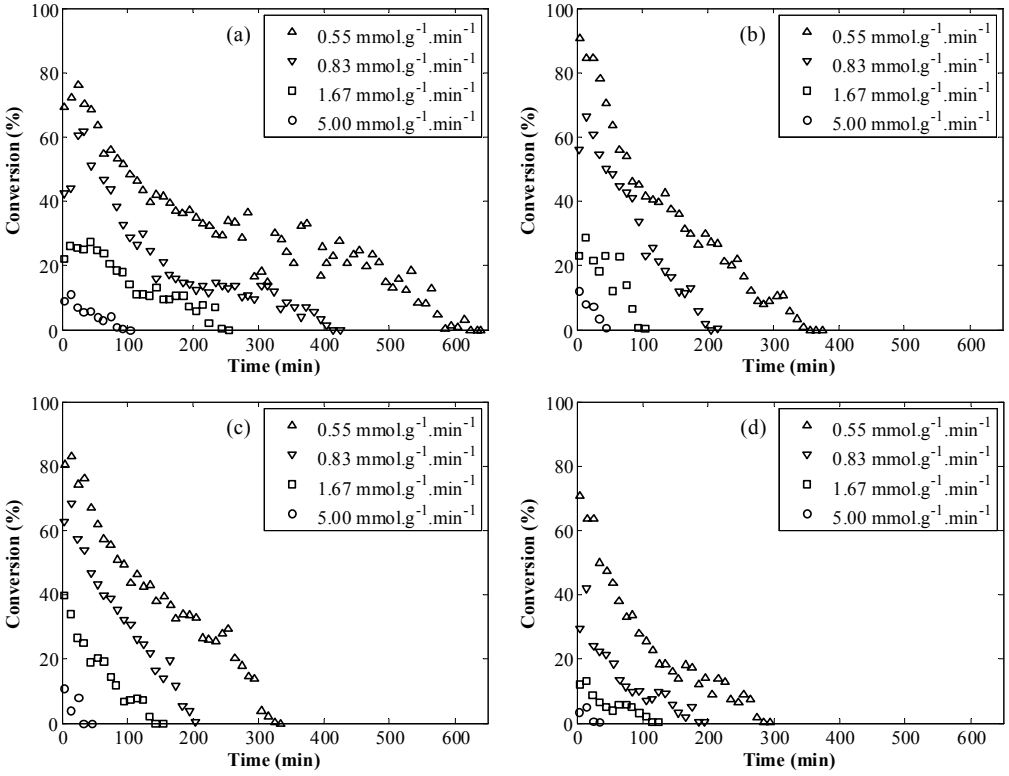

Figure 2: Conversion for reactions carried out at different temperatures and substrate/enzyme ratios: (a) 30 °C, (b) 40 °C, (c) 50 °C and (d) 60 °C.

This scenario is in conformity with the observations made by Abbas and Comeau (2003), who reported flavor synthesis in organic medium (cyclohexane) catalysed by lipase from *Mucor sp.*, free and immobilized on Amberlite IRC 50. The authors carried out reactions of propionic, butyric and caproic acids with methanol, ethanol, allyl, butanol, isoamyl, geraniol, citronellol and farnesol alcohols, in equimolar ratios. The optimum temperature for the immobilized enzyme was, approximately, 40 °C. As the temperature increased, the enzyme's activity decreased. According to the authors, most of the esters were obtained with a conversion greater than 90%. The same temperature was found to be suitable for *Rhizomucor miehei* reactions by Noel and Combes (2003) and the application of higher temperatures resulted in higher deactivation rates.

Using the immobilized lipase Lipozyme® IM-77, Chang *et al*. (2007) reported optimal conditions for hexyl laurate synthesis at the temperature of 45 °C, substrate molar ratio 1:2 and flow rate of 4.5 mL·min^{-1}, yielding a molar conversion of 97%. The reaction was carried out in a continuous packed bed reactor and *n*-hexane was used as solvent.

Furthermore, Ju *et al*. (2008) reported the synthesis of hexyl laurate in a continuous packed bed bioreactor from direct esterification of ethanol and lauric acid using a solvent-free system. The commercial lipase Lipozyme® IM-77, from *Rhizomucor miehei*, was applied. The optimum synthesis conditions were a temperature of 55 °C, flow rate of 0.5 mL·min^{-1}, concentration of lauric acid of 0.3 mol·L^{-1} and production rate of 81.58±1.76 μmol·min^{-1}, yielding a maximum molar conversion of approximately 60%.

Nevertheless, Dahlan *et al*. (2005) reported optimal synthesis of citronellyl butyrate in a packed bed reactor, using *n*-hexane as solvent and immobilized *Candida rugosa* as biocatalyst, at a flow rate of 1 mL·min^{-1} and temperature of 50 °C, yielding a maximum conversion of 95%.

Chen *et al*. (2011b) studied the synthesis of caffeic acid phenethyl ester in an ultrasound-assisted continuous packed bed bioreactor using immobilized enzyme from *Candida antarctica* (Novozym® 435) as biocatalyst. The authors found optimum operational conditions at the temperature of 72.66 °C, flow rate of 0.046 mL·min^{-1} and an ultrasonic power of 1.64 W·cm^{-2}. Moreover, a molar conversion of 92.11% was achieved and the enzyme remained stable for approximately 6 d.

Figure 3 shows the amount of pentyl octanoate accumulated as a function of enzyme loading into the bioreactor.

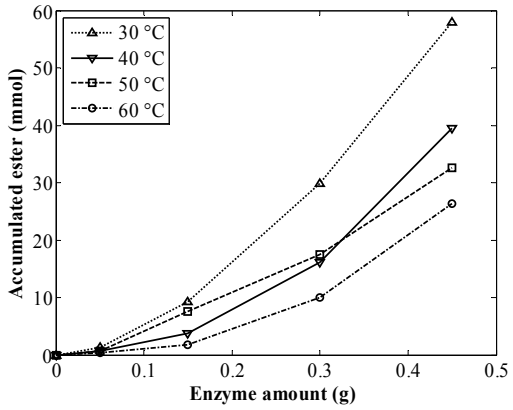

Figure 3: Amount of pentyl octanoate accumulated as a function of temperature and enzyme loading into the reactor. Continuous lines are shown for better visualization of the results.

The highest amount of flavor compound was observed when the reaction was carried out at 30 °C, yielding approximately 60 mmols of ester for an enzyme loading of 0.5 g into the bioreactor. Although the reaction showed higher initial conversions at 40 °C and 50 °C than at 30 °C, in the latter case the enzyme was stable for a longer time interval. Therefore, a balance of the effect of temperature on the reaction performance can be readily observed: while higher temperatures result in a higher specific reaction rate and, consequently, higher velocity, a secondary effect on the stability of the enzyme is triggered, resulting in a higher deactivation rate. As expected, Figure 3 also shows that the amount of ester obtained increased with the addition of enzyme into the bioreactor.

The results obtained confirmed the presence of pentyl octanoate at the outlet of the bioreactor, considering that the two peaks observed at 1709 and 1736 cm^{-1} correspond to the carbonyl groups of octanoic acid and the ester, respectively.

Effect of Temperature and Water on the Conversion of the Reaction

Several models have been proposed for the analysis of enzyme stability. Many of them, however, rely on multiparametric equations, derived from multiple stage mechanisms. In this sense, a two-step mechanistic model, which takes into account first-order kinetics of formation of an intermediate state and its decomposition to form the deactivated state, has been applied by some authors (Cavaille-Lefebvre

and Combes, 1998; Noel and Combes, 2003). On the other hand, simple models are available, which can provide valuable insight about the underlying mechanism, as well as quantitative indications about the system behavior with only a few, but meaningful, parameters. The simplest model proposed for the evaluation of enzymatic deactivation consists of a single step first-order kinetics (Illeova *et al*., 2003; Borda *et al*., 2004; Ladero *et al*. 2006), where the enzyme evolves from its original active state (ε_1) to a deactivated state (ε_2) according to Equation (3):

$$-da/dt = k_d \cdot a, \tag{3}$$

where k_d is the first-order deactivation constant (min^{-1}), which takes into account the effect of the temperature and the water produced by the reaction, a is the activity of the enzyme (dimensionless) and t is time (min). Integration of Equation (3) from an initial state, here defined by α (dimensionless), to a deactivated state in a given time t (min), defined by A_{res} (dimensionless), results in the simple expression given by Equation (4):

$$A_{res} = \alpha \cdot e^{-k_d \cdot t}, \tag{4}$$

Figure 4 shows the results obtained for the fitting of Equation (4) to each set of experimental data, i.e., at each temperature and substrate/enzyme ratio studied.

The adjusted model captured the tendency of the activity decreasing due to temperature and the water formed along the reaction for all the cases studied. In particular, good results were achieved at early times.

Table 1 presents the parameters α and k_d for the deactivation model adjusted to the experimental data.

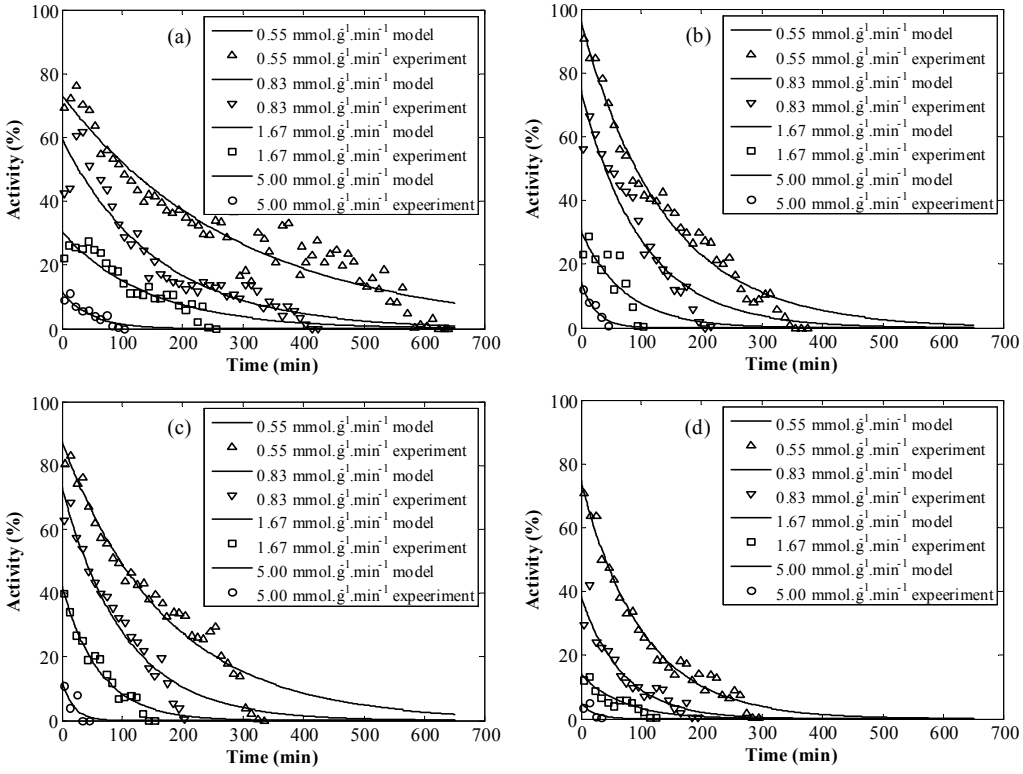

Figure 4: Comparison of predicted and experimental profiles for reactions carried out at different temperatures and substrate/enzyme ratios: (a) 30 °C, (b) 40 °C, (c) 50 °C and (d) 60 °C.

Table 1: Parameters adjusted for the first-order deactivation model due to the temperature and the water produced during the reaction on *Rhizomucor miehei* lipase (RML).

Temperature (°C)	Substrate/enzyme ratio ($mmol_{substrate} \cdot min^{-1} \cdot g^{-1}_{enzyme}$)	α (%)	k_d (min^{-1})
30	0.55	73.25	3.39×10^{-3}
	0.83	59.55	6.35×10^{-3}
	1.67	30.39	7.01×10^{-3}
	5.00	11.62	20.7×10^{-3}
40	0.55	96.10	7.17×10^{-3}
	0.83	74.33	9.94×10^{-3}
	1.67	30.28	13.7×10^{-3}
	5.00	14.41	39.7×10^{-3}
50	0.55	87.45	5.80×10^{-3}
	0.83	73.13	9.68×10^{-3}
	1.67	42.23	16.3×10^{-3}
	5.00	12.34	52.0×10^{-3}
60	0.55	74.62	9.85×10^{-3}
	0.83	38.36	14.3×10^{-3}
	1.67	14.10	17.6×10^{-3}
	5.00	4.978	62.7×10^{-3}

The deactivation constant showed a direct dependence on the temperature and the substrate/enzyme ratio. The higher the temperature and the higher the substrate/enzyme ratio, more pronounced the deactivation that was observed. In particular, an exponential behavior was observed for k_d as the substrate/enzyme ratio increased. However, for low substrate/enzyme ratios, there is a tendency of k_d to collapse to small values, roughly commom for all cases. This analysis can be easily verified in Figure 5.

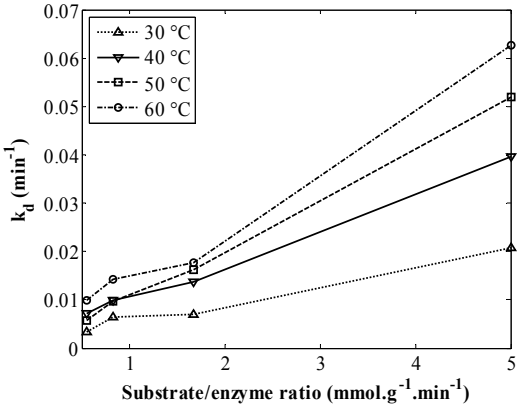

Figure 5: Deactivation constant (k_d) profile for each temperature studied (30, 40, 50 and 60 °C) as a function of the substrate/enzyme ratio. Continuous lines are shown for better visualization of the results.

Noel and Combes (2003) studied the thermal stability of RML in aqueous solution. In particular, a powder dispersion with a concentration of 1 $g \cdot L^{-1}$ was used. The authors reported deactivation constants in the range of 1.2×10^{-3} to 138.6×10^{-3} min^{-1} for temperatures of 40 to 60 °C, respectively. It should be pointed out that, although the systems studied by Noel and Combes (2003) and in this work are inherently different, the values of the thermal deactivation rate are of the same order of magnitude.

As previously discussed, the preparation of the enzyme for use in this reaction medium may have a pronounced effect on its performance (Rodrigues and Fernandez-Lafuente, 2010a). This makes the direct comparison of the results of this paper with other works a complex task (Rodrigues and Fernandez-Lafuente, 2010a). Moreover, it should be noted that different immobilization protocols might further improve the results obtained in this paper.

CONCLUSIONS

Immobilized lipase Lipozyme® RM IM can be applied in continuous bioreactors for ester synthesis. However, deactivation of the biocatalyst can be observed during the reaction as a function of temperature and the substrate/enzyme ratio, as well as water produced by the esterification reaction. Higher conversions were obtained at 40 ºC, while a larger amount of ester was produced when the reaction was carried out at 30 ºC. Further studies aiming to verify the reusability of the enzyme in reaction under the same conditions used in this work are important and will be conducted.

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NOMENCLATURE

Latin Letters

A_{res}	residual activity of the enzyme	dimensionless
a_w	water activity	$g_{water} \cdot g_{enzyme}^{-1}$
C_{A0}	initial concentration of substrate	$mmol \cdot L^{-1}$
k_d	first-order deactivation constant	min^{-1}
m	accumulated amount of ester	mmol
Q	volumetric flow	$L \cdot min^{-1}$
t	time	min
w	amount of enzyme	g
X(t)	conversion of the reaction as a function of time	dimensionless

Greek Letters

α	initial activity of the enzyme, dimensionless
φ	feed ratio, dimensionless

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