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Lipase-catalyzed synthesis of polylactic acid: an overview of the experimental aspects

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Abstract

BACKGROUND: Enzymes have received increasing attention as biocatalysts. The poly(lactic) acid (PLA) has been widely employed in biomedical applications and PLA synthesis by a 'green route' is of particular interest. Here the aim is to prepare PLA using lipases, focusing on optimization of the procedure. The effects of the type and concentration of lipase, type of reaction, solvent, and time on the recovery of solid polyester, conversion rate and molecular weight have been explored. *Pseudomonas cepacia* (PCL), Porcine pancreatic lipase (PPL) and immobilized CAL-B were used as biocatalysts.

RESULTS: CAL-B was the most effective biocatalyst, with 60% LA conversion and 55% recovered solid polymer; PCL and PPL gave rise to poor recovery of polymer. A novel thermal treatment was successfully employed to enhance the molecular weight Mn of PLA.

CONCLUSIONS: This work offers a set of optimal conditions to synthesize PLA as a function of the lipase used. Information of this nature is currently not available in the literature, thus the findings here are a valuable tool for any researcher in this topic and a state-of-the-art contribution in terms of the best biocatalyst and the best conditions for PLA synthesis.

Keywords: biocatalysis; enzymatic polymerization; polylactic acid; enzymes; lipase; lactic acid

INTRODUCTION

Because of its biocompatibility and degradability to non-toxic products, polylactic acid (PLA) based polymers and copolymers have been employed in novel applications, such as absorbable bone plates, artificial skin, tissue scaffolds, and carriers of drugs for controlled release systems.[1]

The synthetic routes to obtain PLA are, basically, direct condensation of the free acid or ring opening polymerization of esters of the acid. The principal disadvantage of the first method is the low molecular weight of the resultant polymer due to the equilibrium among the free acid, the oligomers and the water produced during the reaction (which should be continuously removed).[2] The ring-opening mechanism requires the use of heavy metals based catalysts, such as oxides of Zn and Sn, which commonly contaminate the polymer obtained.[3–5] In addition, high purity monomers are needed and severe conditions of temperature and vacuum must be used.[4] In this context, enzymatic polymerization emerges as one of the most viable alternatives to avoid these difficulties. In brief, two fundamental advantages over the above-mentioned procedures are gained when enzymatic synthesis is used: first, it is an environmentally benign method that can be carried out under mild conditions; second, it is highly specific and provides adequate control of the polymerization process.[5–8]

Lipases are the most versatile biocatalysts because they can be applied to the synthesis of a wide range of substrates with a high stereospecificity and enantioselectivity. In general lipases used in polyester synthesis are of mammalian (Porcine pancreatic lipase (PPL)), fungal (*Candida antarctica* lipase B (CAL)), or bacterial origin (*Pseudomonas cepacia* (PCL)). Supported or immobilized commercial lipases have also been developed in order to modify particular characteristics of enzymes, increasing their activity.[9]

A review of the open literature revealed numerous articles dealing with enzymatic polymerization but the majority of them report the use of lactones, sugars and polycarbonates as monomers.[10,11] Articles focused on the enzymatic synthesis of PLA are still limited. For example, Distel *et al*. reported the synthesis of PLA using an alkaline protease from *Bacillus sp* and immobilized CAL-B (Novozyme 435).The work was oriented to evaluate the activity of the enzymes after a modification to improve their solubility in organic solvents. They explored the effect of reaction temperature and water on the stability of the enzyme.

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An analysis of the effect of such variables on the reaction yield or on the average molecular weight of the PLA obtained was not included in this contribution.[12] Sonwalkar *et al*. investigated the role of silica gel in the polymerization of LA induced by PPL. They studied the influence of different solvents, with different polarities, on the conversion of LA to PLA. They found that the silica gel itself was able to catalyze the polycondensation since the best results, in terms of conversion, were reached using silica gel alone instead of the lipase/silica gel mixture. The authors have not advanced in the characterization of the prepared polyesters.•[13] The contribution of Kiran and Divakar deals with the lipase-catalyzed polymerization of LA using PPL, and two types of immobilized *Rhizomucor miehie* lipases (lipozyme and chirazyme). They evaluated the influence of the substrate/lipase ratio and the initial amount of LA on the average molecular weight of the PLA generated. They observed that PPL was the best biocatalyst to induce polyesterification, from both conversion and molecular weight points of view.[14] Two articles, from Whalberg and Huijser, refer to the copolymerization of LA with caprolactone and glycolic acid, respectively, and describe the structural characteristics of the copolyesters obtained.[15,16]

This contribution proposes a completely different view of the enzymatic polymerization of LA. In the majority of studies the reaction conditions, i.e. nature of lipase, kind of reaction, solvent, reaction time, etc. are stated without detailing the reasons for such a selection. The study tried to clarify those aspects that should be taken into account to reach optimal conditions; and details related to the experimental procedures are provided. Specifically, the recovery of PLA after the reaction, the interaction of lipase with the solvent and the potential mistakes in measurement of the conversion by titration (due to the presence of non-free LA) were explored. This systematic study has found a set of optimal conditions as a function of the selected lipase. For this purpose three different enzymes were employed: two soluble (*Pseudomonas cepacia* (PCL) and porcine pancreatic lipase (PPL)) and one immobilized (Candida Antarctica Lipase B (CAL-B)). Selection of the biocatalysts was based on the literature concerning PLA polymerization and similar enzymatic systems.[12–16]

It is worth noting that, to the best of the authors' knowledge, information of this nature has not been addressed previously in the literature.[11,17]

EXPERIMENTAL

Materials

Commercially available D/L lactic acid (liquid, 85%), was obtained from Sintorgan S.A (Argentina). Immobilized CAL-B, Novozyme 435 was provided by Novo Nordisk A/S, Denmark. PPL from porcine pancreas Type II was supplied by Sigma (St Louis, MO) and PCL were from Amano (Nagoya, Japan).

Analytical grade hexane, absolute ethanol, isopropyl ether and methylene chloride were all provided by Dorwill•. Potassium hydroxide was from Merk•, and biphtalate used for KOH normality determination was from Productos TIMPER•. Special filters (0.45 μm, 47 mm) were purchased from Osmonics• and distilled water was used in the precipitation of PLA.

Polymerization reactions

Polymerization experiments were performed according to the methodology described earlier.[18] 5 mmol of LA, the corresponding amount of enzyme and 8 mL of the appropriated solvent were employed. Bulk reactions were conducted under similar conditions but without solvent.

Product work out procedure

To recover the PLA, the procedure applied in previous work was employed.[18] In brief, the homopolymers were dissolved in 2 mL of CH_2Cl_2 and the lipase was separated by filtration. Then 40 mL of water were added to precipitate the polymer, which was isolated after solvent evaporation.

The experimental conditions (amount of substrate, temperature, time, stirring speed, etc.) were selected on the basis of available literature reports on similar systems and also considering previous experience in the topic.[18,19]

Conversion of LA

The conversion of LA to PLA was evaluated in terms of the percentage of esterified LA during the polymerization reaction, using analytical titration with a KOH solution. This process involves a series of steps, which are different depending on the kind of reaction (solution or bulk). In both cases the first step was sampling. The samples were obtained from a stirred reaction medium. In the case of solution reactions the withdrawn samples (150–250 mg) were diluted in 10 mL of an ethanol/ether solution (50:50 v/v) mixture and titrated with KOH using 15–20 drops of phenolphthalein as the end point indicator.

When the reactions were performed in bulk the withdrawn sample was diluted in 8 mL of the appropriate solvent (hexane or isopropyl ether) in order to provide sampling concentrations of the same order as those obtained in the solution. After this, sampling and titration steps were similar to the above-mentioned procedure.

The conversion in percentage was defined as:

$$\%C = \frac{m_{theo.} - m_{obt}}{m_{theo.}} \times 100$$

where $m_{theor.}$ are the theoretical milliequivalents of LA calculated from the weight of the initial sample, and $m_{obt.}$ are the milliequivalents obtained from titration after the selected reaction time, calculated as follows:

$$m_{obtx} = N \times (V - V^*)$$

where N is the normality of the KOH solution, V is the volume consumed in the sample titration and $V*$ is the solution consumed in the ether/ethanol mixture.

The results reported in this work are the average of three to five replicates.

Determination of the number average molecular weight (Mn)

The Mn of PLA was determined by end-group analysis.[12,20,21] A known amount of the polymer sample (10–30 mg) was diluted in CH_2Cl_2 (5–10 mL) and titrated against standard alcoholic KOH, under stirring. The milliequivalents obtained lead to Mn by applying the following relation:

$$Mn = \frac{w \times n \times 100}{V \times N}$$

where w is the polymer mass, n is the numbers of end functional groups in the polymer (in this case 2), N is the normality of KOH and V is the corrected consumed volume. The data given in this report are the result of three to five replicates of each sample. The error in the measurements was ±2–7%.

FTIR, H^1 and C^{13} NMR spectroscopy

A Nicolet FTIR 520 spectrometer was used for recording transmission spectra in the range 4000–400 cm^{-1}. The PLA spectra were obtained by casting a CH_2Cl_2 solution of the polymer on a KBr window, and assaying after solvent evaporation. To record the lipase spectrum, the solids were dispersed on a KBr window using a mineral oil (nujol). ^1H and C^{13}NMR were used to determine the structure of the homopolymers. Spectra were recorded at 300 and 75 MHz, respectively, on a Varian Innova 300 spectrometer at 25 °C. The solvent employed was deuterated chloroform ($CDCl_3$) and the tetramethylsilane (TMS) signal was taken as the zero chemical shift.

RESULTS AND DISCUSSION

Influence of the kind of lipase

The performance of the three lipases was first evaluated in terms of the conversion of LA to PLA. The results, expressed as percentage (%) conversion, as a function of the selected lipase, are detailed in Table 1. The maximum conversion was obtained using PPL, while the conversion was slightly lower in the case of PCL and dramatically lower for CAL-B.

To verify the catalytic role of the lipases a control reaction was carried out by mixing 5 mmol of LA and the solvent at 60 °C for 96 h. No significant conversion levels were registered at the end of the treatment, as is shown in the Table 1, indicating that the lipases were acting as biocatalysts under the conditions employed.

The data in Table 1 referring to the percentage of recovered solid PLA, estimated from the theoretical amount of PLA that should be obtained, reveal that there is a discrepancy between the conversion values and the amounts of recovered solid. To better illustrate the differences between the three lipases regarding the recovery of polymer, data were additionally expressed as mg solid polyester mg^{-1} lipase h^{-1}, as is also shown in Table 1; the same discrepancy between conversion and recovery may be observed. Higher conversion levels were measured in the case of soluble enzymes, but only traces of solid polyesters were recovered in such cases. In contrast, satisfactory amounts of solid PLA were recovered using immobilized CAL-B, and the conversion was considerably lower than that determined with soluble lipases.

Table 1. Conversion (%), of solid recovered PLA (estimated from the theoretical recovered PLA, calculated on the basis of conversion value and initial concentration of LA), mg solid PLA mg^{-1} lipase h^{-1} and percentage of separated enzyme during the purification process, as a function of the biocatalyst used

	Control reaction	Immobilized CAL-B	PCL	PPL
Conversion (%)	0.88	58	88	96
% of recovered PLA	–	55	12[a]	2
mg solid PLA mg^{-1} lipase h^{-1}	–	5×10^{-3}	–	3×10^{-4}
% of reovered enzyme	–	85	34	90

All reactions included in the table were carried out using 54 mg lipase $mmol^{-1}$ LA.
[a] Liquid oligomers.

The huge differences between LA conversion and gravimetric values of solid PLA recovered with soluble lipases can be ascribed to the presence of very low molecular weight oligomers (lower than 400 Da) originated during polymerization. Compounds of this nature are generally volatile so it is proposed that they may escape from the reaction mixture, leading to the observed mass loss. It is important to emphasize that information about this in the literature is scarce; hence confirmatory studies are required.[22]

Inefficient enzyme/product separation could also be the cause of the poor recovery of solid PLA. FTIR studies were performed aimed to determine the presence of remaining polyester in the lipase moieties and to accurately identify the PLA. The spectra of pure immobilized CAL-B (a) and PLA (b) are shown in Fig. 1. These are compared with the corresponding recovered enzyme after the reactive process (c). As the spectra were recorded in mineral oil (nujol) as dispersant, the nujol spectrum is also included as reference (discontinuous line in Fig. 1).

Comparing the spectra of PLA with that of the pure enzyme, several differences arise. For example, the PLA spectrum presents two sharp bands at 1730 and 1660 cm^{-1} (vs; C=O) while in the pure enzyme a non-well-defined peak is observed that may be associated with the noise signal. The same is observed in the C–O and C–O–C absorption region, where the

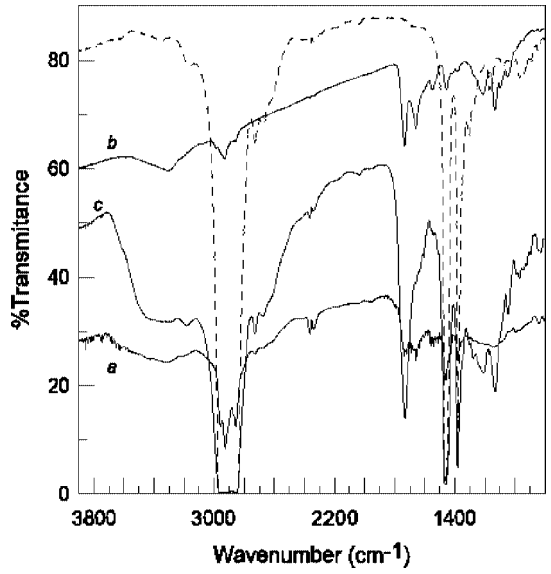

Figure 1. Region between 900 and 3800 cm^{-1} of FTIR spectra of: (a) pure immobilized CAL-B; (b) recovered solid polymer (PLA), (c) recovered enzyme after purification process and nujol spectrum (- - - -).

PLA spectrum exhibits two clear bands at 1200 and 1120 cm^{-1} and no bands are observed in the spectrum of immobilized CAL-B.[23] In contrast, comparing the spectra of PLA and the recovered enzyme, one finds almost the same signals, suggesting that the recovered enzyme contains some residual polymer. These findings reveal that a part of the prepared polyester remained adsorbed in the separated lipase. Further evidence of this phenomenon is presented later (Effect of the lipase concentration).

In Fig. 2 the spectra of PLA oligomer synthesized by PCL (b), PCL separated after reaction (c) and the pure enzyme (a) are shown. Analysis of Fig 2 shows that the spectrum of the recovered enzyme is very similar to that of the pure lipase and these differ significantly from the PLA spectrum. The differences are more evident in the region between 1000 and 1200 cm^{-1}, associated with the absorption of C–O and C–O–C groups, where the enzyme spectra (pure and recovered) exhibit a wide and non-well-defined band. The C=O region, between 1600 and 1800 cm^{-1} is quite different in the PLA spectrum compared with that of the lipase. The former shows two sharp bands at 1680 and 1720 cm^{-1}, while the lipase spectrum shows a faintly detected band at 1680 cm^{-1}. Similar results were achieved using PPL. Therefore, in the case of soluble lipases, the discrepancy between the recovered solid product and the conversion may not be attributed to inefficient separation of lipase/product. This reinforces the hypothesis regarding the generation of volatile low molecular weight oligomers. The characterization and recovery/separation of non-solid products has not been investigated in detail because this research was mainly oriented towards optimization of the experimental procedure to achieve solid materials suitable for the biomedical field as drug delivery carriers.

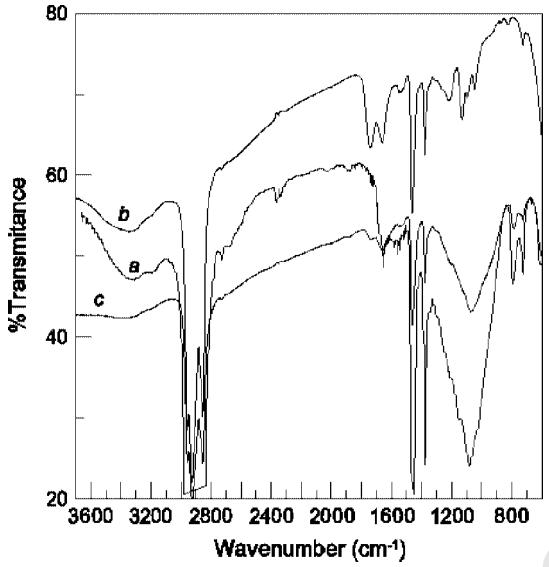

Figure 2. Region between 700 and 3600 cm^{-1} of FTIR spectra of: (a) pure PCL; (b) recovered PLA oligomer and (c) recovered enzyme after purification process.

Characterization of solid homopolymer

To further identify the solid PLA recovered using immobilized CAL-B, H^1 and C^{13} NMR analyses were undertaken. Chemical shift values and assignments are shown in Table 2 and the PLA signals are labelled according to Scheme 1.

The $-CH_3$ signals from hydroxyl terminal LA oligomers (mainly dimer and trimer) appear as a multiplet between 1.18 and 1.30 ppm. Similarly the $-CH_3$ signals from carboxyl terminal LA oligomers and the polymer chain are observed as a multiplet between 1.52 and 1.60 ppm. The –CHO region also reveals the presence of free hydroxyl terminal oligomers by quartets appearing at 4.40 and 4.43 ppm, respectively. Likewise, signals located at 5.23 and 5.30 ppm are from carboxyl terminal lactic oligomers and polymer, respectively.

The information derived from C^{13}-NMR supported the data provided by H^1-NMR in demonstrating the

Table 2. C^{13}- and H^1NMR chemical shifts (ppm) of PLA from lipase-catalyzed polymerization using 54 mg lipase (immobilized CAL-B) $mmol^{-1}$ LA

C^{13}-NMR		
$\delta(CH_3)$	δ(CHO)	δ(CO)
16.9 n 20.4 2	66.7 1 67.0 2 69.1 3 69.7 n	171.2–175.3 3, 4, n 179.1 1

H^1-NMR	
$\delta(CH_3)$	δ(CHO)
1.18–1.30 2 1.52–1.66 3 and n	4.41 (q) 2 5.27 (q) n

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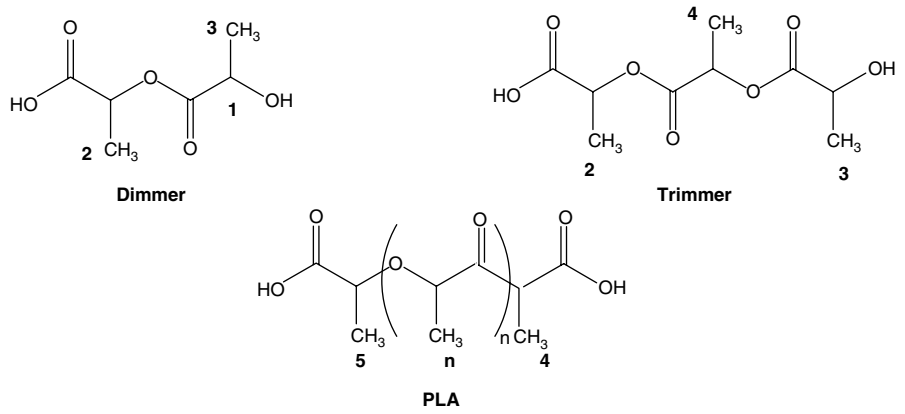

Scheme 1. Chemical structure of PLA and its derivatives originated during enzymatic polymerization.

presence of LA oligomers of different chain length (dimers, trimers) and polymer. For instance three signals located at 16.9, 20.4, 20.5 ppm may be ascribed to $-CH_3$ from the PLA chain and polylactic oligomers, respectively. Signals associated with $-CH-O-$appear at 66.7 and 67.0 ppm from hydroxyl terminated LA oligomers; while at 69.1 and 69.7 ppm, signals ascribed to the PLA chain and carboxyl terminated PLA, respectively, may be observed. In the carbonyl carbon region, a band located at 179.1 ppm is detected and can be attributed to LA dimers; while signals between 171.2 and 175ppm indicate the presence of longer chain oligomeric species as well as polymeric LA moieties.

Finally, the information provided by NMR spectroscopy supports the results obtained from FTIR in confirming the purity of the prepared PLA since no evidence of residual lipase were detected.

Role of water in the reaction media

The first reactions (bulk and solution) were conducted through the procedure described in the experimental section, using 9 mg of lipase per mmol LA. Samples were withdrawn at 18, 24, 48, 72 and 96 h to monitor conversion as a function of time. An initially low conversion, growing as a function of time was expected. However, the data obtained, shown in Fig. 3, do not display the expected behaviour. In the figure the conversion values (%), arising from bulk and solution reactions induced by immobilized CAL-B, as a function of the time are included. The plot shows a high initial conversion that passes through a minimum and starts to rise until the end of the reaction (at 96 h) where the LA conversion achieves maximum. It is worth noting that a comparable trend was observed in the case of soluble lipases.

This unusual behavior is caused by the presence of different chemical species, such as anhydride from LA dimerization and dimers, in the formulation of commercial LA, so the lipase does not find available free-carboxylic acid to start polymerization. The initial step of the reaction is then the hydrolysis of LA derivatives. The formation of acid leads to a fall in the conversion, evidenced at 24 and 48 h, in bulk and solution reactions, respectively. After this, the consumption of LA as a consequence of esterification generates the increasing conversion (Fig. 3).

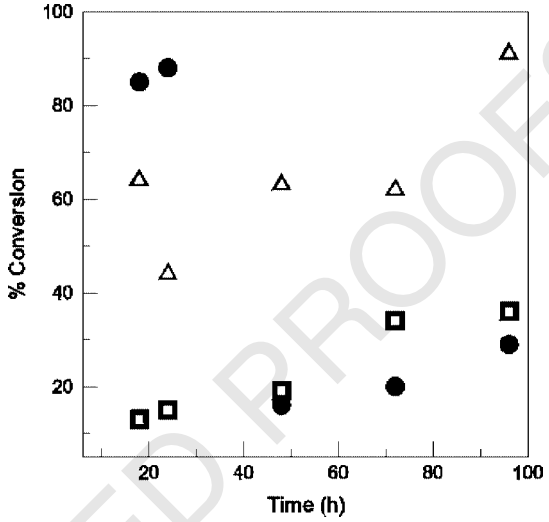

Figure 3. Evolution of conversion (%) as a function of the time (h) for the reactions, in bulk (△) and solution without water (●), and for the reaction in solution with water addition (□). All the reactions were catalyzed by 9 mg lipase (CAL-B) $mmol^{-1}$ LA, at 60 °C for 96 h.

The presence of LA anhydrides and related species as well as the oligomerization of the monomer in commercial solutions of lactic acid has previously been reported in the literature.[12,24–27] In the current case, even when the raw LA contains a percentage of water in its formulation (roughly 15%), evidently it is not enough to promote spontaneous chemical hydrolysis. Therefore it was considered necessary to add water to the reaction mixture to hydrolyze the lactic anhydride and related species to lactic acid in a shorter time and to avoid mistakes in the measured conversion.

Although the role of water is quite controversial, several authors agree that the water is strongly associated with the mechanism of enzymatic polymerization. According to Dong *et al.*, in agreement with other publications, the water participates in the initial step and is consumed in the earlier polymerization stages (150 h). Therefore the authors proposed that an optimal amount of water (ranged between 1 and 4%)

is beneficial for conversion and polymer molecular weight.[28,29] Based on these references the reactions have been carried out according to the procedure described in the Experimental section, with 1.5% w/w of water (with respect to the mass of LA). The data on conversion as a function of time for reactions performed with the addition of water show the expected profile: low initial conversion that grows as a function of time, as is demonstrated by the plot included in Fig. 3. From the conversion point of view this parameter increases from 29% (for the reaction conducted without added water) to 36% for polymerization with 1.5% of added water, confirming the beneficial effect of the water. (Note that only solution reaction data are included in Fig. 3 as a consequence of results presented later).

Selection of reaction time

The reaction time was set at 96 h based on published information on lipase-catalyzed polymerization of similar systems.[12–14,28] This time resulted in reasonable and satisfactory promotion of LA polymerization using immobilized CAL-B and PPL, but some problems were found in the case of PCL.

A linear dependence of conversion on time was observed using 9 mg lipase (PCL) per mmol LA when allowing the reaction for 96 h. However, conversion reduced after 24 h when 18 mg lipase (PCL) per mmol LA was used. This tendency occurred with both solution and bulk reactions, as is clearly shown in Fig. 4.

It was assumed, based on available information, that the hydrolysis (PCL mediated) of the resultant polyester (whose synthesis was also PCL mediated) was the cause of this phenomenon, which seems to be strongly dependent on the concentration of lipase since depolymerization occurred when the quantity of PCL was raised to 18 mg from 9 mg per mmol LA.[9,30] Taking into account these findings the optimal reaction time seems to be 24 h when PCL is the biocatalyst.

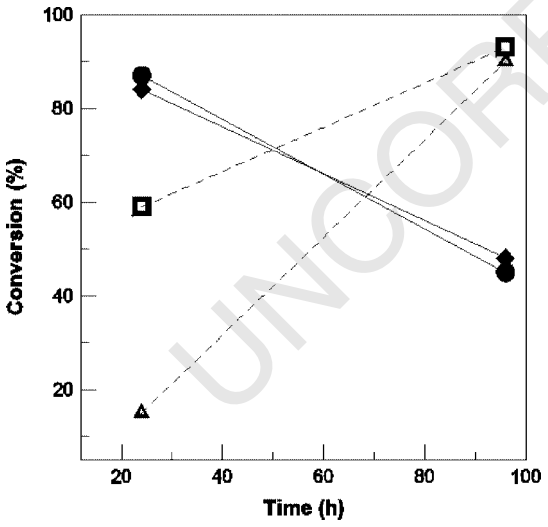

Figure 4. Variation of conversion (%) as a function of the time for reactions catalyzed by 9 mg lipase (PCL) mmol^{-1} LA, in bulk (△) and solution (□); and with 18 mg lipase (PCL) mmol^{-1} LA, in bulk (●) and solution (⧫), at 60 °C for 96 h.

Selection of the reaction approach: bulk versus solution

The most suitable reaction media is the one that ensures high LA conversion values, and more importantly, allows sufficient recovered solid polymer. In this sense the bulk reactions catalyzed by immobilized CAL-B exhibited some problems with regard to PLA isolation. Even when conversion values were high (Fig. 3), only a rubbery resin was recovered from the reaction mixture, independently of the enzyme concentration. In spite of the huge amount of published articles dealing with solvent-free polymerization, there are very few dealing with LA polymerization and they have not employed immobilized CAL-B as biocatalyst.[31] As a consequence, the cause of this behaviour is uncertain. It is hypothesized that the formation of volatile low molecular weight oligomers occurred, and these compounds evaporated during the solvent evaporation step, as observed in the case of soluble lipases. Furthermore, the formation of low molecular weight products instead of solid polymer can be attributed to a fall in the enzyme activity due to deactivation of immobilized CAL-B when in contact with strong polar environments.[25] Pirozzi *et al*. have investigated the performance of immobilized CAL-B as biocatalyst in esterification and transesterification of LA. They observed a severe loss of stability of the enzyme in the presence of LA when operating in a solvent-free environment.[30,32] Therefore the solution reaction appears to be the most viable route to obtain PLA using this lipase.

In the case of PCL, a minimum amount of lipase was separated after polymerization when reactions were conducted in solution, while the bulk allowed satisfactory recovery of the enzyme (Table 1). As obviously the more accurate and simple procedure is preferred, bulk was the selected option, in agreement with several studies that report the use of PCL in solvent-free systems during the polymerization of other monomers.[31,33]

No difficulties were found in the isolation of PPL during solution reactions since two perfectly delimited phases were formed and the lipase was removed by filtration. In contrast, some problems were detected during the bulk, since PPL was almost soluble in LA and in the product. Consequently, a unique uniform solution was observed at the end of the reaction, thus the polyester and the enzyme could not be separated by the addition of CH_2Cl_2. Based on these results the solution reaction was chosen.

Selection of the solvent in solution reactions

Having selected the reaction media, n-hexane was chosen as solvent based on literature reports. However, some difficulties appeared in LA homopolymerization

using immobilized CAL-B. It was verified that n-hexane interacts with the enzyme by reproducing the reaction procedure without LA. Withdrawn samples of the reaction mixture were titrated against KOH standard solution at 18, 24, 48, 72 and 96 h. Consumed KOH (mL mg^{-1} enzyme) as a function of time is shown in Fig. 5 and reveals that hexane extracts some acid components from the lipase formulation, leading to an increase in acidity of the media.

The plot in Fig. 5 also indicates that there is a proportional dependence between acidity of the environment and time. Hence, large errors can occur when calculating the conversion by titration during the reaction period when using hexane. Besides this, Torres and Otero found that hexane minimizes the potential of LA to act as acyl donor to the enzyme. This fact is highly adverse to polymerization, however, it favours esterification of the acid with alcohols.[25]

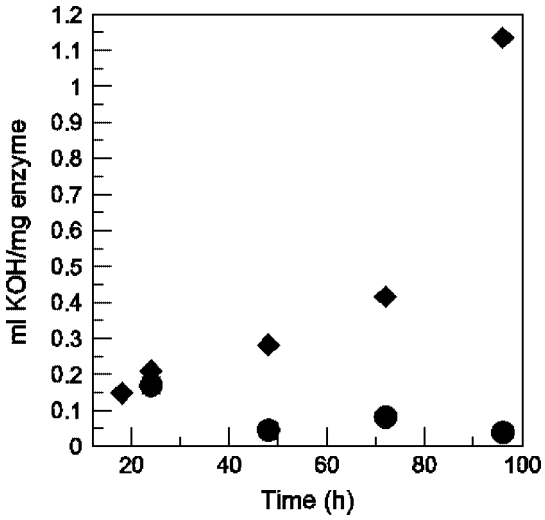

Figure 5. Consumed KOH (mL solution mg^{-1} enzyme) as a function of the treatment time for immobilized CAL-B (9 mg $mmol^{-1}$ LA)/hexane (8 mL); (♦) and CAL-B (9 mg $mmol^{-1}$ LA)/isopro-pyl ether (8 mL) (●) at 60 °C for 96 h.

From the experimental point of view, the difficulty in isolating solid PLA from the reaction mixture was another disadvantage associated with n-hexane/enzyme interaction. Considering these factors and the available literature, isopropyl ether was selected as solvent for polymerization of LA. Insignificant levels of acidity were registered when this solvent and the lipase were contacted at 60 °C for 96 h (Fig. 5). In contrast hexane was shown to be an appropriate solvent to carry out the reaction in the presence of PPL.

Effect of the concentration of lipase

The concentration of lipase affected not only the polymerization yield but also the molecular weight of the products obtained.[9,12,34] In spite of this there is not enough information in the open literature, especially referring to PLA enzymatic synthesis.[13,14] This study explored the range of concentrations between 9 and 54 mg enzyme $mmol^{-1}$ LA using both immobilized and soluble lipases.

The dependence of the conversion on the concentration of lipase is addressed in Table 3. Conversion increased with higher concentrations of immobilized CAL-B, as a consequence, the optimum concentration was 54 mg $mmol^{-1}$. It is worth noting that similar results were reported by other authors studying different systems.[12,33,34]

In the case of PCL, the data in the table point out that conversion grows in proportion to the amount of lipase for reactions conducted in bulk over 24 h. This agrees with results obtained by other authors using PCL in the polymerization of other substrates as well as with current data obtained with immobilized CAL-B.[15]

A substantially different trend is observed in the case of PPL since conversion remains almost constant with increase in lipase concentration. The reasons for this behaviour are uncertain, and comparable trends in the open literature do not offer convincing explanations. For example, Knani *et al*. obtained similar results

Table 3. Conversion (%) of isolated enzyme after reaction, percentage of recovered PLA and Mn (Da) as a function of the concentration of the different lipases (mg lipase $mmol^{-1}$ LA)

Enzyme	Concentration of enzyme	Conversion	% recovered PLA	% recovered lipase	Mn
Imm.CAL-B	9	24	6	Traces	–
	18	27	44	80	2461
	36	47	45	107[c]	925
	54	58	55	85	446
PCL	1.7	22	Traces	Traces	–
	3.3	56	Traces	34	–
	6.7	75	28[a,b]	25	–
	10	88	12[a]	34	400
PPL	9	92	Traces	22	–
	18	92	Traces	80	–
	36	91	Traces	95	–
	54	96	2	90	768

[a] Viscous yellow liquid.
[b] Not enough to characterize.
[c] This surely contains PLA.

for the lipase-catalyzed polymerization of methyl 6-hydroxyhexanoate, a model hydroxyester. They found that increasing the PPL concentration from 1.5 to 3 g $mmol^{-1}$ substrate, changed the yield (%mol) only slightly from 8.3 to 9.1; they did not offer an explanation.[35] The divergence between the LA conversion and the amount of recovered PLA using PPL and PCL has been discussed (see earlier section) and seems to be independent of the concentration of lipase.

To analyse the correlation between the observed tendency and the recovered solid PLA, in the case of immobilized CAL-B, the percentage of recovered PLA was related with the concentration of enzyme. The data, included in Table 3, suggest that 54 mg enzyme $mmol^{-1}$ LA is the optimum concentration to recover acceptable amounts of PLA, in agreement with the conversion results. Here, the phenomenon of polyester adsorption on immobilized CAL-B moieties after reaction is more evident in the percentage of separated lipase in the case of 36 mg $mmol^{-1}$ LA concentration. From these data, the amount of polymer adsorbed can be obtained by gravimetric methods. It is clear from Table 3 that at least 7% of the isolated lipase corresponds to the polymer, meaning that 13% of the prepared PLA (21 mg) could not be separated from the lipase during the filtration step. With lower and higher concentrations of biocatalyst it was not possible to estimate gravimetrically the quantity of adsorbed PLA; thus only the qualitative information provided by FTIR (Fig. 1) was considered. Hence, these observations may partially justify the differences between recovered and theoretical recovered PLA when using immobilized CAL-B.

Although the necessary quantities of lipase to attain satisfactory results are huge compared with metal-based catalysts, economy advantages should be considered. The most relevant are the possibility of reuse of the enzymes and the easier purification of the prepared polymers, especially with regard to materials destined for biomedical devices.

Evaluation of the number average molecular weight (Mn)

The dependence of PLA Mn on the enzyme concentration deserves detailed attention.

The data in Table 3 reveal that the molecular weight decreased with increasing concentration of immobilized CAL-B, in opposition to the observed conversion tendency. This behaviour may be justified by looking at the mechanism of polymerization; since more total chains were formed when larger amounts of lipase were used, the rate of monomer consumption was high, but resulted in products with lower molecular weight. Other authors have found similar results using different systems.[34] For instance, Kiran and Divakar observed a comparable tendency in the polymerization of LA using PPL as biocatalyst. They found an optimum enzyme/substrate (E/S) relation that led to maximum PLA molecular weight (1300 Da), and above this E/S value they observed a fall in Mn of the polymer. These authors did not offer an explanation of this phenomenon. In the current work, linear relationship has been determined between Mn and conversion as a function of the enzyme concentration.[12]

When PPL was employed as biocatalyst, the PLA Mn was 768 Da, which is of the same order as those values reported in the open literature using the same lipase.[12,13] However, a great difference in reaction times were noted between the current work and published articles, since they allowed reactions between 141 and 507 h, providing continuous removal of water by employing molecular sieves.[12,13]

Although the recorded values of Mn for the solid PLA in this research are of the same order as most of the polymers prepared via enzymatic polymerization reported in the literature, they have limited applications as obtained. Therefore, a novel strategy was proposed to raise Mn, which consists basically of a thermal treatment of the LA homopolymers: 50–100 mg of PLA were heated under vacuum at 190 °C, for 20 h, in accordance with the procedure proposed by Kimura *et al*.[35,36] Preliminary results revealed that significant increases in the Mn of PLA were obtained after the thermal treatment; Mn values ranged between 5000 and 6200 Da. Furthermore, an important mass loss, of the order of 34–40% with respect to the initial mass of the sample, was observed after the process. As was stated above, complementary studies are currently underway to explain these observations and the mechanism that resulted in such changes in the Mn. A likely mechanism is thermally induced polycondensation with water loss (and probably some loss of short chain products – dimers and trimers of LA).

The findings of this research are summarized in Table 4 in terms of optimal experimental conditions to attain appropriate amounts of solid PLA (when possible), as a function of the selected lipase.

CONCLUDING REMARKS

The main experimental parameters associated with the polymerization of LA were studied with the goal of optimization of the process. Immobilized CAL-B, PCL and PPL were screened to find the most effective biocatalyst in terms of the conversion of LA (%) to PLA and, importantly, by their ability to generate solid recoverable products.

Table 4. Summary of the best conditions (in terms of recoverability, conversion and Mn of PLA) for LA polymerization, as a function of the lipase selected

Enzyme	Type of reaction	Time (h)	Solvent	Recovered PLA
Imm. CAL-B	Solution	96	Isopropyl ether	Solid
PCL	Bulk	24	–	Liquid
PPL	Solution	96	Hexane	Solid[a]

[a] Minimal amount.

Undoubtedly the most effective biocatalyst was immobilized lipase working at 60 °C and allowing the reaction for 96 h. It was found that the optimal reaction approach was via solution instead of bulk, using isopropyl ether as solvent. Regarding the concentration of biocatalyst, there was an almost linear relationship between conversion and amount of lipase, and the opposite trend was evidenced in the case of Mn. Under these conditions almost 55% of polymer recovery in the solid state was achieved with satisfactory conversion levels. The solid PLAs recovered showed adequate values of Mn (between 400 and 2400 Da); in spite of this a novel thermal treatment was proposed to further increase the Mn, giving promising results.

The findings for PCL and PPL revealed that these enzymes were not effective biocatalysts for LA polymerization (under the conditions explored here) with regard to the recovery of solid product. Volatile low molecular weight oligomers were the principal products of these reactions. On the other hand, satisfactory conversion levels were attained in both cases. PPL was found to be more effective in solution using hexane as solvent, and performing polymerization at 60 °C for 96 h. Enhanced PCL performance was observed for solvent-free reactions at 60 °C for 24 h. The evolution of conversion with concentration of biocatalyst was almost linear in the case of PCL; in the case of PPL, LA conversion was shown to be independent of the amount of added enzyme.

Finally, research into the global effect of water from the mechanistic point of view is currently underway, with the goal of producing recoverable higher molecular weight polymers using soluble lipases.

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REFERENCES

1 Kricheldorf H, Synthesis and application of polylactides. *Chemosphere* **43**:49–61 (2001).
2 Okada M, Chemical Síntesis of biodegradable polymers. *Prog Polym Sci* **27**:87–133 (2002).
3 Sodegard A and Stolt M, Properties of lactic acid based polymers and their correlation with composition. *Prog Polym Sci* **27**:1123–1163 (2002).
4 Albertsson A, Edlun U and Stridsbereg K, Controlled ring opening polymerization of lactones. *Macromol Symp* **157**:39–46 (2000).
5 Saulnier B, Coudane J, Garreau H and Vert M, Hydroxyl – bearing poly(α-hydroxy acid) type aliphatic degradable polyesters prepared by ring opening (co)polymerization of dilactones based on glycolic, gluconic and L-lactic acid. *Polymer* **47**:1921–1929 (2006).
6 Kobayashi S, Kashiwa K, Kawasaki T and Shoda S, Novel method for polysaccharide synthesis using an enzyme: the first *in vitro* synthesis of cellulose via a nonbiosynthetic path utilizing cellulase as catalyst. *J Am Chem Soc* **113**:3079–3084 (1991).
7 Kobayashi S, Shoda S and Uyama H, Enzymatic polymerization and oligomerization *Adv Polym Sci* **121**:1–30 (1995).
8 Blinkovsky A and Dordick M, Peroxidase-catalyzed synthesis of lignin-phenol copolymers. *J Polym Sci Part A:Polym Chem* **31**:1839–1846 (1993).
9 Varma I, Albertsson A, Ritimonti R and Srivastava R, Enzyme catalyzed synthesis of polyesters. *Prog Polym Sci* **30**:949–981 (2005).
10 Kobayashi S, Enzymatic polymerisation: a new method of polymer synthesis. *J Polym Sci Part A: Polym Chem* **37**:3041–3056 (1999).
11 Kumar A and Gross R, Candida antarctioca lipase B-catalyzed transesterification: new synthetic routes to copolyesters. *J Am Chem Soc* **122**:11767–11770 (2000).
12 Kiran K and Divakar S, Lipase-catalysed polymerisation of lactic acid and its film forming properties. *World J Microb Biotechnol* **19**:859–865 (2003).
13 Sonwalker R, Chen C and Ju L, Roles of silica gel in polycondensation of lactic acid in organic solvent. *Bioresource Technol* **87**:69–73 (2003).
14 Distel K, Zhu K and Wang G, Biocatalysis using an organic soluble enzyme for the preparation of poly(lactic acid) in organic solvents *Bioresource Technol* **96**:617–623 (2005).
15 Wahlberg J, Persson P, Olsson T, Hedestrom E and Iversen T, Structural characterization of a lipase catalysed copolymerisation of ε-caprolactone and D,L- lactide. *Biomacromolecules* **4**:1068–1071 (2003).
16 Huijser S, Staal B, Huang J, Duchateau R and Koning C, Topology characterization by MALDI-ToF-MSof enzymatically synthesized poly(lactide-co-glycolide). *Biomacromolecules* **7**:2465–2469 (2006).
17 Kumar A and Gross R, Candida antartica lipase B catalyzed polycaprolactone synthesis: effects of organic media and temperature. *Biomacromolecules* **1**:133–138 (2000).
18 Lassalle V, Barrera Galland G and Ferreira ML, Lipase-catalyzed copolymerization of lactic and glycolic acid with potential as drug delivery devices. *Bioprocess Biosyst Eng* In press.●.
19 Lassalle V and Ferreira M, Enzymatic poly and co-polymerisation of lactic acid. *Proceedings of 8th International Symposium on Biocatalysis and Biotransformations*, 8–13 July,, Oviedo, Spain (2007).
20 Allen P, *Techniques of Polymer Characterization.* Butterworths, London (1959).
21 Wang N, Wu ● and Lujan-Upton H, Synthesis, characterization, biodegradation, and drug delivery application of biodegradable lactic/glycolic acid oligomers: I. Synthesis and characterization. *J Biomater Sci Polym edn* **8**:905–917 (1997).
22 Schliecker G, Schmidt C, Fuchs S and Kissel T, Characterization of a homologous series of D,L-lactic acid oligomers; a mechanistic study on the degradation kinetics *in vitro*. *Biomaterials* **24**:3835 (2003).
23 Hummel D and Scholl D, *Atlas of Polymer and Plastics Analysis*, 2nd edn. Hanser, Munich (1988).
24 From M., Adlercreutz P and Mattiasson B, Lipase catalysed esterification of LA, *Biotechnol Lett* **19**:315–317 (1997).
25 Torres C and Otero C, Part III. Direct enzymatic esterification of lactic acid with fatty acids. *Enzymemicrob Technol* **29**:3–11 (2001).
26 Uyama H, Wada S, Fukui T and Kobayashi S, Lipase-catalyzed synthesis of polyesters from anhydride derivatives involving dehydration. *Biochem Eng J* **16**:145–152 (2003).
27 Dung T, Aspi V, Kolah K, Asthana N, Peereboom L, Lira C, *et al*., Oligomer distribution in concentrated lactic acid solutions. *Fluid Phase Equilibria* **236**:125–135 (2005).
28 Dong H, Cao S, Li Z, Han S, You D and Shen J, Study on the enzymatic polymerization mechanism of lactone and the strategy for improving the degree of polymerisation. *J Polym Sci: Part A: Poym.Chem* **37**:1265–1275 (1999).

29 Bisht K, Henderson L, Gross R, Kaplan D and Swift G, Enzyme-catalyzed ring-opening polymerization of ö-pentadecalactone. *Macromolecules* **30**:2705–2711 (1997).
30 Pirozzi D and Greco Jr G, Activity and stability of lipases in the synthesis of butyl lactate. *Enzyme Microb Technol* **34**:94–100 (2004).
31 Matsumura S, Mabuchi K and Toshima K, Lipase-catalyzed ring-opening polymerization of lactide. *Macromol Rapid Commun* **18**:477–482 (1997).
32 Pirozzi D and Greco Jr G, Lipase-catalyzed transformations for the synthesis of butyl lactate: A comparison between esterification and transesterification. *Biotechnol Progress* **22**:444–448 (2006).
33 Namekawa S, Suda S, Uyama H and Kobayashi S, Lipase catalysed ring opening polymeryzation of lactones to polyesters and its machanistics aspects. *Int J Biol Macromol* **25**:145–151 (1999).
34 Deng F and Gross R, Ring opening bulk polymerisation of ε-caprolactone and trimethylene carbonate catalysed by lipase Novozyme 435. *Int J Biol Macromol* **25**:153–159 (1999).
35 Knani D, Gutman A and Kohn D, Enzymatic polyesterification in organic media. Enzyme-catalyzed synthesis of linear hydroxyesters. II. Ring opening polymerisation of ε-caprolactone. *J Polym Sci Part A:Polym Chem* **31**:1221–1232 (1993).
36 Moon S, Lee C, Taniguchi I, Miyamoto M and Kimura Y, Melt/solid polycondensation of l-lactic acid: an alternative route to poly(l-lactic acid) with high molecular weight. *Polymer* **42**:5059–5062 (2001).
37 Takahashi K, Taniguchi I, Miyamoto M and Kimura Y, •Melt/solid polycondensation of glycolic acid to obtain high-molecular-weight poly(glycolic acid). *Polymer* **41**:8725–8728 (2000).

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