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Abstract

The synthesis of 17-hydroxy-oleic acid based oligomeric esters was investigated with immobilized *Pseudozyma antarctica* Lipase B and hexanediol as co-substrate. The effects of different reaction parameters on velocity and product composition at equilibrium conditions were analyzed. The synthesis of oleic acid esters was used as a reference system for initial evaluation of reaction parameters. The reaction with oleic acid and hexanediol was fastest at an enzyme concentration of 5% at 60 °C and high conversions of > 90 % were achieved in non-polar solvents in the presence of molecular sieves. In heptane an oleic acid conversion of 96 % was reached with a final diester to monoester ratio of > 4:1. In syntheses trials with 17-hydroxy-oleic acid the formation of oligomers was verified with GPC, however; conversion was generally lower than with oleic acid. Removal of hydroxyl fatty acid monomers and dimers and the formation ester functionalities could be verified by GC analysis. An increase of the degree of oligomerization was observed simultaneously by GPC analysis. The number-average molecular weight was around 1400 in the best trials corresponding to a degree of oligomerization of around 4 units of hydroxyl-fatty acid attached to a hexanediol core. Though transformations were not complete, the final oligomer size was in the lower range of polyester diols used for polyurethane manufacturing.

1. Introduction

Linear polyurethanes are obtained from the reaction of di-isocyanates with diol building blocks. The diols can be tailored regarding structure, polarity or molecular weight and determine the polyurethane properties. Typical building blocks are polyether, polycarbonate or polyester derived oligomers of petrochemical origin [1]. Biobased diols were already produced from vegetable oils [2], sugar derived hydroxymethylfurfural [3] or ricinoleic acid (12-hydroxy-oleic acid), which was esterified with a polyethylene glycol core to yield a diol building block for PU synthesis [4].

The aim of this work was the lipase-catalyzed synthesis of oligomeric diols starting from hexanediol and 17-hydroxy-oleic acid according to Fig. 1. The unusual 17-hydroxy-oleic acid can be obtained from yeast derived sophorolipid biosurfactants. Depending on yeast strain subterminally or terminally hydroxylated fatty acids are incorporated into the biosurfactants [5-8]. The best sophorolipid producer is *Starmerella bombicola* with yields exceeding 100 g/l [9-11]. The main structure of its sophorolipid is a lactone with subterminally hydroxylated oleic acid [5,6].

Lipases are versatile biocatalysts for the synthesis of bio-based esters e.g. for food, cosmetics, lubricants and fuel applications [12-15]. Their process stability makes them ideal biocatalysts for solvent based ester synthesis [16,17]. Lipase B from *Pseudozyma antarctica* is the most studied enzyme with an exceptional temperature and solvent stability. Numerous examples of esterification and transesterification reactions were shown with this enzyme, which is commercially available in different formulations including the polyacrylate-immobilized Novozym 435 [18-21]. Polyester synthesis with *Pseudozyma antarctica* lipase can either be done by polycondensation under removal of water [22] or by ring-opening polymerization starting from lactones [23,24]. The immobilized enzyme can easily be removed from the polymerization mixture and used repeatedly [25]. Several polymers were synthesized with immobilized lipase B from *Pseudozyma antarctica* and the manifold approaches are summarized in recent review articles [21,26-29].

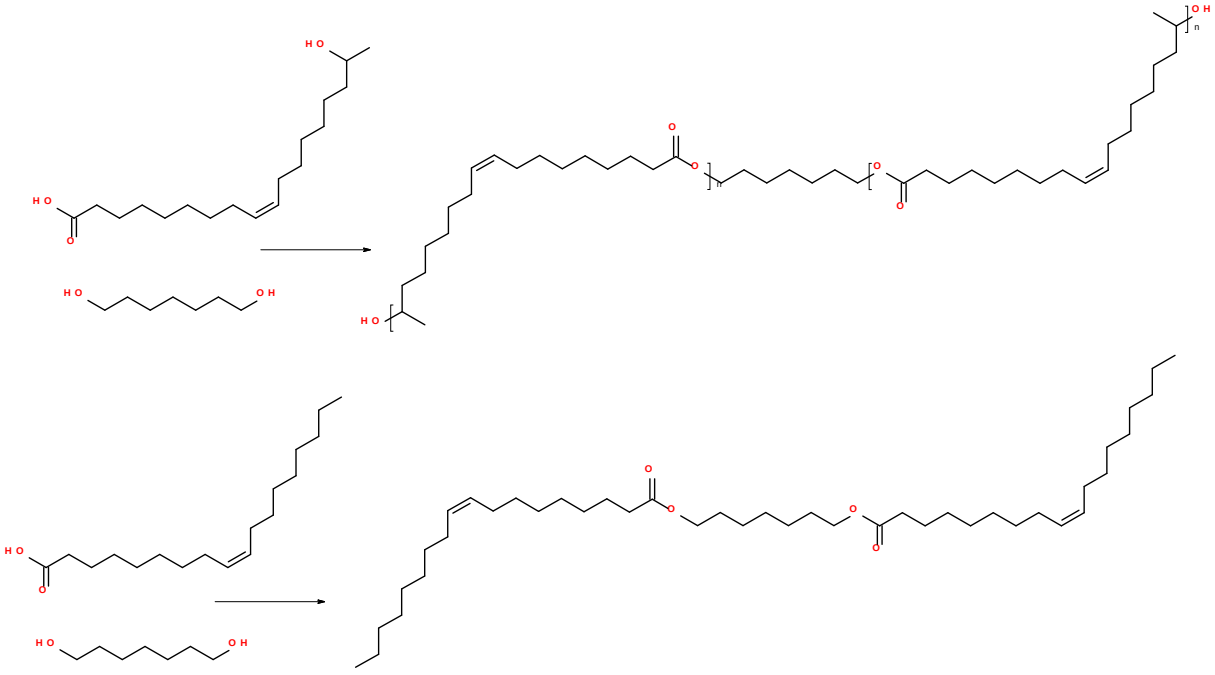


Fig. 1: Lipase catalyzed esterification of hexanediol with 17-hydroxy-oleic acid (top) and oleic acid (bottom)

2. Materials and methods

2.1 Materials

All chemicals were of synthesis grade. BSTFA + 1% TMCS and reference standards for GC calibration were from Carl Roth. Liquid and immobilized lipase B preparations from *Pseudozyma antarctica* (Lipozyme CALBL and Novozym 435) are products from Novozymes and Accurel MP 1000 is a macroporous polypropylene from Membrana. Oleic acid was obtained from DAKO and hexanediol was from Merck. Molecular sieves were obtained from Alfa Aesar. All other chemicals and solvents were from Carl Roth or VWR International. Solvents used in the study were heptane, 2-butanone (MEK) and 2-methyl-2-butanol (2M2B).

2.2 Preparation of immobilized lipase and hydroxylated fatty acids

Lipozyme CALBL was immobilized onto Accurel MP 1000 by adsorption as described in [30]. 2 g of Accurel MP 1000 were soaked for 30 min in 20 mL ethanol. Ethanol was removed and 20 mL of water and 5 mL of Lipozyme CALBL were added and incubated on a rotary shaker overnight at room temperature. The immobilized enzyme was filtered, dried on a sheet of paper and stored at 8 C.

Hydroxylated fatty acids were prepared according to the methods described by Sonnabend et al. [31]. In brief sophorolipids were produced by fermentation of *Starmerella bombicola*, extracted with ethyl acetate and washed with water. The crude sophorolipids were then washed with hexane for fatty acid removal and treated with acid to cleave the glycosidic bonds. The released hydroxylated fatty acids were separated, washed and dried.

2.3 Biocatalytic esterification reactions

The syntheses of oleic acid based diesters and 17-hydroxy-oleic based esters were performed in sealed flasks on a rotary shaker at 250 rpm. Exemplarily 2 eq. oleic acid (8.8 mmol) and 1 eq. hexanediol (4.7 mmol) were dissolved in 15 mL of solvent and the reaction was started by addition of 1 % Novozym-435. After 24h at T = 60 °C the reaction was stopped by filtering off the Novozym-435. Aliquots were taken at different time intervals to monitor the reaction and for final product analysis. The effect of solvents, temperature, enzyme concentration, enzyme support and addition of molecular sieves were analyzed under comparative synthesis conditions. In synthesis trials with 17-hydroxy-oleic acid higher amounts of Novozym 435 of up to 12 % (w/w) were used and the substrates molar ratio was varied from 2:1 to 8:1.

2.4 Analytical methods and calculations

In routine analysis 10 µL of the lipid phase were dissolved in 940 µl of heptane in a GC-vial and 50 µl silylation agent (BSTFA + 1% TMCS) were added. The samples were sealed and incubated in an oven at 80°C for one hour. Analysis was done with a Shimadzu GC 2010 Plus using a MTX Biodiesel TG column (RESTEK, length 14m, ∅ 0.53 mm, film thickness 0.16 µm)

connected to a FID detector with helium as carrier gas and a temperature gradient from 75°C to 410 °C. A split ratio of 5 with an injection volume of 1.5 µl was applied.

Analysis of lipids was done according to the DGF standard method C-V 2 "Acid value and free fatty acid content (Acidity)" with 0.2 – 2 g of lipid sample dissolved in ethanol. Acid values of samples containing organic solvents were normalized to the lipid content in the organic phase. The acid value was determined by titration with 0.1 M KOH solution against phenolphthalein using a Metrohm Dosimat E535 and calculated with the following equation:

$$AV = \frac{\text{ml KOH consumed} \cdot [KOH] \cdot M_{KOH}}{\text{g sample}}$$

Oligomers were analyzed by gel permeation chromatography (GPC), using a PSS polymer safety system with Agilent 1260 hardware modules. The system is equipped with an isocratic pump, a vacuum degasser, a styrene-divinylbenzene copolymer column (5 µm particle size and 1000 Å porosity), a column oven and a standard autosampler. For detection, a refractive index (RI) and UV-visible detectors (250 nm) are used. The column was calibrated with narrow molecular weight distribution polystyrene (ReadyCal Kit standards from PSS polymer). The samples were measured at 30°C and a flow rate of 1 mL min^{-1} with tetrahydrofuran (HPLC grade) as eluent. The sample concentration and injection volumes were 10 mg mL^{-1} and 50 µL respectively.

3. Results and discussion

3.1 Esterification of oleic acid with hexanediol

The esterification of hexanediol with oleic acid was used as a reference system for initial evaluation of suitable reaction conditions. Besides enzyme concentration and temperature the influence of molecular sieves and the solvent system were analyzed. Generally hydrophobic solvents are better suited for esterification reactions; however, the solubility of hexanediol and the 17-hydroxy oleic acid was low in heptane. Therefore different solvent mixtures were analyzed. Addition of 20 % MEK or 2M2B were sufficient to generate a single-phase system.

Reactions were monitored with acid value titration, which allowed the calculation of oleic acid conversion (Fig. 2) and high temperature GC was used for analysis of monoesters and high boiling diesters (Fig. 3+4). In a solvent system containing 80 % heptane and 20 % MEK the reaction velocity at an enzyme concentration of 5 % (w/w substrates) and 60 °C was fastest. Accordingly lipase B from *Pseudozyma antarctica* is known for its exceptional temperature stability. Without addition of molecular sieves the reactions proceeded to a final conversion of approximately 75 %, which is the equilibrium in the heptane / MEK solvent system. Upon addition of molecular sieve (Fig. 2, right) the reaction velocities increased and the equilibrium was shifted towards ester synthesis. In the heptane / MEK solvent system conversions of 91 – 92 % were achieved. The pure heptane system in combination with molecular sieve performed slightly better with a final conversion of 96 %

after 24 h. Greater water retention of the more hydrophilic MEK based solvent system most probably accounts for the differences in equilibrium ester concentration most probably.

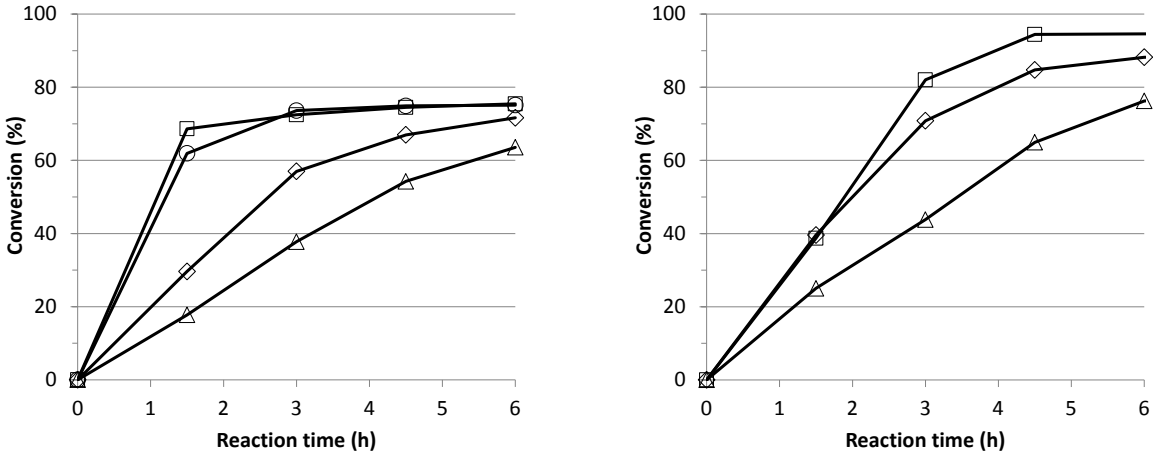


Fig. 2. Conversion of oleic acid esterification analyzed via acid value titration, **left:** analysis of temperature and enzyme concentration with △ = 1 % Novozym 435, 45 °C; ◇ = 5 % Novozym 435, 45 °C; ○ = 1 % Novozym 435, 60 °C and □ = 5 % Novozym 435, 60 °C; **right:** analysis of solvent and molecular sieve effect (5 % each) with 1 % Novozym 435 and △ = 45 °C, heptane/MEK 8:2; ◇ = 60 °C; heptane/MEK 8:2 and □ = 60 °C, 100 % heptane

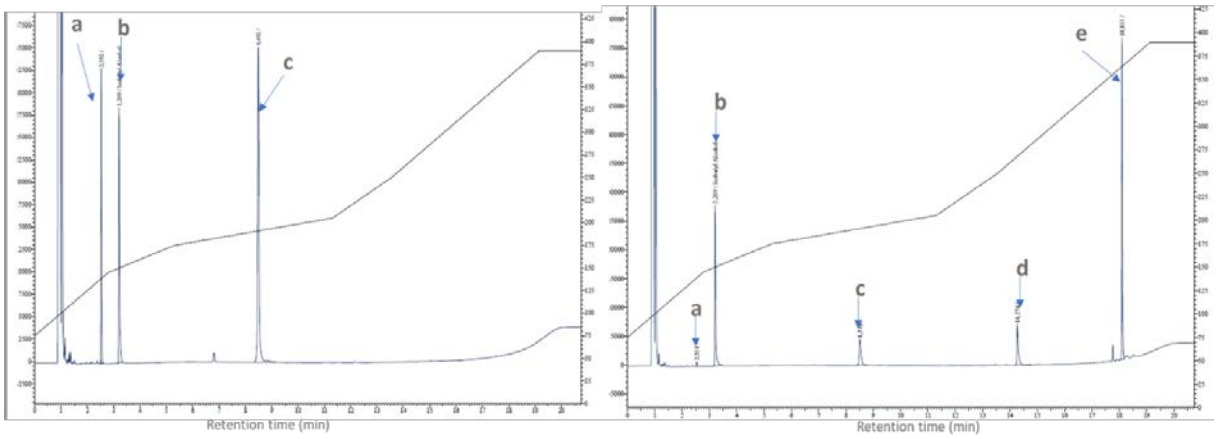


Fig. 3. GC-chromatogram of substrate oleic acid / hexanediol mixture (left) and product mixture after 24 h (right) with a) hexanediol, b) internal standard pentadecane, c) oleic acid, d) hexanediol monoester and e) hexanediol diester.

In GC analysis the course of the reaction was monitored over a period of 24 h and the conversion rates were in good agreement with data obtained from acid value analysis. The reactions at 45 °C without molecular sieve and at 60 °C in the presence of molecular sieves are exemplarily shown in Fig. 4 for the heptane / MEK (8:2) solvent system. In both reactions a fast disappearance of hexanediol was visible, which coincided with an accumulation of the monoester. Diester formation started time-delayed and a monoester maximum of 25 – 35 % after 3 – 4 hours of reaction time was formed in the consecutive reaction. The reaction at 60 °C proceeded significantly faster and a higher final concentration of diesters was achieved

after 24 h in the presence of molecular sieves. The diester to monoester ratio without molecular sieve was 80 : 20, while that with molecular sieve reached 91 : 9. In the pure heptane system a diester to monoester ration of 93 : 7 was reached.

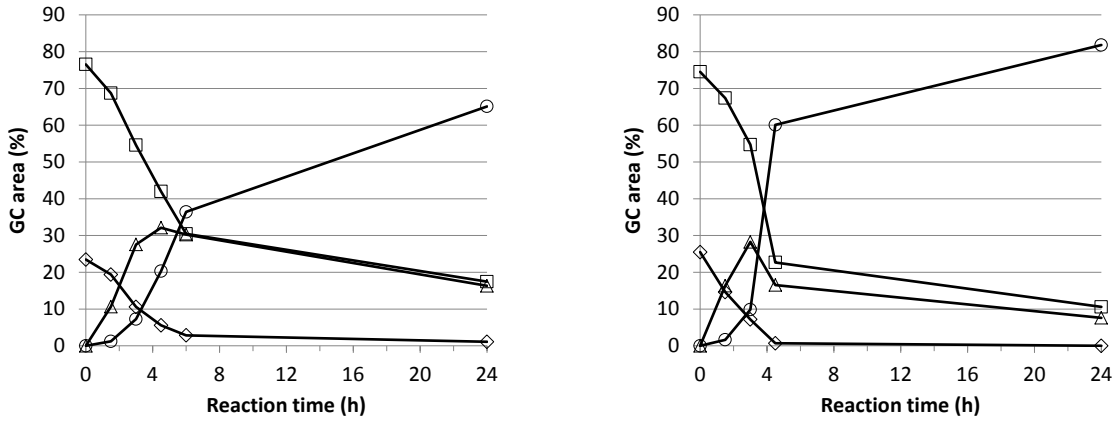


Fig. 4. GC analysis of ester formation in the heptane / MEK 8:2 solvent system with □ = oleic acid, ◇ = hexanediol, △ = monoester and ○ = diester; **left:** reaction with 1 % Novozym 435 at 45 °C without molecular sieve, **right:** reaction with 1 % Novozym 435 at 60 °C in the presence of molecular sieve

3.2 Esterification of 17-hydroxy oleic acid with hexanediol

Analysis of 17-hydroxyoleic acid obtained from acid splitting of *Starmerella bombicola* sophorolipids revealed that the hydroxyl fatty acid was not pure (Fig. 5). Some oligomerization occurred leading e.g. to dimer formation (peak D). From the acid value of 94 it was concluded that intramolecular lactonization as well as formation of higher boiling estolides led to a variety of side products with an average size of a dimer. Upon esterification hexanediol and the monomer peak decreased and new product peaks E and F were formed. Retention time of peaks E correspond to hexanediol monoesters. Peaks in the region of F may either be the monoester of a hydroxyoleic acid dimer or the diester with two hydroxyoleic acid monomers. Higher oligomers could not be detected due to their high boiling points

17-Hydroxyoleic acid was esterified with hexanediol by Novozym 435 as well as by *Pseudozyma antarctica* lipase B immobilized in the hydrophobic polypropylene support Accurel MP 1000 (Fig. 6). In general the reaction was faster with Novozym 435. A clear difference in overall conversion was observed in dependence of the molar ratio of hexanediol to hydroxy fatty acid. In a molar ratio of 1:2 (calculated as monomers) an esterification yield of > 90 % was achieved, while only around 50 % of the acid groups were esterified after 48 h in the trials with 1:8 molar substrate ratios.

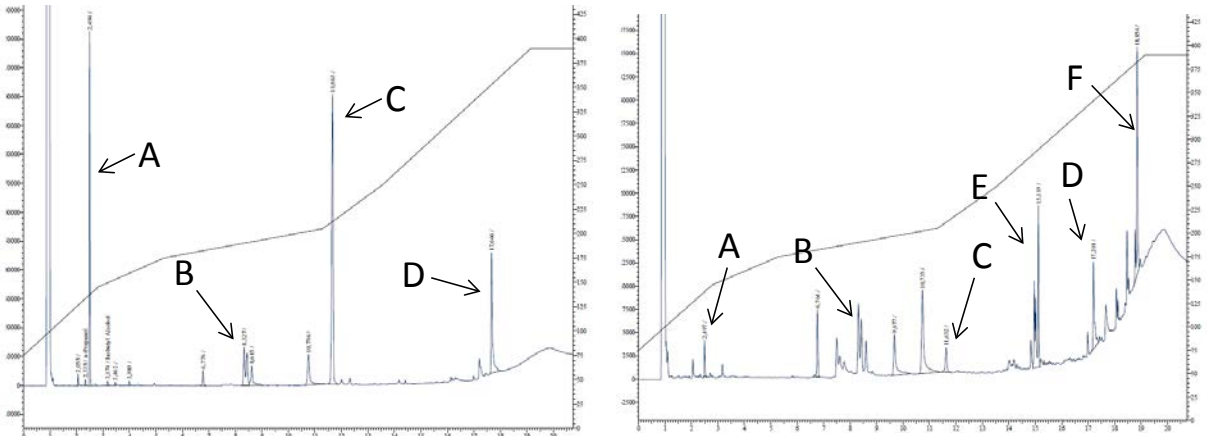


Fig. 5. GC chromatograms of substrate mixture of 17-hydroxyoleic acid and hexanediol (left) and product mixture after transformation with Novo 435 (right), A = hexanediol, B = unknown compounds, C = 17-hydroxyoleic acid, D = dimer of 17-hydroxyoleic acid, E) monoester F) higher esters

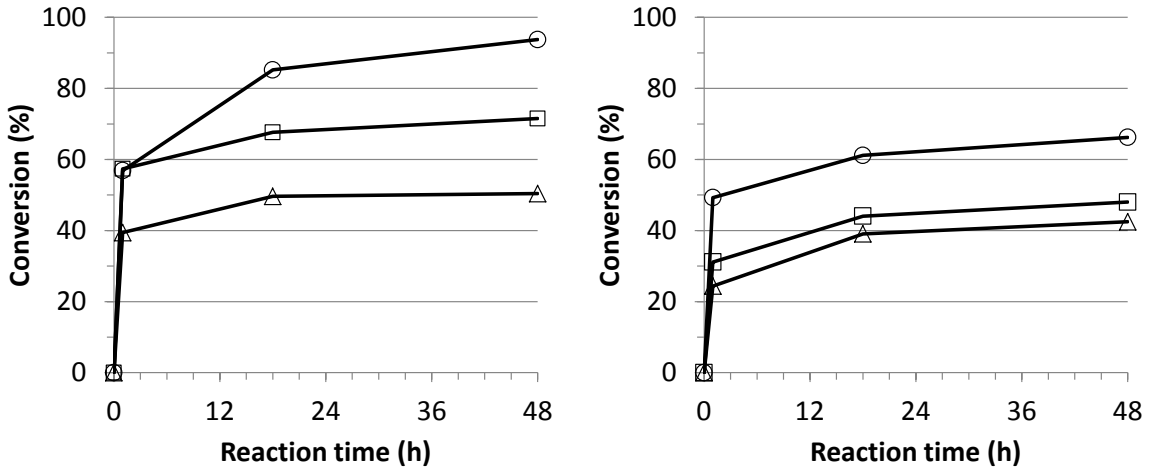
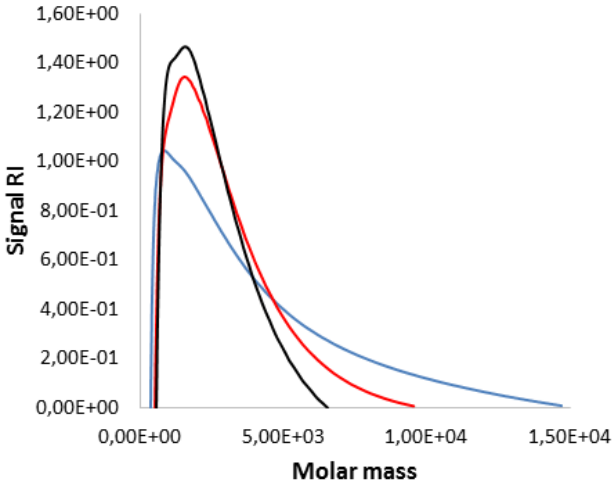


Fig. 6. Top: conversions with Novozym 435 (left) and lipase B immobilized on Accurel MP 1000 with hexanediol to hydroxyl fatty acid substrate ratios of 1:2 (○), 1:4 (□) and 1:8 (△); **right:** GPC overlay of reaction products from conversions with Novozym 435 with black line = 1:2, blue line = 1:4 and red line = 1:8 substrate ratios



The reactions were conducted with higher enzyme concentrations compared to the oleic acid based reference system and thus the initial esterification was very fast. After 1 h the slope of the conversion curves changed and only a slow increase was observed. An explanation for this behavior is the regioselectivity of the lipase. We believe that the terminal hydroxyl groups of 1,6-hexanediol are esterified rapidly, whereas the secondary hydroxyl groups of the 17-hydroxyoleic acid are hardly recognized by the enzyme. A full transformation in a molar 1:2 ratio is possible, because enough primary hydroxyl groups are available. In contrast, in a 1:8 molar ratio a conversion of 50 % can be achieved, when an average substrate size of a dimer is assumed. Accordingly the GPC chromatograms of the reaction products were comparable with a molar mass of around 1.200 – 1.400 g/mol independent of the molar ratios used. The average degree of polymerization was 4 – 5. Upon longer incubation of the reaction mixtures the esterification yield increased to around 60 %; however, oligomers of significantly higher mass were not obtained. To increase molecular weight, further optimization of reaction conditions or utilization of lipase combinations is needed. Alternatively a chemical pre-oligomerization of the hydroxyl fatty acid prior to enzymatic esterification may be applied.

Conclusions

With the reference system hexanediol / oleic acid the synthesis of diesters was achieved in good yield with more than 90 % of diester content. A solvent system with 80 % heptane and 20 % polar solvent was chosen for the esterification of hydroxyl fatty acids. Molar masses of up to 1.400 were reached corresponding to a degree of polymerization of 4 – 5. Oligomer masses were limited because lipase B of *Pseudozyma antarctica* has a strong preference for the esterification of the primary hydroxyl groups of hexanediol in comparison to the secondary hydroxyl groups of 17-hydroxyoleic acid. Optimizing process conditions, selecting lipases with selectivity for the 17-hydroxy group or combining chemical and enzymatic esterification may be approaches to further increase the degree of polymerization of the polyester diols.

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The authors have declared no conflict of interest.

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