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Conference paper

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Optimization of enzymatic ring-opening copolymerizations involving δ-gluconolactone as monomer by experimental design

Abstract: Enzymatic incorporation of carbohydrate-derived monomer units into hydrophobic polyester backbones represents a promising alternative to obtain new biodegradable oligomers and polymers. Immobilized lipases are efficient biocatalysts for copolymerization of β-butyrolactone and δ-gluconolactone, but only a systematic optimization study was able to highlight the influence of the main reaction parameters on the polymerization degree and on the relative copolymer content of the product. Therefore, experimental design was employed for determination of the optimal ring-opening copolymerization conditions in solventless reaction systems, at temperatures up to 80 °C. The obtained products, cyclic and linear polyesters, have been characterized by FT-IR, MALDI-TOF MS, NMR, and TG analysis, demonstrating the incorporation of gluconolactone unit(s) into the hydrophobic backbone of the polyester and the formation of new bio-based products.

Keywords: biocatalysis; experimental design; lipase; modeling; POC-2014; polymerization; δ-gluconolactone.

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Introduction

The enzymatic ring opening polymerization was intensively studied in the recent years as a part of the “green” way to obtain new materials, particularly for biochemical and pharmaceutical applications [1]. Alongside poly-lactic acid, polyhydroxyalkanoates could emerge as the most important bioplastics in the near future, considering their availability in high amounts at relatively low price. Polyhydroxybutyrates (PHB) are natural biopolymers synthesized by bacteria [2, 3] as energy and carbon source, and plants [4] but due to their high crystallinity PHB are rigid and brittle, consequently not suitable for certain applications [5]. Another possible way to obtain PHB, particularly with lower molecular mass, is ring-opening polymerization of β-butyrolactone (BL) [6]. Insertion of a different monomeric unit in the polyhydroxyalkanoate chain could overcome the men-

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tioned drawbacks and induce new functionalities, to yield bio-based polymers with possible industrial and medical applications [7, 8]. Such polymerization reactions can be accomplished by lipase biocatalysis [9]. Lipases have been already proved to catalyze copolymers synthesis by using a variety of substrates such as sugars derivatives, poly(ethylene glycol), alkyl diacids, diols [10, 11], as well as ring opening polymerization of various lactones [12, 13], to obtain polyesters. Insertion of δ-gluconolactone (GL), a sugar derivative, into the PHB backbone formed by polymerization of 3-hydroxybutyric acid [14] as well as of GL units into the polycaprolactone (PCL) backbone [15] using lipase biocatalysis was successfully accomplished, opening the way for extending such studies to other monolactones.

The experimental design methods have been applied in many topical areas including nanoparticles optimization synthesis [16, 17] degradation [18], fermentation [19] and synthesis [20, 21]. The statistical analysis and response surface methodology (RSM) offer the maximum amount of information from the experimental data and is able to establish the influence of selected process parameters (independent variables) on the response variable, which is usually the product yield.

Nevertheless, optimization studies of enzymatic synthesis of polymers using RSM are scarce [22]. In the case of copolymerization catalyzed by lipase, the formation of several linear and cyclic products makes such an optimization more difficult, given that the synthesis of homopolymers cannot be avoided. Therefore, the current work is focused on the systematic study of the synthesis of copolymers of BL and GL via enzymatic ring opening polymerization (ROP), including optimization of the main parameters (i.e., molar ratio of monomers, reaction temperature and enzyme amount), as well as on the characterization of the synthesized copolyesters. Since the copolymers, particularly the linear copolyesters, were the main products (although the PHB by-product is also valuable, as mentioned), the target was to maximize their relative yield, together with an increase of the average molecular weight of the copolymer.

Materials and methods

Materials

β-Butyrolactone (BL), D-glucono-δ-lactone (GL), immobilized *Candida antarctica* lipase B (Novozyme 435) and *Thermomyces lanuginosus* lipase (Lipozyme-TL IM) were from Novozyme. *tert*-Butanol (*t*-BuOH), dimethyl sulfoxide (DMSO), and toluene were purchased from Merck. Polyhydroxybutyrate (PHB) was bought from Sigma-Aldrich, as reference for the thermal analysis. Other reagents were of analytical grade, used without purification.

Enzyme-catalyzed polymerization

Polymerization in organic solvents

BL (5 mmoles) and different amount of lipases were added to GL (1 mmole) dissolved in 1 mL organic medium. The reactions were performed in 4 mL Micro Reaction Vessels, under argon atmosphere, magnetically stirred at 300 rpm at 80 °C. The reactions were stopped by filtration of enzymes.

Polymerization in solventless conditions

To 0.178 g GL different amount of BL (to a final molar ratio from 3:1 up to 5:1), and different amounts of lipase, immobilized by different methods, were added. The reactions were performed into brown 4 mL capped vials at temperatures in the range of 60–80 °C, in argon atmosphere. At the end of reaction 1 mL of methanol:acetone (1:1, v/v) was added to the reaction mixture to decrease the viscosity and allow the separation of the immobilized enzyme by filtration. The polymer was isolated by evaporating the solvent from this filtrate and drying overnight in vacuum, at 60 °C.

Pre-solubilization of gluconolactone

Pre-solubilization of the GL in the BL co-monomer was carried out to increase the copolymer content and to diminish the homopolymer formation in the solventless experiments. BL: GL mixtures, at molar ratios from 1:1 up to 5:1, have been prepared and incubated at 80 °C and 350 rpm. After 24 h a clear solution was formed, provided that the molar ratios were higher than 3:1. In all experimental design experiments this pre-solubilization step has been accomplished and the reactions were started by adding the appropriate amount of lipase.

Experimental design and data evaluation

To establish the effects of the three independent variables, namely the temperature, the enzyme/substrate ratio and the molar ratio of monomers, on the copolymer content, copolymer polymerization degree and the weight average molecular mass (M_w), a 3-factorial-3 level experimental design was carried out using the Unscrambler 9.2 software package (CAMO Software AS) by using the Box-Behnken method [23], choosing two replicates for each sample and three center samples. For each variable 3 different levels (the lowest, the central and highest of their fixed domain) were given. The Unscrambler first response indicated a 27 experiments set (Table 1). The reactions were performed by copolymerization of BL and GL in the presence of a microbial lipase, as described in the experimental section. At the end of reaction the products were analysed by the MALDI-TOF MS technique,

Table 1 Parameter obtained from experimental design and the determined values from MALDI-TOF spectra, using BL and GL as raw materials.

Entry	Temperature	Enzyme amount	Molar ratio	M_w_C experimental	M_w_C predicted
1	60	20	4	663	672
2	60	20	4	672	672
3	80	20	4	650	638
4	80	20	4	641	638
5	60	60	4	716	720
6	60	60	4	711	720
7	80	60	4	658	665
8	80	60	4	683	665
9	60	40	3	771	761
10	60	40	3	769	761
11	80	40	3	716	702
12	80	40	3	684	702
13	60	40	5	736	732
14	60	40	5	734	732
15	80	40	5	693	703
16	80	40	5	696	703
17	70	20	3	726	734
18	70	20	3	734	734
19	70	60	3	796	799
20	70	60	3	799	799
21	70	20	5	750	748
22	70	20	5	752	748
23	70	60	5	765	757
24	70	60	5	759	757
25	70	40	4	770	769
26	70	40	4	770	769
27	70	40	4	768	769

allowing the calculation of the copolymer content, copolymer polymerization degree, and M_w, as described elsewhere [14]. The M_w calculated for the copolymer was used as response variable for the ANOVA analysis, to elucidate the quadratic effects of the three independent variables on the copolymer content and to establish the optimum of the reaction in terms of M_w, based on the response surface graph.

Characterization of the products

Fourier Transform Infrared (FT-IR) spectra of the samples were obtained in attenuated total reflectance (ATR) mode on a Bruker Vertex 70 (BrukerDaltonik GmbH, Germany) spectrometer equipped with a Platinum ATR, Bruker Diamond Type A225/Q. Spectra were collected in the range 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and with 64 co-added scans.

MALDI-TOF MS analysis of products was carried out using Bruker BIFLEX III matrix assisted laser desorption ionization time-of-light mass spectrometry (BrukerDaltonik GmbH, Germany) at an acceleration voltage of 20 kV using 2,5-dihydroxybenzoic acid as matrix. Weight average (M_w) and number average (M_n) molecular weights were calculated, and the product compositions were estimated as described elsewhere [14].

NMR spectra were recorded on a BrukerAvance III spectrometer operating at 500.0 MHz (1H) and 125.0 MHz (13C). The samples were dissolved in DMSO-d6 and the chemical shifts δ are given in ppm from TMS.

Non-isothermal crystallization behavior of PHB, PCL and their copolymers with δ-gluconolactone have been performed using a DSC 204 F1 Phoenix differential scanning calorimeter (Netzsch, Germany). The specimens were heated from 20 to 600 °C under nitrogen atmosphere at a heating rate of 10 °C/min, and cooling rate of 10 °C/min

Thermogravimetric measurements (TGA/DTA) were done using a TG 209 F1 Libra thermogravimetric analyzer (Netzsch, Germany), under nitrogen atmosphere, at a heating rate of 10 °C/min, in the range of room temperature to 600 °C.

Results and discussions

The insertion of GL into the hydrophobic chain of PHB has been carried out, starting from BL, a four membered ring lactone. This study represents the continuation of a previous one, using a seven membered ring lactone (*ε*-caprolactone, ECL) [15]. The optimization of the reaction system was performed by investigation of the effect of three important variables (temperature, enzyme amount and molar ratio of monomers) on the copolymer formation.

Based on the MALDI TOF analysis of the reaction product, the general reaction scheme presented in Fig. 1 was proposed. The polymeric reaction products of BL with GL are linear and cyclic copolymers, together with linear and cyclic homopolymers of the non-sugar co-monomer, also in accordance with the results obtained for ECL with GL [15].

Selection of the reaction medium

The effect of the solvent for the BL-GL system was investigated, to append the study previously carried out for the ECL-GL system [15]. Organic solvents with different polarities, as well as the solventless system were evaluated, using an immobilized lipase (Novozyme 435) as catalyst. The results (Fig. 2) indicate that a more polar reaction medium, as in case of DMSO:*t*-BuOH mixture, resulted in higher relative content of linear copolymer, probably due to the increased solubility of GL co-substrate. At the same time, the presence of DMSO, a solvent with known inhibitory effect on lipases [24], led to the lowest M_n and M_w values among the reaction media tested. Figure 2 also revealed that using DMSO:*t*-BuOH (1:4, v/v), the relative content of cyclic copolymer among the total copolymer (58 %) was higher compared to the ECL-GL reaction products, in the

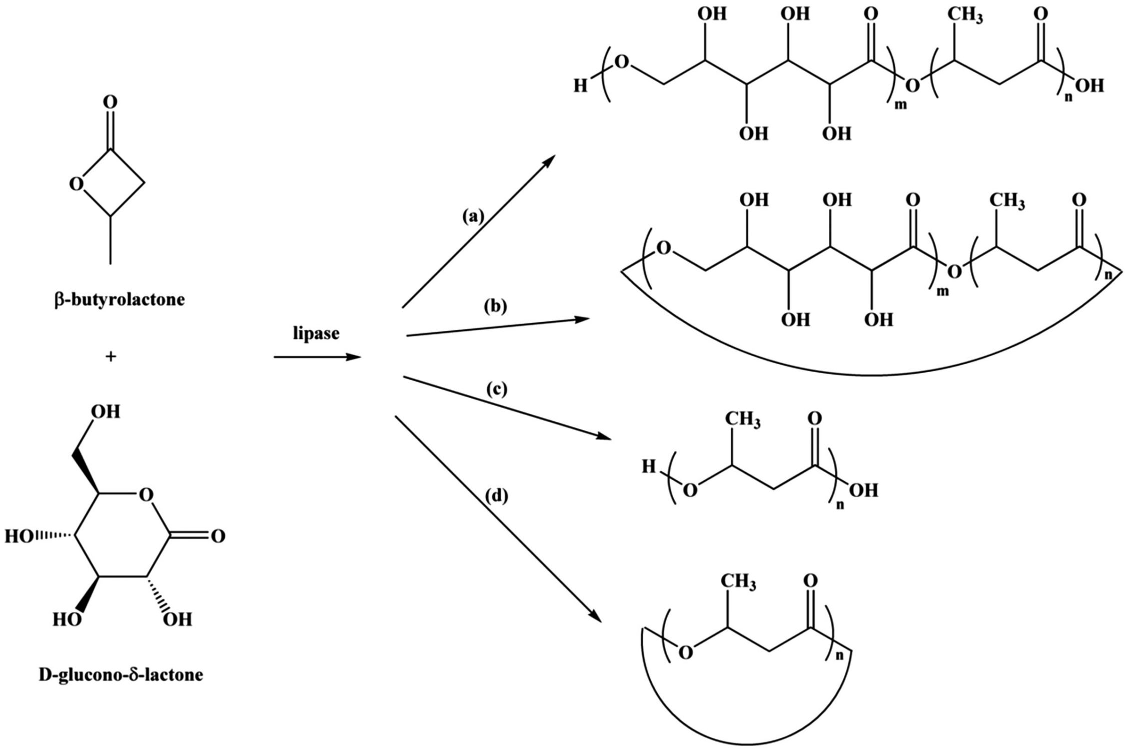

Fig. 1 Reaction products of BL-GL copolymerization, catalyzed by lipase: linear copolymers (a); cyclic copolymers (b) linear homopolymers (c); cyclic homopolymers (d).

same conditions (28 %) [15]. This could also explain the lower values of the M_n and M_w. The other important conclusion was that the reaction can be appropriately carried out in solventless systems. The main reason of organic solvent utilization was to improve homogeneity, given that the reaction mixture contained a non-polar lactone and a polar lactone. A systematic study of pre-solubilization of GL in the BL co-monomer dem-

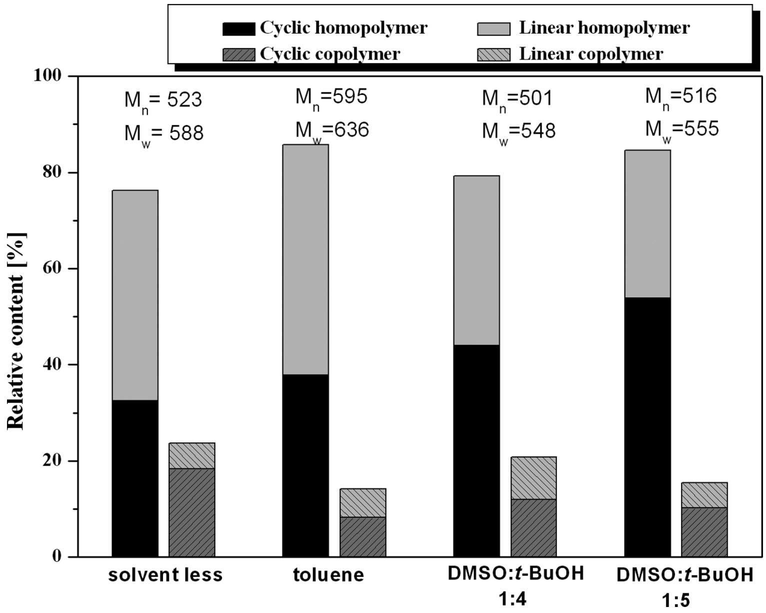

Fig. 2 Influence of the reaction medium on the composition and molecular weight of the product, in the copolymerization reaction of BL with GL catalyzed by lipase.

onstrated that at BL:GL molar ratios higher then 3:1 a homogeneous mixture was achieved at 80 °C, after 24 h stirring. Consequently, the solventless reaction system was considered optimal, yielding the highest amount of copolymer and allowing the easiest isolation of the product, since the removal of DMSO requires high temperature and high vacuum. Therefore, the experimental design was also performed in solventless conditions.

Experimental design

The Box–Behnken designs (BBDs) were intensively studied in past years for optimizing the synthesis of different products, principally because they provide suitable mathematical models (i.e., the quadratic model) by using a small number of experimental runs [25].

In this study BBD was employed for BL-GL copolymer systems. The same study was also performed for the ECL-GL system, showing the same tendencies and giving near results. Therefore, we consider this model appropriate for enzymatic synthesis of aliphatic polyesters by copolymerization of lactones with different ring sizes with GL, but only the results for BL-GL will be discussed in this work. Immobilized lipase from *T. lanuginosus* (Lipozyme-TL IM) was used as biocatalyst, based on the previous lipase screening results [15]. Although lipases from *T. lanuginosus* and *C. antarctica* are quite similar regarding the overall yield of polymer formation, the first was preferred for the experimental design, based on the higher amount of copolymer formed.

The completed matrix, including the experimental weight average molecular weight values calculated for the total copolymer (M_w_C), based on the MALDI-TOF spectra, is presented in Table 1. The first three columns contain the values of the selected independent variables, while the last two columns contain the M_w_C values determined experimentally and predicted by the model.

The obtained experimental data were subjected to statistical analysis by the ANOVA technique and regression analysis. Among the pre-defined models available in the Unscrambler package, the highest R^2 value (96.9 %) was obtained when the quadratic model has been chosen. The results of ANOVA analysis allow the assessment of the models adequacy (Table 2). The pure error in the M_w_C calculated from the repetitions, the results of *t*-tests and *p*-values, as well as the confidence limits for each effect are included among these results.

The p-values for square and interaction terms included in the reduced model were found to be less than 0.05. The p-value for the lack of fit was found to be 0.0511, which indicated that lack of fit was not significant and showed the goodness of fit.

The pure error, i.e. the mean square of deviations of the M_w_C determined for the copolymer, measured at the central points from their mean value, was 70.4 from 3 repetitions, corresponding to a $\sqrt{70.4} = 8.39$ % standard deviation measured at the central point of variables, a satisfactory value.

The appropriateness of prediction is demonstrated in Fig. 3a, where the M_w_C calculated are plotted vs. the measured data. Data points marked by circles indicate the results of 27 experiments carried out according to the experimental design.

The normal probability plot of residuals (Fig. 3b) show that the data are normally distributed, indicating acceptable goodness of fit.

Analysis of effects

Based on the calculation of the relative content of every copolymer type present in the reaction product (from the MALDI-TOF spectra), the significance level of each independent variable (temperature, enzyme amount and molar ratio) was analyzed.

According to the results presented in Table 3, the temperature has the most significant effect and the formation of the copolymer is directly correlated with the variation of the temperature. The overall significance levels of the other two independent variables (enzyme amount and molar ratio) were insignificant. However, certain combinations of these variables (e.g., temperature by molar ratio) could have important effect on the

Table 2 Statistical results generated by ANOVA, setting the weight average molecular weight calculated for the copolymers (M_w_C) as response variable.

	SS	DF	MS	F-ratio	p-Value
Summary					
Model	5.226E+04	9	5.852E+03	58.992	0.0000
Error	1.686E+03	17	99.194		
Adjusted total	5.435E+04	26	2.090E+03		
Variable					
Intercept	1.274E+05	1	1.274E+05	1.284E+03	0.0000
Temperature (A)	7.700E+03	1	7.700E+03	77.627	0.0000
E/S (B)	5.588E+03	1	5.588E+03	56.330	0.0000
Molar ratio (C)	756.250	1	756.250	7.624	0.0134
AB	220.500	1	220.500	2.223	0.1543
AC	435.125	1	435.125	4.387	0.0515
BC	1.596E+03	1	1.596E+03	16.091	0.0009
AA	2.398E+03	1	2.398E+04	241.763	0.0000
BB	5.054E+03	1	5.054E+03	50.952	0.0000
CC	2.422E+03	1	2.422E+03	24.413	0.0001
Model check					
Main	1.404E+04	3	4.681E+03		
Int	2.252E+03	3	750.585	7.557	0.0020
Int+Squ	3.637E+04	3	1.212E+04	122.216	0.0000
Squ	0.000	0	0.000	0.000	0.0000
Error	0.000	0	0.000		
Lack of fit					
Lack of fit	700.625	3	233.542	3.317	0.0511
Pure error	985.667	14	70.405		
Total error	1.686E+03	17	99.194		

SS, sum of error squares; DF, degree of freedom; MS mean value of error squares; F-result of the statistical F test on the studied variable; p-observed significance level.

formation of the polymers. Specifically, the amount of homopolymer formation is reversely correlated with the effect of temperature by molar ratio of the reactants; meanwhile the formation of the copolymer shows a direct dependency with the combined effect of the variables mentioned above.

Response surface analysis and contour plots analysis

In order to check the real importance of the enzyme ratio on M_w_C, the obtained regression surface was plotted as function of M_w_C. These types of plots offer supplementary information. Although the effect of enzyme amount was not significant for the overall process, when M_w_C was plotted as function of both temperature and enzyme amount, an optimum value was obtained (Fig. 4).

The response surface analysis indicate that working at 4:1 molar ratio BL:GL, the highest M_w_C value of 774 will be achieved at the central point corresponding to 68.3 °C and 46.7 mg enzyme amount. This limitation of molecular weight increase is probably due to the high content of cyclic copolymer, which cannot be lowered in the given conditions.

Characterization of the reaction products

Various physico-chemical characterization methods were performed to demonstrate the formation and chemical structure of the polymeric products.

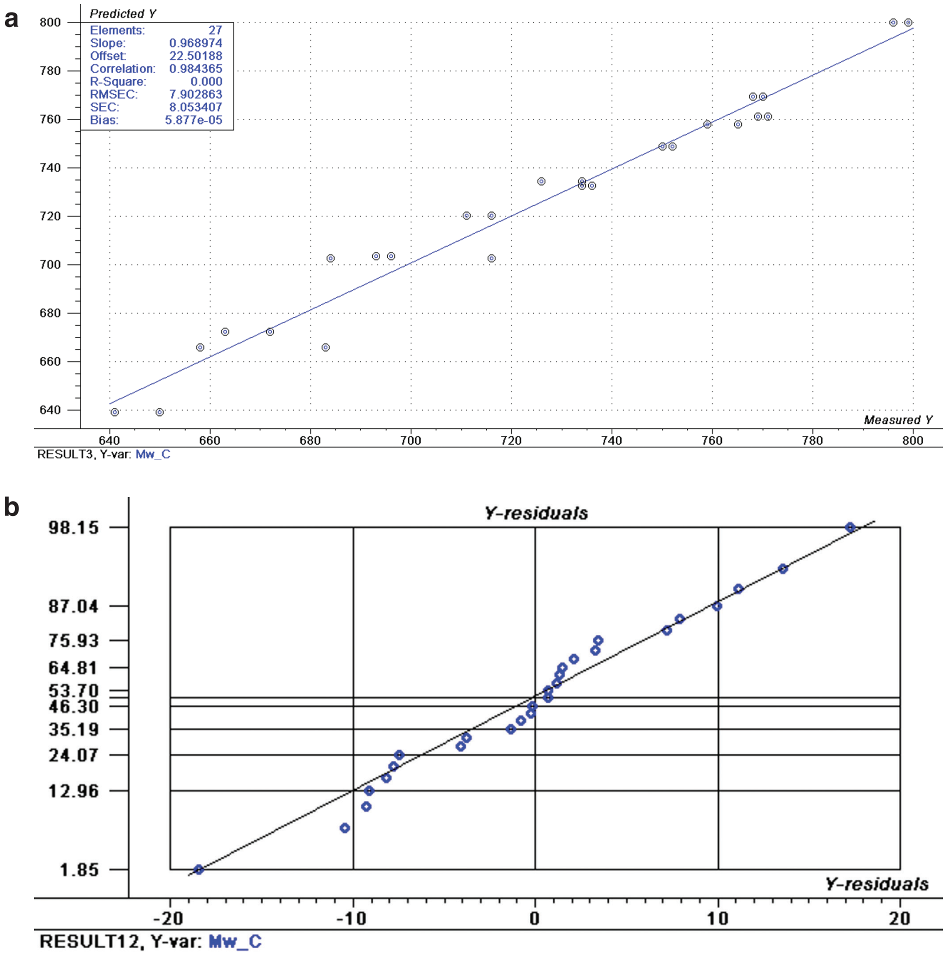

Fig. 3 Comparison of the measured and predicted weight average molecular weight of the copolymer (a), and probability plot of residuals (b).

The functional group changes during the copolymerization reaction were monitored by infrared spectroscopy. The FT-IR spectra of the BL-GL copolymer product, together with the starting monomers, are depicted in Fig. 5. The ester formation is confirmed by the absorption band assigned to the carbonyl group stretching vibration, which was shifted to 1716 cm^{-1}, compared to 1813 cm^{-1} in BL and 1724 cm^{-1} in GL, respectively.

^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-COSY, HSQC, HMBC spectra of reaction products were measured (see the Supplementary material). Due to the complexity of the reaction mixture and the large number of signals, only the most relevant conclusions of the 2D spectra are discussed, which confirm the insertion of the GL into

Table 3 Analysis of effects on the composition of reaction products.

Variable	LH	CH	LC	CC	HP	CP	M_w_C
Temperature (A)	NS	–	–	++	NS	+	NS
Enzyme ratio (B)	NS	NS	NS	NS	NS	NS	NS
Molar ratio (C)	NS	NS	NS	NS	NS	NS	NS
AB	NS	NS	NS	NS	NS	NS	NS
AC	NS	–	NS	+	–	+	NS
BC	NS	NS	NS	NS	NS	NS	NS

LH, linear homopolymer; CH, cyclic homopolymer; LC, linear copolymer; CC, cyclic copolymer; HP, total homopolymer; CP, total copolymer.

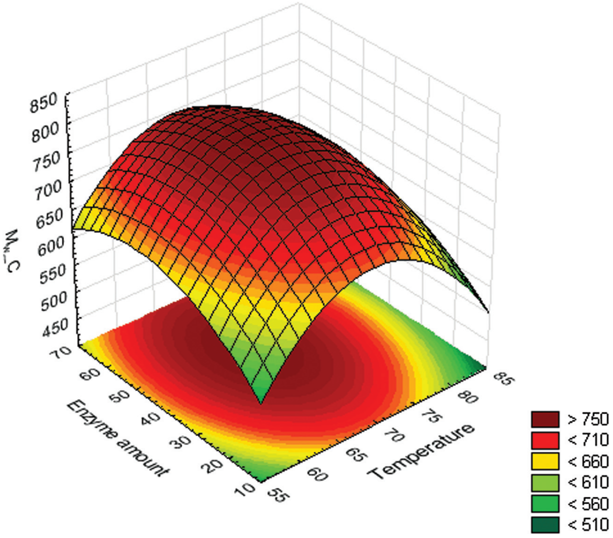

Fig. 4 The combined effect of enzyme amount and temperature on the M_w_C, at 4:1 molar ratio BL:GL.

the PHB backbone, as well as the formation of the ester bond between the primary OH group of the GL the carboxyl group of BL. To ease the discussion, the chemical structure of the linear trimer, one of the possible copolymerization products, is shown in Fig. 6. In the HMBCET spectrum (Fig. 7), coupling at distance of the signal from 5.1 ppm corresponding to the CH (No. 13 in Fig. 7) with 2 types of carbonyl groups, at 168.9 (No. 15) ppm and 171.4 (No. 11) ppm, indicate the insertion of the ring-opened glucono-lactone. The covalent bound between the primary OH group of the GL with BL was confirmed by the HSQC NMR spectra, where the coupling of the proton from 3.58 ppm with the proton from 67.2 ppm, belonging to the carbon from the CH_2 group (C6) (Supplementary material).

The thermal stability of the BL-GL copolymers compared to the commercially available homopolymer PHB and δ-gluconolactone were assessed by thermal gravimetric analysis (TGA), while differential scanning calorimetry (DSC), was used to measure the heat capacity as a function of temperature.

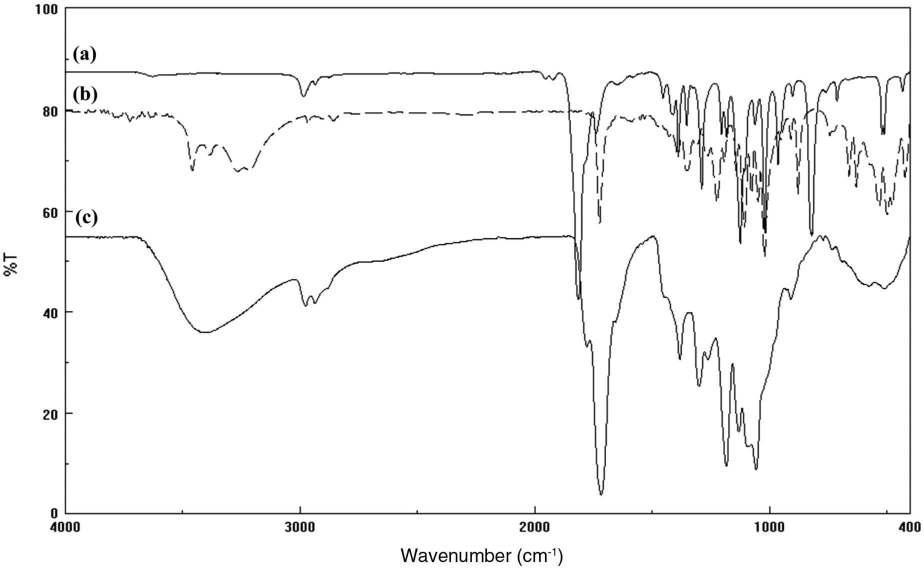

Fig. 5 FT-IR spectra of the monomers and of the reaction product. (a) BL; (b) GL; (c) product obtained at 24 h reaction time, in solventless system.

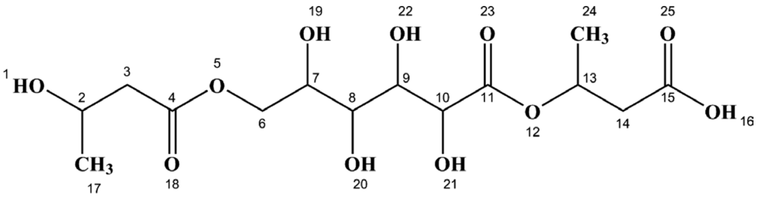

Fig. 6 The proposed chemical structure of the linear trimer product, confirmed by NMR 2D spectrometry.

The TGA curves of the BL-GL copolymers are presented in Fig. 8a (blue line). Compared to the reference homopolymer PHB (red line) a decrease of the thermal stabilities was observed for the copolymer. A loss of mass started at 100 °C, achieving the inflection for the BL-GL at 250.3 °C. The DSC curves (Fig. 8b) indicated for the PHB (red line) two endotherm peaks, at 175.4 °C and 276.5 °C, probably due to some internal transitions that can occur. In the case of the product samples (blue line) the melting temperature (T_m) values were shifted to lower temperatures (about 90 °C). This change of the crystallization behavior of PHB in the copolymers could be attributed to the shorter chain length of the PHB in the copolymers, reducing the number of

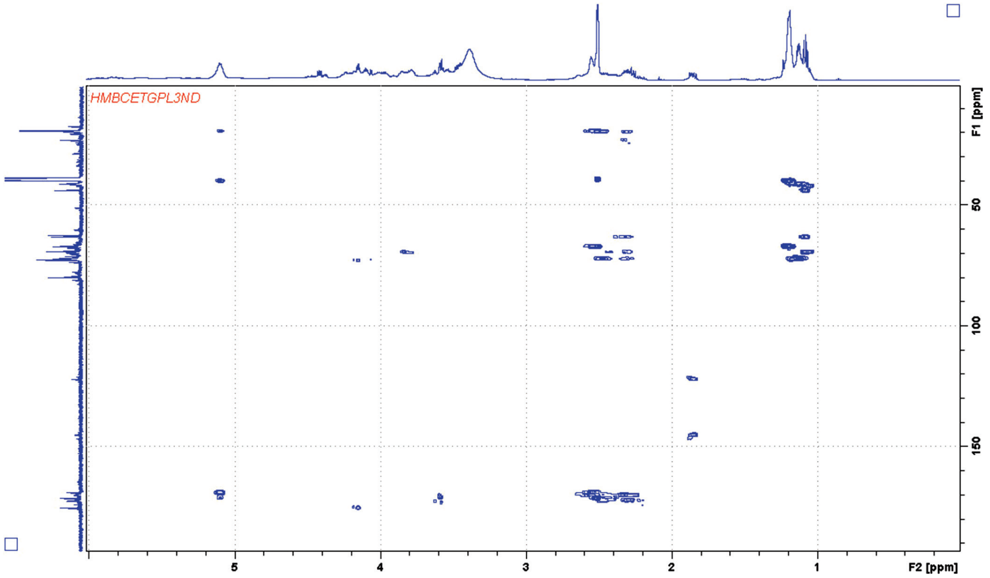

Fig. 7 The HMBCET 2D NMR spectral region (0–6 ppm/0–200 ppm) of the BL-GL reaction product, demonstrating the insertion of ring-opened GL.

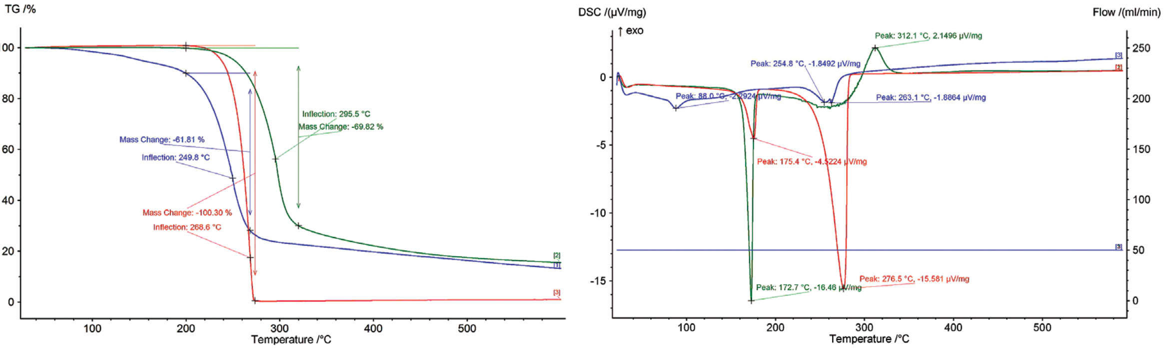

Fig. 8 TG (a) and DSC (b) analysis of the BL-GL copolymerization product (blue), compared with the PHB reference homopolymer (red) and GL (green).

crystallizable segments on the crystal growth front [1]. These results are in accordance with a report on PHB-graft-PEG copolymers, concluding that even though important differences in the TGA curves have not been observed, the thermal properties measured by DSC showed that T_m and glass transition temperature were shifted to lower temperature with the graft percent increasing of PEG [26].

Conclusions

Lipase-catalyzed ring-opening copolymerization of β-butyrolactone and δ-gluconolactone was performed in solventless reaction systems, preceded by solubilization of the carbohydrate lactone in the co-monomer in excess, to ensure homogeneity. To assess the optimal reaction conditions for achieving the highest possible molecular weight of the copolymer, experimental design together with statistical analysis were applied, for the first time in enzymatic copolymerization reactions. It was demonstrated that such a method can be useful to optimize the reaction parameters for the formation of the desired product in a complex mixture of linear and cyclic homopolymers and copolymers. The predictions of the quadratic model were in concordance with the experimental data, demonstrating the effectiveness of this approach. The structure of the copolymer with inserted gluconolactone units in the open form and the ester linkage at the primary OH group was demonstrated by NMR analysis. Although the polymerization degree is limited by the formation of cyclic products and possible stereochemical hindrance in the chain elongation step, the obtained products could be useful in pharmaceutical, food, or medical applications. This method allows to design and obtain oligomeric esters with enhanced hydrophilicity and well-defined structure, for specific applications.

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