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Effect of several reaction parameters in the solvent-free ethyl oleate synthesis using *Candida rugosa* lipase immobilised on polypropylene

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Abstract

Lipase from *Candida rugosa* was immobilised onto polypropylene powder by physical adsorption. The immobilised catalyst (CR/PP) was used in the enzymatic synthesis of ethyl oleate in solvent-free medium. The influence of the initial water content, acidity of the aqueous media added, mass of catalyst, reaction temperature, substrate ratio, etc., on enzymatic activity, has been studied. Comparison of specific enzymatic activities achieved using the prepared catalyst and the crude lipase demonstrated that *C. rugosa* lipase was interfacially activated upon its adsorption on polypropylene. Besides, immobilisation of the lipase led to enhanced thermal stability.

Experimental data reported in this manuscript do not belong to equilibrium data but to enzymatic activity attained in the first 2 h of reaction. After this period, it was shown that, in the current synthesis, deactivation/agglomeration/inhibition of the catalyst prevented further CRL activity. However, 2 h measurements allowed fulfilling the aim of this work: the determination of the best conditions for ethyl oleate production in short periods of time, using an immobilised derivative of a relatively cheap lipase as it is *C. rugosa* lipase. Best results were achieved in the reaction performed at 45 °C and 350 rpm, with an initial stoichiometric ratio of substrates, 20% of aqueous content, and mediated by 50 mg of CR/PP (0.0585 mmol/mg of CR-h). The deleterious effect of ethanol excess and agglomeration of the native and immobilised catalyst have been analysed.

Keywords: Ethyl oleate synthesis; Lipase; Reaction parameters; Aggregation; Inhibiting effect

1. Introduction

The wide variation in composition and properties of esters makes them one of the most important classes of organic compounds. They find widespread uses as perfumes, cosmetics, solvents, plasticisers, flavours, in medicine and in the pharmaceutical industry. Ester synthesis by means of either free or immobilised lipases, is becoming a topic of growing interest and has recently received greater consideration relative to the traditional chemical synthetic methods, particularly in the production of natural flavours and fragrances [1]. This is due to the regio- and stereospecificity expressed by most lipases, the mild operation conditions, the degree of purity of the obtained products, and their acceptability in the food industry. Moreover, since enzymes are active at moderate temperature, pressure and pH conditions, energy costs become lower and no corrosion-resistant equipment is needed.

Candida rugosa, formerly *Candida cylindraceae*, has been the microorganism most frequently used for lipase-catalyzed synthesis [2]. *C. rugosa* lipase (CRL) is an attractive biocatalyst because of its high activity not only in hydrolysis but also in synthesis reactions. Moreover, it is one of the cheapest lipases available. Immobilisation of lipases allows their use in continuous processes, easy separation of product from enzyme and lipase reuse. Among the immobilisation methods, adsorption is the one most commonly used with CRL. In particular, adsorption onto polypropylene has been reported [3,4].

Lipases are known to contain an amino acid lid covering their active site, which opens in presence of a hydrophobic/hydrophilic interface. This phenomenon, called interfacial activation, has been found for *C. rugosa* lipase upon its

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adsorption onto hydrophobic supports [5]. The immobilised lipase is then fixed in an "open conformation" and enhanced enzymatic activity is achieved. In this contribution, lipase from *C. rugosa* has been successfully activated by its adsorption onto a non-porous polypropylene powder.

To improve the economic feasibility of the process, reactors for enzymatic esterifications must be small and the degree of conversion of substrates to products high. One possibility for increasing the efficiency is to perform the reaction without addition of solvents, whenever possible. This makes the reactor volume smaller, the substrate concentrations higher, and avoids additional solvent-recovery operations. However, when solvent-free systems are studied, fluidodynamics of the reaction media must be carefully addressed.

Each ester synthesis is a specific problem. This work is aimed to optimise the conditions for ethyl oleate synthesis catalysed by lipase from *C. rugosa* immobilised onto low-molecular weight polypropylene powder in the absence of solvent. Water content, reaction temperature, catalyst mass, substrate ratio and alcohol chain length, are some of the variables that have been investigated in order to find the best experimental conditions to achieve high yields of ethyl oleate using PP-supported-*C. rugosa* lipase. The role of water (initially added or generated in reaction) in the formation of a biphasic system is discussed and analysed. In the open literature there are no reports about the use of PP-supported-*C. rugosa* lipase for this ester synthesis performed in solvent-free (SF) media. Moreover, at the author's knowledge, this is the first time that a biphasic system for CRL-mediated-ethyl oleate synthesis has been analysed at this level of detail.

2. Experimental

2.1. Materials

Low molecular weight non-porous polypropylene powder (30 000 g/mol–23 m^2/g, particle size from 590 to 1100 μm) was obtained by polymerization using metallocenes. *C. rugosa* AY lipase (65 000 g/mol) was kindly donated by Amano Enzyme Inc. Oleic acid (99%) was purchased from J.T. Baker. The commercial biocatalyst Novozyme 435 (10 000 PLU/g), was a gift of Novozymes, Denmark. Absolute ethanol (99%) and sulphuric ether (99%) were both purchased from Dorwil. Buffer solution of pH 7 (di-sodium hydrogenophosphate), and potassium hydroxide were both from Merck. The solution is 2.5 mmol/kg KH_2PO_4 and 2.5 mmol/kg Na_2HPO_4. Octane used in preactivation experiments was provided by Laboratory Ceblaco.

2.2. Definitions

The initial molar ratio N is defined as initial mol of alcohol/initial mol of fatty acid (FA, in this case, oleic acid). The initial water content percentage, W, is defined as initial mass of water/initial mass of fatty acids (initial g water/g FA × 100). When SFS are analysed, low contents of water such as 1% (g/g FA × 100) reach water activities (a_w) near 1 [6]. Therefore, since for the water percentages used in this contribution a_w is near 1 in all cases, data is presented in terms of concentration of water (W) instead of water activity.

Biocatalyst mass (w_0) refers to the total mass of *free lipase* used (in the case of CR/PP the amount of effective lipase present in the total catalyst mass around 20–25% (w/w) of CR/PP [7]).

2.3. Reporting the performance of CR (free and immobilised)

Generally, in enzymatic reactions, data are reported in terms of initial specific enzymatic activity or initial rate for each condition. Conversion data are obtained for the first minutes of the reaction, linearized, and from the slope reaction rates are obtained. However, comparisons must be done in more general terms. A catalyst can be initially very active but easily deactivated, especially a biocatalyst including a lipase, and, most importantly, in a highly concentrated system as it is a solvent-free medium.

The use of conversion to present data does not allow complete understanding of catalyst performance either. Two catalysts may attain the same conversion but if the time needed to do it is not reported, fair comparisons cannot be make. Besides, the same conversion can be obtained with 200 mg of a catalyst than 50 mg of another catalyst achieves in the same time, and performance differences would not become evident unless data per unit of mass of catalyst are presented.

In view of the previous concepts, in this contribution data have been presented in terms of specific enzymatic activities calculated as follows:

$$\text{specific enzymatic activity } \left(\frac{\text{mmol converted fatty acids}}{\text{h mg}}\right) \cong \frac{(N_{FA})^0 \times (X_{ac})_t}{t w_0}$$

where $(N_{FA})^0$is the initial mmol of fatty acid (in this case mmol of oleic acid); $(X_{ac})_t$ is the conversion of fatty acids at time t; t is the time (hours of reaction); w_0 is the CRL present in total biocatalyst mass. Except for kinetic studies, data of reactions catalysed by CRL or by CR/PP belong to 2 h measurements. This period of time assured that the maximum activity attainable with CR derivatives for the present synthesis had been reached.

2.4. Immobilisation procedure

Four hundred milligrams of CRL were added to 50 ml of buffer solution of pH 7, and subjected to strong stirring during 30 min in order to solubilize lipase. A filtering step was performed in order to retain carbohydrates and other insoluble compounds. A gram of ethanol pre-treated PP with particle diameter in the range of 590–1180 μm [8] was added

to lipase solution and immobilisation began. Adsorption was performed at room temperature in a neutral buffer medium of ionic strength equal to 0.014 M. Samples from supernatant solution were periodically withdrawn, filtrated with special filters for small particle's powder, and diluted with buffer of pH 7 up to 5 ml, for later UV–vis analysis of lipase content. At the end of the immobilisation period, the catalyst was washed with distilled water, separated from solution by filtration and dried to constant weight at 45 °C. UV–vis measurements revealed a lipase content of 20–25% (w/w).

2.5. Esterification reaction

Typical reaction consisted in the direct esterification of 3 g of oleic acid and 0.5 g of ethanol ($N = 1$). 0.6 g of buffer of pH 7 (W = 20% = initial mass of aqueous solution/initial mass of oleic acid × 100) were typically added to reaction mixture. The reactors were 10 ml vials, which were kept at 45 °C and stirred at 350 rpm for 2 h (except when kinetics studies were performed). Reaction began when the biocatalyst was added to the reactants. Two-hour-samples (near 0.1 g) were withdrawn using a needle inserted through the stopper of the reactor-vial. They were dissolved in an ethanol-sulphuric ether 50/50% (v/v) mixture (5 ml/5 ml), and analysed titrimetrically by duplicate or even triplicate for the residual acid content using a basic solution of potassium hydroxide (0.05N) standardised with dried potassium-monoacid biphtalate. Phenolphthalein was used as the end-point indicator. The percentage of conversion of fatty acids was calculated comparing the obtained value of fatty acid with that at the beginning of the reaction. Results were then converted to units of specific enzymatic activity.

2.6. Kinetic studies

Kinetic studies were performed with CR/PP previously treated with octane–buffer 5%/95% (v/v) mixture at 45 °C by 30 min. A recent work from our group demonstrated that this pretreatment reduces agglomeration of supported lipases and interfacially activates CRL [9]. The use of just a few samples of three individual reproducible experiments allowed kinetics building with several points, with no need of additional scale change in order to keep reaction mixture mass (4.1 g) approximately constant. Data are reported in terms of converted mmol of oleic acid per mg of CR.

2.7. Equilibrium data

Literature generally calls and reports equilibrium values for when no further catalytic activity is found. However, it must be considered that enzyme-catalysed reactions can stop because of lipase deactivation, a phenomenon caused by different factors such as temperature, shear stresses, exposure to interfaces, and chemical denaturants like alcohols. Therefore, if the biocatalyst deactivates catalysed reactions stop even if *true reaction equilibrium is far from been achieved*. At least in the conditions assayed in this work, this was the case of CR derivatives. Inhibition due to ethanol and fatty acid stopped the progress of the catalysed reaction at conversions (10–14%) far from equilibrium values (80–85% depending on conditions).

With the aim of experimentally determining equilibrium conversions, the commercial immobilised catalyst Novozyme 435 (free of the strong deactivation effects exhibited by CRL), was used. Fifty milligrams of Novozyme catalysed the SF-synthesis of ethyl oleate at 45 °C, with W in the range of 0–20%. Every time conversion measurements showed that activity had stopped, 50 mg of fresh catalyst were added. The operation was repeated until the addition of fresh catalyst did not led to further conversion increment.

2.8. Parameter study

To analyse the effect of different parameters on ethyl oleate production catalysed by CR/PP, operation conditions of typical reaction (for details see Section 2.5) were varied each at a time. Results were compared with data from the synthesis mediated by crude CRL, for equivalent lipase content. Interfacial activation of the adsorbed lipase was shown.

2.8.1. Effect of water content

W was varied from 0 to 20%. The effect of water content was studied using 10 mg of free CRL, 50 mg of CR/PP, and 50 mg of Novozyme 435, at 45 °C and 350 rpm. To test the influence of aqueous media acidity on specific enzymatic activity, distilled water was replaced by a neutral phosphate buffer solution.

2.8.2. Effect of temperature

The effect of temperature on the ethyl oleate synthesis esterification reaction was examined at the range of 35–75 °C with both free (10 mg) and immobilised lipase (50 mg), the last one pre-treated with an octane–buffer 5%/95% (v/v) mixture for 30 min at room temperature [9].

2.8.3. Amount of catalyst

Different masses of free and immobilised catalyst were added to the so-called "typical reaction", consisting of 3 g of oleic acid, 0.5 g of ethanol, and 0.6 g of pH 7 buffer (20%), carried out at 45 °C and 350 rpm for 2 h time. Biocatalyst agglomeration was studied.

2.8.4. Substrate ratio, N

Two values of N (1 and 1.5) – defined as mol of ethanol per mol of oleic acid – were assayed on the typical reaction using 50 mg of CR/PP. In other experiments, keeping these two values of N, the corresponding ethanol volume was added to the reaction mixture in two steps: at the beginning and after 1 h of reaction. Deactivation due to alcohol excess was studied.

3. Results and discussion

3.1. Kinetics and equilibrium

Fig. 1 shows the kinetics of ethyl oleate synthesis catalysed by 50 mg de octane–buffer 5%/95% (v/v) pre-treated CR/PP at 45 °C. Data shown in Fig. 1 reveal that only 30 min were enough to reach the maximum oleic acid conversion achievable in the current system using pre-treated CR/PP (0.134 mmol/mg of CR—equivalent to 14% of conversion). After this time, reaction proceeds with no catalyst intervention. Deactivation of CR/PP and not equilibrium issues, accounts for the absence of additional conversion of oleic acid. In the same conditions, after 2 h of reaction, Novozyme 435 showed conversions as high as 86%, proving that conversion found with CR/PP is not the equilibrium conversion, but the maximum conversion achievable with this catalyst in this system and under the specified operating conditions.

In reference to the determination of true equilibrium values, 50 mg of Novozyme were periodically added to reaction media with increasing aqueous content. When the adding of fresh Novozyme did not lead to further activity increment, measured conversion was assumed as equilibrium conversion. Results of equilibrium conversions achieved are summarized in Table 1.

3.2. Influence of aqueous content

Literature about lipase-mediated synthesis in non-aqueous media agrees with the idea that although reaction media may consist mainly of organic solvent or substrates (solvent-free systems), water is unavoidable in order to keep the enzyme active. Apart form maintaining the active tertiary structure of the enzyme, water is mechanistically involved in the progress of the reaction, and its content controls the activity demonstrated by the lipase, be either hydrolysis or synthesis [10]. Therefore, water content effect studies are of crucial importance in organic media synthesis catalysed by hydrolytic enzymes.

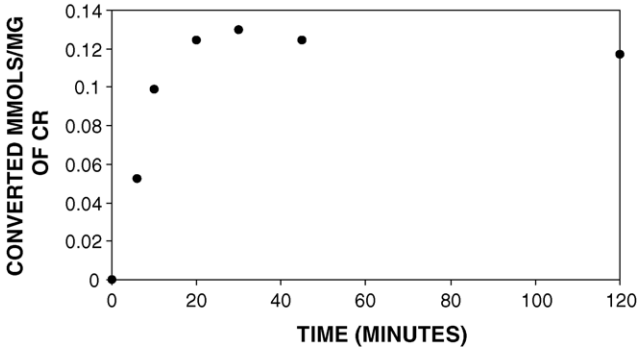

Fig. 1. Kinetics of ethyl oleate synthesis catalysed by 50 mg of octane–buffer 5%/95% (v/v) pretreated CR/PP. Temperature: 45 °C, stirring: 350 rpm. Twenty percentage of initial pH 7 buffer.

Table 1
Equilibrium conversion of ethyl oleate synthesis achieved using increasing amounts of Novozyme 435

W (%)	Equilibrium conversion (%)
0.0	86.5
4.8	82.3
9.9	81.9
22.3	79.7

Temperature: 45 °C, stirring: 350 rpm, $N = 1$.

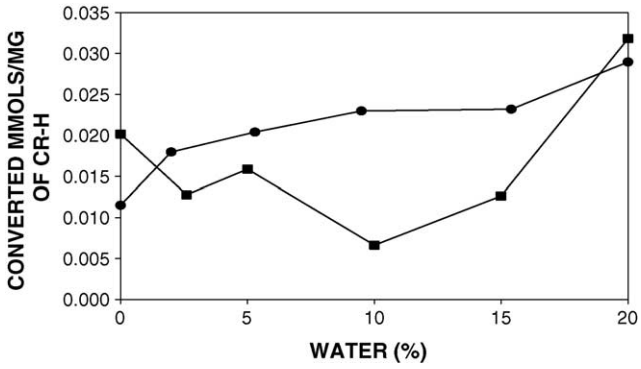

Fig. 2. Effect of initial water content in SF ethyl oleate synthesis catalysed by 10 mg of CRL (■) and 50 mg of CR/PP (●). Specific enzymatic activities (converted mmol of oleic acid/mg of lipase-hour) were measured after 2 h of reaction at 45 °C and 350 rpm.

Fig. 2 illustrates the variation of the specific enzymatic activity (converted mmol/mg of CR-h) with *W*. Most of the published works dealing with lipase-mediated reactions *in organic media* report lipase optimum synthetic activities for very low water contents (below 1–2%) [11,12]. However, in this solvent-free system, crude and immobilised *C. rugosa* lipase, exhibit highest activity at 20% water content. Increasing *W* up to 30% led to even higher conversions but, since emulsion nature prevented the withdrawn of representative samples, in the following experiments up to 20% of initial aqueous media was used.

It has been reported that, to make accurate comparisons, substrate concentration influence on optimum water content should be taken into account. By varying concentrations, different kinds of activity profiles can by obtained by the same enzyme. Wehtje and Adlercreutz showed that low substrate concentrations gave activity optimum at low water activity but at high substrate concentration the activity was enhanced with increasing water activity [13]. When using high substrate concentrations, reactions are not carried out in “pure” solvent, and this will affect the reaction medium. In the present solvent-free media, where “media is made of substrates”, the high substrate concentration seems to affect in a very important way the expected dependence of enzyme activity on water content.

Moreover, in this work we propose that the importance of water presence mainly deals with the number and kind of phases formed upon its addition to the mixture of substrates. While media with no water addition looked transparent and just one phase was observed, media with $W = 20\%$ looked opaque and two phases were clearly distinguished. Microphо-

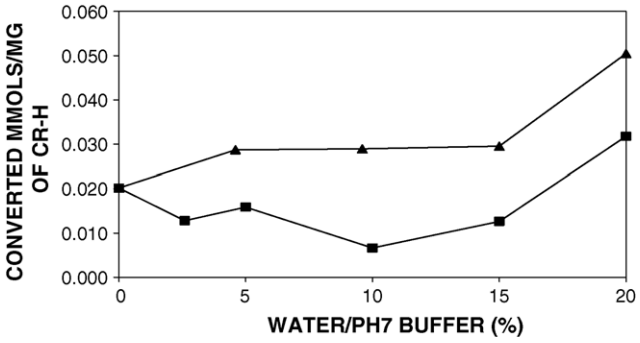

Fig. 3. Dependence on aqueous media content of ethyl oleate synthesis catalysed by 10 mg of CRL. Increasing amounts of water (■) and pH 7 buffer solution (▲) were added. Specific enzymatic activities were measured after 2 h of reaction at 45 °C and 350 rpm.

tographs demonstrated that for *W* above 5% the system becomes a polidisperse emulsion, with the size of the droplets increasing with the increase of water content. Then, when *W* higher than 5% are added the system we are dealing with is completely different from the monophasic systems found in synthesis performed in organic solvents, and even different from this solvent-free system with no water initially added. Therefore, the analysis is not a minor task and the effect of water cannot be considered only in the simple LeChatelier sense (water is a product of reaction, therefore increasing water would lead to lower ester generation).

The analysis of the true equilibrium constant in biphasic systems is really difficult and not totally developed yet. The main conclusion of references of Martinek et al. [14], Eggers et al. [15] and Voutsas et al. [16] is that the biphasic system composition affects the "equilibrium constant of a reaction", which is no longer a constant value. As Martinek et al. demonstrated several years ago, "biphasic equilibrium constants" differ from monophasic equilibrium constants, being the former sometimes several orders of magnitude higher than the latter [14]. Evidently, for the system under study, the formation of a second phase leads to a notorious increase in the conversion of reactants.

3.3. Influence of aqueous media acidity

According to data supplied by Amano Enzyme Inc., for a standardised esterification reaction performed in an aqueous system, CRL exhibits optima activity at pH 7. In the CR/PP-mediated typical reaction, when pH 7 buffer was used instead of distilled water (pH = 5.8), enzymatic activity was enhanced in more than 50% (see Fig. 3). Profiles obtained with both water and pH 7 buffer were very similar. However, it is evident that pH of the aqueous media contacted with lipase greatly influenced its activity.

It has been reported that free CRL exists in different aggregation forms depending on the pH of the medium in aqueous solution. At acidic pH, the relative proportion of high-molecular-weight forms of the enzyme is higher than at pH 7.0 [17]. In this way distilled water pH (5.8) would promote higher enzyme agglomeration than the neutral solution. Catalyst agglomeration decreases the effective number of lipase molecules available for catalysis, leading to a reduction in the measured enzymatic activity.

3.4. Effect of reaction temperature

The effect of temperature in ethyl oleate synthesis is shown in Fig. 4 for both CRL and CR/PP pre-treated with 5%/95% (v/v) octane–buffer mixture. For all reaction temperatures specific enzymatic activity developed by CR/PP pre-treated with octane–buffer was superior to the one obtained with the native lipase, showing the interfacial activation of lipase due to its adsorption onto an hydrophobic support.

Temperature maximum showed by enzymatic reactions is the result of two opposite consequences of temperature increase: reaction rate increment and deactivation rate increment. Due to its higher activation energy, when temperature gets higher, inactivation rate increases much more quickly than catalysis rate does.

For CRL, temperature maximum predicted for enzymatic reactions was found at about 65 °C. Above 65 °C CRL's activity severely decreased, showing at 75 °C a reduction of activity of 50%. For the adsorbed lipase, however, there was no clear evidence of temperature maximum. It has been widely reported that thermal deactivation of lipase can be reduced considerably by its immobilisation. Arroyo et al. [18] and Moreno et al. [19] have studied thermal deactivation of lipase B from *Candida antarctica* (CALB) and lipase from *C. rugosa*, in their native and immobilised forms. For both lipases, deactivation of the immobilised enzyme was much slower than that of native enzyme. In this work, immobilisation-induced rigidity achieved by CR adsorption onto PP, promoted thermal stability enhancement. Evidently, at least after 2 h of reaction, the stronger structure conferred by PP, allowed CR/PP to keep high activity for all temperatures assayed, even at 75 °C, a temperature level at which CRL underwent deactivation.

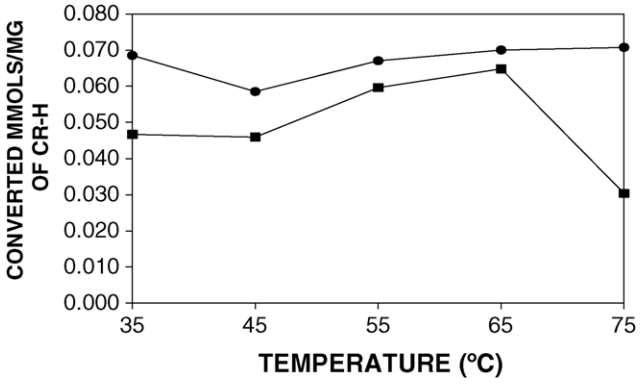

Fig. 4. Dependence on temperature of ethyl oleate synthesis catalysed by 10 mg of CRL (■) and 50 mg of CR/PP (●) pretreated with 5%/95% (v/v) octane/buffer mixture. Twenty percentage of pH 7 buffer was initially added to reaction media. Specific enzymatic activity was measured after 2 h of reaction at 350 rpm.

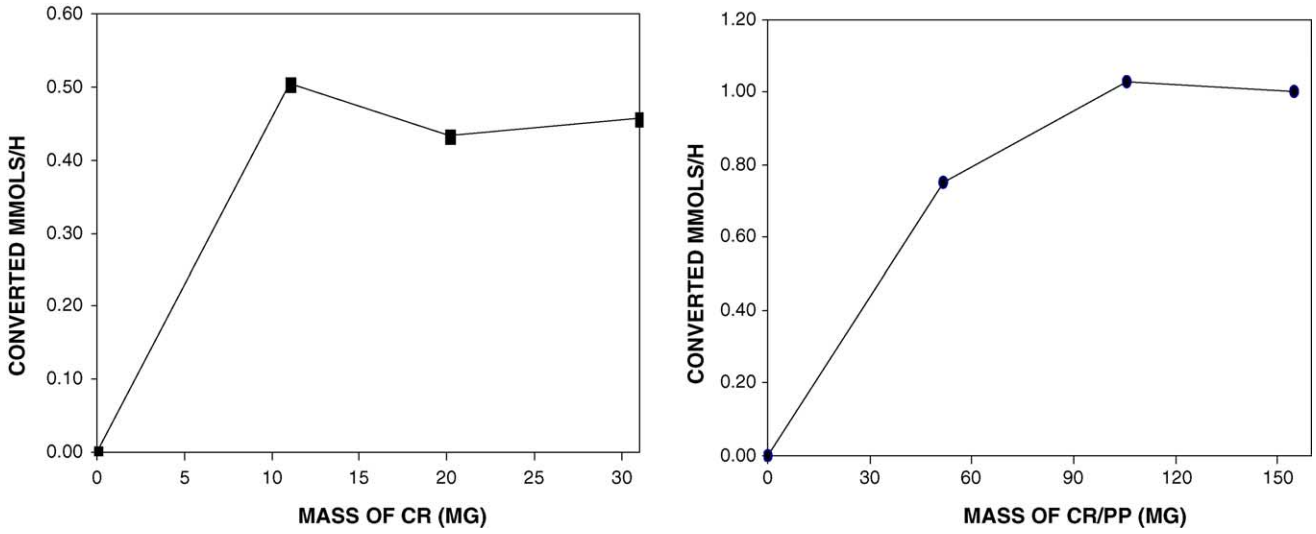

Fig. 5. Catalyst mass effect on 2 h enzymatic activity in ethyl oleate-CRL (■) and CR/PP (●)-mediated synthesis. CR/PP was pretreated with octane–buffer 5%/95% (v/v) mixture, 20% of pH 7 buffer was initially added to reaction media, $T = 45\,^{\circ}C$ and stirring rate = 350 rpm.

Comparing specific enzymatic activity response to temperature for CRL with the profile supplied by Amano Enzyme Inc., a notorious shift of temperature optimum is evidenced. According to Amano Enzyme Inc. CRL shows optimum activity between 40 and 50 °C. In this work, however, CRL exhibits highest activity in the range of 55–65 °C. Enhanced temperature optima determined for CRL in this system is a consequence of the different reaction media used in both experiments. While Amano Enzyme Inc. tested CRL in aqueous media, in the present study ethyl oleate synthesis was carried out in solvent-free media, a far more viscous reaction media than the one used by Amano Enzyme Inc. High viscosity of solvent-free media may maintain the lipase structure more rigid, preventing its unfolding and denaturation. Besides, in this kind of medium, higher temperatures are needed in order to reduce mass transfer resistances. Similar tendencies were found for CR/PP. Several publications that use immobilised CRL in organic media, report temperature optimums at about 40 °C [20,21].

3.5. Influence of catalyst mass

Linear relation between catalyst mass and conversion was found in a short range of catalyst concentration. Then, for both, native and immobilised lipase, enzymatic activity reached a plateau showing no increment for higher amounts of catalyst mass used (see Fig. 5). Linko et al., in the synthesis of butyl oleate with 3.2% of added water found the same behaviour for CRL. Increasing amounts of lipase led to higher yields only up to 1.5% of lipase, then, higher catalyst amounts did not lead to higher conversion yield [22]. Enzyme aggregation accounts for the finding. Intrinsic tryptophan fluorescence, surface hydrophobicity and dynamic light scattering (DLS) experiences demonstrated that CRL aggregation in aqueous media dramatically increases with enhanced protein concentration [23].

With immobilisation efficiency previously determined (around 200 mg of CR/g CR/PP), the effect of increasing amounts *of effective lipase mass* on enzymatic activity can be compared (see Fig. 6). Lipase activation due to its immobilisation onto PP is inarguable. Fig. 7 shows the effect of the mass of catalyst for reactions with 20% of distilled water and with 20% of buffer of pH 7. The beneficial effect of using neutral buffer as aqueous medium was verified for all amounts of catalyst tested.

3.6. Enzyme aggregation

Agglomeration using lipases free and immobilised CRL in solvent-free systems has been reported from several years ago [23]. Results shown in Figs. 5 and 6 confirmed the clumping CRL and CR/PP in solvent-free ethyl oleate synthesis. Aggregation of CRL in aqueous media has been reported [23,24].

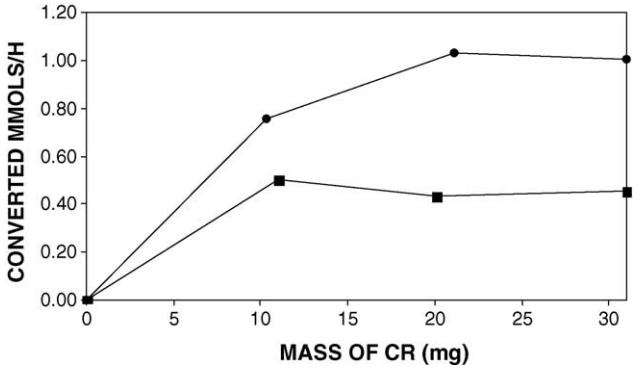

Fig. 6. Effect of effective CR present on the biocatalyst on activity of ethyl oleate synthesis using soluble CRL (■) and adsorbed CR (●). CR/PP was pretreated with octane–buffer 5%/95% (v/v) mixture, 20% of pH 7 buffer was initially added to reaction media, $T = 45\,^{\circ}C$ and stirring = 350 rpm. Effective mass of was calculated taking into account the amount of immobilised lipase (near 200–250 mg/g of insoluble catalyst).

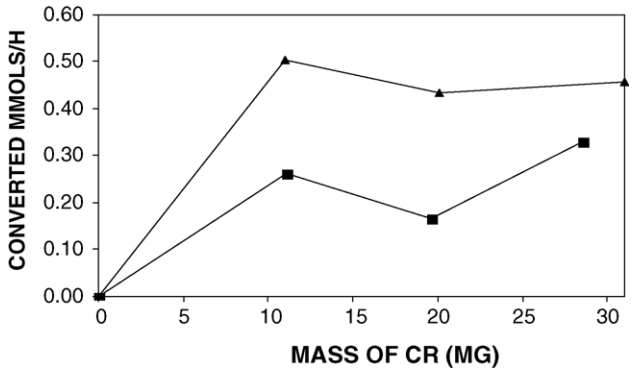

Fig. 7. Influence of CRL mass on ethyl oleate synthesis with initial 20% of water (■) and 20% of pH 7 buffer (▲). Specific enzymatic activities were measured after 2 h of reaction at 45 °C and 350 rpm.

Also in a non-aqueous environment, where attractive forces between enzymes are stronger than the ones responsible for their dispersal, particle formation has been reported [25]. In the present work, the high percentage of water added (20%), might have acted as a binding agent favouring agglomeration of particles while subjected to agitation in oleic acid.

Clumping leads to aggregate formation and inhomogeneous enzyme distribution. The enzymes molecules on the outer surface of such particles are exposed to high substrate concentrations (especially high when solvent-free media is used), but mass transport into a particle of clumped catalyst (free or immobilised) can severely limit the concentration of substrate inside the particle. Then, just the fraction of the catalyst added that remains on the outer surface of the agglomerates, is truly available for catalysis. Substrates have reduced access to catalytic molecules inside agglomerates, being the efficiency of the milligram of catalyst added to reaction mixture seriously reduced.

It has been reported that enzyme clumping can be overcome by its immobilisation on a support, which offers a high surface area to spread the enzyme over a larger area [24,25]. In this way, lipase is exposed to a much more homogeneous substrate concentration. Even though in these experiments immobilisation on PP did not completely eliminate catalyst clumping, the phenomenon was clearly reduced (see Fig. 6). Incubation of CR/PP with 5%/95% octane/buffer pH 7 by 30 min at 45 °C also helped in reducing CR/PP agglomeration.

3.7. The medium initially faced by lipase

The medium initially faced by lipase greatly influences lipase behaviour. When 30 mg of free CR lipase were added to 0.6 g of water, and typical amounts of reactants were added immediately after, 4 h of reaction at 45 °C and 650 rpm of stirring led to an specific enzymatic activity 30% lower than the one obtained in a second experiment in which lipase was added to *the whole reaction mixture* (instead of being previously contacted with water). Apparently, if lipase is contacted with a pure aqueous medium, much higher aggregation of catalyst occurs, and no strong mixture shaking (650 rpm) is capable of reversing aggregate formation.

The importance of the medium initially faced by lipase on its agglomeration has been previously introduced in the esterification of octanoic acid with methanol in cyclohexane with 2% (v/v) of water [24]. In this synthesis *simultaneous* addition of CRL and silica gel to reaction mixture, showed that silica was able to reduce lipase agglomeration. However, if silica gel was added *after* the addition of the enzyme powder, no reversal of lipase agglomeration was detected, with similar activity to the one find with no silica gel addition [24].

3.8. Influence of substrate ratio, N (EtOH mol/oleic acid mol)

Specific enzymatic activity found in typical reaction was strongly affected by the use of substrate molar ratios higher than the stoichiometric ratio ($N = 1$). The use of a substrate ratio of 1.5 reduced the specific enzymatic activity by nearly a half (see Fig. 8), showing *C. rugosa* lipase inhibition due to ethanol excess. The loss of activity of the biocatalyst with increasing ethanol concentrations might be concerned with dehydrating effects on the protein molecule [26]. Besides, a deleterious effect on the activated active site of lipase because of side reactions of ethanol (and even oleic acid) with the catalytic triad can also be responsible for the reduced enzymatic activity measured.

Experiments in which ethanol was added to the reaction mixture in two steps (at the beginning and after 1 h of reaction) were performed for $N = 1$ and 1.5. In the first case, dosing of ethanol lead to a measured enzymatic activity 20% lower than the one obtained when all ethanol was added at the beginning. When N was kept in 1.5, the specific enzymatic activity achieved was the lowest (three times lower than $N = 1$ and one alcohol addition). Reduction of the initial rate of reaction due to the initial addition of a substoichiometric relationship of ethanol, and higher lipase inhibition due to ethanol excess in the second hour of reaction explains the observed decrease in activity.

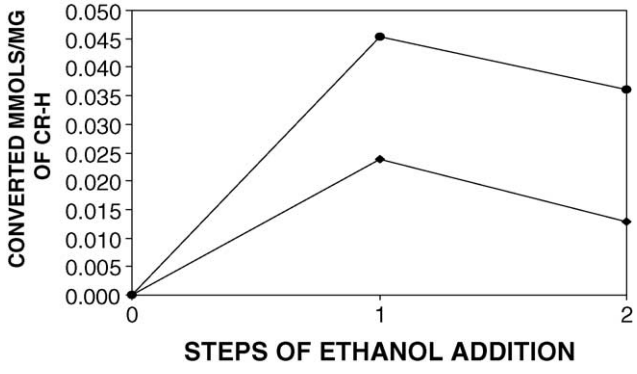

Fig. 8. Dependence of specific enzymatic activity in ethyl oleate synthesis with substrate ratio, $N = 1$ (●), $N = 1.5$ (♦). Effect of ethanol dosification. Reaction was carried out at 45 °C and 350 rpm, 50 mg of CR/PP, 20% of initial pH 7 buffer.

Among the tested conditions, stoichiometric substrate ratio addition at the beginning of reaction showed to be the best option, which is in agreement with most literature dealing with lipase-mediated esterifications [25,27–29]. Ethanol substoichiometric substrate ratios reduced rate of reaction and prohibit total oleic acid conversion. On the other hand, substrate ratios higher than unity stopped enzymatic reaction due to lipase inhibition caused by ethanol.

4. Conclusion

C. rugosa lipase demonstrated to be active in oleic acid esterification carried out in solvent-free systems. When immobilised by simple adsorption onto powdered polypropylene, the catalyst showed enhanced activity and higher thermal stability, with no catalyst deactivation detected up to 75 °C. Even if conversions are low, operation in solvent-free highly concentrated systems led to important ester formation in just 30 min of reaction. The addition of relatively high amounts of aqueous medium caused the formation of an emulsioned system in which both, native CRL and CR/PP, developed maximum activity. In particular, the highest specific enzymatic activity was obtained using 50 mg of supported CRL, 20% of pH 7 buffer solution, $N = 1$, 45 °C and 350 rpm.

Agglomeration effects due to water, and deactivation due to ethanol inhibition are the most important problems to solve in order to increase the specific enzymatic activity achieved in a solvent-free system like the one studied in this contribution, using free and PP-immobilised CRL.

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