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"Green" Poly(butylene succinate-*co*-dilinoleic succinate) Copolymers Synthesized Using *Candida Antarctica* Lipase B (CAL-B) as Biocatalyst †

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Abstract: Driven by the increasing demand to reduce energy consumption and greenhouse gas emission biobased segmented block copolymer, poly(butylene succinate-co-dilinoleic succinate) (PBS-DLS) with 70:30 (wt%) ratio of hard to soft segments was synthesized using *Candida antarctica* lipase B (CAL-B) as a biocatalyst. During two-step synthesis in diphenyl ether, biobased diethyl succinate was polymerized with renewable 1,4–butanediol and dimer linoleic diol to obtain "green" copolyester as sustainable alternative to petroleum-based materials. ^{1}H NMR measurements confirmed that using enzyme as a catalyst we were able to obtain multiblock copolyester. The GPC analysis revealed number averaged molecular mass to be 25,000 g/mol. Furthermore, DSC measurements indicated two-phase morphology with low temperature glass transition (T_g) of soft segments and high melting point (T_m) of hard segments. Moreover, cytotoxicity test using mouse fibroblast cells L929 was performed on saline extracts from PBS-DLS copolymer indicating excellent biocompatibility *in vitro*.

Keywords: enzymatic catalysis; polyesters; renewable resources

1. Introduction

With the growing environment pollution and energy shortage caused by the petroleum consumption, developing biodegradable polymeric materials from renewable sources has gained a lot of attention [1]. Due to the increasing awareness of sustainability, traditional plastic materials have become less attractive in comparison to more eco-friendly alternatives. Aliphatic polyesters seem to be a good candidate to aim this goal as they can be produced using monomers fully derived from biomass feedstock [2]. They are suitable for conventional materials replacement due to the wide range of advantageous features, good mechanical properties and variable transition temperatures, thus they can be processed by injection moulding or extrusion into different forms. Among this group, segmented block copolyesters are triggering great attention as they consist of different types of building blocks possessing various properties and transition temperatures, thus being capable of forming hard and soft sequences. Hard segments are responsible for the dimensional, thermal and mechanical stability of the polymer and the soft segments are designed to impart the elasticity to the material. With appropriate selection of these building sequences it is possible to manufacture biobased copolyesters with desirable properties and wide spectrum of potential applications such as: fibers, films, scaffolds and drug delivery systems [3–5].

Lipases are enzymes which are extensively explored in biocatalytic synthesis of polymeric materials and *Candida antarctica* lipase B (CAL-B) is an important member of this family as it possesses

many beneficial features including: high regio-, chemio-, stereo- and enantioselectivity as well as catalytic activity which enables to obtain highly structure-regulated materials [6,7]. Additionally, CAL-B exhibit broad substrate specificity and it works well under mild conditions with various monomers and organic solvents. It is already known that lipases can be successfully employed to produce polymeric materials such as: polyesters, polyamides and polycarbonates etc. and be a good replacement for organometallic catalysts, which can have undesirable impact on environment [8–11]. All these facts together show that biocatalysis is an effective platform for developing a "green" and sustainable polymer chemistry and this approach is even more appealing since both monomers and catalysts are derived from biomass [4].

To extend the library of biobased copolyesters synthesized using enzymes as biocatalyst, we perform two-stage polycondensation of diethyl succinate, 1,4–butanediol and dilinoleic acid diol (DLA-OH) in diphenyl ether. Obtained material was further characterized regarding the chemical structure, molecular weight, crystalline and thermal properties.

2. Experiments

2.1. Materials

Dichloromethane (DCM: ≥ 99.5%) and diphenyl ether (≥ 99%) were purchased from Sigma Aldrich (Poznan, Poland), diethyl succinate (DS: ≥ 99%) was purchased from Matrix Fine Chemicals (Sevelen, Switzerland). 1,4-butanediol (BD: ≥ 99%) was purchased from Alfa Aesar (Kandel, Germany). Dimer linoleic diol (DLA-OH) Pripol™ 2033 (dimer alcohol: ≥ 96.5%) was kindly provided by Croda Coatings & Polymers (Gouda, The Netherlands). Chloroform ($CHCl_3$: ≥ 98.5%) was purchased from Chempur (Piekary Slaskie, Poland), methanol (MeOH: ≥ 99.8%) was purchased from Stanlab (Lublin, Poland). Lipase B from *Candida antarctica* (CAL-B), covalently immobilized on polyacrylate beads (300–500 μm > 95%, Fermase CALB™ 10000), with a nominal activity of 10 000 PLU/g (propyl laurate Units per gram dry weight), was purchased from Fermenta Biotech Ltd. Mumbai and from Enzyme Catalyzed Polymers LLC Akron (OH, USA).

2.2. Polymer Synthesis

We synthesized poly(butylene succinate-co-dilinoleic succinate) with a 70:30 wt% ratio of hard to soft segments (abbreviated as PBS-DLS 70:30). The dilinoleic-succinate soft segments (DLS) were built using dilinoleic diol (DLA-OH) and butylene-succinate (PBS) hard segments were built using 1,4-butanediol both reacting with ethyl succinate. Briefly, CAL-B (10 wt% of total monomers) was added to a round-bottom flask containing 1,4-butanediol (BD), diethyl succinate (DS) and dimer linoleic diol (DLA-OH) in diphenyl ether (200 wt% of total monomers). The prepared solution was placed into oil heated bath and the first step was carried out under inert gas flow at atmospheric pressure at an initial temperature of 80°C. After 1 h, when the reaction mixture was homogeneous, the temperature was slowly increased to 95° C and the collection of ethanol was proceeded (3 h). Further oligomerization was conducted under pressure of 600 Torr for 21 h, this step was applied to allow for conversion of monomers into non-volatile oligomers. In next step, the pressure has been gently reduced to 2 Torr, while still maintaining the reaction temperature at 95 °C for 96 h. Upon completion, the product mixture was dissolved in chloroform and filtered to remove enzyme. The obtained chloroform solution was then concentrated and added dropwise to cold methanol. The precipitated product was filtered, washed three times with cold methanol, collected and dried in vacuo at 40 °C for 24 h.

2.3. Material Characterization

Nuclear magnetic resonance (^{1}H NMR) and Fourier Transform infrared spectroscopy (ATR-FTIR) were used to assess the chemical structure of the synthesized copolymer. A TM Bruker DPX 400 spectrometer (400 MHz) was used to record spectra of ^{1}H NMR (128 scans, 1 s relaxation delay). The samples were dissolved in $CDCl_3$ and tetramethylsilane (TMS) was used as internal reference. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectra were recorded on Bruker

APLHA spectrometer across the spectral range 400 to 4000 cm^{-1} with a 2 cm^{-1} resolution. Spectra were averaged from 32 scans.

The number average molecular weight (M_n), weight average molecular weight (M_w) as well as dispersity index (Đ) were determined by gel permeation chromatography (GPC). Measurements were performed in a set-up consisting of a Viscotek VE 1122 pump, column system and detector. The separation system consisted of two, 300 × 75 mm high resolution Styragel columns with a mixed bed PLgel 5 µm Mixed C (Polymer Laboratories). Chloroform with a flow rate of 1 mL/min was used as the eluent in a thermostatic system at 35 °C. Each time 100 µL of analyzed samples were injected into the chromatograph in the form of chloroform solutions with a 3% (*w*/*v*) concentration where the Shodex SE 61 differential refractometer was used as the detector. The M_n, M_w and Đ values were assessed based on a calibration curve determined for narrow polystyrene standards.

The thermal behavior of the obtained PBS-DLS 70:30 copolyester was investigated using TA Instruments DSC Q2500 Discovery differential scanning calorimeter (DSC). Measurements were performed in a heating-cooling-heating cycle over the temperature range from −90 to 200 °C at heating/cooling rate of 10 °C/min and under nitrogen atmosphere. Glass transition temperature (T_g) was derived as a midpoint of the transition in a second heating cycle.

Indirect contact cytotoxicity tests were performed according to ISO10993-5 using mouse fibroblast cells line L929. 150 mg of PBS-DLS 70:30 copolymer was placed into a well of 24-well culture plate. Then 2 mL of complete growth media (DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin) was added to the well and the plate was incubated in a CO_2 incubator intended for cell culture at 37°C for 24 h. In parallel a 96-well plate was prepared and 10.000 of L929 mouse fibroblast cells was plated at each well (5% CO_2, 37 °C, 24 h). After 24 h of culture in complete growth media, the medium was replaced with 100 µL of sample extract (6 technical repetitions were performed) and the plate was incubated again for 24 h. After incubation, cell viability was assessed by phase contrast microscopy and resazurin viability assay, where 20 mL of resazurin solution (0,15 mg/mL in PBS) was added to test well and plates were transferred to incubator (5% CO_2, 37°C) for another 4 h. Fluorescence (Em: 540, Ex: 590) was measured by BioTek Synergy HTX multifunctional plate reader.

3. **Results and Siscussion**

Chemical structure of obtained PBS-DLS copolyester was verified by nuclear magnetic resonance (NMR). Structural analysis of ^{1}H-NMR spectra and peaks assignments are presented in Figures 1–2.

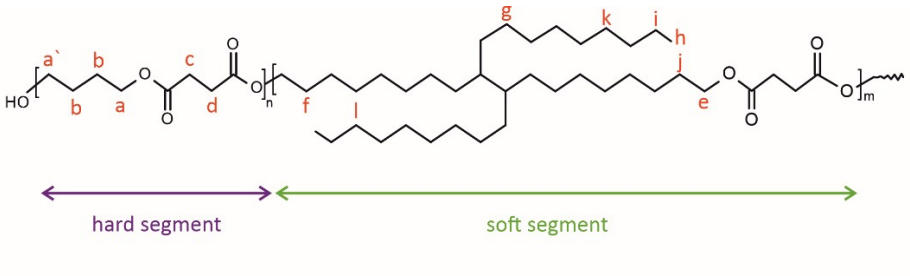

Figure 1. Chemical structure of poly(butylene succinate-co-dilinoleic succinate) (PBS-DLS).

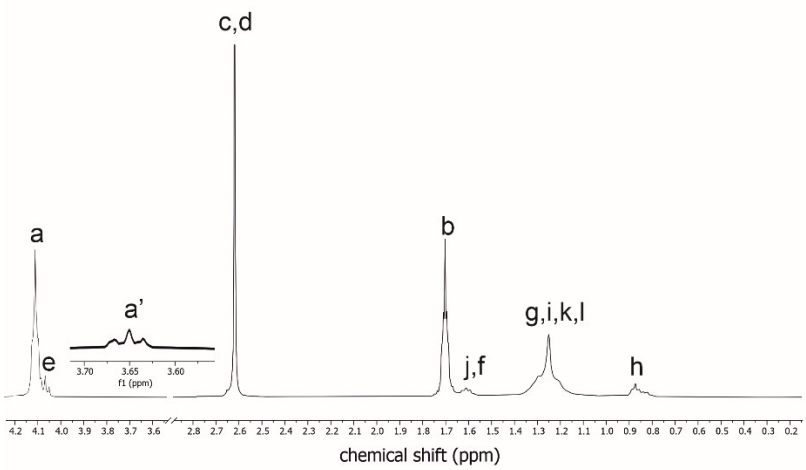

Figure 2. H NMR spectra of the obtained aliphatic polyester PBS-DLS 70:30.

The following signal assignments for PBS-DLS 70:30 from ^{1}H NMR in $CDCl_3$ were registered (Figure 2). Chemical shifts at δ^1H = 4.12 ppm (a) confirmed the formation of BD-DS ester bond while signals at δ^1H = 4.06–4.08 ppm (e) indicated on ester bond formation between DLA-OH and DS. Signals at δ^1H = 3.66–3.69 ppm (a') are ascribed to the protons from the methylene end groups of 1,4-BD in hard segments and methylene protons on DS and the four internal protons on 1,4-BD are located at δ^1H = 2.63 ppm (c, d,) and δ^1H = 1.71 ppm (b), respectively. The six terminal protons on DLA-OH methyl end groups are detected at δ^1H = 0.85 ppm (h). The appearance of (f), (g), (i), (j), (k), (l) and (e) signals indicating the presence of long aliphatic chains from DLS sequence. ^{1}H NMR analysis confirmed the expected chemical structure of PBS-DLS 70:30 copolyester. Furthermore, based on the ^{1}H NMR spectra, the sample composition was assessed following method presented in [12] and showed in Table 1.

Table 1. Sample composition, number average molecular mass calculated from ^{1}H NMR, number average molecular mass, weight average molecular mass and dispersity index calculated from GPC.

Copolymer	Composition: wt% [mol%]		GPC [b]		
	Theoretical	Calculated [a]	M_n [g/mol]	M_w [g/mol]	Đ
PBS-DLS 70:30	70/30 [89.4/10.6]	68/32 [88.6/11.4]	25 200	205 600	8.2

M_n—number average molecular mass, M_w—weight average molecular mass, Đ—dispersity index, [a] value calculated from ^{1}H NMR [b] values determined from GPC.

Table 1 shows the final polymer composition. As it can be observed, we were able to obtain good match between theoretical values and those calculated from ^{1}H NMR spectra, however, a greater amount of DLS sequence has been introduced into copolymer, in comparison to the initial feed and probably this may be caused by the evaporation of 1,4-butanediol during vacuum assisted step. DLA-OH is more difficult to be removed due to its long aliphatic chain and high molecular weight (540 g/mol), therefore we assume that it remains constantly in the reaction media. Furthermore, the number (M_n) and weight (M_w) average molecular masses as well as dispersity index (Đ) of PBS-DLS 70:30 copolyester were assessed by GPC measurements. Taking under consideration similar reported methods in which PBS-based copolymers were synthesized by biocatalysis [2,9], we obtain quite high M_n (25,200 g/mol) and M_w (205,600 g/mol) values, however, dispersity index was expected to be equal or grater than 2, which is quite usual for random step-growth polycondensation [8]. Herein, Đ value is much higher which may indicate that CAL-B exhibit a tendency to produce material with different macromolecules distribution and size.

Chemical structure of PBS-DLS copolymer was further confirmed by ATR-FTIR spectroscopy, as showed in Figure 3. Peaks at 2920 cm^{-1} and 2855 cm^{-1} are ascribed to the asymmetric and symmetric stretching vibrations of the –CH_2–, respectively. The strong band at 1710 cm^{-1} is ascribed to the C=O

carbonyl vibrations. Bands at 1210 cm^{-1} and 1150 cm^{-1} are assigned to the asymmetric and symmetric C–O–C stretching vibrations of ester groups, respectively. Furthermore, peaks observed at 1445–1470 cm^{-1} and 1390–1425 cm^{-1} are ascribed to the deformation and wagging vibrations of the $–CH_2–$ groups, respectively. Peaks appearing at region 1000–500 cm^{-1} are assigned to the in-plane and out-of-plane deformation vibrations of C–H and C–C groups.

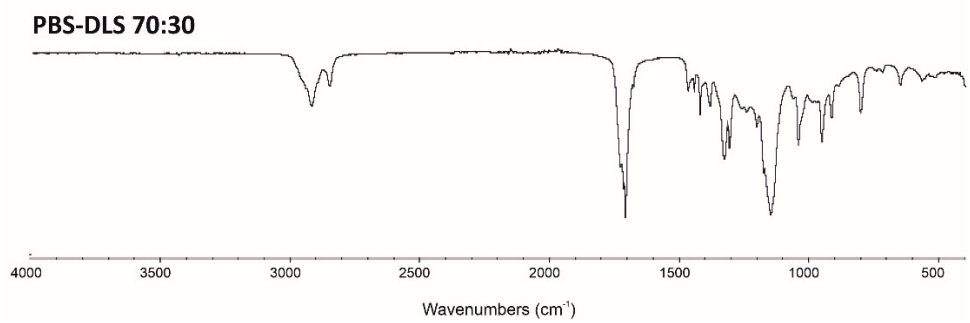

Figure 3. ATR-FTIR spectrum of PBS-DLS 70:30 copolymer.

The thermal behavior of the PBS-DLS 70:30 copolymer was characterized by DSC, as summarized in Table 2. During measurement we were able to observe T_g, T_m and T_c which indicate on material semicrystallinity. We found that glass transition temperature (T_g) of copolymer is noticeably lower in comparison to neat PBS sample, which proves that amorphous DLS soft segments were well incorporated into material structure. Furthermore, T_c, ΔH_c and X_c values also decreased due to the presence of DLS sequences, which obviously are hindering the crystallization transition during cooling from the melt. This fact can be explained by the greater distances appearing between PBS rigid blocks which are responsible for material crystallinity. Moreover, the addition of DLS soft segments contributed to the reduction in melting enthalpy (from 81 to 49,52 J/g) and T_m (from 110 to 98 °C) which also indicate that quality, perfection and degree of crystalline phase decreases due to the weaker intermolecular interactions [13].

Table 2. DSC results for PBS-DLS 70:30 copolymer.

Material	T_g [°C]	T_c [°C]	ΔH_c [J/g]	T_m [°C]	ΔH_m [J/g]	X_c [%]
PBS-DLS 70:30	−45	53	50.99	98	49.52	44.9
PBS [a]	−34	70	86	110	81	62

T_g—glass transition temperature; ΔH_m—melting enthalpy of the hard segments; T_m—melting temperature; T_c—crystallization temperature; X_c—crystalline phase content in the copolymer [a] numerical values obtained from [14].

In order to assess the cytotoxicity of the synthesized PBS-DLS 70:30 copolymer, biological tests were carried out using indirect contact method. Performed tests revealed that material is not inducing any cytotoxic response and ensure an adequate cell proliferation as can be seen on Figure 4. Furthermore, values obtained from the measurements with the multifunctional plate reader showed that the viability of mouse fibroblast cells L929 in presence of extract from analyzed materials was 98% which indicate on material high biocompatibility *in vitro*.

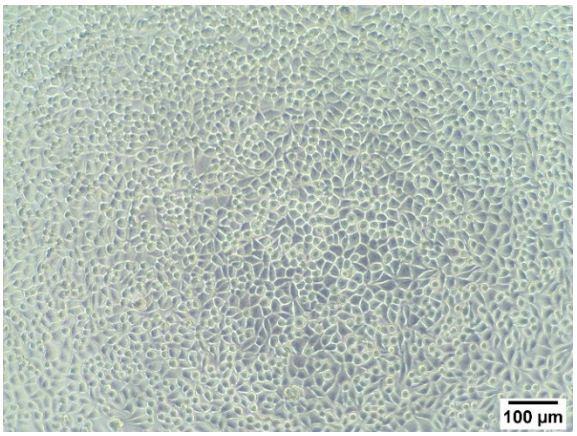

Figure 4. Confluent L929 mouse fibroblast cells with the presence of extracts from PBS-DLS 70:30 (magnification: 10x).

4. Conclusions

In this work, we prove that the two-stage enzymatic polycondensation of diethyl succinate, 1,4-butanediol and dimer linoleic diol enable to produce sustainable aliphatic polyester, poly(butylene succinate-co-dilinoleic succinate) PBS-DLS 70:30 with the M_n value of 25,000 g/mol. ^{1}H NMR analysis also revealed that obtained material possess desirable molar composition and chemical structure which indicate on the good process control. Focusing on the thermal properties we found that the X_c, T_m and T_g of PBS-DLS copolyester decreased after incorporation of amorphous DLS soft sequences. Finally, biocompatibility tests conducted with L929 mouse fibroblast cells highlighted the excellent in vitro biocompatibility of PBS-DLS copolyester which is very beneficial for developing bio- and eco-friendly synthesis approach and increases the range of potential applicability of these materials, for example in biomedical applications.

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References

1. Hillmyer MA, Tolman WB. Aliphatic Polyester Block Polymers: Renewable, Degradable, and Sustainable. Acc Chem Res 2014;47:2390–6. https://doi.org/10.1021/ar500121d.
2. Jiang Y, Woortman AJJ, Alberda Van Ekenstein GOR, Loos K. Environmentally benign synthesis of saturated and unsaturated aliphatic polyesters via enzymatic polymerization of biobased monomers derived from renewable resources. Polym Chem 2015;6:5451–63. https://doi.org/10.1039/c5py00660k.
3. Jawad H, El Fray M, Boccaccini AR, Harding SE, Wright JS, Chen Q, et al. Nanocomposite Elastomeric Biomaterials for Myocardial Tissue Engineering Using Embryonic Stem Cell-derived Cardiomyocytes. Adv Eng Mater 2010;12:B664–74. https://doi.org/10.1002/adem.201080078.
4. Jiang Y, Woortman AJJ, Alberda van Ekenstein GOR, Loos K. Enzyme-catalyzed synthesis of unsaturated aliphatic polyesters based on green monomers from renewable resources. Biomolecules 2013;3:461–80. https://doi.org/10.3390/biom3030461.
5. Penczek P, Czub P, Pielichowski J. Unsaturated Polyester Resins: Chemistry and Technology, 2005, p. 1–95. https://doi.org/10.1007/b136243.
6. Kobayashi S. Recent Developments in Lipase-Catalyzed Synthesis of Polyesters. Macromol Rapid Commun 2009;30:237–66. https://doi.org/10.1002/marc.200800690.
7. Anderson EM, Larsson KM, Kirk O. One Biocatalyst–Many Applications: The Use of Candida Antarctica B-Lipase in Organic Synthesis. Biocatal Biotransformation 1998;16:181–204. https://doi.org/10.3109/10242429809003198.
8. Azim H, Dekhterman A, Jiang Z, Gross RA. Candida antarctica Lipase B-Catalyzed Synthesis of Poly (butylene succinate): Shorter Chain Building Blocks Also Work 2006:3093–7. https://doi.org/10.1021/bm060574h.
9. Sonseca A, El Fray M. Enzymatic synthesis of an electrospinnable poly(butylene succinate-co-dilinoleic

succinate) thermoplastic elastomer. RSC Adv 2017;7:21258–67. https://doi.org/10.1039/c7ra02509b.
10. Kobayashi S, Makino A. Enzymatic Polymer Synthesis: An Opportunity for Green Polymer Chemistry. Chem Rev 2009;109:5288–353. https://doi.org/10.1021/cr900165z.
11. Ikeda R, Uyama H, Kobayashi S. Novel Synthetic Pathway to a Poly(phenylene oxide). Laccase-Catalyzed Oxidative Polymerization of Syringic Acid. Macromolecules 1996;29:3053–4. https://doi.org/10.1021/ma951810b.
12. Sokołowska M, Stachowska E, Czaplicka M, El Fray M. Effect of enzymatic versus titanium dioxide/silicon dioxide catalyst on crystal structure of 'green' poly[(butylene succinate)- co -(dilinoleic succinate)] copolymers. Polym Int 2020:pi.6104. https://doi.org/10.1002/pi.6104.
13. Mincheva R, Delangre A, Raquez J-M, Narayan R, Dubois P. Biobased Polyesters with Composition-Dependent Thermomechanical Properties: Synthesis and Characterization of Poly(butylene succinate-co -butylene azelate). Biomacromolecules 2013;14:890–9. https://doi.org/10.1021/bm301965h.
14. Sonseca A, McClain A, Puskas JE, El Fray M. Kinetic studies of biocatalyzed copolyesters of poly(butylene succinate)(PBS)containing fully bio-based dilinoleic diol. Eur Polym J 2019;116:515–25. https://doi.org/10.1016/j.eurpolymj.2019.04.038.

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