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The environmental influence in enzymatic polymerization of aliphatic polyesters in bulk and aqueous mini-emulsion

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ABSTRACT

The catalytic effect of enzymes in different environments has been compared. Biodegradable polyesters and corresponding nanoparticles have been synthesized by an "eco-friendly" technique; enzyme-catalyzed ring-opening polymerization of lactones in bulk and in an aqueous mini-emulsion. Lipases from *Burkholderia cepacia* (lipase PS), *B. cepacia* immobilized on ceramic, *Pseudomonas fluorescens* and *Candida Antarctica* have been used as catalysts in the polymerization of L-Lactide (LLA), pentadecanolide (PDL) and hexadecanolide (HDL). The reaction conditions during the bulk polymerization of LLA were varied by adding different amounts of ethylene glycol at 100 °C or 125 °C. A number average molecular weight (M_n) of 78,100 was obtained when lipase PS was used at 125 °C. Lipase PS had a high catalytic activity in an aqueous environment with 100% conversion in 4 h, and the nanoparticles obtained from mini-emulsion polymerization were between 113 and 534 nm in size. The amount of hydrophobe affected the size of the PDL nanoparticles produced, less than the amount of surfactant in both systems.

1. Introduction

The environment in which an enzyme operates has an impact on its catalytic effect. Enzymes used for ring-opening polymerization (ROP) are lipases that in nature hydrolyse fatty acids in an aqueous environment [1,2], but it has been shown that some lipases are stable in organic solvents and at higher temperatures and can be used for esterification reactions [3,4]. Our group has worked extensively on the organometallic ROP of polyesters such as L-lactide (LLA), ε-caprolactone (CL) and 1,5-dioxepan-2-one (DXO), and lipases are now an attractive alternative as an environment-friendly way to synthesize polyesters [5–7]. Lipase from *Candida Antarctica* (lipase CA) is widely recognized as a versatile catalyst for ROP, and homopolymers from lactones have been synthesized using this enzyme [8,9]. The first report of the bulk synthesis of polylactide (PLA) using an enzyme as a catalyst was published by Matsumura et al [10,11]. They used lipase PS to polymerize L-, D-, and D, L-lactide in the temperature range from 80 °C to 130 °C, and obtained PLA with a number average molecular weight of up to 270,000. Branched PLAs alter the physical, thermal and mechanical properties depending on the number of branches and they have been synthesized with lipase by using multifunctional alcohols e.g. inositol, pentaerythritol, glycerol, and polyglycerine [12–16]. Recently PLAs with different numbers of branches, molecular weights and stereochemistry were synthesized using enzymes [17], and the enzymatic hydrolysis rates of these PLAs were studied [18]. Enzymatic synthesis of larger lactones, such as pentadecalactone has been reported with a M_n 34,000 when lipase CA was used [19,20].

Since the natural environment for enzymes is water, they should have the highest catalytic efficiency in an aqueous environment. Various ring-sized lactones have been synthesized in an aqueous medium catalyzed by lipase [21,22]. It has been showed that enzymes are good catalysts in the mini-emulsion polymerization of cyclic esters [23–25], and they obtained nano-sized particles. Biodegradable nanoparticles are becoming very interesting for their possible applications in the medical field such as drug delivery and tissue engineering. It is important for these applications that the end products are non-toxic, and the technique to obtain these particles is to use polymers and an emulsification method. However, as the polyester polymerizations are often catalyzed by stannous 2-ethylehexanoate ($Sn(Oct)_2$), there are difficulties in removing all traces of the organometallic catalysts and organic solvents [26]. Mini-emulsion polymerization is a method where nanoparticles are formed and, by using an enzyme as a catalyst, the need for tedious purification methods is reduced. In addition, whereas the suspension and dispersion polymerization processes give micron-sized particles, mini-emulsion polymerization gives stable particles in the nanometer size range, typically particles with a diameter of between 50 and 500 nm [23].

The objective was to use the environment influence on the enzymes and assess the effects on the reaction rate, molecular

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weight and particle size. The polymerizations were performed either in bulk using ethylene glycol as initiator or as mini-emulsion polymerization in an aqueous environment. We synthesized aliphatic polyesters using lipase from *Burkholderia cepacia* (lipase PS), lipase from *B. cepacia* immobilized on ceramic (lipase PS-imob), lipase from *Pseudomonas fluorescens* (lipase AK) and lipase CA as catalysts.

2. Experimental section

2.1. Materials

Toluene (Merck, Germany) was dried over a Na-wire before use. Lipases from *P. fluorescens* (lipase AK), *B. cepacia* (lipase PS), *B. cepacia* immobilized on ceramic (lipase PS-imob) and *C Antarctica* Novoenzyme 435 (lipase CA) were purchased from Sigma–Aldrich, Sweden and dried for 2 days under vacuum at room temperature. Hexadecane and Brij 58 (Sigma–Aldrich, Sweden) was used as received. Ethylene glycol (Merck, Germany), chloroform (LabScan, Ireland), hexane (LabScan, Ireland) and methanol (BDH, United Kingdom) were used as received. L-lactide, (LLA) (Serva Feinbiochemica, Germany), was purified by recrystallization in dry toluene. The monomer was then dried for 24 h under reduced pressure at room temperature. Pentadecanolide (PDL), hexadecanolide (HDL) and sodium dodecyl sulphate (SDS) were purchased from Sigma–Aldrich, Sweden and used as received. The monomers were stored under nitrogen atmosphere before use.

2.2. Polymerization technique: enzymatic polymerization in bulk

The polymerizations of poly(L-lactide acid) (PLLA) were performed in bulk using an enzyme as catalyst, and ethylene glycol was used as co-initiator when M/I was 100:1 and 500:1. The amount of lipase was 10 wt% of monomer. The monomer, co-initiator, and lipase were weighed into silanized round-bottom flasks under a nitrogen atmosphere in a drybox (Mbraun MB 150B-G-I). The round-bottom flask was fitted with mechanical stirring and sealed. The polymerizations were started by immersing the flask in a thermostated oil bath (100 °C and 125 °C) and were allowed to proceed for 7 days. For conversion studies, samples were taken out under nitrogen pressure every other day and analyzed with Nuclear Magnetic Resonance (NMR). After polymerization, the enzyme was filtered of and the polymer was precipitated in a mixture of cold hexane and methanol (95:5). After drying under vacuum, the PLLA polymers were stored under dry conditions.

2.3. Mini-emulsion

The mini-emulsion was prepared according to earlier published procedure [24]. In a basic recipe, 2.40 g of pentadecanolide 100 mg of hexadecane (hydrophobe) and 10.0 g of a 1.0 wt% Brij 58 solution in water were stirred for 1 h at 40 °C. The mini-emulsion was prepared by ultrasonicating the mixture for 120 s at 40% amplitude (High Intensity Ultrasonic processor, 400 W, Sweden). A suspension of 50 mg of lipase in 5.0 g of surfactant solution was added and the mini-emulsion was stirred for up to 24 h. The reaction was stopped with sodium dodecyl sulphate (SDS). The temperature and the amounts of monomer, lipase, surfactant and hydrophobe were varied.

3. Characterization

3.1. Nuclear magnetic resonance (NMR)

The degree of monomer conversion was determined by ^{1}H NMR spectroscopy, comparing the relative intensities of the peaks originating from the resonance peaks from the monomer and polymer.^{1}H NMR spectra were obtained using a Bruker Avance DPX-400 Nuclear Magnetic Resonance Spectrometer operating at 400.13 MHz.Non-deuterated chloroform was used as an internal standard ($\delta = 7.26$ ppm).

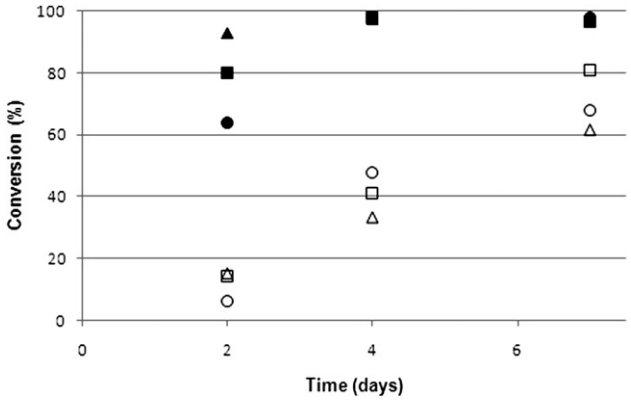

Fig. 1. Conversion as a function of time for the bulk polymerization of LLA catalyzed by lipase from *Burkholderia cepacia* (lipase PS) with and without EG (M/I 100, 500:1) as initiator ○ 100 °C ● 125 °C △ EG100 100 °C ▲ EG100 125 °C □ EG500 100 °C ■ EG500 125 °C.

3.2. Size exclusion chromatography (SEC)

SEC was used to monitor the molecular weights of the polymers after polymerization. The polymers were analyzed with a Waters 717 plus auto-sampler and a Waters model 515 apparatus equipped with three PLgel 10 μm mixed B columns, 300 × 7.5 mm (Polymer Labs., UK). Spectra were recorded with a PL-ELS 1000 evaporative light-scattering detector (Polymer Labs., UK). Millenium^{32} version 3.05.01 software was used to process the data. Chloroform was used as an eluent, at a flow rate of 1.0 mL/min. Polystyrene standards with a narrow molecular weight distribution in the range of 4000–900,000 were used for calibration.

3.3. Dynamic light scattering (DLS)

The size of the polymer particles was determined using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK) with a 514.5 nm red laser collecting the scattered light at an angle of 90°. The measurements were made at a temperature of 22 °C. The samples were diluted 50 times (0.1 g emulsion + 5.0 g deionized water) prior to the analysis. Each sample was analyzed three times.

4. Results and discussion

4.1. Enzymatic bulk polymerization of L-lactide

An attractive alternative and environment-friendly way to synthesize lactones is using enzymes as catalysts in ring-opening

Table 1
Effect of M_o/I ratio on bulk polymerization of LLA by lipase PS with and without EG at 100 °C and 125 °C after 7 days.

M_o/I	T (°C)	Conversion[a] (%)	M_n[b]	PDI
–	100	68.0	1200	3.3
–	125	98.0	78,100	1.4
100	100	61.7	11,800	1.2
100	125	98.1	16,700	1.7
500	100	80.8	11,600	1.2
500	125	96.4	47,000[c]	1.4

[a] Determined by ^{1}H NMR in $CDCl_3$.
[b] Determined by SEC using narrow polystyrene standards and $CHCl_3$ as eluent.
[c] Determined by SEC using polystyrene standards and CH_2Cl_2 as eluent.

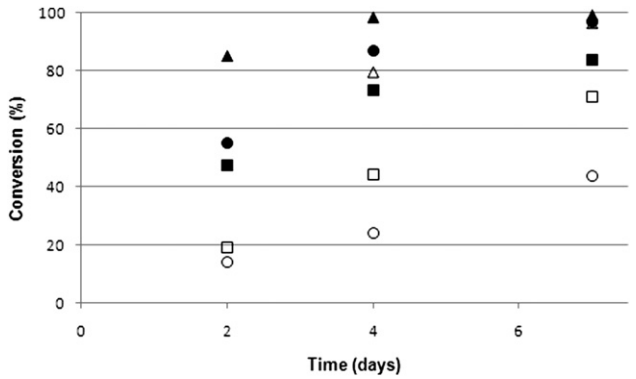

Fig. 2. Conversion as a function of time for the bulk polymerization of LLA catalyzed by immobilized lipase PS with and without EG (M/I 100, 500:1) as initiator ○ 100 °C ● 125 °C △ EG100 100 °C ▲ EG100 125 °C □ EG500 100 °C ■ EG500 125 °C.

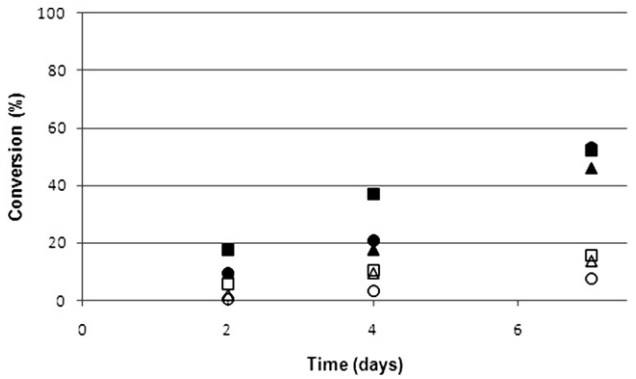

Fig. 3. Conversion as a function of time for the bulk polymerization of LLA catalyzed by lipase AK with and without EG (M/I 100, 500:1) as initiator ○ 100 °C ● 125 °C △ EG100 100 °C ▲ EG100 125 °C □ EG500 100 °C ■ EG500 125 °C.

polymerization (ROP). Bulk polymerizations of L-lactide (LLA) have been performed using three different enzymes, lipase from *B. cepacia* (lipase PS), *B. cepacia* immobilized on ceramic (lipase PS-imob), *P. fluorescens* (lipase AK) and *C. Antarctica* (lipase CA) as catalyst with or without ethylene glycol (EG) as initiator (M/I 100, 500:1). The conversion is shown as a function of time in Fig. 1 for LLA polymerization using lipase PS (10 wt%) as catalyst with and without alcohol as initiator.

The difference in reaction rate between 100 °C and 125 °C is obvious, as the higher temperature gives a higher conversion rate and 100% is reached in 4 days. A large addition of EG at 125 °C resulted in an increased reaction rate during the first two days, without EG and EG100, the conversion was 64% and 93% respectively. The same trend was not seen at 100 °C and the conversion was lower than at 125 °C. It was not higher than 80% even after 7 days. Control bulk experiments of LLA were performed without enzyme present and the conversion after 7 days was insignificant. It is therefore concluded that the reactions are catalyzed purely by the enzyme.

Table 1 summarizes the results of the bulk polymerization of LLA catalyzed by lipase PS and initiated by EG with different M_o/I_o ratios at 100 and 125 °C. The number average molecular weight (M_n) of the synthesized poly(L-lactide) (PLLA) was highest at 125 °C without any initiator present. The more initiator present in the reaction, the lower was the M_n. The water molecules present in the enzyme seem thus to be sufficient to initiate the polymerization of LLA, and no extra initiator is needed. The poly dispersity index (PDI) values do not change with added increasing of EG.

Our group has previously worked with Novoenzyme 435 from *C. Antarctica* [5,6], it is an immobilized enzyme known to have a high catalytic effect towards lactones, and an immobilized version of lipase PS was therefore investigated. A ceramic-immobilized lipase PS (lipase PS-imob) was used as catalyst to evaluate whether the polymerization rate and molecular weight of LLA would be affected. The results for the polymerization of LLA catalyzed by lipase PS-imob (10 wt%) with and without EG are presented in Fig. 2.

A large amount of added EG resulted in a faster reaction at both temperatures, and it is clear that at 100 °C more EG is needed to achieve a high conversion. EG 100,100 °C reached 80% conversion after 4 days and there was almost full conversion after 7 days. There was no great difference in reaction rate between lipase PS and lipase PS-imob, except for M_o/I 100 at 100 °C where 96% conversion was reached with lipase PS-imob compared to 62% with lipase PS. The molecular weight of the polymer increased with low or no EG present and at a higher temperature, as seen in Table 2. For the polymerization of LLA, lipase PS-imob as a catalyst did not give as high a molecular weight as when lipase PS was used.

Fig. 3 shows the conversion as a function of time for the bulk polymerization of LLA using lipase AK (10 wt%) as catalyst with and without EG as initiator. The highest conversion was reached with no EG added at 125 °C. At 100 °C, the conversion was very low, and the addition of EG did not affect the rate. The conversion rates were generally much lower than those with lipase PS and lipase PS-imob. LLA is, in other words, not a suitable substrate for this enzyme.

The resultant molecular weights of the polymers are shown in Table 3 and high molecular weights were obtained without EG at 100 °C. When EG was added, the molecular weights decreased with a higher amount of EG at a higher temperature. M_o/I 100 at 125 °C gave satisfactory molecular weights.

Bulk polymerizations of LLA with lipase from *C. Antarctica,* Novoenzyme 435 (lipase CA) as catalyst were performed under the same conditions as above. The highest conversion was 94%, but the highest M_n was only 9400 and this was obtained for M_o/I 100 at 125 °C. The results thus suggest that lipase PS is the best enzyme for the bulk polymerization of LLA and that the addition of initiator is an advantage.

Table 2
Effect of M_o/I ratio on bulk polymerization of LLA by immobilized lipase PS with and without EG at 100 °C and 125 °C, 7 days.

M_o/I	T (°C)	Conversion[a] (%)	M_n[b]	PDI
–	100	43.5	9200	1.2
–	125	97.1	12,900	1.3
100	100	96.4	6200	1.2
100	125	99.3	8600	1.3
500	100	71.1	10,600	1.4
500	125	83.9	11,300	1.2

[a] Determined by ^{1}H NMR in $CDCl_3$.
[b] Determined by SEC using narrow polystyrene standards and $CHCl_3$ as eluent.

Table 3
Effect of M_o/I ratio on the bulk polymerization of LLA by lipase AK with and without EG at 100 °C and 125 °C.

M_o/I	T (°C)	Conversion[a] (%)	M_n[b]	PDI
–	100	7.6	72,500	1.2
–	125	53.0	39,100	1.2
100	100	13.6	2200	1.3
100	125	46.2	48,000	1.2
500	100	15.7	2000	1.3
500	125	52.1	6200	1.4

[a] Determined by ^{1}H NMR in $CDCl_3$.
[b] Determined by SEC using narrow polystyrene standards and $CHCl_3$ as eluent.

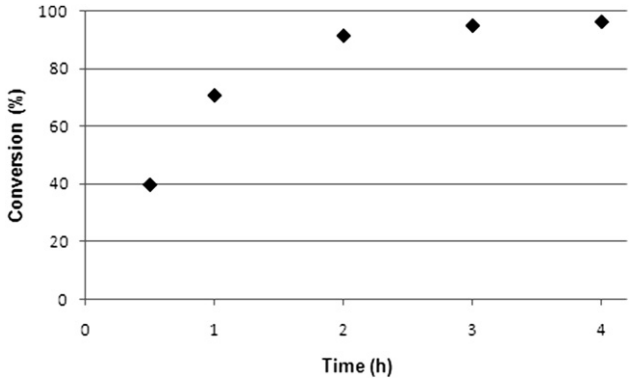

Fig. 4. Conversion as a function of time for the kinetic study of mini-emulsion of HDL catalyzed by lipase PS at 45°C.

4.2. Mini-emulsion

The enzymatic activity was also evaluated in an aqueous environment, in a mini-emulsion ring-opening polymerization. Mini-emulsion with LLA as monomer was not successful and in order to achieve mini-emulsion the monomer was shifted from LLA to a hydrophobic cyclic esters, hexadecanolide (HDL), two enzymes, lipase PS and lipase AK were tested with this monomer. A kinetic study was performed with the 16-membered ester monomer HDL and lipase PS in a mini-emulsion system, Fig. 4. The conversion was above 96% after 4 h at 45 °C and the M_n varied between 1250 and 9300. The M_n and PDI results determined by SEC for HDL polymerized with lipase PS and lipase AK is shown in Table 4 and Table 5 respectively.

Previously published data for lipase PS and pentadecanolide (PDL) indicated that a reaction time of 24 h was needed to reach a high conversion [24]. When PDL and HDL were polymerized in this mini-emulsion system, a conversion close to 100% was reached under 5 h, as shown in Fig. 5 (Figs. S1 and S2 show the NMR data for PDL and HDL). The reactions were compared with bulk polymerizations at 45 °C using 10 wt% lipase PS and it was obvious that the mini-emulsion polymerization worked much better than the bulk system. This agrees with earlier published data [23] and indicates that lipase PS is most efficient when it is operating in its natural environment.

The same conversion studies were carried out using lipase AK. In the bulk system, a conversion of about 20% was reached after 3.5 h as shown in Fig. 6. In contrast, for the same reaction time and a lower reaction temperature, a conversion of close to 80% was reached in the mini-emulsion system.

Table 4
Mini-emulsion polymerization of HDL using lipase PS.

Surfactant [wt%]	Hydrophobe [g]	Reaction time [h]	Conversion HDL [%][a]	M_n[b]	PDI[b]	Z-average size [d nm][c]	PDI[c]
5	0.1	22	100	1250	1.5	534	0.555
0.5	0.1	22	100	1640	2.4	–	–
1	0.01	22	100	1280	2.1	270	0.917
1	1.0	22	100	1540	2.4	199	0.721
1	0.1	5	100	2320	2.4	121	0.064
5	0.1	5	95.9	1710	2.0	197	0.792
0.5	0.1	5	96.7	1520	2.7	355	0.492
1	0.01	5	100	9330	5.6	115	0.111
1	1.0	5	100	2290	2.3	114	0.172

[a] Calculated from ^{1}H NMR spectrum.
[b] determined by SEC using narrow polystyrene standards.
[c] obtained from DLS measurements.

Table 5
Mini-emulsion polymerization of HDL using lipase AK.

Surfactant [wt%]	Hydrophobe [g]	Reaction time [h]	Conversion HDL [%][a]	M_n[b]	PDI[b]	Z-average size [d nm][c]	PDI[c]
5	0.1	22	100	1390	1.6	315	0.610
0.5	0.1	22	98.2	1360	1.9	–	–
1	0.01	22	100	1740	2.3	–	–
1	1.0	22	100	1450	2.1	–	–
1	0.1	5	94.9	1730	1.7	178	0.296
5	0.1	5	94.9	1230	2.0	185	0.377
0.5	0.1	5	91.6	1440	2.2	–	–
1	0.01	5	96.8	1550	2.6	–	–
1	1.0	5	90.0	1770	2.5	244	0.473

[a] Calculated from ^{1}H NMR spectrum.
[b] determined by SEC using narrow polystyrene standards.
[c] obtained from DLS measurement.

Mini-emulsion polymerization using either the lipase AK or the lipase PS enzyme offers an interesting alternative to the more conventional bulk system.

4.3. Mini-emulsion polymerization of HDL using lipase PS

The influences of different parameters in the mini-emulsion polymerization of HDL using lipase PS were assessed by vary the hydrophobe and surfactant concentrations. The amount of surfactant was either 0.5 or 5 wt% while the amount of the hydrophobe was either 0.01 or 1.0 g. The reaction time was set to 5 h or 22 h. Each combination was run three times and the values reported in Table 4 are average values from these three replications. High conversions of 100% or close to it were obtained, independent of the amounts of surfactant, and hydrophobe or the reaction time. The dynamic light scattering (DLS) data showed that the Z-average size was in the range of 113–534 nm, although the PDI was very large in many cases. The high PDI might be a result of non-spherical particles. Lemon-shaped particles have for example been observed using a similar system at higher temperatures [24]. It is never the less obvious from the results that 22 h was too long a reaction time, resulting in a high PDI. The amount of hydrophobe affected the size of the PDL nanoparticles produced less than the amount of surfactant. Larger particles were obtained by decreasing the amount of surfactant.

4.4. Mini-emulsion polymerization of HDL using lipase AK

The influence of surfactant and hydrophobe amount and reaction time on the conversion and particle size were also evaluated using lipase AK as catalyst. The results are presented in Table 5, where it is evident that and lipase AK is not a suitable catalyst for this mini-emulsion polymerization system.

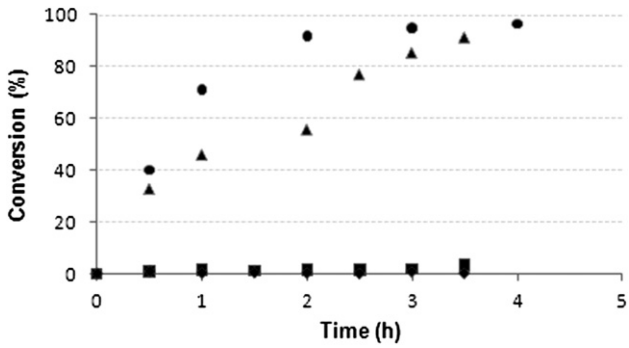

Fig. 5. Conversion as a function of time for the bulk polymerization of PDL catalyzed by lipase PS ■ PDL bulk lipase PS-imob, 45 °C ◆ PDL bulk lipase PS, 45 °C ▲ PDL mini-emulsion, 45 °C ● HDL mini-emulsion, 45 °C.

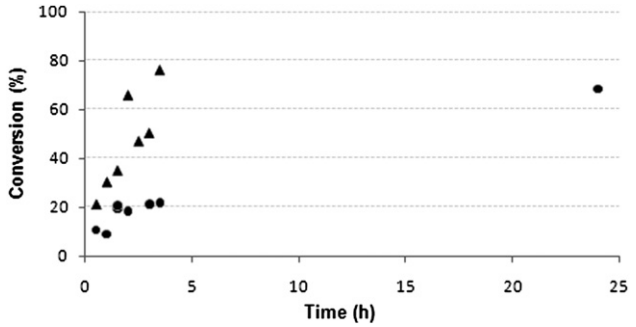

Fig. 6. Conversion as a function of time for the bulk polymerization and mini-emulsion of PDL catalyzed by lipase from lipase AK ▲ PDL mini-emulsion, 45 °C ● PDL bulk, 45 °C.

The Z-average size was in the range of 185–315 nm. The gaps in the table represent reactions which had a very high PDI, were the particle size was very large and the values were accordingly not valid. The PDI values presented are also high, but no trend similar to that with lipase PS could be seen. The amount of hydrophobe affected the size of the PDL nanoparticles less produced than the amount of surfactant.

5. Conclusion

The eco-friendly enzymatic polymerization of aliphatic polyesters clearly shows that lipase from *B. cepacia* (lipase PS) gave the best result in both bulk and in mini-emulsion polymerization. Polymerization of L-lactide (LLA) using lipase PS resulted in a higher number average molecular weight (M_n) and a higher reaction rate than when *P. fluorescens* (lipase AK) or *C. Antarctica* (lipase CA) were used, regardless of whether or not ethylene glycol was used as an initiator. There was no great difference between the reaction rates of lipase PS and *B. cepacia* immobilized on ceramic (lipase PS-imob), but the molecular weights were generally much lower in the case of lipase PS-imob. A M_n of 78,100 was obtained with lipase PS at 125 °C. Lipase CA gave a high conversion after 7 days, but the molecular weight did not increase accordingly. In all the systems, the addition of ethylene glycol increased the reaction rate while the molecular weight decreased. Lipase PS had a much better catalytic efficiency than lipase AK in mini-emulsion polymerization. A reaction time of 22 h was, for both systems, too long, and 5 h was sufficient to reach 100% conversion. The particle size was in the range of 100–500 nm and the amount of surfactant influenced the particle size more than the amount of hydrophobe. High Polydispersity index (PDI) values gave in many cases inaccurate data, but in general lipase PS was evidently more suitable for the aqueous environment. Larger particles were obtained by decreasing the amount of surfactant. These results were better than when lipase AK was used. The environment definitely influence the activity of the enzymes and lipase PS was most adaptable to the environment of enzymes compared.

Acknowledgements

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Appendix. Supplementary data

Supplementary data associated with this article can be found, free of charge, in the online version at doi:10.1016/j.polymer.2010.09.016.

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