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Biocatalytic synthesis of polyesters from sugar-based building blocks using immobilized *Candida antarctica* lipase B

David I. Habeych[a,b,*], P. Benjamin Juhl[c], Jürgen Pleiss[c], Diana Vanegas[d], Gerrit Eggink[a,b], Carmen G. Boeriu[b,e,*]

[a] Food and Bioprocess Engineering Group, Wageningen University and Research Centre, P.O. Box 8129, 6700 AA Wageningen, The Netherlands
[b] Food & Biobased Research, Division Biobased Products, Wageningen University & Research Centre, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[c] Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
[d] Universidad Pontificia Bolivariana, Chemical Engineering Department, Circular 1 70-01, Medellín, Colombia
[e] University "A. Vlaicu", Department of Chemical and Biological Sciences, Arad, Romania

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ABSTRACT

The synthesis of linear ester oligomers (LEOs) and cyclic ester oligomers (CEOs) from non-activated succinic acid (A) in combination with di-anhydro hexitols (B, DAH) in a toluene based medium using immobilized *Candida antarctica* lipase B (CAL B), was studied. The conversion is highest for isomannide and decreases in the order isomannide > isosorbide ≫ isoidide. These experimental results were corroborated by substrate-imprinted docking indicating that the hydroxyl group oriented inwards the "V"-shaped plane of the DAHs (*endo*-hydroxyl) is preferred over the outwards oriented hydroxyl group (*exo*-hydroxyl) by CAL B. The maximum conversions under optimized conditions were 88.2% and 93.7% for succinic acid and isomannide, respectively. MALDI-TOF detected products at 24 h were a mixture of cyclic (35.1%) and linear ester oligomers (64.9%). Cyclic ester oliogomers were the most abundant products during the first 8 h of reaction (32.5–48.7%), where the first cyclic of the series (CEO_1) was the most predominant cyclic product (23–40%).

1. Introduction

The sugar based economy, together with technical developments is leading to the production of novel raw materials that could be applied for innovative oil-independent polymer synthesis. Di-anhydro hexitols (DAH): 1,4:3,6-dianhydro-D-glucitol or isosorbide; 1,4:3,6-dianhydro-D-mannitol or isomannide; and 1,4:3,6-dianhydro-L-iditol or isoidide are good examples of such raw materials, which are produced from the glucose of natural feedstock (i.e. starch). Currently isosorbide is the only bulk-produced and the least high-priced among the DAH. Nevertheless, the growing application of the DAH isomers has triggered efforts of industrial producers to implement the synthesis of all three isomers at reasonable price [1]. The most significant differences among the DAHs isomers are the orientation of the two hydroxyl and proton groups that confer variations in spatial configuration, physical and chemical properties (see Scheme 1). The bond orientation (chirality) in the DAHs is a fundamental parameter for modulation of polyesters properties based on them [1–4].

Polyesters derivative from DAH can be synthesized via polycondensation or ring opening polymerization. The latter has several advantages over the quality of the final product, but the limited availability of raw materials (cyclic ester oligomers—CEOs) has hampered its application for the synthesis of polyesters. Therefore, study of new synthetic routes to CEOs is of great interest. The condensation of aliphatic dicarboxylic acid dichloride with isosorbide using pyridin as catalyst and chloride acceptor by dropwise addition of the substrates (pseudo-high-diluted conditions) has been studied [5]. Interestingly, condensation of adipoyl dichloride led to formation of 100% CEOs with reaction yields above 96%. The authors used MALDI-TOF as a mean to estimate the relative occurrence of the different species [5,6]. The occurrence of cyclic ester oligomers is not only a feature of halo-derivatives. The formation of CEOs has been also found during chemical synthesis of linear poly(isosorbide succinate) [4]. The first known enzymatic-catalyzed esterification that used DAH as substrate was reported in 1993 [7]. Immobilized lipase (Lipozyme IM-20) and oleic acid in a tubular reactor in a solvent-free system was used. After 13 h of the operation of the system 95% of the initial hydroxyl derivatives were consumed. Jacobson and Stockmayer [8] proposed a statistical mechanistic model for the equilibrium among linear and cyclic

* Corresponding authors at: Food & Biobased Research, Division Biobased Products, Wageningen University & Research Centre, P.O. Box 17, 6700 AA Wageningen, The Netherlands. Tel.: +31 317 48 01 68; fax: +31 317 48 30 11.
E-mail addresses: david.habeychnarvaez@wur.nl (D.I. Habeych), Carmen.boeriu@wur.nl (C.G. Boeriu).

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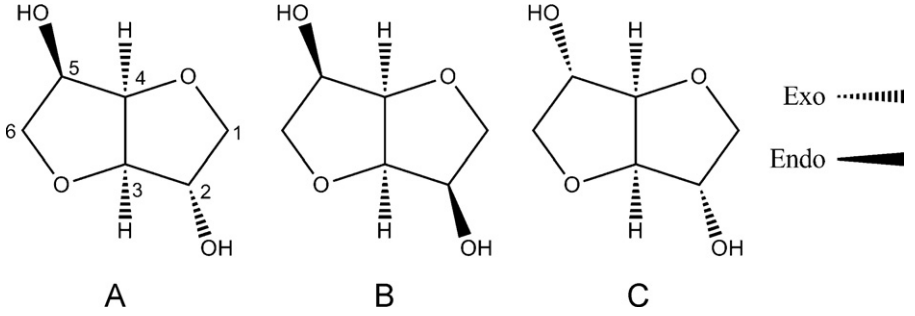


Scheme 1. (A) (3R,3aR,6S,6aR)-hexahydrofuro[3,2-b]furan-3,6-diol; 1,4:3,6-dianhydro-D-sorbitol, or isosorbide; (B) (3R,3aR,6R,6aR)-hexahydrofuro[3,2-b]furan-3,6-diol; 1,4:3,6-dianhydro-D-mannitol, or isomannide; (C) (3S,3aR,6S,6aR)-hexahydrofuro[3,2-b]furan-3,6-diol; 1,4:3,6-dianhydro-L-iditol, or isoidide.

species (LEOs ⇔ CEOs) in a thermodynamically controlled reaction and in the presence of chemical catalyst. In a kinetically controlled reaction and in the absence of side reactions, however, all the products will be cycles when conversions approaches 100% [9]. Results in agreement with this statement (Kricheldorf's theory) have been found recently by other authors [10]. They found that condensation of 4,4-difluorodiphenylsulfone with DAH in DMSO/toluene system at 140 °C and K_2CO_3 as catalyst, led to the formation of CEOs after 24 h and with a conversion above 95%. To the best of our knowledge nothing has been reported over *in vitro* enzymatic synthesis of ester oligomers based on DAH and non-activated di-acids. Therefore, the aim of the present work was to explore the synthesis of CEOs and LEOs from sugar derivatives (DAHs) together with a non-activated di-acid (succinic acid) in the presence of a suitable biocatalyst as basis for synthesis of biodegradable polyesters. We illustrate and discuss the origin of reactivity differences found among the DAH isomers. The reaction conditions were improved by experimental design of the most relevant factors and further water removal from the reaction medium. Furthermore, substrate-imprinted docking was used to model the formation of the esters by the enzyme and to understand the molecular basis of observed CAL B specificity [11].

2. Experimental

2.1. Materials

Lipases and solvents were obtained from Sigma–Aldrich. Novozym® 435 was provided by Novozyme (The Netherlands). Chirazyme® enzymatic kit was purchased from Roche Molecular Biochemicals. The rest of enzymes were purchased from Fluka. All solvents were equilibrated in 4 Å molecular sieves (Sigma–Aldrich) for at least 24 h before use. *trans*-2-[3-(4-*tert*-Butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB) and potassium trifluoroacetate were obtained from Sigma–Aldrich. All solvents were purchased from Merck. Isosorbide, isomannide and isoidide were gently provided by Roquette Frères S. A. (France).

2.2. Enzymatic activity of Candida antarctica lipase B (CAL B)

The esterification activity was determined using *n*-butyric acid and *n*-butanol as substrates in toluene:tert-butanol solution (70:30, wt%) at 65 °C, based on the method reported by Kiran and collaborators [12]. The consumption of the acid and the formation of the ester were followed by GC and the activity was reported in UA. We define the units of esterification activity (UA) as the mmole of butyric acid that are esterified per hour. Typically immobilized *C. antarctica* lipase B has a esterification activity of 0.06 UA mg^{-1}.

2.3. Enzymatic esterification

In a typical reaction non-activated succinic acid (50 μmol) and the DAH (50 μmol) were dissolved in 5 mL of toluene and *tert*-butanol (70:30, wt%), in 10 mL glass tubes. The reaction was performed at 65 °C and 24 h, unless otherwise stated. The reaction was initiated by addition of the lipase (1.2 UA, 4 g L^{-1}). Reactions were stopped by rapid cooling on ice bath. Subsequently, the enzyme was separated by centrifugation at 14,000 rpm and the supernatant was collected and stored for further analysis.

2.4. Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

Samples were analyzed by MALDI-TOF spectrometry in an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) in the positive mode and outfitted with a laser (337 nm). The matrix, *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB), was prepared at 40 mg mL^{-1} and the dopant, potassium trifluoroacetate at 5 mg mL^{-1}, both in tetrahydrofuran. Sample, matrix and dopant solutions were mixed (10:5:5) and 0.3 μL of mixture was placed in MALDI-TOF plates (5 mm in diameter) and dried in air. Maltodextrins and polyethylene glycol (600, 1000, and 2000 Da) were used for calibration. Ions were accelerated with a 25 kV voltage (delayed extraction time of 200 ns). At least three spectrums with a total of 200 shoots per spot were collected using the lowest possible laser intensity that led to a good quality spectrum [13,14]. Within each MALDI-TOF spectrum, the intensity of all signals obtained by the potassium-adduct per chemical specie were added up and subsequently the relative contribution of each species was calculated. It was assumed that there are no significant differences between the response factors of the molecules [6,13].

2.5. Substrate analysis

Unreacted substrate was followed with a Waters 1525 binary HPLC pump implemented with a refraction index detector and an ionic exchange column Alltech OA-100 (sulfonated polystyrene-divinylbenzene) eluted with 3 mM sulfuric acid aqueous solution (0.4 mL min^{-1}). Samples and standards solutions (1000 μL) were dried under reduced pressure. The remained solid was dissolved in Milli-Q water (100 μL) and warm up in a bath (70–80 °C). Insoluble solid was separated by centrifugation and the supernatant (10 μL) was injected in the column and data were recorded. Similar procedure was done for standards of succinic acid and DAHs.

2.6. Oligomer analysis

The reaction samples (10 μL) were injected in a HPLC Waters 2690, outfitted with a PDA detector and a 250 mm × 3 mm ODS-2 Intersil (Varian Inc.) column at 40 °C. Elution of oligomers was

done in a gradient flow, phase A: acetonitrile (Merck) and phase B: water. Both mobile phases were supplemented with trifluoroacetic acid (0.1%, v/v). Gradient elution started at 5:95 (v/v) A/B and ended at 95:5 (v/v) A/B with a flow rate of 0.5 mL min^{-1} and the sample injection volume of 10 μL.

2.7. Fourier transform infrared analysis (FT-IR)

Between 2 and 10 μL liquid samples were dried on a zinc selenite microplate of a Varian 1000, Scimitar TM series FT-IR system. A total of sixty-four infrared scans were recorded and averaged with a resolution of 2 cm^{-1}.

2.8. Experimental design and statistical analysis

A three level full factorial design with additional three center points was used. The three factors chosen were temperature (A, 50–80 °C), initial concentration of non-activated succinic acid and DAH (B, 10–100 mM), and enzyme concentration (C, 4–10 mg mL^{-1}). Response variable was the substrate conversion after 24 h. The substrate ratio was 1:1 (mol). All the reactions were conducted as independent duplicates and each duplicate was done twice. Statistic results were analyzed by Statgraphics Plus 5.0 at 95% confidence interval ($\alpha = 0.05$). Analysis of variance (ANOVA) was used to evaluate significance of factors, interactions among factors, and presence of autocorrelation in the residuals of the regression analysis. Hence, Probability test (*P*-test), Fisher–Snedecor test (*F*-test) and Durbin–Watson test (DW-test) were applied.

2.9. Substrate-imprinted docking

The docking procedure consists of five steps. First, a tetrahedral reaction intermediate is covalently docked into an enzyme structure. Second, the best scoring substrate placement is used to construct an enzyme–substrate complex. Third, the geometry of the enzyme–substrate complex is optimized by energy minimization (200 steps steepest descent, 800 steps conjugate gradient). Fourth, the substrate is removed from the optimized complex resulting in an optimized enzyme structure. Fifth, the optimized enzyme structure is used for a second covalent docking of the same substrate. Final docking results are analyzed for geometric filter criteria necessary for the catalytic mechanism, their docking score, and the maximum overlap volume used during the docking.

The CAL B X-ray structures 1LBS, 1LBT [15], 1TCA, 1TCB, and 1TCC [16] were used in this study. The substrates were modelled as tetrahedral reaction intermediates of isosorbide, isomannide, and isoidide esters with butyric acid. Because of symmetry, one ester was modelled for isomannide and isoidide, while it was necessary to model two different esters for isosorbide, as the two stereocenters that feature the hydroxyl groups are not identical. Butyric acid and succinic acid are both readily converted by CAL B, and therefore butyric acid was used during the docking as a less complex model substrate.

3. Results and discussion

3.1. Factors that influence the reaction of CAL B with DAHs and non-activated succinic acid

Initial experiments to determine the catalytic capacity of *C. antarctica* lipase B, CAL B Novozym® 435, in the reaction of the three DAHs and non-activated succinic acid were carried out under thermodynamic controlled conditions as described in Section 2. Experiments with isoidide (Scheme 1C) as substrate did not show positive outcome. Conversion of isoidide in the presence of CAL B was less than 5% and further experiments with other enzymes such as *Candida rugosa*, porcine pancreas, wheat germ, and *Thermomyces lanuginose* lipase showed almost no conversion (data not shown). Isosorbide and isomannide (Scheme 1A and B) showed better results in the presence of CAL B (conversion higher than 15%). Furthermore, an experimental design was applied to find an improvement in the reaction when CAL B was the biocatalyst. Three variables were considered as the most relevant to find the best conditions of the esterification. We chose temperature, initial substrate concentration, and enzyme concentration as the main factors and substrate (DAH) consumption after 24 h as response variable to evaluate the performance of CAL B. The reaction was carried out in 10 mL tubes under the similar conditions described in Section 2.

Table 1
ANOVA analysis for reaction of isosorbide and non-activated succinic acid in the presence of *C. antarctica* lipase CAL B.

Isosorbide	Sum of squares	DF	Mean square	*F*-ratio	*P*-value
A: Temperature	252.8	1	252.8	13.69	0.0005
B: Substrate conc.	27.2	1	27.2	1.46	0.232
C: Enzyme conc.	48	1	48	2.58	0.114
AB	0.9	1	0.88	0.05	0.828
AC	1.9	1	1.85	0.1	0.754
BC	187.4	1	187.4	10.07	0.003
Blocks	3.9	1	3.9	0.21	0.649
Total error	967.5	52	18.6		

DF: degree of freedom; *F*-ratio: Fisher–Snedecor test; *P*-value: Probability test.

The analysis of variance (Table 1) for non-activated succinic acid consumed in the esterification with isosorbide in the presence of CAL B showed that the temperature is the only important variable. The largest value of the sum of squares (252.8) as well as the lowest *P*-value (0.0005) corresponds to the temperature of reaction. The large experimental *F*-ratio value (13.69) confirms the above stated when it is compared with the *F*-test value (4.02) from the tables for a degree of freedom (1, 52). A similar analysis of variance (Table 2) for non-activated succinic acid in the esterification with isomannide showed that not only the reaction temperature, but also the initial substrate concentration is an important variable. The two larger values of the sum of squares (1284.2 and 4554.5) as well as the lowest *P*-value (0.003 and 0) correspond to the temperature and the initial substrate concentration, respectively. The large experimental *F*-ratio values (10.09 and 35.78) confirm the above stated when it is compared with the *F*-test value (4.02) from the tables for a degree of freedom (1, 52). The evaluation of second order interactions among factors was also done. The conversion of isosorbide showed a small interaction between initial substrate and enzyme concentration (data not shown). Isomannide conversion, however, exhibited an interaction among temperature and initial substrate concentration as well as initial substrate and enzyme concentration (data not shown). Nevertheless, due that the initial enzyme concentration was not an important factor these interactions should not be taken into account in practical terms. The Durbin–Watson

Table 2
ANOVA analysis for reaction of isomannide and non-activated succinic acid in the presence of *Candida antarctica* lipase B.

Isomannide	Sum of squares	DF	Mean square	*F*-ratio	*P*-value
A: Temperature	1284.2	1	1284.2	10.09	0.003
B: Substrate conc.	4554.5	1	4554.5	35.78	0
C: Enzyme conc.	0.6	1	0.6	0	0.945
AB	426.3	1	426.3	3.35	0.073
AC	47.2	1	47.2	0.37	0.545
BC	188.69	1	188.69	1.48	0.229
Blocks	70.36	1	70.36	0.55	0.461
Total error	13190.2	52	127.3		

DF: degree of freedom; *F*-ratio: Fisher–Snedecor test; *P*-value: Probability test.

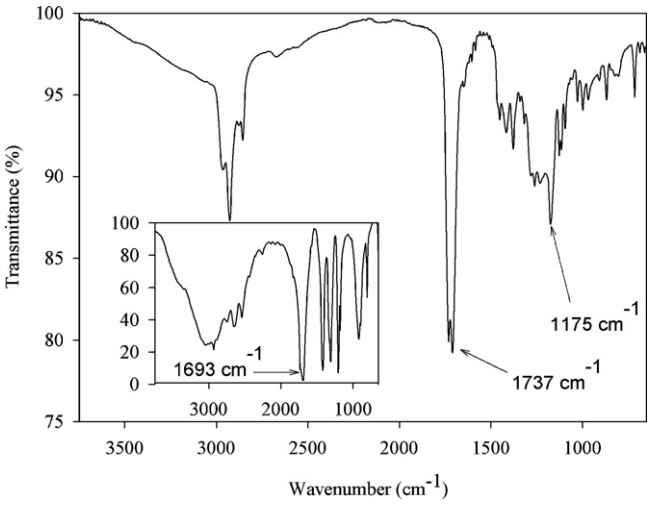


Fig. 1. FT-IR spectrum of non-activated succinic acid (A) and isomannide (B) reaction mixture in the presence of *Candida antarctica* lipase B after 24 h (main figure). Succinic acid spectrum (inserted figure).

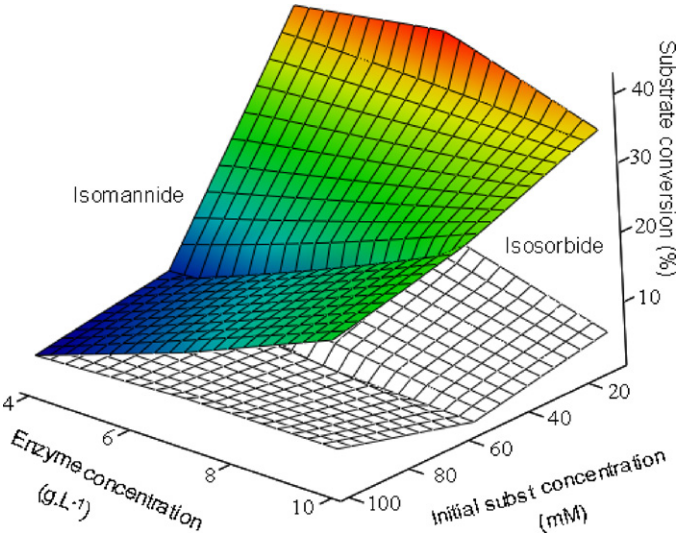


Fig. 3. Response surface for isomannide and isosorbide at 65 °C.

test showed values of 2.37 and 1.85 for isomannide and isosorbide, respectively. These values are close to 2.0, which indicate that there is independence of the errors (no serial autocorrelation).

The formation of the ester bond after 24 h reaction in the presence of *C. antarctica* lipase B using DAH and non-activated succinic acid as substrate, was confirmed by FT-IR. Initial succinic acid carbonyl (C=O) stretching at 1680 cm^{-1} shifts after reaction to 1710 cm^{-1} and 1737 cm^{-1} corresponding to carbonyl from the ester bonds formed at LEOs and CEOs, respectively (Fig. 1). A new strong C–O stretching band at 1175 cm^{-1} confirms also the formation of esters. Effects of the initial substrate and enzyme concentration on consumption of the chosen DAH at 50 and 65 °C after 24 h reaction are shown in Figs. 2 and 3, respectively. Isomannide showed higher conversions than isosorbide at 50 °C (Fig. 2) and 65 °C (Fig. 3). At the levels evaluated in this experimental design the initial enzyme concentration did not have a significant effect, while the temperature and the initial substrate concentration did. The low significance for the enzyme concentration suggests that the reaction occurs under thermodynamically controlled conditions. The

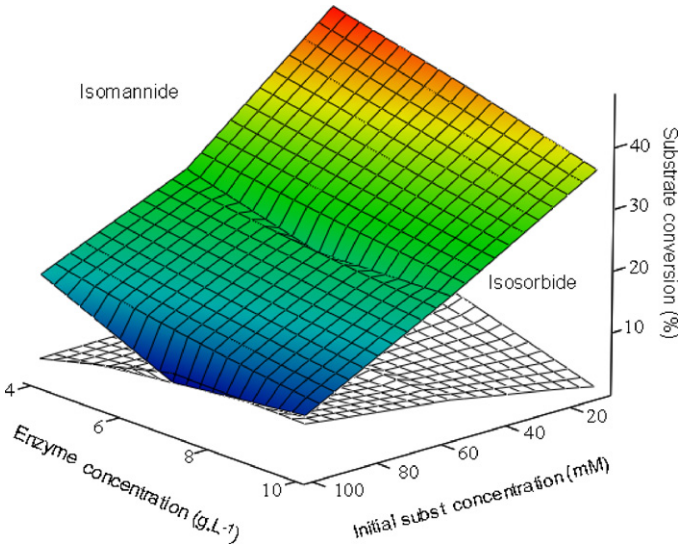
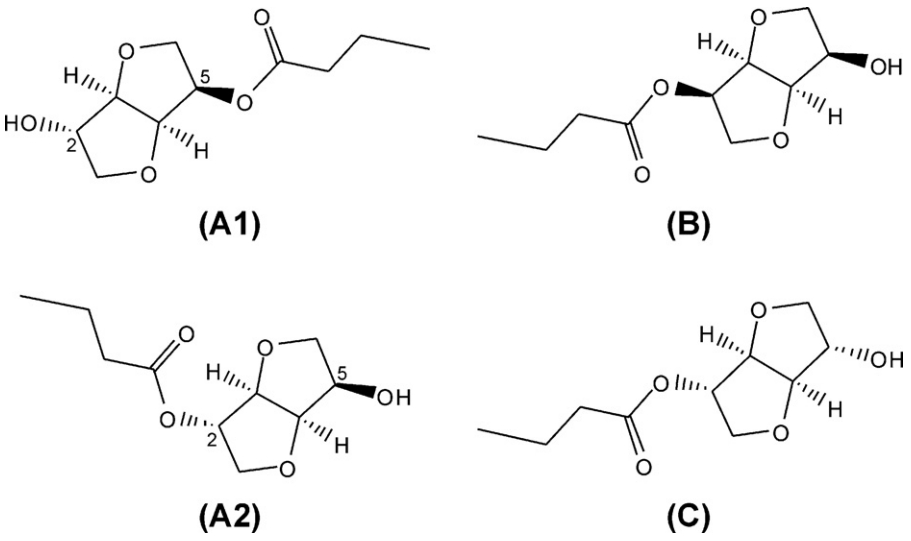


Fig. 2. Response surface for isomannide and isosorbide at 50 °C.

highest isomannide conversion was 47.4% when the initial isomannide concentration was 10 mM and the enzyme activity units were 1.2 UA (4 g L^{-1}). The highest isosorbide conversion was 16.3% when the initial isosorbide concentration was 10 mM and the enzyme activity units were 1.2 UA (4 g L^{-1}). There were no significant differences in conversions at 50 and 65 °C. The maximum conversions for isomannide were 47.4% at 50 °C and 41.9% at 65 °C, while maximum conversions for isosorbide were 13.1% at 50 °C and 16.13% at 65 °C. The reaction conducted at 80 °C, however, differs with the results at 60 °C and 65 °C for both, isomannide and isosorbide. The isomannide conversion at 80 °C ranged from 3.7% to 24.2%, while the isosorbide conversion at the same temperature ranged from 0% to 4% (data not shown). These results are in agreement with the reported data of CAL B thermostability in non-aqueous media [17–19].

There are three interesting observations from this study: (i) that the conversion of isoidide was not favoured by CAL B, (ii) the conversion of isomannide was higher than the conversion of isosorbide for most of the conditions tested, and (iii) the differences among the three DAHs is the endo and exo hydroxyl groups at C2 and C5 (Scheme 1). These three facts indicate that CAL B must have a preference for *endo*-hydroxyl groups (attached to C5 in isosorbide and C2 and C5 in isomannide, Scheme 1) rather than for *exo*-hydroxyl group (attached to C2 in isosorbide and C2 and C5 in isoidide, Scheme 1). This will explain also the low reactivity of isoidide (two *exo*-hydroxyl groups, Scheme 1) found in earlier experiments. The steric configuration of the DAHs has been also suggested as the main factor of the reactivity for polyester synthesis [1,4]. In contrast, an opposite effect to what we found with CAL B has been observed when chemical catalyst was used. For example, Noordover et al. [3,4] prepared succinic acid and isosorbide polyesters in the presence of titanium (IV) *n*-butoxide in the melt. The authors found a limitation in the final molecular weight, reaching a value up to 2500 g mol^{-1}. Such limitation in the chemical catalyzed polymerization of isosorbide is thought due to the low reactivity of the *endo* hydroxyl group (Scheme 1A position 5). Such low reactivity is owing to the formation of hydrogen bonds in the *endo* position, while the *exo* hydroxyl group is exposed and ready to react. In addition, they found by different means that the ratio in the terminal hydroxyl groups (*endo*/*exo*) was 6:4. This confirmed the hypothesis that *exo*-hydroxyl group in isosorbide is significantly more reactive than its *endo* counterpart under chemical catalyzed conditions. This feature of the *exo*-hydroxyl group has been observed by other



Scheme 2. (A1) (3R,3aR,6S,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate, (A2) (3S,3aR,6R,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate, (B) (3R,3aR,6R,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate, (C) (3S,3aR,6S,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate.

authors not only with chemical catalyst but also with biological degradation of polyesters [1].

A more thorough analysis of the DAH structure could explain our results. The rings are *cis* orientated in "V" shape and inclined to one another in an angle of approximately 120°. The orientation of the *endo*-hydroxyl group or (R)-configurated hydroxyl group attached to C5 in isosorbide, present as well in C2 and C5 in isomannide, and absent in isoidide (Scheme 1A–C) must play a key role on the enzymatic stereospecificity of CAL B. Moreover, due that the *endo*-hydroxyl group ((R)-configurated) generates an intramolecular hydrogen bond with the adjacent oxygen atom in the cyclic ether ring, the carbon attached to it must be more positively charged that the carbon attached to an *exo*-hydroxyl group ((S)-configurated) [4,20]. Hence, the positive charge in the C5 of isosorbide (C2 and C5 in isomannide) must favour the nucleophilic attack that leads to the formation of the ester bond. This explanation is in agreement with the enzymatic esterification mechanism described by Zaks and Klibanov [21]. Furthermore, Boulif et al. [22] reported recently the monosubstitution of isosorbide with ricinoleic acid with 95% conversion of the *endo*-hydroxyl group ((R)-configurated) and less than 1% conversion of the *exo*-hydroxyl group ((S)-configurated) in the presence of CAL B at 63 °C. Reetz and Schimossek [23] use CAL B for N-acylation of (R,S)-phenylethylamine with ethyl acetate. CAL B only reacted with the (R)-phenylethylamine reaching 77% conversion and synthesizing the (R)-configurated amide enantiomerically pure (ee = 99%). Likewise, Overmeyer et al. [24] used CAL B for transesterification of (R,S)-1-phenylethanol with vinylacetate in supercritical CO_2 environment. They reached a conversion above 45% of the (R)-enantiomer in few hours with enantiomeric excess above 99%. Interestingly, Hilker et al. [25] used CAL B-catalyzed dynamic kinetic resolution with in situ chemical racemization to produce chiral polyesters. They started from the racemic mixture of 2,2-dimethyl-1,4-benzenedimethanol as the di-ol and dimethyl adipate as the di-acyl donor. The reaction led to the formation of polyesters containing only the (R)-enantiomer with a conversion of 92% after 70 h. Hence, these evidences together with our results suggest that *C. antarctica lipase* B (CAL B) exhibits higher reactivity for (R)-configurated groups than for (S)-configurated ones. These results are also in agreement with ours, where *exo*-hydroxyl group exhibits less reactivity than *endo*-hydroxyl group. Therefore, the reactivity of DAH is featured by the type of catalyst used and also due to the mechanism involved in the reaction. As a result, the esterification in the presence of CAL B biocatalyst shows a potential in the synthesis of novel biobased polyesters beyond the possibilities of the chemical catalyst.

3.2. Docking of the substrates into the active site

To rationalize the molecular basis for the observed CAL B preference of isomannide over isosorbide and over isoidide, substrate-imprinted docking was used to dock butyric acid esters of the DAHs into five CAL B structures (Scheme 2). Productive docking solutions were always found if the ester bond is formed with an *endo*-hydroxyl group, as found in isomannide and one of the isosorbide hydroxyl groups (C5, Schemes 1 and 2), while no productive docking solutions could be found for esters with *exo*-hydroxyl groups, as found in isoidide and one of the isosorbide hydroxyl groups (C2, Schemes 1 and 2). This is in agreement with the experimentally observed preference of CAL B for the *endo*-hydroxyl group. An analysis of the substrate placements in the active site generated by docking shows, that esterification at the *endo*-hydroxyl group leads to a reaction intermediate that fits into the CAL B alcohol pocket without clashes of the two rings with the enzyme (Fig. 4C) and forms the hydrogen bonds required for stabilization of the transition state [26] (Fig. 4A).

The analysis of the non-productive substrate placements achieved for C (Fig. 4B) shows that C can be fitted into the binding pocket of CAL B, but not in a pose that forms the required hydrogen bonds for catalysis (Fig. 4B and D). C is placed in the CAL B binding pocket in a way that puts the alcohol oxygen of the ester beyond hydrogen bond distance to the NH-group of H224 (3.5 Å) and additionally points the free electron pairs of the alcohol oxygen away from H224, making a hydrogen bond even unlikely if the distance was smaller. Therefore, the CAL B preference of isomannide over isosorbide over isoidide is a direct result of the preference for *endo*-hydroxyl groups, which is due to the transition state of esters with *exo*-hydroxyl groups not forming all the required hydrogen bonds for catalysis. If these bonds are not formed properly, catalysis is either very slow or does not occur at all.

3.3. Reaction yield optimization

Additional experiments were conducted to increase the conversion yield in the biocatalyzed reaction of non-activated succinic

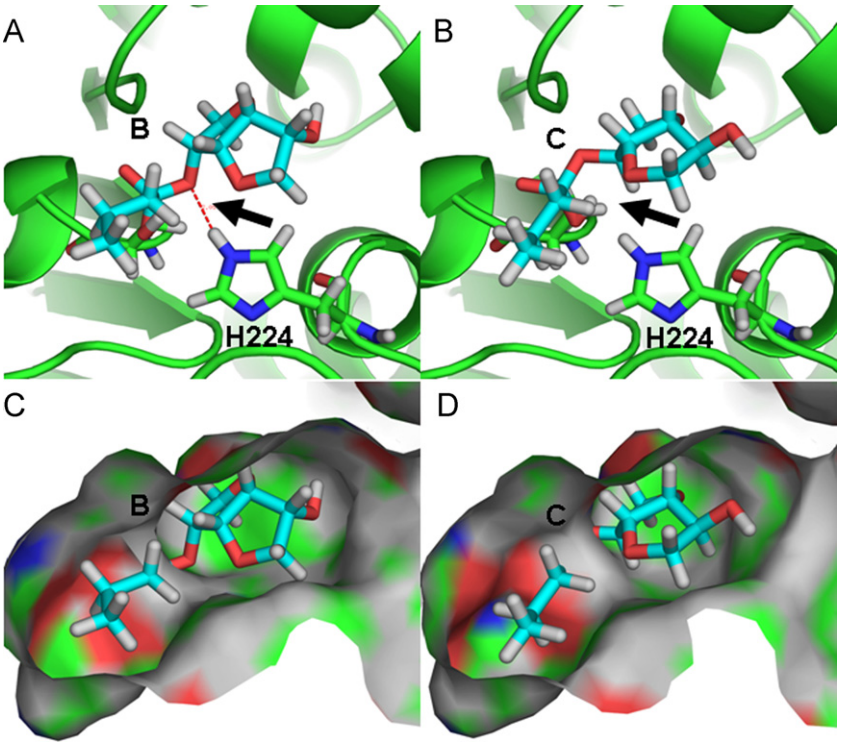


Fig. 4. (A and C) (3R,3aR,6R,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate; (B) docked into CAL B (PDB-ID: 1LBS). H224 forms a hydrogen bond with the alcohol oxygen of the ester (red dashed line, black arrow). (B and D) (3S,3aR,6S,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate (C) docked into CAL B (PDB-ID: 1LBS). The necessary hydrogen bond between H224 and the alcohol oxygen of the ester cannot be formed (black arrow).

acid and isomannide. We mentioned before that water produced in the reaction plays a key role in the biosynthesis of esters, shifting the equilibrium from ester formation toward ester hydrolysis with consequent reaction yield diminishing. Hence, we decided to perform a reaction improvement in terms of water control in the presence of molecular sieves. Enzymatic reactions were carried out in 200 mL round bottom glass reactor with a working volume of 50 mL and in the presence of *C. antarctica* lipase B (4 g L^{-1}, 12 UA). The reactor was equipped with a flat paddle impeller at 200 rpm and the same conditions mentioned at Section 2. Molecular sieves (5 g) dried for at least 24 h at 120 °C were added at the beginning of the reaction. Samples were withdrawn from the reactor every hour during the first 8 h and a final sample was taken at 24 h of reaction for further analysis. Two control reactions were set up. The first control included both substrates without CAL B and without molecular sieves, while the second control contained substrate with molecular sieves and without CAL B. In both control reactions we did not observe quantifiable substrate conversion or product synthesis. 43.7% of initial succinic acid was converted together with 47.4% isomannide when the water produced was not removed (no molecular sieves added, see Fig. 3). Conversion of 88.2% of succinic acid and 93.7% of isomannide were observed after 24 h enzymatic reaction in the presence of molecular sieves (Fig. 5).

3.4. Product characterization

Since CAL B exhibits more reactivity toward isomannide than toward its homologous isomers, we continued our work on characterization of the reaction products only for reactions with isomannide. The preparative synthesis of isomannide succinate oligomers from non-activated succinic acid (A) and isomannide (B) was conducted at a 50 mL scale in the presence of molecular sieves as described in Section 2. No conversion occurred in the absence of the enzyme (data not shown). Samples were taken every hour up to 24 h of reaction and a MALDI-TOF analysis was performed.

The most abundant synthesized products shown in Scheme 3 are summarized in Table 3. Oligomers up to octadecamers (AA_8) were detected in the reaction mixture. Low molecular weight CEOs and LEOs were produced during all the time course analysis. The results show that during the first 8 h of reaction the most abundant products were the CEOs (32.5–48.7%, Entries 45 and 50). The abundance of LEOs follows the order AA_n (19.5–24.8%, Entries 28 and 32) ≈ AB_n (13.7–28.9%, Entries 1 and 3) > BB_n (9.9–16.2%, Entries 41 and 40). Interestingly, the prevalent specie was the CEO_1 (23.2–40%, Entries 45 and 50) reaching the highest abundance at 8 h. The formation

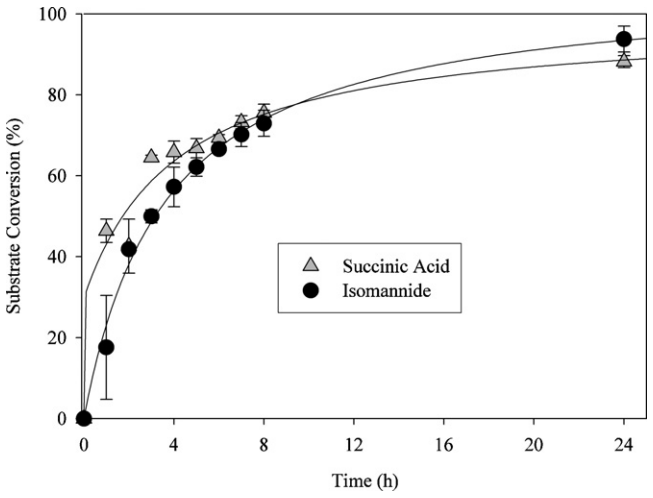


Fig. 5. Substrate conversion of non-activated succinic acid and isomannide in the presence of *Candida antarctica* lipase B and molecular sieves. Error bars represent the standard deviation between duplicates.

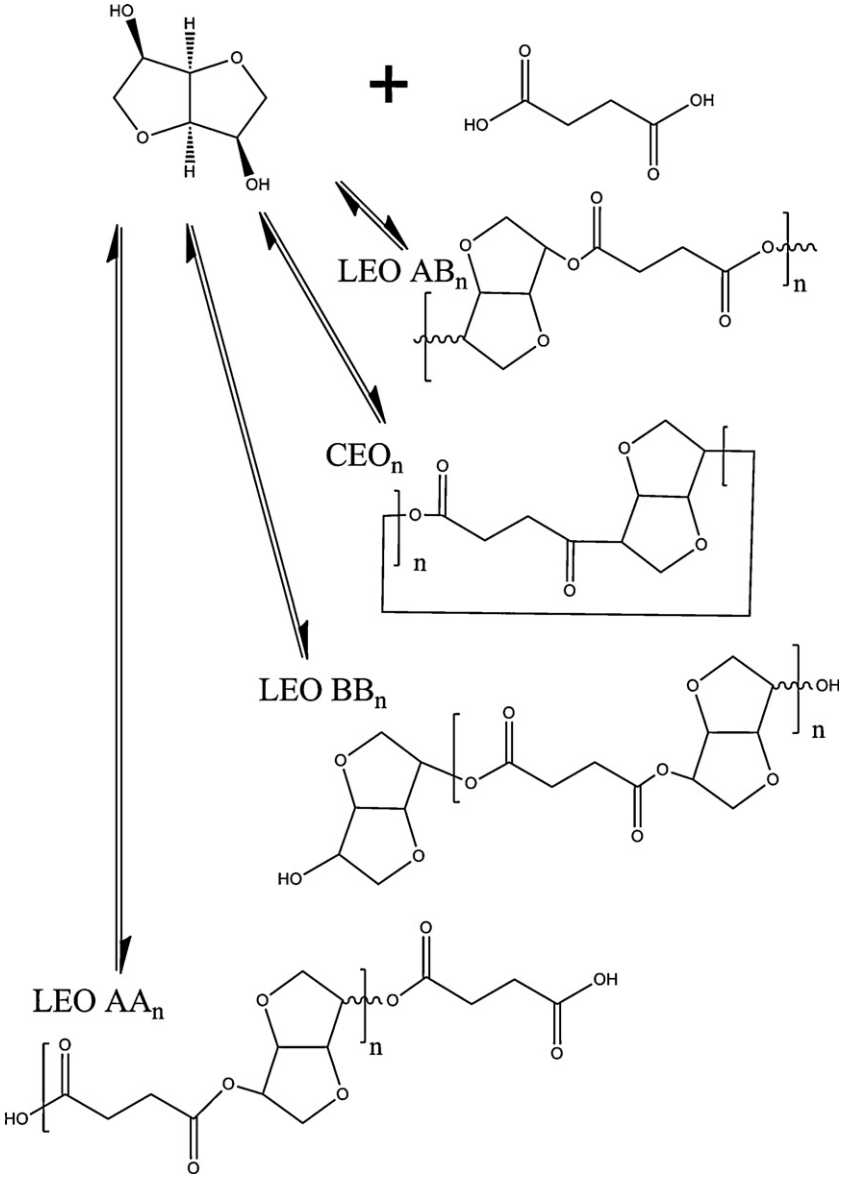
Table 3
MALDI-TOF time course analysis of main products of oligo(isomannide succinate) synthesized in the presence of *Candida antarctica* lipase B. CEO_n. CEO_n states for the cyclic ester oligomer with *n*-times succinic acid (A) and isomannide (B). Similarly AB_n states for the linear ester oligomer with *n*-times succinic acid (A) and isomannide (B). AA_n states for the dicarbonyl terminal linear ester oligomers with *n*-times repeated for $(AB)_nA$, the linear $(n+1)$-emer; thus, AA_3 is the linear ester oligomer (AB)(AB)(AB)A. BB_n state for dihydroxyl terminal linear ester oligomer with *n*-times repeated for $B(AB)_n$; thus BB_3 is the linear ester oligomer B(AB)(AB)(AB).

Entry	Total oligomer	Time (h)	AB_1		AB_3		AB_4		AB_5							
			Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.
1	13.7	1			12.4	1.5			1.3	0.0						
2	22.7	2	3.3	0.2	17.9	2.5			1.5	0.2						
3	28.9	3	5.8	1.6	20.9	3.9	2.3	0.8								
4	26.8	4	4.2	0.3	19.1	4.0	2.6	0.3	1.1	0.1						
5	23.5	5	2.3	0.1	18.0	1.0	3.2	0.2								
6	19.7	6	2.7	0.2	12.0	5.1	3.7	0.9	1.3	0.2						
7	23.4	7	5.1	2.6	14.4	4.1	2.8	0.9	1.1	0.3						
8	19.0	8			15.1	3.2	2.9	0.6	1.0	0.2						
24	13.5	24	1.3	0.3	9.0	3.8	2.2	0.5	1.0	0.2						

Entry	Total oligomer	Time (h)	AA_1		AA_2		AA_4		AA_5		AA_6		AA_7		AA_8	
			Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.
25	24.5	1	13.1	1.2	1.8	0.1	1.1	0.0	7.7	0.7					0.7	0.1
26	20.9	2	6.5	1.1	3.1	1.0	1.5	0.3	7.7	1.3	2.1	0.1				
27	23.5	3	5.5	1.2	10.4	5.8	1.4	0.2	4.3	1.8	1.1	0.1	0.8	0.1		
28	24.8	4	7.4	1.8	9.8	2.6			5.8	0.8			1.1	0.1	0.7	0.1
29	19.8	5	6.7	0.4	3.7	0.4	1.3	0.1	5.9	0.5	1.4	0.1			0.8	0.0
30	23.0	6	7.8	1.0	2.5	0.2	1.7	0.5	7.8	1.6	1.5	0.1	1.7	0.1		
31	20.6	7	7.2	2.7	4.3	1.1	1.3	0.4	6.4	2.1			1.4	0.4		
32	19.5	8	7.9	1.8	2.8	0.6	1.1	0.2	6.3	1.2	1.4	0.2				
33	44.2	24	32.9	8.2	2.8	0.7	0.9	0.2	5.5	1.4	1.1	0.4	0.9	0.2		

Entry	Total oligomer	Time (h)	BB_1		BB_2		BB_3		BB_5		BB_6		BB_7			
			Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.
34	11.8	1			2.2	0.1	2.7	0.2	2.1	0.2	2.0	0.3	2.8	0.4		
35	13.2	2	3.3	0.2			2.9	0.4	2.1	0.2	2.0	0.2	2.9	0.2		
36	12.0	3	2.6	0.3	4.6	3.2	2.4	0.4			1.2	1.7	1.1	1.0		
37	13.2	4	2.4	0.2	4.1	0.6	2.2	0.3	1.7	0.1	1.0	0.1	1.9	0.3		
38	13.1	5			4.4	0.6	3.3	0.2	1.8	0.1	0.9	0.0	2.6	0.1		
39	12.3	6			2.2	0.3	3.1	0.6	2.3	0.5	2.0	0.8	2.7	0.4		
40	16.2	7	5.7	1.5	2.1	0.6	2.4	0.8	1.8	0.6	1.7	0.6	2.5	0.8		
41	9.9	8			2.9	0.5	2.8	0.5	1.9	0.3			2.3	0.6		
42	7.3	24					1.9	0.5	1.8	0.4	1.4	0.3	2.3	0.5		

Entry	Total oligomer	Time (h)	CEO_1		CEO_3		CEO_5		CEO_6							
			Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.	Abundance (%)	St. Dev.
43	47.7	1	37.7	3.0	2.0	0.1	7.1	0.3	1.0	0.0						
44	41.8	2	30.8	5.2	1.7	0.1	8.0	0.8	1.3	0.1						
45	32.5	3	23.2	7.5	4.5	2.9	4.8	2.0								
46	35.1	4	24.8	6.0	3.8	0.6	5.6	0.7	0.8	0.1						
47	42.0	5	30.8	5.5	3.8	0.5	6.5	0.4	0.9	0.0						
48	44.4	6	33.3	6.0	2.3	0.2	7.8	1.5	0.9	0.1						
49	38.0	7	29.6	8.4	1.9	0.5	6.5	1.9								
50	48.7	8	39.9	7.9	2.6	0.5	6.3	1.2								
51	35.1	24	28.3	7.2	1.2	0.3	5.5	1.3								



Scheme 3. Isomannide succinate oligomers from non-activated succinic acid (A) and isomannide (B) in the presence of *Candida antarctica* CAL B lipase. Example LEO BB_2 stands for the di-hydroxyl terminal oligomer BABAB.

of CEO_1 can be partially explained for the *cis* spatial configuration of the rings in a "V" shape together with the *cis* orientation of the hydroxyl groups (Scheme 1), which may favour the formation of the ester bond at both sides of succinic acid molecule leading to the synthesis CEO_1 as stable product. In many enzyme-catalyzed reactions of alkyl building blocks under thermodynamically controlled conditions, however, has been demonstrated that CEO_1 is less abundant product than, for example, CEO_2 [27,28]. In contrast, formation of cyclic species of terephthalate, isophthalate and phthalate dimethyl ester has been investigated was shown to depend on the geometry of the rigid, aromatic monomer [29]. The authors found that only dimethyl isophthalate only not was capable to form the first CEO of the series (CEO_1), but also CEO_1 was the most abundant product among all the CEOs formed. These results are comparable with ours, since the configuration of isomannide may favour the stable formation of CEO_1.

At 24 h the abundance of CEOs decreases dramatically (Table 3). AA_n becomes the predominant species (44.2%, Entry 33), where AA_1 constitutes the 74% of the total AA_n (32.9%, Entry 33). Fig. 6 summarizes the results at 24 h. The total CEOs reach a concentration of 1.4 mM, where the first (CEO_1) and fifth (CEO_5) cyclic esters were 1.1 mM and 0.24 mM, respectively. These concentrations represent more than 96% of the total synthesized CEOs. Hydroxy-carbonyl terminal oligomers (AB_n) reach a concentration of 0.57 mM, where AB_3 (0.40 mM) is more than the 9% of the total products and the 67% of the total AB_n detected. Total di-hydroxy terminal oligomers (BB_n) present the lowest concentration (0.3 mM) of the total detected products. Di-carbonyl terminal LEOs (AA_n), on the other hand, are the most abundant products at 24 h (1.8 mM). Interestingly, AA_1 (1.3 mM) presents not only the highest concentration in the di-carbonyl terminal series, but also among total the detected products. This fact suggests that the system undergoes a product hydrolysis between 8 h and 24 h.

4. Conclusions

The esterification of di-anhydro hexitols together with non-activated succinic acid in a toluene based medium in the presence of *C. antarctica* lipase B (CAL B) was studied. Temperature and initial substrate concentration were the most important factors over the

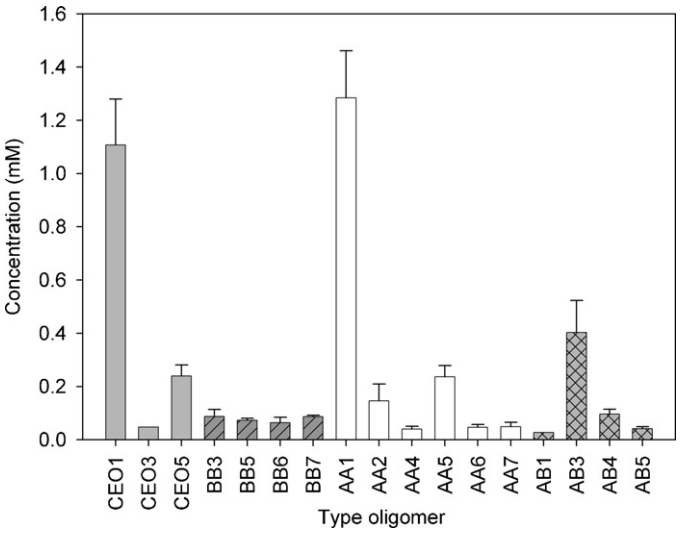


Fig. 6. Ester oligomers formed after 24 h enzymatic conversion of non-activated succinic acid and isomannide in the presence of CAL B (see Table 3). Error bars indicate the standard deviation between duplicates. The concentration of products was calculated taking into account the abundance of each compound, the water theoretically produced, and the substrate conversion.

reaction yields. Interestingly, CAL B had preference for isomannide over isosorbide and over isoidide; such an enantiopreference was not only found experimentally, but also supported by substrate-imprinted docking analysis. This fact set isomannide as a preferable substrate among the DAHs for further enzymatic studies due to difficulties in reactivity found with chemical catalyst. Finally, a characterization of products in the thermodynamically controlled system was conducted with the positive outcome that the studied system promotes the formation of CEOs as well as the synthesis of di-carbonyl terminal LEOs (AA_n). Therefore, esterification of non-activated succinic acid and isomannide in the presence of CAL B shows potential in the biosynthesis of novel building blocks (CEOs and LEOs) from ready-to-use biobased raw materials and is advantageous in respect to chemical ones due to the specificity already discussed.

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