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Enzymatic Poly(octamethylene suberate) Synthesis by a Two-Step Polymerization Method Based on the New Greener Polymer-5B Technology

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Abstract: Here, we report a new two-step enzymatic polymerization strategy for the synthesis of poly(octamethylene suberate) (POS) using an immobilized *Pseudozyma antarctica* lipase B (IMM-PBLI). The strategy overcomes the lack of enzymatic POS synthesis in solvent-free systems and increases the final polymer molecular weight. In the first step, the direct polycondensation of suberic acid and 1,8-octanediol was catalyzed by IMM-PBLI at 45 °C, leading to the production of prepolymers with molecular weights (MWs) of 2800, 3400, and 4900 g mol^{-1} after 8 h in miniemulsion, water, and an organic solvent (cyclohexane: tetrahydrofuran 5:1 *v/v*), respectively. In the second polymerization step, wet prepolymers were incubated at 60 or 80 °C, at atmospheric pressure, in the presence of IMM-PBLI, and without stirring. The final POS polymers showed a significant increase in MW to 5000, 5800, and 19,800 g mol^{-1} (previously synthesized in miniemulsion, water, or organic solvent, respectively). FTIR analysis of the final polymers confirmed the successful POS synthesis and a high degree of monomer conversion. This innovative two-step polymerization strategy opens up a new opportunity for implementing greener and more environmentally friendly processes for synthesizing biodegradable polyesters.

Keywords: enzymatic polycondensation; aqueous media; organic solvent media; bulk polymerization; two-step polymerization

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1. Introduction

Plastics have considerable importance in modern society. However, they are rarely produced by green processes or considered to be environmentally friendly. Process sustainability and polymer biodegradability are critical requirements for the next generation of plastics. The new biodegradable plastics should be hydrolyzable to their corresponding monomers, recycled/reused, or easily degraded by environmental microorganisms [1,2]. Polyester-based biodegradable plastics have gained interest due to the easy hydrolysis of their ester bonds and their potential use in a broad range of applications, from food packaging materials to industrial and biomedical applications [3–5].

The increasing demand for biodegradable polyesters has enhanced interest in the study of poly(alkylene dicarboxylate)s, such as poly(octamethylene suberate) (POS) [6,7]. The synthesis of such biodegradable polyesters remains a challenge due to the requirement for high temperatures and organometallic catalysis, which leads to low selectivity and specificity, toxicity, and environmental concerns [6]. Furthermore, special attention to atom

economy must be paid as it is now desirable to produce plastic with as little waste as possible [8].

To overcome these adverse effects, biocatalysis using free or immobilized enzymes is gaining attention as an alternative to the conventional organic chemistry polymerization processes. The nature of the polymerization medium and reaction conditions significantly influence the polyester characteristics and biocatalyst stability [9–12]. However, these enzymatic polyester syntheses continue to operate under unfavorable polymerization conditions, such as using organic solvents and high temperatures, or even when a low vacuum is used to remove the generated by-product (e.g., water), which may inhibit or denature the enzyme [13–16]. Compared to organic solvent media, the use of water as a solvent in polymerization has economic and environmental benefits [16,17].

Researchers have previously explored the potential of enzymatic polymerization in a miniemulsion system to obtain aliphatic polyesters, comparing water and organic solvent as alternative reaction systems [17]. The lipase-catalyzed polycondensation described occurs in a renewed aqueous polymerization medium, presenting excellent results for polyester synthesis and biocatalyst reutilization without the need to use organic solvents for polyester recovery [17]. This technology uses immobilized lipases or esterases to perform polycondensation with high concentrations of insoluble monomers (precipitate) in the aqueous medium and the synthesized polyesters. For this reason, this polycondensation strategy was classified as "from solid to solid via biocatalysis" at a low temperature in a sustainable and environmentally friendly process and as part of the Polymer-5B technology [18].

The molecular weight (MW) of polyesters determines their functionality and applicability. Polyesters with MWs above 10,000 g mol^{-1} form an important class of polymers widely used in producing fibers, films, and 3D structures [19]. Polyesters with MWs below 10,000 g mol^{-1} are used as plasticizers, thickeners, or softeners for skin creams and cosmetics, due to their adhesive properties. They are also used in microelectronics and as precursor building blocks or prepolymers for the copolymerization of different polyesters and polyamides and for polyurethane manufacturing.

The synthesis of polyesters by polymerization in solvent-free reaction systems (bulk polymerization) has been successfully used in chemical synthesis with temperatures between 150 and 280 °C and in enzymatic synthesis at lower temperatures, between 70 and 120 °C [20–23]. For example, Hunsen, et al. [24] and Azim et al. [25] tested *Humicola insolens* cutinase immobilized on a polymeric support (Lewatit) to synthesize polyesters by bulk polymerization. The polycondensation occurred between adipic acid and diols from C4 to C8 at 70 °C over a 48 h period. The experiment used low-vacuum conditions and tetrahydrofuran solvent and resulted in the production of polymers with MW between 2700 and 12,000 g mol^{-1} [24,25].

A more careful analysis of polymerization strategies, particularly direct polycondensation and bulk polymerization in the synthesis of polyesters, revealed several severe energetic and environmental drawbacks. These pose a limiting factor for industrial applications if polyester production is performed via enzymatic synthesis [10,26]. For example, they require the use of organic solvents, high temperatures, and low-vacuum conditions (0.1 to 100 mmHg) for the removal of by-products (water and alcohols). High concentrations of the active enzyme are required in the initial reaction medium, the polymerization reaction time is longer, and the enzymes can only be used once due to the low stability of the biocatalyst under these reaction conditions [10,27]. For these reasons, until now, there has been no enzyme polymerization synthesis implemented on a large scale.

For industrial applications, polymer molecular weight is an important consideration. An increase in polymer MW can improve the thermal and mechanical properties specific to a practical application. Two-step polymerization is a simple and efficient methodology to increase the polymer MW, and Vouyiouka et al. (2013) propose a green route for the preparation of aliphatic polyesters in two-step polymerization [28].

In previous a work, the authors presented an innovative enzymatic polymerization strategy for the synthesis of poly(octamethylene suberate) (POS) in water and miniemulsion. It allowed for the reutilization of the enzyme multiple times, which could be advantageous for industrial processes [17,18]. Here, the authors describe the continuation of these studies on poly(octamethylene suberate) (POS) synthesis, using an innovative two-step polymerization strategy catalyzed by immobilized *Pseudozyma antarctica* lipase B (IMM-PBLI). They combine direct polycondensation in three different reaction media (miniemulsion, water, and organic solvent) (first step), with thermal incubation in the presence of the same biocatalyst (IMM-PBLI) (second step).

2. Materials and Methods

2.1. Materials

2.1.1. Chemical Reagents

Suberic acid (99%, Acros Organics, Geel, Belgium) and 1,8-Octanediol (98%, Acros Organics, Geel, Belgium) monomers were used for polyester synthesis. Hexadecane (99%, Sigma Aldrich, Darmstadt, Germany) and Triton-X100 (Merck) were used to obtain stable miniemulsions. Cyclohexane (99.5%, Merck, Darmstadt, Germany) and Tetrahydrofuran (THF) (99%, contains 250 ppm BHT as an inhibitor) from Honeywell, Charlotte, NC, USA, were mixed (5:1 *v/v*, respectively) and used as the organic solvent. Sodium and potassium hydroxide solution, 5 M, 0.5 M, and 12.5 mM, were prepared from standards (Merck, Darmstadt, Germany). Tributyrin (98%, Sigma Aldrich, Darmstadt, Germany) and NaCl (99.5%, Panreac Química SLU, Barcelona, Spain) were used for enzyme activity assays.

2.1.2. Commercial Enzyme Preparations

Pseudozyma antarctica lipase B (PBL), previously known as *Candida antarctica* lipase B (CALB) powder (Chiral Vision, Den Hoorn, The Netherlands), was dissolved and tested in the free form. Several commercial immobilized enzyme preparations were used for POS synthesis, such as *Pseudozyma antarctica* lipase B adsorbed on acrylic carriers (IMMCALB-T1-350, Chiral Vision, Den Hoorn, The Netherlands), identified in this work as IMM-PBLI; a cutinase (lipase NZ 51032, Novozymes) covalently immobilized on acrylic support IB-150A (IMML51-T2-150, Chiral Vision, Den Hoorn, The Netherlands); a *Candida antarctica* lipase B immobilized by adsorption on methacrylic support (Novozym 435, Novozymes, Bagsvaerd, Denmark); and a *Candida antarctica* lipase B expressed in *Aspergillus niger* immobilized in macroporous acrylic resin (CALB-Sigma, Sigma, Darmstadt, Germany).

The beads of the different immobilized enzyme preparations were washed with Milli-Q water (Merck Millipore, Darmstad, Germany) and left to dry at room temperature before use, as per the supplier's recommendations.

2.2. Methods

2.2.1. Activity Assay by Titration Method of Tributyrin Hydrolysis

The activity assay of the free and immobilized enzyme preparations was performed through a titration method using an emulsion system. Tributyrin (30 mM) in 25 mM phosphate buffer with NaCl (100 mM) and 3.5% (*v/v*) of Triton X-100 was hydrolyzed with enzyme preparation at 30 °C, pH 8.0. The released butyric acid was automatically titrated with the alkaline reagent contained in Methrom's Titrino 702 SM syringe. The pH (8.0) was maintained with NaOH solution.

The activity (Act) of the enzymes, in free or immobilized forms, was expressed in (TBU/g) and determined by the following Equation (1):

$$\text{Act} = ((\Delta V_{NaOH}/\Delta t) \times M)/m \quad (1)$$

where $\Delta V_{NaOH}/\Delta t$ is the slope between the volume of standard NaOH solution added and consumed to maintain constant pH (8.0) as a function of the hydrolysis time expressed in $mL_{NaOH}\ min^{-1}$; M is the molarity of the NaOH of the titration solution expressed in mM;

and m is the mass of biocatalyst with free or immobilized enzyme expressed in g of the powder formulation used in the free enzyme preparation and g of the biocatalyst with the immobilized enzyme on a solid support, respectively (Table 1).

Table 1. The assayed activity of free and immobilized enzyme preparations according to the titration method of the tributyrin hydrolysis (TBU—µmol butyric acid per min).

Biocatalysts	Activity (TBU/g)
Free *Pseudozyma antarctica* lipase B (PBL)	5478
Immobilized *Pseudozyma antarctica* lipase B (IMM-PBLI)	1225
Immobilized *Candida antarctica* lipase B (Novozym 435)	533
Immobilized *Candida antarctica* lipase B (CALB Sigma)	674
Immobilized cutinase (lipase 51032) (IMML51-T2-150)	5667

2.2.2. Enzymatic Polycondensation of Suberic Acid and 1,8-Octanediol

The enzymatic polymerization of suberic acid and 1,8-octanediol was carried out using three modes: direct polycondensation, two-step polymerization, and polycondensation in the solvent-free system (bulk polymerization).

Preparation of the Polymerization Media in Miniemulsion, Water, and Organic Solvent

The suberic acid and 1,8-octanediol were added in equimolar concentrations (0.5 M) to the polymerization medium (i.e., water, miniemulsion, and organic solvent) and then used for poly(octamethylene suberate) synthesis. The direct polycondensation was carried out in 20 mL capped flasks used as a reactor with 10 mL of working polymerization volume, inside a sand bath to control the temperature. All reagents were used without any additional modification except the 1,8-octanediol. To obtain a better dispersion in the aqueous polycondensation media, 1,8-octanediol was homogenized using a pestle and mortar to produce a finer powder.

To prepare for polyester synthesis, the polymerization medium was mixed for 5 min to obtain a dense suspension of the monomers in water, and a homogenized solution in organic solvent (cyclohexane: tetrahydrofuran 5:1 *v/v*). The initial pH of the water polymerization medium was adjusted to 5.0 using NaOH (0.5M). The miniemulsion system used for enzymatic polyester synthesis was composed of monomers (0.5 M), a Triton X-100 surfactant, and water (16.8%:1.6%:81.6%, respectively). To obtain the miniemulsion, compounds were homogenized by direct magnetic stirring (500 rpm, 25 °C) for 1 h, then subjected to ultrasonication for 120 s (pulses of 5 s on/10 s off) at 50% amplitude (SONOPLUS, Bandelin, tip MS72) [17]. The initial pH of the miniemulsion system was 3.3 and was corrected to 5 using NaOH before polymerization.

Direct Polycondensation (First Step)

The direct polycondensation of suberic acid and 1,8-octanediol in the different polymerization media (miniemulsion, water, or organic solvent) were performed at 45 °C and started with the addition of the biocatalyst, in the form of the free enzyme (6 mg/mL) or immobilized enzyme (8 mg/mL). Each polymerization medium was then maintained with magnetic stirring (250 rpm) to homogenize the biocatalyst in the medium. The polymerization was allowed to occur for 2, 4, 8, or 48 h.

At the end of the reaction, the polymerization medium with the biocatalyst was removed from the reactor and centrifuged at room temperature, 10,000 g for 1 min (Eppendorf, Centrifuge 5810 R) to separate the wet solid from the liquid phase. The wet solid phase, with a more than 75% liquid content, was made up of POS polymer and trace monomers as solid precipitates and the biocatalyst.

Parallelly, the polymerization medium was previously separated from the biocatalyst by simple filtration using a stainless-steel mesh (pore equivalent to 200 µm), which retained the particle size of the immobilized enzyme preparations (size much larger than 200 µm)

and let pass the insoluble particles, the monomers that had not been consumed, and the synthesized polyester. Finally, the polyester, biocatalyst, and unreacted monomers present as solids in the filtrate were concentrated by centrifugation.

Two-Step Polymerization

An enzymatic two-step polymerization process using IMM-PBLI as biocatalyst was investigated. The 1st step of the polymerization (direct polycondensation) was carried out for 2, 4, or 8 h. The 2nd step of the polymerization was performed as a typical discontinuous drying process in an oven with air circulation (Memmert). The wet prepolymer obtained after centrifugation was incubated at 60 or 80 °C for 2, 4, or 24 h in an open vessel in the presence or absence (biocatalyst previously removed by filtration) of the biocatalyst (IMM-PBLI).

A schematic overview of enzymatic two-step polymerization is illustrated in Figure 1.

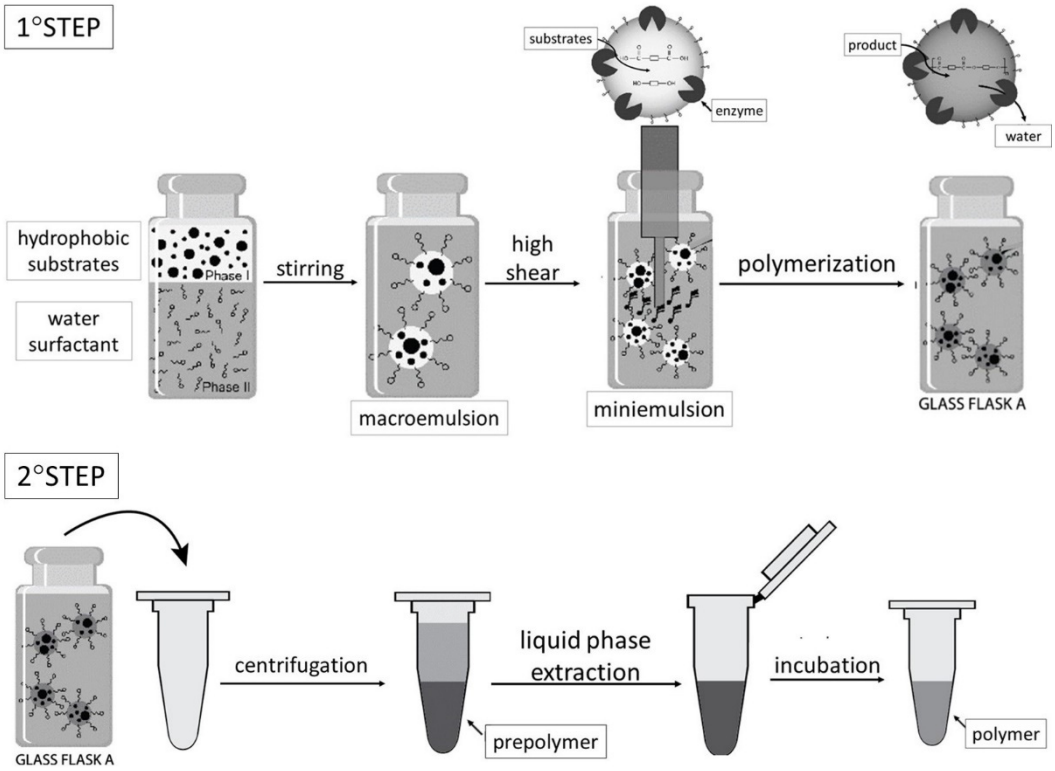

Figure 1. Schematic overview of POS synthesis using an enzymatic two-step polymerization process in miniemulsion.

Polycondensation in the Solvent-Free System (Bulk Polymerization)

Equimolar amounts of the monomers (suberic acid and 1,8-octanediol) were used in the polycondensation media for the solvent-free system to synthesize poly(octamethylene suberate). The bulk polymerization was carried out in 20 mL capped flasks inside a sand bath to control the temperature. The temperature was increased to 65–70 ºC to promote enzyme and monomer interactions. The bulk polycondensation started with the addition of the immobilized enzyme preparation (IMM-PBLI) for the same weight proportion of monomers and biocatalyst used in direct polycondensation (21 g of both monomers/g biocatalyst).

2.3. Determination of Acidity and Polymerization Conversion

The conversion (%) was calculated using the initial and final acidity assay expressed in mg KOH g^{-1} in each polycondensation medium, according to the AOCS Official Method Te1a-64. The remaining acid titration with a standard KOH solution (0.5 M) was performed

in triplicate, using a Methrom Titrino 702 SM. The acidity assay was carried out in triplicate under these conditions, and the average values are presented in the text.

Samples were taken directly from the polycondensation media, weighed, and then diluted in 5 mL of ethanol. Acidity and the conversion of dicarboxylic acid were calculated using the following equations:

$$\text{Acidity (mg KOH/g)} = \frac{\text{V} \times \text{M} \times \text{MW(KOH)}}{\text{m}} \quad (2)$$

$$\text{Conversion (\%)} = \frac{\text{Initial acidity} - \text{final acidity}}{\text{initial acidity}} \cdot 100 \quad (3)$$

where V is the consumed volume of KOH solution in mL, M is the molarity of KOH solution in M, m is the weight of the sample (g), and MW (KOH) is the molecular weight of KOH (56.1 g mol^{-1}). The experiment was repeated in triplicate.

2.4. Characterization of Synthesized Poly(octamethylene suberate) (POS)

2.4.1. Sample Preparation

The wet solid phases containing polyesters and monomers not consumed in the polymerization steps were washed with water or organic solvent. They were then dried in an oven (Memmert) with air circulation at 30 °C before characterization by Size Exclusion Chromatography (Section 2.4.2), FTIR (Section 2.4.3), and Thermogravimetric analysis (Section 2.4.4).

2.4.2. Size Exclusion Chromatography (SEC)

The average molecular weight (MW) of the synthesized polyesters was determined by Size Exclusion Chromatography (SEC) using a High-Performance Liquid Chromatography (LaChrom HPLC) apparatus equipped with a refractive index detector (Merck LaChrom RI Detector L-7490) and with a Polystyrene/Polydivinylbenzene column (ResiPore Agilent). The elution solvent was THF at a flow rate of 0.5 mL min^{-1}, and experiments were performed at 40 °C. The calibration curve was determined using polystyrene molecular weight standards (660 to 482,400 g mol^{-1}, EasiCal from Agilent). The standards and dried samples were solubilized in THF, submitted to a thermal shock at 40 °C for 5 min, and then centrifuged at room temperature before supernatant injection. Experiments were repeated in triplicate.

2.4.3. FTIR Analysis

Fourier transform infrared (FTIR) spectra were obtained at room temperature using a Bruker IFS 66/S FTIR Spectrometer (Bruker Optics, Ettlingen, Germany) in the range of 520–4000 cm^{-1}. The spectra were taken as the average of 32 scans at a resolution of 4 cm^{-1}. The experiments were performed in triplicate.

2.4.4. Thermogravimetric Analysis (TGA)

Through the thermogravimetric analysis (TGA 92-16.18 Setaram), the thermal stability of polyesters was evaluated. Melting temperature (T_m), beginning temperature (T_{onset}), and temperature at maximum weight loss (T_{max}) were also determined. The heating ramp was 10 °C min^{-1}, with the temperature varying between 20 and 600 °C. The analysis was performed under a nitrogen atmosphere (60 mL min^{-1}).

3. Results and Discussion

3.1. Direct Polycondensation in Water

In a previous work, immobilized PBL (IMM-PBLI) was shown to be an excellent biocatalyst for the polycondensation of suberic acid and 1,8-octanediol using a miniemulsion system and water [17]. In this work, other commercial biocatalysts based on immobilized

PBL and a cutinase were tested in the synthesis of poly(octamethylene suberate) by the direct polycondensation of suberic acid and 1,8-octanediol in water, as listed in Table 2.

Table 2. Molecular weights (MW) of poly(octamethylene suberate) synthesized from 0.5 M suberic acid and 1,8-octanediol in water at pH 5.0, 45 °C, for 48 h.

	Molecular Weight—MW (g mol^{-1})	
Biocatalysts	**Free Enzyme**	**Immobilized Enzyme**
Pseudozyma antarctica lipase B free form—PBL	3550	
Pseudozyma antarctica lipase B immobilized on acrylic carriers— IMM-PBLI		7600
Candida antarctica lipase B immobilized on Novozym 435	-	6200
Candida antarctica lipase B immobilized on CALB-Sigma	-	6300
Cutinase (lipase NZ 51032) immobilized on IMML51-T2-150	-	7000

The characterization of the different polyesters synthesized in this work showed that high yields of between 60 and 98% were obtained with MWs of between 3500 and 7600 g mol^{-1}, depending on the biocatalysts used. The polyester synthesis performed with the immobilized enzyme preparations, Novozym 435, CALB-Sigma, IMML51-T2-150, and IMM-PBLI, were more efficient than those performed with the free enzyme preparation (PBL). In this work, the efficiency of the POS synthesis was extended to other commercial immobilized CALB preparations and for a cutinase (NZ 51302 lipase from Novozymes) immobilized on the IB-150A acrylic support (IMML51-T2-150). According to several authors, these biocatalysts are not characterized by the interface activation mechanism [29].

Surprisingly, the yields and polymer MW observed in this work are in opposition to what is typically reported by several authors. For example, Meyer, T. (2002) [30] stated that it was expected that the use of immobilized enzymes would result in severe problems of diffusion and resistance to the external and internal mass transfer of monomers and polymers, around and inside the solid support, respectively. However, in this work the commercial immobilized enzyme preparations tested showed a higher catalytic efficiency than the respective free enzymes (soluble form).

One possible explanation for the lower efficiency of the free enzyme (PBL) compared to the immobilized enzyme preparations would be that the strong adsorption of soluble enzyme to the surface of the precipitated monomers decreases the concentration and activity of the free enzyme upon the whole polymerization medium. A second hypothesis is that the soluble enzyme becomes deactivated under the polymerization conditions used, such as at 45 °C or at pH 5.0, a phenomenon previously observed by authors for other lipases [31].

The enzyme screening presented in this section showed that higher MW and yield regarding POS synthesis was obtained using IMM-PBLI, so the further experiments were performed with this enzyme.

3.2. Polymerization in a Solvent-Free System (Bulk Polymerization)

The synthesis of POS has been successfully performed via organic synthesis using polymerization in a solvent-free system by Gasti et al. 2008 [6] and Gasti et al. 2009 [7]. These authors carried out POS synthesis using a bulk polymerization strategy. They obtained polymers with MWs of 35,500 g mol^{-1} using titanium tetrabutoxide as an organometallic catalyst, with 72% yield at 150 °C for the first 6 h, and then in a vacuum at 180 °C for 18 h. This last step was explicitly carried out to remove the by-products (e.g., water), as even minimal amounts can negatively affect the thermodynamic equilibrium and consequently the polymerization process via organic synthesis. In the end, the polyester was dissolved

in chloroform, precipitated with ethanol to separate the excess of monomers, and finally dried. The bulk polymerization conditions via organic synthesis may have been possible; however, it cannot be considered a green process or environmentally friendly [6,7].

In this work, enzymatic polycondensation in a solvent-free system was initially tested on POS synthesis with IMM-PBLI at 45 °C, but no monomer fusion was observed. At 65–70 °C, partial fusion occurred, probably of 1,8-octanediol with a melting point of 57 °C, but after 24 h, no significant polyester synthesis had occurred. This polymerization strategy failed due to the low temperature used, preventing significant fusion of the monomers; in particular, suberic acid with a melting point between 141 and 144 °C. Another possible reason for this negative result is the low solubility of the suberic acid in 1,8-octanediol at this range of temperatures. This would prevent the diffusion and contact of the soluble suberic acid with the active site of the immobilized enzymes, in contrast to what happens in water, despite the low monomer solubility, as previously observed by the authors of [17]. Another possible explanation for these results is the lack of the minimum water content to keep the biocatalysts active and functional under these bulk polymerization conditions.

3.3. Two-Step Polymerization Strategy

Bulk polymerization can also occur in two consecutive steps. The first step is to achieve a certain degree of monomer conversion and synthesis of prepolymers (oligomers) using temperatures between 50 and 100 °C. Then, in a second step, the by-products of the polymerization are removed (e.g., water) by applying a high vacuum (0.1 to 100 mmHg) or by the bubbling of dry air in the polymerization medium. With the removal of these by-products, polyesters of a higher molecular weight can be synthesized in the presence of a biocatalyst or, as is more common, with a chemical catalyst, such as diisocyanide or diisocyanate, as described in a patent by Kumar R. et al., 2005 [32].

The bulk polymerization strategy based on two consecutive steps was adapted and investigated further in this work. The first step of the polymerization includes the poly(octamethylene suberate) (POS) synthesis by direct polycondensation in miniemulsion, water, or organic solvent using the selected biocatalyst (IMM-PBLI). This first step resulted in the synthesis of low-MW prepolymers at 45 °C after polymerization for 2, 4, or 8 h, and the high stability of the biocatalyst (Figure 2) [17]. At the end of the first step, the concentrated wet solid phase, obtained by centrifugation, was found to contain POS prepolymer, unreacted monomers, and the biocatalyst, presenting a lower liquid content ($\geq$75%) in relation to the solvent content of the original polymerization medium, which was important to keep the equilibrium with a polyesterification reaction and not hydrolysis.

The second step of the polymerization was performed in an oven, like a typical discontinuous drying process, where the wet solid phase containing mainly the synthesized POS prepolymer and the biocatalyst (IMM-PBLI) were exposed to 60 or 80 °C to obtain a POS polymer with a higher molecular weight.

The POS prepolymers obtained after 2, 4, and 8 h of the first step of polymerization were then submitted to the second step, which was incubation at a higher temperature (60 or 80 °C) for 2, 4, or 24 h. This range of temperatures was selected as it was observed that the biocatalyst (IMM-PBLI) showed high stability at 65 °C in water and miniemulsion but much lower stability in the organic solvent [17]. The experiments performed and the conditions used are outlined in Table 3. For example, in the experiment labelled as POS 2/24, 2 is the time (h) of the first step, and 24 is the time (h) of the second step of polymerization. The different polymerization media and first-step polymerization times were analyzed with regard to their effect on the times and temperatures required for the second step of polymerization.

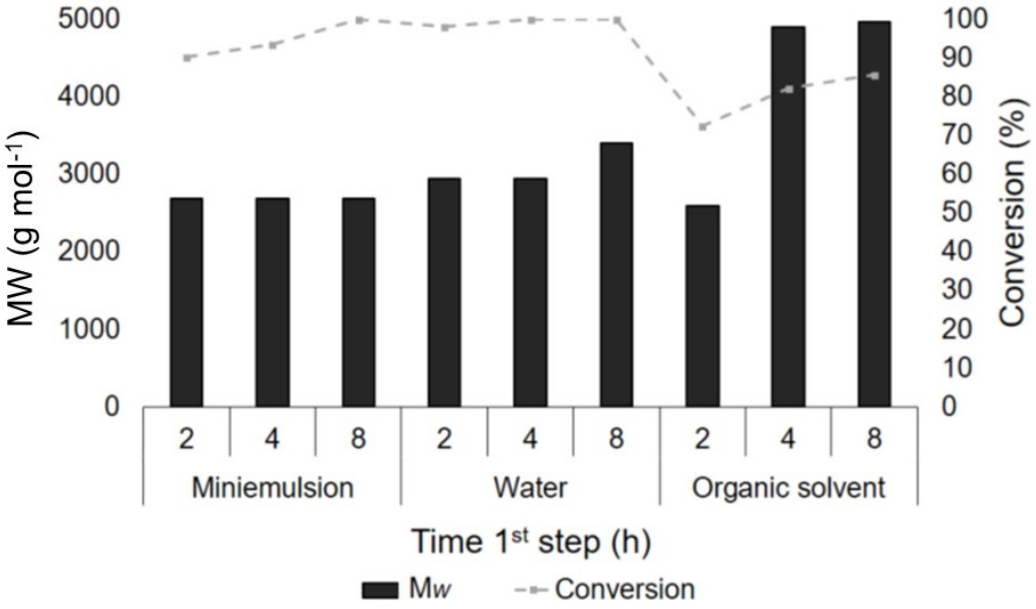

Figure 2. POS molecular weight (MW) and conversion (%) obtained in the first step of polymerization in miniemulsion, water, or organic solvent for 2, 4, and 8 h. Reaction conditions: vessel scale in batch operation mode with 10 mL of polymerization media; 0.5 M equimolar concentration of 1,8-octanediol and suberic acid; the organic solvent was a mixture of cyclohexane—THF (5:1 *v/v*); pH corrected to 5.0 in miniemulsion and water media; 250 rpm; 45 °C; 8 mg mL^{-1} of IMM-PBLI.

Table 3. Experimental outline of poly(octamethylene suberate) (POS) synthesis regarding the time of the first and second steps of polymerization.

	2nd Step Time (h)		
1st Step Time (h)	**2**	**4**	**24**
2	POS 2/2	POS 2/4	POS 2/24
4	POS 4/2	POS 4/4	POS 4/24
8	POS 8/2	POS 8/4	POS 8/24

3.3.1. Step of Polymerization

The MW and conversion results obtained with the first step of polymerization via biocatalysis (IMM-PBLI) in miniemulsion, water, or organic solvent are shown in Figure 2.

The MW of the POS prepolymers formed in miniemulsion did not present significant variation over time (around MW ~2800 g mol^{-1}), and a conversion of 98% was reached after 8 h. In water, the POS prepolymers had an increased MW, achieving a MW of 3400 g mol^{-1} after 8 h and a conversion of around 97%. The POS prepolymers synthesized in organic solvent showed higher MWs after 4 and 8 h of 4900 and 5000 g mol^{-1}, respectively, compared to miniemulsion and water (Figure 2). However, the percentage conversion in the organic solvent for the first step of polymerization was lower than in miniemulsion and water, achieving a maximum conversion of 86% at 8 h (13% less conversion than the other two polymerization media).

3.3.2. Second Step of Polymerization of Wet POS Prepolymers Synthesized in Miniemulsion

No significant variation was observed in the POS MW after the second step of polymerization using the prepolymer obtained in miniemulsion regarding the temperature tested, i.e., 60 vs. 80 °C (Figure 3). The incubation time of the first polymerization step did not significantly influence the MW obtained at the end of second step of polymerization (24 h) (Figure 3). However, an apparent increase in the polymer MW (2 h < 4 h < 24 h) was correlated with the time of exposure to both high temperatures in the second step of

polymerization, i.e., the POS MW seemed to increase significantly from 2 to 24 h. This MW profile was not observed for the POS prepolymer synthesized for 2, 4, and 8 h in the first step of polymerization in miniemulsion, as a similar MW of 2800 g mol^{-1} was obtained for all the reaction time conditions (Figure 2).

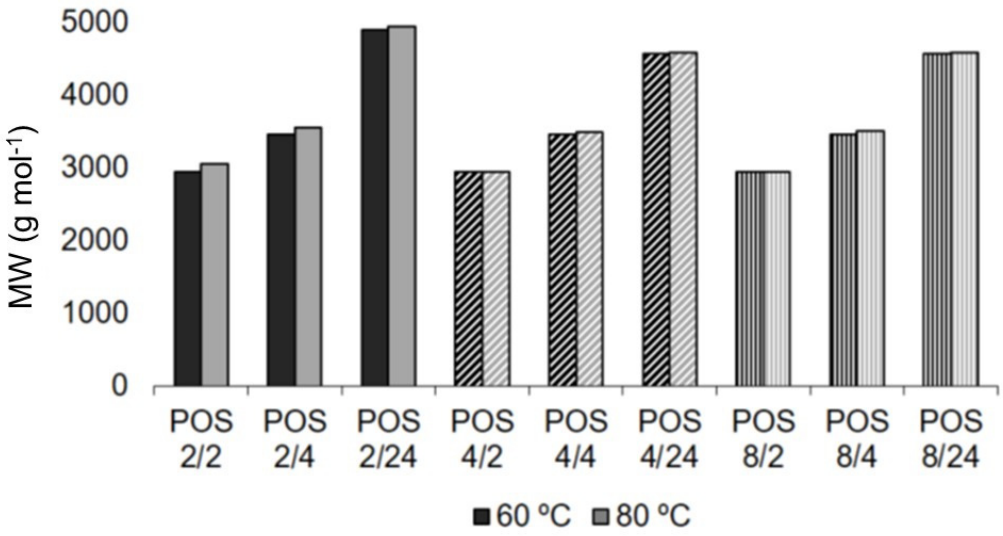

Figure 3. POS molecular weight (MW) of the second step of polymerization obtained from the wet prepolymer synthesized in miniemulsion. Reaction conditions: 60 °C or 80 °C (black and grey bars, respectively), without stirring but in the presence of the biocatalyst (IMM-PBLI).

The increase in POS MW obtained from the prepolymer synthesized in miniemulsion was highly related to the exposure time in the second step of polymerization. The temperature difference in step one (60–80 °C) only slightly influenced the POS MW (Figure 3). Low melting points of 56.7 °C (Section 3.4) characterize the low MWs of the POS prepolymers synthesized in the first step of polymerization. When these prepolymers were submitted to higher temperatures of 60 or 80 °C in the second step of polymerization, it is likely that melting occurred, enabling their diffusion to the active site of the immobilized enzyme and the formation of POS with a higher MW, despite the absence of stirring.

3.3.3. Second Step of Polymerization of Wet POS Prepolymers Synthesized in Water

The wet prepolymers synthesized in water for 2, 4, and 8 h led to slight polyester MW variations observed at the end of the second step of polymerization (Figure 4). The polyester MW variation was more significant in the wet prepolymers exposed to 60 °C and 80 °C in the second step (POS 2/2, POS 2/4, POS 4/2, POS 4/4, POS 8/2, and POS 8/4) compared to those synthesized in miniemulsion. Additionally, the polyester MW seemed to increase significantly with exposure time and higher temperatures used in the second step of polymerization, similarly to the behavior observed for miniemulsion. Maximal MW values were achieved with the longest incubation time for step two (24 h) (Figure 4).

The high temperatures of 60 or 80 °C in the second polymerization step melted the prepolymers synthesized in water (T_m 58.2 °C, Section 3.4) and shifted the polyesterification equilibrium for polymer formation, resulting in POS with a higher MW. Additionally, these results could be due to the lower moisture content of the wet prepolymer synthesized in the first step of polymerization in water after centrifugation. Furthermore, the partial removal of the water present inside the structural wet prepolymer chain by evaporation at 60 or 80 °C influenced the obtained data.

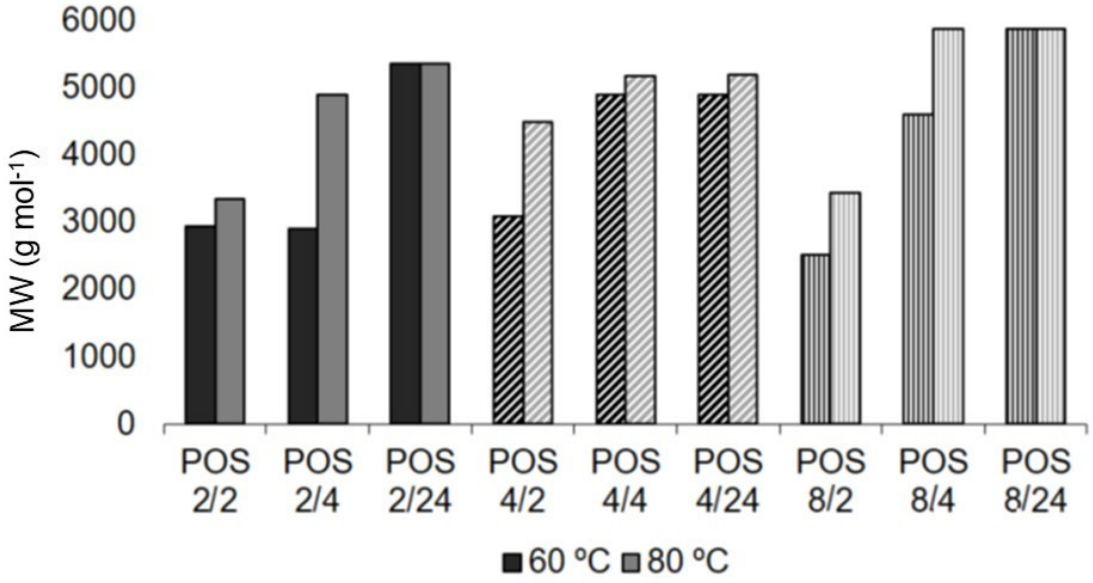

Figure 4. POS MW after the second polymerization step using prepolymer synthesized in water. Reaction conditions: 60 °C and 80 °C (black and grey bars, respectively), without stirring but in the presence of the biocatalyst (IMM-PBLI).

3.3.4. Second Step of Polymerization of Wet POS Prepolymers Synthesized in Organic Solvent

The use of the organic solvent in the first polymerization step resulted in POS of higher MW (16,200 and 19,800 g mol^{-1}) at the end of the second polymerization step compared to those returned when miniemulsion or water were used in the first step (Figure 5). In the second polymerization step, an increase in temperature from 60 to 80 °C also led to an increase in POS MW from 16,000 to 19,800 g mol^{-1} after 24 h (Figure 5).

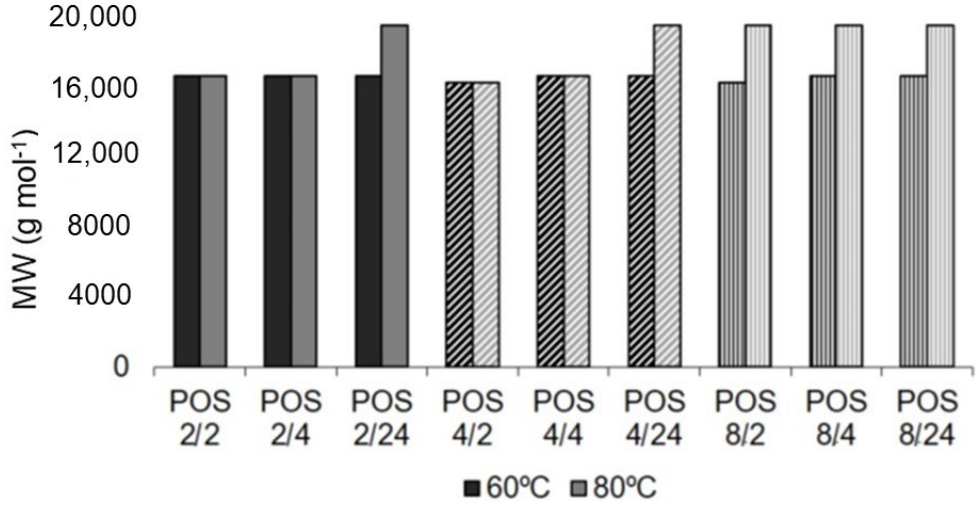

Figure 5. POS MW of the second polymerization step obtained from the prepolymer synthesized in organic solvent. Reaction conditions: 60 and 80 °C (black and grey bars, respectively), without stirring but in the presence of the biocatalyst (IMM-PBLI).

The significant increase in polyester MW due to the increased incubation time (2–24 h) seen with miniemulsion or water was not observed when organic solvent was used in the first step. In fact, a maximum polyester MW of 16,000 g mol^{-1} was achieved at 60 °C for all incubation times and when the first step was 2 or 4 h and the second step 24 h. Polyesters of 19,000 g mol^{-1} were obtained in all experiments where the first polymerization step was 8 h

and the second step was performed at 80 °C, indicating that the second step incubation time was irrelevant in this case (Figure 5). These results suggest that thermodynamic equilibrium was achieved quickly under these experimental conditions. Another possible explanation is the partial or total evaporation of the cyclohexane and THF (boiling points of 80.7 and 66.1 °C, respectively) in the second polymerization step. In this case, the polymers formed would not be able to diffuse and contact the active site of the immobilized enzyme in the absence of stirring, leading to a halt in polymer formation and thus no further increase in MW. An additional hypothesis is that the biocatalyst (IMM-PBLI) was deactivated quickly in this organic solvent, as was previously reported by the authors of [17] at 65 °C.

For all the experiments performed using this novel two-step polymerization strategy, it is possible to conclude that the wet prepolymer synthesized in miniemulsion and water induced the formation of oligomers with a lower MW for 2 to 8 h (Figures 3 and 4) than those obtained from the prepolymer synthesized in organic solvent (Figure 5). The presence of a higher amount of water in the prepolymer (obtained by miniemulsion and water) may have led to hydrolysis (inverse polyesterification reaction) for a higher temperature range (60 to 80 °C). For this reason, there was no accumulation of polyesters with a higher MW than 5800 g mol^{-1} under these experimental conditions, in contrast to the 7600 gmol^{-1} polyester obtained at 45 °C after 48 h of direct polycondensation in water (Table 2). The incubation time of the first polymerization step did not influence the polymer MW as significantly as the incubation time of the second step of polymerization.

The MW of the POS polymers obtained at the end of the second step of polymerization from the wet POS prepolymers synthesized in the organic solvent in the first step corresponded to about half of the value (35,500 g mol^{-1}) obtained by Gasti et al. 2008 [6].

3.3.5. Second Polymerization Step in the Absence of the Biocatalyst

A control experiment was performed in the absence of the biocatalyst (removed by filtration) to clarify whether or not the thermal polymerization of the wet prepolymers synthesized in the first step occurred during the second polymerization step.

In this group of experiments, the variation of and increase in the polymer MW was analyzed after the second step of polymerization had been allowed to occur for 24 h at 80 °C. The wet POS prepolymers used were synthesized after 8h in the first step using miniemulsion, water, or organic solvent (Figure 6). The MW of the polyesters obtained at the end of the second step did not differ from those obtained after the first step, independent of the polymerization media used.

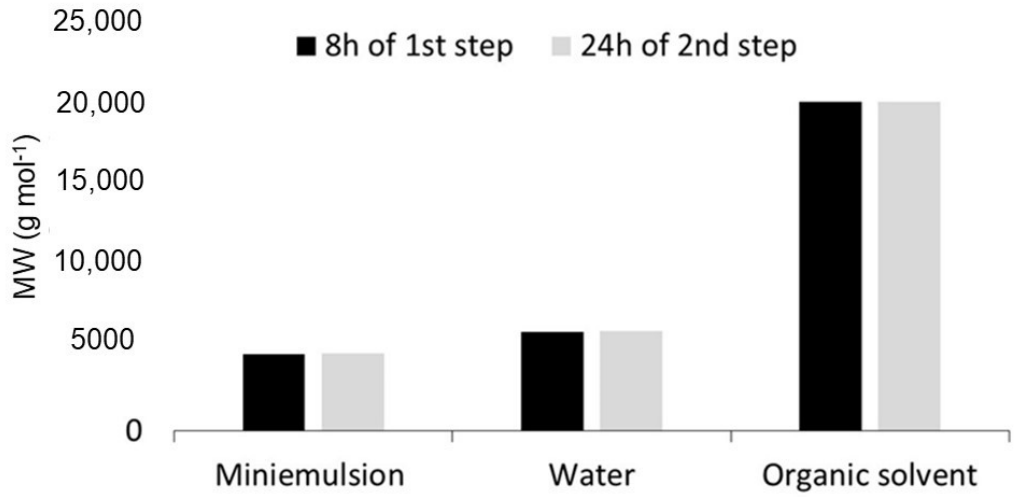

Figure 6. Molecular weights (MWs) of POS 8/24 obtained at the end of the second step of polymerization after 24 h at 80 °C in the absence of biocatalyst. Wet prepolymers were synthesized after 8 h of the first step of polycondensation in miniemulsion, water, or the organic solvent.

This group of experiments proves that thermal polymerization did not occur at 60 to 80 °C and that the biocatalyst was active and functional during the second step of polymerization in the POS polymer synthesis despite the absence of stirring in the previous group of experiments (Sections 3.3.2–3.3.4).

3.4. Thermal Properties of POS Polymer Synthesized in Two-Step Polymerization Strategy

The POS polymers obtained at the end of the two-step polymerization strategy were characterized by TGA analysis and compared to the wet prepolymers synthesized at the end of the first step in miniemulsion, water, and organic solvent. These thermal properties can be seen in the thermograms (Figure 7), and the data of the maximum temperature (T_{max}), the temperature at the extrapolated beginning (T_{onset}), and the melting temperature (T_m) were obtained through the TGA analysis (Table 4).

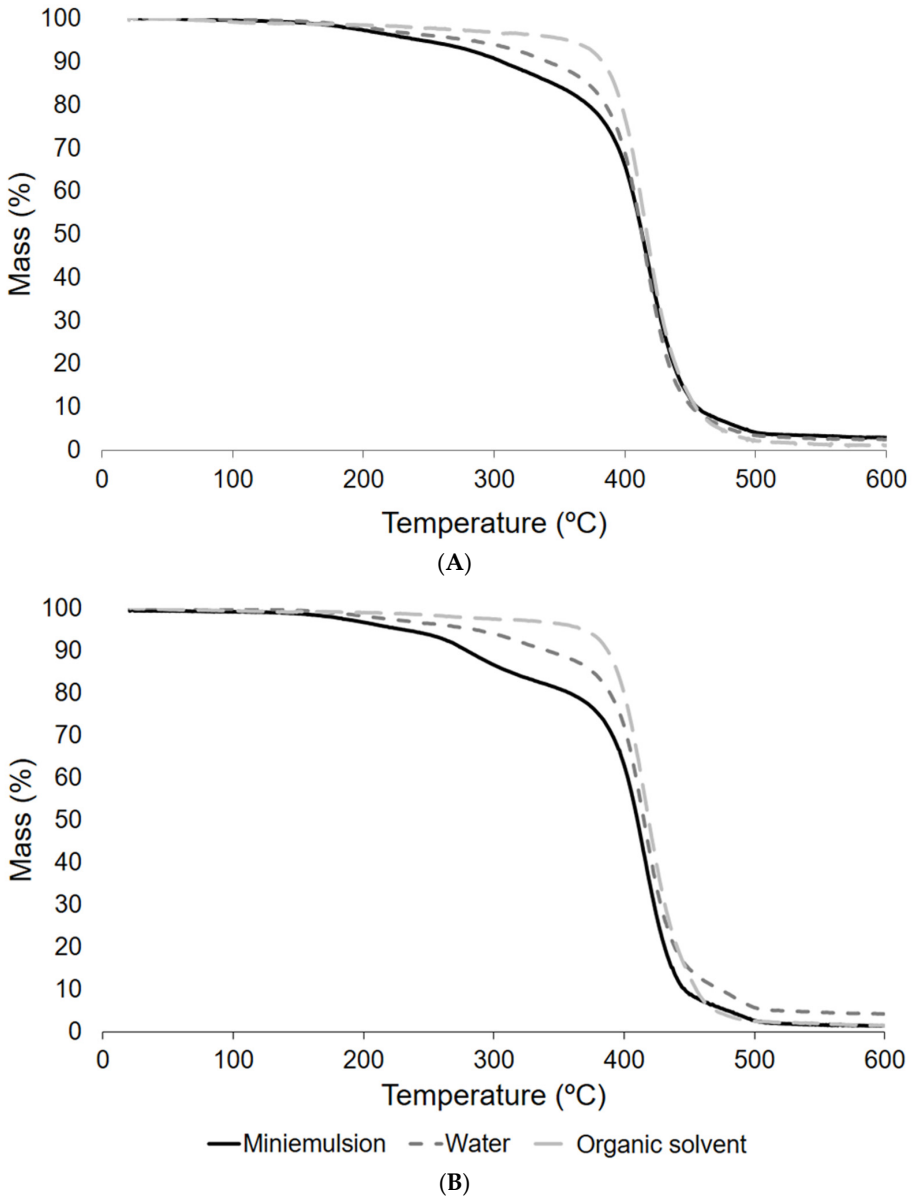

Figure 7. Thermograms of thermogravimetric analysis (TGA) of POS in miniemulsion, water, and the organic solvent (cyclohexane: THF (5:1 *v/v*)): (**A**) at the end of the 2 h of the first step of polymerization and (**B**) at the end of the 24 h of the second step of polymerization, i.e., the POS polymers identified by POS 2/24.

Table 4. Maximum degradation temperature (T_{max}), stability temperature or extrapolated beginning temperature (T_{onset}), melting temperature (T_m), and maximum weight loss obtained in TGA analysis of POS prepolymer synthesized in first step at 2 h (Figure 7A) by direct polycondensation in the different polymerization media (miniemulsion, water, and the organic solvent) and the respective POS polymers obtained at the end of 24 h for the second step of polymerization for 80 °C (Figure 7B), i.e., the POS polymers identified by POS 2/24.

Polymerization Medium	Stage	T_{max} (°C)	T_{onset} (°C)	T_m (°C) [2]	Weight Loss (%)
Miniemulsion	1st step	424.3	386.0	56.7	97.5
	2nd step	508.2	384.9	59.7	98.7
Water	1st step	416.4	386.7	58.2	97.4
	2nd step	498.7	386.9	62.9	96.7
Organic solvent [1]	1st step	422.0	386.5	65.4	98.4
	2nd step	482.5	384.9	75.5	99.2

[1] Mixture of cyclohexane: THF (5:1 *v/v*). [2] Values obtained from the heating curve during TGA analysis.

In the thermograms of the POS prepolymers synthesized in the first step in miniemulsion and water and the POS polymer obtained at the end of the second step of polymerization (Figure 7A,B, respectively), no initial degradation at a low temperature due to the presence of an excess of water was observed, contrary to previous observations by the authors of [17]. In this work, this lower weight loss of the POS prepolymers obtained at the end of the first step was due to the longer time used during the drying in the oven at 30 °C, which reduced the water content inside the structural polymer chain. The lower weight degradation of all the POS polymers obtained at the end of the second step of polymerization (24 h) compared to the first step was due to the prolonged polymer exposure at high temperatures (80 °C) that also reduced the water content.

The weight loss for all the POS prepolymers and polymers between 120 and 280 °C may have been due to traces of monomers in the sample, since their flash and boiling points are near this temperature range, namely, 120 °C and 272 °C and 210 °C and 230 °C for 1,8-octanediol and suberic acid, respectively. The weight loss observed between 280 and 400 °C could have been due to polymer molecules with lower molecular weights present in the polymer samples.

The presence of the surfactant in the POS prepolymer obtained by miniemulsion at the end of the first step and in the POS polymer at the end of the second step of polymerization was noticed due to the degradation temperature of around 450 °C.

The T_{max} and T_m in the second step of polymerization increased compared to those obtained in the first step (Table 4). The T_{max} increase may have been due to the larger polymer size and also, in the case of the miniemulsion used as a polymerization medium, the presence of impurities such as the surfactant (Table 4). Furthermore, the T_m of the POS prepolymers synthesized in water in the first step (58.2 °C) and of the respective POS polymer obtained in the second step (62.9 °C) were higher than those obtained in miniemulsion (56.7 °C and 59.7 °C, respectively), which were in agreement with the higher molecular weights of the POS polymers obtained in water. The presence of impurities such as the surfactant (Triton-X100) could decrease the melting point of the polyester (59.7 °C) obtained in the miniemulsion, compared with the melting points of 62.9 °C and 75.5 °C obtained for water and organic solvent, respectively. According to the TGA analysis, a similar effect was also observed by other authors [33].

3.5. POS Polymer Characterization by FTIR

In a previous work, the chemical structure of poly(octamethylene suberate) was analyzed by the authors using ^{1}H NMR [17]. In this work, the POS samples synthesized in the miniemulsion, water, and the organic solvent were assayed by FTIR analysis to confirm the polymer molecular structure and polymer purity (Figure 8).

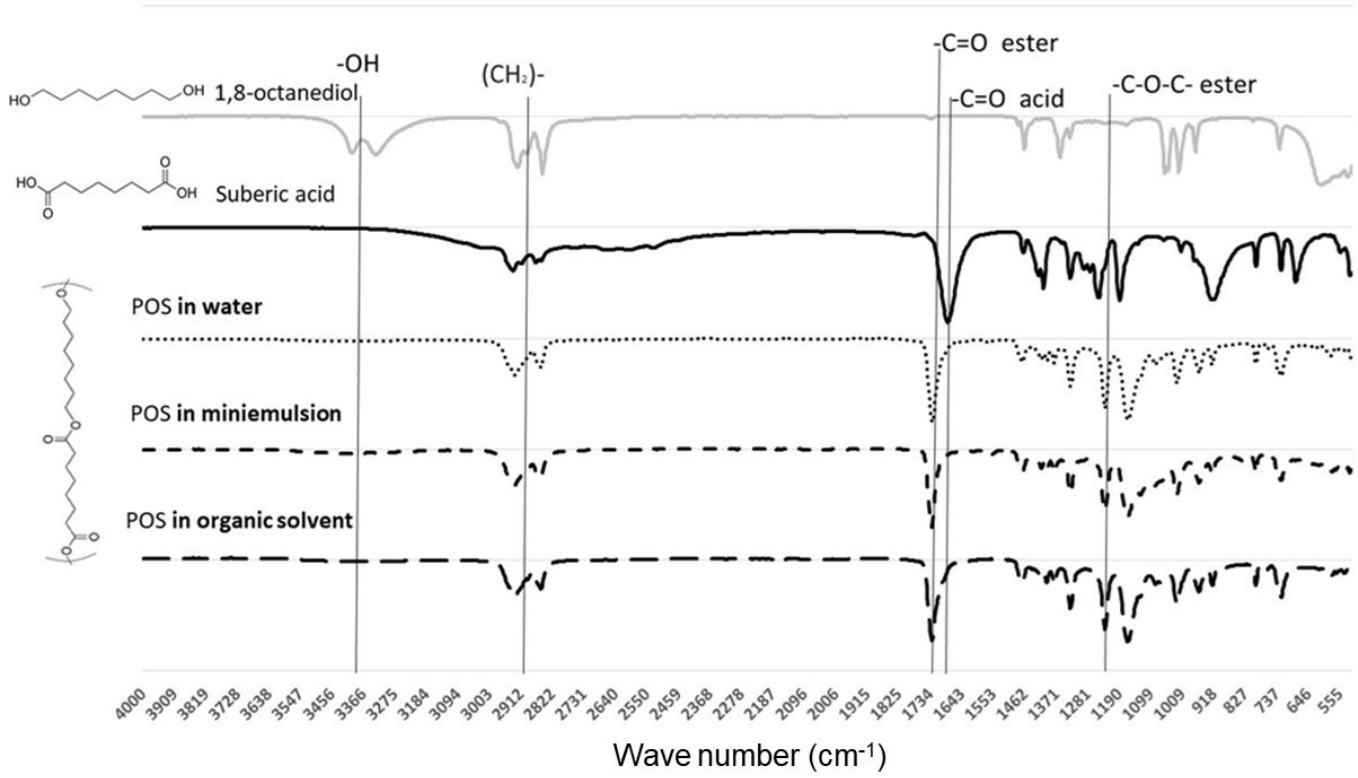

Figure 8. FTIR spectra of 1,8-octanediol, suberic acid, and poly(octamethylene suberate) samples synthesized in water, miniemulsion, and the organic solvent.

The FTIR spectra confirmed the polyester structure of the POS by comparison with the spectra of the monomers 1,8 octanediol and suberic acid. The typical -OH band of diol at 3360 cm^{-1} and the -C=O stretching band of suberic acid were present at 1683 cm^{-1}. The peaks at 2858 and 2929 cm^{-1} correspond to the symmetrical stretching vibration of $-CH_2$ groups in the monomers and polyesters. The characteristic bands of the ester carbonyl group appeared at 1726 cm^{-1}, and the -C-O-C- peak appeared around 1232 cm^{-1} [34,35]. The FTIR spectrum of the polyester illustrated the absence of a detectable band at 1683 cm^{-1} from the -C=O stretching vibration of carboxylic acid groups and the -OH band at 3360 cm^{-1}, confirming a high degree of conversion.

4. Conclusions

Various commercial immobilized lipases based on *Pseudozyma antarctica* lipase B (previously named *Candida antarctica* lipase B) and one cutinase (lipase NZ 51032, Novozymes) were successfully utilized to catalyze the synthesis of poly(octamethylene suberate) (POS) by direct polycondensation in water.

An innovative two-step polymerization strategy for the synthesis of poly(octamethylene suberate) was successfully developed in this work. In this strategy, a low-molecular-weight prepolymer was synthesized (first step) by direct polycondensation in a miniemulsion system, water, or organic solvent over a period of 2, 4, or 8 h. In the second step of polymerization, the wet prepolymer was incubated in a typical discontinuous drying process in an oven at 60 or 80 °C and at atmospheric pressure for 2, 4, or 24 h, in an open vessel in the presence of the biocatalyst (IMM-PBLI). Even without stirring, the MW of the POS polymers obtained in the first polymerization step (miniemulsion, water, or organic solvent) increased 1.8-, 1.7-, and 3.4-fold during the second polymerization step, respectively.

According to this experimental work, the optimization of water or organic solvent content in the wet prepolymers, the geometry of the reactor for different impellers, and the

selection of stable biocatalysts at 60 or 80 ºC are fundamental to obtain some guidelines for a scale-up. Future work is expected to increase the POS polymer MW to higher than 20,000 g mol^{-1} for prepolymers synthesized in aqueous media, particularly in water, as they are an important class of polymers widely used in producing fibers, films, and 3D structures.

This innovative concept of a two-step polymerization strategy clearly contributes to the development of a greener and more environmentally friendly process that constitutes part of the core of the new Polymer-5B technology.

5. Patents

The results reported in this manuscript are partially included in the patent approved in Portugal, PT 116045, 31 December 2019, (https://inpi.justica.gov.pt/LinkClick.aspx?fileticket=sgUnJNjP5vY%3d&portalid=6, accessed 7 December 2021). They were more recently identified as PCT/PT2020/050051, Synthesis of polyesters in aqueous polymerization media "from solid to solid" via biocatalysis, published as WO 2021/137711 A1, (https://patentscope.wipo.int/search/pt/detail.jsf?docId=WO21137711, 7 December 2021).

Author Contributions: Conceptualization, analytical work, data handling, and writing (original draft, review, and editing)—A.C.D.P.; conceptualization, writing (review and editing), analytical work, and supervision—D.P.C.d.B.; writing (review), resources, and supervision—A.O.; conceptualization, methodology, data handling, writing (review and editing), resources, funding acquisition, and supervision—L.P.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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