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Lipase-catalyzed syntheses of linear and hyperbranched polyesters using compressed fluids as solvent media

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ABSTRACT

Enzyme-mediated syntheses of polyester structures are described using green compressed fluids as solvent media, thus avoiding the use of toxic volatile organic compounds or metal catalyst. Supercritical carbon dioxide is presented as a adequate medium for enzymatic synthesis of relatively high molecular weight poly(δ-valerolactone). Alternatively, liquid 1,1,1,2-tetrafluoroethane (R-134a) is a green, non-toxic, relatively polar and hydrophobic solvent, which enhances the solubility of many substrates of interest along with sustained lipase activity for successful syntheses of linear and hyperbranched polyesters. Moreover, liquid R-134a functions under mild reaction conditions (25 bar and 65 °C).

1. Introduction

The development of biocompatible and biodegradable macromolecules with defined structures and properties aiming at food or biomedical applications leads to advanced polymer synthesis throughout non-toxic methodologies [1–3]. Enzymatic ring-opening polymerizations (eROP) of small and medium-sized (4-, 6-, and 7-membered), as well as large-sized (12- to 16-membered) lactones, macrolides and cyclic oligomers proved successful in low polar and hydrophobic solvents [4–9]. Among them, eROP of the seven-member ring lactone, *epsilon*-caprolactone (CL) is widely reported using *Candida antartica* lipase B (CALB) biocatalyst. The linear polyester from the six-member non-substituted lactone, δ-valerolactone (VL), is also an interesting material but still relatively unexplored compared to its parent poly-ε-caprolactone (PCL). eROP of VL is reported in bulk and in common hydrophobic organic solvents with long reaction times (up to 10 days) mediated by lipase from *Pseudomona fluorescens* (lipase PF), lipase from *Candida cylindracea* (lipase CC), porcine pancreatic lipase (PPL), *Rhizopus japonicus* lipase (lipase RJ) and also by CALB to attain in all cases low molecular weight poly-δ-valerolactone (PVL) (<2000 g/mol) [5,10,11].

On the other hand, eROP to afford hyperbranched polyesters is a challenging field of research. The interest in hyperbranched structures arises from their compactness and their enhanced segment density and functionality [12,13]. Properties of hyperbranched polymers are often affected by the nature of the backbone and the chain end functional groups, degree of branching (DB) or molecular weight distributions. The reported procedures for the synthesis of hyperbranched polyesters to date require temperatures exceeding 150 °C and the use of toxic catalysts or initiators [14]. There are several reports on enzyme-mediated polyesters with achieved branched structures in bulk reactions [15,16]; however, reports on enzymatic synthesis of hyperbranched polyesters in the open literature are restricted to the work of Frey and co-workers [17]. These authors described an enzymatic method for the preparation of a hyperbranched polyester by co-polymerization of CL with 2,2-bis(hydroxymethyl)butyric acid (BHB) as AB_2 core [17]. The main drawback of the reaction was the low solubility of the core molecule in low polar toluene. Addition of 1,4-dioxane, which is classified by the IARC as a Group 2B carcinogen, in order to increase substrate solubility had to be kept to minimum as it deactivates the lipase. Besides, enzymatic synthesis of a hyperbranched PVL had yet not been reported. Many polar polymers, such as hyperbranched polyester structures, or some of their monomer precursors present low solubility in the common hydrophobic solvents used in lipase-catalyzed polyester syntheses. Alternatively, we aim at reporting the use of the polar compressed 1,1,1,2-tetrafluoroethane (R-134a) as solvent to achieve novel polyester structures by enzymatic means. R-134a has nowadays extended use as refrigerant or pro-

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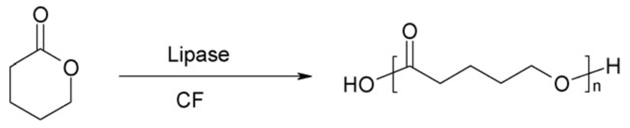

Fig. 1. Reaction scheme for eROP of VL in CF.

pellant and its liquid state is easily reached at moderate pressures (<10 bar, at 25 °C) [18]. Compressed R-134a is regarded as green solvent since it is inert, non-toxic and non-flammable, it has no ozone depletion potential (ODP) and it is classified as generally recognized as safe (GRAS). R-134a is also manufactured to current good manufacturing practice (cGMP) standards for use in metered-dose inhaler applications in the pharmaceutical industry or other medical applications [19]. Further advantage relies on its polarity, which is comparable to tetrahydrofuran and dichloromethane, in which lipases present poor enzymatic activities. Instead, liquid R-134a is hydrophobic, thus allowing for the retention of water molecules on the active site of lipases to sustain activity [20–22]. Generally, compressed fluids technologies allow the complete re-use of the solvent media by recirculation in closed-loop systems, thus having the potential to be environmentally benign and economical alternative to VOCs.

2. Materials and methods

2.1. General considerations

VL and CL were distilled under vacuum (30 mmHg, 90 °C) prior to use over CaH_2 and stored over 3 Å molecular sieves at 5 °C. BHB (Aldrich, US) was used as received. The enzyme catalyst Novozym 435 was a kind gift from Novozymes (Mexico) and consisted of ~10% by wt of CALB supported on an acrylic resin with a specific activity of 7000 PLU/g. Tetrahydrofuran spectrophotometric grade was purchase from J.T. Baker (Mexico). Carbon dioxide (research grade 99.8%) was provided by Infra-Air products (México) and used as supplied. R-134a standard cylinder (Dupont, SUVA, 98% purity, US) was supplied by Refacciones Star (Mexico). Chloroform and methanol (Química Barsa, México, technical grade) were used as supplied. ^{1}H NMR spectra were recorded in a Varian Unity Innova spectrometer operating at 400 MHz using deuterated chloroform and tetramethylsilane as internal reference. DB was obtained following the equation reported elsewhere [17]. The number and weight-average molecular weights of PVL and hyperbranched polyesters were measured by SEC with two columns (PLgel 10 μm MIXED-B LS 300 × 7.5 mm, Polymer Laboratories, VARIAN, US) mounted on a Agilent 1200 series thermostat (25 °C) (US). The columns were eluted with THF (flow rate of 1 mL/min) by a HP Series 1100 isocratic pump and calibrated with polystyrene standards. An evaporative light scattering PL-ELS 1000 (Polymer Laboratories, US) operating at gas flow = 1.0 (neb. temp. = 40 °C; evap. temp. = 80 °C) was used as detector. MALDI-TOF spectra were recorded in a MALDI-TOF/TOF 4800 Plus^{TM} Analyzer of Applied Biosystems (US). Hyperbranched polymers (4 mg) were dissolved in dioxane (1 mL). A volume of 10 μL of this solution was mixed with an equal volume of matrix (10 mg/mL 2,5-dihydroxybenzoic acid (DHB) in methanol/water (1:1, v/v)). An aliquot (0.5 μL) was applied to the sample probe, which was inserted into the spectrometer after evaporation of the solvent under reduced pressure. Spectra were taken in the positive ion mode. Products having m/z values below 500 were disregarded in the evaluation of spectra to avoid matrix signals. Differential scanning calorimetry (DSC) measurements were carried out in a DuPont DSC 2100 (US) in the range −100 to 100 °C at a heating rate of 10 °C/min, under nitrogen atmosphere. The thermogravimetric analyses (TGA) were conducted on a TA Instruments Hi-Res TGA 2950 (US) in the 25–300 °C at a heating rate of 10 °C/min, under nitrogen atmosphere.

2.2. Solubility experiments and polymerization procedures

A typical procedure for the synthesis of the linear PVL and hyperbranched PVL and PCL in compressed fluids: Biocatalyst beads (1.4 g) were placed in 40 mL stainless steel reactors equipped with a crossbar magnetic stirrer driven by external stirring plates. Thermally inactivated enzyme was used in control experiments. BHB (1.4 g, 9.4 mmol) was added for hyperbranched polyester syntheses. The reactor and its contents were dried overnight using a high vac-

Table 1
Results of the lipase-catalyzed synthesis of PVL in CF and in bulk.

Entry	Time (h)	Reaction media	Yield[b] (%)	M_n[c] (×10^{-3} g/mol)	PDI[c]
1	12	R-134a	21.4	5.7	1.6
2	12	$scCO_2$	–	–	–
3	12	Bulk	–	–	–
4	24	R-134a	52.0	11.3	1.5
5	24	$scCO_2$	55.7	13.1	1.8
6	24	Bulk	–	–	–
7	48	R-134a	65.1	8.3	1.7
8	48	$scCO_2$	66.5	18.4	1.6
9	48	Bulk	14.3	1.7	1.4
10	72	R-134a	66.3	7.9	1.7
11	72	$scCO_2$	68.6	28.0	1.6
12	72	Bulk	69.8	5.0	1.3
13	96	R-134a	63.9	6.6	1.7
14	96	$scCO_2$	74.9	22.7	1.7
15	96	Bulk	70.5	5.3	1.3
16	120	R-134a	71.2	6.2	1.7
17	120	$scCO_2$	89.4	16.4	1.8
18	120	Bulk	70.4	4.3	1.3
19	144	R-134a	69.9	9.1	1.6
20	144	$scCO_2$	50.1	7.7	1.7
21	168	R-134a	60.1	5.2	1.4
22	168	$scCO_2$	25.3	4.2	1.8
23	48	Control polymer[a]	0	–	–

[a] Control polymer was carried out using steam-sterilized enzyme.
[b] Determined by weight.
[c] Determined by SEC calibrated with polystyrene standards.

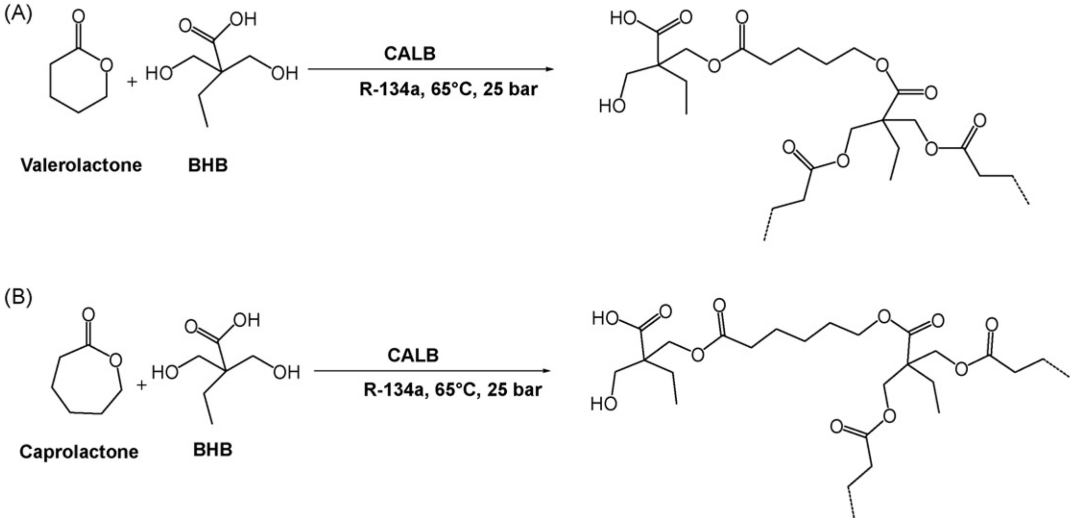

Fig. 2. Reaction scheme for eROP of hyperbranched polyesters in liquid R-134a.

uum oil pump (Vacubrand, GER). Then, VL (14 mL, 150.8 mmol) or CL (14 mL, 126.3 mmol) when indicated, were vaccum transferred to the reactor. The reactor was loaded with liquid R134a or carbon dioxide throughout an ISCO high-pressure syringe pump (ISCO Corp., US). The pressurizing column of the ISCO syringe pump was cooled by a refrigerant bath (Julabo, EC12). Two independent thermocouples, one measuring the temperature at the heating jacket and the other one measuring the temperature inside the reactor

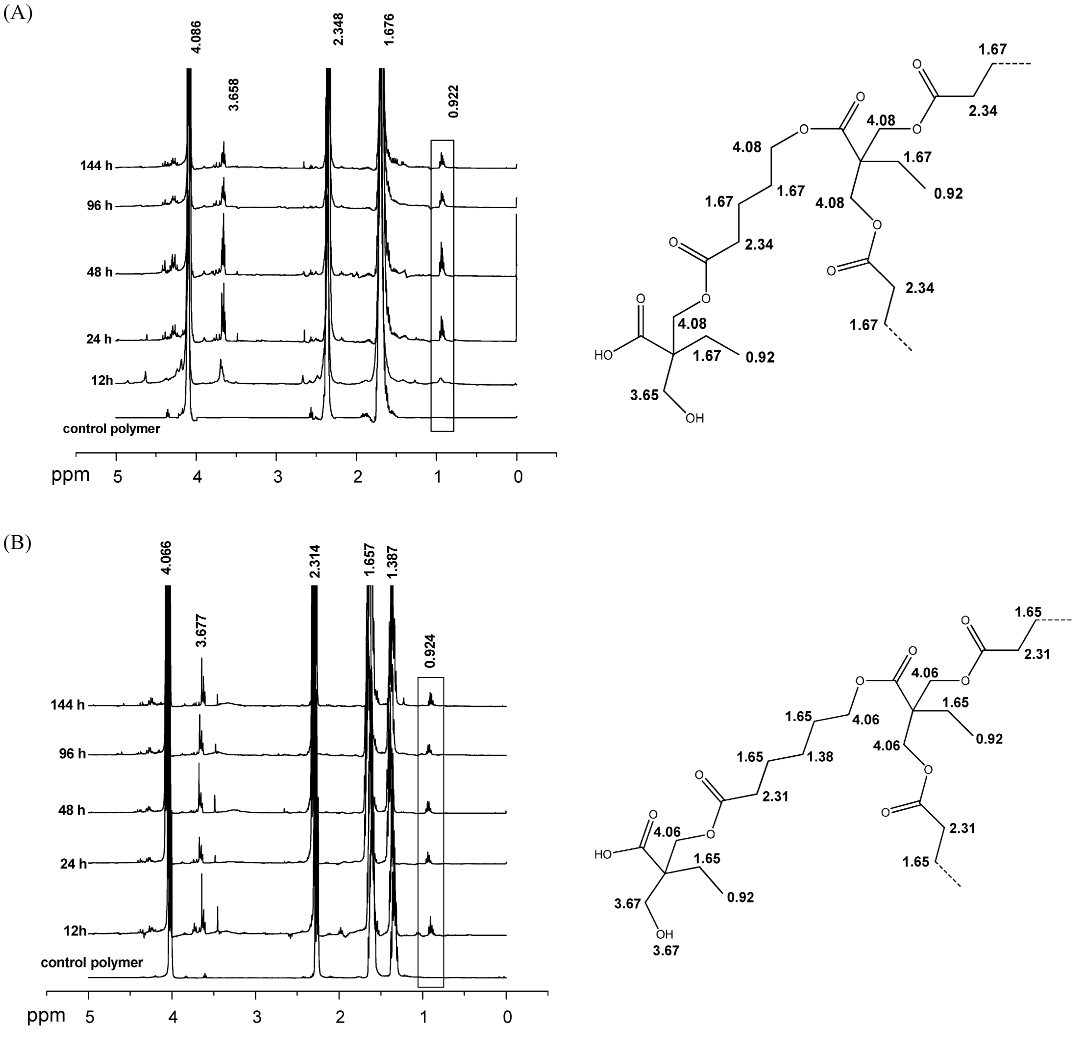

Fig. 3. (A) ^{1}H NMR spectra for poly-(VL-co-BHB) upon reaction times and control homopolymer PVL; (B) ^{1}H NMR spectra for poly-(CL-co-BHB) upon reaction times and control homopolymer PCL. Molecular drawings show estimated ^{1}H NMR shifts (δ/ppm) determined by Chem office software (Cambridgesoft).

Table 2
Characterization data of the hyperbranched polyesters prepared with VL (6 mol% BHB).

Entry	Time (h)	Copolymer fraction	Yield[b] %	DB[c]	M_n[d] ($\times 10^{-3}$ g/mol)	PDI[d]	T_m[e] (°C)	T_d[f] (°C)
1	12	1st	34.6	0.02	1.6	1.6	54.9	250.3
		2nd	9.6	0.07	0.9	1.5	48.9	247.4
2	24	1st	34.7	0.03	1.9	1.5	55.5	251.8
		2nd	10.9	0.06	0.9	1.5	55.5	243.2
3	48	1st	39.1	0.06	2.0	1.6	55.6	257.4
		2nd	15.0	0.07	1.1	1.5	50.9	255.4
4	96	1st	35.7	0.07	2.1	1.5	55.9	259.7
		2nd	15.1	0.07	1.2	1.5	50.6	242.3
5	144	1st	28.7	0.07	2.0	1.5	53.7	251.9
		2nd	4.9	0.09	0.9	1.5	48.8	241.8
6	48	Control polymer[a]	65.1	–	8.3	1.7	56.1	270.0

[a] Control polymer was carried out using only VL at 48 h of reaction.
[b] Determined by weight.
[c] BHB% and DB was determined from the integration of characteristic ^{1}H NMR signals.
[d] Determined by SEC calibrated with polystyrene standards.
[e] Measured by DSC at a heating rate of 10 °C/min.
[f] Degradation temperature corresponding to 5% weight loss of material measured by TGA at a heating rate of 10 °C/min.

carefully monitored the reaction temperature. Operating temperature and pressure for liquid 134a were adjusted at 65 ± 1 °C and 25 ± 2 bar, respectively, for all polimerization experiments. Operating pressure and temperature for all experiments with $scCO_2$ was 300 ± 5 bar and 65 ± 1 °C, respectively. Depressurization of the reactor to atmospheric pressure at the end of each experiment proceeded after cooling the reactor to room temperature prior to opening. Bulk eROP of VL (14 mL) at 65 °C was also carried out with the biocatalyst (1.4 g). Reactor contents were dissolved in chloroform and the insoluble biocatalyst beads were removed by filtration. Liquid fractions were precipitated in cold methanol 1/10 (v/v) to yield the linear polyesters or hyperbranched polyesters. For hyperbranched polyesters the metabolic phase was cooled to 5 °C for 24 h in order to precipitate a second fraction, which was recovered by filtration. Polyester samples were dried under vacuum (4×10^{-4} mbar, 2–3 h) and weighed to determine the product yield.

^{1}H NMR ($CDCl_3$) hyperbranched PVL (δ/ppm): 4.08 ($–CH_2O–$); 3.65 ($–CH_2OH$); 2.34 ($–CH_2COO–$); 1.67 ($–CH_2CH_2–$); 0.92 ($–CH_3$).

^{1}H NMR ($CDCl_3$) hyperbranched PCL (δ/ppm): 4.06 ($–CH_2O–$); 3.67 ($–CH_2OH$); 2.31 ($–CH_2COO–$); 1.65 ($–CH_2CH_2COO–$); 1.38 ($–CH_2CH_2CH_2–$); 0.92 ($–CH_3$).

Solubility measurements. Solubility of selected monomer mixtures BHB with CL and BHB with VL in the CF was carried in a cylindrical 40 mL stainless steel reactor equipped with sapphire windows and a cross-bar magnetic stirrer driven by external stirring plates. Heat was applied by external ceramic jackets. Temperature was controlled by two independent thermocouples one measuring the temperature at the heating jacket and the other one measuring the temperature inside the reactor. CF loads proceed by ISCO pump similarly to the described polymerization procedures. The operating pressure and temperature were 25 ± 2 bar and 65 ± 1 °C for Liquid R-134a and 300 ± 5 bar and 65 ± 1 °C for $scCO_2$, respectively.

3. Results and discussion

3.1. eROP of VL in liquid R-134a and in $scCO_2$

The relatively high ring-strain of the 7-member CL enhances de production of higher molecular weight polyesters by eROP (40,000–80,000 g/mol) than the six-member ring VL, which lipase-mediated polymerization has only been reported in low propagation (<2000 g/mol) using conventional hydrophobic solvents [5,10,11,22]. However, PVL can also be achieved in relatively high molecular weight by eROP using liquid R-134a or $scCO_2$ as solvent media and CALB biocatalyst (Fig. 1). The results on the eROP of VL in CF media are shown in Table 1. The experiment of entry 23 in Table 1 for thermally inactivated enzyme demonstrates that no conversion was obtained in the absence of biocatalyst activity.

Comparative bulk polymerization experiments (Table 1) were halted at 120 h by the solidification of the media; in addition an induction period of 48 h was needed to attain the polymer product, whereas a maximum M_n of 28,000 g/mol with a PDI of 1.6 was

Table 3
Results of the hyperbranched polyesters prepared with CL (7 mol% BHB).

Entry	Reaction time (h)	Copolymer fraction	Yield[b] %	DB[c]	M_n[d] ($\times 10^{-3}$ g/mol)	PDI[d]	T_m[e] (°C)	T_d[f] (°C)
1	12	1st	30.5	0.02	2.5	1.6	60.2	270.0
		2nd	12.3	0.10	0.8	1.7	57.1	160.0
2	24	1st	42.3	0.02	2.7	1.6	59.3	200.0
		2nd	15.0	0.09	0.9	1.7	49.3	220.0
3	48	1st	54.1	0.05	2.4	1.5	57.5	260.0
		2nd	13.5	0.09	1.1	1.6	55.2	250.0
4	96	1st	61.7	0.04	2.7	1.5	56.5	250.0
		2nd	9.1	0.11	0.8	1.7	52.2	260.0
5	144	1st	40.7	0.04	2.7	1.5	57.0	270.0
		2nd	7.0	0.09	0.9	1.7	52.8	255.7
6	48	Control polymer[a]	67.0	–	18.6	4.5	58.9	280.0

[a] Control polymer was identically carried out but without BHB addition.
[b] Determined by weight.
[c] DB was determined from the integration of characteristic ^{1}H NMR signals.
[d] Determined by SEC calibrated with polystyrene standards.
[e] Measured by DSC at a heating rate of 10 °C/min.
[f] Degradation temperature measured by TGA corresponding to 5% weight loss of material at a heating rate of 10 °C/min.

achieved using $scCO_2$ ($T = 65\,^\circ C$, $P = 300$ bar, $t = 72$ h). Then, the maximum molecular weight attained was more than 10-fold higher than those reported earlier for eROP of VL [5,10,11]. Highest polymer yield (*ca.* 90%) was also achieved using $scCO_2$ media (120 h). As can be observed in Table 1, the use of liquid R-134a (25 bar, 65 °C) gave 21% polyester yield in the first 12 h, whereas at this time there was no recovered material using $scCO_2$. Both media showed a decrease in polymer yield at longer reaction times attributed to polymer degradation, being more dramatic for $scCO_2$ (25.3% at 168 h). Depressurization of the reactor to atmospheric pressure at the end of each experiment proceeded easily in experiments carried out with R-134a after cooling the reactor at 5 °C for few minutes or at room temperature for 30 min. Whereas, in the case of $scCO_2$, depressurization took longer times and further refrigeration at −78 °C for 1 h was needed prior to a safe opening.

3.2. *eROP of hyperbranched VL and CL polyesters*

Dissolution of the BHB (10 wt%) in the presence of VL and CL in the liquid R-134a media at the experimental conditions (65 °C, 25 bar) was initially corroborated by solubility experiments. However, poor solubility of this compound (BHB <3 wt%) was observed at identical concentrations but using $scCO_2$ (65 °C, 300 bar), which did not encourage its use for the synthesis of the target materials. The results on eROP of VL and CL with the concurrent BHB polycondensation in liquid R-134a is presented herein, thus leading to the new enzyme-mediated hyperbranched structure poly(VL-co-BHB), see Fig. 2. As well, enzyme-mediated poly(CL-co-BHB) was achieved avoiding the use of toxic volatile organic solvents reported elsewhere [17]. ^{1}H NMR spectra for hyperbranched co-polyesters shown in Fig. 3 confirms the incorporation of BHB.

The materials were initially precipitated in methanol to yield the first fractions with the lowest DB (see Tables 2 and 3), but after filtration, the methanolic phases were kept at 5 °C (5 h) to allow the precipitation of a second fraction with highest DB. Data compiled in Table 2 and Table 3, for poly(VL-co-BHB) and poly(CL-co-BHB), respectively, shows that yields in both hyperbranched polyester fractions tend to increase in the first 48 h, followed by a decrease, which might be attributed to polymer degradation. Thermal properties of the attained hyperbranched copolymers were investigated by means of DSC and TGA (Tables 2 and 3). DSC traces displayed similar T_m values for both linear and hyperbranched materials with acceptable degradation temperatures (T_d).

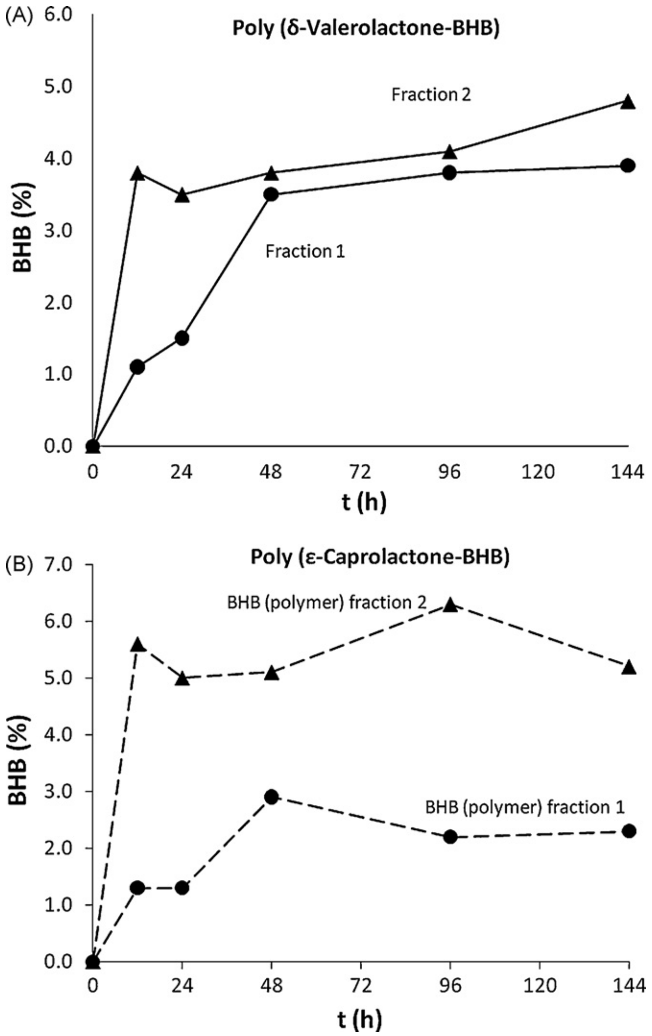

Fig. 4. (A) BHB% (polymer)-time relationships (● fraction 1, ▲fraction 2) for poly-VL-co-BHB; (B) BHB% (polymer)-time relationships (● fraction 1, ▲fraction 2) for poly-VL-co-BHB.

Incorporation of the AB_2 monomer in the copolymers was determined from the integration of the characteristic ^{1}H NMR signals. The incorporated BHB units show a characteristic signal at 0.9 ppm that can be used to quantify their fraction by comparison to that assigned to the $–OOC–CH_2$ group of the VL and CL repeat units ($\delta = 2.3$ ppm) [17]. The first fractions exhibit a maxi-

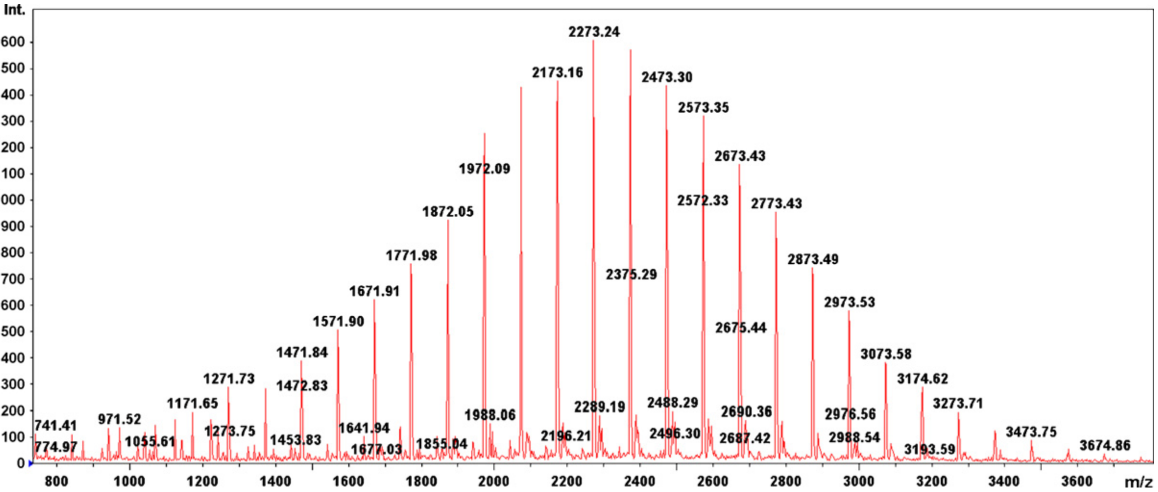

Fig. 5. MALDI-TOF spectrum of the sample of hyperbranched PVL with the AB_2 core from entry 2 (2nd fraction) in Table 2.

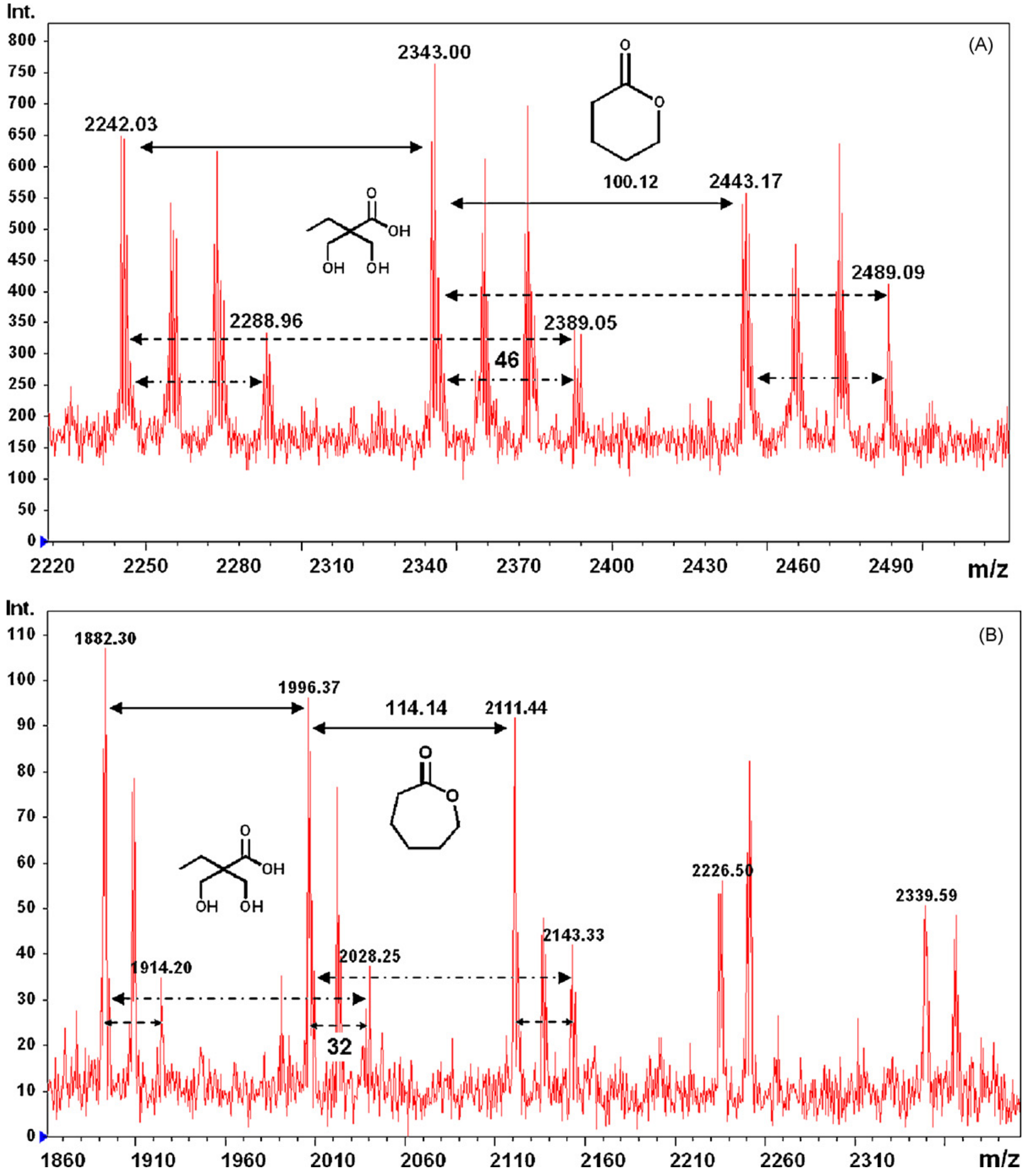

Fig. 6. Representative section of the MALDI-TOF spectra of hyperbranched polyesters (samples 48 h) recorded in linear mode (A) VL and (B) CL first fractions.

mum DB at 48 h reaction, whereas, the second fractions showed no DB variations with reaction time. In all cases, BHB incorporation and DB were higher on the second fractions than in the firstly precipitated products. Fig. 4 shows BHB incorporation percentage versus time for both types of polyester structures. The experimental evidences pointed out that the co-polymerization of VL and CL with BHB undergo rapidly (12 h) and yields and molecular weights did not display substantial variations over time in both fractions.

The M_n values determined by means of SEC fall between 1300 and 3200 g/mol for hyperbranched VL and from 1,400 to 4300 g/mol for poly(CL-co-BHB). Interestingly, all the prepared polymers showed relatively narrow PDI in the range of 1.5–1.6 and 1.5–1.7 for co-polymerizations using VL and CL, respectively.

MALDI-TOF mass spectrometry data corroborated the molecular structure of the hyperbranched polyesters. Fig. 5 shows the MALDI-TOF spectrum acquired for poly(VL-co-BHB) as an example.

As observed in Fig. 6A as a representative section of spectrum, signals were observed at regular intervals of 100 m/z corresponding to the molecular weight of a VL unit. The difference between adjacent peaks equals 46 m/z were assigned to the exchange of a VL unit for a BHB unit, which confirms a random incorporation of each unit in the copolymers. Observed minor series of signals with the loss of 18 units (H_2O) from the major peaks might indicate that macromolecular cyclization also occurred to a certain extent, as reported elsewhere [17]. In the case of poly(CL-co-BHB), the difference between adjacent pick equals 32 were attributed to the exchange of a CL unit (114 g/mol) for a BHB unit (Fig. 6B). MALDI-TOF spectra displayed higher molecular weights than those obtained by SEC, being up to 5500 g/mol for poly(VL-co-BHB) and *ca.* 6000 g/mol for poly(CL-co-BHB). Therefore, on the basis of our results on molecular weight determinations similar propagation can be obtained in our system also with the less strained cyclic lactone.

4. Conclusions

Lipase-mediated synthesis of relatively high molecular weight PVL is reported in supercritical carbon dioxide and liquid R-134a solvent media. $scCO_2$ might be useful for green polymer synthesis whenever possible based on its low cost and availability, as well as good physical properties. However, new experimental evidences shown in this work demonstrate that the selected monomer substrates to achieve the hyperbranched polyesters present low solubility in compressed CO_2 and good solubility in liquid R-134a. Herein, novel lipase-mediated hyperbranched poly(VL-co-BHB) has been achieved, as well as poly(CL-co-BHB), by a non-toxic method. Then, research on liquid R-134a or other non-toxic and polar CF to exploit sustainable and non-toxic polymerization routes shows great potential.

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