Novel Enzymatic Synthesis of 3-hydroxybutyric Acid Oligomers with Inserted Lactobionic Acid Moieties

SÁNDOR KAKASI-ZSURKA[1,3], ANAMARIA TODEA[1], ANDRADA BUT[1], CRISTINA PAUL[1], CARMEN BOERIU[2,4], LAJOS NAGY[3], CORNELIU MIRCEA DAVIDESCU[1], FRANCISC PÉTER[1*]

[1] University "Politehnica" of Timişoara, Faculty of Industrial Chemistry and Environmental Engineering, C. 6 Telbisz, 30001 Timişoara, Romania
[2] Wageningen UR, Food & Biobased Research, Division of Biobased Products, Wageningen, The Netherlands
[3] University of Debrecen, Department of Applied Chemistry, H-4010 Debrecen, Hungary
[4] University "Aurel Vlaicu", Department of Chemical and Biological Science, Arad, Romania

3-Hydroxybutyric acid and lactobionic acid yielded linear and cyclic oligomers in a lipase-catalyzed condensation polymerization reaction, performed at 80°C in bulk and organic solvent systems. Novozyme 435 was the most efficient biocatalyst, and a mixture of t-butanol and dimethylsulfoxide in 80:20 (v/v) ratio provided the highest copolymer conversions. The highest degree of polymerization reached 7 in case of copolymers with inserted lactobionic acid moiety and 11 for the 3-hydroxybutyric acid homopolymer by-product.

Keywords: lactobionic acid, 3-hydroxybutyric acid, copolymers, lipase, biocatalysis

Although natural polymers synthesized by enzymes (e.g. proteins, starch, cellulose) are products with massive industrial and economic impact, the interest toward *in vitro* synthesis of polymeric compounds by enzymatic catalysis arised only in the past few decades. The main advantages of a biocatalytic way to produce natural or unnatural polymers consists of mild reaction conditions and high selectivity control, including regioselectivity, chemo-selectivity, and enantioselectivity [1-2]. Recent developments in enzymatic polymerization have been targeted mainly to lipases, esterolytic enzymes able to catalyze the reverse esterification reaction as well, due to their high activity and stability in nonaqueous reaction media. Two reaction routes are possible to realize polyester synthesis by lipase-catalyzed reactions: ring-opening polymerization of lactones and condensation polymerization of either dicarboxylic acids with diols or oxyacids [3-5].

Lipases have also demonstrated catalytic efficiency in synthesis of sugar esters with selectivity for the primary hydroxyl groups, but several difficulties have been caused by the lower reactivity of sugars compared to fatty alcohols, and the low solubility of raw sugars in nonpolar solvents that are particularly suitable for lipase-catalyzed esterification reactions. Thus, utilization of polar organic solvents, like *t*-butanol, pyridine or dimethylsulfoxide was necessary [6-7]. Polymerization reactions of sugars catalyzed by hydrolases have the same bottlenecks, needing acid derivatives with highly reactive leaving groups, like *bis*(2,2,2-trifluoroethyl)-adipate, used as co-monomer for synthesis of a poly(sucrose adipate) in anhydrous pyridine [8]. To avoid the complex and multi-step synthesis of activated monomers, carbohydrate 1,5-lactones have been employed as starting materials for functionalized aliphatic polyesters. Ring-opening polymerization of tetra-*O*-acetyl-D-glucono-1,5-lactone gave an oligoester with 1,4-butanediol, initiated by a metal alcoxide [9]. Although ring-opening polymerization of lactones catalyzed by enzymes was intensively studied

[10], synthesis of copolymers involving ring-opening of a carbohydrate lactone was not yet reported, excepting our preliminary data [11].

Lactobionic acid (LBA), obtained by oxidation of lactose, is a compound already used in specialty products for biomedical applications, but could also become a bulk chemical in the future, depending on its price [12]. Synthesis of LBA graft copolymer with chemically modified chitosan was recently reported [13]. However, to our best knowledge, there are no reports about inclusion of LBA in a polymer chain by polycondensation. The present study was focused on synthesis of such a copolymer, using 3-hydroxybutyric acid (3HBA) as co-monomer and lipase biocatalyst. 3-HBA is, like LBA, a "green" raw material, available by depolymerization of bacterial poly(3-hydroxybutyrate by a specific depolymerase enzyme [14].

Experimental part
Materials and methods
(*R,S*)-3-hydroxybutyric acid (3HBA), *t*-butanol, toluene, and dimethylsulfoxide were analytical grade reagents purchased from Merck. Immobilized *Candida antarctica* B lipase on acrylic resin (Novozyme 435) and *Rhizomucor miehei* lipase (Lipozyme-RM IM) were from Novozymes. *Candida antarctica* B lipase was bought from (Germany). Molecular sieves (4Å) were provided by Acros Organics, and lactobionic acid (LBA) by Sigma.

Polymerization in organic solvents
In 5 mL Micro Reactions Vessels LBA (0.358 g, 1 mmole) was solved/suspended in 2 mL organic medium and then 3HBA (0.465 mL, 5 mmole) and 50 mg lipase were added. The reaction was magnetically stirred at 300 rpm under argon atmosphere, at 80°C, with activated 4Å molecular sieves for removal of water by-product. After separation of enzyme by filtration, the products were precipitated into methanol (10:1, v/v). The precipitate was separated by centrifugation at low temperature (4°C) and 5000×g relative centrifugal force for 60 min, and washed twice

with methanol. A white solid was obtained after drying in vacuum at 60ºC. The total conversion of 3HBA was assayed by gas-chromatographic analysis of a sample taken from the reaction mixture, derivatized with N,O-*bis*(trimethylsilyl)-trifluoracetamide, and analysed as presented in our previous study [16].

Polymerization in bulk

In a 4 mL vial 0.465 mL 3-HBA, 50 mg Novozyme 435, and 358 mg of LBA were added. The mixture was heated at 80°C under argon atmosphere. At the end of the fixed reaction time the mixture was diluted with chloroform and the enzyme removed by filtration. The polymer product was obtained by evaporation of chloroform and was dried in vacuum at 60ºC.

Products characterization

FT-IR Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) spectra were obtained from a JASCO FT/IR 430 spectrometer on 500-4000 cm^{-1} range at 4 cm^{-1} spectral resolution. Solid samples were prepared as KBr pellets and liquid samples as films, using KBr windows.

MALDI-TOF MS analysis

A Bruker BIFLEX III matrix assisted laser desorption ionization time-of-light mass spectrometer ((Germany) was utilized for MALDI-TOF MS analysis of products. The analysis conditions were set as follows: acceleration voltage of 20 kV, and 2,5-dihydroxybenzoic acid as matrix. Samples (10 mg/mL), the matrix (2,5-dihydroxybenzoic acid, 20 mg/mL) and the ionization agent sodium trifluoroacetate (5 mg/mL) were individually dissolved in an appropriate organic solvent. 10 μL of sample solution and 5 μL of ionization agent solution were mixed with 50 μL matrix solution, and an aliquot (0.3 μL) was applied to the sample plate. Within one MALDI-TOF spectrum, the intensities of all signals (generated by the sodium adduct ions) of the present molecular species were summed and the total set as 100%. Separately, a sum of signal intensities was calculated for every type of linear and cyclic copolymer present in the analysed product, as well as for the identified 3HBA homopolymers. We calculated the relative composition of the reaction product, assuming that the response factor was the same for every oligomeric type present in the mixture. Although this method cannot be used for precise quantitation, it is useful to estimate the relative distribution of the synthesized products, being already utilized in this respect for fructose oligosaccharide-lauryl ester oligomers [15].

Results and discussions

Polyhydroxyalkanoates (PHA) of microbial origin are important natural biopolymers, but could also be utilized as raw materials to obtain the constituent chiral hydroxy acids. Poly(3-hydroxybutyrate), the most studied PHA, is a rigid and brittle material, consequently not suitable for certain applications. 3HBA, obtained by depolymerization of P3HB, could be a valuable monomer for synthesis of new biodegradable oligomers and polymers, like homopolymers with lower molecular weight, or various copolyesters. In the present study, enzymatic poly-condensation of 3HBA with LBA was carried out in various reaction conditions. The most probable copolymers possible to be formed by insertion of LBA in the P3HB chain are shown in figure 1.

Lipase-catalyzed copolymerization of 3HBA and LBA

Evidence of ester product formation was provided by FT-IR measurements. Figure 2 shows the FT-IR spectra of raw materials (3HBA and LA) and a 3HBA-LA reaction product. The FT-IR spectrum of the product confirm the ester formation by shift of the absorption band of the C=O group stretching vibration from 1741 cm^{-1} in LA and 1724 cm^{-1} in 3HBA to 1732 cm^{-1} in the product. It also can be seen the shift on the large absorption band, which
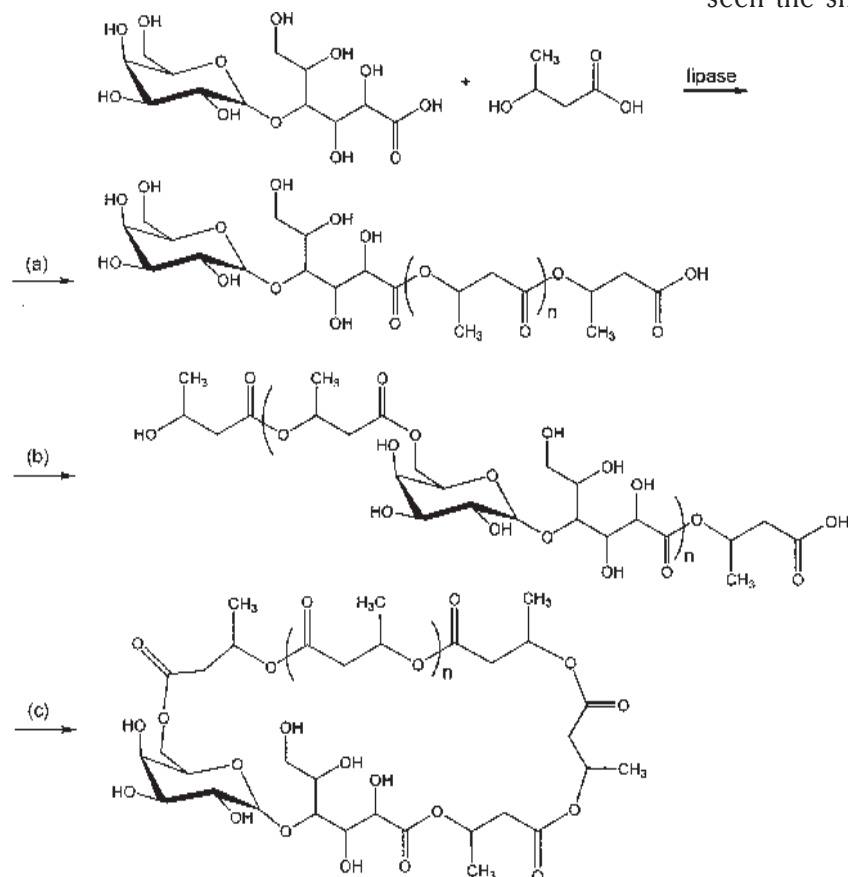

Fig. 1. Copolymerization scheme of 3-hydroxybutyric and lactobionic acid yielding (a) linear copolymer with a galactose end unit; (b) linear copolymer with inserted LBA-derived moieties; (c) cyclic copolymer
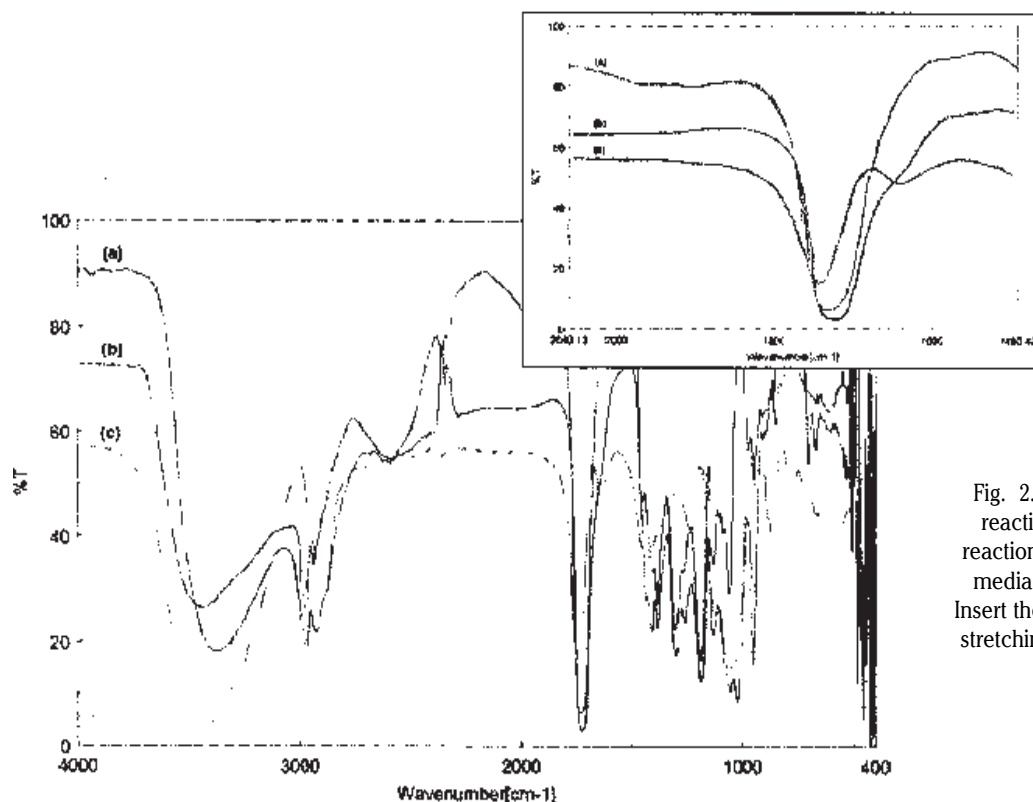
Fig. 2. FT-IR spectra of (a) 3HBA-LA reaction product obtained at 7 days reaction time in DMSO:*t*-BuOH reaction media, (b) raw 3HBA and (c) raw LA. Insert the 1500-2100 cm^{-1} region, with the stretching vibration band of C=O group

corresponds to the OH groups stretching vibration, to a lower wavenumber value of 3423 cm^{-1}.

The structure of the enzymatic synthesis products was analyzed by MALDI-TOF MS spectrometry. The theoretical molecular masses of sodium adduct ions of copolymer with lactobionic and 3-hydroxybutyric acid units were calculated in order to identify them in the real spectrum of the product. Figure 3 shows a typical MALDI-TOF mass spectrum of the product synthesized by copolymerization of 3-hydroxybutyric acid with LBA in DMSO:*t*-BuOH reaction medium at 80°C, using Novozyme 435.

Although the P3HB homopolymer species have significant presence in the spectrum, we also identified linear and cyclic oligomers containing inserted LBA moieties. Table 1 presents the proposed structures for a few selected oligomers from the MALDI-TOF spectrum, based on the similarity of their calculated and experimental molecular masses.

Based on these results we can demonstrate, for the first time, that copolymerization of 3HBA with lactobionic acid is possible by lipase-catalyzed reaction.

Selection of the enzyme

Three different microbial lipases have been tested as biocatalyst: two were *Candida antarctica* lipase B lipases, in immobilized (Novozyme 435) and native form (C-Lecta),
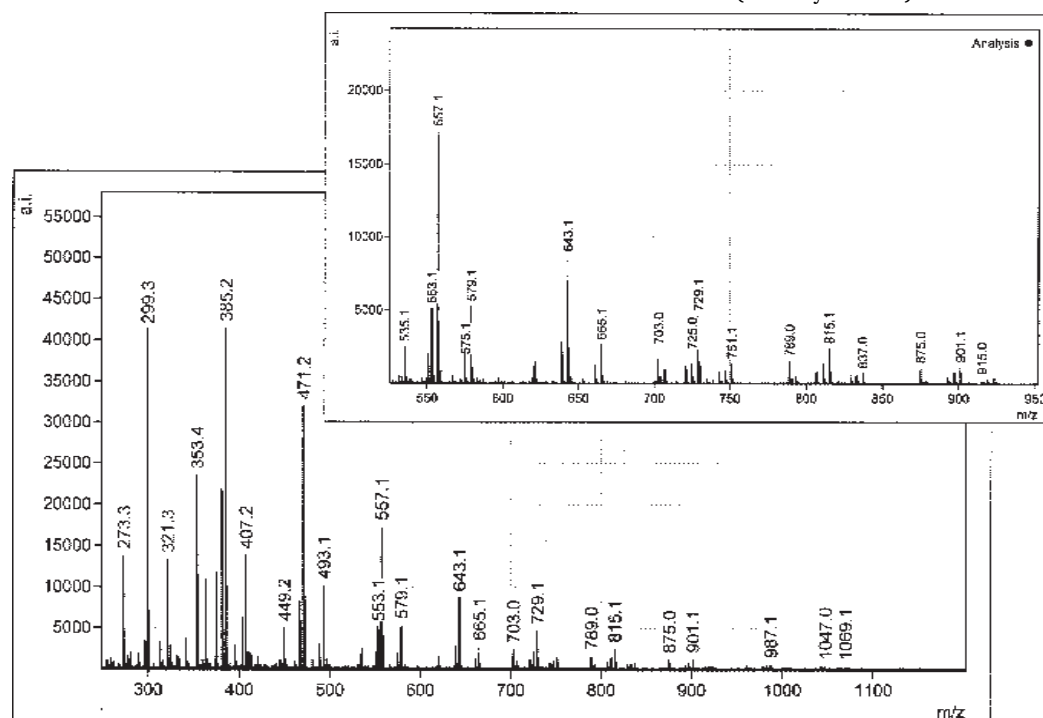

Fig. 3. MALDI-TOF MS spectrum of copolymerization reaction product of 3HBA and lactobionic acid (at 5:1 molar ratio), catalyzed by Novozyme 435 at 80°C and 7 days reaction time, in DMSO:*t*-BuOH medium

Table 1
MOLECULAR MASSES OF TYPICAL COPOLYMERS IDENTIFIED IN
THE MALDI-TOF SPECTRUM

Proposed structures	Molecular mass of Na adduct	
	Calculated	Experimental
3HBA-LBA-3HBA	553.50	553.1
LBA-3HBA-LBA-3HBA	893.99	893.0
Cyclic 3HBA-LBA-3HBA-LBA	875.79	875.0
3HBA-LBA-(3HBA)$_2$-3HBA	725.71	725.0
Cyclic 3HBA-LBA-(3HBA)$_2$-3HBA	707.71	707.0
3HBA-(3HBA)$_5$-3HBA	643.73	643.1
3HBA-(3HBA)$_6$-3HBA	729.83	729.1
3HBA-(3HBA)$_7$-3HBA	815.94	815.1
3HBA-(3HBA)$_8$-3HBA	902.05	901.1

and the third was *Rhizomucor miehei* lipase (Lipozyme-RM IM). This selection was motivated by the specificity of *Candida antarctica* B lipase for 3HBA [16] and the known ability of *Rhizomucor miehei* lipase to catalyze esterification reactions. The reactions have been carried out in the conditions presented in the Experimental Part, using DMSO:*t*-BuOH (20:80/v:v) as reaction medium, under argon atmosphere.

The relative compositions did not show significant differences between copolymer and homopolymer content
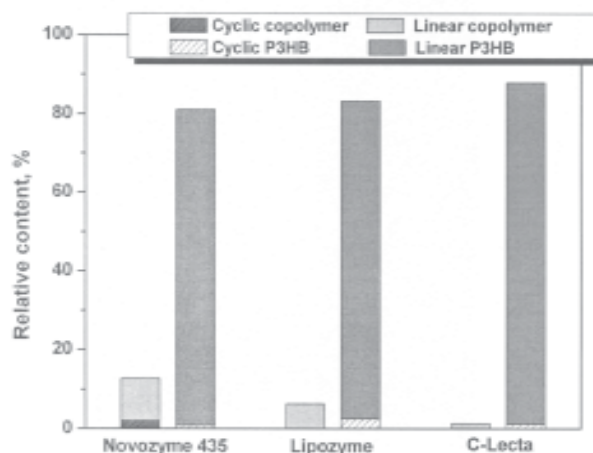

Fig. 4. Relative composition reaction product 3-hydroxybutyric acid copolymers with lactobionic acid (5:1 molar ratio), catalyzed by different microbial lipases, at 80°C and 7 days reaction time, in DMSO:*t*-BuOH (20:80, v:v) reaction medium

of the reaction products obtained with different enzymes (fig. 4). The P3HB homopolymer synthesis was facilitated in all cases, compared to the synthesis of copolymers including LBA moieties. However, higher amounts of linear and cyclic copolymers were obtained when Novozyme 435 was the biocatalyst.

The better catalytic performance of this enzyme is more accurately demonstrated by the highest copolymer polymerization degree obtained in the reactions carried out in DMSO:*t*-BuOH (20:80, v:v) reaction medium (table 2). Although the average molecular weights were not significantly different, only using Novozyme 435 as catalyst the DP of copolymer with LBA reached 7 in the linear

Enzyme	Mn[a]	Mw[b]	PDI[c]	Highest DP of copolymer
Novozyme 435	407.1	453.5	1.11	7
Lipozyme	398.1	434.9	1.09	3
C-Lecta	405.6	441.7	1.08	2

Table 2
AVERAGE MOLECULAR WEIGHT CHARACTERISTICS OF THE REACTION PRODUCTS SYNTHESIZED FROM 3HBA AND LBA, CATALYZED BY DIFFERENT LIPASES

[a]Number-average molecular weight

[b]Weight-average molecular weight

[c]Polydispersity index

Reaction medium	3HBA conversion %	Mn[a]	Mw[b]	PDI[c]
Solventless	50.7	309.4	419.0	1,35
Toluene	57.5	408.9	450.5	1,10
t-BuOH/DMSO 80/20 (v/v)	> 99	407.1	453.5	1,11
t-BuOH/DMSO 50/50 (v/v)	>99	402.3	448.8	1.11

Table 3
INFLUENCE OF REACTION MEDIUM ON 3HBA TOTAL CONVERSION AND MOLECULAR WEIGHT OF THE PRODUCT, IN THE COPOLYMERIZATION REACTION WITH LA CATALYZED BY NOVOZYME 435 LIPASE

[a]Number-average molecular weight

[b]Weight-average molecular weight

[c]Polydispersity index

copolymer with 897.1 molecular mass of the Na adduct. Copolymers with more than one inserted sugar unit were also formed when this enzyme has been used. In contrast, Lipozyme IM and C-Lecta lipases yielded only copolymers with DP not higher than 3 and 2, respectively. In fact, with C-Lecta lipase only the ester of LBA with 3-HBA was synthesized.

Influence of the reaction medium

The low solubility of lactobionic acid encumbered the lipase-catalyzed synthesis of polyesters, requiring the investigation of various solvent systems, able to dissolve totally or partially the sugars, and nontoxic for the enzymes. Our selection of reaction medium was based on literature data for similar reaction systems. Polymerization of lactones was reported in toluene [17,18], while mixtures of t-BuOH and DMSO, with a DMSO content up to 20% (v/v), were used in enzymatic synthesis of sugar esters [15]. Alongside toluene and mixtures of DMSO and t-BuOH, we also studied the copolymerization reaction in solventless system. The average molecular weight characteristics of the reaction product and the 3HBA total conversion in four different reaction media are presented in table 3.Although the 3HBA conversion was higher in the *t*-BuOH/DMSO mixture which can dissolve higher concentrations of sugars without important decrease of enzyme activity, the polydispersity index was not influenced. The highest polymerization degree did not exceed 7 for the copolymer and 11 for the homopolymer.

3HBA conversion has limited relevance for the copolymerization reaction, while the MALDI-TOF MS spectrum indicated that an important part of 3HBA was converted to homopolymers. The analysis of product composition might give more accurate information on the reaction course and influence of the reaction medium. Such an estimation was possible considering the MALDI-TOF MS data, as described in the Experimental part. Accordingly, we considered four types of products: cyclic and linear copolymers, and homopolymers (fig. 5).

As expected, the copolymerization was facilitated in DMSO:*t*-BuOH reaction media, due to the higher solubility of LBA. The slight difference between the copolymer yields at different DMSO:*t*-BuOH ratios was determined by the higher enzyme inactivation effect at increased DMSO content in this solvent mixture. The high content in homopolymer (at least, 80%) in the reaction product is probably explained by the large 3HBA excess used and the
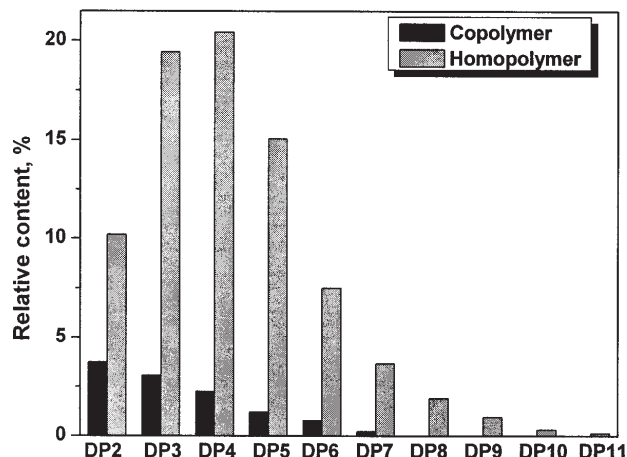
Fig. 6. Relative composition of the reaction mixture after incubation of 3HBA and GL with Novozyme 435 lipase for 7 days, at 80°C, in bulk (a), and in *t*-BuOH/DMSO (80/20, v/v) reaction medium, calculated from MALDI-TOF MS spectral data at different polymerization degrees (DP)

faster reaction rate of P3HB synthesis, regardless to the reaction medium.

The relative product composition at different DP is also important with regard to the mechanism of the process. Such a dependence is presented in figure 6 for the copolymers and homopolymers obtained in DMSO:*t*-BuOH (80:20, v/v) reaction medium. The relative composition of the obtained product at different polymerization degrees were calculated based on the MALDI-TOF MS spectrum from figure 3. The highest amounts of oligomers with lactobionic acid units inserted were found at DP2 and DP3, and with increasing DP the elongation was achieved by addition of a 3HBA repetitive unit to the previous oligomer. Considering the low polymerization degree, it is obvious that most of the formed copolymers contain no more than one inserted sugar unit. In case of the homopolymer, the maximal relative content was found at a higher polymerization degree value.

Conclusions

For the first time, lipase-catalyzed synthesis of 3-hydroxybutiric oligomers with inserted lactobionic acid moieties has been accomplished in organic and solvent-free reaction systems. Lipase from *Candida antarctica* B had proved highest polyesterification activity, and the copolymer formation was facilitated in *t*-BuOH/DMSO reaction medium. The insertion of the hydrophilic lactobionic acid residues could improve the physical and functional properties of P3HB polymers and represent the starting point for new biomaterials.

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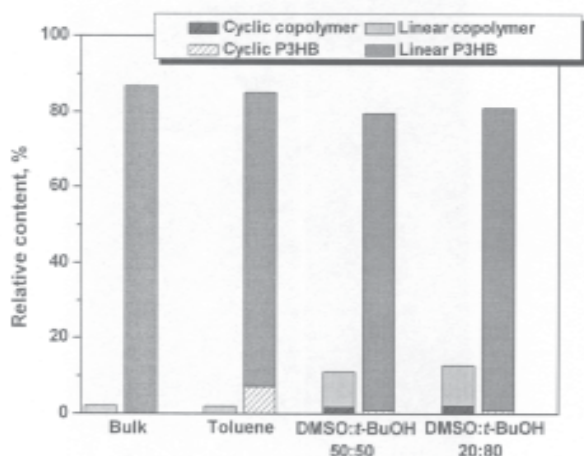5. KOBAYASHI, S., UYAMA, H., KIMURA, S., Chem. Rev., **101**, 2001, p.3793

Fig. 5. Relative composition of the reaction product of 3HBA copolymerization with LBA (5:1 molar ratio), catalyzed by lipase from *Candida antarctica* B (Novozyme 435), at 80°C and 7 days reaction time, in bulk and various organic solvent reaction media

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