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Solvent-free ethyl oleate synthesis mediated by lipase from *Candida antarctica B* adsorbed on polypropylene powder

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Abstract

The enzymatic synthesis of ethyl oleate by direct esterification of oleic acid and ethanol in solvent-free media has been studied. Native lipase from *Candida antarctica B* and *Candida antarctica B* lipase adsorbed on powdered polypropylene were used with promising results. The influence of different parameters such us temperature, mass of lipase and aqueous content of reaction medium, on conversion profiles has been carefully studied. High water contents that ensured the co-existence of two liquid phases gave the best results, with conversions of up to 78.6% in 7 h of reaction. Pre-treatment with octane/buffer mixtures significantly reduced agglomeration of the immobilized catalyst, leading to important increments in specific enzymatic activity, when compared with non-pre-treated biocatalyst. Lipase desorption from PP has also been studied.

Keywords: Ethyl oleate; Lipase; Reaction parameters; Agglomeration; Desorption

1. Introduction

In the last years, enzymes have shown to be powerful catalysts for a number of reactions. In comparison with chemical catalysts, enzymes show higher selectivity, they work in milder conditions, and they are environmentally friendlier. Lipases (EC 3.1.1.3) are a family of enzymes that in their natural environment catalyze the hydrolysis of fats. However, in the appropriate reaction media, lipases have shown to be very active in synthetic bio-catalysis of reactions of esterification of fatty acids, alcoholysis and *trans*-esterification [1–4].

Synthetic reactions catalyzed by lipases may be performed in aqueous media, in organic solvents, in supercritical fluids [5], in ionic liquids [6] or, alternatively, in solvent-free systems [7–9]. Solvent-free systems (SFS) are highly concentrated media, economically and operationally interesting for industrial processes. In this kind of systems not only the cost of the solvent itself is avoided, but also its separation from un-reacted substrates and products, and the cost of recycle as well.

In the current work, the enzymatic synthesis of ethyl oleate has been studied. Ethyl oleate is a fatty ester which finds wide application in cosmetic and food additives industries, in the production of tailored triglycerides or in diesel fuel additives [10–11]. No solvent has been added to reaction medium consisting only of oleic acid, ethanol, variable percentages of added water, and native or immobilized lipase B from *Candida antarctica* (CALB). CALB is an interesting lipase with potential application in a number of industrial processes such as the synthesis of optically active compounds in the pharmaceutical industry [12], in the pulp and paper industry for pitch removal and de-inking processes [13] or in the synthesis of esters used in the flavor industry [14]. CALB has been mostly used in an immobilized form, commercially available from Novo Nordisk (*Novozyme 435*). However, this report concerns with the solvent-free synthesis of ethyl oleate catalyzed by CALB immobilized on a cheaper support such as polypropylene (PP) powder. CALB lipase has been previously adsorbed on EP100 with interesting results in the synthesis of 6-*O*-glucose palmitate [15].

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With the aim of optimizing the conditions for ethyl oleate synthesis, the influence of reaction temperature, mass of catalyst and water was carefully studied. The trends obtained in CALB-mediated ethyl oleate synthesis have been compared with a previous work performed using *Candida rugosa* lipase, native and supported on PP.

2. Experimental

2.1. Materials

Native lipase B from *Candida antarctica B* (5000 U/ml) was kindly supplied by Novozyme. Oleic acid (99%) was purchased from J.T. Baker. Absolute ethanol (99%) and sulphuric ether (99%) were both purchased from Dorwil. Buffer solution of pH 7 (di-sodium hydrogen-phosphate) and potasium hydroxide were both from Merck. Octane used in pre-treatment experiments was provided by Laboratory Ceblaco.

Low-molecular-weight polypropylene powder (30,000g/mol, BET area: 23 m^2/g) was obtained by polymerization using metallocenes. Commercial PP obtained with Ziegler-Natta catalysts (controlled particle diameter between 590 and 1190 um), and glass spheres of 1 mm diameter (purchased from Científica Nacional, Argentina) covered with PP, were both used as supports.

2.2. Immobilization procedure

The immobilization of lipase B from *Candida antarctica* was performed at room temperature for 7 h with 350 rpm stirring. 2.4 ml of the enzyme commercial solution (12,000 U) were diluted up to 50 ml with standard buffer of pH 7 and contacted with 1 g of ethanol pre-treated polypropylene (PP), following the method previously described in the immobilization of *Candida rugosa* lipase [16]. After the desired contact time, the insoluble material was recovered by filtration and washed with distilled water. Finally, the biocatalyst (CA/PP) was dried to constant weight at 45 °C. The same batch of CA/PP was used in the whole range of conditions tested.

2.3. Esterification reaction

In all experiments performed, reaction medium consisted of the stoichiometric mixture of substrates (N = moles of ethanol/moles of oleic acid), and different percentages of added water. Reaction was started by the addition of the biocatalyst (CALB or CA/PP) to reaction mixture, kept at 45 °C in 10 ml vials stirred at 350 rpm. During reaction (7 h) several samples were withdrawn and analyzed by titration for the residual acid content with a basic solution of potassium hydroxide. Phenolphthalein was used as the end-point indicator. The percentage of conversion of fatty acid at a definite t, was determined according to:

$$X(\%) = \frac{\text{initial moles of oleic acid} - \text{moles of oleic acid at time } t}{\text{initial moles of oleic acid}} \times 100 \quad (1)$$

2.4. Parameter study

2.4.1. Influence of the water content

When SFS are analyzed, low contents of water such as 1% (g of water/g of fatty acids × 100) reach water activities (a_w) near 1 [17]. Therefore, since for the water percentages used in this contribution a_w is near 1 in all cases, we have chosen to present data in terms of concentration of water (W: initial mass of water/initial mass of fatty acid × 100) instead of using water activities. The effect of increasing the initial water content of reaction medium was studied using 300 U of native lipase and 50 mg of CA/PP, at 45 °C and 350 rpm.

2.4.2. Influence of temperature on reaction

The effect of temperature on conversion achieved in ethyl oleate synthesis was examined at the temperature range of 35–75 °C with both free and immobilized lipase. For all reactions performed, W was kept in 20%.

2.4.3. Influence of the amount of lipase

In the case of the immobilized lipase (CA/PP) the amount of biocatalyst added to reaction medium was varied from 50 to 150 mg. In both experiments temperature was kept at 45 °C and W was 20%. Agglomeration effects were analyzed.

2.4.4. Pre-treatment of CA/PP with "oil–water" interfaces

Immobilized lipase (50 mg) were typically contacted with 5 ml of octane/buffer of pH 7 mixture for 30 min, at 45 °C and 1000 rpm. According to previous results obtained for *Candida rugosa* lipase adsorbed on PP, the mixture of octane/buffer in a volumetric ratio of 5/95 provided the immobilized catalyst with an "oil–water" interface that induced lipase activation [18]. After 30 min, the pre-treated catalyst was recovered by filtration and dried at 45 °C for 1 h. Increasing amounts of the pre-treated biocatalyst were used in ethyl oleate synthesis preformed at 45 °C with W = 20%. Activation of lipase was analyzed.

2.5. Desorption of lipase

Being CALB immobilized upon PP by simple adsorption, the desorption of lipase was checked. Reuse of CA/PP was assayed in order to determine if huge activity of CA/PP was due to the adsorbed lipase, or if it was actually caused by desorbed lipase catalyzing reaction in its native form. Several experiments using CA/PP, CALB adsorbed on PP obtained using commercial Ziegler-Natta catalysts, and CALB adsorbed on PP linked to glass spheres, were

performed. PP/glass support was prepared according to the procedure previously reported [19].

3. Results and discussion

3.1. Determination of the amount of CALB immobilized on PP

The extremely high discrepancy in the amount of immobilized lipase quantified by UV–vis methods, reported by different authors (using equal lipases and supports) has been previously pointed out [20]. A rigorous study of our group on the different ways to determine the amount of lipase immobilized on polypropylene powder revealed that UV–vis methods, (even the most simple one (at 280 nm) and also the most used ones (Bradford, Lowry, commercial kits for protein determinations)) have several drawbacks that make it difficult to obtain reproducible results of commercial lipase uptake when using PP as adsorbent. Due to the powdered nature of the solid, colloidal particles of PP generated during adsorption (stirring at 350 rpm) could not be removed from samples withdrawn from the immobilization vial by filtration or even by centrifugation techniques. Light dispersion due to remaining disperse/colloidal PP particles increased the values of absorbance of the whole UV–vis spectrum, leading to values unexpectedly high for proteins. When dealing with powdered solids, lipase adsorption of buffer ions during immobilization also occurred. Lipases have plenty of lateral groups, which may ionize and adsorb ions from the buffer, shifting buffer equilibrium. Since buffer adsorption bands appear in the same range as the protein bands (from 200 to 500 nm), the absorbance decrease due to lipase adsorption on PP cannot be isolated from the reduction caused by the change of concentration of buffer ions [20].

On the other hand, selective adsorption of lipase onto hydrophobic supports also prohibits accurate lipase quantification through elemental determination of N in solution, or through FTIR methods. Commercial preparations include other compounds apart from lipase which are not adsorbed at the immobilization step. Those other compounds present in the enzymatic preparation, but absent in the supported biocatalyst, do not account for nitrogen content, leading to a total nitrogen percentage lower than expected. ‘‘Purification’’ experimented by lipase when it was adsorbed on PP, was also illustrated through IR bands of the enzymatic commercial preparation much wider than the ones found in CR/PP and attributed to ‘‘purified’’ lipase only [20]. In the case of commercial powdered lipases (this is not the case of the CALB solution used in this contribution), inhomogeneities in the composition of the powdered preparation also occur.

The exhaustive analysis of the different methods for quantification of lipase adsorbed on PP clearly demonstrated that the best way to address the amount of lipase immobilized is a reaction. Esterification of fatty acids has been proposed as an adequate method to characterize immobilized lipases, especially CALB which is known by its synthetic properties. In this contribution, we have used the ethyl ester reaction to check the amount of lipase immobilized on PP. In this way, it has been found that 50 mg of CA/PP have the same activity than 0.06 ml of free CALB (300 U provided by the supplier (Novo)). This result demonstrated that about 50% of the lipase contacted with PP was effectively adsorbed (equivalent to 6000 lipase Units per gram of PP), a result previously obtained for the adsorption of *Candida rugosa* lipase on PP powder in equivalent conditions [20].

Considering the lipase Units provided by the supplier 50 mg of CA/PP have equivalent units than 0.06 ml of free CALB (300 U).

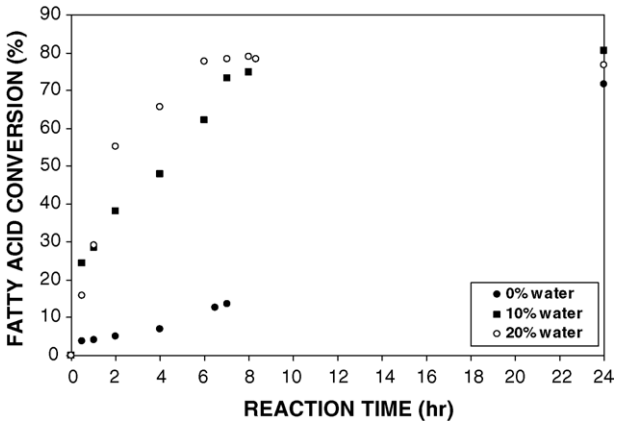

Fig. 1. Influence of water content (initial mass of water × 100/initial mass of oleic acid). CALB (300 U), 45 °C, 350 rpm, $N = 1$.

3.2. Influence of the water content

The effect of initial water content on the conversion to ethyl oleate catalyzed by native CALB is shown in Fig. 1. Presented data reveal two clearly different patterns. Reactions with 10 and 20% of water initially added show high initial rates, reaching final conversion after 8 h of reaction. Further time of reaction did not contribute to significant conversion increment. The lowest curve shows the time course for reaction with no water added (except for the water present in absolute ethanol, 0.2% v/v). The pattern developed shows lower initial rate and reduced conversions for the first hours of reaction, a time in which the experiments with added water reached final conversion. However, 24 h-conversion demonstrated a sudden increase in reaction rate, leading to a measured conversion similar to conversion found in the other experiments. Similar results were observed for native CALB in the esterification of (R, S)-ibuprofen in isooctane pre-equilibrated at different initial a_w values [21]. In that work, authors found highest esterification rates for initial a_w of the system higher than 0.8. The progress curve of reactions pre-equilibrated at an

initial $a_w = 0.35$ suddenly changed it shape above 20% conversion. The ''activation'' effect was explained in terms of the water production during reaction. In the same reaction, *Candida rugosa* lipases also showed greater esterification rates for initial a_w over 0.8 [22].

At this point it is worth discussing the system in which reactions took place. While reactions with $W = 10$ or $W = 20$ were conducted in a liquid–liquid system from the beginning of reaction, the experiment with no water added is, 'at least initially', a homogeneous system with a unique liquid phase in which ethanol and products of the reaction are soluble in the oleic acid.

Data has showed that for this synthesis in absence of solvents, the formation of a second aqueous phase allows greater reaction rates, maybe through the extraction of the water produced from the organic phase. The thermodynamics of biphasic systems have been scarcely studied [23,24]. Authors that analyzed these systems agree on the fact that biphasic systems can allow higher yields because of the shift of equilibrium conversion induced. For $W = 0$, thermodynamic equilibrium calculations predict the split into two phases at a conversion of 16% [25], letting us presume that the formation of a second aqueous phase is the responsible for rate acceleration after 7–8 h of reaction. In this way, with the advance of reaction, the system in which the synthesis is taking place changes, presumably from monophasic to microemulsioned. Microemulsions are macroscopically homogeneous and isotropic dispersions of one liquid phase into another, stabilized by an interfacial film of surfactant (in this case, oleic acid). However, on a microscopic scale microemulsions are two-phase systems structured into aqueous and oil microdomains. In the case of study, even though the aqueous phase produced is not visible at simple sight, a microemulsion is formed and thermodynamics of biphasic media applies.

The pattern of conversion developed by CALB adsorbed on PP (CA/PP) showed a notably similar behavior. Fig. 2 shows the conversion profile for the first 8 h of reaction catalyzed by CA/PP for increasing *W*.

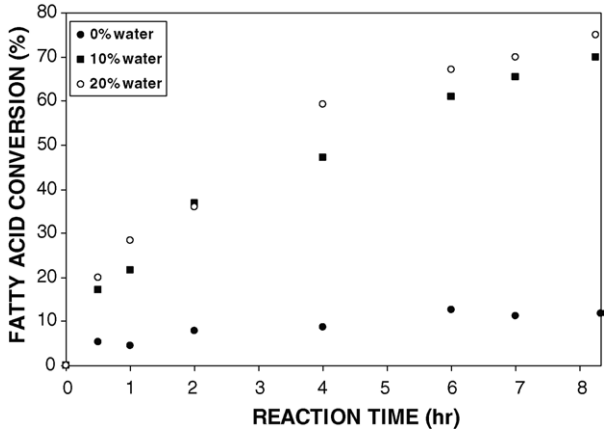

Fig. 2. Influence of water content (initial mass of water × 100/initial mass of oleic acid). CA/PP (50 mg), 45 °C, 350 rpm, $N = 1$.

Again, initially biphasic systems achieve final conversions in 8 h, while reaction with $W = 0$ enhanced its rate only after conversions higher than 10% were achieved (data not shown). Previous studies with lipase from *Candida rugosa* (native and adsorbed on PP) also showed highest conversions at $W = 20\%$ [26].

From Figs. 1 and 2 it is clear that with 300 U from CALB solution and with 50 mg of CA/PP *almost the same results in final conversion after* 8 h *are obtained with* 0, 10 *and* 20% *water*. Assuming that the immobilized lipase is in the same conformation than soluble lipase, we may consider that the same amount of CALB is present in 300 U of solution and in 50 mg of CA/PP.

3.3. Influence of reaction temperature

The pattern of temperature effects in an enzymatic reaction in low-water media is the same as in aqueous media. The initial rate of reaction increases with temperature, in the usual Arrhenius fashion. However, the stability of the enzyme will decline with temperature, and at high enough temperature catalytic activity will be lost rapidly before significant conversion is reached. The effect of temperature in ethyl oleate synthesis mediated by 300 U of native CALB is shown in Fig. 3.

For native lipase temperature optimum was found at 45 °C. Reaction at 35 and 65 °C gave also high conversions. At 75 °C, however, CALB's activity severely decreased, showing five-fold reduction in 7 h-conversion when compared with maximum yield. Evidently, operation at temperatures equal or higher than 75 °C considerably enhance deactivation rate leading to a drastic reduction of biocatalyst activity in short periods of time (note that at 75 °C conversion found in the first 60 min of reaction is almost the same that final conversion).

It has been said that thermal deactivation of lipase can be reduced considerably by its immobilization [27]. Fig. 4 shows the time courses of reaction catalyzed by CA/PP. It appears that adsorption of lipase onto PP, does not change the temperature optimum found for native CALB.

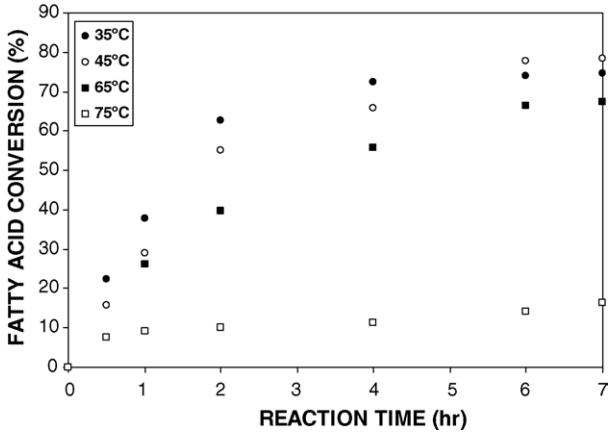

Fig. 3. Influence of temperature, 300 U CALB, $W = 20\%$, 350 rpm, $N = 1$.

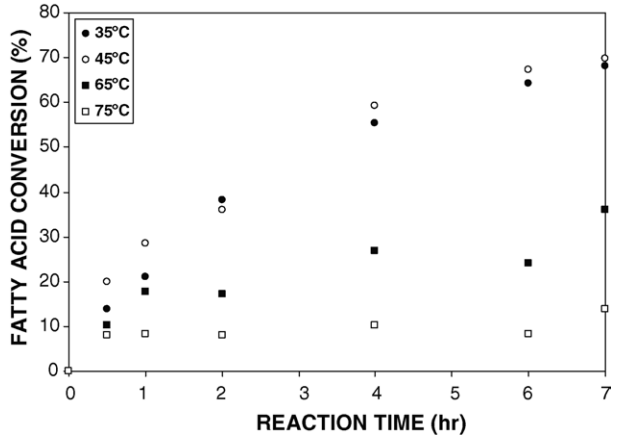

Fig. 4. Influence of temperature, 50 mg CA/PP, $W = 20\%$, 350 rpm, $N = 1$.

Thermal stability enhancement due to lipase adsorption on PP has been previously found for lipase from *Candida rugosa* in ethyl oleate synthesis [26]. However, in the same reaction mediated by CA/PP, the adsorbed lipase showed higher deactivation than free lipase did. Denaturing of CA/PP is specially noticeable at 65 °C, with conversion reduction seriously higher than the one shown by native CALB. Being lipase from *Candida antarctica* such an active catalyst, it appears that the induced rigidity conferred by PP far from enhancing lipase stability, it reduces lipase ability to show high activity at high temperatures. In the synthesis of 6-*O*-glucose palmitate by CALB/EP100 temperatures above 40 °C led to enzyme deactivation, with almost 35% loss in activity at 70 °C [15].

3.4. Influence of the amount of lipase

The mass of immobilized biocatalyst used in reaction at $W = 20\%$ and 45 °C, was increased from 50 to 150 mg. Fig. 5 shows that using twice more catalyst the specific enzymatic activity during reaction course is reduced to less than a half.

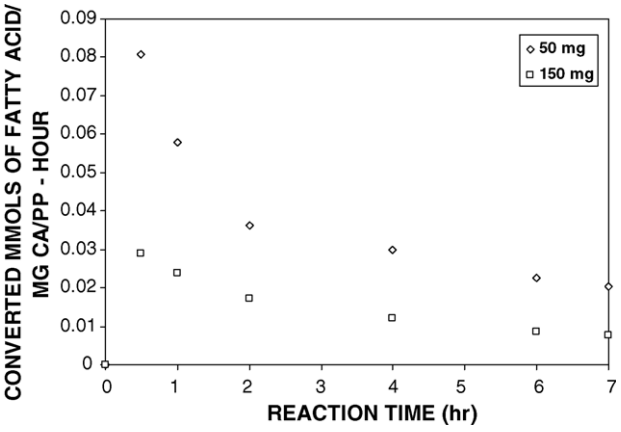

Fig. 5. Influence of increasing immobilized biocatalyst mass on specific enzymatic activity, $W = 20\%$, 45 °C, 350 rpm, $N = 1$.

Even if immobilized, agglomeration of the biocatalyst reduces its efficiency at masses higher than 50 mg. *Candida rugosa* lipase adsorbed on PP showed the same behavior [26]. Agglomeration using free and immobilized lipases in solvent-free systems has been reported from several years ago [28]. The phenomenon of 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 immobilized) can severely limit the concentration of substrate inside the particle. Lower activity of a fraction of biocatalyst reduces global yield, decreasing the efficiency per the mass unit of biocatalyst.

3.5. Octane/buffer pre-treatment

Maruyama et al., found that *Rhizopus japonicus* lipase activity was enhanced by oil–water activation with aliphatic hydrocarbons/water mixtures [29]. *Candida rugosa* lipase also showed an increment in conversion of near 40% when pre-treated with octane/buffer 5/95 v/v mixtures [18]. The immobilized form of *Candida antarctica* B lipase was contacted for 30 min with the mentioned octane/buffer mixture. Increasing amounts of the pre-treated biocatalyst catalyzed the esterification reaction. In Fig. 6, 2 h-conversion results are compared with non-pre-treated CA/PP data.

Different from *Candida rugosa* lipase [18], conversion achieved with 50 mg pre-treated CA/PP demonstrated that no activation took place. The difference in behavior observed is explained by the structure of the compared lipases. While the active site of *Candida rugosa* lipase is found at the end of an amino acid tunnel covered by a lid, CALB's active site is *not covered* by any amino acid flap. In the presence of hydrophobic/hydrophilic inter-phase, the

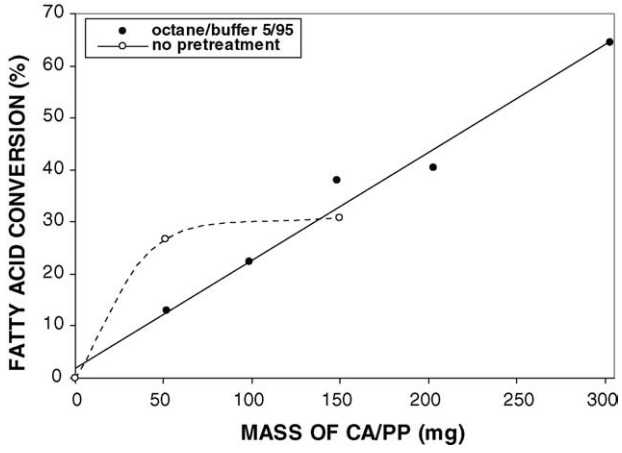

Fig. 6. Influence of immobilized biocatalyst mass. White circles correspond to untreated catalyst and black ones to octane/buffer 5/95 v/v pre-treated CA/PP: 2 h of reaction, $W = 20\%$, 45 °C, 350 rpm, $N = 1$.

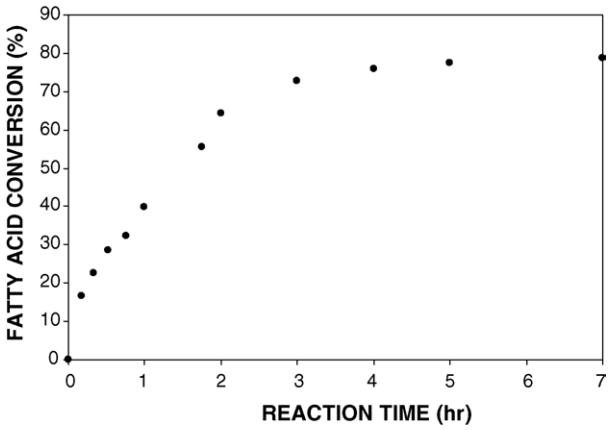

Fig. 7. Conversion profile with 300 mg of CA/PP pre-treated with 5 ml of octane/buffer of pH 7 5/95 v/v mixture: $W = 20\%$, 45 °C, 350 rpm, $N = 1$.

lid covering the active site of lipase from *Candida rugosa* is shifted allowing substrates access. Oil–water pre-treatments like the octane/buffer mixture assayed provided this lipase with the interface needed for so-called *interfacial activation*. On the other hand, lipase from *Candida antarctica* B, does not undergo interfacial activation [30,31]. Its tunnel is shorter than the amino acid tunnel of *Candida rugosa* lipase, and it contains two different pockets: one for the acyl type group (an elliptical funnel) and another for the alcohol [32].

The structure of CALB justifies the absence of any improvement in conversion upon pre-treatment with the octane/buffer mixture. However, for higher amounts of catalysts Fig. 6 clearly shows that octane/buffer pre-treatment reduces agglomeration of biocatalyst found for non-treated CA/PP (please go back to Fig. 5). The full line in Fig. 6 clearly depicts the reduction in the adverse clumping effects achieved with octane/buffer pre-treatment. While a conversion plateau is achieved for non-treated biocatalyst mass above 50 mg, increasing amounts of pre-treated CA/PP lead to greater conversions without agglomeration effects noticeable.

According to data presented, at least 300 mg of pre-treated CA/PP can be used with no conversion reduction due to biocatalyst's aggregation. Fig. 7 shows the complete conversion profile found with 300 mg of pre-treated CA/PP, at 45 °C with $W = 20\%$.

3.6. Desorption of lipase

In order to assure that no important lipase desorption from PP is taking place, reuse of CA/PP was tried. Table 1 shows the conversion achieved in 6 h of reaction in the use and first reuse of 120 mg of CA/PP. The aim of reuse experiments was to prove that the high conversion found in CA/PP-mediated reactions was due to the immobilized biocatalyst and not due to desorbed lipase acting as soluble CALB. However, the powdered nature of PP obtained with metallocenes made it difficult to recover the solid from

Table 1
Conversion achieved in ethyl oleate synthesis catalyzed by CA/PP or CA/PP/glass, $W = 20\%$, 45 °C, 350 rpm, $N = 1$

	CA/PP (120 mg)	CA/PP/glass (200 mg)
First use (%)	74.65	16.81
Reuse 1 (%)	34.98	9.62
Reuse 2 (%)	–	5.24

reaction medium. Strong stirring during 7–8 h of reaction deeply reduced catalyst size, and just a fraction of solid added to reaction medium could be retained by filtration, even with special filters for very small particle diameters. The difficulties found in the recovery of the solid also prohibited the quantification of lipase desorption trough measurement of enzymatic activity in solution.

As it is shown in the second column of Table 1, the solid recovered from the first reaction and reused in the same conditions led to a conversion of near 47% of the conversion achieved in the first use of the catalyst. These results let us assure that definitely *not all lipase has been desorbed* from PP in its first use in reaction. However, since as it has been pointed out, just a fraction of CA/PP could be recovered and used in the first reuse reaction. Therefore, the reduction in conversion achieved cannot be completely attributed to partial desorption or inactivation of lipase, but to a combined effect of desorption/inactivation and the fact that in the second reaction a lower mass of catalyst was used.

In order to overcome the problem of the low recovery of metallocenes–PP, PP obtained with Ziegler Natta catalysts was used as support. Comparing conversions achieved in the same reaction (around 22% in 7 h of reaction with 200 mg of catalyst), it was evidenced that lipase adsorbed on PP from Ziegler-Natta was notably less than the amount of lipase loaded onto metallocenes–PP. Alternatively, glass spheres chemically linked to metallocenes–PP were assayed as support. It was expected that this support had the advantages of PP obtained with metallocenes and, additionally, recovery

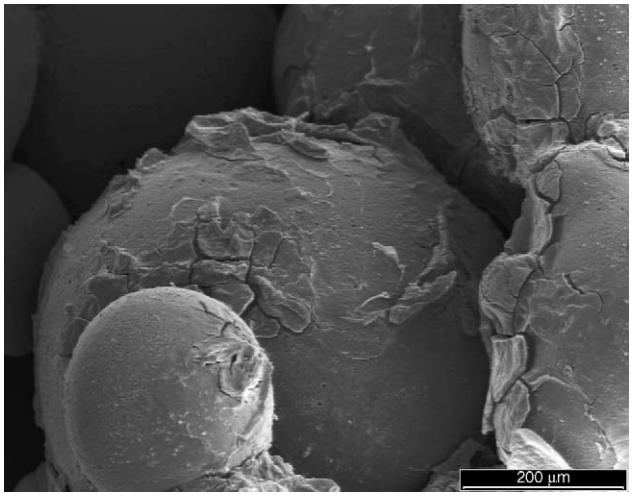

Fig. 8. Sem image of PP/glass spheres.

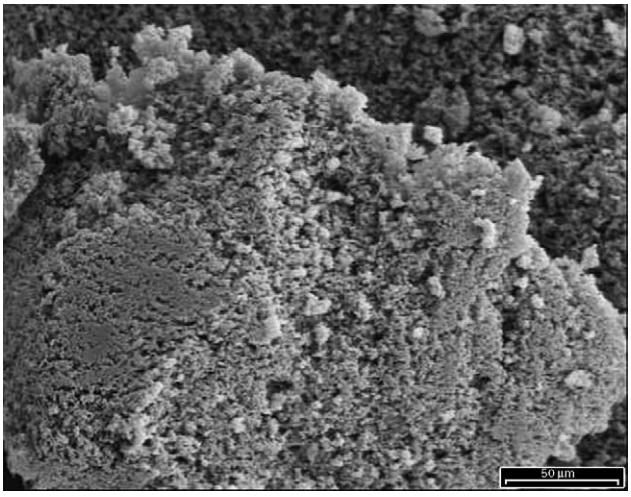

Fig. 9. Sem image of CA/PP.

problems would be overcome. Fig. 8 shows a SEM image of the PP/glass spheres. A SEM Photomicrography of CA/PP is also provided (Fig. 9).

Results achieved in ethyl oleate synthesis with 200 mg of CA/PP/glass catalyst are also shown in Table 1. It is clear from ''first use'' data that, (as it happened with Ziegler-Natta PP), much more active lipase was adsorbed to powdered PP than to PP/glass spheres. Anyway, relative reduction of conversion achieved in reuses experiments, confirmed that, although desorption or inactivation of lipase adsorbed on PP actually exists, the phenomenon *does not involve all lipase* immobilized, but just a fraction of it. Even though further efforts in order to find a way to efficiently recover CA/PP must be done, desorption experiments performed let us confirm that conversion achieved in reaction mediated by CA/PP is definitely due to the immobilized lipase and not to desorbed lipase acting as native enzyme.

Comparison of the effect of temperature on reaction mediated by CALB and CA/PP (Figs. 3 and 4) also demonstrates that lipase adsorbed on PP is actually acting in its adsorbed form, since temperature profiles, specially the ones found at 65 °C, are very different.

4. Conclusion

Native and immobilized lipase from *Candida antarctica* B, have shown to be highly active in the synthesis of ethyl oleate in solvent-free medium. The parameter study performed let us to infer the best conditions for ester production. In particular, the presence of an organic/aqueous two-phase liquid–liquid system, showed to be a good alternative for conversion enhancement. In reference to temperature, both native and adsorbed lipase showed highest activity at 45 °C. Operation at temperatures above 65 °C drastically deactivate both CALB and CA/PP. Biocatalyst agglomeration detected for CA/PP, was overcome through pre-treatment of the immobilized derivative with an octane/buffer mixture. This pre-treatment drastically reduced the aggregation phenomenon allowing more efficient use of CA/PP. The optimized experimental conditions found for the synthesis of ethyl oleate were the following: the mass of catalyst of 300mg pre-treated with octane/buffer 5/95 v/v, at 45 °C, with 20% of water. Under these conditions 78.6% of fatty acid conversion was achieved after 7 h of reaction. In reference to lipase desorption from PP, some degree of inactivation/desorption of lipase actually exists, but the phenomenon is low enough to allow the use of the catalyst two to three times with still high conversions. Further studies in order to overcome metallocenes–PP size reduction are currently been done.

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