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Biocatalytic Approach for Novel Functional Oligoesters of ε-Caprolactone and Malic Acid

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Abstract: Biocatalysis has developed in the last decades as a major tool for green polymer synthesis. The particular ability of lipases to catalyze the synthesis of novel polymeric materials has been demonstrated for a large range of substrates. In this work, novel functional oligoesters were synthesized from ε-caprolactone and D,L/L-malic acid by a green and sustainable route, using two commercially available immobilized lipases as catalysts. The reactions were carried out at different molar ratios of the comonomers in organic solvents, but the best results were obtained in solvent-free systems. Linear and cyclic oligomeric products with average molecular weights of about 1500 Da were synthesized, and the formed oligoesters were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The oligoester synthesis was not enantioselective in the studied reaction conditions. The operational stability of both biocatalysts (Novozyme 435 and GF-CalB-IM) was excellent after reutilization in 13 batch reaction cycles. The thermal properties of the reaction products were investigated by thermogravimetric (TG) and differential scanning calorimetry (DSC) analysis. The presence of polar pendant groups in the structure of these oligomers could widen the possible applications compared to the oligomers of ε-caprolactone or allow the conversion to other functional materials.

Keywords: enzymatic polymerization; oligoesters; malic acid; ε-caprolactone; lipase; green polymers; biobased monomers

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1. Introduction

The synthesis of polymers, which belong to the most important synthetic materials manufactured at the industrial scale, began about 100 years ago, using chemical catalysts. Homogeneous and heterogeneous chemical catalysts, including acids, bases, radical-generating compounds, metal-based catalysts, etc., still represent the most important option for efficient and selective polymer production, showing remarkable progress in the last years in the development of controlled radical polymerizations [1] or the utilization of metal-organic framework materials [2]. Albeit large-scale utilization and continuous development of the "classical" catalytic pathway of polymer synthesis still continues, enzyme-catalyzed polymerization has emerged as a valuable approach since the early 1980s, particularly in the case of specialty polymers, leading to green polymer chemistry [3].

As stated by Kobayashi, enzymatic polymerization can be defined as "the in vitro polymerization of artificial substrate monomers catalyzed by an isolated enzyme via non-biosynthetic (nonmetabolic) pathways" [4]. The recent developments of the biocatalytic way of polymerization have been achieved using enzymes from several classes, particularly

oxidoreductases (e.g., peroxidases and laccases); transferases (e.g., glycosyl transferases); and hydrolases (e.g., lipases, proteases, and glycosidases) [5], but lipases were the more versatile enzymes for this aim. They have several advantages, as easy accessibility, high activity against non-natural substrates and in organic media, mild reaction conditions coupled with the retention of activity at higher temperatures and broad substrate specificity [6–10], which can also be exploited in the synthesis of polymers. The green synthesis of polyesters using lipases as biocatalysts was the subject of extensive research, demonstrated by the continuously increasing number of publications in this field, as reviewed by Kobayashi [4,11,12] and other authors [13,14].

The ring-opening polymerization (ROP) of ε-caprolactone (ECL), as well as copolymerizations involving ECL, attracted growing interest in the past years, because polycaprolactone (PCL), a semicrystalline polymer with a low glass transition temperature and melting point, can be used in several fields as tissue engineering, drug delivery systems, microelectronics, or packaging, while block copolymers, including PCL, have excellent properties for various technological applications, e.g., copolymers with polyethylene glycol are good transporters for the controlled delivery of hydrophobic drugs [15]. Lipases are particularly valuable for the ROP reactions of lactones [16], allowing the synthesis of polymers/oligomers and copolymers/co-oligomers in controlled reaction conditions and suited for biomedical applications, avoiding the toxicity effects of trace amounts of the metal catalyst, e.g., PCL-b-PEG-b-PCL triblock copolymers with polyethylene glycol [17]. The previous works of our group demonstrated the synthetic possibilities towards various co-oligomers of ECL with δ-gluconolactone [18–20], 5-hydroxymethyl-2-furancarboxylic acid [21], and fatty hydroxy acids [22–24]. The importance of copolymers derived from ECL is emphasized by novel reports on the possibilities to obtain this monomer from phenol by a chemoenzymatic process [25]. As phenol could be available from lignin [26], the whole process of ECL copolymer synthesis can be considered biobased when the other co-monomer is also obtained from renewable raw materials.

Apart from fatty hydroxy acids, natural hydroxy acids with a shorter chain could also be of great interest for the synthesis of copolymers/co-oligomers with ECL. The copolymer of D,L-lactide (the lactone cyclic diester derived from D,L-lactic acid) with ECL, using *Candida antarctica* B lipase, was reported already in 2003 [27], but the utilization of lactic acid, or other natural hydroxy acids like malic acid, as monomers in enzyme-catalyzed processes looks much more difficult to accomplish, despite the perspectives of these copolymers. Hollmann et al. demonstrated that the utilization of natural edible acids, like tartaric and L-malic acid as acyl donors in transesterification reactions catalyzed by Novozyme 435, was impeded by the high acidity of these compounds that led to irreversible inactivation of the biocatalyst [28].

Malic acid is a chemical present in many natural resources (grapes and apples) but is manufactured mainly as racemic D,L-malic acid by chemical synthesis from fossil resources at a market volume range currently between 60,000 and 200,000 ton yr^{-1}. Ranked among the main platform chemicals, the biotechnological production of malic acid got considerable interest, based on the ability of microorganisms from several species of *Aspergillus*, *Ustilago*, or *Aureobasidium*, to synthesize L-malic acid [29]. Consequently, the interest towards malic acid as a biobased monomer is also increasing, due to the dicarboxylic structure with a hydroxyl pendant group, which allows many possibilities to obtain homo- or heteropolymers.

Like poly(lactic acid), malic acid-based polymers can be synthesized by two chemical methods: polymerization through polycondensation and ring-opening polymerization. Malic acid, whether natural or synthetic, resembles biological activity, acting especially as a specific inhibitor of DNA polymerase [30]. Compared to the conventional aliphatic polyesters, those containing malic acid units contain free carboxyl or hydroxyl functional groups along the macromolecular chains. Hydroxyl groups influence the crystallinity, degradation behavior, and polarity of the resulting polyester. Functional polyesters of malic acid, with pendant hydroxyl groups, have a high potential for medical applications

due to their biodegradability and biocompatibility [31]. Poly(malic acid) has been used in the synthesis of controlled release drug delivery systems and, subsequently, tested against tumor cells corresponding to breast cancer [32].

There are many reports on the chemical synthesis of poly(malic acid) or functional polyesters containing malic acid. Hyperbranched crosslinked copolymers of L-malic acid and β-cyclodextrin were synthesized, allowing a predefined molecular weight and retention of the optical activity [33]. Poly(ε-caprolactone-b-β-malic acid-b-ε-caprolactone) triblock copolymers were synthesized by chemical catalysis from benzyl-β-malolactonate and ECL, involving protection and deprotection steps [34]. However, such processes are hindered by the well-known disadvantages of the chemical catalysts, and the utilization of enzymes could resolve many of these problems, as discussed before. The literature data on co-polyesters from L-malic acid, adipic acid, and 1,8-octanediol in organic media, with Novozyme 435 as the biocatalyst, indicated that the malic acid units were incorporated into the macromolecular chains exclusively through their carboxyl groups [35]. Malic acid has been also used in enzymatic polymerizations with 1,8-octanediol, and a decrease in the average molecular weights was observed as the malic acid content introduced in the reaction was reduced [36]. Therefore, polymerization reactions involving one carboxyl and the hydroxyl groups of malic acid and leaving the second carboxyl group free would represent a new category of biobased functional polymers. The aim of this study was the enzymatic synthesis of new functional oligoesters containing ε-caprolactone (ECL) and D,L/L-malic acid (D,L-MA/L-MA). The main reaction products are linear and cyclic co-oligomers containing ECL and MA, as shown in Figure 1.

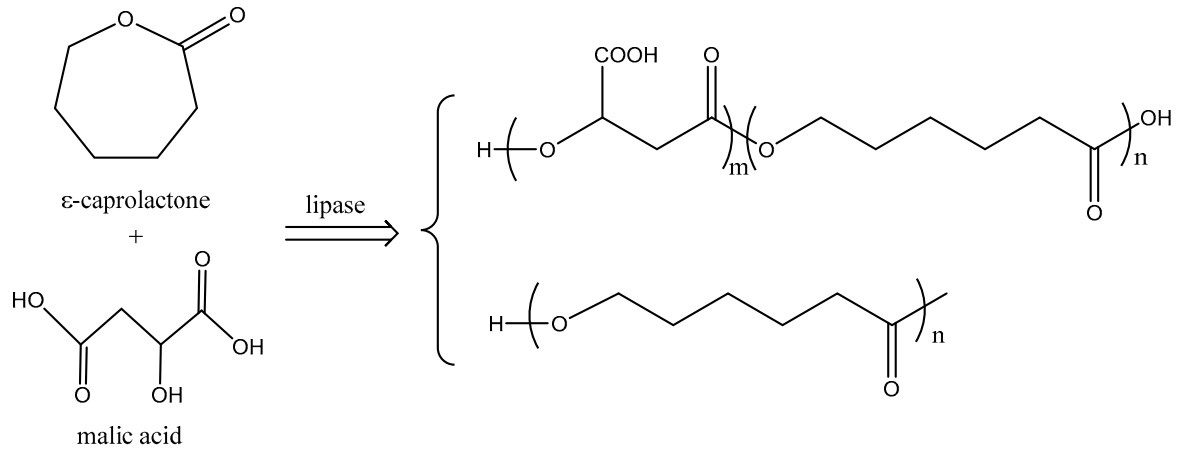

Figure 1. Reaction scheme of the polycondensation reaction of ε-caprolactone with malic acid, catalyzed by lipase, yielding oligoester products.

The structural characterization was performed by a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis and infrared spectroscopy, while the thermal properties were evaluated by thermogravimetric (TG) and differential scanning calorimetry (DSC). Two immobilized lipases were used as the catalyst.

To the best of our knowledge, this is the first report on the successful enzymatic synthesis of such co-oligomers. The insertion of malic acid units with pendant carboxyl groups in the PCL polymer chain can obviously widen the possible applications of these materials. The utilization of lipases (particularly from *Candida antarctica* B) for the synthesis of esters with higher polarity by the attachment of chains carrying the appropriate functional groups was already demonstrated as a powerful tool to increase the bioavailability of the active ingredient for applications in the agri-food [37] or biomedical [38] fields. Apart from high molecular weight polymers, oligoesters have lower melting points, being mostly liquid or semisolid at room temperature, depending on the type of the monomers used [39]. Consequently, biobased oligoesters can be applied in the formulation of drug delivery

carriers, plasticizers, soft tissue implants, and cosmetics, as well as building blocks for polymer synthesis [40].

2. Materials and Methods

2.1. Materials

ε-Caprolactone (ECL, ≥98%), DL-malic acid (D,L-MA, 99%), L-malic acid (L-MA, 99%), toluene (>99%), tetrahydrofuran (THF 99.8%), methanol (>99.6%), acetonitrile (ACN 99%), 2-methyl-tetrahydrofuran, and lipase B from *Candida antarctica* immobilized on acrylic resin (Novozyme 435) were Sigma-Aldrich products acquired from Merck KgaA (Steinheim am Albuch, Germany), while lipase B from *Candida antarctica* immobilized on microporous ion exchange resin (GF-CalB-IM) was kindly provided by GenoFocus (Daejeon, Republic of Korea). All reagents were used as purchased.

2.2. Methods

2.2.1. Oligoester Synthesis in Organic Solvent Media

ECL and D,L-MA/L-MA, at different molar ratios (total of 1.5 mmoles) were homogenized in 2-mL Eppendorf tubes with 1-mL organic solvent (toluene, 2-methyl-tetrahydrofuran, and acetonitrile). The immobilized biocatalyst (15 mg) was added to the reaction mixture, and the reactions were performed under continuous stirring at 1200 rpm for 24 h at 70 °C using an Eppendorf Thermomixer Comfort heating shaker (Eppendorf, Hamburg, Germany). These experimental methodology and reaction parameters were set based on the previous results concerning polymerization reactions of hydroxy acids with ECL [21,23]. At the end of the reaction, the enzyme was separated from the reaction mixture by filtration, followed by removal of the solvent by vacuum-drying at room temperature. The unreacted malic acid was separated from the product by washing with 5-mL methanol in three portions and filtration. Vacuum-drying at room temperature was used again to remove the methanol traces. The oligoester products were analyzed by FT-IR spectroscopy and MALDI-TOF MS spectrometry, while the unreacted malic acid was assessed by HPLC chromatography in the reunited methanolic solutions.

2.2.2. Oligoester Synthesis in Solvent-Free System

Mixtures of ECL and D,L-MA/L-MA, at fixed molar ratios (total 1.5 mmoles), were stirred for 24 h in 2-mL Eppendorf tubes at 80 °C and 1200 rpm for complete homogenization; then, a 15-mg biocatalyst (Novozyme 435 or GF-CalB-IM biocatalysts) was added. The reason for using the same amount of both biocatalysts was that they displayed very close activity values in the standard reaction of *p*-nitrophenyl palmitate hydrolysis (8.7 and 8.1 U/mg, respectively). Thus, the initial enzyme/substrate ratio was 87-U/mmol monomers for Novozyme 435 and 81-U/mmol monomers for GF-CALB-IM, in all experiments. The mixture was incubated for 24 h at 80 °C and 1200 rpm for polymerization using an Eppendorf Thermomixer Comfort heating shaker (Eppendorf, Hamburg, Germany). The higher temperature, compared to the reactions carried out in organic solvent media, was justified by the necessity to have a homogeneous system at the beginning of the reaction. After completing the process, the products were dissolved in THF and separated from the biocatalyst by filtration. The rest of the work-up process was the same as described for the oligoester synthesis in organic solvents in Section 2.2.1.

2.2.3. Time Course of the Oligomer Synthesis

The time course of the reaction of ECL (1 mmol) with D,L-MA (0.5 mmol) was investigated using 15-mg Novozyme or GF-CalB-IM as the biocatalyst in a solvent-free system at a 2:1 ECL:D,L-MA molar ratio. The reaction was carried out in Eppendorf tubes at 80 °C and 1200 rpm using the Eppendorf Thermomixer Comfort heating shaker (Eppendorf, Hamburg, Germany). Samples were taken after 2, 4, 6, 8, 12, 24, and 48 h and dissolved in tetrahydrofuran. The work-up was performed as described in Section 2.2.1. The reaction

products were analyzed through MALDI-TOF MS spectrometry, while the conversion of the malic acid monomer was assayed by HPLC.

2.2.4. Operational Stability of the Biocatalyst

Novozyme and GF-CalB-IM biocatalysts were tested in 13 repeated cycles of the polymerization reaction between ECL (1 mmol) and D,L-MA (0.5 mmol) in a solvent-free system. The reaction was performed at 80 °C and 1200 rpm for 24 h with 15 mg of biocatalyst. After 24 h, the reaction products were dissolved in tetrahydrofuran and separated from the biocatalyst. Subsequently, the biocatalyst was washed 3 times with tetrahydrofuran, dried, and reused in a new reaction cycle. The work-up of the product was performed as described in Section 2.2.1.

2.2.5. HPLC Chromatography Analysis

The conversion of malic acid was determined by HPLC analysis using a Jasco HPLC system equipped with a PU-2089 Plus quaternary pump and UV-2070 Plus detector (JASCO International Co., Hachioji, Japan) on a reverse-phase Synergy 4-µm Hydro-RP 80A, 250 mm × 4.6 mm column (Phenomenex, Torrance, CA, USA). Malic acid was eluted using acetonitrile/water/o-phosphoric acid, 99:1:0.1 (*v*/*v*/*v*), isocratic for 12 min, and a flow rate 0.8 mL/min, with UV-detection achieved at 210 nm. Quantitative analysis was performed using a calibration curve with pure (>99%) D,L-malic acid.

2.2.6. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR of the monomers and oligomer samples was performed by the attenuated total reflection (ATR) mode using a Bruker Vertex 70 spectrophotometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a Platinum ATR module, Bruker Diamond Type A225/Q.I. For each sample, 128 coadded scans were recorded in the range of 4000 to 400 cm^{-1}.

2.2.7. MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) Analysis

MALDI-TOF MS analysis of the reaction products was performed using a Bruker UltrafleXtreme spectrometer (Bruker Daltonik GmbH, Bremen, Germany) at an acceleration voltage of 20 kV using trans-2-(3-(4-t-butyl-phenyl)-2-methyl-2-propenylidene) malononitrile (DCTB) as a matrix and sodium trifluoroacetate (NaTFA) as an ionizing agent. Then, 10 µL of sample (10 mg/mL) were homogenized with 10 µL of matrix solution (40 mg/mL) and 3 µL of NaTFA (5 mg/mL). Approximately 1 µL of the mixture was added onto the plate and measured in the positive ion mode. The calibration was performed with polyethylene glycol (PEG) solutions of different molecular weights (600, 1000, and 2000), and the results were processed with the FlexControl and Flex Analysis software packages (Brucker Daltonics, Bremen, Germany).

To characterize the structure of the oligomers, the average molecular masses (numerical and gravimetric) and the polydispersity were determined based on the MALDI-TOF MS spectra, as previously described [41]. Using the MALDI-TOF MS spectra, it was possible to identify the synthesized cyclic and noncyclic copolymers and homopolymers based on the similarity between the calculated and identified molecular masses of the oligomeric products from the reaction mixture. Estimation of the amounts of linear copolymers, cyclic copolymers, linear homopolymers, and cyclic homopolymers was also possible, although MALDI-TOF MS is not considered a quantitative analysis method [42].

2.2.8. Thermal Analysis

The synthesized reaction products were characterized by thermogravimetric (TG) and differential scanning calorimetry (DSC) analysis. Thermogravimetric analyses were performed using the TG 209 F1 Libra thermogravimetric analyzer (Netzsch, Selb, Germany)

in a nitrogen atmosphere with a heating rate of 10 K/min, and the chosen temperature range was 20–500 °C.

DSC analyses were performed using the DSC 204 F1 Phoenix differential scanning calorimeter (Netzsch, Selb, Germany) under nitrogen atmosphere in the temperature range 20–150 °C with a heating rate of 10 K/min. The recorded data were processed with Netzsch Proteus-Thermal Analyses version 6.1.0. (Netzsch-Geraetebau GmbH, Selb, Germany).

3. Results and Discussion

Malic acid is 2-hydroxybutanedioic acid and belongs to the class of β-hydroxy acids but can be also considered α-hydroxy acid, having the hydroxyl group in the α-position to one carboxyl group and in the β-position to the other carboxyl group [43]. In the case of the synthesis of malic acid polymers/oligomers with diols, this structural characteristic is not important, the polyester formed with the participation of both carboxyl groups, leaving the hydroxyl group as a pendant. The polycondensation process with ECL is different, because the participation of the hydroxyl group is compulsory (excepting when malic acid is the terminal unit) and is obvious that the carboxyl group in the β-position to the hydroxyl group will be included in the polymer chain, the other hydroxyl group remaining free. As a result, a new type of functional oligoesters, with pendant carboxyl groups, can be obtained.

Together with the copolymers, linear and cyclic homopolymers of both monomers can be synthesized as possible by-products, but the formation of poly(malic acid) by enzymatic catalysis, in the reaction conditions of this study, was not detected (and was not included in Figure 1 as a possible side reaction).

3.1. Characterization of the Oligoester Products by MALDI-TOF MS

The formation of the co-oligoesters was demonstrated by MALDI-TOF MS spectrometry. A typical MALDI-TOF MS spectrum of the reaction products obtained in a solventless system in the presence of GF-CalB-IM at a 2:1 molar ratio of ECL:D,L-MA is presented in Figure 2. The m/z values of several peaks can be assigned to the Na^+ adducts of the oligoesters formed in the polymerization reaction. For example, the series of peaks with m/z 842, 956, 1070, and 1185 were assigned to the Na^+ adducts of linear co-oligoesters containing one MA unit and between seven and 10 ECL units. Cyclic co-oligoesters were also identified at m/z values 822, 936, 1050, and 1068, corresponding to Na^+ adducts of the oligoesters containing one MA unit and between seven and 10 ECL units. Co-oligoesters with more than one MA unit were also identified, demonstrating the inclusion of MA in the polymeric chain, e.g., the values of the peaks with m/z 844, 958, 1072, and 1186 correspond to the Na^+ adducts of the linear co-oligoesters containing two MA units, together with seven to 10 ECL units.

3.2. The Effect of the ε-Caprolactone/Malic Acid Molar Ratio

The effect of the molar ratio on the monomers in the formation and composition of the oligomeric products was evaluated using three different ECL:MA molar ratios: 1:2, 1:1, and 2:1, with Novozyme 435 or GF-CalB-IM as the catalyst. The reactions were performed for both D.L-MA and L-MA in solventless systems, according to the method presented in Section 2.2.2, at 80 °C. The reaction temperature was set based on the previous results of the synthesis of ECL co-oligomers with hydroxy acids [21]. The relative composition of the oligomerization products calculated based on the MALDI-TOF MS spectra, as well as the conversions of malic acid, are presented in Tables 1 and 2 for Novozyme 435 and GF-CalB-IM, respectively.

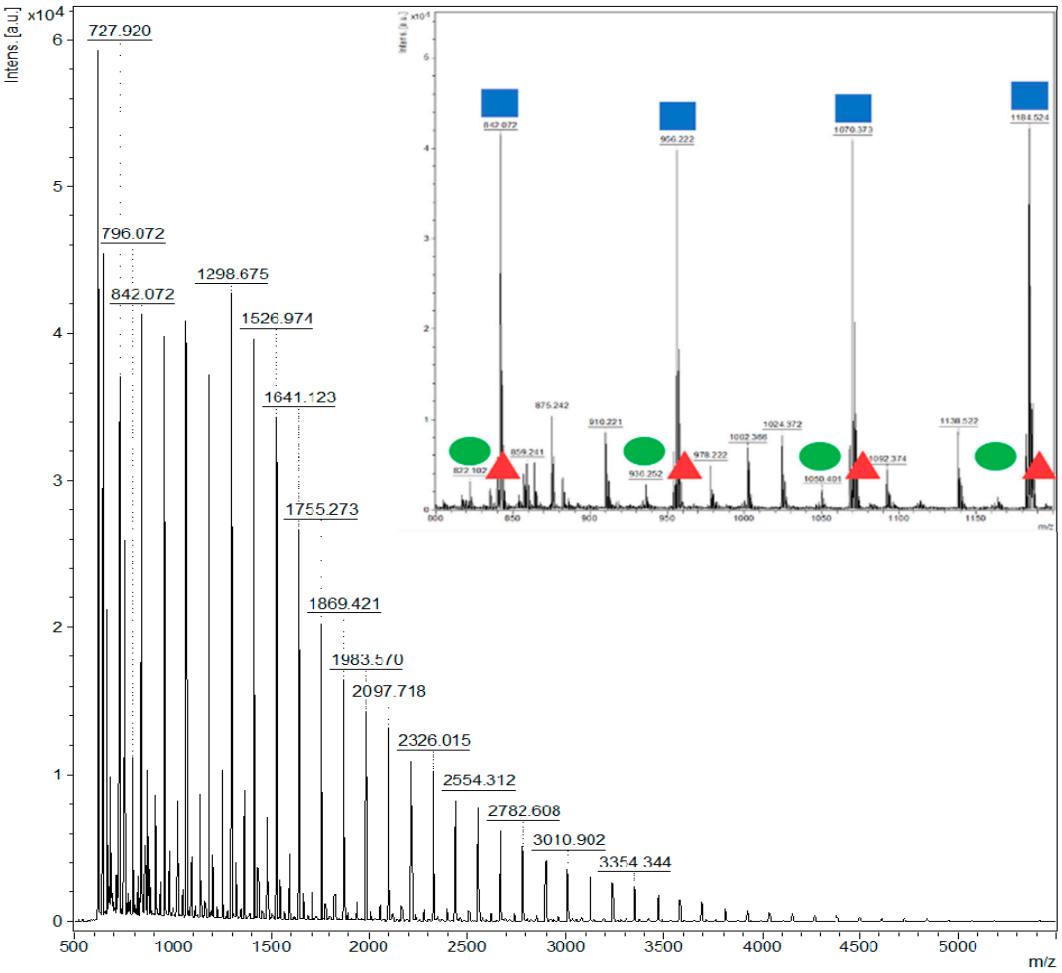

Figure 2. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF-MS) spectrum of reaction products synthesized from ε-caprolactone (ECL) and L-malic acid (L-MA) at a 2:1 molar ratio using GF-CalB-IM as the biocatalyst in solventless system at 80 °C. Inset: Detailed view of the m/z range 800–1200, highlighting the oligomers of interest (■ are linear oligoesters with 1 unit of MA, ● are cyclic oligoesters with 1 unit of MA, ▲ and are linear oligoesters with 2 units of MA).

Table 1. Influence of the ε-caprolactone: malic acid molar ratio on the composition of the reaction products obtained in the presence of Novozyme 435 in a solventless system.

Co-Monomer	Malic Acid Conversion (%)	Molar Ratio [a]	M_n (Da)	M_w (Da)	$Đ_M$	Relative Composition of the Reaction Products (%)				DP Max [f]
						LC [b]	CC [c]	LH [d]	CH [e]	
D,L-malic acid	20.41	1:2	782	831	1.20	63.4	1.37	23.6	11.6	14
	57.93	1:1	924	1038	1.12	88.6	2.6	2.1	6.7	18
	99.77	2:1	1343	1582	1.18	82.1	1.0	8.0	8.8	26
L-malic acid	46.82	1:2	807	849	1.05	84.7	5.0	5.8	4.5	13
	68.93	1:1	952	1053	1.11	86.6	1.3	1.3	10.9	16
	97.87	2:1	1079	1247	1.18	82.7	1.5	3.5	12.3	21

[a] ECL:MA molar ratio, [b] LC: linear co-oligomer, [c] CC: cyclic co-oligomer, [d] LH: linear homo-oligomer, [e] and CH: cyclic homo-oligomer. [f] Maximal degree of polymerization of the co-oligomer. $Đ_M$: dispersity.

Table 2. Influence of the ECL:MA molar ratio on the composition of the reaction products obtained in the presence of GF-CalB-IM in a solventless system.

Co-Monomer	Malic Acid Conversion (%)	Molar Ratio [a]	M_n (Da)	M_w (Da)	$Đ_M$	Relative Composition of the Reaction Products (%)				DP Max [f]
						LC [b]	CC [c]	LH [d]	CH [e]	
D,L-malic acid	26.22	1:2	800	881	1.20	57.0	3.4	18.5	21.1	15
	75.11	1:1	1166	1266	1.09	78.6	2.9	2.2	16.3	20
	95.61	2:1	1383	1642	1.19	78.0	0.6	11.4	9.9	29
L-malic acid	45.00	1:2	936	1019	1.09	76.9	1.8	-	21.3	15
	63.07	1:1	1068	1245	1.17	78.4	1.0	6.7	13.9	23
	94.91	2:1	1214	1523	1.25	78.3	0.5	9.7	11.4	30

[a] ECL:MA molar ratio, [b] LC: linear co-oligomer, [c] CC: cyclic co-oligomer, [d] LH: linear homo-oligomer, and [e] CH: cyclic homo-oligomer. [f] Maximal degree of polymerization of the co-oligomer. $Đ_M$: dispersity.

Regarding the results in terms of the enantioselectivity of the biocatalysts for the malic acid co-substrate, important differences were not observed between racemic D,L-malic and the natural L-malic acid isomer. It can be concluded that the oligomerization process is not enantioselective, although the lipase from *Candida antarctica* B is well-known for its high enantioselectivity in reactions involving racemic acids and their esters [44,45]. In addition to the highest medium molecular weights, higher conversions and relative copolymer contents were obtained when ECL was used in excess. The decrease of polymerization efficiency at higher amounts of malic acid could be explained by two different perspectives: the higher viscosity of the reaction mixture and the inhibitory effect of the higher concentrations of malic acid for lipases, previously reported by Li et al. [36]. This presumption was confirmed also by the low MA conversion values determined by the HPLC analysis. Using ECL in excess, the conversion of MA exceeded 99% with Novozyme as the biocatalyst and was slightly lower in the case of the GF-CalB-IM lipase.

The overall results indicate a slightly better catalytic efficiency of Novozyme 435 compared to the GF-CalB-IM lipase. However, they demonstrate that the GF-CalB-IM lipase, which is by far less-known and utilized than Novozyme 435, can be used with excellent results for the oligoester synthesis process, creating the possibility of designing the process development also based on the physical characteristics and availability of the immobilized lipase at a possible lower cost.

The relative content of the linear copolymer obtained in the presence of GF-CalB-IM did not exceed the 80% value. A direct correlation of the MA content increasing with the decrease of the medium molecular weight was also observed for the enzymatic synthesis of terpolymers synthesized using MA, adipic acid, and octandiol, probably due to the higher hydrophilicity of MA and the difusability hinderance in the active site of the lipase [35].

An example of the MALDI-TOF MS spectrum of the reaction product obtained in the presence of the GF-CalB-IM lipase is presented in Figure S1 (Supplementary Materials). The formation of the linear co-oligomers with 1 MA units was highlighted. Based on these results, the following experiments were performed using racemic D,L-malic acid.

3.3. The Influence of Organic Solvents on the Oligoester Synthesis

The presence of organic solvents in the reaction system could have a significant effect on the catalytic efficiency of lipases, particularly by controlling the partition of the substrates and products between the enzyme and the reaction medium. On the other side, they can negatively affect the activity and selectivity, especially the hydrophilic solvents, by stripping the essential hydration water shell from the enzyme molecule. In this study, two common organic solvents, acetonitrile, and toluene, as well as 2-methyl-tetrahydrofuran (Me-THF), a green solvent [46], were selected to evaluate their influence on the enzymatic synthesis of oligoesters from ECL and D,L-MA. This selection was based on preliminary experiments regarding their solvation ability for malic acid (data not shown). The reactions were performed in the presence of Novozyme 435 for 24 h at 1200 rpm and an ECL:D,L-MA

molar ratio of 2:1. The temperature was set at 70 °C, a lower temperature than in the solventless experiments, to prevent the evaporation of the solvent. A solventless system, in the same reaction conditions, was considered as the reference. The average molecular weights and relative contents in the oligomeric products, calculated based on the MALDI-TOF MS spectra, are presented in Table 3. In terms of the relative copolymer contents, the highest values were obtained in the solventless system, followed by the green solvent Me-THF. The average molecular weights, the copolymer content, and the conversion of malic acid were lower when acetonitrile was used as the reaction media, compared to toluene or Me-THF. The utilization of acetonitrile had a major negative effect on the average molecular weight, also resulting in a significant decrease of the relative copolymer content (37%). Among the three investigated organic solvents, the highest average molecular weight was obtained in toluene, but in this solvent, the PCL homopolymer was the main reaction product.

Table 3. Effects of organic solvents on the ECL-to-D,L/L-malic acid (D,L-MA)-based oligoester formation.

Solvent	Malic Acid Conversion(%)	M_n (Da)	M_w (Da)	$Đ_M$	Relative Composition of the Reaction Products (%)				DP Max [e]
					LC [a]	CC [b]	LH [c]	CH [d]	
-	98.72	1357	1644	1.21	78.0	1.3	1.1	9.6	28
Acetonitrile	42.85	842	961	1.14	35.9	0.4	59.4	4.3	25
Me-THF	98.08	970	1053	1.09	53.4	0.96	36.4	9.0	17
Toluene	51.34	1023	1138	1.11	32.4	0.4	47.9	19.2	19

[a] LC: linear co-oligomer, [b] CC: cyclic co-oligomer, [c] LH: linear homo-oligomer, and [d] CH: cyclic homo-oligomer. [e] Maximal degree of polymerization of the co-oligomer. $Đ_M$: dispersity. Me-THF: 2-methyl-tetrahydrofuran.

The highest average molecular weights for terpolymers from adipic acid, 1,8-octanediol, and L-malic acid were reported in hydrophobic solvents (isooctane and n-hexane), which cannot dissolve the substrates [35]. Since the monomers were preincubated at 120 °C to homogeneity, it is possible that these reactions did not occur in the organic solvent but at the interface of the monomer mixture and the non-miscible solvent, presuming that the oligomer was partially soluble in that solvent. Our approach was different, based on the selection of solvents that can dissolve the monomers and, also, in view of the possible scaling-up that could be more easily controlled in such conditions. Obviously, the highest relative copolymer content was obtained in the solventless system, but the good results obtained in the green solvent Me-THF are also promising in view of further development of the process, e.g., in a continuous flow system. Although these results suggest a reaction system composed only from the monomers and the enzyme as the most advantageous possibility for the scaling-up, the continuous system could perform better in the presence of an organic solvent. The most important conclusion is that the possibility of fine-tuning of the reaction engineering will allow the selection of the best process parameters in both batch and continuous-flow mode, as was demonstrated by previous studies from the literature [47,48]. The work-up of the reaction product involves the utilization of solvents, but these solvents can be selected without any connection to the oligomerization process only by the criteria of efficiency in the work-up and biocatalyst recovery stages. For this reason, in the following experiments, the solventless system was preferred.

3.4. Time Course of the Oligomerization Reaction

The malic acid oligoester formation was evaluated up to 48h of reaction time. Samples were collected at predetermined time intervals. The results (Figure 3a,b) indicate that the maximum conversion of malic acid was reached after 24 h. At higher reaction times, significant changes were not observed, concerning the relative contents of the copolymers and homopolymers. In terms of malic acid conversion, the first 12h were slightly higher in the presence of Novozyme 435.

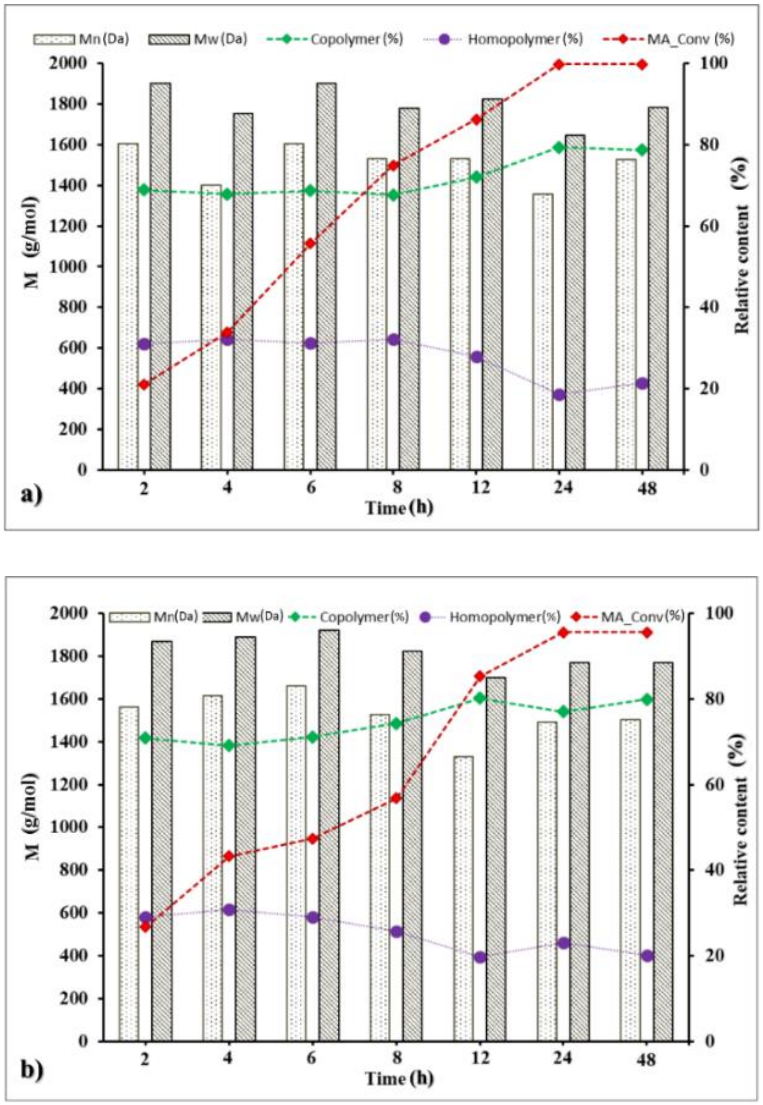

Figure 3. Time course of the oligoester synthesis reaction catalyzed by Novozyme 435 (**a**) and GF-CalB-IM (**b**). Reaction parameters: ECL:MA molar ratio 2:1 in a solventless system at 80 °C.

3.5. Operational Stability of the Biocatalyst in Repeated Reaction Cycles

The operational stability of both immobilized biocatalysts was evaluated in 13 batch utilization cycles performed at the ECL:MA molar ratio of 2:1 in a solventless system for 24 h. The results indicate the excellent operational stability of both catalysts. As shown in Figure 4a,b, after 13 reaction cycles, the average molecular weight and relative oligoester content were not significantly affected. The malic acid conversion, determined by HPLC (data not shown), was also not affected by the repeated use of the biocatalyst, being 92.76% in the case of Novozyme 435 and 92.79% for GF-CalB-IM after 13 reactions cycles each. Novozyme 435 is the most efficient biocatalyst used for polyester synthesis, but there are relatively few studies related to its operational stability in copolymerization reactions. Although the reactions were performed in a solventless system and the biocatalysts recovery involved the utilization of a medium polar organic solvent (tetrahydrofuran), the activity was not affected.

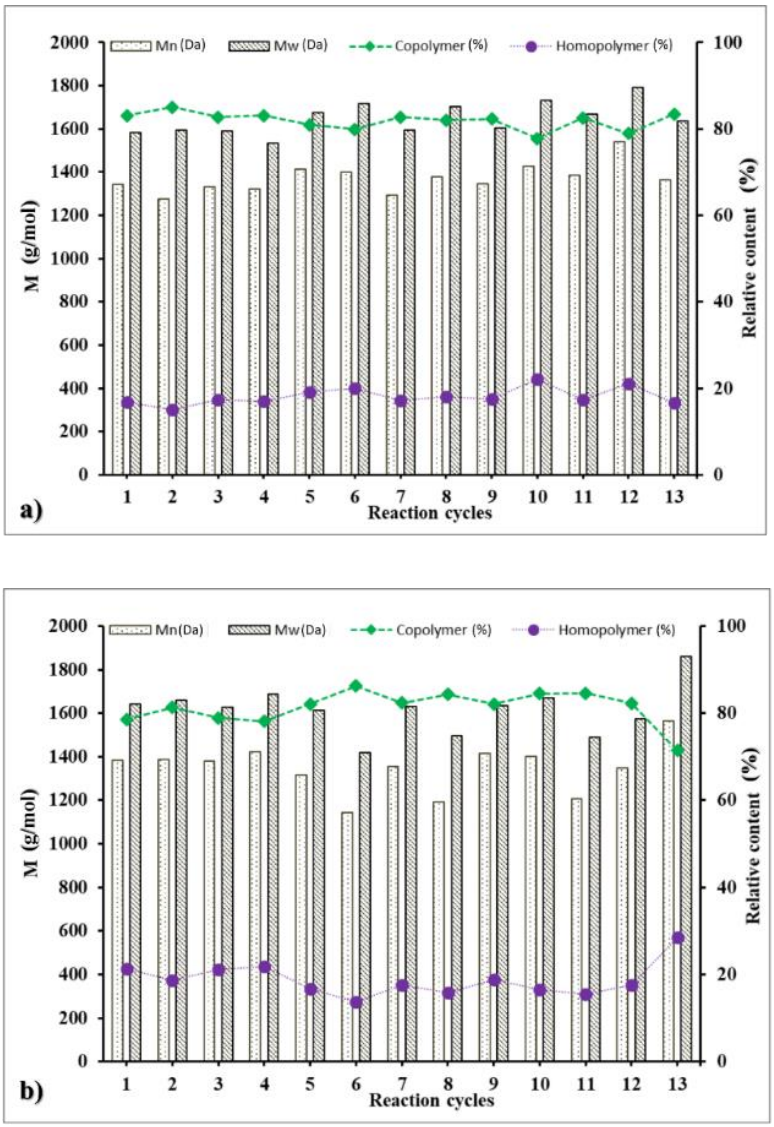

Figure 4. The operational stability of Novozyme 435 (**a**) and GF-CalB-IM (**b**) in repeated reaction cycles for the synthesis of oligoesters containing malic acid units. Reaction parameters: ECL:MA molar ratio 2:1 in a solventless system at 80 °C for a 24-h reaction time.

3.6. Characterization of the Oligomerization Products by FTIR Spectroscopy

The oligomerization products, along with the starting monomers, were characterized by FTIR spectroscopy. In Figure S2 (Supplementary Materials), the overlapping FTIR spectra of a selected products are presented. Beyond the presence of the absorption bands characteristic for the main functional groups coming from the monomers (not assigned, as they are obvious), the shift of the absorption band corresponding to the carbonyl group valence vibration from 1722 cm^{-1} in the ECL spectrum and 1674 cm^{-1} in the MA spectrum to a split band with values at 1685 cm^{-1} and 1720 cm^{-1} in the product spectrum can be considered structural evidence of the oligoester formation. Moreover, the presence of the adsorption band at 1685 cm^{-1} in the oligoester spectrum can be attributed to the presence of the free C=O group from the unreacted α-COOH of MA.

3.7. Thermal Stability of the Malic Acid-Based Oligoesters

The thermal properties of the copolymers, in contrast to the properties of the PCL homopolymer and the MA monomer, were evaluated by TG and DSC. The thermograms (Figure 5) indicate a decrease in the thermal stability of the ECL_MA (green line) co-

polyester compared to the PCL (blue point line) homopolymer. The mass loss in the case of the copolymer starts at about 210 °C. The details about the mass loss at different temperature intervals are presented in Table 4. Compared to PCL, more than 75% of the oligoester mass is lost at a temperature lower than 400 °C.

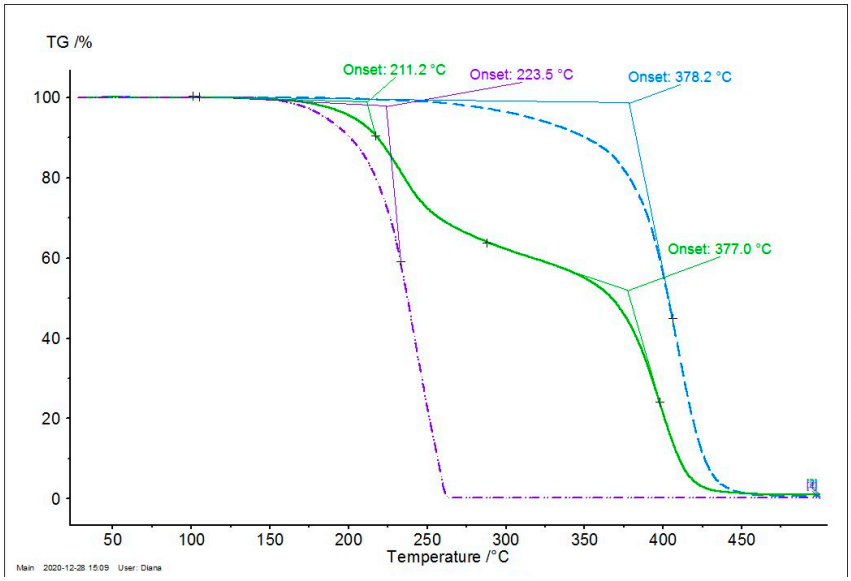

Figure 5. Thermograms of the ECL_MA co-polyester (green line), polycaprolactone (PCL) homopolymer (blue point line), and MA monomer (purple dashed line).

Table 4. Mass losses over different temperature ranges of the ECL_MA co-polyesters, polycaprolactone (PCL) homopolymer, and MA monomer, respectively.

Compound	Mass Loss (%)			
	20–200 °C	**20–300 °C**	**20–400 °C**	**20–500 °C**
ECL_MA	4.44	37.84	78.74	98.50
PCL	0.30	3.72	42.94	99.29
MA	9.64	99.79	99.79	99.79

As seen in Figure 5, compared to the PCL homopolymer and the MA monomer, which degrade in a single step at 378.2 °C and 223.5 °C, respectively, the ECL_MA copolymer degrades in two stages, a fact confirmed by the existence of two inflection points at 211.3 °C and 377.0 °C, respectively.

Figure 6 shows the DSC thermograms of the co-oligomer compared to the DSC thermograms of the homopolymers PCL and MA. After the first heating cycle, the reaction product (green line) exhibits two endothermic peaks at 58.0 °C and 126.9 °C, situated at a temperature difference of about 10 °C from the peaks corresponding to the PCL homopolymer and the MA monomer, respectively.

The DSC thermograms registered after two heating cycles are presented in Figure S2. No clear glass transition temperature was observed, and the results are in concordance with the previously reported data for the chemically synthesized products [31].

In Table 5 are presented the results obtained of the DSC analysis for the copolymer, PCL homopolymer, and the MA monomer. The values of the melting enthalpies (ΔH_f) decreased compared to the value obtained for the PCL due to the presence of malic acid in the oligoester chain. The results are in concordance with the previously reported results for malic acid-containing terpolymers, where a decrease of the melting enthalpies (ΔH_f) was observed with the increasing of the MA content [35,36]. The crystallinity of the co-oligomer was determined according to Huang et al. [49] for PCL derivatives. The results indicate

that the MA unit affects the crystallinity of the product compared to PCL, and it can be affirmed that the MA-based oligoester is semicrystalline.

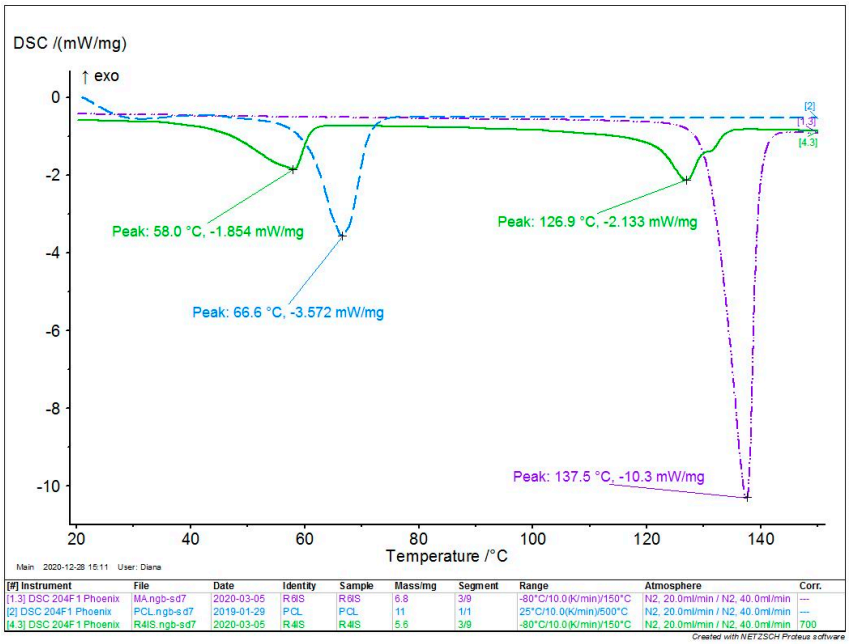

Figure 6. Differential scanning calorimetry characterization (DSC) curves of the ECL_MA co-polyesters (green line), PCL homopolymer (blue point line), and MA monomer (purple dashed line).

Table 5. Differential scanning calorimetry (DSC) parameters of the ECL_MA co-polyesters, PCL homopolymer, and MA monomer.

Compound	T_i (°C)	T_f (°C)	ΔT (°C)	T_{pk} (°C)	ΔH_f (J/g)	χ_c (%)
MA	122.0	144.5	22.5	137.5	305.4	n.d.
PCL	45.7	77.6	31.9	66.6	142.4	100
ECL_MA	34.6	64.3	29.7	58	72.29	50.76
	113.3	135.7	22.4	126.9	57.35	40.27

T_i: initial peak temperature; T_f: final peak temperature; $\Delta T = T_f - T_i$; T_{pk}: peak temperature of endothermic peak; ΔH_f melting enthalpy, χ_c crystallinity, determined from the first heating scan.

Unlike the MA and the PCL homopolymers, each one exhibiting a single endothermic peak, the copolymer has two endothermic peaks situated at a temperature difference of about 10 °C, compared to the peaks of MA and the PCL homopolymers.

4. Conclusions

Oligoesters containing malic acid and ε-caprolactone were successfully synthesized by enzymatic route. Two commercially available immobilized Cal B lipase-based products were tested as biocatalysts in four different reaction media. A higher efficiency of the polymerization reactions was obtained in a solventless system at 80 °C. The optimal reaction time was determined at 24 h, when the conversion of malic acid exceeded 95%. Excellent operational stability of the biocatalysts after 13 reaction cycles was demonstrated. Compared to the correspondent homopolymer PCL, the thermal properties of the oligoesters revealed a decrease of crystallinity at 50%.

From the perspective of the availability of ε-caprolactone from lignocellulosic raw materials and the biotechnological production of L-malic acid, the whole process of oligoester synthesis presented in this work can be considered green. Consequently, a new type of biobased functionalized oligomeric esters will be available for evaluation in various applications. Biobased oligoesters could fulfill an important request from the industry for

such products, particularly for cosmetics and biomedical utilizations, also reducing the use of nonrenewable carbon sources.

Supplementary Materials: The following are available online at https://www.mdpi.com/2227-9717/9/2/232/s1: Figure S1. MALDI TOF-MS spectrum of the reaction products obtained from ECL and D,L-MA synthesized in a solventless system at 80 °C in the presence of GF-CalB-IM at a molar ratio of ECL:MA = 1:1. Figure S2. The FTIR spectrum of the MA (purple), ECL (blue), and ECL_MA co-oligomers (green). Figure S3. The DSC melting endotherms in the range −80 °C to +150 °C (first heating cycle: red and second heating cycle: green) of the reaction products obtained from ECL and D,L-MA synthesized in a solventless system at 80 °C in the presence of Novozyme 435 at a molar ratio of ECL:MA = 2:1.

Author Contributions: Conceptualization, D.M.D., A.T., and F.P., methodology, D.M.D., I.C.B., and I.B.; validation, D.M.D., A.T., and F.P.; formal analysis, E.Ș. and M.P., investigation, D.M.D., I.C.B., and I.B.; writing—original draft preparation, D.M.D., A.T., and F.P.; writing—review and editing, A.T. and F.P.; supervision, F.P.; and funding acquisition, A.T. and F.P. All authors have read and agreed to the published version of the manuscript.

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