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Biocatalytic synthesis of new copolymers from 3-hydroxybutyric acid and a carbohydrate lactone

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ABSTRACT

Lipase-catalyzed reaction of 3-hydroxybutyric acid with D-glucono-δ-lactone at 5:1 molar ratio and 80 °C yielded a mixture of moderate molecular weight linear and cyclic oligomers. The most efficient biocatalyst, *Candida antarctica* B lipase (Novozyme 435), allowed the synthesis of new oligomeric compounds with ring-opened gluconolactone units included in the oligomeric chain, without previous derivatization of the sugar, or activation of the acid monomer. The reaction medium nature had an important influence on the product composition. Although the main copolymer amount was synthesized in tert-butanol/dimethylsulfoxide medium, the highest polymerization degrees, up to 9 for the copolymer, and 10 for the 3-hydroxybutyric acid homopolymer co-product, were achieved in solventless conditions.

1. Introduction

Like many other important compounds, biodegradable polymers based on renewable resources are products of industrial (white) biotechnology [1]. Three routes are achievable to obtain polymeric substances using biotechnological procedures: (i) isolation of natural biopolymers produced in plants; (ii) production by microorganisms; (iii) synthesis from bio-derived monomers by *in vitro* enzymatic catalysis. While the first two are based on biosynthetic pathways and basically limited to synthesis of naturally occurring polymers by *in vivo* enzymatic catalysis, the third offer several possibilities to design new synthetic strategies for existing polymers, or even to obtain new polymeric compounds that are difficult to be synthesized by conventional chemical catalysis. Enzymatic polymerizations also display the well-known advantages of biocatalytic processes, like mild reaction conditions and avoiding toxic chemicals as catalysts [2]. Even though the most important biomacromolecules are nucleic acids, proteins, and polysaccharides, polyesters have also gained constantly increasing interest. Polyesters, both aliphatic and aromatic, are already used for manufacturing of several industrial and consumer products, but likewise they are considered emerging biomaterials for medical applications, or carriers for controlled drug release [3].

The enzymes already investigated for enzymatic synthesis of polyesters were mainly hydrolases, with lipases demonstrating special ability to catalyze such reactions. Essentially, they are two applicable polymerization routes: ring-opening polymerization (ROP) of lactones and polyester condensation polymerizations, both having been intensively investigated during the past decade, as reviewed by Kobayashi [4], Albertsson and Srivastava [5], and Gross et al. [6] Recently, another hydrolase, cutinase from *Humicola insolens*, was successfully used for cell-free polyester synthesis [7]. In a special case, polymerization of (*R*)-β-hydroxyalkanoate monomers, polyhydroxyalkanoate (PHA) synthases of bacterial origin have also been demonstrated their capability to work *in vitro* [8].

Synthesis of polymeric compounds that contain carbohydrates had also captured considerable interest, by reason of their new functional capabilities able to induce specific properties and applications. Sugar-containing poly(acrylate)-based biocompatible hydrogels suitable for biomedical and membrane applications

Abbreviations: ROP, ring-opening polymerization; PHA, polyhydroxyalkanoate; 3-HBA, 3-hydroxybutyric acid; P3HB, poly(3-hydroxybutyrate); GL, D-glucono-δ-lactone; RO-GL, ring-opened gluconolactone; [Bmim][PF_6], 1-butyl-3-methylimidazolium hexafluorophosphate; *t*-BuOH, *tert*-butanol; DMSO, dimethylsulfoxide; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

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have been obtained by a chemoenzymatic method, but only the 6-acryloyl sugar monomer was synthesized using a lipase-catalyzed biocatalytic procedure, the polymerization being performed in traditional manner [9]. Vinyl sugar esters suitable for polymerization reactions were also obtained by regioselective transesterification in the 6 position of several hexoses with vinyl adipate in dimethylformamide, catalyzed by an alkaline protease [10].

Enzyme-catalyzed direct inclusion of a sugar-derived moiety in the polymer backbone is not an easy task, taking into account the difficulty to find and enzyme having specificity for both monomers, or the very low solubility of non-derivatized sugars in the solvents normally employed as media for biocatalytic polycondensation or ROP reactions. Good results were reported with protected sugars, like for lipase-catalyzed acroylation of a sugar derivative with protected anomeric position, followed by ROP of ε-caprolactone initiated by the acryl-sugar intermediate, in toluene [11]. ROP of ε-caprolactone was reported to be induced by the hydroxyl group in the 6-position of methyl-glucopyranoside, at 60 °C, without solvent, with Novozyme 435 lipase as the catalyst [12]. Sugar-containing polymers were also synthesized by enzymatic polycondensation reactions, but activation of the employed ester co-monomers was necessary to shift the reaction equilibrium toward the polymer product. Polycondensation of sucrose with *bis*(2,2,2-trifluoroethyl)-adipate was catalyzed by an alkaline protease in anhydrous pyridine, yielding a polyester that contained ester linkages at 6 and 1′ positions of the sucrose [13].

(*R*)-3-Hydroxybutyric acid (3HBA), derived from poly[(*R*)-3-hydroxybutyrate] (P3HB), could be a valuable monomer for the synthesis of new biodegradable oligomers and polymers. Although microorganisms synthesize polymers and copolymers of 3HBA, the monomer could be also obtained *via* an enzymatic process. Production of chiral 3-hydroxycarboxylic acids directly in the culture broth by *in vivo* depolymerization of the corresponding polyhydroxyalkanoates was already demonstrated [14]. P3HB can be degraded by various intracellular and extracellular depolymerases [15]. Using a thermophilic *Streptomyces* sp. MG with strong hydrolytic activity for depolymerization of PHB, the process was considered economically feasible, even in terms of 3HBA recovery [16]. As a monomer, 3HBA could be a precursor for the synthesis of pure biodegradable P3HB with desired molecular weight, or for the synthesis of different copolyesters.

Lipase-catalyzed ROP of lactones, mainly ε-caprolactone, and polycondensation of hydroxy acids, like lactic or ricinoleic acid, was subject of numerous investigations in the past decade [4]. By contrast, literature data about reactions involving 3HBA and β-butyrolactone are scarcer. Lipase-catalyzed ROP of (*R*)-β-butyrolactone was used to obtain optically active linear and cyclic P3HBs, with molecular weights centered around 2100 Da for the cyclic and 3200 Da for the linear forms, when 5% porcine pancreatic lipase was employed as catalyst at 100 °C, for 24 h in a solventless system [17]. Porcine pancreatic lipase was used for polymerization of 3HBA in organic solvents, yielding oligomers [18]. Ionic liquids could be an alternative for organic solvents as reaction media for the synthesis of polyhydroxyalkanoates by ROP or polycondensation reactions. *Candida antarctica* B lipase catalyzed the ROP of several lactones in 1-butyl-3-methylimidazolium *bis*(trifluoromethane) sulfonimide ([Bmim]Tf_2N) at 60 °C [19].

Carbohydrates are attractive as highly functionalized renewable monomers for the synthesis of various polymers by lipase-catalyzed condensation polymerization reactions, but they require utilization of protected alditols or activated aldaric esters or chlorides. Therefore, ROP polymerization of carbohydrate 1,5-lactones emerged as an easier approach to obtain aliphatic polyesters, but their polymerization was reported until now only by chemical catalysis. *Tetra*-O-acetyl-D-gluconolactone, reacted with 1,4-butanediol in the presence of a metal alcoxide initiator, yielded a low molecular weight oligoester [20]. A structurally different D-gluconolactone derivative, subjected to ROP in the presence of stannous butyrate initiator, yielded polyesters with number-average molecular weights ranging from 1800 to 7300 Da [21]. After our best knowledge, enzyme-catalyzed ROP of carbohydrate lactones or incorporation of non-derivatized carbohydrate units in polyhydroxybutyrate structures was not yet reported by other groups. Therefore, the target of this work was to include gluconolactone-derived units in the enzymatically synthesized oligomers or polymers of 3HBA.

2. Experimental

2.1. Materials

(*R,S*)-3-Hydroxybutyric acid (3HBA), D-glucono-δ-lactone (GL), *tert*-butanol (*t*-BuOH, ∼99% pure), 1-butanol (>99% pure), butyric acid (>99% pure), isooctane (>99% pure), toluene (>99%), dimethylsulfoxide (DMSO, ∼99.7% pure), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]), were purchased from Merck. Optically active (R)-3-hydroxybutyric acid (R3HBA, >98%) was a product of Aldrich. Immobilized *Candida antarctica* lipase B on acrylic resin (Novozyme 435) and *Rhizomucor miehei* lipase (Lipozyme-RM IM) were from Novozymes, *Burkholderia cepacia* (Amano PS) and *Pseudomonas fluorescens* (Amano AK) lipases from Aldrich, lyophilized *Candida antarctica* lipase B (CALB-Lecta) from C-Lecta (Leipzig, Germany), *Aspergillus niger* and porcine pancreatic lipase (PPL) from Sigma. Molecular sieves (4 Å) were provided by Acros Organics.

2.2. Methods

2.2.1. Lipase-mediated esterification of 3HBA

Different lipases have been tested to select the suitable enzyme for the forthcoming polymerization reactions. The reactions were performed in a 5 mL glass vials containing 3HBA/1-butanol (1:2 molar ratio) in 2 mL isooctane, and 25 mg of enzyme. The mixture was stirred using an orbital shaker (MIR-S100, Sanyo, Japan) at 300 strokes/min and 40 °C (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland). After 24 h reaction time, samples were drawn out for gas chromatographic analysis.

2.2.2. Polymerization in organic solvents

3HBA (0.465 mL, 5 mmole) and *Candida antarctica* lipase B (50 mg, Novozyme 435) were added to GL (0.178 g, 1 mmole) dissolved in 2 mL organic medium. The reactions were performed in 5 mL Micro Reactions Vessels, under argon atmosphere, with activated 4 Å molecular sieves, magnetically stirred at 300 rpm and 80 °C. The reactions were stopped by filtration of enzyme. The polymers were isolated by precipitation into methanol (10:1, v/v), and centrifuged at 4 °C and a relative centrifugal force of 5000 × *g* for 60 min. The supernatant was removed and the resulting precipitate was washed twice with methanol. The precipitated polymer was dried in vacuum at 60 °C, resulting in a white solid.

2.2.3. Polymerization in ionic liquid

The same protocol as for reactions in organic solvents was applied, excepting the use of 1 mL IL instead of organic solvent. The products were isolated from the IL by extraction with toluene, evaporation of toluene, and vacuum-drying at 60 °C.

2.2.4. Polymerization in bulk

50 mg Novozyme 435 and 178 mg GL were added to 0.465 mL 3-HBA placed in a 4 mL vial. The reaction was carried out in the same

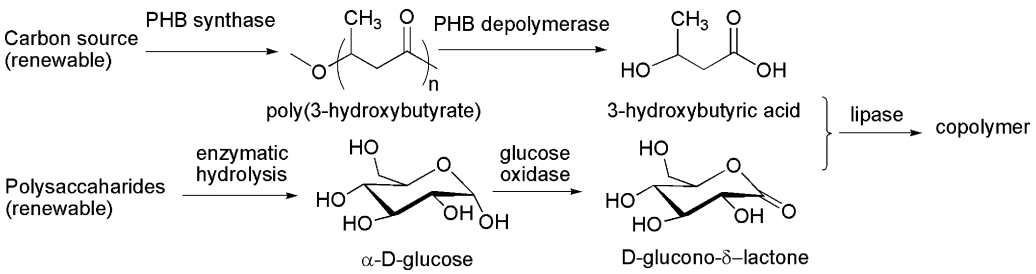

Fig. 1. Biocatalytic route for the synthesis of sugar-containing copolymers of 3-HBA based on renewable resources.

conditions as in organic solvents. After extraction with chloroform, the reaction product was worked up by removal of the enzyme by filtration, evaporation of the extraction solvent, and vacuum-drying at 60 °C.

2.3. Product characterization

2.3.1. Gas chromatography

Gas chromatography (GC) analysis of 3HBA esterification products was performed by a Dani 86.10 (Dani S.p.A., Italy) chromatograph equipped with flame ionization detector, and a 15 m × 0.32 mm BPX-5 capillary column (SGE, Australia). The analysis conditions were set as follows: oven temperature: 70–250 °C with 10 °C/min heating rate, injector temperature 300 °C, detector temperature 350 °C, carrier gas (helium) flow 1.45 mL/min. The conversions were calculated based on the internal standard method (n-dodecane was used as standard) using a calibration curve for 1-butyl-3-hydroxybutyrate, obtained and purified in our laboratory. The total conversion of 3HBA was also assayed by GC. A filtered sample (20 μL) from the reaction mixture was derivatized by addition of 20 μL N,O-*bis*(trimethylsilyl)-trifluoracetamide, incubated at 80 °C for 1 h, and analyzed in the same conditions as for the butyl esters.

2.3.2. FT-IR spectroscopy

Fourier Transform Infrared Spectroscopy (FT-IR) spectra were obtained from a JASCO FT/IR 430 spectrometer (JASCO Corp., Japan), 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.

2.3.3. MALDI-TOF MS analysis

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of products was carried out using Bruker BIFLEX III matrix assisted laser desorption ionization time-of-light mass spectrometry (Bruker Daltonik GmbH, Germany) at an acceleration voltage of 20 kV using 2,5-dihydroxybenzoic acid as matrix. Samples (10 mg/mL), the matrix (20 mg/mL) and the ionization agent NaTFA (5 mg/mL) were individually dissolved in organic solvent. 10 μL of sample solution, 5 μL of ionization agent solution were mixed with 50 μL of matrix solution and an aliquot (0.3 μL) was applied to the sample plate.

Within one MALDI-TOF spectrum, the intensities of all signals (originated from sodium adduct ions) were added and the total set as 100%. Separately, a sum of signal intensities was calculated for every type of oligomer present in the analyzed product. We calculated the relative composition of the reaction product, assuming that the ionization efficiency was the same for every oligomeric type present in the mixture. Such estimations have been already used to calculate the relative distribution of fructose oligosaccharide-lauryl ester oligomers [22].

3. Results and discussion

3HBA obtained from microbial polyhydroxyalkanoates can be a valuable starting material for biodegradable copolymers with sugar derivatives, obtained entirely from easily available renewable resources by biocatalytic pathways. In the present study, we used GL as non-derivatized co-monomer, but other derivatives of sugars, like lactobionic acid, could be also appropriate (Fig. 1).

3.1. Screening of lipases as 3-hydroxybutyric acid esterification catalysts

The catalytic efficiency of different microbial lipases, as well as porcine pancreatic lipase, was tested in the esterification reaction of 3HBA with 1-butanol, in organic solvent medium (Table 1). Control reactions were run with n-butyric acid, in the same conditions, only for the most efficient enzymes. The presence of a 3-hydroxy substituent in the carboxylic acid molecule was obviously detrimental to the enzyme esterification activity. The ester yields obtained at 24 h reaction time were low for most of the tested enzymes. The best performing lipase, Novozyme 435, also showed significantly lower esterification activity for 3HBA than for the unsubstituted butyric acid. Gorke et al. [19] reported an 11% faster reaction rate for butyric acid than for 3HBA in the esterification reaction with both 1- and 2-butanol, in ionic liquid medium, catalyzed by Novozyme 435 lipase. They suggested that 3HBA fits poorly in the acid binding site of lipase, compared to butyric acid. The slower reaction rate observed for 3HBA could be explained by the more complicate reaction mechanism than in case of a linear carboxylic acid, as well. In a previous study of 3HBA esterification with different alcohols [23], we demonstrated using GC–MS data that polymerization of the 3HBA monomer and esterification of the formed acid oligomers occurred competitively with the normal esterification reaction. Even if the esters of acid dimer and trimer subsequently undergone transesterification reactions with the alcohol, yielding finally the same 3HBA ester product, the over-

Table 1
Screening of lipases for catalytic efficiency in esterification reactions of racemic and optically active 3-HBA. The reactions have been carried out at 40 °C, with 3-HBA/1-butanol at 1:2 molar ratio, in isooctane.

Lipase	Acid	Reaction time (h)	Ester yield (%)
Candida antarctica B (Novozyme 435)	3HBA	24	52
Candida antarctica B (Novozyme 435)	R3HBA	24	27
Candida antarctica B (C-Lecta)	3HBA	24	42
Mucor miehei (Lipozyme-RM IM)	3HBA	24	2
Burkholderia cepacia (Amano PS)	3HBA	24	<1
Pseudomonas fluorescens (Amano AK)	3HBA	24	<1
Candida cylindracea	3HBA	24	9
Porcine pancreatic lipase	3HBA	24	<1
Aspergillus niger	3HBA	24	<1
Candida antarctica B (Novozyme 435)	Butyric acid	3	98
Mucor miehei (Lipozyme-RM IM)	Butyric acid	3	35

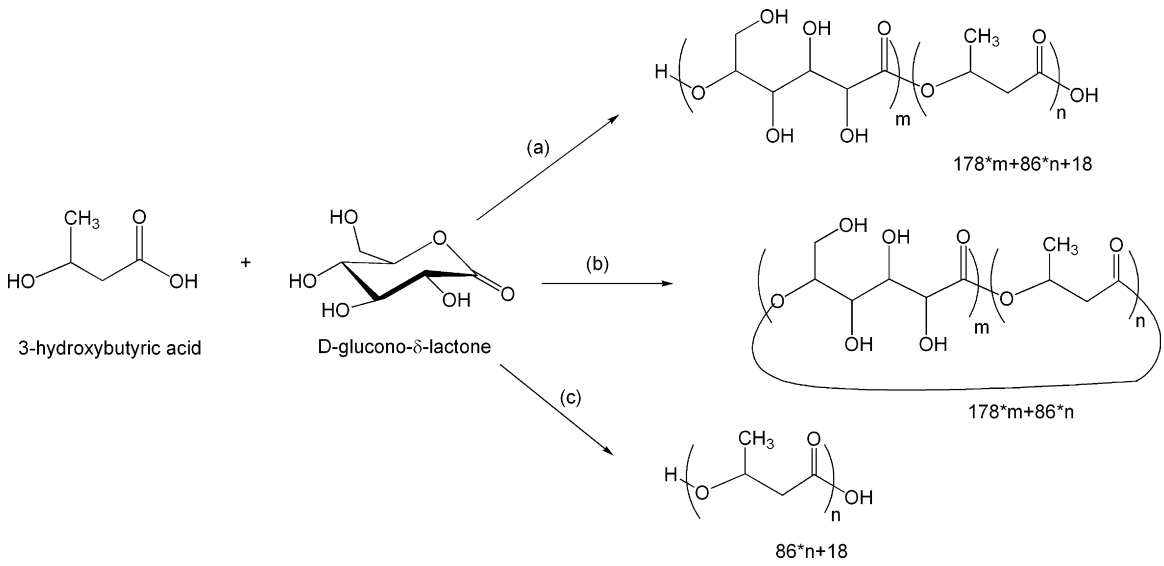

Fig. 2. Reaction scheme of the enzymatic polymerization reaction. The equations indicate the masses of the corresponding oligomers, *m* and *n* meaning the numbers of GL and HBA repeat units, respectively. 178 and 86 are the masses of the GL and HBA units, respectively, and 18 is the mass of the end-group H_2O.

all process was slower. 3-Hydroxybutyric acid is a chiral compound, and the lipase enantioselectivity could also influence the ester yields. However, carrying out the same reaction with the best performing enzyme and optically active (*R*)-3-hydroxybutyric acid as substrate, the obtained yield was lower than in the case of racemic acid (Table 1), demonstrating that not enantioselectivity was the limiting factor of this reaction. Among the tested lipases, Novozyme 435 has proven the highest esterification activity and was further employed as polymerization catalyst.

3.2. Lipase-catalyzed copolymerization of 3HBA and GL

Insertion of sugar-derived polyhydroxylic units in the P3HB backbone is difficult by traditional chemical methods, but looks more reliable using a biocatalytic route. We investigated the lipase-catalyzed synthesis of such oligomeric compounds, to exploit both the regioselectivity of lipases in the esterification of sugars and their ability to catalyze ring-opening polymerization of lactones.

An easily available carbohydrate, 1,5-lactone, D-glucono-δ-lactone (GL), was selected as sugar derivative. The insertion of GL in the polyester chain of P3HB can be accomplished in two possible ways, yielding linear (a), or cyclic (b) copolymers, together with possible homopolymers (c) of 3HBA (Fig. 2).

In the typical experimental protocol, GL was dissolved or suspended in the reaction medium, 3HBA, enzyme, and molecular sieves added, and the reaction mixture stirred for several days in argon atmosphere. The product was isolated after removing the enzyme and molecular sieves by filtration.

Infrared spectroscopy was used to monitor the functional group changes during the polymerization reaction. The FT-IR spectra of the raw materials and a reaction product are shown in Fig. 3. The absorption bands in the FT-IR spectrum of the product confirm the

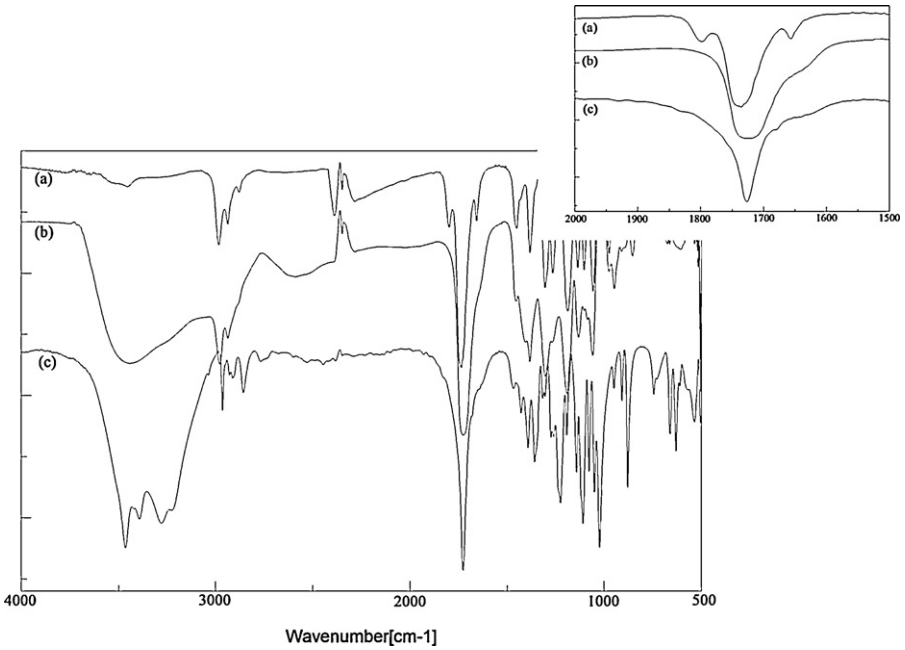

Fig. 3. FT-IR spectra of (a) 3HBA-GL reaction product, obtained at 7 days reaction time in [Bmim]PF_6 reaction medium, (b) pure 3HBA and (c) pure GL. Inset the expanded view of 1600–2200 cm^{-1} region, with the stretching vibration band of C=O group.

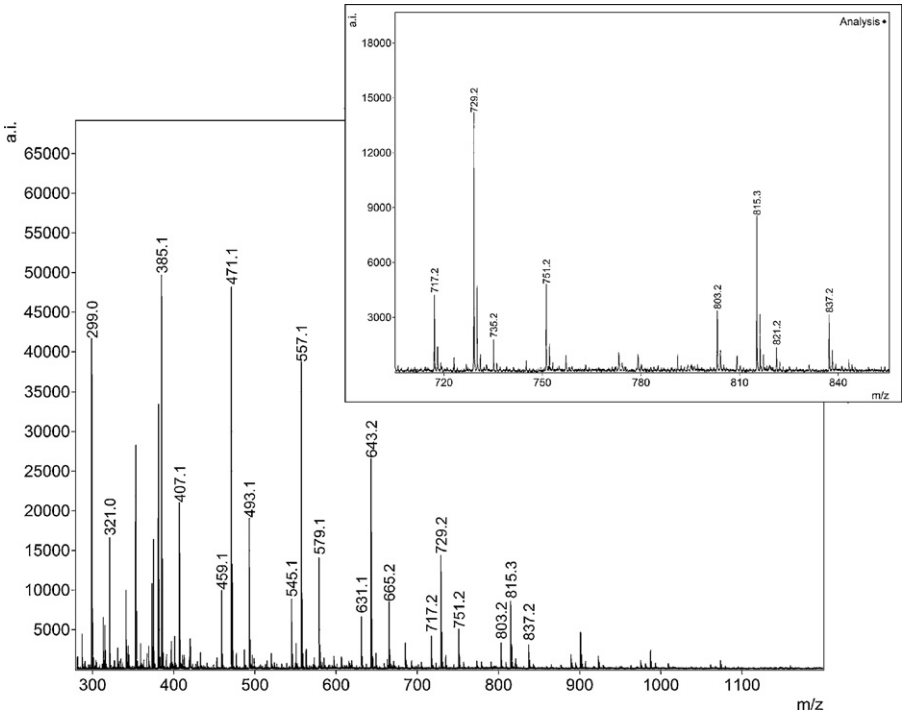

Fig. 4. MALDI-TOF MS spectrum of copolymerization reaction product of 3HBA and GL (at 10:1 molar ratio), catalyzed by Novozyme 435 at 80 °C in bulk, at 7 days reaction time. Inset the expanded view of the 710–850 *m/z* region.

ester formation, by shifting the band assigned to the carbonyl group stretching vibration from 1724 cm^{-1} in 3HBA and GL, to 1734 cm^{-1} in the product. In the special case of reaction accomplished in IL medium, the FT-IR spectrum also indicates that only the 3HBA homopolymer was obtained, by the important decrease of the hydroxyl group absorption band intensity in the 3300–3400 cm^{-1} region.

The products were identified by MALDI-TOF mass spectrometry. Fig. 4 shows a typical MALDI-TOF mass spectrum of the reaction product obtained by copolymerization of 3HBA and GL in bulk, using Novozyme 453 at 80 °C. The spectrum contains a characteristic series of peaks, which can be assigned to the sodium adduct ions of different oligomer series ($[M+Na]^+$).

According to Fig. 4, linear (as shown in Fig. 2a) and cyclic (Fig. 2b) oligomers containing inserted GL unit, as well as P3HB homopolymers (Fig. 2c) were found in the MALDI-TOF MS spectrum. For example, the peak at *m/z* 735.2 corresponds to the sodium adduct ion of a linear oligomer with $n = 6$ and $m = 1$ (i.e., one inserted GL unit). In addition, P3HB homopolymer series were also found, e.g. the peak at *m/z* 729.2 is due to the presence of oligomer with $n = 8$ and $m = 0$. Furthermore, the presence of cyclic oligomers with one inserted GL unit can also be recognized, e.g. the Na adduct of the cyclic oligomer with $n = 6$ and $m = 1$ was visible at *m/z* 717.2. Interestingly, $[M-H+2Na]^+$ adducts of linear homopolymers are also present in the MALDI-TOF MS spectra at a 22 *m/z* distance from the P3HB peaks.

Based on these results, it can be demonstrated that copolymerization of 3HBA with a nonderivatized sugar lactone is possible by lipase catalyzed reaction, yielding oligomers with inserted 2,3,4,5,6-pentahydroxy-caproic acid units, derived from the GL.

3.3. Influence of reaction medium

The reaction medium has an essential role in biocatalytic processes. A major drawback of lipase-catalyzed biotransformation of polyhydroxylic compounds is their low solubility in nonpolar organic solvents that are particularly suitable for lipase-catalyzed synthetic reactions. Therefore, the copolymerization of 3HBA and GL should be strongly influenced by the solubility of raw materials (mainly GL) and synthesized oligomers (Table 2).

Based on the literature data reported for comparable reaction systems, we investigated several media for the copolymerization reactions: toluene, mixtures of *t*-BuOH and DMSO, the IL $[Bmim]PF_6$, as well as the solventless system. It is likely to presume that even a smaller amount of dissolved sugar might be enough to ensure the progress of reaction, if the formed oligomeric or polymeric product is soluble. 3HBA has much better solubility in organic solvents, therefore only the sugar derivative solubility looks restrictive for the reaction.

All experiments were carried out in the same conditions: 80 °C, argon atmosphere, magnetic stirring, molecular sieves for removal of water by-product, and 7 days reaction time. The number-average and weight-average molecular weights were calculated for the entire reaction product, based on MALDI-TOF MS analysis, as indicated in Section 2. The conversion of 3HBA was calculated based on unreacted acid, determined by GC analysis. The copolymerization reaction was not significantly influenced by the reaction medium in regard of average polymerization degree (Table 1). Although the 3HBA total conversions (in both copolymer and homopoly-

Table 2
Influence of reaction medium on 3HBA total conversion and average molecular weight of the product, in the copolymerization reaction with GL catalyzed by Novozyme 435 lipase.

Reaction medium	3HBA total conversion (%)	Mn[a]	Mw[b]	PDI[c]
Solventless	74.6	451.3	504.7	1.12
Toluene	83.3	394.5	434.9	1.10
t-BuOH/DMSO, 80/20 (v/v)	>99	384.3	422.7	1.10
$[Bmim]PF_6$	>99	469.1	565.8	1.20

[a] Number average molecular weight.
[b] Weight average molecular weight.
[c] Polydispersity index.

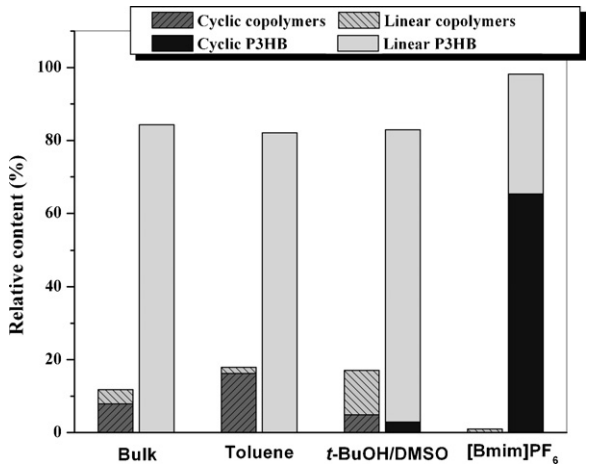

Fig. 5. Relative composition 3-hydroxybutyric acid copolymers with δ-gluconolactone (10:1 molar ratio), catalyzed by lipase from *Candida antarctica* B (Novozyme 435), 7 days, at 80 °C, in bulk and in various reaction media.

mer) were high, the highest polymerization degree did not exceed 10, even for the homopolymer. These results agree with findings of other groups for reactions of 3HBA and β-butyrolactone. In an earlier report concerning enzymatic synthesis of P3HB from 3HBA, Shuai et al. [18] reported number-average molecular weights of 660 and 720 in hexane and diethyl ether, respectively, but these solvents are not appropriate for copolymerization with GL. Studying enzymatic polymerization of several lactones in IL media, Gorke et al. [19] obtained the lowest DP (up to 5) in case of β-butyrolactone. The explanation of the relatively low DP could be the not accurate structural conformity between the 3HBA molecule and the active center of lipase, or the reduced solubility of the higher DP oligomer in the reaction medium.

Hindrance of the enzyme activity by insufficiency of water could be another possible cause of limitation of molecular weight increase of the product. A more accurate water control throughout the reaction will be realized in the forthcoming studies, in order to increase the polymerization degree.

An emerging question is the influence of reaction medium on the obtained copolymer composition. Such information will help us to fine tune this composition in the desired direction: a linear copolymer of 3HBA and RO-GL, P3HB end-functionalized with a sugar unit, cyclic copolymer, etc. The estimation of the relative composition of the product was possible based on MALDI-TOF MS data, as described in Section 2. Between the two possible structures having the same molecular mass (P3HB end-functionalized with a GL unit and cyclic copolymer with inserted RO-GL) we assumed the formation of cyclic oligomers, as cyclization results in lower polymerization degree, unless the cyclization product itself is a substrate for the lipase. As only MALDI-TOF data cannot prove this hypothesis, NMR analysis will be performed in our forthcoming studies to elucidate the exact structure of all obtained oligomers.

As shown in Fig. 5, the reaction medium had important influence on product composition. In all reaction media employed, an important part (at least 80%) of the initial 3HBA was recovered in the products as P3HB, caused probably by the higher reaction rate of homopolymer formation and the important 3HBA excess used.

In a solventless system, in which excess 3HBA has the role of reaction medium, the obtained copolymer was formed mainly from cyclic oligomers, linear oligomers with inserted RO-GL representing about one third of the product amount. Formation of cyclic oligomers as major product was also reported by Matsumura et al. [17] for lipase-catalyzed ROP of (*R,S*)-β-butyrolactone, in bulk.

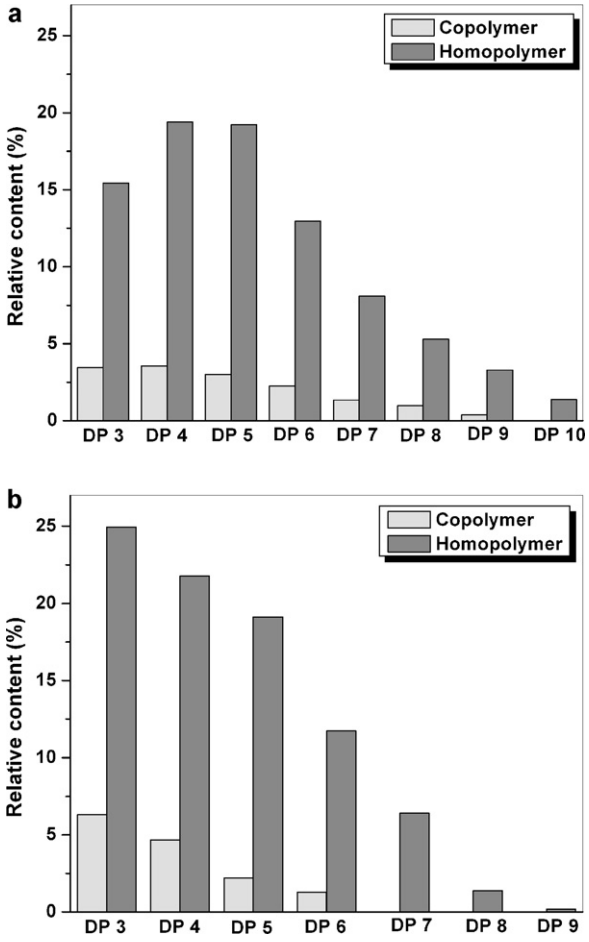

Fig. 6. Relative composition of the reaction mixture after incubation of 3HBA and GL with Novozyme 435 lipase for 7 days, at 80 °C, (a) in bulk, and (b) in *t*-BuOH/DMSO (80/20, v/v) reaction medium, calculated from MALDI-TOF MS spectral data at different polymerization degrees (DP).

Toluene could be an appropriate organic medium for lipase-catalyzed polymerization reactions, as previously demonstrated for copolymerization of BL with 12-hydroxydodecanoic acid [24]. Utilization of this solvent in the studied process resulted in a very high relative amount of cyclic product.

Sugars and sugar derivatives are soluble only in highly polar solvents as DMSO, pyridine, or dimethylformide [25], but these solvents can lead to an important decrease of lipase activity [26]. To avoid such inactivation, mixtures of DMSO with *t*-BuOH were used in the enzymatic synthesis of sugar esters [22]. In the present study, an organic medium composed of *t*-BuOH and DMSO in 80/20 (v/v) ratio was the most appropriate reaction medium, yielding more than 70% relative amount of ring-opened form in the obtained copolymer.

Another option to increase homogeneity of the reaction system could be the utilization of ILs with an imidazolium cation, as they have polarities on the Reichard's scale in the range of the most polar organic solvents [27]. Among several ILs investigated, we detected polymerization products only in [Bmim]PF_6 reaction medium, but almost exclusively homopolymers of 3-HBA, and mainly in the cyclic form. The explanation is most likely related to the solubility of GL. Gorke et al. [19] reported that water-immiscible ILs were superior to water-miscible ILs, as reaction media in the ROP of lactones. Although [Bmim]PF_6 is water-immiscible, its solvation capacity for GL was probably low and the polymerization reaction was effective only for 3HBA. An investigation of a much larger spectrum of ILs will be necessary to exploit the advantages of such reaction media.

Based on the appropriate MALDI-TOF MS spectral data, the relative composition of the obtained product at different polymerization degrees was also calculated, as showed in Fig. 6. In bulk (Fig. 6a), they were no significant differences between the relative ratio of copolymer and homopolymer at different DP. Oligomers with DP in the range 4–5 have been detected at highest content in the product, in accordance with the calculated average molecular weight. In *t*-BuOH/DMSO (Fig. 6b), the majority of oligomers with RO-GL were found at DP3, and the overall polymerization process showed a slower increase of the obtained molecular mass than in the bulk system. In both cases, the increase of the copolymer DP was achieved mostly by addition of a 3HBA repetitive unit to the previous oligomer, as we identified only small amounts of oligomers with more than one RO-GL included.

4. Conclusions

Insertion of a sugar-derived monomeric unit in the P3HB chain induces new functionalities and could be a route to bio-based polymers with possible industrial or medical applications.

For the first time, copolymerization of 3HBA with GL was achieved in a lipase-catalyzed reaction, yielding oligomers containing inserted 2,3,4,5,6-pentahydroxy-caproate units. Novozyme 435 lipase demonstrated the highest esterification activity for the 3HBA substrate, while the highest selectivity toward formation of copolymers with inserted RO-GL was obtained in a *t*-BuOH/DMSO (4/1, v/v) reaction medium. Extension of this novel polyesterification reaction to copolymers with inserted lactobionic acid units is currently in study.

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