Immobilized lipases from *Yarrowia lipolytica* useful for polyester synthesis

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1. Introduction

The use of *in vitro* enzyme catalysis has shown great promise for the preparation of monomers, oligomers, and polymers [1]. Lipases are enzymes that catalyze hydrolysis of fats (fatty acid triglycerides) in living cells. In non aqueous medium, on the other hand, lipase can act as catalyst for esterification and transesterification. This characteristic property has been applied to lipase-catalyzed ring-opening polymerization and polycondensation under mild reaction conditions to produce biodegradable polyesters and polycarbonates [2]. Lipases catalyze the ring-opening polymerization of lactones (small to large rings), cyclic diesters (lactides) and cyclic carbonates to produce polyesters or polycarbonates. The condensation polymerization of hydroxy acid, diacids or polyanhydrides with diols is also catalyzed by lipase. Lipase catalyzed polymerization is an eco-friendly technique for the preparation of useful polyesters by polycondensation as well as polyaddition (ring-opening) reactions [3]. In view of the increased interaction of polymer science and biotechnology, lipases immobilized on polymeric supports are experiencing increased use in many industrial applications. The extent of the immobilization and activity of immobilized lipases is affected by the nature of the support and by environmental factors such as pH, temperature, and nature of the reaction medium. Thus, to design a suitable support for lipase immobilization, all these aspects have to be considered. Immobilized lipase performs better in hydrophobic environments [4].

In this work, we describe the preparation of immobilized biocatalysts from the extracellular lipase *Lip2* from an over producing strain of the yeast *Yarrowia lipolytica* and this application as biocatalyst in the polymerization of polyesters derived from adipic acid and glycerol to form dendritic polymers and from ε-caprolactone to obtain poly (ε-caprolactones).

2. Experimental conditions

Chemicals and supports were obtained from Sigma-Aldrich (Mexico) and comercial immobilized lipases were kindly donated by Novozymes; *Yarrowia lipolytica* strain used for lipase production was JMY329, an engineered strain carrying the lipase gene in about 16 copies per genome [5]. Lipase immobilization was carried out from previous reports [6]: one gram of immobilization support was gently stirred into dialyzed lipase solution (10-95mL) at 4°C for 12 hours, washed and freeze dried. Protein adsorption was followed using the Bradford reagent and BSA as standard. Synthesis of dendrimers was performed at the following conditions: 5 mL of a solution of 40 g/L of adipic acid and an equimolar quantity of glycerol in *t*-butanol was reacted using 100 mg of immobilized lipase at 50°C. PCL synthesis was carried out in 10 mL vials previously dried and purged with dry nitrogen. In a typical run, monomer (ε-CL, 0.5g), catalyst (enzyme, 50 mg) were added under dry nitrogen atmosphere. Vials were stoppered with a rubber septum and placed in a thermostated bath at predetermined temperatures (125 and 150°C) and time periods (6 and 24 h). Final polymer was crystallized from chloroform/methanol, separated from the insoluble enzyme by filtration through 10-15 μm glass-fritted filters and dried under vacuum. Molecular weights and conversions during reaction were monitored by ^{1}H-NMR.

3. Results and discussion

Lip2 production, immobilization optimization and lipase activity measurements results were recently reported by Sandoval, *et al* [7]. Lipases with highest activity were used in the polymer synthesis. Polymers were synthesized under mild conditions as described in the methodology section. FT-IR spectra for dendrimers peak patterns shows three main signals, at 3471 (OH), 2946 (aliphatic C-H stretch) and 1729 (C=O) cm^{-1}. By DSC it was detected a glass transition temperature *(Tg)* of -34.94, -34.87 and -34.82 ºC for dendrimers synthesized by Lip2p on Accurel, N-435 and Lip2p on Lewatit respectively. Chemical shifts for ^{1}H-NMR (200 MHz): δ 1.576 (m, -CH_2-CH_2-CH_2-CH_2-), 2.29 (m, -CH_2-CH_2-CH_2-CH_2-), 3.47 (m, -CH_2-CH-CH_2-), 3.54-3.61 (broad m, -CH_2-CH-CH_2-), 3.75 (m, -CH_2-CH-CH_2-), 4.04 (m, -CH_2-CH-CH_2-), 4.17 (m, -CH_2-CH-CH_2-), 4.35 (m, -CH_2-CH-CH_2-), 5.17 (m, -CH_2-CH-CH_2). ^{13}C NMR: δ 173.64 (COOR), 173.37 (COOR), 172.93 (COOR), 75.43 (CH), 69.46 (CH), 65.42 (CH_2), 62.22 (CH_2), 60.55 (CH_2), 33.54 (CH_2), 33.275 (CH_2), 24.15 (CH_2). These

results agree for those obtained by chemical polymerization reported previously [8]. In figure 1 the ^{1}H-NMR spectrum of dendrimer obtained by biocatalysis is shown.

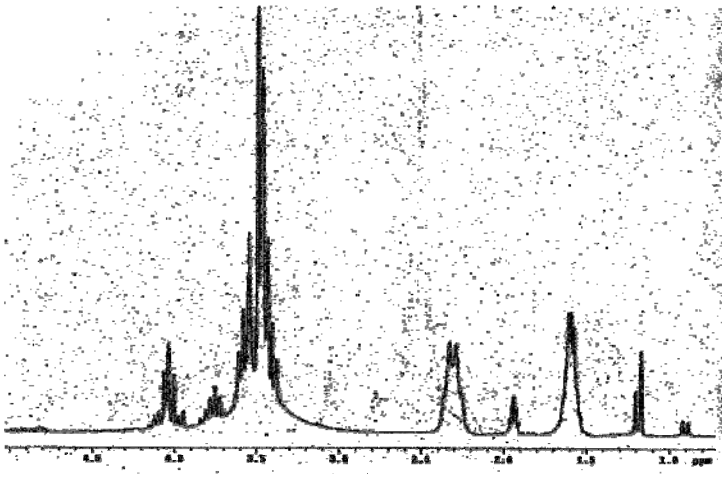

Figure 1. *^{1}H-NMR spectrum of dendrimer obtained with Y.lipolytica lipase immobilized on lewatit in CD_3CN. R= 5 mL of a solution of 40 g/L of adipic acid and an equimolar quantity of glycerol in t-butanol /100 mg lipase. With gentle stirring. T= 50 ℃. t= 48 h.*

Results for PCLs are shown in table 1. We can observe that higher molecular weights are obtained with *Y. lipolytica* lipase in free form and immobilized on lewatit. Higher conversion values and molecular weights are obtained at higher temperatures compared to those obtained by N-435, this can be attributed to a higher thermostability presented by Lip2 lipase and to the denaturation of N-435 at high temperatures.

Table 1. *Molecular weights and degrees of crystallinity of PCLs obtained by ROP with Y. lipolytica lipase.*

Entry	Conversion (%)*	Molecular Weight (Da)*	Crystallinity (%) **
1	91	1201	66.5
2	74	1358	85.04
3	6.3	601	ND
4	3	653	ND
5	100	1585	58.1
6	100	1564	71.2

*Determined by ^{1}H-NMR. **Determined by DSC. ND not determined.

1. R= 0.12 mL ε-CL/12 mg free lipase. T= 150 °C. t= 6 h.
2. R= 0.12 mL ε-CL/12 mg immobilized on lewatit lipase. T= 150 °C. t= 6 h.
3. R= 0.12 mL ε-CL/12 mg N-435 lipase. T= 150 °C. t= 6 h.
4. R= 0.12 mL ε-CL/12 mg immobilized on accurel lipase. T= 150 °C. t= 6 h.
5. R= 0.5 g ε-CL/50 mg free lipase. T= 125 °C. t= 24 h.
6. R= 0.5 g ε-CL/50 mg free lipase. T= 125 °C. t= 24 h with gentle stirring.

The ^{1}H-NMR spectrum for poly (ε-caprolactone) synthesized in vitro (Figure 2), signals for methylene end groups *b* [-CH_2COOH, δ 2.36] and *q* [-CH_2OH, δ 3.64] are clearly seen. The other peaks in the spectrum are assigned to other methylenes of the [-CO-$(CH_2)_5$-O-] repeating unit.

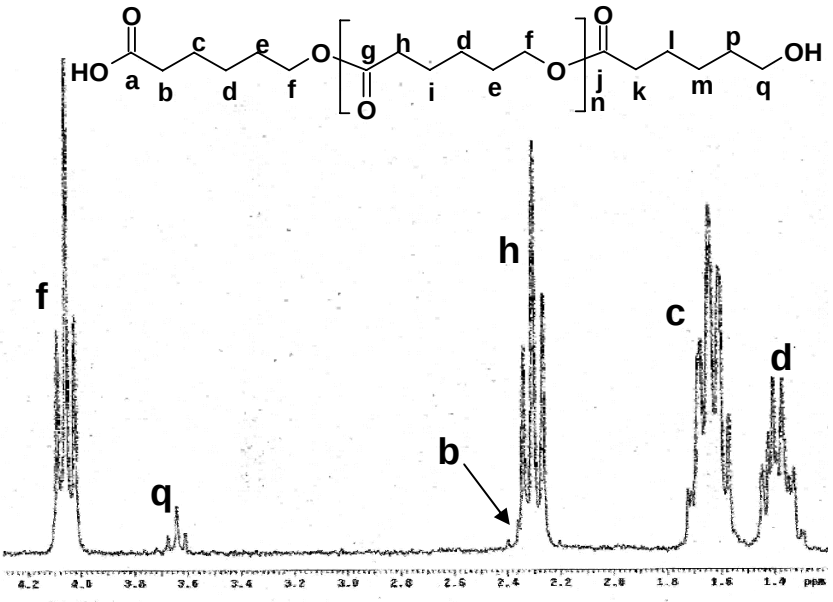

***Figure 2**. ^{1}H-NMR spectrum of poly(ε-CL) obtained with Y. lipolytica lipase immobilized on lewatit in $CDCl_3$. R= 0.12 mL ε-CL/12 mg immobilized on lewatit lipase T= 150 °C. t= 6 h, Mn(NMR)=1358 Da.*

4. Conclusions

Lewatit resulted to be the best suited support to obtain a high activity biocatalyst. Optimized Lip2 immobilized derivatives are good biocatalysts for dendritic polymers and PCLs synthesis. Lip2 showed a higher thermostability at high reaction temperatures compared to Novozyme 435. Molecular weight characterization of synthesized dendrimers is in course. PCLs synthesized are asymmetric telechelic α-hydroxy-ω-carboxylic acid as determined by proton and carbon-13 NMR.

5. References

[1] Nakaoki, T., Mei, Y., Millar, L.M., Kumar, A., Kalra, B., Miller, M.E., Kirk, O., Christensen, M., Gross, R.A., *Industrial Biotechnology* **1(2)** (2005) 126-134.

[2] Uyama, H., Kuwabara, M., Tsujimoto, T., Kobayashi, S., *Polymer Journal* **34(12)** (2002) 970-972.

[3] Varma, I.K., Albertsson, A.C., Rajkhowa, R., Srivastava, R.K., *Prog. Polym. Sci.* **30** (2005) 949–981.

[4] Chauhan, G.S., Chauhan, S., Kumar, Y., Thakur, U. S., Kanwar, S.S., Kaushal R., *Journal of Applied Polymer Science* **105** (2007) 3006–3016.

[5] Madzak, C., Gaillardin, C. and Beckerich, J. M. *Journal of Biotechnology* **109(1-2)** (2004) 63-81.

[6] Al-Duri, B. and Yong, Y. P., *Biochemical Engineering Journal,* **4(3)** (2000) 207-215.

[7] Sandoval, G., Mejía, F., Marty, A., Barrera-Rivera, K. A., *Revista Mexicana de Ingeniería Química* (2007) Submitted.

[8] Carnahan, M. A., Grinstaff, M. W., *Macromolecules* **39(2)** (2006) 609-616.