SYNTHESIS OF OLIGOESTERS FROM BIOBASED BUILDING BLOCKS BY *Candida antarctica* LIPASE B

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BIOCATALYTIC SYNTHESIS OF CYCLIC ESTER OLIGOMERS FROM BIOBASED BUILDING BLOCKS

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Dedicado a mi padre, mi madre y al gato

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INTRODUCTION

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1. INTRODUCTION

Polyesters are an important class of polymers with exceptional physical and mechanical properties and numerous applications. Polyester materials are used as fibres, plastics and coating or as components of films, in composites and elastomers. Most of the polyesters applied today are built up from monomers derived from fossil fuel. Polyethylene terephtalate (PET), polybutylene succinate (PBS), polyacrylates are only few examples of largely used polyesters produced from petrochemical feedstocks. With oil resources forecasted to exhaust by year 2050, the permanent fluctuations of the oil price marked by political issues, increasing environmental awareness worldwide and the development of new technologies, there is growing interest for biobased polyesters produced from renewable feedstocks.

Currently, there are only few biobased polyesters. Representative examples of natural occurring polyesters are polyhydroxyalkanoates (PHA) which are linear polyesters produced by microbial fermentation from sugars or lipids. Typical building blocks of PHA are 3 (or 4)-hydroxy butyrate, 3-hydroxy valerate, and 3-hydroxy hexanoate. Polymers made of PHA were industrially marketed by ICI/Zeneca and later by Monsanto. The industrial production became unsustainable after 1995 (Gross and Kalra 2002). In the past decade much effort has been put to develop high production strains, to advance the technology for PHA production, to increase the production yield and to improve the downstream processing. Research on production of functionalized PHA with reactive groups in the aliphatic side chain is in progress. In 2005, Metabolix restarted the commercialization of microbial PHAs and currently develops transgenic crops to improve the production.

Another example of a commercially produced biobased semisynthetic polyester is polylactic acid. The building blocks of PLA, L-lactic acid, is nowadays directly obtained by fermentation of sugars using microorganism (i.e. *lactobacillus sp.*). Industrial

manufacture of high molecular weight PLA is carried out via polycondensation (PC) (implemented by Mitsui Toatsu Chemicals) and ring-opening polymerization (ROP) from the cyclic ester dimer (lactide) (implemented by Cargill). PLA is the first renewable polymer produced on an industrial scale. In April 2002, Cargill opened PLA production plant with an estimated capacity of 150.000 tonne per year. PLA has properties similar with the conventional polymers polyethylene and polypropylene, and is completely biodegradable (compostable). PLA has a wide range of applications, such as fabrics production, disposable plastic materials. Blends of PLA have a good resistance to deformation and transparency, making them useful for disposable tableware, and packaging applications. More specialized products are sutures, stents, dialysis media, and drug delivery devices.

The development of the technology to produce diversity of biobased building blocks is a way to increase the number of biobased polyesters with wide spectrum of chemical, physical and mechanical properties. This new generation of biobased polyesters will replace the petroleum derived polymers (Scheme 1). These new building blocks could eventually be associated with green, biocatalytic technologies for polyesterification. The latest is the topic of this thesis.
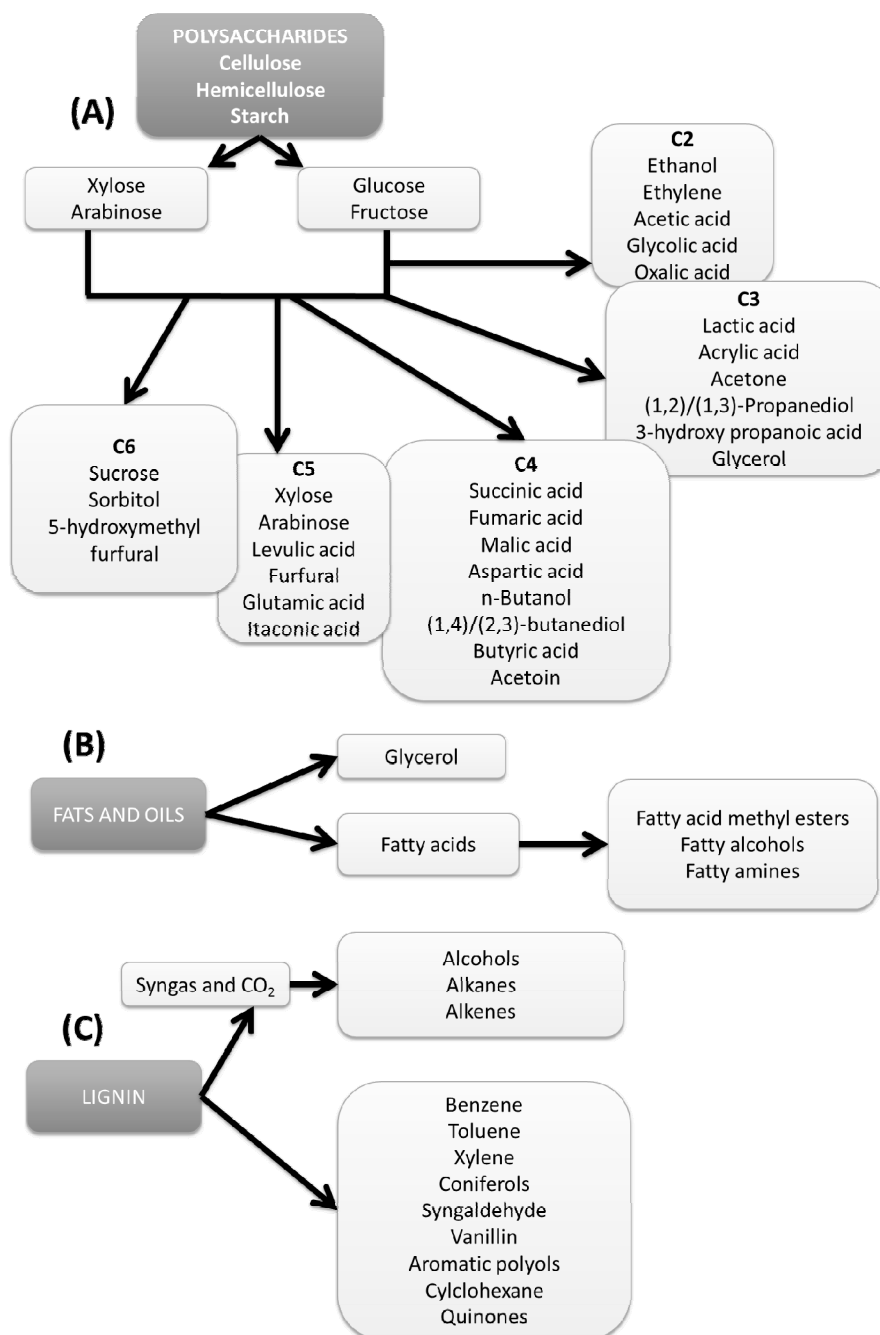
Scheme 1 Products obtained by renewable resources. Biorefinery products. (adapted from chemistry innovation website)

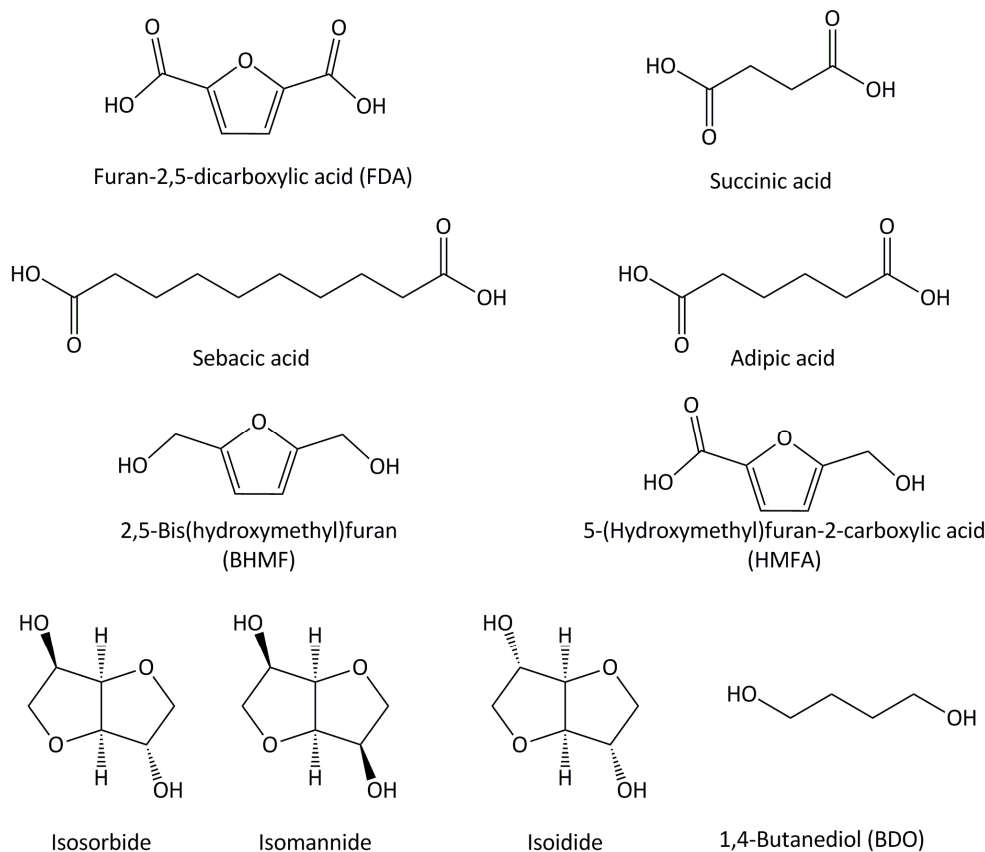## 2. BIOBASED BUILDING BLOCKS USED IN THIS WORK.

Biobased building blocks are obtained by biorefining of biomass (Scheme 1).

From the multitude of compounds that are produced in the biorefinery, some

of them are already produced industrially and have found industrial application for polyester synthesis, like L-lactic acid and 1,3-propanediol. For others, like succinic acid, the technology for their production is still under development. Nevertheless, the availability of bio-succinic acid will allow the production of a fully bio-polybutylene succinate (PBS) (since the co-monomer 1,4-butanediol can also be produced from succinic acid) that can replace the petroleum-based PBS that is currently commercialized. Also other compounds can be produced from C6 sugars that are emerging as building blocks for the polyester synthesis, namely, dianhydrohexitols and the furan-based monomers, like furan-2,5-dicarboxylic acid (FDA), 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA), and 2,5-bis (hydroxyl methyl)furan (BHMF). These building blocks form the core of this thesis; therefore, we will focus below on some aspects of their production (Scheme 2).

2.1. 1,4-Butanediol (BDO) and Succinic Acid (1,4-Butanedioic Acid)

Succinic acid and 1,4-butanediol can be fully synthesized using sugar-based feedstock by bacterial fermentation (Hofvendahl and Hahn–Hagerda 2000; Zeikus et al. 1999). *A. succiniciproducens* can utilize several substrates as carbon sources (Lee et al. 1999; Lee et al. 2001). High production yields (1.3 mole/mole) and low concentration of by-products can be obtained in the synthesis of succinic acid by *A. succiniciproducens* using glycerol as substrate (Lee et al. 2001).

Furan-2,5-dicarboxylic acid (FDA)

Succinic acid

Sebacic acid

Adipic acid

2,5-Bis(hydroxymethyl)furan
(BHMF)

5-(Hydroxymethyl)furan-2-carboxylic acid
(HMFA)

Isosorbide   Isomannide   Isoidide   1,4-Butanediol (BDO)

Scheme 2 Building blocks used in this work for the synthesis of biobased low molecular weight polyesters.

A. succinogenes metabolizes carbon sources to succinic acid, acetic acid, and formic acid and tolerates a larger concentration of succinic acid than any other strain (Guettler et al. 1996a; Guettler et al. 1996b). One of the drawbacks of fermentation of *A. succinogenes* is the high concentrations of by-products like acetic acid, propionic acid, and pyruvic acid which results in a high downstream processing costs. Succinic acid yield with variants of this strain is around 1 mol/mol glucose and has a productivity of 0.79 g.(L.h)$^{-1}$ (Guettler et al. 1996a).

Recently, a metabolically engineered *E. coli* strain has been reported to produce aerobically 58.3 g.L^{-1} succinic acid with a yield of 0.85 mol/mol glucose. The synthesis of by-products, however, could not be avoided (Lin et al. 2005a; Lin et al. 2005b). The purification of succinic acid from fermentative synthesis normally amounts more than

50% of the total production costs. Furthermore, the purification of succinic acid has been explored using electrodialysis, precipitation, and extraction with purities between 80 and 99% and efficiencies between 70 and 80% (Datta et al. 1992; Glassner and Datta 1992; Huh et al. 2006). Succinic acid can be directly modified to other biobased building blocks or to precursors with a potential use at industrial scale. Three succinate derivatives with major applications are obtained through hydrogenation routes. These are γ-butyrolactone (GBL), 1,4-butanediol (BDO), and tetrahydrofuran (THF). From these three, BDO has a wide application as industrial solvent and recently it has been used as raw material for polyesters, such as polybutylene terephthalate and polybutylene succinate (GS Pla®, Bionolle®), resins, automotive, and electrical parts. The potential market for products based on succinic acid is estimated at 270 ktonne.yr^{-1} (Willke and Vorlop 2004), while BDO has a global market of 1,300 ktonne.yr^{-1} (Delhomme et al. 2009).

2.2. 1,4:3,6-Dianhydrohexitols (DAH)

1,4:3,6-Dianhydrohexitol (DAH) is the name for 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 (Scheme 2 and 3). DAHs are produced mainly from C6 carbohydrates like glucose and mannose, which are obtained from polysaccharides such as starch, cellulose, and mannans extracted from cereals (Fenouillot et al. 2010). Hydrogenation of these sugars produces polyols (i.e. sorbitol and mannitol), which undergo a subsequent dehydration step by an acid catalyst (Flèche and Huchette 1986; Sanborn 2008). Typically the products will be isosorbide and isomannide. The synthesis of isoidide is done by isomerisation of these DAHs. The potential forecast market for isosorbide as polyethylene terephthalate (PET) replacement according to the US Department of Energy is estimated at 45.4 ktonne.yr^{-1} in 2020 (Carde 2001). The importance of the DAHs has increased recently due to the stereoconfiguration that confers specific properties of synthesized polycondensates (Kricheldorf 1997). DAH has been applied as biobased building blocks for the synthesis not only of polyesters, but also polyamides, poly(ester

amides), polyimides, polycarbonates, polyethers, and polyurethanes (Fenouillot et al. 2010; Galbis and García-Martín 2010).
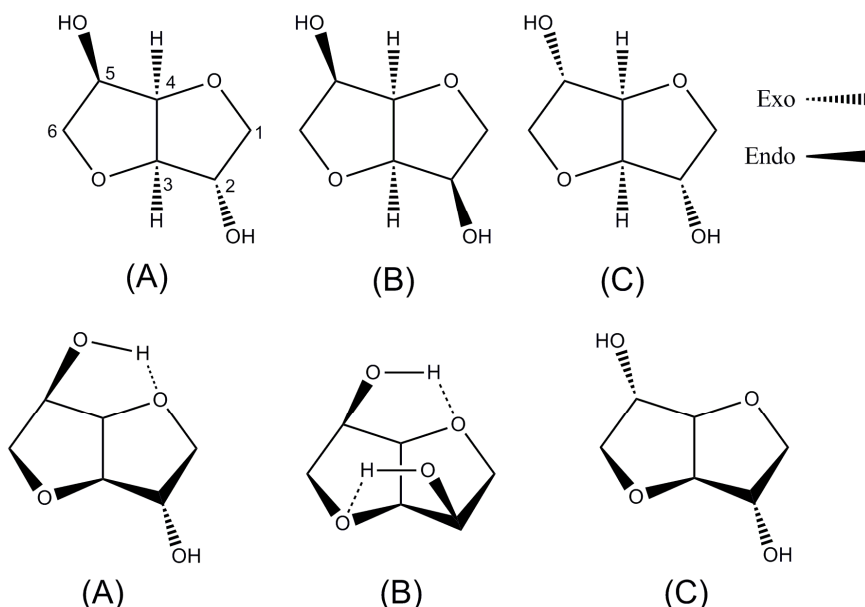
Scheme 3 1,4:3,6-dianhydrohexitols (DAH). (A) 1,4:3,6-dianhydro-D-glucitol or isosorbide; (B) 1,4:3,6-dianhydro-D-mannitol or isomannide; and (C) 1,4:3,6-dianhydro-L-iditol or isoidide

2.3. Aliphatic Dicarboxylic Acids (Sebacic Acid and Adipic Acid)

Adipic acid (1,6-hexadecanoic acid, Scheme 2) is one of the most important organic diacids industrially applied. The remarkable work of Carothers in the 30's led to the synthesis of Nylon, which remains one of the most important produced fibre made of adipic acid and hexamethylene diamine (Nylon 6.6). The pioneer work of Carothers encouraged Du Pont to invest in the industrial production of adipic acid in 1937 (Castellan et al. 1991). The industrial production of adipic acid is traditionally carried out by hydrogenation of benzene to cyclic intermediates and subsequent oxidation of cyclohexanol or cyclohexane under strong acid conditions (Castellan et al. 1991; Rohl et al. 1970). Adipic acid, however, can be alternatively synthesized using biobased building blocks. For example biobased succinic acid can be readily hydrogenated to 1,4-butanediol, which can be further carbonylated to adipic acid (Zeikus et al. 1999). Niu *et al.* synthesized adipic acid with 97% conversion starting from a biobased *cis,cis*-muconic

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acid (Niu et al. 2002). *Cis,cis*-muconic acid was obtained by a recombinant *E. coli* strain that produces 36.8 g.L^{-1} of the acid with a yield of 0.22 mol/mol glucose. The estimated market of adipic acid is close to the 2200 ktonne per year. Adipic acid has industrial uses as intermediate in the chemical, pharmaceutical, food and perfume industries, among others. The production of adhesives, plasticizers, gelatinizing agents, hydraulic fluids, lubricants, cosmetics, replacement of acidulants in the food industry, polyurethane foams, leather tanning, and urethane belongs to the portfolio of products made of adipic acid. Sebacic acid (Scheme 2), on the other hand, is manufactured by saponification of castor oil at high temperatures (Vasishtha et al. 1990). Although the sebacic acid yields are low, this route has been found to be industrially competitive. Sebacic acid has found application in the synthesis of Nylon in combination with hexamethylene diisocyanate. Sebacic acid is also applied to synthesize plasticizers, jet and air-cooled combustion motors lubricants, resins, cosmetics, candles, antiseptics, painting materials, plasticizers, and polyesters (Vasishtha et al. 1990).

2.4. Furan Derivatives (FD)

Furan-2,5-dicarboxylic acid (FDA), 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA), and 2,5-bis (hydroxyl methyl)furan (BHMF) (Scheme 2) are a set of furan derivatives of potential importance in the polymer industry. The chemical similarity of the furan ring to the phenyl ring opens the possibility to replace phenyl-based polymers such as polyethylene terephthalate (PET) for furan-based polymers.

Furan monomers are produced chemically from the furfural (2-furan carboxaldehyde) and 5-hydroxymethyl 2-furan carboxaldehyde (HMF) which are generated by acid-catalyzed thermal dehydration of pentoses (C5) and hexoses (C6) (Corma et al. 2007; Gandini 2008; Gandini and Belgacem 1997; Gandini et al. 2009a; Moreau et al. 2004). The worldwide production of furfural has a market of 300 ktonne.yr^{-1} with the largest

application in the production of furfuryl alcohol as commodity for the synthesis of diverse polymers and resins (Gandini 2008). In addition, furfuryl alcohol can be formylated to synthesized 2,5-bis-(hydroxyl methyl)furan (BHMF). Alternatively, the reduction of HMF leads also to the production of BHMF. This reaction is simpler but its industrial application so far has been limited due to the low availability of HMF. Synthesis of furan-2,5-dicarboxylic acid (FDA) can be carried out by oxidation of the alcohol and aldehyde groups in HMF, among other methods. All these furan biobased building block have been explored during the last decades for the synthesis of different sorts of polymers (Gandini 2008; Gandini and Belgacem 1997; Gandini et al. 2009b). As an example, the furan homologous of polyethylene terephthalate (PET) has been chemically synthesized (Gandini et al. 2009b). Synthesis of poly(2,5-ethylene furandicarboxylate) (PEF) from FDA dichloride and dimethyl ester and ethylene glycol, reached a DP between 250-300 by Sb_2O_3 catalyzed reaction in the melt at 220 °C and vacuum (Gandini et al. 2009b). The product had properties comparable to PET (i.e. thermostability, glass transition and crystallinity). Recently, a microbial route using and engineered *Pseudomonas* strain has been reported for the production of FDA by oxidation of HMF (Koopman et al. 2010). This process employs *Pseudomonas putida* S12, expressing a specific heterologous oxidase. This whole-cell biocatalyst provides a robust alternative to the poisoning-sensitive catalysts employed in chemical FDA synthesis. In addition, the conversion of the precursor HMF to FDA proceeds to completion, resulting in FDA titres up to 40 g l^{-1} with < 0.1% mono-carboxylated furans. In addition, the biocatalytic production of BHMF from hydroxyl methyl furfural with conversion superior to 90% using either enzymes or whole cells in a single step process has been described (Boeriu et al. 2008).

3.   ENZYME-CATALYZED POLYESTER SYNTHESIS

Polyester synthesis from biobased building blocks can be carried out in two basic ways: (i) condensation polymerization or polycondensation (PC) also called step-growth polymerization and (ii) ring-opening polymerization (ROP, Scheme 4). The chemical process for the synthesis of polyesters by polycondensation reaction involves heating the mixture of dialcohols and diacids or diesters at high temperatures in the presence of organometallic or acid catalyst. To allow the progress in this equilibrium reaction towards the formation of the polymer, the formed water or alcohol must be removed by reduced pressure, azeotropic distillation, or in the presence of water removal agents such as molecular sieves (Varma et al. 2005). The high temperatures used lead often to undesired side reactions such as degradation of the substrate, formation of intermolecular bonds, and oxidation of the final product with the consequent colour development, among others (Moore and Kelly 1978; Noordover et al. 2007; Noordover et al. 2006). In addition, chemical catalysts are organometallic compounds that could leave residuals in the final products. Enzyme-catalyzed polymerization, in contrast to chemically catalyzed polymerization, is a benign process, can be carried out at low pressures and temperatures, and can be regio-, enantio-, and stereoselective. These advantages allow the synthesis of chiral polymers with exceptional features. Typically the enzyme-catalyzed reactions are performed using lipases (or esterase). The advances on enzymatic polycondensation (PC) and ring-opening polymerization (ROP) will be further discussed.
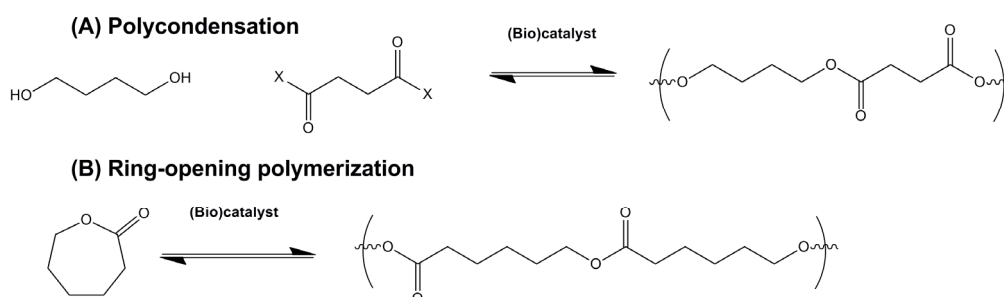
Scheme 4 Polyester synthesis via (A) polycondensation and (B) Ring-opening polymerization.

4. ENZYMATIC POLYCONDENSATION (PC)

PC involves the use of either free diacids or activated carboxyl derivatives (halo-, alkyl-, alkylene-) and diols (Scheme 4A). *In vitro* enzyme catalyzed synthesis of polyester was reported for the first time in 1984 using lipase from several organisms for the synthesis of ester and lactones (Gatfield and Sand 1984). Since then the number of publications on enzymatic synthesis of polyesters increased significantly. Lipases and esterases have been the classical enzymes used for the biobased synthesis of polyesters, although, cutinase and serine-protease have been reported as potential biocatalyst (Gatfield and Sand 1984; Zaks and Klibanov 1988). Binn *et al.* reported the enzyme-catalyzed reaction of adipic acid and BDO. The authors found a polymerization degree of 20 (M_w ~ 4172-4645 Da), and low polydispersity index (1.11) (Binns et al. 1993). The use of free diacid-diol system with dimethyl ester-dialcohol was compared. Transesterification performed under equivalent conditions using dimethyl adipate was substantially slower than the free-diacid system, generating species over 7100 Da but with a lower average molecular weight, M_w 2343 Da. In addition, enzyme-catalyzed esterification can also be carried out by modification of the leaving groups. For example, the enzymatic synthesis of polyesters from vinyl esters with diols has been studied (Kline et al. 2001). CALB-catalyzed polymerization of divinyl adipate and BDO led to formation of a high molecular weight product 20.000 Da (DP=100). Furthermore, models to explain the effect of the enzyme and substrate concentration, the length of the initial substrates (adipate, BDO and oligomers obtained from them), and substrate stoichiometry on the reaction behaviour have been developed (Chaudhary et al. 1998). Most of the enzyme-catalyzed polymerizations are carried out in liquid medium, however, the synthesis of several diacids and dialcohols in a free-solvent has also been reported (Kobayashi et al. 1998). In all the solvent-based biocatalyzed systems, the nature of the solvent in terms of polarity influences the activity and stability of the enzyme to a large extent (Cabral et al. 1994). The dielectric constant of the medium would alter the residues on the surface of the

enzyme, altering the native configuration with the consequent loss of the enzymatic synthetic capacity. Hence, properties of the solvents such as the dielectric constant as well as the water/octanol partition coefficient (log(P)) have been used as a mean to characterize the catalytic efficiency of the lipases in polyesterification reactions. As a general rule, the application of non-polar solvents promotes the synthesis of the polyesters. For example, the synthesis of poly(1,6-hexanediyl succinate) in the presence of porcine pancreas lipase was tested using acetonitrile (log(P)=-0.33), toluene (log(P)=2.5), and ethyl benzene (log(P)=2.6). Almost total substrate conversion was reached when ethyl benzene and toluene were used as reaction medium, while the reaction performed in acetonitrile exhibited a conversion lower than 80% (Berkane et al. 1997). Similar solvent effects have been observed in the synthesis of polyesters during the polymerization of linear ω-hydroxyesters in the presence of porcine pancreas lipase (Knani 1993). The water/octanol partition coefficient and the degree of polymerization exhibited a direct relation. The more polar solvents allow the formation of dimers, while the reaction performed in non-polar solvents led to oligomers with a DP of 10. The typical reaction medium for the synthesis of polyesters has been toluene, due to the easy dissolution of the substrate, availability, and low polarity, even though the use of ionic liquids, supercritical carbon dioxide, and emulsified systems has been also reported as media for polymerization (Blattner et al. 2006; Halling 1992; Halling 1984; Uyama et al. 2002; Yang and Gulari 1994). The group of Halling *et al.* has studied the effect of water in enzyme-catalyzed polymerization carried out in dried hydrophobic organic solvent. Aspects such as water activity and partition coefficient in water-solvent systems were discussed (Halling 1992; Halling 1984; Halling 1989; Halling 1990; Halling 1994; Kvittingen 1994; Kvittingen et al. 1992). The effect of water is mostly studied in terms of water activity (a_w), although, control of water activity during a reaction is neither easy at laboratory nor at industrial scale. The water is needed into the reaction system to keep the enzyme active, although high concentrations of water (or a_w) are in detriment of the enzyme catalytic capacity.

Reaction temperature is also an important parameter that determines the conversion of the substrate in enzyme-catalyzed polymerization. Gandhi *et al*. found that temperature combined with polar solvents contributes to the thermal deactivation of lipase from *Mucor miehei* (<u>Zaks and Klibanov 1985</u>). Temperature affects the native structure of the enzymes by promoting the unfolding of the molecule with the consequent disruption of the forces that maintain its structure (including hydrogen-bonding, ionic, van der Waals, and hydrophobic interactions) and evidently with the reduction of the catalytic performance (<u>Gandhi et al. 2000</u>).

Cyclization is a specific case of polycondensation. The enzymatic synthesis of CEOs occurs in both, chemical and enzymatic-catalyzed reactions. In general, cyclization reactions can be divided in two groups, (i) homocondensation of α,ω-hydroxy acids and (ii) heterocondensation of diacid (diester) and diol. The former one has been more widely studied due to the importance of these CEOs in polymer industry together with the easy solubility in conventional reaction media. The factors that affect the enzymatic cyclization are similar for both cases. In 1984 Gatfield *et al.* patented the first methodology for synthesis of CEOs derived from 15-hydroxypentadecanoic using esterase from *Mucor miehei* (<u>Gatfield and Sand 1984</u>). Later Makita *et al.* explored the cyclization of 10-hydroxydecanoate in non-polar solvents with different lipases and solvents, being the most efficient (yield >70%) porcine pancreas lipase and *Pseudomonas spp.* lipase (<u>Makita et al. 1987</u>). Similarly, enzymatic cyclization of α,ω-di-acids (C4-C14) and di-ols (C7-C18) with lipases of different origins in non-polar solvents was promoted at low substrate concentration and high temperatures (<u>Zhi-wei and Charles J. 1988</u>; <u>Zhi-wei et al. 1988</u>). The cyclization reaction can be accompanied by the formation of linear ester oligomers (LEOs). Lalot *et. al.* determined by kinetic studies based on the molecular model of Jacobson-Stockmaeyer that CEOs formation in lipase-catalyzed reactions decreases by increasing the length of the CEO (<u>Berkane et al. 1997</u>; <u>Jacobson and Stockmayer 1950</u>; <u>Lalot and Marechal 2001</u>). In contrast, chemical cyclization of activated suberic acid (dichloride) with isosorbide using pyridine as catalyst and HCl

acceptor led to the formation of 100% CEOs with a substrate conversion of 96% (Kricheldorf et al. 2003). Studies on thermodynamically and kinetically controlled cyclization have been addressed, as well as the ring - ring and the chain - ring equilibrium (Kricheldorf 2003; Kricheldorf and Schwarz 2003). The authors states that an ideal thermodynamically controlled polycondensation is characterized by three main aspects: (1) the ring-chain equilibria automatically include ring-ring equilibria, which become decisive for the thermodynamical properties of the system at high conversions, (2) at 100% conversion, all reaction products are cycles, and (3). the chain growth is limited by the thermodynamical properties of the ring-ring equilibrium (Kricheldorf 2003; Kricheldorf and Schwarz 2003).

In summary there are several factors that trigger the enzymatic formation of CEOs over LEOs: (i) equimolar substrates ratio, (ii) low substrate concentrations, (iii) affinity biocatalyst - substrate, (iv) type of the reaction media, and (v) efficiency in the removal of by-products such as water and alcohols.
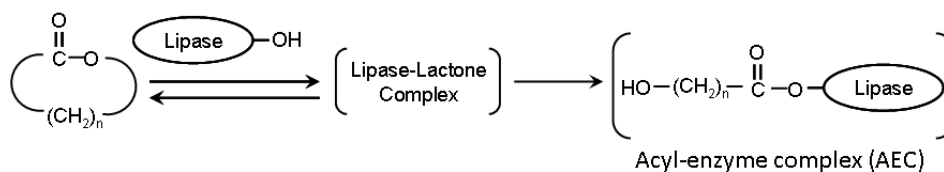
5. ENZYMATIC RING-OPENING POLYMERIZATION (ROP).

ROP (Scheme 4B) in the polyester synthesis has been extensively studied and several papers have reviewed the characteristics and limitations in enzyme-catalyzed reactions (Kobayashi 2010; Kobayashi et al. 2006; Kricheldorf et al. 2003). The first reported studies on enzymatic ROP were done using ε-caprolactone and δ-valerolactone as building blocks. Both lactones were polymerized by *Pseudomona fluorescens* lipase at several temperatures. The former one reached a conversion of 92% and a molecular weight of 7.700 Da when polymerized at 70 ^{o}C, while the latter did not show significant differences in the final product molecular weight (1600 Da at 45 ^{o}C and 1900 Da at 60 ^{o}C) with a monomer conversion of 95% for both conditions (Uyama and Kobayashi 1993). Knani *et al*. studied the influence of several variables in the ROP of ε-caprolactone (Knani 1993). The reaction was performed in hexane and methanol was used as nucleophile initiator. The reaction yield (94-95%) was similar for all monomer/methanol ratios

(50/1, 3/1, and 1/1), although the average molecular weight of the final polymer decreased with the increase of the methanol concentration. Albertsson and Srivastava published a review with an extensive list of references on ROP of lactones such as β-butyrolactone, γ-butyrolactone, ε-caprolactone, α-methyl-ε-caprolactone, γ-methyl-ε-caprolactone, γ-ethyl-ε-caprolactone, γ-propyl-ε-caprolactone, γ-caprolactone, δ-caprolactone, δ-decalactone, 12-dodecanolide, α-methyl-12-dodecanolide, and δ-dodecalactone among others (Albertsson and Srivastava 2008). They showed that enzyme-catalyzed reactions via ROP have both high molecular weight and high monomer conversion, and therefore could compete with chemical-catalyzed reactions with the additional advantages of enzyme-catalyzed systems. The common synthesis of PLA involves the production of the lactic acid lactone - lactide - with the subsequent polymerization using chemical-based catalysts via ROP. Alternatively, ROP of lactide with lipase PS in the presence of either water or methanol as initiators has been applied. The final product reached a molecular weight of 126 kDa with total monomer (*meso*-lactide) conversion (Matsumura et al. 1997). The mechanism for the lipase-catalyzed ROP involves the initial ring opening with formation of acyl-enzyme complex (AEC) at the serine residue of the catalytic site of lipase (Scheme 5). The process can be divided in two main stages (i) initiation and (ii) propagation. The attack by the nucleophile (i.e. water or alcohol) on the acyl carbon of the AEC yields the ω-hydroxycarboxylic acid, which is the expected propagation species (initiation step). Subsequently, AEC is attacked by the terminal hydroxyl group of the growing polymers (propagation) leading to the formation of an additional unit into the polymer chain (Kobayashi 2010; Matsumura 2002).

6. *Candida antarctica* LIPASE B (CALB).

In most of the studies related to the synthesis of polyesters by polycondensation and ring opening polymerization, a lipase was used as catalyst. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of ester chemical bonds in water-insoluble lipid substrates. Naturally most lipases act at a specific position on the glycerol backbone of lipid substrates (Garrett and Grisham 2008).
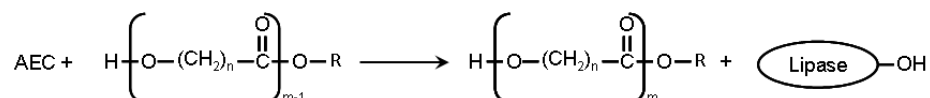
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(i) Initiation
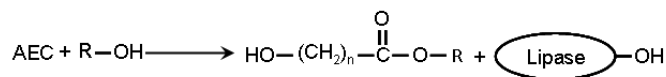
R = H, Alkyl chain

(ii) Propagation

Scheme 5 Ring-opening polymerization mechanism.

Lipases has been widely applied in non-conventional media not only at laboratory scale, but also in industrial resolution of racemic mixtures and pharmaceuticals i.e. production of chiral compounds (Anderson et al. 1998). Among the commercial lipases, *Candida antarctica* lipase B (CALB) is found to be highly attractive for organic synthesis due to the stereo-, regio-, and enantioselectivity and the capacity to accept a wide range of substrates (Kobayashi 2010). CALB has been used in polymerization reactions of sensitive moieties such as vinyl and (met)acrylates. Furthermore, CALB accepts nucleophiles such as amino-, hydroperoxy-, amide- and thiol-groups (Anderson et al. 1998). The tridimensional and primary structure of CALB is well known (Scheme 6). CALB is constituted by 317 amino acids with a molecular weight of 33.273 Da and an isoelectric point of 6.0. A fascinating feature of lipases is the presence of a mobile lid covering the catalytic site. It has been demonstrated that the opening of the lid led to the active site accessibility and the formation of the oxyanion hole that is involved in the catalytic mechanism (Brzozowski et al. 1991). The lid is a key feature of lipases to form hydrophobic environment for catalytic processes and therefore rules the catalytic activity of *Candida antarctica* lipase B (Uppenberg et al. 1994). The active centre has a catalytic triad serine (Ser105), histidine (His224), and aspartic acid (Asp187) similar to

serine proteases. CALB exhibits two channels at the active site pocket. It is assumed that they are the housing of the acyl and the alcohol leaving groups during natural hydrolysis of fatty acid glycerol esters. The broader channel is associated to the acyl chain, while the nucleophile moiety (alcohol) is associated with the narrower one (Uppenberg et al. 1995).
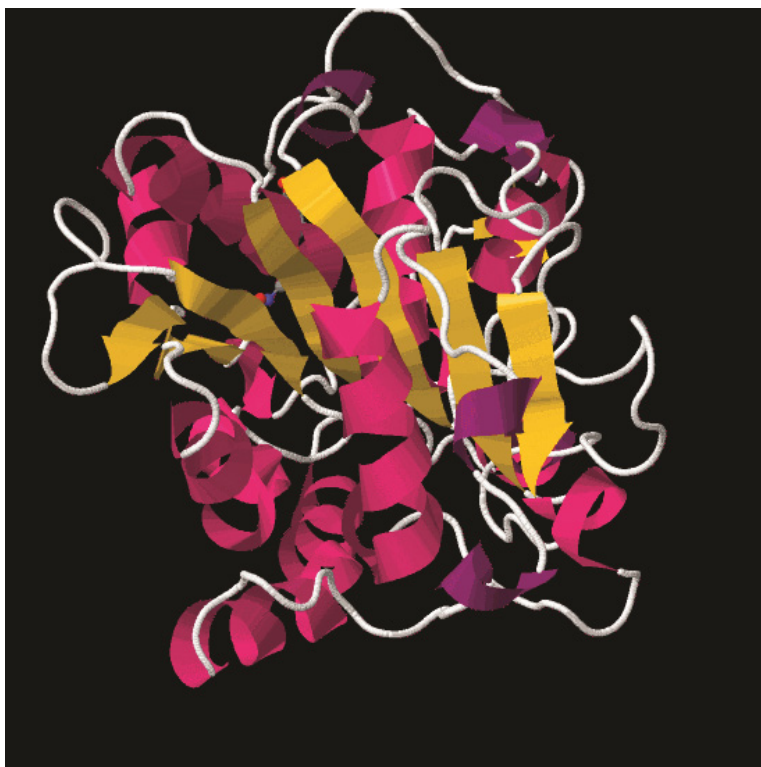
Scheme 6 Tridimentional structure of *Candida antarctica* lipase B. RCSB Protein Data Bank Uppenberg *et al.* (1995).

The general catalytic mechanism of CALB involving the catalytic triad is depicted in Scheme 7. The imidazole ring of the histidine catalytic triad in the free enzyme forms two hydrogen bonds: one hydrogen bond with the aspartic acid residue and the other one with the serine residue. During the esterification reaction the acyl substrate disrupts the hydrogen bond between the serine residue and the histidine residue forming the acyl-enzyme complex (AEC, acylation step) with the concomitant leaving of the weak nucleophile (R-OH). The oxyanion hole stabilizes the docking of the substrate into the

catalytic site of the enzyme. The transition-state is stabilized by four hydrogen bonds between the N-H-groups of the oxyanion hole and the substrate (tetrahedral intermediate). A covalent bond between the oxygen from the serine residue and the carbon present at the carbonyl group of the acid (ester) is formed. The subsequent step is the nucleophilic attack of the alcohol. The histidine residue gets involved in the destabilization of the acyl-enzyme complex, such destabilization promotes the nucleophilic attack of the hydroxyl substrate (nucleophile) with the formation of the ester bond and the simultaneous regeneration of the hydroxyl group at the serine residue (deacylation step). The two nucleophiles are present in the reaction, water and alcohol, therefore the basicity of the hydroxyl containing substrate and the spatial configuration determines also the progress of the esterification (Kobayashi 2010).

The application of enzymes for polyester synthesis has many advantages: polymerization can be performed under mild conditions, regio-, stereo- and enantioselectivity can be induced in the end products, opening novel directions towards speciality polymers, and the enzymatic process is environmentally friendly. The lipases can be used in non-conventional media and can catalyze other reactions than the natural ones. Application of lipase in polyester synthesis makes use of its ability to catalyze esterification reaction in dry organic solvents.

7.   Research aim and thesis outline

The potential of enzyme catalysis for the synthesis of polyesters via both polycondensation and ROP has been showed; as well the potential for the synthesis of cyclic ester oligomers. Advantages of ROP vs. polycondensation open the question for the production of CEOs as starting materials for polyesters via ROP.
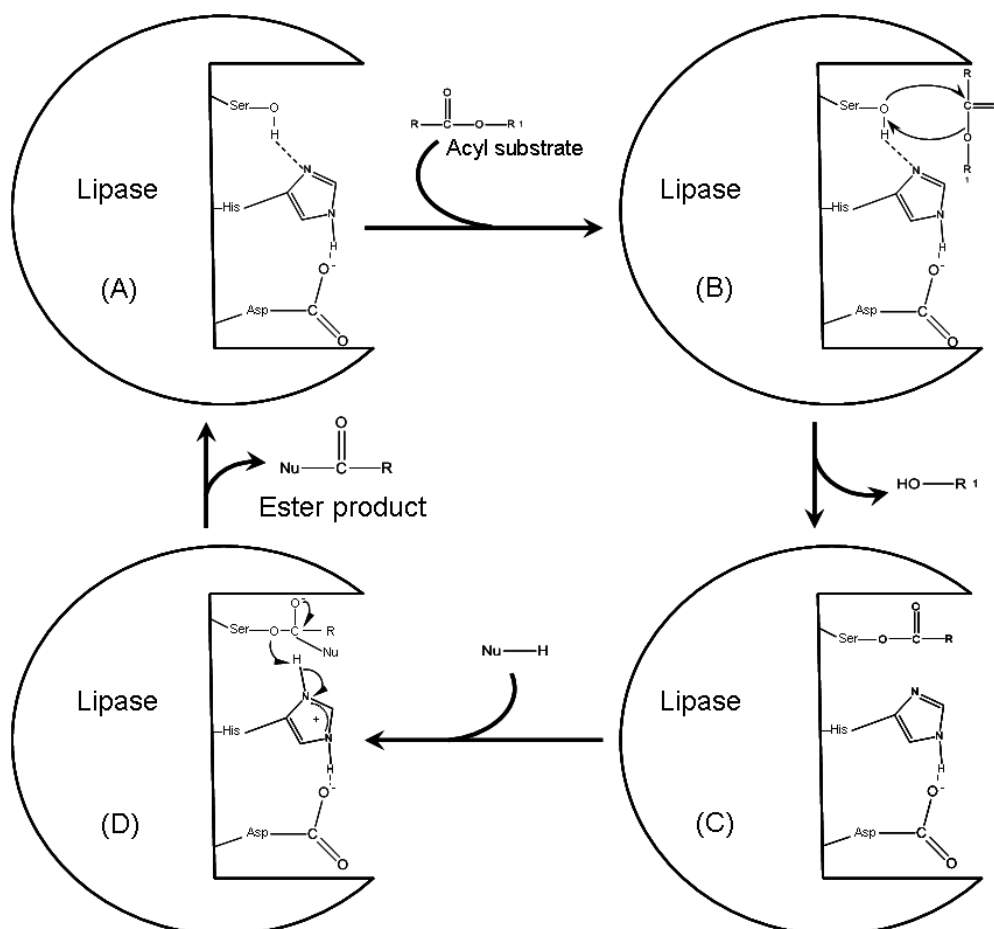
Scheme 7 Esterification mechanism of *C. antarctica* lipase B. (A) CALB in the native state. (B) acylation of CALB. (C) acyl-enzyme complex configuration. (D) CALB deacylation step.

The research on the biobased building blocks above discussed is triggered either for the special features that these substrates confer to the synthesized polyesters or for the difficulties found by conventional polymerization routes (i.e. PC). For example, polyesters prepared from DAH are semi-crystalline materials and have molecular weights and thermal properties suitable for thermoset coating applications. In addition, these materials show good chemical and mechanical stability. Furthermore, more specialized polymers such as liquid crystalline polymers can be synthesized from DAH. The properties of the final polymer are dependent of the chirality of the substrate (i.e. endo- and exo-hydroxyl groups). Polyesters from FDA have been already demonstrated to have

similar properties as PET. The synthesis of furan derivatives via polycondensation is problematic because of the thermolability of these biobased building blocks; therefore, it is interesting to produce CEOs that can be applied as raw material for ROP. Also, enzyme-catalyzed synthesis of polyesters from succinic acid and BDO leads to products with much higher molecular weight and crystallinity than chemical-catalyzed polycondensations. Therefore, the goal of this thesis was to evaluate the possibilities to produces oligoesters, especially cyclic ester oligomers (CEOs) from the biobased building blocks above discussed. The combination of very flexible substrates such as 1,4-butanediol (BDO), adipic acid, sebacic acid, and succinic acid with more rigid ones such as 2,5-furandicarboxylic acid (FDA), 1,4:3,6-dianhydrohexitols (DAH, isomannide, isosorbide, and isoidide), 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA), and 2,5-bis-(hydroxymethyl) furan (BHMF) in the presence of *Candida antarctica* lipase B have been explored.

Chapter 2 addresses a procedure to synthesize ester oligomers from non-activated succinic acid and BDO. A model for the formation of CEOs is proposed. The use of pulse fed-batch bioreactor is demonstrated as a manner to improve the prevalence of CEOs over LEOs. In addition, a comparison between the system with and without water removal is conducted.

Chapter 3 focuses on the enantioselectivity of CALB in enzyme-catalyzed oligomerization of the three DAHs with succinic acid. Different reaction conditions are studied by a factorial experimental design. The enantioselectivity found by CALB was also confirmed by imprinted docking modelling of the enzyme with the three sugar-based substrates. An analysis of the products in time is also shown.

Chapter 4 explores the possibilities to synthesize oligoesters from FDA, BHMF, sebacic acid, adipic acid, succinic acid and HMFA. Comparison among the reaction yields and the diversity of products for the different combination of these building blocks is presented.

Chapter 5 exposes the utilization of different bioreactor operation conditions to evaluate the formation of CEOs with all the substrates used in the previous chapters. We evaluate fed-batch, pulse fed-batch, plug-flow, and batch operation as bioreactor models. Productivities, yields of reaction and type of products are analysed.

Chapter 6 combines the conclusions of the preceding chapter into a general conclusion and from this prospective discusses the possibilities for further applications and current limitations that open new areas of research in the field of CEOs synthesis for ROP production.

LIST OF SYMBOLS

Summary of the symbols and abbreviations used in this thesis:

A   The dicarboxyl monomer

AA_n Linear ester oligomer dicarboxyl terminal with DP = n; for example, AA_3 is the oligoester ABABABA (DP = 7)

AB_n Linear ester oligomer hydroxyl and carboxyl terminal with DP = 2n+1; for example, AB_3 is the oligoester ABABAB (DP = 6)

B   The dihydroxyl monomer

BB_n Linear ester oligomer diol terminal with DP = 2n+1; for example, BB_3 is the oligoester BABABAB (DP = 7)

BDO   1,4-Butanediol

BHMF   2,5-bis-(hydroxymethyl) furan

CALB   Immobilized lipase B from *Candida antarctica*

CEO_n Cyclic ester oligomer of DP 2n

CEOs   Cyclic ester oligomers

DAH   1,4:3,6-dianhydrohexitols

DP   Degree of polymerization according to the first IUPAC definition (*i.e.* AB_1 has a DP = 2 and AA_2 has a DP=5). "*The number of monomeric units in a macromolecule or oligomer molecule, a block or a chain*" (IUPAC, 1997, see http://old.iupac.org/goldbook/D01569.pdf)

FDA   2,5-Furandicarboxylic acid

HMFA   5-(hydroxymethyl)furan-2-carboxylic acid

LEO_n Linear ester oligomer of DP 2n(+1)

LEOs   Linear ester oligomers

PBS   poly(butylene succinate)

PC   Polycondensation

ROP   Ring-opening polymerization

REFERENCES

Albertsson A-C, Srivastava RK. 2008. Recent developments in enzyme-catalyzed ring-opening polymerization. Advanced Drug Delivery Reviews 60(9):1077-1093.

Anderson EM, Larsson KM, Kirk O. 1998. One Biocatalyst - Many Applications: The Use of *Candida antarctica* B-Lipase in Organic Synthesis. Biocatalysis and Biotransformation 16(3):181-204.

Berkane C, Mezoul G, Lalot T, Brigodiot M, Marechal E. 1997. Lipase-Catalyzed Polyester Synthesis in Organic Medium. Study of Ring-Chain Equilibrium. Macromolecules 30(25):7729-7734.

Binns F, Roberts SM, Taylor A, Williams F. 1993. Enzymic polymerisation of an unactivated diol/diacid system. J. Chem. Soc., Perkin Trans. 1:899-904.

Blattner C, Zoumpanioti M, Kroner J, Schmeer G, Xenakis A, Kunz W. 2006. Biocatalysis using lipase encapsulated in microemulsion-based organogels in supercritical carbon dioxide. The Journal of Supercritical Fluids 36(3):182.

Boeriu CG, Es DSv, Frissen AE, Haveren Jv. 2008. Chemoenzymatic synthesis of 2,5-disubstituted furan monomers for novel polyesters. Proceedings of the "Bioproduction" symposium. Thessaloniki (Greece). p 131-134.

Brzozowski AM, Derewenda U, Derewenda ZS, Dodson GG, Lawson DM, Turkenburg JP, Bjorkling F, Huge-Jensen B, Patkar SA, Thim L. 1991. A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. 351(6326):491-494.

Cabral JMS, Best D, Boross L, Tramper J. 1994. Applied Biocatalysis. Amsterdam: Hardwood Academic Publishers. 478 p.

Carde T. 2001. New Continuous Isosorbide Production From Sorbitol. US Department of Energy (USA).

Castellan A, Bart JCJ, Cavallaro S. 1991. Industrial production and use of adipic acid. Catalysis Today 9(3):237-254.

Chaudhary AK, Beckman EJ, Russell AJ. 1998. Nonequal reactivity model for biocatalytic polytransesterification. AIChE Journal 44(3):753-764.

Corma A, Iborra S, Velty A. 2007. Chemical Routes for the Transformation of Biomass into Chemicals. Chemical Reviews 107(6):2411-2502.

Datta R, Glassner D, Jain M, Vick Roy J; Mitsui Toatsu Chemicals, Incorporated USPTO 5,168,055, assignee. 1992. Fermentation and purification process for succinic acid United States of America.

Delhomme C, Weuster-Botz D, Kühn FE. 2009. Succinic acid from renewable resources as a C4 building-block chemical : a review of the catalytic possibilities in aqueous media. Green Chemestry 11:13-26.

Fenouillot F, Rousseau A, Colomines G, Saint-Loup R, Pascault JP. 2010. Polymers from renewable 1,4:3,6-dianhydrohexitols (isosorbide, isomannide and isoidide): A review. Progress in Polymer Science 35(5):578-622.

Flèche G, Huchette M. 1986. Isosorbide. Preparation, Properties and Chemistry. Starch - Stärke 38(1):26-30.

Galbis J, García-Martín M. 2010. Synthetic Polymers from Readily Available Monosaccharides. [Without Title]. p 1-30.

Gandhi Nn, Patil Ns, Sawant Sb, Joshi Jb. 2000. Lipase-Catalyzed Esterification. Catalysis Reviews 42(4):439-480.

Gandini A. 2008. Polymers from Renewable Resources: A Challenge for the Future of Macromolecular Materials. Macromolecules 41(24):9491-9504.

Gandini A, Belgacem MN. 1997. Furans in polymer chemistry. Progress in Polymer Science 22(6):1203-1379.

Gandini A, Coelho D, Gomes M, Reis B, Silvestre A. 2009a. Materials from renewable resources based on furan monomers and furan chemistry: work in progress. Journal of Materials Chemistry 19(45):8656-8664.

Gandini A, Silvestre AJD, Neto CP, Sousa AF, Gomes M. 2009b. The furan counterpart of poly(ethylene terephthalate): An alternative material based on renewable resources. Journal of Polymer Science Part A: Polymer Chemistry 47(1):295-298.

Garrett RH, Grisham CM. 2008. Biochemistry. Hall P, editor: Prentice Hall. 1059 p.

Gatfield I, Sand T; Haarmann & Reimer GmbH, assignee. 1984. Enzyme-mediated synthesis of esters and lactones. USPTO 4.451.565.

Glassner D, Datta R; Mitsui Toatsu Chemicals, Incorporated USPTO 5,143,834, assignee. 1992. Process for the production and purification of succinic acid United States of America.

Gross RA, Kalra B. 2002. Biodegradable Polymers for the Environment. Science 297(5582):803-807.

Guettler M, Jain M, Bhupendra S; Mitsui Toatsu Chemicals, Incorporated USPTO 5,504,004, assignee. 1996a. Process for making succinic acid, microorganisms for use in the process and methods of obtaining the microorganisms United States of America.

Guettler M, Jain M, Rumler D; Mitsui Toatsu Chemicals, Incorporated USPTO 5,573,931, assignee. 1996b. Method for making succinic acid, bacterial variants for use in the process, and methods for obtaining variants United States of America.

Halling P. 1992. Salt hydrates for water activity control with biocatalysts in organic media. Biotechnology Techniques 6(3):271.

Halling PJ. 1984. Effects of water on equilibria catalysed by hydrolytic enzymes in biphasic reaction systems. Enzyme and Microbial Technology 6(11):513.

Halling PJ. 1989. Organic liquids and biocatalysts: theory and practice. Trends in Biotechnology 7(3):50.

Halling PJ. 1990. High-affinity binding of water by proteins is similar in air and in organic solvents. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1040(2):225.

Halling PJ. 1994. Thermodynamic predictions for biocatalysis in nonconventional media: Theory, tests, and recommendations for experimental design and analysis. Enzyme and Microbial Technology 16(3):178-206.

Hofvendahl K, Hahn–Hagerda B. 2000. Factors affecting the fermentative lactic acid production from renewable resources. Enzyme and Microbial Technology 26(87-

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107).

Huh YS, Jun Y-S, Hong YK, Song H, Lee SY, Hong WH. 2006. Effective purification of succinic acid from fermentation broth produced by Mannheimia succiniciproducens. Process Biochemistry 41(6):1461-1465.

Jacobson H, Stockmayer WH. 1950. Intramolecular Reaction in Polycondensations. I. The Theory of Linear Systems. The Journal of Chemical Physics 18(12):1600-1606.

Kline BJ, Lele SS, Beckman EJ, Russell AJ. 2001. Role of diffusion in biocatalytic polytransesterification. AIChE Journal 47(2):489-499.

Knani D, Arie L. Gutman David H. Kohn. 1993. Enzymatic polyesterification in organic media. Enzyme-catalyzed synthesis of linear polyesters. I. Condensation polymerization of linear hydroxyesters. II. Ring-opening polymerization of &epsis;-caprolactone. Journal of Polymer Science Part A: Polymer Chemistry 31(5):1221-1232.

Kobayashi S. 2010. Lipase-catalyzed polyester synthesis – A green polymer chemistry. Proceedings of the Japan Academy, Series B 86(4):338-365.

Kobayashi S, Ritter H, Kaplan D, Matsumura S. 2006. Enzymatic Synthesis of Polyesters via Ring-Opening Polymerization. Enzyme-Catalyzed Synthesis of Polymers: Springer Berlin / Heidelberg. p 95-132.

Kobayashi S, Takeya K, Suda S, Uyama H. 1998. Lipase-catalyzed ring-opening polymerization of medium-size lactones to polyesters. Macromolecular Chemistry and Physics 199(8):1729-1736.

Koopman F, Wierckx N, de Winde JH, Ruijssenaars HJ. 2010. Efficient whole-cell biotransformation of 5-(hydroxymethyl)furfural into FDCA, 2,5-furandicarboxylic acid. Bioresource Technology 101(16):6291-6296.

Kricheldorf HR. 1997. "Sugar diols" as Building Blocks of Polycondensates. Polymer Reviews 37(4):599-631.

Kricheldorf HR. 2003. Macrocycles. 21. Role of Ring-Ring Equilibria in Thermodynamically Controlled Polycondensations. Macromolecules 36(7):2302-2308.

Kricheldorf HR, Sabe C, Schwarz G, Krüger RP. 2003. Macrocycles 27: Cyclic aliphatic

polyesters of isosorbide. Journal of Polymer Science Part A: Polymer Chemistry 41(21):3414-3424.

Kricheldorf HR, Schwarz G. 2003. Cyclic Polymers by Kinetically Controlled Step-Growth Polymerization. Macromolecular Rapid Communications 24(5-6):359-381.

Kvittingen L. 1994. Some aspects of biocatalysis in organic solvents. Tetrahedron 50(28):8253-8274.

Kvittingen L, Sjursnes B, Anthonsen T, Halling P. 1992. Use of salt hydrates to buffer optimal water level during lipase catalysed in synthesis in organic media: a practical procedure for organic chemists. Tetrahedron 48(13):2793-2802.

Lalot T, Marechal E. 2001. Enzyme-catalyzed Polyester Synthesis. J. Polymeric Materials 50:267-286.

Lee PC, Lee WG, Kwon S, Lee SY, Chang HN. 1999. Succinic acid production by Anaerobiospirillum succiniciproducens: effects of the H2/CO2 supply and glucose concentration. Enzyme and Microbial Technology 24(8-9):549-554.

Lee PC, Lee WG, Lee SY, Chang HN. 2001. Succinic acid production with reduced by-product formation in the fermentation of Anaerobiospirillum succiniciproducens using glycerol as a carbon source. Biotechnology and Bioengineering 72(1):41-48.

Lin H, Bennett GN, San K-Y. 2005a. Fed-batch culture of a metabolically engineered Escherichia coli strain designed for high-level succinate production and yield under aerobic conditions. Biotechnology and Bioengineering 90(6):775-779.

Lin H, Bennett GN, San K-Y. 2005b. Metabolic engineering of aerobic succinate production systems in Escherichia coli to improve process productivity and achieve the maximum theoretical succinate yield. Metabolic Engineering 7(2):116-127.

Makita A, Nihira T, Yamada Y. 1987. Lipase catalyzed synthesis of macrocyclic lactones in organic solvents. Tetrahedron Letters 28(7):805-808.

Matsumura S. 2002. Enzyme-Catalyzed Synthesis and Chemical Recycling of Polyesters. Macromolecular Bioscience 2(3):105-126.

Matsumura S, Mabuchi K, Toshima K. 1997. Lipase-catalyzed ring-opening polymerization

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of lactide. Macromolecular Rapid Communications 18(6):477-482.

Moore JA, Kelly JE. 1978. Polyesters Derived from Furan and Tetrahydrofuran Nuclei. Macromolecules 11(3):568-573.

Moreau C, Belgacem MN, Gandini A. 2004. Recent Catalytic Advances in the Chemistry of Substituted Furans from Carbohydrates and in the Ensuing Polymers. Topics in Catalysis 27(1):11-30.

Niu W, Draths KM, Frost JW. 2002. Benzene-Free Synthesis of Adipic Acid. Biotechnology Progress 18(2):201-211.

Noordover BAJ, Duchateau R, van Benthem RATM, Ming W, Koning CE. 2007. Enhancing the Functionality of Biobased Polyester Coating Resins through Modification with Citric Acid. Biomacromolecules 8(12):3860-3870.

Noordover BAJ, van Staalduinen VG, Duchateau R, Koning CE, van B, Mak M, Heise A, Frissen AE, van Haveren J. 2006. Co- and Terpolyesters Based on Isosorbide and Succinic Acid for Coating Applications: Synthesis and Characterization. Biomacromolecules 7(12):3406-3416.

Rohl H, Eversmann W, Hegenberg P, Hellemanns G; 1970. Process For The Continuous Production Of Saturated Aliphatic Dicarboxylic Acids

Sanborn A; 2008. Process for the production of anhydrosugar alcohols. US.

Uppenberg J, Hansen MT, Patkar S, Jones TA. 1994. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from Candida antarctica. Structure 2(4):293-308.

Uppenberg J, Oehrner N, Norin M, Hult K, Kleywegt GJ, Patkar S, Waagen V, Anthonsen T, Jones TA. 1995. Crystallographic and molecular-modeling studies of lipase B from Candida antarctica reveal a stereospecificity pocket for secondary alcohols. Biochemistry 34(51):16838-16851.

Uyama H, Kobayashi S. 1993. Enzymatic Ring-Opening Polymerization of Lactones Catalyzed by Lipase. Chemistry Letters 22(7):1149-1150.

Uyama H, Takamoto T, Kobayashi S. 2002. Enzymatic Synthesis of Polyesters in Ionic Liquids. Polymer Journal 34(2):94-96.

Varma IK, Albertsson A-C, Rajkhowa R, Srivastava RK. 2005. Enzyme catalyzed synthesis of polyesters. Progress in Polymer Science 30(10):949.

Vasishtha A, Trivedi R, Das G. 1990. Sebacic acid and 2-octanol from castor oil. Journal of the American Oil Chemists' Society 67(5):333-337.

Willke T, Vorlop KD. 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. Applied Microbiology and Biotechnology 66(2):131-142.

Yang C-L, Gulari E. 1994. Enzymic Esterification of Diols in Reverse Micellar Media. Biotechnology Progress 10:269-276.

Zaks A, Klibanov AM. 1985. Enzyme-catalyzed processes in organic solvents. Proceedings of the National Academy of Sciences of the United States of America 82(10):3192-3196.

Zaks A, Klibanov AM. 1988. Enzymatic catalysis in nonaqueous solvents. J. Biol. Chem. 63(7):3194-3201.

Zeikus JG, Jain MK, Elankovan P. 1999. Biotechnology of succinic acid production and markets for derived industrial products. Applied Microbiology and Biotechnology 51(5):545-552.

Zhi-wei G, Charles J. S. 1988. Enzymic synthesis of macrocyclic lactones. J. Am. Chem. Soc. 110:1999-2001.

Zhi-wei G, Ngooi TK, Scilimati A, Fulling G, Sih CJ. 1988. Macrocyclic lactones via biocatalysis in non-aqueous media. Tetrahedron Letters 29(44):5583-5586.

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Biocatalytic Synthesis of Oligoesters from Succinic Acid and 1,4-Butanediol

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ABSTRACT

The lipase-catalyzed synthesis of cyclic ester oligomers from non-activated succinic acid (A) and 1,4-butanediol (B) in the presence of immobilized *Candida antarctica* lipase B was investigated. Batch and pulse fed-batch systems were implemented to increase the formation of cyclic ester products. The substrate conversions after 24 h were 86% and 95% under batch and fed-batch operation, respectively and the product of the reaction was, for both systems, a mixture of cyclic (CEOs) and linear (LEOs) ester oligomers. Fed-batch operation afforded a product containing 71% cyclic ester oligomers (CEOs) as compared with only 52% CEOs in batch operation. Cyclic ester oligomers accumulated as the reaction progressed, with the dimer CEO_1 the most preponderant product (i.e. 50% of the total products formed in fed batch operation). The pulse fed-batch operation system was superior to the batch operation not only because higher substrate conversion and more CEOs were obtained, but also because it resulted in products with higher degree of polymerization (DP up to 7). Cyclic ester oligomers are produced from the early stage of the reaction simultaneously with the linear ester oligomers by a ring-closure reaction on the active site of the enzyme, and not as a result of ring-chain equilibria.

1. INTRODUCTION

Enzymatic polymerization is a field of remarkable interest for the production of green plastics (biopolymers) with new properties and many potential applications in chemical, medical and pharmaceutical industry among others (Knani 1993). An important feature of these biopolymers supporting their future success is the fact that they can be synthesized from renewable resources in a sustainable manner. Polyesters, one important class of renewable biopolymers, are manufactured industrially via polycondensation (PC) and ring-opening polymerization (ROP). ROP has several advantages over PC, but the scarce availability and high manufacturing costs of cyclic ester oligomers (CEOs) at industrial scale has prevented its general application for polyester synthesis. Therefore, feasible technical-economical routes for CEOs synthesis are of great interest (Brugel and Di Cosimo 2005; Brugel and Dicosimo 2006; Knani 1993; Lalot and Marechal 2001; Matsumura 2002; Sugihara et al. 2006).

Several strategies have been studied for the synthesis of CEOs, including condensation of the monomers using metal complexes or enzymes as catalysts and by depolymerization of long chains (Berkane et al. 1997; Lalot and Marechal 2001; Matsumura 2002; Sugihara et al. 2006). Enzymatic synthesis of CEOs has the advantage of mild reaction conditions and high selectivity. The factors that can regulate (or promote) the cyclization reaction are the type of acid (ester) and alcohol, the type of enzyme, the substrate and enzyme concentrations, temperature, reaction medium, and removal of by-products. For example, the reaction between dimethyl terephthalate and diethylene glycol in the presence of *Candida antarctica* lipase (CALB) leads to the formation of linear ester oligomers (LEO) and one cyclic tetramer as the unique cyclic product (Hilker et al. 2008; Lavalette et al. 2002). The enzymatic cyclization of α,ω-di-acids (C4-C14) and di-ols (C7-C18) with lipases of different origins was carried out under different conditions. The authors reported that high dilution conditions, high temperatures and non-polar solvents promoted the synthesis of the macrocycles (Zhi-wei and Charles J. 1988). Osanai *et. al.*

reported CALB-catalyzed degradation of poly(3-hydroxy butanoate) (Osanai et al. 2003). The CALB-catalyzed reaction was carried out in toluene using 500 % CALB respect to poly(3-hydroxy butanoate) at 40 ^{o}C in toluene. The product was a mixture of CEOs and LEOs, where the LEOs amount 95%. Similarly, the CALB-catalyzed depolymerization of poly(butylene succinate) (PBS) and poly(caprolactone) toward the formation of CEOs as unique sort of product has been demonstrated (Kondo et al. 2008). The final mixture had mainly the dimer (CEO$_2$) and the trimer (CEO$_3$) when PBS was the substrate, while dimer was the main product when poly(caprolactone) was the substrate. Furthermore, The enzymatic cyclization of poly(hexamethylene succinate) and poly(octamethylene succinate) has been studied as equilibrium between CEOs and LEOs. The authors reported two interesting findings: (1) The logarithm of the molar cyclization equilibrium constant K (mol.L^{-1}) decreases proportional to the number of times (log(n)) that the repeating unit is present in the formed rings, (2) the formation of CEOs as a result of the hydrolysis and cyclization of LEOs was demonstrated. This finding fits with the equilibrium exposed by Jacobson-Stockmaeyer (Jacobson and Stockmayer 1950; Lalot and Marechal 2001).

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Recently the synthesis of PBS starting from a mixture of cyclic butylene succinate oligomers via ROP using *Candida antarctica* B lipase as biocatalyst was reported (Sugihara et al. 2006). The enzyme-synthesized PBS product had an average molecular weight of M_w 130 kDa and a polydispersity index of 1.6, which was significantly higher than that obtained in the same study for PBS produced by the direct polycondensation of the diol and dimethyl succinate by lipase catalysis , *i.e.* M_w of 45 kDa and an M_w/M_n of 3.7. The mixture of cyclic butylene succinate oligomers (DP = 4 - 10, M_n 390) was obtained by transesterification of dimethyl succinate with 1,4-butanediol using also immobilized lipase B from *Candida antarctica* (Novozym435) (100 wt.-%) in toluene at 90 ^{o}C for 48 h (Sugihara et al. 2006). Azim *et. al.* reported the CALB-catalyzed synthesis of PBS from diethyl succinate and BDO in toluene (Azim et al. 2006). The initial attempts of polymerization were conducted using the succinic acid and BDO with unsuccessful results

due to the low solubility of the acid in toluene, therefore the diethyl ester was preferred. The reaction performed in diphenyl ether allowed to reach an average molecular weight of 38 kDa with a polydispersity coefficient of 1.39. PBS and its copolymers have become important biopolymers due not only to the physical properties such as thermal stability, high heat deflection temperature, good toughness, but also due to the flexibility upon application on extrusion, injection, blowing and blister moulding. Therefore, the PBS and copolymers can be applied for the production of film, paper, laminate, sheets and tape, among others (Fujimaki 1998). Most of the commercial polymer based on PBS is industrially carried out by the polycondensation of succinic acid or its methyl ester and 1,4-butanediol (BDO) obtained from fossil resources, although it is possible to be obtained from renewable ones (Bechthold et al. 2008; Zeikus et al. 1999). The synthesis is carried out using chemical catalysts. Although, PBS exhibits good thermal and mechanical properties similar to polyethylene and polypropylene, the commercial PBS is usually a copolymer with other diacids, diols or eventually α,ω-hydroxyl acids copolymer, such as adipic acid, ethylene glycol, and ε-caprolactone (Cao et al. 2002; Fujimaki 1998; He et al. 2000; Mazzocchetti et al. 2009; Oishi et al. 2006). For example, Bionolle® is the commercial name of an aliphatic polyester produced by polycondensation of glycols or BDO in combination with succinic acid or adipic acid using a chemical-catalyzed reaction (Fujimaki 1998). Oishi *et. al.* reported the chemical-catalyzed synthesis of poly(butylene succinate) copolymers (Oishi et al. 2006). The polymerization was carried out from succinic acid in combination with diglycollic acid and 1,4-butanediol in the presence of titanium based chemical catalyst. The polymers produced exhibited number-average molecular weight higher than 65 kDa with an improvement of tensile properties due to the glycollate moiety of the synthesized polyester.

As mentioned before, the synthesis of PBS by enzymatic ROP from both purified cyclic butylene succinate dimer and crude cyclic ester oligomers with DP from 2 to 10 was successful, and PBS with high molecular weight and low polydispersity was obtained (Sugihara et al. 2006). The cyclic butylene succinate oligomers were synthesized by

enzymatic cyclization from dimethyl succinate and 1,4-butanediol. In this study, we report the biocatalytic synthesis of oligo(butyl succinate) from non-activated succinic acid and 1,4-butanediol (BDO) in a binary solvent system consisting of toluene and *tert*-butanol based medium using immobilized *Candida antarctica* lipase B (Novozym 435) as catalyst. A process for cyclic ester oligomers starting from non-activated succinic acid instead of the dimethyl or diethyl esters might be technological and economical

sustainable, since the biobased succinic acid produced by fermentation could be directly used for polymerization, without an additional esterification step. The polymerization of succinic acid with 1,4-butanediol with Novozym 435 at 65 ^{o}C in the binary solvent system produced 52% cyclic ester oligomers with DP 2 to 8, with the butylene succinate dimer as the major product, after 24h. When the reaction was conducted in a pulse fed-batch operation mode, the yield in CEOs was increased to 71%, and thus to a route to improve the prevalence of CEOs over LEOs is demonstrated. To the best of our knowledge, this is the first time that pulse fed-batch bioreactor has been reported as a way to improve the synthesis of CEOs in an enzyme-catalyzed reaction. A pathway for CEOs synthesis is proposed. A route to improve the prevalence of CEOs over LEOs is demonstrated and a mechanism for CEOs synthesis is proposed.

2. EXPERIMENTAL SECTION

2.1. Materials

2,5-dihydroxybenzoic acid (2,5-DHB), *n*-butyric acid, *n*-butanol, sodium acetate, molecular sieves and solvents were obtained from Sigma – Aldrich. Novozym$^{®}$ 435, the immobilized lipase B from *Candida antarctica* (Novozym 435) was donated by Novozyme (The Netherlands). In the text, the enzyme is referred as CALB.

2.2. Esterification Activity of immobilized *Candida antarctica* Lipase B (CALB)

The esterification activity was determined using *n*-butyric acid and *n*-butanol as substrates in toluene : *tert*-butanol solution (70:30 % wt) at 65 ^{o}C. The activity was based on the method reported by Kiran and collaborators (Kiran et al. 2000). 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 millimoles of butyric acid that are esterified per hour. The enzymatic activity of CALB at the conditions above mentioned was 0.06 UA.mg^{-1}.

2.3. Enzymatic oligo(butylene succinate) Synthesis

A typical enzymatic reaction was performed as follows: 5.9 mg (50 μmol) of succinic acid and 4.5 mg (50 μmol) of 1,4-butanediol were dissolved in 5 mL of solvent mixture consisting of toluene and *tert*-butanol 70:30 (wt), in 10 mL glass tubes, and kept at 65 ^{o}C. The reaction was initiated by addition of 20 mg of the immobilized enzyme (1.2 UA corresponding to 0.24 UA/ml). The tubes were gently stirred with magnetic bars in thermostatic IKA bath. Reactions were stopped by rapid cooling after 24 h incubation at 65 ^{o}C. The insoluble enzyme was separated by centrifugation at 14,000 rpm and the solvent was evaporated under reduced pressure. 50 mL - Scale reaction was performed in a 200 mL round bottom glass reactor outfitted with a flat paddle impeller at 200 rpm and similar concentration of substrates (10 mM) and enzyme (12 UA, respectively 0.24 UA/ml solution). Molecular sieves were dried for at least 24 h before use. 5 g were added to 50 mL medium at the beginning of the reaction, unless otherwise stated.

2.4. Matrix Assisted Laser desorption/Ionization Time-of-Flight Mass spectrometry (MALDI-TOF MS)

MALDI-TOF analysis was done on mass spectrometer Ultraflex workstation (Bruker Daltonics, Bremen, Germany) outfitted with a 337 nm laser. 2,5-dihydoxybenzoic acid (DHB) was the matrix and sodium acetate the dopant. DHB was prepared at 20 mg.mL^{-1} acetonitrile: methanol (90:10 v/v). The dopant was also dissolved at 5 mg.mL^{-1} in the mixture acetonitrile:methanol. The sample, matrix and dopant solutions were mixed in 10:5:5 (v/v). Subsequently, 1 μL of mixture containing from 0.75 to 1.0 mg.mL^{-1} sample, 5 mg.mL^{-1} of DHB and 1.25 mg.mL^{-1} of dopant were deposited in polished MALDI-TOF stainless steel plates (5 mm in diameter) and dried before analysis. The mass spectrometer was operated in the positive mode and calibrated with a mixture of maltodextrins (mass range 250-2500 Da). The ions were accelerated by a 25 KV voltage and the laser irradiance was set between 20 to 40% of the full laser power. The ions were detected using the reflector mode. A total of 100 laser shots were compiled for each spectrum.

2.5. Gel permeation chromatography (GPC)

Oligomers were analyzed in a Viscotek GPCmax VE 2001 system outfitted with a Viscotek internal pump and a Viscotek TDA 302 triple detector array for refraction index and viscosity detection. An external Waters Lambda max UV detector at 230 nm was also used. The data were recorded at Omnisec 4.5 station. The products were separated with MZ-Gel SD*Plus* series columns of 50 Å, 100 Å, 1000 Å and 10.000 Å operating at 40 ^{o}C. Samples of 150 μL were eluted with chloroform at 1 mL.min^{-1}. The average molecular weight was calibrated using a standard mixture of polystyrene.

2.6. HPLC Analysis

The samples were analyzed to determine the unreacted substrates by HPLC using a Waters 717 Plus auto-sampler outfitted with a Waters 1525 binary HPLC pump and a

Waters 2414 refraction index detector. Samples and standards in toluene / *tert*-butanol were dried under reduced pressure (1000 mbar) at 40 ^{o}C. The solid was dissolved in Milli-Q water:*tert*butanol (4:1). 20 µl of final solution were eluted in an ionic exchange column Alltech OA-100 (sulfonated polystyrene-divinylbenzene) with 3 mM sulfuric acid aqueous solution. The procedure above mentioned was done not only for the samples, but also for the standard curve.

2.7. Chromatographic Analysis (HPLC –MS)

HPLC analysis was carried out using a Waters 2690 equipment provided with a pump, degasser, autosampler, column oven, diode-array detector, and coupled to an MS system, outfitted with an electrospray probe. The analyses were performed with a 250 x 3 mm ODS-2 Intersil (Varian Inc.) at 40 ^{o}C. The elution phase A consisted of acetonitrile (Merck) and the elution phase B was ultra-pure water. Both mobile phases were supplemented with 0.1% (v/v) trifluoroacetic acid (TFA). Gradient elution was held during 5 minutes at 5% (v/v) A and ramping up to 95% (v/v) A. The flow rate was 1.0 mL.min^{-1} and the injection volume was 10 µL. 0.2 mL.min^{-1} of the outlet flow were pumped into an atmospheric pressure chemical ionization mass spectrometer (APCI-MS) (LCQ, Thermo Finnigan, San Jose, USA). Quantitative analysis was based on integration of the peak areas in the chromatogram assuming that the response factors for all products observed is one.

2.8. Fourier Transform Infrared analysis (FT-IR)

5 µl liquid of sample were dried on zinc selenite microplate of a Varian 1000, Scimitar TM series FT-IR system. 64 interferograms were recorded and averaged with resolution of 2 cm^{-1}.

2.9. Nuclear Magnetic Resonance Analysis (NMR)

Samples were dried at 40 ^{o}C in vacuum. The solid was suspended in CDCl$_3$ for further analysis. NMR spectra were recorded on a Bruker Avance DPX300 spectrometer operating at 300 MHz (^{1}H).

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3.   RESULTS AND DISCUSSION

3.1. Oligomer synthesis and characterization in batch system

The reaction between succinic acid and 1,4-butanediol was carried out using immobilized *Candida antarctica* lipase B (CALB) as catalyst in a reaction medium containing toluene (70% wt) and *tert*-butanol (30% wt.). *tert*-Butanol was used as co-solvent to enhance the solubility of the substrates, and in particular of that of succinic acid which is insoluble in toluene. Molecular sieves were used as water entrapment. The reaction was carried out at low substrate concentration and equimolar substrate ratio, to promote the formation of cyclic esters. HPLC analysis after 24 h of incubation at 65 ^{o}C showed a conversion of succinic acid of 86% (Figure 1). Three control reactions were also set up. The first two control reactions contained only the substrates and the substrates with molecular sieves, both without the enzyme. In both controls no quantifiable conversion of the substrates nor product synthesis was observed, confirming that the consumption of succinic acid measured in the reaction studied is due only to the action of the biocatalyst. The third control reaction contained the substrates and the enzyme, without molecular sieves, typical for the thermodynamic controlled enzymatic esterification without equilibrium shift. In this latest case, the conversion of succinic acid at 24 h was 37% (Figure 1). From Figure 1, we see that at early stage (i.e. up to 10% substrate conversion) the initial rate of the control reaction (0.47 mmole.(L.min)$^{-1}$) is significantly higher than that of the reaction with *in situ* water entrapment (0.33 mmole.(L.min)$^{-1}$) but then decreases with the progress of the reaction, being practically 0.0 mmole.(L.min)$^{-1}$ at 24 h. This suggests

that in the thermodynamic controlled enzymatic esterification of 1,4-butanediol with succinic acid the equilibrium is reached at a substrate conversion of about 37%. Comparable values for the substrate conversion at the thermodynamic equilibrium have been reported in the enzymatic polymerization of dimethyl succinate and 1,4-butanediol in toluene with lipozyme (46%) and Novozym435 (56%), respectively (<u>Mezoul et al. 1995</u>). In the presence of molecular sieves, the rate of reaction at 24 h was 0.23 mmole.$(L.min)^{-1}$.
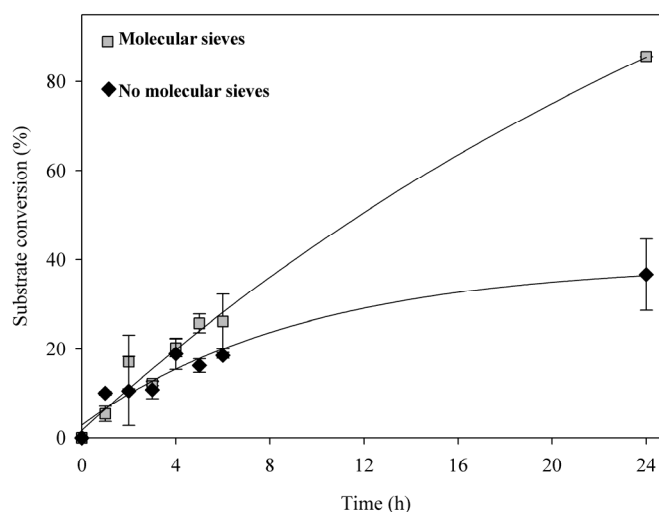

Figure 1 Time course analysis of the substrate during the CALB-catalyzed synthesis of oligo(butylene succinate).

The presence of esters in the reaction product was demonstrated by FT-IR and NMR spectroscopy. In the FT-IR spectrum (Figure 2) the appearance of an intense peak at 1728 cm^{-1} (C=O stretch) and a new strong C-O stretching band at 1163 cm^{-1} clearly show the formation of the ester. The ^{1}H NMR ($CDCl_3$) spectrum (Figure 3) contains peaks associated to the succinate ester moiety: δ = 1.61-1.71 ppm (m, 4H) corresponding to the central methylene protons in the alcohol chain, $-OCH_2-CH_2CH_2-CH_2O-$), 2.60-2.64 ppm (m, 4H) corresponding to the methylene protons associated to the succinate ester moiety ((-$COCH_2$-)) and 4.11-4.12 ppm (m, 4H) for the terminal methylene protons in the alcohol chain (-CH_2O-). GPC (Figure 4) and HPLC-MS (Figure 5) showed that the product of the reaction is a mixture of butyl succinate oligomers (Mn = 730 Da, with a polydispersity

index of 1.5). Low molecular weight linear oligoesters with dicarboxyl (AA_1) and dihydroxyl (BB_1, BB_2) end groups and cyclic ester oligomers with DP ranging from 2 to 8 were the main products identified by HPLC-MS. The dimer ester AB_1 was also identified. MALDI-TOF-MS analysis confirmed the LC-MS results and evidenced the presence of longer linear oligomers such as the hexamer AB_3 (534 Da), the octamer AB_4 (706 Da) and the decamer AB_5 (878 Da), but in very low concentration (data not shown).
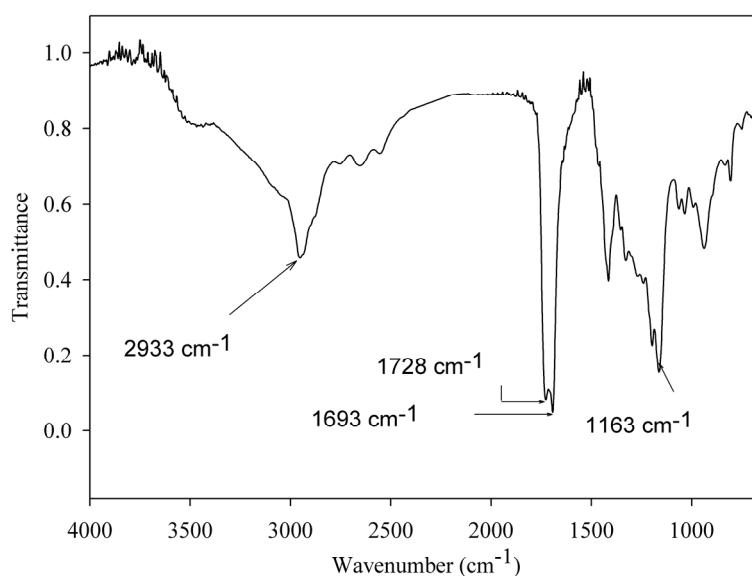
Figure 2 FT-IR spectrum of succinic acid (A) and 1,4-butanediol (B) reaction mixture in CALB-catalyzed reaction.

The most abundant cyclic and linear ester oligomers formed are illustrated in Scheme 1. Table 1 summarizes the products formed after 24 hours of reaction and their abundance, expressed as the area percentage of the corresponding peaks in the chromatogram of the HPLC-MS analysis. In the presence of molecular sieves (reaction equilibrium shift by *in situ* water removal), the product mixture contained comparable amounts of linear (47.6%) and cyclic (52.4%) ester oligomers. The most abundant CEOs were the dimer (CEO_1) and tetramer (CEO_2), which form together 34.7% of all products and 72% of the

total cyclic oligoesters formed. The cyclic hexamer (CEO$_3$, 13.9%, Entry 8) and octamer (CEO$_4$, 3.8%, Entry 9) were also found.
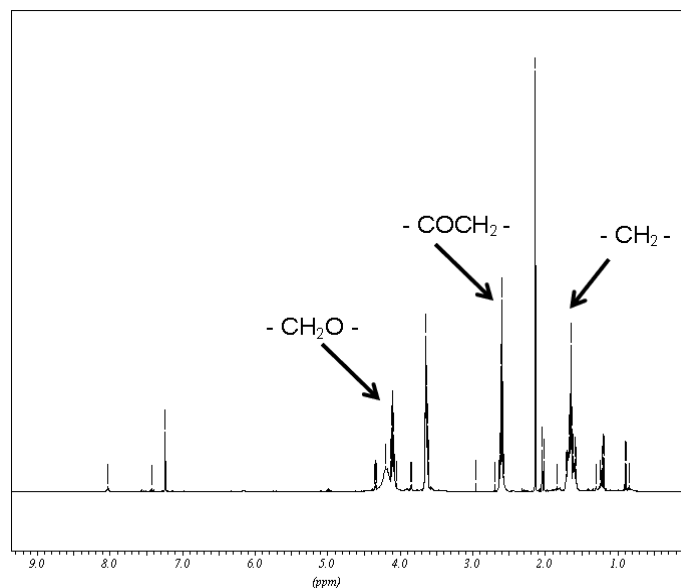
56

Figure. 3 ^{1}H NMR of oligobutylene succinate) synthesized in CALB-catalyzed reaction.
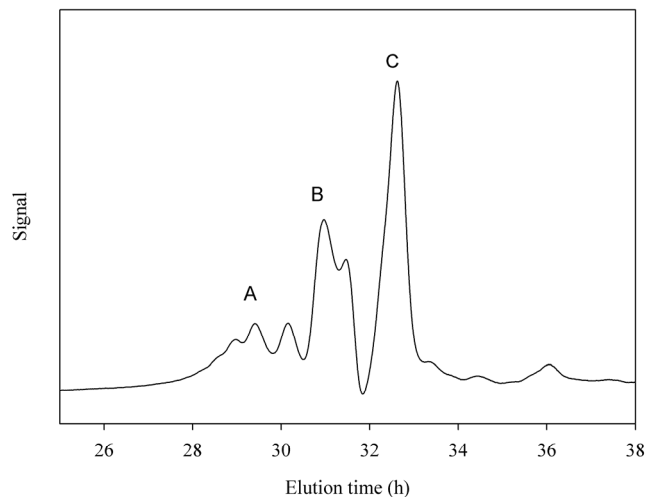

Figure 4 Gel permeation chromatogram (GPC) of oligo(butylene succinate) synthesized in CALB-catalyzed reaction: A (1050 Da), B (550 Da) and C (280 Da)

The high concentration of cyclic oligoesters in the product suggests that the rate of ring opening in subsequent polymerization reactions is lower than the rate of ring formation,

and this effect seems to be more pronounced for the small cycles (CEO_1 and CEO_2). A similar behaviour was observed for cyclic ester oligomers derived from 1,4-hexanediol and adipic acid, during the lipase-catalyzed ring-opening polymerization of pure cyclic oligomers produced by the enzymatic degradation of polybutylene adipate (Kondo et al. 2008). The cyclic butyl adipate dimers, trimers and tetramers polymerized faster than the equivalent monomer during the polymerization reaction catalyzed by Novozym435 (the same enzyme as the one used in this study), in toluene at 60 °C.

The most abundant LEO was the first linear α,ω-dicarboxyl trimer AA_1 (34.8%, Entry 3, Table 1), followed by the linear α-hydroxyl-ω-carboxyl dimer AB_1 (9%, Entry 1). The rest of the linear ester oligomers were the α,ω-dihydroxy tri- (BB_1, Entry 4), penta- (BB_2, Entry 5) and hepta- (BB_3, Entry 6) oligomers and the α-hydroxyl-ω-carboxyl tetramer AB_2 (Entry 4), all in very low concentration. Moreover, the linear α,ω-dicarboxyl trimer AA_1 was the only oligoester α,ω-dicarboxyl terminal oligoester (AA_n) both by LC-MS and MALDI-TOF-MS analysis. The composition of the product mixture of the equilibrium reaction (i.e. control reaction with no molecular sieves added) is comparable (Table 1). The main difference is that in the reaction with equilibrium shift, longer oliogoesters are formed. A time-course analysis gave more insight into the fate of individual oligoesters formed during the progress of the reaction. Figure 6D, 6E, and 6F shows the formation of some oligomers in time, when the enzyme-catalyzed reaction was done in the presence of molecular sieves. The concentration of the linear α-hydroxyl-ω-carboxyl dimer AB_1 increased during the first hour and stayed constant in time for the whole duration of the reaction (Figure 4D). This suggests that the rate of formation of the AB_1 linear dimer and the cumulative rate of its consumption in subsequent chain-elongation and cyclization reactions are comparable. The α-hydroxyl-ω-carboxyl tetramer AB_2, however, accumulates in the first 3 h and then is rapidly consumed, reaching the minimum level at 6h (Figure 6E). A similar behaviour was observed for the α,ω-dixydroxy- terminal oligoesters BB_1 and BB_2 (Figure 6D and 6E). The α,ω-dixydroxy- terminal oligoester AA_1, however, accumulated in the first 6 h of the reaction, and then decreased with low pace

reaching moderately high levels at 24 h (data not shown). In contrast to the linear ester oligomers, the concentration of cyclic esters CEO_1, CEO_2, and CEO_3 increased continuously in time (Figure 4F, 4C)

Table 1 Products detected by HPLC-MS during esterification of succinic acid (A) and 1,4-butanediol (B) at 24 h CALB-catalyzed reaction. The molecular weight was detected by MS as protonated form. The values are average among duplicates. The area is calculated as percentage over the total area of detected products at 24 h reaction.

Entry	Compound	Area (%) No mol. sieves	Area (%) Mol. sieves	m/z + 1
1	AB_1	2.3	9	190+1
2	AB_2	1.3	0.6	362+1
3	AA_1	24.5	34.8	262.1
4	BB_1	0.8	0.8	226+1
5	BB_2	8.8	1.3	434+1
6	BB_3	N.D	1.1	606+1
7	$CEO_1 + CEO_2$	42.7	34.7	172+1 and 344+1
8	CEO_3	19.8	13.9	516+1
9	CEO_4	N.D	3.8	688+1
	Total area	100	100	

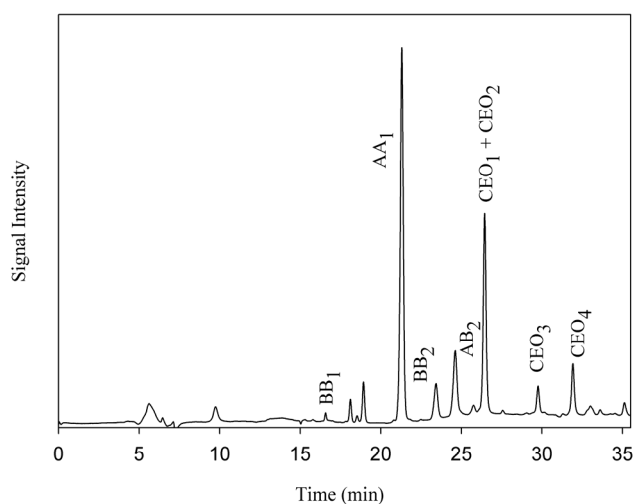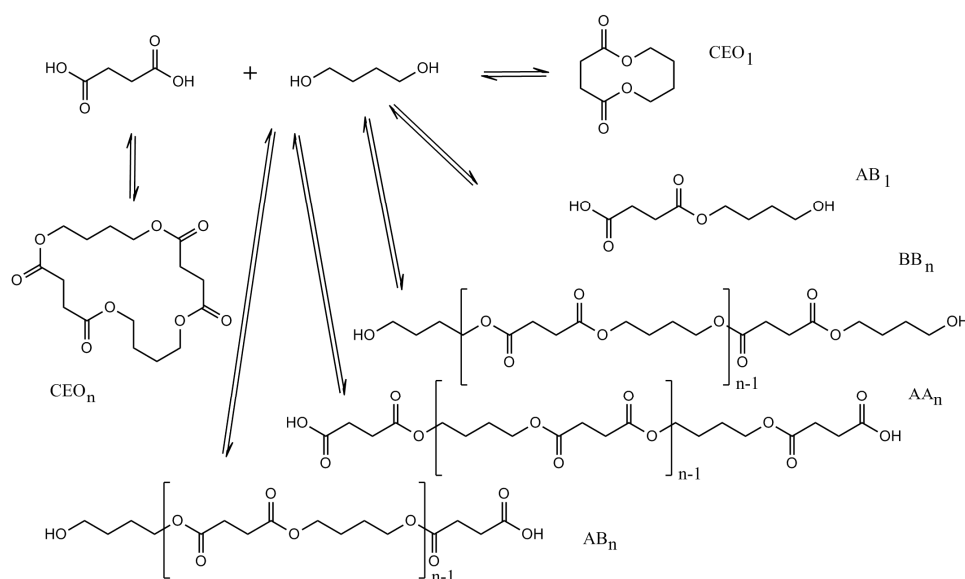*Area based on HPLC chromatograms.

Figure 5 HPLC chromatogram of oligo(butylene succinate) synthesized in CALB-catalyzed reaction.

Scheme 1 Products from succinic acid (A) and 1,4-butanediol (B) polycondensation in CALB-catalyzed reaction.

This supports the assumption discussed before that, in a complex reaction system containing both cyclic and linear oligoesters, the rate of enzymatic ring opening is slow. This might be due to a lower affinity of the enzyme for cyclic oliogoesters than for the linear oligoesters, both competing for the same binding site. As a consequence, cyclic ester oligomers accumulate in time and become the major products of the reaction. Surprisingly, a similar profile of the variation of the concentration of the cyclic monomer, dimer and trimer was obtained when the polymerization reaction was conducted under equilibrium conditions (Figure 6C). Under equilibrium conditions, however, all linear ester oligomers AB_n, AA_n and BB_n are formed at early stages of the reaction and reach the highest level at about 6 h, when actually the equilibrium is attained (Figure1, 6A, and 6B). From this point on, no further chain elongation was observed, due to the fact that the water produced in the polyesterification reactions and which accumulates in the reaction medium is stronger nucleophile than the α-hydroxyl terminal group of both, the 1,4-butanediol substrate and the oligoesters formed, and consequently it hydrolyzes the enzyme-acyl complex. Based on these results, we can conclude that the water produced

during the polyesterification reaction under equilibrium conditions reduces the substrate conversion, the total product yield and the molecular weight of the polyesters formed, but does not significantly affect the ratio between the cyclic and the linear oligoesters formed.
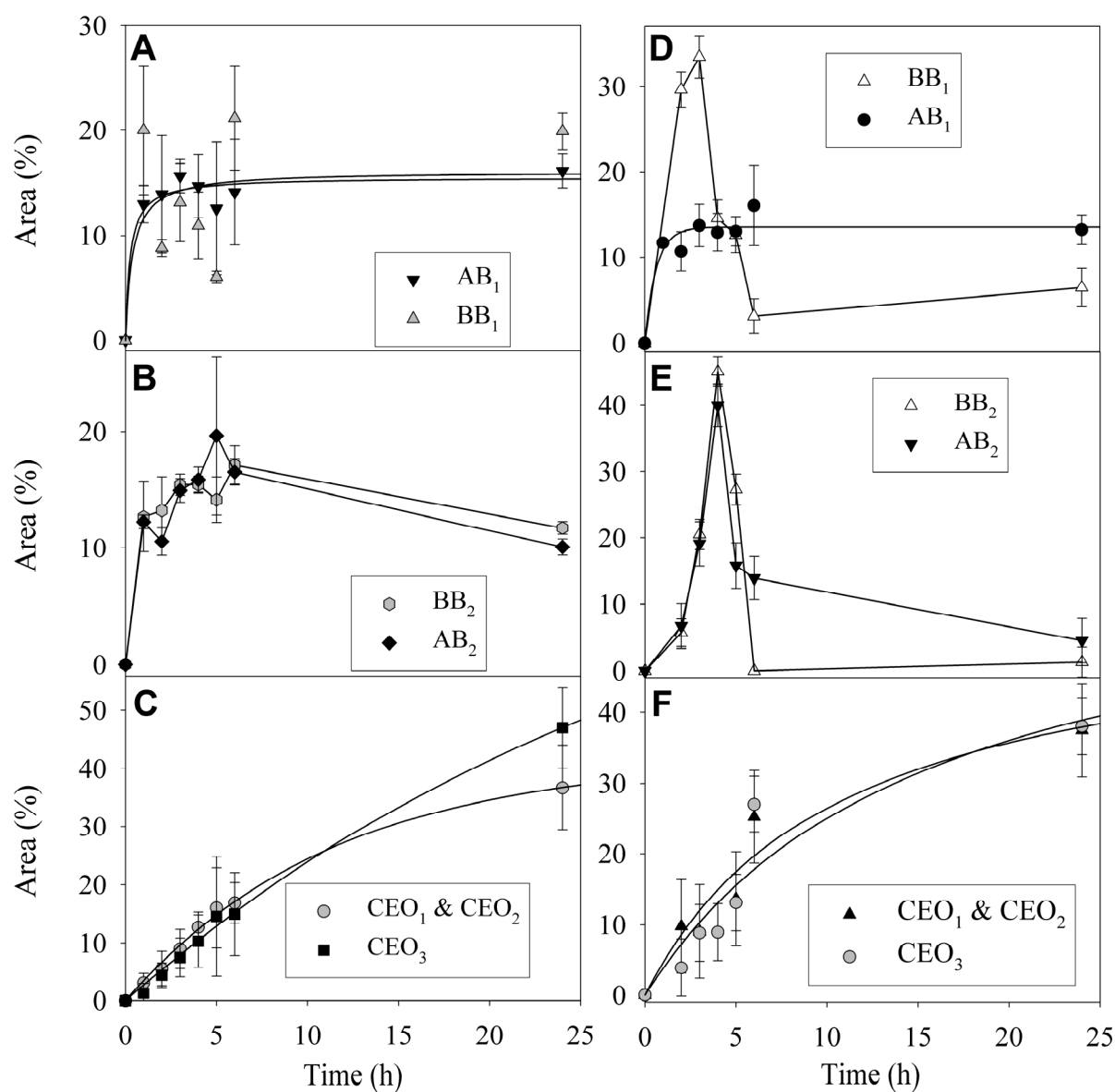
60

Figure 6 Time course analysis of cyclic and open ester oligomers (LEOs and CEOs). Absence of molecular sieve (A, B, and C), presence of molecular sieve (D, E, and F). Error bars represent the standard deviation between duplicates. The Area (%) is expressed as a percentage over the maximum area per specie.

3.2. Pulse Fed-batch Approach

In further studies we have explored the enzymatic polymerization reaction with *in situ* water removal in a fed-batch operation mode, to test if the dilution of the system triggers the prevalence of cyclic ester oligomers (CEOs) at the end of the biosynthesis. A 200 mL round bottom glass reactor outfitted with a flat paddle was initially loaded with

reaction medium free of substrate, molecular sieves and CALB. Drop-wise addition of 1 mL of equimolar solution of succinic acid (50 mM) and 1,4-butanediol (50 mM) was done every hour during the first 9 h and the solution was left overnight at 65 °C with stirring. Samples were taken at 0.1, 4.5, 8 and 24 hr. Neither succinic acid nor BDO were detected by GC or by HPLC five minutes after the addition of the substrate. This results is in agreement with the initial rate of the reaction in the batch system (0.47 mmole.(L.min)$^{-1}$). The total substrate conversion after 24 hr was 94.5%, significantly higher than in the batch reaction. Results of MALDI-TOF analysis of the product are shown in Table 2. The fed-batch reaction exhibited a larger spectrum of products than the batch experiments. CEOs from dimer (CEO_2) up to dodecamers (CEO_6) were detected. In all the type of LEOs (AB_n, BB_n and AA_n) the longest chains found under fed-batch experiments were two subunits (AB) larger than those found in batch reaction. Concentration of the products after 24 h reaction is summarized in Figure 7. The total CEOs concentration amount to 71.1%, where CEO_1 counts for 51.4 % of the total products and it is the 72% of the total cyclic products. The open ester AB_1 was 14.6%, while α,ω-dicarboxyl and α,ω-dihydroxyl terminal oligomers represent less than 11% of the total detected products. Figure 8 shows the relative abundance of products in the time course analysis. Five minutes after the start of the reaction the most abundant formed products were the linear α-hydroxy-ω-carboxy oligoesters AB_n (56%, 0.1 h), where AB_1 corresponded to the 97% of the total detected AB_n oligomers (Figure 8).

Table 2 MALDI-TOF analysis in pulse fed-batch reaction.

Entry	Time (h)	CEO_1 172	CEO_2 344	CEO_3 516	CEO_4 688	CEO_5 861	CEO_6 1033		
1	0.07*	+	+	+	+				
2	4.5	+	+	+					
3	8	+	+	+	+				
4	24	+	+	+	+	+	+		

		AB_1 190	AB_2 362	AB_3 534	AB_4 706	AB_6 1051			
5	0.07	+	+	+	+				
6	4.5	+	+	+					
7	8	+	+	+	+				
8	24	+	+	+	+	+			

		AA_1 290	AA_2 462	AA_3 635	AA_4 807	BB_1 262	BB_2 434	BB_3 606	BB_4 779
9	0.07	+	+	+		+	+	+	+
10	4.5	+	+	+	+	+	+	+	+
11	8	+	+		+	+	+	+	+
12	24	+	+	+	+	+	+	+	+

*Time = 0.07 represents five minutes after the first pulse of the substrates.

CEOs amounted to 35%, while the rest (less than 10%) were the linear ester oligomers AA_n and BB_n. The concentration of the cyclic ester oligomers increased in time at the expense of LEOs AB_n. The ratio between the relative abundance of CEO_n / AB_n in time (R^2=0.999) and also the ratio CEO_1/ AB_1 in time (R^2=0.990) fitted in a straight line (data not shown). This finding indicates that the CEOs are formed exclusively by closure of the AB_n and not by back-biting.
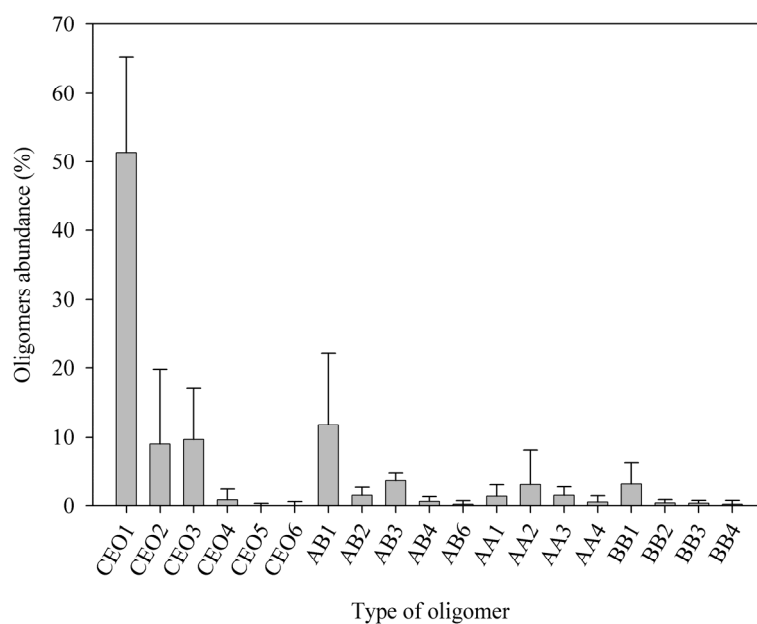
Figure 7 Abundance of products after 24 h reaction in CALB-catalyzed reaction in pulse fed-batch operation. Error bars represent the standard deviation between duplicates.
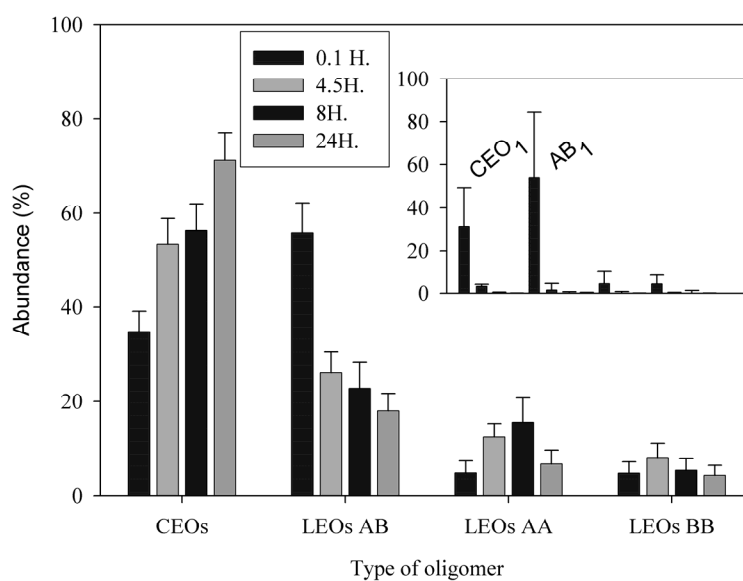

Figure 8 Time course analysis of total ester oligomers in pulse fed-batch experiments (Main). Abundance of oligomers vs. type of oligomers after 5 minutes (0.1 h) of reaction (Insert). The error bars indicate the standard deviation between duplicates).
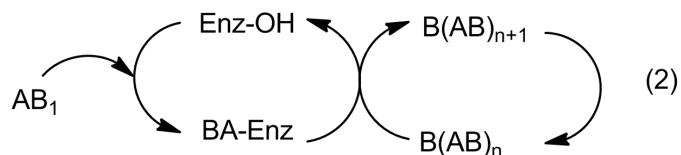
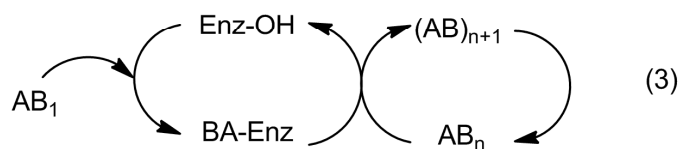## 3.3. Pathways for Cyclic and Linear Ester Oligomers

The results of this study clearly show that during the enzymatic polyesterification of an non-activated diacid with a diol carried out in solution phase under diluted conditions, cyclic ester oligomers are main products of the enzyme-catalyzed reaction along with linear ester oligomers. Based on the concentration of each product species in the mixture at a given time "t" during the progress of the reaction determined from time course experiments both in batch and pulse fed-batch experiments, we can propose a comprehensive mechanism for the formation of cyclic oligoesters and linear chain esters (Scheme 2). The pathway proposed here is based on the well-known mechanism for enzymatic (poly)esterification (Henderson et al. 1996), but differs from other reported mechanisms (Binns et al. 1998; Lalot and Marechal 2001) by the fact that it takes into account the change in the concentration of all intermediate species identified in the reaction mixture during the course of the reaction. In other words, we consider the competition between the various reactive intermediates in the system, and we suggest that the type of products formed during the propagation and chain elongation steps is controlled by (a) the concentration of the different oligoester species in the system at a time "t" and (b) the affinity of the enzyme for both, the acylating oligomer and the nucleophile oligomer, respectively. The enzymatic polyesterification follows the classical mechanism of lipase catalysis for esterification reaction; the reaction is initiated by the formation of the simple monoester between succinic acid and 1,4-butanediol, termed AB_1, via the activated acyl-enzyme complex (A-Enz, eq. 1 in Scheme 2).
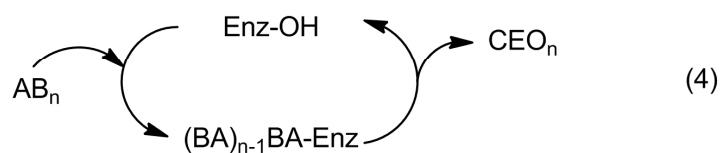
Inititation

$$A \longrightarrow \text{Enz-OH} \quad B$$
$$\text{A-Enz} \quad AB_1$$

(1)
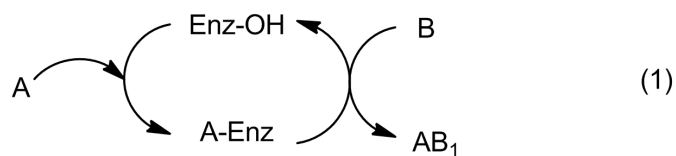
Chain elongation

Growing polymer chain, odd (n = 0, 1,...)

$$AB_1 \longrightarrow \text{Enz-OH} \quad B(AB)_{n+1}$$
$$\text{BA-Enz} \quad B(AB)_n$$

(2)

Growing polymer chain, even (n = 1, 2,...)

$$AB_1 \longrightarrow \text{Enz-OH} \quad (AB)_{n+1}$$
$$\text{BA-Enz} \quad AB_n$$

(3)

Cyclization (n= 1, 2...)

$$AB_n \longrightarrow \text{Enz-OH} \quad CEO_n$$
$$(BA)_{n-1}\text{BA-Enz}$$

(4)

Growing polymer chain, odd (n = 1, 2,...)

$$A \longrightarrow \text{Enz-OH} \quad A(BA)_n$$
$$\text{A-Enz} \quad AB_n$$

(5)

Growing polymer chain (n=1, 2,...)

$$A(BA)_n \longrightarrow \text{Enz-OH} \quad AB_{2n}$$
$$(AB)_n\text{A-Enz} \quad B(AB)_n$$

(6)
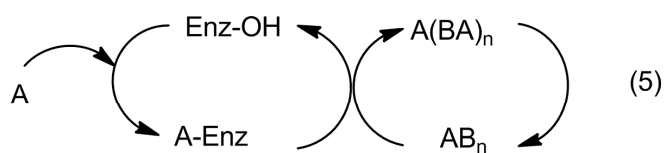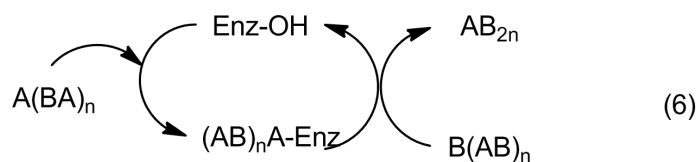
Scheme 2 Mechanism for the CALB-catalyzed synthesis of ester oligomers from BDO and succinic acid.

AB_1 is a bifunctional monomer, i.e. an α-hydroxy-ω-carboxyl monoester, and will initiate several reaction pathways by formation of a new activated acyl-enzyme complex, (BA-Enz, eq. 2, Scheme 2) to promote chain elongation. In one pathway (eq. 2), proved by the odd-number α,ω-dihydroxy oligomers $B(AB)_{n+1}$, n = 0, 1, 2,... chain elongation occurs by the nucleophilic attack of the terminal hydroxyl group of the diol on the acyl-serine bond of the BA-Enz active complex formed. This pathway is similar with the step-growth polymerization mechanism earlier proposed by Binns and collaborators for the enzymatic polymerization of adipic acid and 1,4-butanediol (Binns et al. 1998). The second pathway, given by eq. 3 in Scheme 2, shows the formation of the even number series of α-hydroxy-ω-carboxyl oligomers AB_n, n= 1, 2,... by the intermolecular nucleophilic attack of the terminal hydroxyl group of the AB_n on the acyl-serine bond of the BA-Enz active complex. This pathway proceeds with rates comparable with that of the $B(AB)_n$ route, since similar patterns for the formation and the levels of the $B(AB)_n$ and $(AB)_n$ oligomers were observed. The intramolecular nucleophilic attack of the terminal hydroxyl group of BA-Enz at the acyl-serine bond on the active centre results in ring closure and the formation of the cyclic esters CEO_n n= 1, 2,… (eq. 4, Scheme 2). The cyclic oliogomers CEO_n, n = 1, 2, 3 are formed from the early stages of the reaction, concomitantly with the linear oligomers AB_n, and BB_n, and this suggests that ring closure occurs on the catalytic site of the enzyme, and not from ring-chain equilibria. In addition, the presence of CEO_4 (Table 2) and absence of AA_4 at 5 minutes after the addition of the substrate in pulse fed-batch suggest that at early stages of the CALB-catalyzed reaction AB_4 is not synthesized by addition of B to ABABABA (AA_3, step-growth oligomerization) but by addition of AB_1 to AB_3 with the further cyclization of AB_4 in a similar way like shown by eq. 4 (Scheme 2). Furthermore, a similar pathway can be proposed for longer AB-type oligomers, leading to macrocyclic oligomers like trimers, tetramers, etc. The formation of cyclic ester oligomers by ring closure was earlier proposed by Lalot and Marechal (Lalot and Marechal 2001) for the CALB-catalyzed polymerization of 1,4-hexanediol and dimethyl succinate. In addition, AB_1 can react as nucleophile with the succinyl-enzyme complex, A-Enz, to form the ABA (AA_1) oligoester and a series of AA_n via a step-growth

polymerization (eq. 5 in Scheme 2). ABA (AA_1) and longer AA_n oligomers are products of the batch and pulse fed-batch polymerization, respectively. The α,ω-dicarboxy oligomer $A(BA)_n$ (AA_n, i.e. AA_1) can generate a new acyl-enzyme complex, $(AB)_nA$-Enz and thus initiate new chains of elongation by reacting with diol oligomers of the type BB_n. $(B(AB)n$ in eq. 6, Scheme 2). However, this last chain reaction occurs at longer reaction times, when the total conversion of succinic acid is higher than 60%, and the concentration of other acylating oligomers is low. This observation is more evident in the pulse fed batch experiment. In the pulse-fed batch operation mode, the monomers (i.e. succinic acid and 1,4-butanediol) are consumed in the first 5 minutes after addition, and as a consequence the pathway 4 becomes more important. Longer oligomers $A(BA)_2$, $A(BA)_3$, $A(BA)_4$ are found in the product mixture in a later stage of the reaction. Figure 6D, E and F show that the different oligoester species are produced and consumed with different rates. The first produced are the synthon AB_1 and the products of step-growth elongation $B(AB)_n$ and AB_n, and the cycles, while the oligoester ABA (AA_1) accumulates at lower pace (data not shown). Formation of $B(AB)_n$ and AB_n follows analogous pattern. Similarly, the oligoesters BB_n and AB_n are the first consumed, while AA_1 is consumed with low rate at later stage of the reaction, when more than 60% of the succinic acid was consumed. The cyclic ester oligomers are not consumed, suggesting a lower enzyme affinity as compared to the linear chain oligomers. Based on this these observations, we suggest that during the enzymatic solution polymerization of succinic acid with 1,4-butanediol under diluted conditions, the formation of products is controlled by the affinity of the enzyme for the acylating monomer or oligomer and by the concentration of the acyl reagents in solution. The enzyme affinity is higher for the α-hydroxy-ω-carboxyl oligomers (i.e. AB_1,…AB_n) than for the α,ω-dicarboxyl oligomers $A(BA)_n$, and for the cyclic ester oligomers CEO_n. The affinity decreases in the order:

Acylation: $A \approx AB > AB_n \gg AA_n > CEO_n$

The affinity of the enzyme for the hydroxyl nucleophiles, namely the α-hydroxy-ω-carboxy oligomer, AB_n, and the α,ω-dihydroxy oligomers $B(AB)_n$ is comparable.

4.   CONCLUSIONS

Novozym 435 lipase B from *Candida antarctica* was a suitable biocatalyst for the oligomers synthesis from non-activated di-acid / di-alcohols systems. When the reaction was conducted in a batch reaction in a binary solvent at 65 ^{o}C, 86% of the monomers were consumed in 24h and a product contained 52% cyclic ester o with DP 2, 4, 6, 8. The butylene succinate dimer was the major cyclic ester formed. Similar results were reported for the production of CEOs from dimethyl succinate and 1,4-butanediol with the same catalyst, but at higher temperature (90 ^{o}C, and 48 h reaction in toluene(Sugihara et al. 2006)). Furthermore, implementation of fed-batch strategy for synthesis of oligo(butyl succinate) demonstrated to be an effective way to increase the formation of cyclic ester oligomers. Substrate conversion in the fed-batch was 94.5% after just 24 h of reaction in the presence of molecular sieves and CALB. The product of the reaction consisted of 71% cyclic ester oligomers. We have shown that under diluted conditions CEOs are formed from the even-number α-hdroxy-ω-carboxy oligomers by ring closure on the active center of the enzyme, and not from back-biting from the linear ester oligomers nor from chain-ring equilibria. Therefore, an optimized CALB-catalyzed esterification of a non-activated succinic acid / BDO system under water removal conditions and operated in fed-batch could eventually lead to the synthesis of CEOs as an unique family of products.

REFERENCES

Azim H, Dekhterman A, Jiang Z, Gross RA. 2006. *Candida antarctica* Lipase B-Catalyzed Synthesis of Poly(butylene succinate): Shorter Chain Building Blocks Also Work. Biomacromolecules 7(11):3093-3097.

Bechthold I, Bretz K, Kabasci S, Kopitzky R, Springer A. 2008. Succinic Acid: A New Platform Chemical for Biobased Polymers from Renewable Resources. Chemical Engineering & Technology 31(5):647-654.

Berkane C, Mezoul G, Lalot T, Brigodiot M, Marechal E. 1997. Lipase-Catalyzed Polyester Synthesis in Organic Medium. Study of Ring-Chain Equilibrium. Macromolecules 30(25):7729-7734.

Binns F, Harffey P, Roberts SM, Taylor A. 1998. Studies of lipase-catalyzed polyesterification of an unactivated diacid/diol system. Journal of Polymer Science Part A: Polymer Chemistry 36(12):2069-2080.

Brugel E, Di Cosimo R; E. I. du Pont de Namours and Co., assignee. 2005. Manufacture of Certain Cyclic Ester Oligomers. United States.

Brugel E, Dicosimo R; E. I. du Pont de Namours and Co., assignee. 2006. Continuous Bioreactor Process for the Preparation of Polyester Cyclic Oligomers. United States.

Cao A, Okamura T, Nakayama K, Inoue Y, Masuda T. 2002. Studies on syntheses and physical properties of biodegradable aliphatic poly(butylene succinate-co-ethylene succinate)s and poly(butylene succinate-co-diethylene glycol succinate)s. Polymer Degradation and Stability 78(1):107-117.

Fujimaki T. 1998. Processability and properties of aliphatic polyesters, BIONOLLE, synthesized by polycondensation reaction. Polymer Degradation and Stability 59(1-3):209-214.

He Y, Asakawa N, Masuda T, Cao A, Yoshie N, Inoue Y. 2000. The miscibility and biodegradability of poly(3-hydroxybutyrate) blends with poly(butylene succinate-co-butylene adipate) and poly(butylene succinate-co-[var epsilon]-caprolactone). European Polymer Journal 36(10):2221-2229.

Henderson LA, Svirkin YY, Gross RA, Kaplan DL, Swift G. 1996. Enzyme-Catalyzed Polymerizations of ε-Caprolactone: Effects of Initiator on Product Structure, Propagation Kinetics, and Mechanism. Macromolecules 29(24):7759-7766.

Hilker I, Schaafsma AEJ, Peters RAH, Heise A, Nijenhuis AJ. 2008. Insight into lipase-catalyzed formation of macrocyclic oligoesters. European Polymer Journal 44(5):1441-1450.

Jacobson H, Stockmayer WH. 1950. Intramolecular Reaction in Polycondensations. I. The Theory of Linear Systems. The Journal of Chemical Physics 18(12):1600-1606.

Kiran KR, Hari Krishna S, Suresh Babu CV, Karanth NG, Divakar S. 2000. An esterification method for determination of lipase activity. Biotechnology Letters 22(19):1511-1514.

Knani D, Arie L. Gutman David H. Kohn. 1993. Enzymatic polyesterification in organic media. Enzyme-catalyzed synthesis of linear polyesters. I. Condensation polymerization of linear hydroxyesters. II. Ring-opening polymerization of ε-caprolactone. Journal of Polymer Science Part A: Polymer Chemistry 31(5):1221-1232.

Kondo A, Sugihara S, Kuwahara M, Toshima K, Matsumura S. 2008. Lipase-Catalyzed Ring-Opening Polymerization of Molecularly Pure Cyclic Oligomers for Use in Synthesis and Chemical Recycling of Aliphatic Polyesters. Macromolecular Bioscience 8(6):533-539.

Lalot T, Marechal E. 2001. Enzyme-catalyzed Polyester Synthesis. J. Polymeric Materials 50:267-286.

Lavalette A, Lalot T, Brigodiot M, Marechal E. 2002. Lipase-Catalyzed Synthesis of a Pure Macrocyclic Polyester from Dimethyl Terephthalate and Diethylene Glycol. Biomacromolecules 3(2):225-228.

Matsumura S. 2002. Enzyme-Catalyzed Synthesis and Chemical Recycling of Polyesters. Macromolecular Bioscience 2(3):105-126.

Mazzocchetti L, Scandola M, Jiang Z. 2009. Enzymatic Synthesis and Structural and Thermal Properties of Poly(ω-pentadecalactone-co-butylene-co-succinate). Macromolecules 42(20):7811-7819.

Mezoul G, Lalot T, Brigodiot M, Maréchal E. 1995. Enzyme-catalyzed synthesis of aliphatic polyesters in organic media: Study of transesterification equilibrium shift and characterization of cyclic compounds. Journal of Polymer Science Part A: Polymer Chemistry 33(16):2691-2698.

Oishi A, Zhang M, Nakayama K, Masuda T, Taguchi Y. 2006. Synthesis of Poly(butylene succinate) and Poly(ethylene succinate) Including Diglycollate Moiety. 38(7):710-715.

Osanai Y, Toshima K, Matsumura S. 2003. Enzymatic degradation of poly(R,S-3-hydroxybutanoate) to cyclic oligomers under continuous flow. Green Chemistry 5(5):567-570.

Sugihara S, Toshima K, Matsumura S. 2006. New Strategy for Enzymatic Synthesis of High-Molecular-Weight Poly(butylene succinate) via Cyclic Oligomers. Macromolecular Rapid Communications 27(3):203-207.

Zeikus JG, Jain MK, Elankovan P. 1999. Biotechnology of succinic acid production and markets for derived industrial products. Applied Microbiology and Biotechnology 51(5):545-552.

Zhi-wei G, Charles J. S. 1988. Enzymatic synthesis of macrocyclic lactones. J. Am. Chem. Soc. 110:1999-2001.

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

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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 (CALB), was studied. The highest conversion was 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 CALB. 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%).

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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 (Fenouillot et al. 2010). The most significant differences among the DAHs isomers is 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 (Fenouillot et al. 2010; Kricheldorf 1997; Noordover et al. 2007; Noordover et al. 2006).

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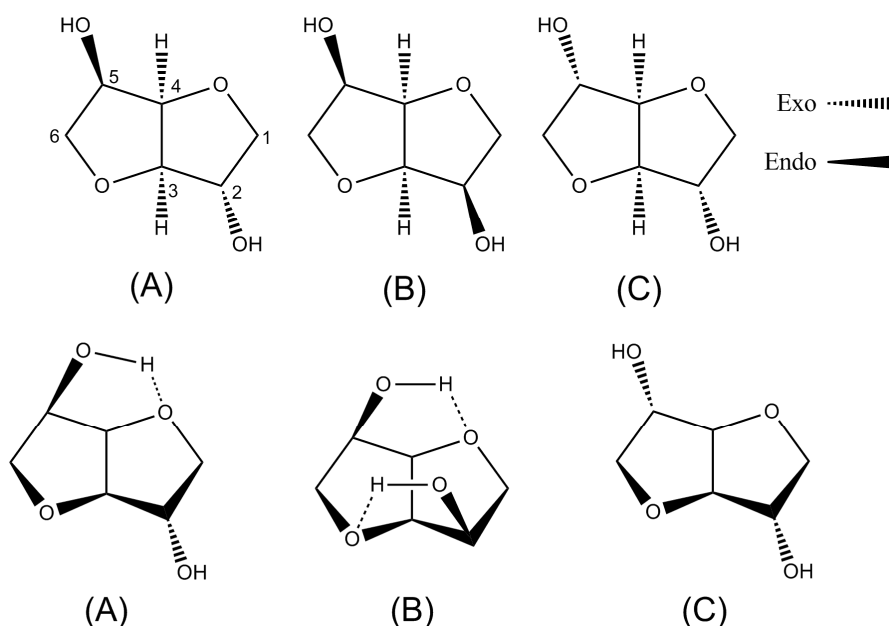
Scheme 1 (A). (3R,3aR,6S,6aR)-Hexahydrofuro[3,2-b] furan-3,6-diol; 1,4:3,6-dianhydro-D-sorbitol, or isosorbide; **(B)**. (3R,3aR,6R,6aR)-Hexahydrofuro[3,2-b] furan-3,6-diol; 1,4:3,6-dianhydro-D-mannitol, or isomannide; **(C)**. (3S,3aR,6S,6aR)-Hexahydrofuro[3,2-b] furan-3,6-diol; 1,4:3,6-dianhydro-L-iditol,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

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 (Kricheldorf et al. 2003). 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 (Kricheldorf et al. 2003; Williams et al. 1997). 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) (Noordover et al. 2006). The first known enzymatic-catalyzed esterification that used DAH as substrate was reported in 1993 (Mukesh et al. 1993). 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 (Jacobson and Stockmayer 1950) proposed a statistical mechanistic model for the equilibrium among linear and cyclic 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 approache 100% (Kricheldorf and Schwarz 2003). Results in agreement with this statement (Kricheldorf's theory) have been found recently by other authors (Chatti et al. 2009). They found that condensation of 4,4-difluorodiphenylsulfone with DAH in DMSO/toluene system at 140 ^{o}C and K$_2$CO$_3$ 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 CALB specificity (Juhl et al. 2009).

2.   EXPERIMENTAL SECTION

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 hours 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 (CALB)

The esterification activity was determined using *n*-butyric acid and *n*-butanol as substrates in toluene:*tert*-butanol solution (70:30 % wt) at 65 ^{o}C, based on the method reported by Kiran and collaborators (Kiran et al. 2000). 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 *Candida 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 ^{o}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 analysed 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 (Laine et al. 2001; Mezoul et al. 1995). 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 among the response factors of the molecules (Laine et al. 2001; Williams et al. 1997).

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 ^{o}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 x 3 mm ODS-2 Intersil (Varian Inc.) column at 40 ^{o}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 to 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 $^{\circ}$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 CALB X-ray structures 1LBS, 1LBT (Uppenberg et al. 1995), 1TCA, 1TCB, and 1TCC (Uppenberg et al. 1994) were used in this study. The substrates were modelled as tetrahedral reaction intermediates of isosorbide, isomannide, and isoidide esters with butyric acid. Due to the 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 CALB, 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 CALB with DAHs and non-activated succinic acid

Initial experiments to determine the catalytic capacity of *Candida antarctica* lipase B, CALB Novozym® 435, in the reaction of the three DAHs and non-activated succinic acid were carried out under thermodynamic controlled conditions as described in the Experimental Section. Experiments with isoidide (Scheme 1C) as substrate did not show positive outcome. Conversion of isoidide in the presence of CALB 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 CALB (conversion higher than 15 %). Furthermore, an experimental design was applied to find an improvement in the reaction when CALB 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 CALB. The reaction was carried out in 10 mL tubes under the similar conditions described in the Experimental Section. The analysis of variance (Table 1) for non-activated succinic acid consumed in the esterification with isosorbide in the presence of CALB 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) correspond 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) 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). 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 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).

Table 1 ANOVA analysis for the reaction of isosorbide and non-activated succinic acid in the presence of *C. antarctica* lipase CALB. DF: degree of freedom; F-ratio: Fisher-Snedecor test; P-value: Probability test.

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		

The formation of the ester bond after 24 h reaction in the presence of *Candida 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 (Figure 1). A new strong C-O stretching band at 1175 cm^{-1} confirms also the formation of esters.

Table 2 ANOVA analysis for reaction of isomannide and non-activated succinic acid in the presence of *C. antarctica* lipase B. DF: degree of freedom; F-ratio: Fisher-Snedecor test; P-value: Probability test.

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		

The Effects of the initial substrate and enzyme concentration on the consumption of the chosen DAH at 50 and 65 °C after 24 h reaction are shown in Figure 2 and Figure 3 respectively. Isomannide showed higher conversions than isosorbide at 50 °C (Figure 2)

and 65 ^{o}C (Figure 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 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 ^{o}C. The maximum conversions for isomannide were 47.4% at 50 ^{o}C and 41.9 % at 65 ^{o}C, while maximum conversions for isosorbide were 13.1% at 50 ^{o}C and 16.13% at 65 ^{o}C. The reaction conducted at 80 ^{o}C, however, differs with the results at 60 ^{o}C and 65 ^{o}C for both, isomannide and isosorbide. The isomannide conversion at 80 ^{o}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 CALB thermostability in non-aqueous media (Feng et al. 1999a; Feng et al. 1999b; Kumar and Gross 2000). There are three interesting observations from this study: (i) the conversion of isoidide was not favoured by CALB, (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 CALB must have 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).
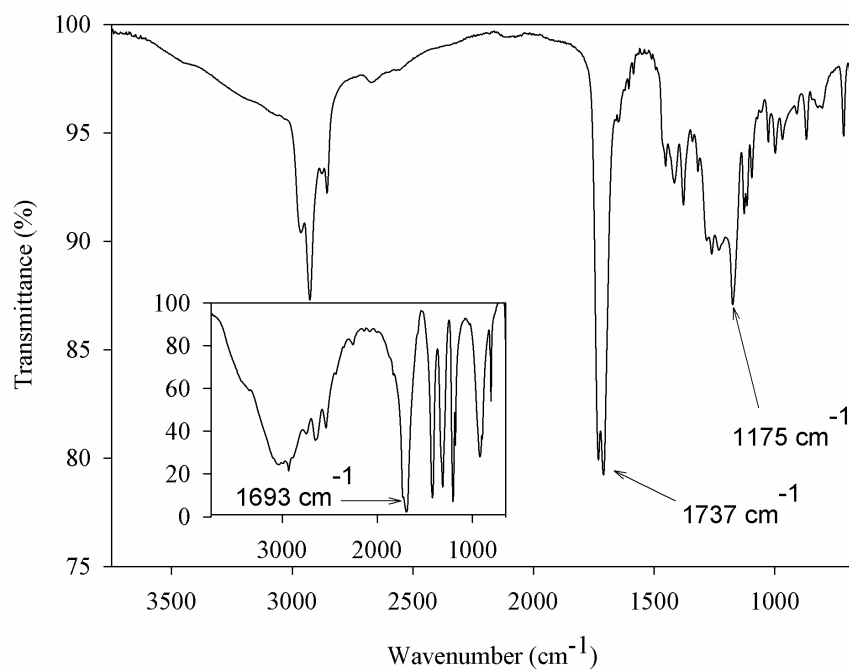
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Figure 1 FT-IR spectrum of non-activated succinic acid (A) and isomannide (B) reaction mixture in the presence of *C. antarctica* lipase B after 24 hours (Main). Succinic acid spectrum (Inserted).
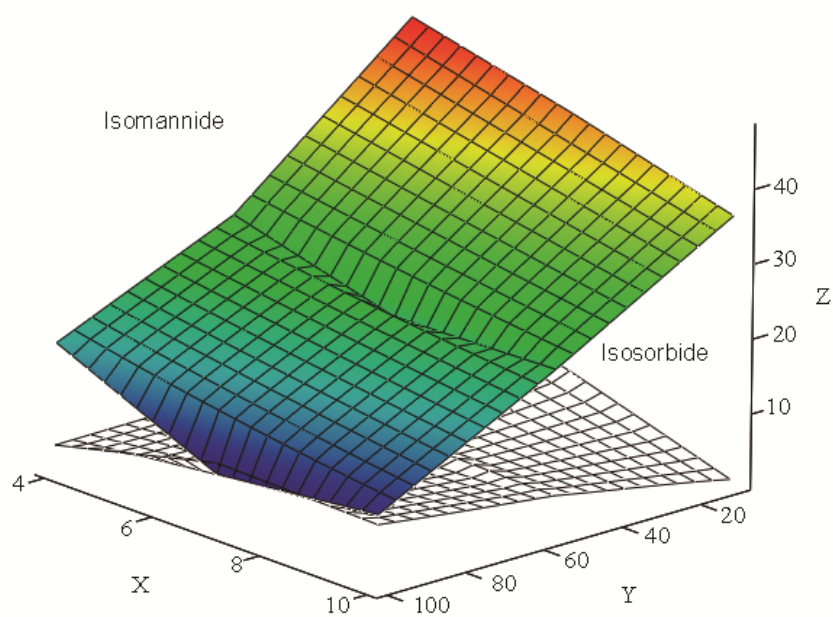

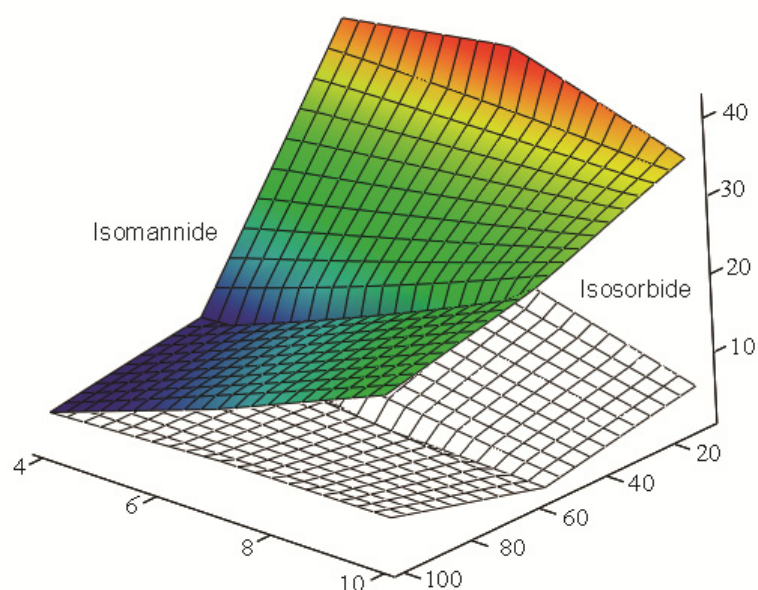**Figure 2** Response surface for isomannide and isosorbide at 50 ^{o}C.

Figure 3 Response surface for isomannide and isosorbide at 65 ^{o}C.

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 (Fenouillot et al. 2010; Noordover et al. 2006). In contrast, an opposite effect to what we found with CALB has been observed when chemical catalyst was used. For example, Noordover *et al* (Noordover et al. 2007; Noordover et al. 2006) 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 authors not only with chemical catalyst but also with biological degradation of polyesters (Fenouillot et al. 2010).

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, B and C) must play a key role on the enzymatic stereospecificity of CALB. 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) (Noordover et al. 2006; Wright and Brandner 1964). 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 (Zaks and Klibanov 1985). Furthermore, Boulif *et al* (2010) (Boulifi et al. 2010) 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 CALB at 63 $^{\circ}$C. Reetz *et al* (Reetz and Schimossek 1996) use CALB for N-acylation of (R,S)-phenylethylamine with ethyl acetate. CALB only reacted with the (R)-phenylethylamine reaching 77% conversion and synthesizing the (R)-configurated amide enantiomerically pure (ee=99%). Likewise, Overmeyer *et al* (Overmeyer et al. 1999) used CALB for transesterification of (R,S)-1-phenylethanol with vinylacetate in supercritical CO_2 environment. They reached a conversion above 45% of the (R)-enatiomer in few hours with enantiomeric excess above 99%. Interestingly, Hilker *et al* (Hilker et al. 2006) used CALB-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)-enatiomer with a conversion of 92% after 70 h. Hence, these evidences together with our results suggest that *Candida antarctica lipase* B (CALB) 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 CALB biocatalyst shows a potential in the synthesis of novel biobased polyesters beyond the possibilities of the chemical catalyst.
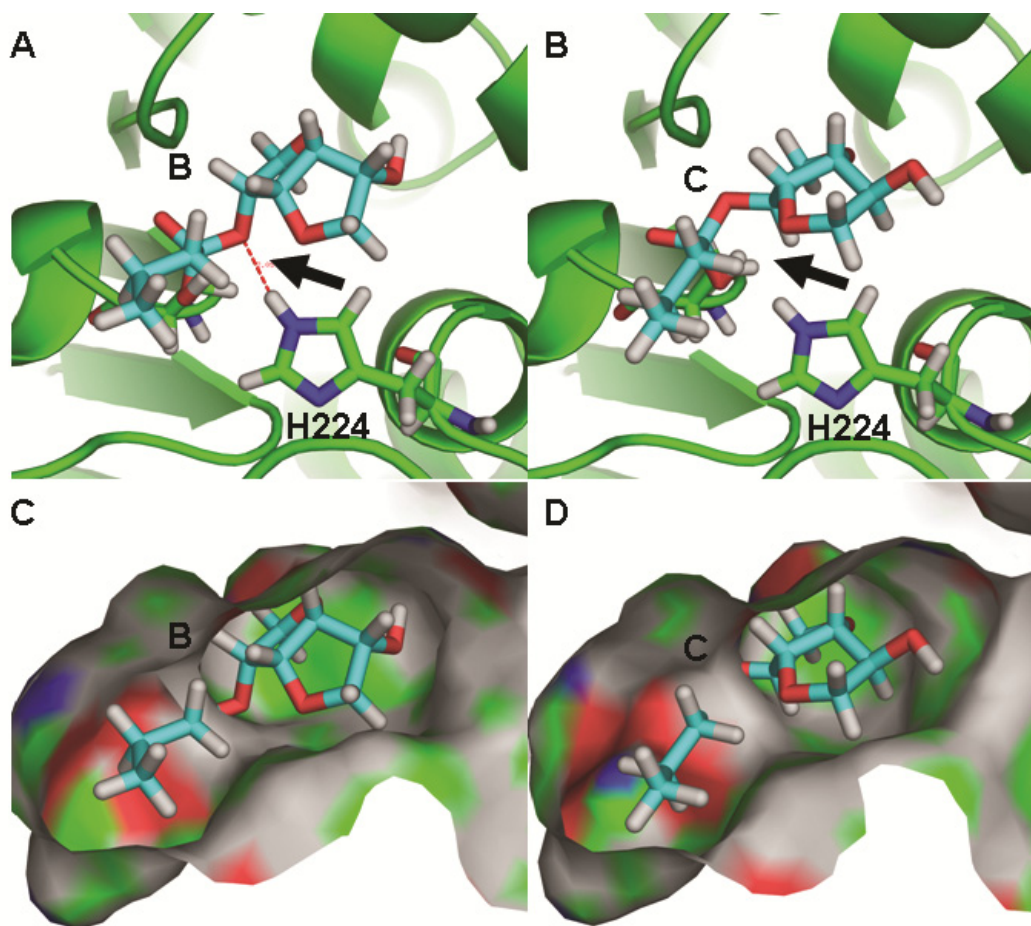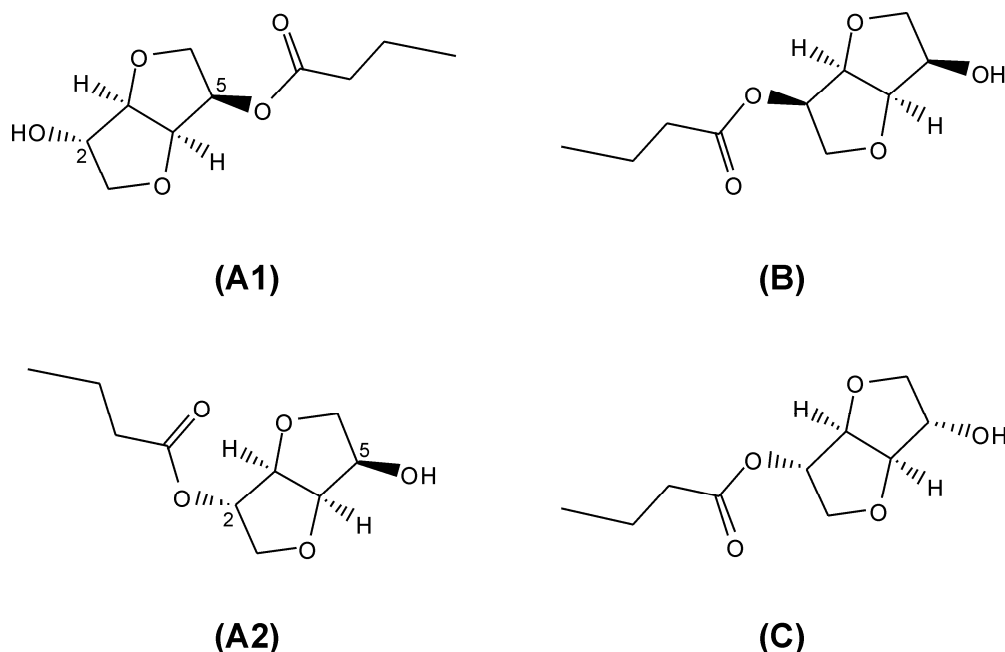
Figure 4 (A, C) (3R,3aR,6R,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate; (B) docked into CALB (PDB-ID: 1LBS). H224 forms a hydrogen bond with the alcohol oxygen of the ester (red dashed line, black arrow). (B,D) (3S,3aR,6S,6aR)-6-hydroxyhexahydrofuro[3,2-b]furan-3-yl butyrate (C) docked into CALB (PDB-ID: 1LBS). The necessary hydrogen bond between H224 and the alcohol oxygen of the ester cannot be formed (black arrow).

3.2. Docking of the substrates into the active site

To rationalize the molecular basis for the observed CALB preference of isomannide over isosorbide and over isoidide, substrate-imprinted docking was used to dock butyric acid esters of the DAHs into five CALB 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, Scheme 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, Scheme 1 and 2). This is

in agreement with the experimentally observed preference of CALB 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 CALB alcohol pocket without clashes of the two rings with the enzyme (Figure 4C) and forms the hydrogen bonds required for stabilization of the transition state (Schulz et al. 2000) (Figure 4A). The analysis of the non-productive substrate placements achieved for C (Figure 4B) shows that C can be fitted into the binding pocket of CALB, but not in a pose that forms the required hydrogen bonds for catalysis (Figure 4B, 4D). C is placed in the CALB 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 CALB 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.

(A1)

(B)

(A2)

(C)

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.

3.3. Reaction Yield Optimization

Additional experiments were conducted to increase the conversion yield in the biocatalyzed reaction of non-activated succinic 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 towards 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 *Candida 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 the Experimental Section. Molecular sieves (5 g) dried for at least 24 h at 120 ^{o}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 CALB and without molecular sieves, while the second control contained substrate with molecular sieves and without CALB. 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 Figure 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 (Figure 5).

3.4. Product Characterization

Since CALB 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 Experimental Section. No conversion occurred in the absence of the enzyme (data not shown). Samples were taken every hour up to 24 hours 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 8h of reaction the most abundant products were the CEOs (32.5 % - 48.7 %, Entry 45 and 50).
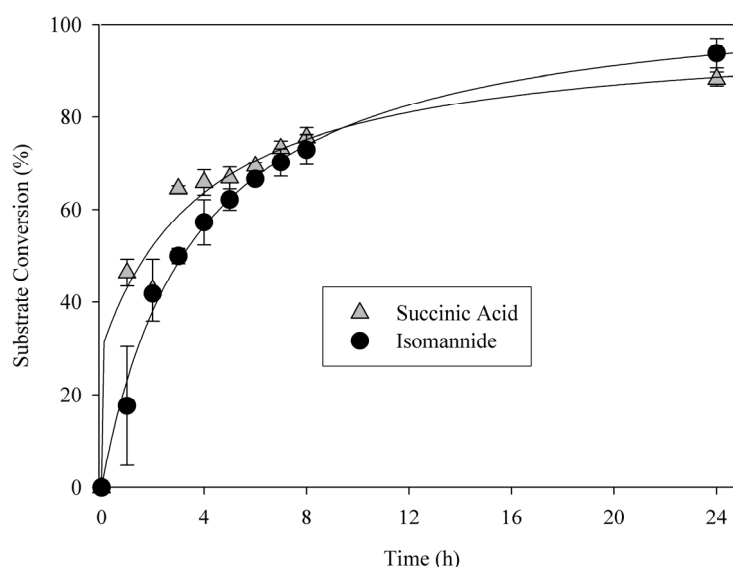

Figure 5 Substrate conversion of non-activated succinic acid and isomannide in the presence of *C. antarctica* lipase B and molecular sieves. Error bars represent the standard deviation between duplicates.

The abundance of LEOs follows the order AA_n (19.5 % - 24.8 %, Entry 28 and 32) $\approx AB_n$ (13.7 % - 28.9 %, Entry 1 and 3) > BB_n (9.9 % - 16.2 %, Entry 41 and 40). Interestingly, the prevalent specie was the CEO_1, (23.2 % - 40 %, Entry 45 and 50) reaching the highest abundance at 8 h. The formation 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. (Berkane et al. 1997; Lalot and Marechal 2001). 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. (Hamilton et al. 1998). The authors found that only dimethyl isophthalate was not only 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). Figure 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.

Table 3 MALDI-TOF time course analysis of main products of oligo(isomannide succinate) synthesized in the presence of *C. 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 BABABAB.

Entry	Time (h)	Total oligomer	AB_1 Abund (%)	AB_1 St.Dev	AB_2 Abund (%)	AB_2 St.Dev	AB_3 Abund (%)	AB_3 St.Dev	AB_4 Abund (%)	AB_4 St.Dev	AB_5 Abund (%)	AB_5 St.Dev
1	1	13.7			12.4	1.5			1.3	0.0		
2	2	22.7	3.3	0.2	17.9	2.5			1.5	0.2		
3	3	28.9	5.8	1.6	20.9	3.9	2.3	0.8				
4	4	26.8	4.2	0.3	19.1	4.0	2.6	0.3	1.1	0.1		
5	5	23.5	2.3	0.1	18.0	1.0	3.2	0.2				
6	6	19.7	2.7	0.2	12.0	5.1	3.7	0.9	1.3	0.2		
7	7	23.4	5.1	2.6	14.4	4.1	2.8	0.9	1.1	0.3		
8	8	19.0			15.1	3.2	2.9	0.6	1.0	0.2		
24	24	13.5	1.3	0.3	9.0	3.8	2.2	0.5	1.0	0.2		

Entry	Time (h)	Total oligomer	AA_1 Abund (%)	AA_1 St.Dev	AA_2 Abund (%)	AA_2 St.Dev	AA_3 Abund (%)	AA_3 St.Dev	AA_4 Abund (%)	AA_4 St.Dev	AA_5 Abund (%)	AA_5 St.Dev	AA_6 Abund (%)	AA_6 St.Dev	AA_7 Abund (%)	AA_7 St.Dev	AA_8 Abund (%)	AA_8 St.Dev
25	1	24.5	13.1	1.2	1.8	0.1	1.1	0.0			7.7	0.7					0.7	0.1
26	2	20.9	6.5	1.1	3.1	1.0	1.5	0.3			7.7	1.3	2.1	0.1				
27	3	23.5	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	4	24.8	7.4	1.8	9.8	2.6					5.8	0.8	1.1	0.1	1.1	0.1	0.7	0.1
29	5	19.8	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	6	23.0	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	7	20.6	7.2	2.7	4.3	1.1	1.3	0.4			6.4	2.1			1.4	0.4		
32	8	19.5	7.9	1.8	2.8	0.6	1.1	0.2			6.3	1.2	1.4	0.2				
33	24	44.2	32.9	8.2	2.8	0.7	0.9	0.2			5.5	1.4	1.1	0.4	0.9	0.2		

Table 3 Continuation.

Entry	Total oligomer	Time (h)	BB₁ Abund. (%)	St.Dev.	BB₂ Abund. (%)	St.Dev.	BB₃ Abund. (%)	St.Dev.	BB₅ Abund. (%)	St.Dev.	BB₆ Abund. (%)	St.Dev.	BB₇ Abund. (%)	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					2.8	0.5	1.9	0.3			2.3	0.6
42	7.3	24			2.9	0.5	1.9	0.5	1.8	0.4	1.4	0.3	2.3	0.5

Entry	Total oligomer	Time (h)	CEO₁ Abund. (%)	St.Dev.	CEO₃ Abund. (%)	St.Dev.	CEO₅ Abund. (%)	St.Dev.	CEO₆ Abund. (%)	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		
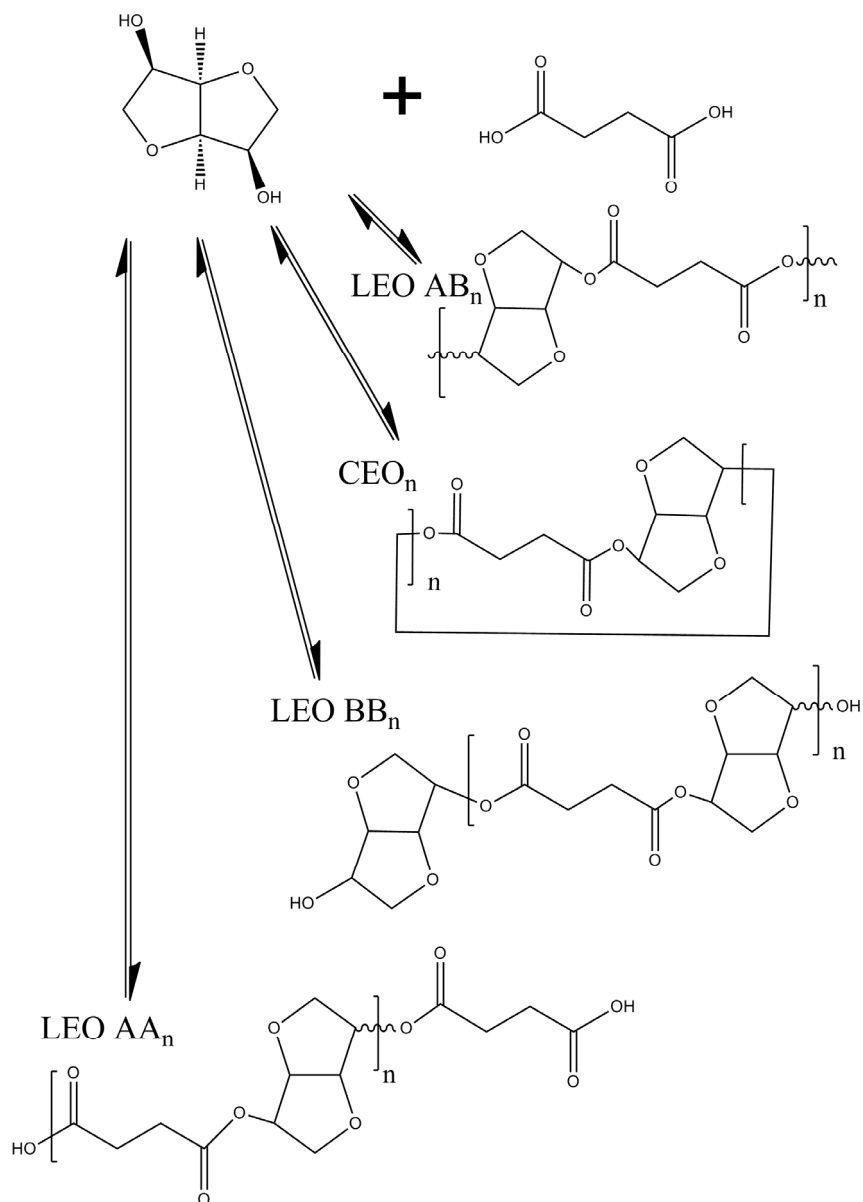
Scheme 3 Isomannide succinate oligomers from non-activated succinic acid (A) and isomannide (B) in the presence of *C. antarctica* CALB lipase. Example LEO BB2 stands for the di-hyrdoxyl terminal oligomer BABAB.

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4.   CONCLUSIONS

The esterification of di-anhydro hexitols together with non-activated succinic acid in a toluene based medium in the presence of *Candida antarctica* lipase B (CALB) was studied. Temperature and initial substrate concentration were the most important factors over the reaction yields. Interestingly, CALB 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.
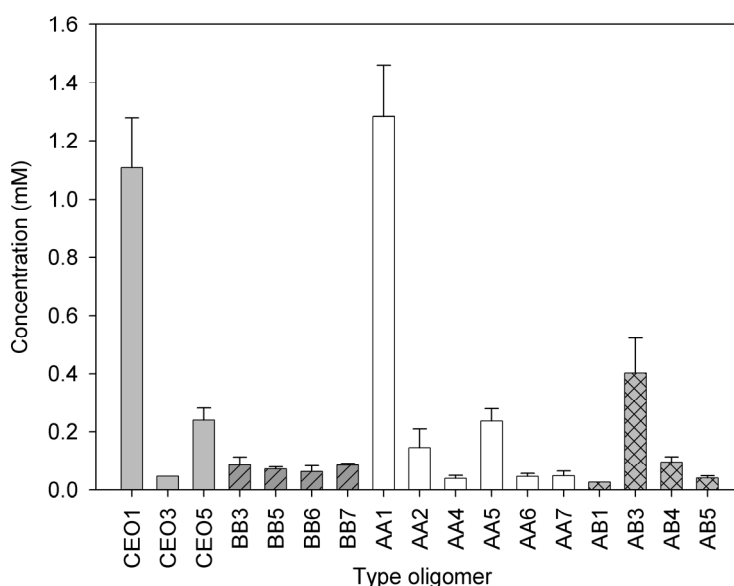
Figure 6 Ester oligomers formed after 24 h enzymatic conversion of non-activated succinic acid and isomannide in the presence of CALB (see Table 3). Error bars indicates the standard deviation between duplicates. The concentration of products was calculated taking into account the abundance of each compound, the water theoretically produced, and the substrate conversion.

Finally, a characterization of products 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 CALB 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.

102   **REFERENCES**

Berkane C, Mezoul G, Lalot T, Brigodiot M, Marechal E. 1997. Lipase-Catalyzed Polyester Synthesis in Organic Medium. Study of Ring-Chain Equilibrium. Macromolecules 30(25):7729-7734.

Boulifi NE, Aracil J, Martínez M. 2010. Lipase-catalyzed synthesis of isosorbide monoricinoleate: Process optimization by response surface methodology. Bioresource Technology In Press, Corrected Proof.

Chatti S, Hani MA, Bornhorst K, Kricheldorf HR. 2009. Poly(ether sulfone) of Isosorbide, Isomannide and Isoidide. High Performance Polymers 21:105-118.

Feng Y, Knüfermann J, Klee D, Höcker H. 1999a. Enzyme-catalyzed ring-opening polymerization of 3(S)-isopropylmorpholine-2,5-dione. Macromolecular Rapid Communications 20(2):88-90.

Feng Y, Knüfermann J, Klee D, Höcker H. 1999b. Lipase-catalyzed ring-opening polymerization of 3(S)-isopropylmorpholine-2,5-dione. Macromolecular Chemistry and Physics 200(6):1506-1514.

Fenouillot F, Rousseau A, Colomines G, Saint-Loup R, Pascault JP. 2010. Polymers from renewable 1,4:3,6-dianhydrohexitols (isosorbide, isomannide and isoidide): A review. Progress in Polymer Science 35(5):578-622.

Hamilton SC, Semlyen JA, Haddleton DM. 1998. Cyclic polyesters: Part 8. Preparation and characterization of cyclic oligomers in six aromatic ester and ether-ester systems. Polymer 39(14):3241-3252.

Hilker I, Rabani G, Verzijl GKM, Palmans ARA, Heise A. 2006. Chiral Polyesters by Dynamic Kinetic Resolution Polymerization13. Angewandte Chemie International Edition 45(13):2130-2132.

Jacobson H, Stockmayer WH. 1950. Intramolecular Reaction in Polycondensations. I. The Theory of Linear Systems. The Journal of Chemical Physics 18(12):1600-1606.

Juhl PB, Trodler P, Tyagi S, Pleiss J. 2009. Modelling substrate specificity and enantioselectivity for lipases and esterases by substrate-imprinted docking. BMC Struct Biol 9:39.

Kiran KR, Hari Krishna S, Suresh Babu CV, Karanth NG, Divakar S. 2000. An esterification method for determination of lipase activity. Biotechnology Letters 22(19):1511-1514.

Kricheldorf HR. 1997. "Sugar diols" as Building Blocks of Polycondensates. Polymer Reviews 37(4):599-631.

Kricheldorf HR, Sabe C, Schwarz G, Krüger RP. 2003. Macrocycles 27: Cyclic aliphatic polyesters of isosorbide. Journal of Polymer Science Part A: Polymer Chemistry 41(21):3414-3424.

Kricheldorf HR, Schwarz G. 2003. Cyclic Polymers by Kinetically Controlled Step-Growth Polymerization. Macromolecular Rapid Communications 24(5-6):359-381.

Kumar A, Gross RA. 2000. Candida antartica Lipase B Catalyzed Polycaprolactone Synthesis: Effects of Organic Media and Temperature. Biomacromolecules 1(1):133-138.

Laine O, Österholm H, Seläntaus M, Järvinen H, Vainiotalo P. 2001. Determination of cyclic polyester oligomers by gel permeation chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 15(20):1931–1935.

Lalot T, Marechal E. 2001. Enzyme-catalyzed Polyester Synthesis. J. Polymeric Materials 50:267-286.

Mezoul G, Lalot T, Brigodiot M, Maréchal E. 1995. Enzyme-catalyzed syntheses of poly(1,6-hexanediyl maleate) and poly(1,6-

hexanediyl fumarate) in organic medium. Macromolecular Rapid Communications 16(8):613-620.

Mukesh D, Sheth D, Mokashi A, Wagh J, Tilak JM, Banerji AA, Thakkar KR. 1993. Lipase catalysed esterification of isosorbide and sorbitol. Biotechnology Letters 15(12):1243-1246.

Noordover BAJ, Duchateau R, van Benthem RATM, Ming W, Koning CE. 2007. Enhancing the Functionality of Biobased Polyester Coating Resins through Modification with Citric Acid. Biomacromolecules 8(12):3860-3870.

Noordover BAJ, van Staalduinen VG, Duchateau R, Koning CE, van B, Mak M, Heise A, Frissen AE, van Haveren J. 2006. Co- and Terpolyesters Based on Isosorbide and Succinic Acid for Coating Applications: Synthesis and Characterization. Biomacromolecules 7(12):3406-3416.

Overmeyer A, Schrader-Lippelt S, Kasche V, Brunner G. 1999. Lipase-catalysed kinetic resolution of racemates at temperatures from 40 °C to 160 °C in supercritical CO_2 Biotechnology Letters 21(1):65-69.

Reetz MT, Schimossek K. 1996. Lipase-Catalyzed Dynamic Kinetic Resolution of Chiral Amines: Use of Palladium as the Racemization Catalyst. CHIMIA International Journal for Chemistry 50(12):668-669.

Schulz T, Pleiss J, Schmid RD. 2000. Stereoselectivity of Pseudomonas cepacia lipase toward secondary alcohols: a quantitative model. Protein Sci 9(6):1053-62.

Uppenberg J, Hansen MT, Patkar S, Jones TA. 1994. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. Structure 2(4):293-308.

Uppenberg J, Ohrner N, Norin M, Hult K, Kleywegt GJ, Patkar S, Waagen V, Anthonsen T, Jones TA. 1995. Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols. Biochemistry 34(51):16838-51.

Williams JB, Gusev AI, Hercules DM. 1997. Characterization of Polyesters by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. Macromolecules 30(13):3781-3787.

Wright LW, Brandner JD. 1964. Catalytic Isomerization of Polyhydric Alcohols.1 II. The Isomerization of Isosorbide to Isomannide and Isoidide. The Journal of Organic Chemistry 29(10):2979-2982.

Zaks A, Klibanov AM. 1985. Enzyme-Catalyzed Processes in Organic Solvents. PNAS 82(10):3192-3196.

Biocatalytic Synthesis of Oligoesters from Alkyl and Furan Derivatives Using Immobilized *Candida antarctica* Lipase B

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ABSTRACT

Biocatalytic synthesis of low molecular weight polyesters from furan based building blocks was performed with immobilized *Candida antarctica* B lipase (Novozym 435) as biocatalyst in a toluene-based medium. Total conversion of 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA) in the homo-polycondensation reaction was reached after 24 h. Polyesters with a degree of polymerization (DP) of 23 (M_w = 2854 Da) were found at the end of the reaction, of which 58.8% were cyclic esters. Condensation polymerization of 2,5-bis (hydroxyl methyl)furan (BHMF) in combination with succinic acid, adipic acid, and sebacic acid was also studied. The highest conversion of BHMF at 8 h was obtained in the reaction with adipic acid (43.3%). BHMF conversion was lower for diacid monomers with both longer (sebacic acid, 39.1%) and shorter (succinic acid 35.4%) alkyl chain. The product of each reaction was a mixture of linear and cyclic ester oligomers. Both the amount of cyclic ester oligomers (CEOs) and the DP decreased with the increase of the aliphatic chain of the diacid (36.9% CEOs, DP up to 16 for succinic acid, 11% CEOs, and DP up to 8 for adipic acid and 15% CEOs and DP 4 for sebacic acid). The conversion of furan-2,5-dicarboxylic acid (FDA) during the biocatalytic condensation polymerization with 1,4-butanediol (BDO) attained 84.2% at 24 h and increased at longer reaction times to 98.4%. Cyclic (36.9%, DP from 6 to 20) and linear (63.1%, DP from 5 to 21) ester oligomers were obtained. This work is a proof of concept for the biocatalytic condensation (co)polymerization of furan-based building blocks. It also shows, for the first time, the biocatalytic synthesis of furan-derived cyclic ester oligomers, with potential application as building blocks for the synthesis of novel (co)polymers via ring opening polymerization (ROP).

1.   INTRODUCTION

The interest and the search for polymers from renewable resources with comparable or better performance than commonly used petrochemical polymers has increased enormously in the past decade, in the context of the transition to a sustainable biobased economy as consequence of fossil fuel depletion and global climate change. We witness a revival on the biotechnological production of biopolymers like polyhydroxyalkanoates (PHAs) and also intensive research on the development and improvement of (bio)synthetic processes for the production of polymers derived from renewable resources. This runs parallel with the research on the biotechnological production of biobased building blocks for polymers, of which lactic acid, succinic acid and 1,3-propanediol are the most known success stories. Furan derivatives (FD) represent one group of building blocks that have acquired significant prominence during the past years (Gandini et al. 2009a; Gandini et al. 2009b; Moreau et al. 2004). Synthesis of these compounds can be done within the concept of biorefinery from the non-food competitive lignocellulosic raw material. The thermo-catalytic dehydration of the C5 and C6 carbohydrates products of the chemo-enzymatic depolymerization of lignocelluloses produces furfural and 5-hydroxymethylfurfural, which can be easily converted via very well known routes into furan-derived monomers (Gandini et al. 2009a; Gandini et al. 2009b; Mahapatro et al. 2003; Moreau et al. 2004). Furan-2,5-dicarboxylic acid (FDA), 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA), and 2,5-bis-(hydroxyl methyl)furan (BHMF) among FD have high potential for application in the synthesis of polyesters. FDA is widely recognised as an important bio-based building block, which could serve as an alternative for petrochemically derived terephthalic acid (TA). TA is used as a monomer in a wide range of materials, ranging from polyester, like polyethylene terephtalate (PET) and polybutylene terephtalate (PBT), to high strength fibres (aramids). The potential of FDA for PET-like polyesters has already been proven. Gandini *et al*.(Gandini et al. 2009b; Moreau et al. 2004) reported the chemical synthesis of poly(2,5-ethylene furandicarboxylate) (PEF) by both the polyesterification of FDA dimethyl ester and its

dichloride homologous with ethylene glycol and by bulk transesterification of 2,5-dihydroxyethyl furandicarboxylate obtained. The solution-phase polyesterification reaction used pyridine as catalyst and resulted in a low molecular weight PEF, with a degree of polymerization (DP) of about 70. However, the bulk polytransesterification of the 2,5-dihydroxyethyl furandicarboxylate, carried out in the melt (at temperature of 220 °C), using Sb_2O_3 as catalyst and high vacuum to remove the liberated ethylene glycol, produced PEF with high molecular weight and a DP of about 250-300 (Gandini et al. 2009b). The PEF thus obtained had properties comparable to PET (i.e. thermostability, glass transition temperature, and crystallinity). Elemental analysis and ^{13}C and ^{1}H NMR analysis confirmed the structure and the high DP of the PEF but showed also the presence of few ether bridges, which were synthesized as effect of the harsh reaction conditions. HMFA and BHMF are other members of the furan family with high potential for application for polyester synthesis. Whereas the thermally stable 2,5-FDA could still be polymerized under high temperature conditions, this would be impossible for HMFA and BHMF, which are thermolabile. This was first shown in late 70s of the past century, in a study on the chemical synthesis of furan polyesters based on FDA (and its dichloride) in combination with BHMF, when many degradation products were formed at temperatures usually applied for either liquid phase and melt polymerization (Moore and Kelly 1978).

Enzymatic polymerization in non-conventional media could offer the solution for the synthesis of furan based polyesters, and in particular for polymers derived from thermolabile furan building blocks, due to the mild reactions conditions of the *in vitro* biocatalytic reactions. The potential of *in vitro* enzyme catalysis for the synthesis of several polyesters other than furan-based, has been demonstrated. Enzymatic (trans)esterification reactions between diesters or diacids and diols have been used with success to prepare polyesters based on C4 to C10 dicarboxylic acids (i.e. succinic acid, adipic acid, sebacic acid) and C4 to C18 diols (Kobayashi 2006; Mahapatro et al. 2003; Mahapatro et al. 2004; Zhi-wei and Charles J. 1988; Zhi-wei et al. 1988). The efficiency of

lipase for the synthesis of polycaprolactone (PC) by ring opening polymerization (ROP) of ε-caprolactone has also been demonstrated (Kumar and Gross 2000). Polycaprolactone with high molecular weight (Mn up to 44800 g.mol^{-1}) was obtained in toluene, at 70 °C, using immobilized Lipase B from *Candida antarctica* (Novozym 435). *In vitro* enzymatic catalysis was also established as a valuable tool for the synthesis of cyclic oligoesters (CEOs) derived from a variety of aliphatic and aromatic monomers (Kricheldorf and Schwarz 2003; Lalot and Marechal 2001; Matsumura 2002; Sugihara et al. 2006). This finding is of high importance, since feasible routes for production of CEOs open new opportunities for the implementation of ROP for polyester manufacturing (Brugel and Di Cosimo 2005). It has been shown that in an enzyme-catalyzed reaction, the preferential formation of CEOs from a diacid - dialcohol system over step-growth polymerization is triggered by several factors: (i) equimolar substrates ratio, (ii) low substrate concentrations, (iii) affinity biocatalyst - substrate, (iv) type of reaction media, and (v) efficiency in the removal of by-products (Zhi-wei and Charles J. 1988; Zhi-wei et al. 1988). For example, the enzymatic reaction of terephthalic acid and ethylene glycol leads to the formation of one cyclic tetramer as unique cyclic product (Hilker et al. 2008; Lavalette et al. 2002). All lipase-catalyzed condensation polymerization reactions described are metal free and can be performed at moderate temperatures.

This Chapter seeks to explore the potential of biocatalysts for the synthesis of furan-based polyesters by polycondensation of non-activated diacids with diols in the presence of immobilized lipase B *Candida antarctica* (Novozym 435), in organic solvents. The diols studied include 2,5-bishydroxymethyl furan (BHMF) and 1,4-butanediol (BDO). The diacids include succinic, adipic and sebacic acids (HOOC(CH$_2$)$_n$COOH, where n = 2, 4 and 8) and 2,5-furandicarboxylic acid (FDA). Also, the homo-polymerization of 5-hydroxynethyl-2-furoic acid (HMFA) was studied. The progress of each reaction as a function of time is reported and products were characterised to determine the molecular weight and the end-group structure. To the best of our knowledge, this is the first time that the enzymatic production of furan-polyesters has been addressed.

2.   EXPERIMENTAL SECTION

2.1. Materials

Solvents were obtained from Sigma – Aldrich. Novozym® 435 was a kind gift by Novozymes (The Netherlands). All solvents were purchased from Merck and equilibrated in 4 Å molecular sieves (Sigma-Aldrich) for at least 24 hours before use. *Trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenyl-diene] malononitrile (DCTB, >99%) and potassium trifluoroacetate (>99%) were obtained from Sigma – Aldrich. 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA, >99% GC tested) was purchased from Matrix Scientific. 2,5-bis(hydroxymethyl)furan (BHMF, >99% GC tested) and furan-2,5-dicarboxylic acid (FDA, >99%) were kindly synthesized and donated by the group of Dr. Daan van Es (Agrotechnology and Food Sciences Group, Biobased Products Division). The rest of chemicals (>99%) were purchased from Merck.

2.2. HPLC Analysis

The concentrations of furan derivatives (BHMF, FDA and HMFA) were determined by HPLC, using a ODS-2 Intersil (250 x 3 mm, Varian Inc.) column at 40 $^\circ$C. Elution of compounds 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, > 99%). 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. BHMF was detected at 240 nm, while FDA was detected at 280 nm.

2.3. GC-MS

The alkyl derivatives (BDO, succinic acid, adipic acid, and sebacic acid) were detected by

GC-MS provided with a Restek (RXI® -5ms) capillary column (30 m x 0.25 μm i.d.). The injection volume was 1 μL, injection temperature 300 ^{o}C, FID temperature 300 ^{o}C, flow rate of the carrier gas (helium) 1.5 mL.min^{-1}. The initial oven temperature was 60°C and heating up to 300 ^{o}C at a rate of 10 ^{o}C.min^{-1}.

2.4. Assay of the Esterification Activity of Lipase in Organic Media

The esterification activity was determined using *n*-butyric acid and *n*-butanol as substrates in toluene : *tert*-butanol solution (70:30 % wt) at 65 ^{o}C. The activity was based in a method reported elsewhere (Kiran et al. 2000). 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 millimoles of butyric acid that are esterified per hour.

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

The samples (10 - 20 μL) were mixed with 10 μL a solution of matrix (DCTB, 160 mM) and 2.5 μL trifluoroacetate as dopant (33 mM). Matrix and dopant were dissolved in tetrahydorfuran and the final mix. MALDI-TOF analysis was done on mass spectrometer Ultraflex workstation (Bruker Daltonics, Bremen, Germany). Polyethylene glycol (600, 1000, and 2000 Da) was used for calibration. 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 (ter Haar et al.). Within each MALDI-TOF spectrum, the intensity obtained by the potassium-adduct per specie were added up and the relative contribution of each one was calculated. It was assumed that there are no significant differences between the response factors of the molecules (ter Haar et al.).

2.6. Fourier Transform Infrared analysis (FT-IR)

5 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}.

2.7. General procedure for the enzymatic oligomerization of furan and alkyl derivatives

A typical reaction procedure is as follows: non-activated diacid (succinic, adipic, sebacic or FDA, 50 µmol) and non-activated diol (1,4-butanediol or BHMF, 50 µmol) were dissolved in 5 mL of toluene and *tert*-butanol solution (70:30 % wt) in 10 mL glass tubes. The reaction takes place at 65 ^{o}C during 24 h, otherwise stated. The biocatalyst used was immobilized *Candida antarctica* lipase B (CALB, 50 mg or 3 UA). The reactions were stopped by quick cooling on ice bath and the enzyme was separated by centrifugation at 14000 rpm. The supernatant was collected and stored for further analysis.

3.   RESULTS AND DISCUSSION

3.1. Enzyme Selection and Conditions for the Polycondensation Reaction
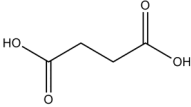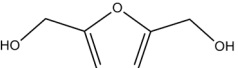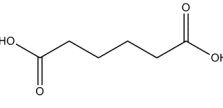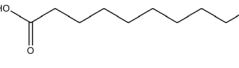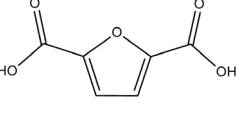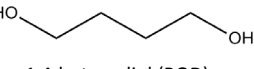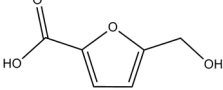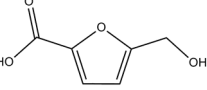
Novozym 435, the lipase B from *Candida antarctica* immobilized on a macroporous acrylic resin was selected as the catalyst for this work. Preliminary screening experiments using FDA and 1,4-butanediol as substrate picked out the immobilized lipase B from *Candida antarctica* as the most active catalyst of all enzymes tested (data not shown). The other enzymes tested were fungal lipases (i.e. lipase from *Candida rugosa, Thermomyces lanuginosa, Mucor javanicus*, *Mucor miehei*, *Rizhopus oryzae, Aspergillus oryzae*, and *Aspergillus niger* and the free lipase B from *Candida antarctica*), wheat germ lipase and lipase from porcine pancreas. Novozym 435 has already been demonstrated to be a versatile and efficient catalyst in the polycondensation of a wide

range of aliphatic and aromatic monomers (Anderson et al. 1998; Hilker et al. 2008; Lavalette et al. 2002). Previous work in our laboratory (Habeych et al. 2011) showed that the esterification activity of Novozym 435 in a model reaction (i.e. synthesis of butyl butyrate) displayed high values in the temperature range from 50 to 70 °C, so 65 °C was used throughout this study. As reaction medium, we have selected a mixture of toluene and tert-butanol containing 70% (wt) toluene and 30% (wt) tert-butanol. This mixture of solvents combines the stabilizing effect of toluene, a hydrophobic solvent with a log P of 2.5, which already has been shown to promote polycondensation reactions catalyzed by lipase (Mahapatro et al. 2003), with the increased solubility of the monomers used in this study in the hydrophilic tert-butanol (log P = 0.35 (Du et al. 2007)). The polycondensation reactions of furan monomers were carried out using molecular sieves to ensure the removal of water by-product liberated during the reaction. The following reactions were studied: (a) 2,5-bishydroxymethyl furan (BHMF) with succinic acid, adipic acid and sebacic acid, respectively; (b) 2,5-furandicarboxylic acid (FDA) with 1,4-butanediol (BDO); and c) homo-polymerization of 5-hydroxymethyl-2-furoic acid (HMFA) (see Table 1). For each monomer combination, two control reactions were set up. The first control contained only the substrates without enzyme and without molecular sieves, while the second control contained the substrates with molecular sieves, but without the biocatalyst. None of the control reactions showed any quantifiable substrate conversion or product synthesis. Therefore, all conversions obtained in reactions in the presence of Novozym 435 are only due to the catalytic efficiency of the biocatalyst. Reactions carried out in the presence of the enzyme, with or without molecular sieves did show substrate conversions. Nevertheless, the conversion of the substrate when only enzyme was used was 2 to 2.5 fold lower than when both enzyme and molecular sieves were added to the system (results not showed). In the presence of Novozym 435 and molecular sieves, at 24 h reaction, substrate conversions between 35.4% (Table 1, entry 1) and 99% (Table 1, entry 5) were obtained, depending on the substrates used. The highest conversion was reached for HMFA (99%) in the homo-polymerization reaction, followed by FDA (84.2%) and BHMF, in polycondensation reactions with aliphatic diols and diacids, respectively.

Nevertheless, when BHMF was reacted with aliphatic α,ω-dicarboxylic acids with different alkyl chain length, the conversion of BHMF was influenced by the length of the alkyl chain. The BHMF conversion was highest in combination with adipic acid (C6; 43.3%) and sebacic acid and decreased slightly for both longer (sebacic acid, C10; 39.1%) and shorter chains (succinic acid, C4; 35.4%). A similar trend in the reactivity of C4, C6 and C10 diacids was reported during copolymerization reactions with aliphatic diols catalyzed by CALB (Mahapatro et al. 2003).

FT-IR and LC-MS analysis confirmed the formation of the ester products. Figure 1 illustrates the FT-IR spectra of the products of the reaction between BHMF with succinic acid (Figure 1A), with adipic acid (Figure 1B) and with sebacic acid (Figure 1C), respectively, and between FDA and BDO (Figure 1D), as compared with the spectra of the mixture of substrates in the corresponding control reaction without enzyme. In all cases, the disappearance of the characteristic peaks of the free, protonated carboxylic group and the appearance in the spectrum of absorptions specific for the ester group is observed. Initial succinic acid carbonyl (C=O) stretching at 1689 cm^{-1} shifts after reaction to 1724 cm^{-1} corresponding to carbonyl from the newly formed ester bonds (Figure 1A). A new strong C-O stretching band at 1049 cm^{-1} confirms also the formation of esters. Adipic acid presents a narrow peak at 1685 cm^{-1} before the reaction with BHMF (Figure 1B). The latter peak shifted to a new value of 1722 cm^{-1} due to the formation of the ester bond. Similarly, the disappearance of the band of 930 cm^{-1} (C-O stretch in acids) and the appearance of the peak at 1147 cm^{-1} (C-O stretch in esters) confirm the formation of the ester bond.

Table 1 Structures, conversions and initial rate of reaction of the furan-based monomers during the enzymatic polymerization reaction with aliphatic co-monomers.

SUBSTRATES		RESULTS	
Acid	**Alcohol**	**Conversion of monomer at 24 h (%)**	**Initial rate (mmol (L min)$^{-1}$)**
Succinic acid	BHMF	35.4	0.063
Adipic acid	BHMF	43.3	0.39
Sebacic acid	BHMF	39.1	0.12
FDA	1,4-butanediol (BOD)	84.2	0.018
HMFA	HMFA	99.9	N.A

In the case of BHMF-sebacic acid reaction, a slight shift of the carbonyl stretching band in the acid (1687 cm^{-1}) is observed (1693 cm^{-1}) together with the formation of a low intensity peak at around 1720 cm^{-1}, that appears as a shoulder of the major peak at 1693 cm^{-1} (Figure 1C, insert). For FDA (Figure 1D), the shift of the absorption of the free caboxyl group at 1664 cm^{-1} to 1723 cm^{-1} proves the formation of esters. LC-MS analysis showed the formation of low molecular weight oligoesters (not shown). The conversion of the furan monomers as a function of time was monitored for reaction times ranging from 5 minutes to 96 hours. Aliquot samples were withdrawn from each reaction mixture at 5 min, at every hour during the first 8 hours of the reaction and at 24 h, 48 h and 96 h. Figure 2A shows the time course of the reaction of BHMF with succinic acid, adipic acid

and sebacic acid, respectively, in the presence of Novozym 435 and molecular sieves, at 65 °C. The conversion of BHMF in all three reactions increased very fast in the first hour and reached the maximum value after 8 h. The increase of reaction time to 24 h, 48 h and 96 h did not result in an increase of BHMF conversion (not shown), and this might be due to an inhibition of the enzyme by the products formed. The initial rates of the BHMF conversion during the polymerization with the alkyl diacids varied significantly as a function of the length of the aliphatic chain of the diacid co-monomer (Table 1). The most reactive monomer combination proved to be BHMF-adipic acid. The initial rate for the BHMF-adipic acid reaction was 6-fold higher than for BHMF-succinic acid and 3-fold bigger than that of BHMF-sebacic acid.
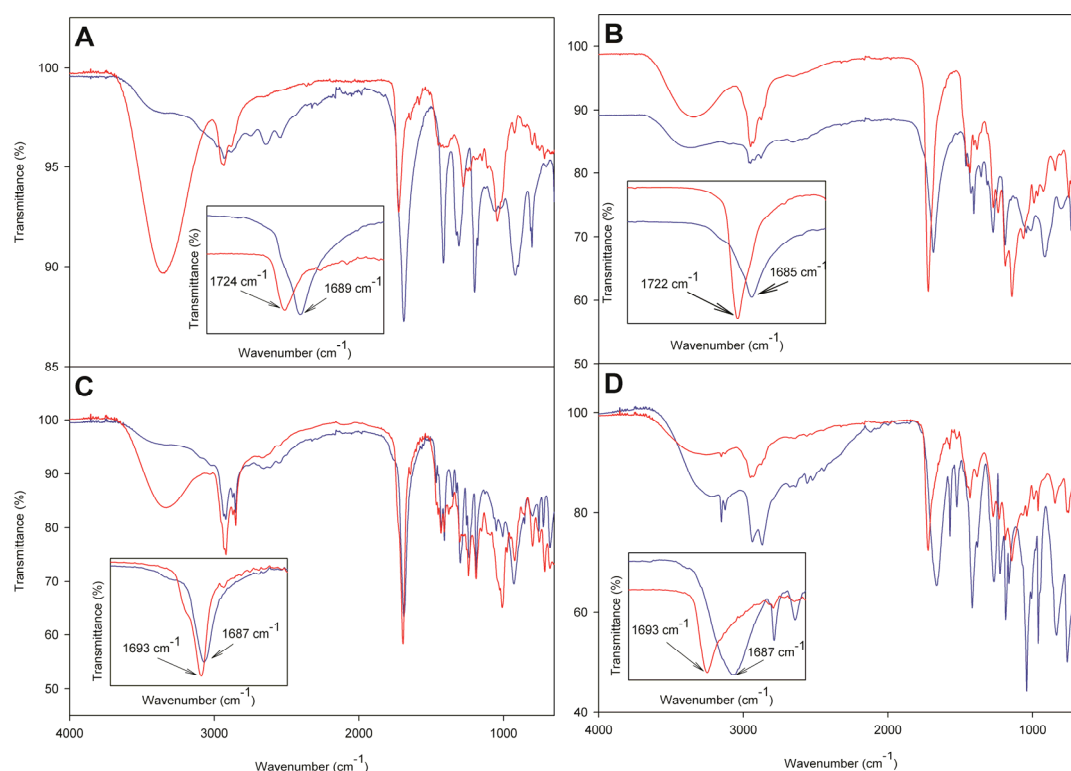
Figure 1 FT-IR spectra of non-activated alkyl diacids and non-activated furan derivatives in the presence of immobilized *Candida antarctica* lipase CALB. (A) control (blue) and reaction (red) of succinic acid and BHMF; (B) control (blue) and reaction (red) of adipic acid and BHMF ; (C) control (blue) and reaction (red) of sebacic acid and BHMF; and (D) control (blue) and reaction (red) of FDA and BDO. The inserts show an enlargement of the spectral region between 1500 – 1900 cm^{-1}.

Based on these results, we can sum up that the reactivity of BHMF in the enzymatic

polyesterification reaction with aliphatic dicarboxylic acids decreases as function of the chain length of the diacid as follows: C6 > C10 > C4. The conversion of FDA in combination with BDO in the presence of Novozym 435 and molecular sieves increased slowly in time, reaching 48.4% at 8 h, 84.2% at 24 h and a maximum level of 98.2% at 48 h reaction (Figure 2B). The initial reaction rate of FDA was 0.018 mmol $(L min)^{-1}$, which is 20 times lower than the initial reaction rate of BHMF with adipic acid. Moreover, the initial reaction rate of FDA with 1,4-butanediol, a C4 diol is 3.5 times lower than the initial rate of BHMF reaction with succinic acid, a C4 diacid, suggesting a higher catalytic activity of the enzyme for BHMF than for FDA. Figure 2B. also shows a comparison between the time course of the FDA-BDO enzymatic polymerization with and without molecular sieves. The ratio between FDA conversions in the presence of molecular sieves vs. FDA conversion in the absence of molecular sieves was constantly between 2 and 2.5 for the whole duration of the reaction (shown as • in Figure 2B).

3.2. Product Characterization

To identify the products of the condensation polymerization reactions described above, the reactions were conducted at 65 °C, in toluene/*tert*-butanol 70/30 (% wt) with 0.6 $UA.mL^{-1}$ catalyst and molecular sieves, for 24 h. MALDI-TOF-MS analysis showed that the product of all reactions investigated was a mixture of linear and cyclic ester oligomers, and that the degree of polymerization and the ratio between the cyclic and linear oligoesters was different for each reaction.
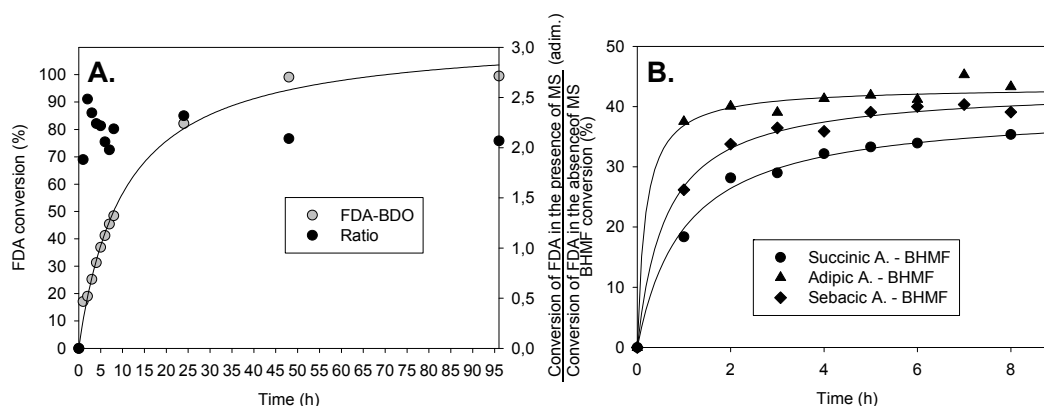
Figure 2 Monomer conversion as a function of reaction time for the CALB-catalyzed polymerization of (A) BHMF with C4, C6 and C10 dicarboxylic acids and (B) FDA and 1,4-butanediol.

The type of products formed as function of the furan monomer and the length of the chain of the aliphatic diacids is discussed below.

3.2.1. Low Molecular Weight Polymers Produced by Homocondensation 5-(Hydroxymethyl) Furan-2-Carboxylic Acid (HMFA).
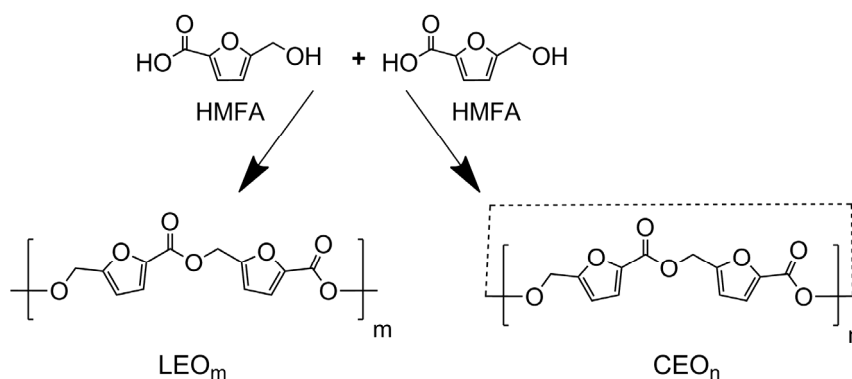
Scheme 1. Synthesis of oligoesters by homo-polycondensation of 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA).

The enzymatic homo-polycondensation of HMFA in the presence of Novozym 435 produced a mixture of cyclic (CEOs) and linear (LEOs) ester oligomers with a degree of polymerization (DP) from 6 to 23 (Figure 3), corresponding to a molecular weight between 863 to 2743 Da for LEOs and 745 to 2854 Da for CEOs. The homo-polycondensation reaction of HMFA is shown in Scheme 1. Figure 3 shows the products identified using MALDI-TOF-MS and their relative abundance in the product mixture. CEOs were the most abundant products formed (58.8%). Among the cyclic oligoesters, the dodecamer (CEO_{12}) and the decamer (CEO_{10}) were the most abundant species, counting for about 50% of the total CEOs. The linear ester oligomer LEO_{15} was the most abundant LEO.
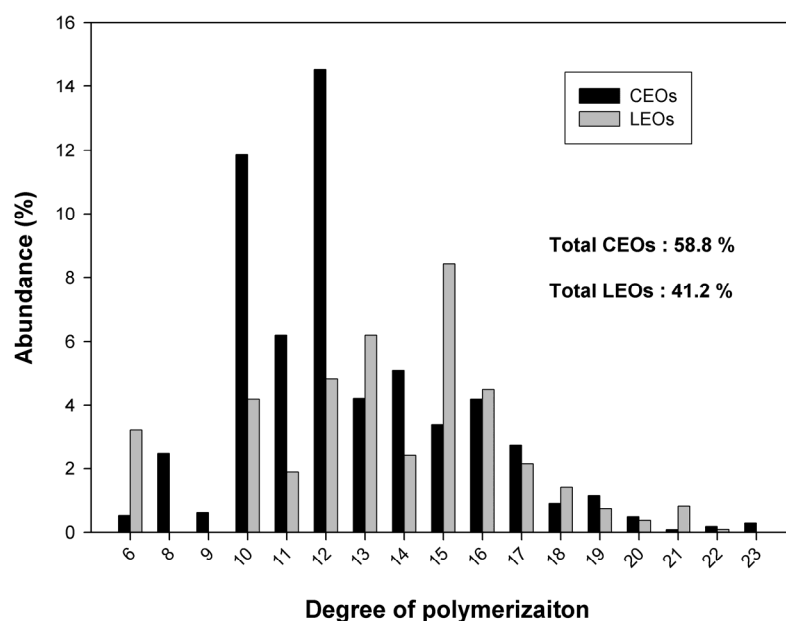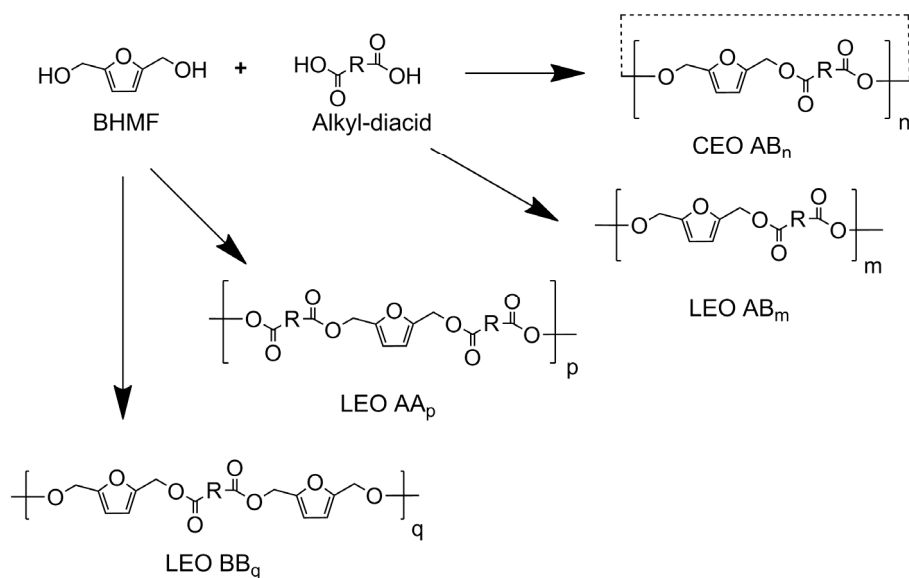
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Figure 3 Products obtained by homo-polycondensation of HMFA in the presence of CALB.

3.2.2. Low Molecular Weight Polymers Produced from Succinic Acid, Adipic Acid and Sebacic Acid in Combination with 2,5-Bis-Hydroxymethyl Furan (BHMF). Effect of Alkyl Chain Length.

Scheme 3. Synthesis of oligoesters by esterification of 2,5-bis (hydroxyl methyl)furan (BHMF) and alkyl-diacids of structure $HOOC(CH_2)_nCOOH$, where n is 2, 4 and 8

The products of the enzymatic polycondensation of BHMF with aliphatic dicarboxylic acids were cyclic ester oligomers (CEOs) and linear ester oligomers (LEOs) with either dicarboxyl (AA_n), dihydroxyl (BB_n) and hydroxyl-carboxyl (AB_n) end groups, as illustrated in Scheme 2. Figure 4 gives all the oligoesters identified using MALDITOF-MS analysis and their relative abundance for the reaction BHMF-succinic acid (Figure 4A), BHMF-adipic acid (Figure 4B) and BHMF-sebacic acid (Figure 4C). The results clearly show the pronounced effect of the diacid chain length on both the type of the oligomers formed (cyclic vs. linear, hydroxyl terminal vs. carboxyl terminal) and the length of the oligomers. Although the polymerization reaction was faster for the reaction of BHMF with longer chain diacids, as discussed before, the length of the polymer products decreased with the increase of the chain length. BHMF-succinic acid produced oligomers with a DP up to 19 (AA_9 and BB_9, Figure 4A), while the maximum DP obtained for BHMF-adipic acid and BHMF-sebacic acid was DP = 8 (AB_4 and CEO_4, Figure 4B) and DP = 5 (BB_2, Figure 4C), respectively. Among the formed products, BHMF-succinic acid produced the highest amount of cyclic ester oligomers (36.9%), which is three times the amount of total CEOs obtained in reactions with adipic acid (11% CEOs) and 2.4 times more than in reaction with sebacic acid. In the BHMF-succinic acid reaction, small cycles (CEO_1 to CEO_4) were the most abundant cyclic oligomers formed (Figure 4A), probably due to highest thermodynamic stability and reduced strain. Moreover the first cyclic member CEO_1, was the most abundant product (17.6%).

The most abundant types of products of the reaction of BHMF with the aliphatic diacids were the linear chain oligomers. The total amount of LEOs increased with the increase of the diacid chain length from C4 to C10, but the degree of polymerization (DP) decreased with the increase of the chain length (Figure 4.). Not only the length of the polymers depended on the alkyl chain length, but also the end group. In the BHMF-succinic acid reaction, linear ester oligomers from DP 2 (M_w AB_1, 228.2 Da) to DP 19 (AA_9, M_w 2009.7 Da and BB_9, M_w 2019.8) were formed, being the most abundant the low molecular weight species with DP $\in$ (2-7). Linear oligoesters with all possible end group

combinations were found (i.e. dicarboxy-, dihydroxy-, and hydroxyl- & carboxy-), and the amount decreased in the order $AA_n > AB_n \approx BB_n$. The most abundant linear ester oligomer was the dicarboxyl terminal AA_1, 11.8%. On the contrary, the linear oligomers produced in the reaction of BHMF with adipic acid and sebacic acid, respectively, were shorter oligomers, with the dihydroxy terminal oligoesters as the most abundant. The amount of the various end-terminal oligoesters decreased as follows $BB_n \gg AB_n \gg AA_n$, for both C6 and C10 acids. For both reactions, the predominant linear oligomer was the first member of the dihydroxy-terminal series, BB_1, namely 38.5% for BHMF-adipic acid and 50.6% for BHMF-sebacic acid. These results, together with the observation that the initial reaction rate increases with the diacid chain length, allows us to conclude that the initiation of the enzymatic polymerization reaction is favoured by longer alkyl chains, while the chain elongation not. It is possible that the low molecular weight linear ester oligomers BB_1 and AB_1 of BHMF with C6 and C10 diacids form non-reactive complexes with the enzyme, thus preventing the growth of the polymer chain.

3.2.3. Low Molecular Weight Polymers Produced from Furan-2,5-Dicarboxylic Acid (FDA) in Combination with 1,4-Butanediol (BDO).

The reaction between FDA and BDO and the type of products formed is illustrated in Scheme 3. The results of the MALDI-TOF-MS analysis of the isolated products are given in Figure 5. The product of the enzymatic condensation polymerization reaction between FDA and BDO was a mixture of cyclic ester oligomers (CEOs, 36.9%, Figure 5) ranging from DP 6 to DP 20 (M_w up to 2101.8 Da) and linear ester oligomers (LEOs, 63.1% Figure 5) with broader DP range, from DP 5 to 21 (M_w up to 2192.0 Da, BB_{10}). Among the cyclic ester oligomers, CEO_3 (17.0%) was the most abundant. The most abundant linear oligomers were the dicarboxy-terminal (AA_n, 39.5%), followed by dihydroxy-terminal (BB_n, 13.7%) and AB_n (9.9%), and the low molecular weight oligomer AA_2 (M_w 576.5 Da) was the most abundant LEO. The fact that at 24 h most of the FDA monomer was consumed and that most of the product consists of lower molecular weight oligomers

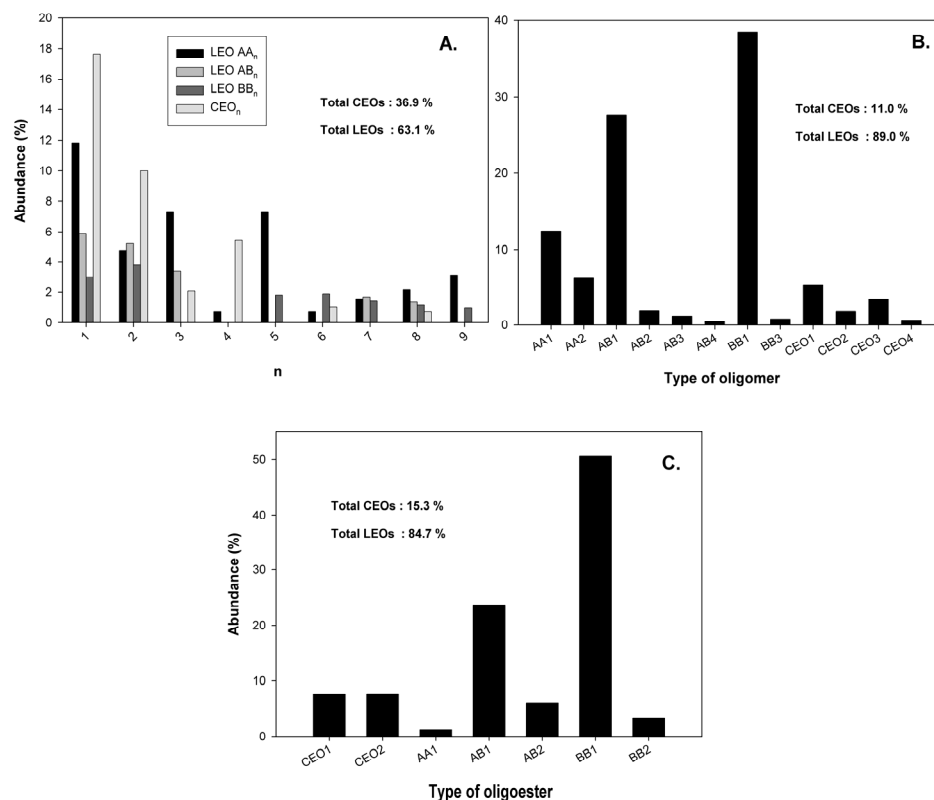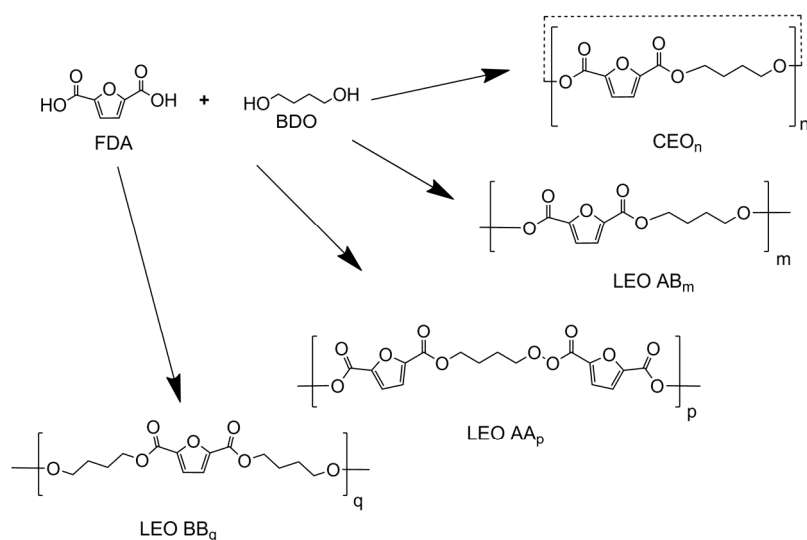suggests that the chain elongation is the slowest reaction.

Figure 4. Products of the condensation polymerization of 2,4-bis(hydroxymethyl)-furan (BHMF) with alkyl diacids. (A) BHMF-succinic acid; (B) BHMF-adipic acid; (C) BHMF-sebacic acid.

Scheme 4 Synthesis of oligoesters by esterification of furan-2,5-dicarboxylic acid (FDA) in combination with 1,4-butanediol (BDO).

4. Conclusions

We report, for the first time, a metal-free enzymatic route to synthesize polyesters based on non-activated furan building blocks and alkyl chain co-monomers, at moderate temperature (65 °C). All three furan monomers studied in this work, namely 5-hydroxymethylfuran-2-carboxylic acid (HMFA), 2,5-furan dicarboxylic acid (FDA) and 2,5-bis-(hydroxymethyl)furan (BHMF), were substrates for the immobilized lipase from *Candida antarctica* (Novozym-435). The products of the reactions were cyclic (CEOs) and linear (LEOs) ester oligomers.
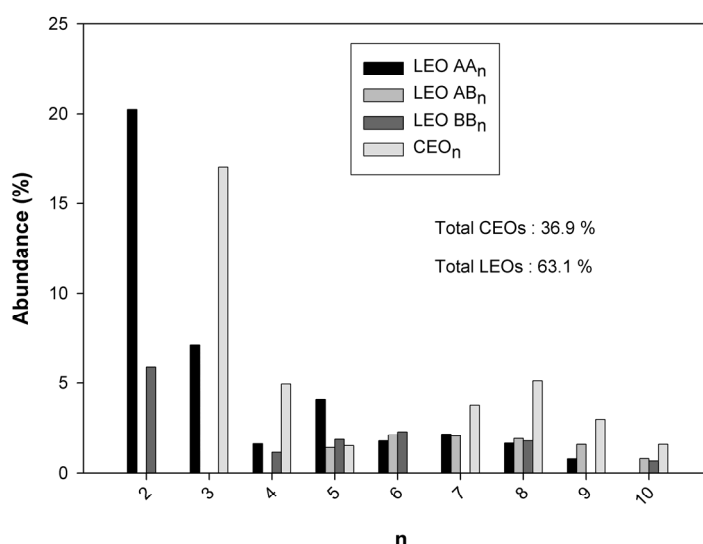
Figure 5 Products obtained by reaction of FDA (A) in combination with BDO (B) after 24 h reaction in the presence of CALB.

The degree of polymerization, the ratio CEOs vs. LEOs formed and the end group of the LEOs species are a function of the furan monomer used and the length of the aliphatic chain of the diacids (in the case of BHMF). The major products of the homo-polymerization of HMFA were cyclic ester oligomers, which are important building blocks for the synthesis of high molecular weight (co)polyesters via (bio)catalytic ring opening polymerization (ROP). Because to the best of our knowledge enzymes in general and CALB in particular, have not been used for the synthesis of polyesters derived from furan

building blocks, the good activity obtained for the polymerization performed here is very promising.

REFERENCES

Anderson EM, Larsson KM, Kirk O. 1998. One Biocatalyst-Many Applications: The Use of *Candida antarctica* B-Lipase in Organic Synthesis. Biocatalysis and Biotransformation 16(3):181-204.

Brugel E, Di Cosimo R; E. I. du Pont de Namours and Co., assignee. 2005. Manufacture of Certain Cyclic Ester Oligomers. United States.

Du W, Liu D, Li L, Dai L. 2007. Mechanism Exploration during Lipase-Mediated Methanolysis of Renewable Oils for Biodiesel Production in a tert-Butanol System. Biotechnology Progress 23(5):1087-1090.

Gandini A, Coelho D, Gomes M, Reis B, Silvestre A. 2009a. Materials from renewable resources based on furan monomers and furan chemistry: work in progress. Journal of Materials Chemistry 19(45):8656-8664.

Gandini A, Silvestre AJD, Neto CP, Sousa AF, Gomes M. 2009b. The furan counterpart of poly(ethylene terephthalate): An alternative material based on renewable resources. Journal of Polymer Science Part A: Polymer Chemistry 47(1):295-298.

Habeych DI, Juhl PB, Pleiss J, Vanegas D, Eggink G, Boeriu CG. 2011. Biocatalytic synthesis of polyesters from sugar-based building blocks using immobilized *Candida antarctica* lipase B. Journal of Molecular Catalysis B: Enzymatic 71(1-2):1-9.

Hilker I, Schaafsma AEJ, Peters RAH, Heise A, Nijenhuis AJ. 2008. Insight into lipase-catalyzed formation of macrocyclic oligoesters. European Polymer Journal 44(5):1441-1450.

Kiran KR, Hari Krishna S, Suresh Babu CV, Karanth NG, Divakar S. 2000. An esterification method for determination of lipase activity. Biotechnology Letters 22(19):1511-1514.

Kobayashi S. 2006. Enzymatic Ring-Opening Polymerization of Lactones by Lipase Catalyst: Mechanistic Aspects. Macromolecular Symposia 240(1):178-185.

Kricheldorf HR, Schwarz G. 2003. Cyclic Polymers by Kinetically Controlled Step-Growth Polymerization. Macromolecular Rapid Communications 24(5-6):359-381.

Kumar A, Gross RA. 2000. Candida antartica Lipase B Catalyzed Polycaprolactone Synthesis: Effects of Organic Media and Temperature. Biomacromolecules 1(1):133-138.

Lalot T, Marechal E. 2001. Enzyme-catalyzed Polyester Synthesis. J. Polymeric Materials 50:267-286.

Lavalette A, Lalot T, Brigodiot M, Marechal E. 2002. Lipase-Catalyzed Synthesis of a Pure Macrocyclic Polyester from Dimethyl Terephthalate and Diethylene Glycol. Biomacromolecules 3(2):225-228.

Mahapatro A, Kalra B, Kumar A, Gross RA. 2003. Lipase-Catalyzed Polycondensations: Effect of Substrates and Solvent on Chain Formation, Dispersity, and End-Group Structure. Biomacromolecules 4(3):544-551.

Mahapatro A, Kumar A, Gross RA. 2004. Mild, Solvent-Free δ-Hydroxy Acid Polycondensations Catalyzed by *Candida antarctica* Lipase B. Biomacromolecules 5:62-68.

Matsumura S. 2002. Enzyme-Catalyzed Synthesis and Chemical Recycling of Polyesters. Macromolecular Bioscience 2(3):105-126.

Moore JA, Kelly JE. 1978. Polyesters Derived from Furan and Tetrahydrofuran Nuclei. Macromolecules 11(3):568-573.

Moreau C, Belgacem MN, Gandini A. 2004. Recent Catalytic Advances in the Chemistry of Substituted Furans from Carbohydrates and in the Ensuing Polymers. Topics in Catalysis 27(1):11-30.

Sugihara S, Toshima K, Matsumura S. 2006. New Strategy for Enzymatic Synthesis of High-Molecular-Weight Poly(butylene succinate) via Cyclic Oligomers. Macromolecular Rapid Communications 27(3):203-207.

ter Haar R, Schols HA, van den Broek LAM, Saglam D, Frissen AE, Boeriu CG, Gruppen H. 2010. Molecular sieves provoke multiple substitutions in the enzymatic synthesis of fructose oligosaccharide-lauryl esters. Journal of Molecular Catalysis B: Enzymatic 62(2):183-189.

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Zhi-wei G, Charles J. S. 1988. Enzymic synthesis of macrocyclic lactones. J. Am. Chem. Soc. 110:1999-2001.

Zhi-wei G, Ngooi TK, Scilimati A, Fulling G, Sih CJ. 1988. Macrocyclic lactones via biocatalysis in non-aqueous media. Tetrahedron Letters 29(44):5583-5586.

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Process Strategies to Improve the CALB-Catalyzed Synthesis of Ester oligomers from Biobased Building Blocks

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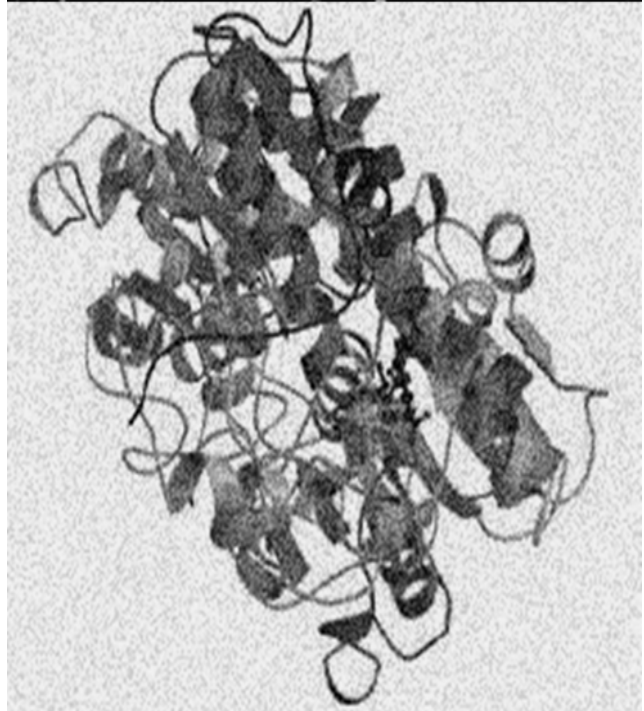
ABSTRACT

Biocatalytic synthesis of oligoesters from biobased building blocks, succinic acid, 1,4-butanediol (BDO), isomannide, and 2,5-furan dicarboxylic acid (FDA) were performed with immobilized CALB as biocatalyst in toluene-based medium. The formed products is a mixture of linear and cyclic ester oligomers. Different bioreactor types were investigated to improve the substrate conversion and the formation cyclic ester oligomers (CEOs). Succinic acid - BDO showed the maximum conversion in pulse fed-batch bioreactor (99.6%) as well as the largest abundance of CEOs (71%), while the amplest product types were found in fed-batch bioreactor (DP up to 21). Isomannide - succinic acid reached the maximum conversion (94%) in batch bioreactor. Plug-flow bioreactor led to the synthesis products with the largest DP (DP = 25) and the largest formation of CEOs occurred in pulse fed-batch bioreactor (48.9%). Almost full substrate conversion (99.1%) for FDA - BDO in pulse fed-batch bioreactor, while the plug-flow bioreactor showed formation of oligomers up to DP 25. the largest and comparable CEOs were found in batch (37%) and plug-flow bioreactors (33%). This work paves a way to improve substrate conversion and formation of CEOs from biobased building blocks as a function of the bioreactor type.

1. INTRODUCTION

Polyesters derived from building blocks obtained from renewable resources have attracted high interest from both academia and the industrial world. Biobased polyesters are considered the most promising polymers of the future. Although the biobased polymers still must confirm the high expectations in terms of performance, economic and technologic feasibility and sustainability, the research into new polymers and new green technologies for their production must continue. Green chemistry together with the concept of biorefinery and the new process technology advances are the frame for the future developments of biobased building blocks and their polymers. Among all of new building blocks, sugar (C5 and C6) based compounds are currently attracting enormous attention because of their potential in the biobased economy and ubiquitous presence in nature. Succinic acid, 1,4-butanediol (BDO), di-anhydro hexitols (DAH), and furan derivatives are examples of such compounds. All of them can be used as basis for the synthesis of new polyesters either via ring-opening polymerization (ROP) or polycondensation, also called step-growth polymerization (Delhomme et al. 2009; Gandini 2008; Kricheldorf 1997; Schwarz and Kricheldorf 1996). Despite that polycondensation has been the most widely applied way to produce polyesters, some drawbacks have been evident such as the formation of the undesired products, degradation of the substrate, development of color in the final product as effect of the partial oxidation of either the substrate or the product, synthesis of low molecular weight products due to limitations in the chemical catalyst specificity, losses of the initial reaction material due to the high temperatures and the low pressure that are required to perform the reaction, among others (Gandini et al. 2009; Moore and Kelly 1978). The ring-opening polymerization, however, has been demonstrated as an improved process due to the possibilities to be carried out under milder conditions of temperature and pressure, in the presence either of chemical catalyst or enzymes, the formation of products with higher molecular weight and lower polydispersity, among others

(Albertsson and Varma 2003; Kobayashi et al. 2006; Sugihara et al. 2006). For example, enzyme-catalyzed ring-opening polymerization of β-propiolactone was done in the presence of lipase from *Candida rugosa* (*Candida cylindracea*) at 60 ^{o}C and in batch bioreactor. The final product reached a molecular weight of 49.100 Da and the reaction yield was 99% (Matsumura et al. 1996). Similarly, ROP of D-L-lactide (*meso*-lactide) using a PS lipase (*Pseudomona spp.*) was fully converted and the final molecular weight of the polyester was 126.000 Da (Matsumura et al. 1997). Yamamoto *et al.* reported the synthesis of poly(tetramethylene carbonate) (PTMC) and poly(hexamethylene carbonate) (PHMC) via ROP using CALB. The final product presented an average molecular weight of 119 and 399 kDa, respectively (Yamamoto et al. 2009). Furthermore, high-molecular-weight poly(butylene succinate) (PBS) using ROP in the presence of CALB has been also done (Sugihara et al. 2006). The reaction was performed with CALB as biocatalyst and the final average molecular weight of the polyester was 130 kDa. This molecular weight was significantly higher than that produced by the direct polycondensation of the diol and di-acid with CALB and also with conventional chemical catalyst. The last examples demonstrates that it is needed to invest efforts in routes to synthesize cyclic ester oligomers (CEOs) that become the building blocks for ROP.

There are factors that trigger the enzymatic formation of CEOs from a diacid - diol system over step-growth polymerization: (i) equimolar substrates ratio, (ii) low substrate concentrations, (iii) affinity biocatalyst - substrate, (iv) type of reaction media, and (v) efficiency in the removal of by-products. For example, the enzymatic reaction of terephthalic acid and ethylene glycol leads to the formation of one cyclic tetramer as unique cyclic product under batch conditions (Hilker et al. 2006; Lavalette et al. 2002). The enzymatic formation of CEOs from α,ω-di-acids (C4-C14) and diols (C7-C18) with lipases from different origins (*Candida rugosa, porcine pancreas,* and *Pseudomonas spp.*) was carried out at different conditions. Low substrate concentrations (10 mM) and high temperatures (> 45 ^{o}C) of reaction together with the use of a non-polar reaction medium (isooctane, hexane, cyclohexane, and carbon tetrachloride), promoted the synthesis of

the two first CEOs up to 72% over the total synthesized products. The addition of water (2%) into the reaction medium reduced the prevalence of CEOs from 38% to 22% together with an increase of the linear oligomers (Zhi-wei and Charles J. 1988). CEOs synthesis can be carried out not only by direct polycondensation of free diacid (diesters) and diols, but also by enzymatic depolymerization of long chains. This approach finds application in the full recycle of the synthesized polymers (Matsumura 2002; Sugihara et al. 2006).

Most of the examples of small scale polyesters synthesis by chemical or enzymatic catalyst are carried out in batch stirred tank bioreactors (STR), which is a good approach for laboratory scale and kinetic study. In addition, enzyme recovery and regeneration for long term reuse is difficult and costly at large scale. In a batch operation system mixing and substrate dispersion are achieved by mechanical agitation, this requires a relatively high input of energy per unit volume and contributes to the physical damage of the support of the enzyme. Furthermore, the operation requires the load of the substrates and the biocatalyst at time zero. Hence, not only the highest substrate concentrations are found at the beginning of the reaction, but also there is not any controllability on the substrate concentration during the course of the reaction. Therefore, it is interesting to explore other possibilities that could lead to better control on the substrate concentration into the bioreactor while keeping good reaction conversion (Doran 1995). For example, fed-batch bioreactor offers a way to have more control on the substrate concentration. The continuous addition of the substrate with the simultaneous consumption into the bioreactor could favor the prevalence of CEOs in a reasonable frame of time due to the dilution factor of the substrate into the reactor. The application of fed-batch operation has been described as a way to increase the formation of CEOs over LEOs. Kricheldorf *et al.* (Kricheldorf et al. 2003) reported the synthesis of CEOs from isosorbide and adipoyl dichloride as acyl donor and pyridine as strong proton donor. The system consisted of the drop-wise addition of the reactant to the medium (pseudo-high-diluted conditions). This strategy can be considered a fed-batch operation mode in a

chemical catalyzed esterification. The chemical catalyzed reaction led to formation of 100% CEOs with a conversion above 96%. (Kricheldorf et al. 2003; Williams et al. 1997). Another possible fed-batch operation mode is by the addition of the substrates by pulses, in that manner the substrate can be kept, at low concentration in the reaction medium with the expected prevalence of the CEOs over LEOs in the product formed. This bioreactor operation mode has not been explored in enzyme-catalyzed reactions. This strategy, however, has shown positive outcome in the fermentative production of the natural insecticide from *Bacillus thuringensis*. Habeych et al. reached higher cell density and an increase of the volumetric productivity in seven-fold by the reduction of the substrate inhibition effect by pulse fed-batch operation compared to batch fermentation (Habeych et al. 2010). In an analogous way, the pulse feeding of the substrate in biocatalyzed reaction may reduce any negative effect associated with the substrate concentration. Ding and Tan reported a comparative study of lactic acid production using different fed-batch feeding strategies (Ding and Tan 2006). Batch, pulse fed-batch, constant fed-rate fed-batch, and exponential fed-batch were used for the synthesis of the acid. Fed-batch culture when the substrate was supplied in exponential feeding was the most effective method for the fermentation of *L*-lactic acid. A *L*-Lactic acid concentrations of 180 g.L^{-1}, productivity of 2.14 g.(l.h)$^{-1}$, and yield of 90.3 % where the maximum values reached, while batch operation mode exhibited lower product concentration (112.5 g.(l.h)$^{-1}$), productivity (1.34 g.(l.h)$^{-1}$), and yield (88.6 %). Hence, comparing with the traditional batch culture, the exponential feeding glucose showed 56.5% improvement in L-lactic acid production, 68.6% improvement in dry cell weight, and 59.7% improvement in productivity, respectively (Ding and Tan 2006).
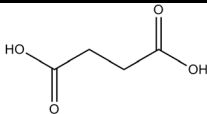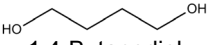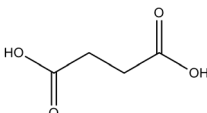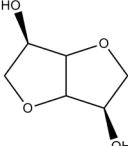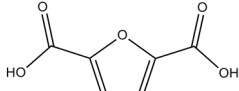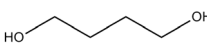
In addition to batch and fed-batch bioreactor there is possible to carry out the enzymatic esterification in a reactor with continuous feed of substrate, namely continuous reactor operation. In this type of operation, the amount of material that can be processed over a given period is represented by the flow rate. Therefore, for a given throughput, the reactor size and operating costs are minimized when the retention time is as small as

possible (Doran 1995). Evidently the retention times is strongly affected by the catalytic characteristics of the system. A variant of the continuous process is the plug-flow operation (packed bed bioreactor). This system can be used for the synthesis of ester oligomers in a lipase bed. Some reaction system could have problems due to the clogging that occurs as a consequence of the product insolubilization, such as during the enzymatic modification of fatty acids or the synthesis of high molecular weight polyesters (Okajima et al. 2003). There are few examples of enzyme-catalyzed enantioselective processes carried out in continuous bioreactors. Most of these studies were applied for the synthesis of chiral pharmaceutical intermediates using immobilized lipases in packed-bed reactor. Experimental design has been applied for the optimization of enzyme-catalyzed (Lipozyme IM from *Rhizomucor miehei)* acidolysis of several oils in packed bed reactor arrangement (Xu et al. 2000a; Xu et al. 2000b). Caprylic acid (*n*-octanoic acid) was the acyl donor and factors such as residence time, reaction temperature, and substrate molar ratio were taken into consideration for the experimental design. High incorporation of acyl donor and retention of high levels of eicosapentaenoic and docosahexaenoic acids in original menhaden oil were obtained. All parameters studied had positive effects on the incorporation of caprylic acid, but only residence time and substrate molar ratio had negative effects on the content of eicosapentaenoic plus docosahexaenoic retained (Xu et al. 2000a). Likewise, the synthesis of triacylglycerols by the same lipase (Lipozyme IM) in a packed bed bioreactor used the approach above mentioned (Xu et al. 2000b). The column had an internal diameter of 47 mm and 50 cm length. The minimum flow rate (1 mL.min^{-1}) that led the reactor to the equilibrium, as well as the maximum water (0.08%) tolerated within the system were found. The preparation of *trans*-(2R,3S) methyl (4-methoxyphenyl)glycidate with enantiomeric excess (e.e.) >99% has been carried out by enantioselective hydrolysis of the racemic glycidate ester by a commercial phospholipase (Lecitase®). The reaction was conducted in a packed column, using toluene as reaction medium (Mishra et al. 2011). The enzyme activity was almost invariant for the first 15 days of use in the plug-flow bioreactor. The selective production of (S)-γ-fluoroleucine ethyl ester by CALB was

carried out in batch and plug-flow bioreactor (Truppo et al. 2008). The plug-flow bioreactor exhibited 20-fold reduction in enzyme to substrate ratio and an increased product yield (>90%) and ee (86%) compared with the batch operation mode (yield of 79% and ee of 78%).

The benefits associated with a particular bioreactor operation mode depends of the kinetic of the reaction, as well as perfect mixing, low mass transfer limitation, among others. For example, since it is thought that low substrate concentrations lead to the prevalence of CEOs over LEOs, we could expect that pulse fed-batch bioreactor and fed-batch bioreactor increases the formation of CEOs compared with the batch bioreactor, although it is not the unique factor that trigger the formation of CEOs. In previous Chapters we demonstrate that the esterification carried out in batch operation mode for several biobased building blocks is feasible, but most of the experiments were carried out in batch reactor. The application of pulse fed-batch bioreactor in the synthesis of oligoesters from BDO and succinic acid, however, demonstrated that an increase in CEOs accumulation could be reached by changing the bioreactor operation mode. Therefore, The current Chapter describes different bioreactor operation strategies to evaluate lipase-catalyzed oligomerization between several biobased diacids (succinic acid and 2,5-furan dicarboxylic acid) and diols (1,4-butanediol and 1,4:3,6-dianhydro-D-mannitol or isomannide) using immobilized *Candida antarctica* lipase B (CALB). The enzyme-catalyzed reactions were performed without activation of the biobased building blocks (Table 1). To the best of our knowledge this is the first report that addresses the use of different bioreactor operation mode as a strategy to increase the synthesis of oligoesters of these biobased building blocks.

Table 1. Biobased building blocks used in this work.

Reaction pairs	
diacid	**diol**
Succinic acid	1,4-Butanediol (BDO)
Succinic acid	Isomannide
2,5-furan dicarboxylic acid (FDA)	1,4-Butanediol (BDO)

2. EXPERIMENTAL SECTION

2.1. Materials

Solvents were obtained from Sigma – Aldrich. Novozym® 435 was kindly donated by Novozyme (The Netherlands). All solvents were equilibrated in 4 Å molecular sieves (Sigma-Aldrich) for at least 24 hours before use. Isomannide (>95%), succinic acid (>99.9), *Trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2- propenylidene] malononitrile (DCTB, >99.9%), and potassium trifluoroacetate (>99.9%) were obtained from Sigma – Aldrich. Furan-2,5-dicarboxylic acid (FDA, >99%) was kindly synthesized and donated by the group of Dr Daan van Es (Wageningen UR Food & Biobased Research, Biobased Products Division).

2.2. Analytical Methods

2.2.1. HPLC: 2,5-Furan Dicarboxylic Acid (FDA) Analysis.

The concentration of FDA was followed using a Waters 717 Plus auto-sampler outfitted with a Waters 1525 binary HPLC pump and a Waters 2414 refraction index detector. The column was an ODS-2 Intersil (250 x 3 mm, Varian Inc.) operated at 40 $^{\circ}$C. Elution of compounds was done in a gradient flow, acetonitrile and water (from 5:95 (v/v) to 95:5 (v/v)). Both mobile phases were supplemented with trifluoroacetic acid (0.1% v/v, > 99%). Eluent flow rate of 0.5 mL min^{-1} and the sample injection volume of 10 μL. FDA was detected at 280 nm.

2.2.2 Gas Chromatography (GC) of Other Biobased Building Block Analysis

GC analysis was performed on a Hewlett-Packard 5890 Series II Gas Chromatograph equipped with an automatic injection system (HP7673 GC/SFC Injector and Controller). Injection volume 1 mL. Split ratio 1:20. Column pressure 150 kPa helium. GC column: Varian CP-FFAP (free fatty acids), 25 m x 0.32 mm x 0.30 mm. Detector: FID at 280 $^{\circ}$C. Injection port temperature 300 $^{\circ}$C. GC program: hold 2 min at 60 $^{\circ}$C, ramp 10 $^{\circ}$C.min^{-1} to 300 $^{\circ}$C.

2.2.3. Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

The samples (10 μL) were mixed with 10 μL of the matrix solution (DCTB, 160 mM) and 2.5 μL trifluoroacetate as dopant (33 mM). Matrix and dopant were dissolved in tetrahydorfuran and 0.3 μL of the final mixture were spotted and dried on a polished stainless steel plate. MALDI-TOF analysis was done on mass spectrometer Ultraflex workstation (Bruker Daltonics, Bremen, Germany). Polyethylene glycol (600, 1000, and

2000 Da) was used as molecular weight standard. Three spectra with 200 shoots per spot were collected using the lowest possible laser intensity that led to a good quality spectrum. Within each MALDI-TOF spectrum, the intensity obtained by the ions-adduct per specie were added up and the relative contribution of each compound was calculated. It was assumed that there are no significant differences between the response factors of the molecules. A similar procedure has been used by other authors (Haar et al. 2010; Huang et al. 2007).

2.2.4. Enzymatic Activity of *Candida antarctica* Lipase B (CALB)

The esterification activity was determined using *n*-butyric acid and *n*-butanol as substrates in toluene:*tert*-butanol solution (70:30 % wt) at 65 ^{o}C. The activity was based in the method reported by Kiran and collaborators (Kiran et al. 2000). 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. Immobilized *Candida antarctica* lipase B (Novozym 435) showed an enzymatic activity of 0.06 UA.mg^{-1}.

2.2.5. Biocatalytic Synthesis of Polyesters

Stirred Tank Batch Reaction. Batch reactions were run in a 250 mL glass bioreactor. Batch reactions were carried out in 50 mL of toluene : *tert*-butanol solution (70:30 % wt) containing 500 μmol of one of the non-activated diacid (succinic acid or FDA) and 500 μmol of one of non-activated diol (BDO or isomannide) at 65 ^{o}C and in the presence of molecular sieves to retain the water formed during the reaction (Table 2). Mixing was done with overhead stirring via a paddle blade impeller and was just sufficient to suspend the immobilized CALB and the molecular sieves.

Stirred tank Pulse Fed-Batch Reaction. Pulse fed-batch reactions were carried out in the same vessel as the batch. 50 mL of toluene:*tert*-butanol solution (70:30 % wt) together with dried molecular sieves and immobilized CALB (Table 2) were placed into the bioreactor and every hour 1 mL of stock solutions with the different substrates were added (Table 2). After the final addition at 8 h, the reaction was allowed to proceed up to 24 h.

Stirred Tank Fed-Batch Reaction. Fed-batch reactions were carried out in the same vessel as the batch reactions. 50 mL of toluene:*tert*-butanol solution (70:30 % wt) together with dried molecular sieves and immobilized CALB (Table 2) were placed into the bioreactor. The reaction was performed at 65 ^{o}C and a constant flow of 4.9 ± 0.1 mL.h^{-1} of stock solution (Table 2) was fed into the bioreactor during 24 h.

Plug-Flow Bioreactor. The reactions were run in a 77 mm x 200 mm polypropylene column. The column was packed with 2.30 ± 0.05 g Novozym® 435 and 8.2 ± 0.1 g of 4 Å dried molecular sieves. At the top and at the bottom the column was provided with tight frits to minimize the fluidization of the bed. The column was kept at 65 ^{o}C and the substrate stock solutions were pumped through the column at 4.92 ± 0.1 mL.hr^{-1} (Table 2).

Table 2 Polyesterification conditions for the different bioreactor operation mode.

Bioreactor operation mode	Substrate	Stock (mM)	Volume* (mL)	Enzyme*** (UA.mL^{-1})	Inflow (mL.h^{-1})	Mol. sieves (g.L^{-1})	Temp. (°C)
Batch							
	BDO succinic acid	10	50	0.22	-	100	65
	Isomannide succinic acid	10	50	0.22	-	100	65
	FDA BDO	10	50	0.43	-	100	65
Pulse Fed-batch bioreactor							
	BDO succinic acid	50	50	0.22	1	100	65
	Isomannide succinic acid	50	50	0.22	1	100	65
	FDA BDO	50	50	0.43	1	100	65

Table 2 Polyesterification conditions for the different bioreactor operation mode. (Continuation)

Bioreactor operation mode	Substrate	Stock (mM)	Volume* (mL)	Enzyme*** (UA.mL⁻¹)	Inflow (mL.h⁻¹)	Mol. sieves (g.L⁻¹)	Temp. (°C)
Fed-batch bioreactor	BDO succinic acid	14	50	0.22	4.92	100	65
	Isomannide succinic acid	14	50	0.22	4.92	100	65
	FDA BDO	20	50	0.43	4.92	100	65
Plug-flow bioreactor (Packed bed bioreactor)	BDO succinic acid	20	9**	5.8	4.92	342	65
	Isomannide succinic acid	20	9**	5.8	4.92	342	65
	FDA BDO	20	9**	5.8	4.92	342	65

* Initial reaction volume; ** Empty volume into the column (porosity, e = 0.38); ***Activity per final reaction volume.

3.   RESULTS AND DISCUSSION

The biocatalytic synthesis of polyesters from the biobased substrates was performed in different type of bioreactors to evaluate the effect of the reactor type on the substrate conversion and the type of products obtained. From previous experiments in our laboratory we proved that all reactions showed significant conversion of substrates, except the reaction of FDA in combination with isomannide (data not shown). Therefore, the experiments were carried out with the substrate pairs shown in Table 1: (i) 1,4-butanediol (BDO) in combination with succinic acid, (ii) 1,4-butanediol (BDO) in combination with 2,5-furan dicarboxylic acid (FDA), (iii) isomannide in combination with succinic acid, and (iv) 2,5-furan dicarboxylic acid (FDA) in combination with isomannide. The polyesterification of the three reaction systems was carried out in the presence of immobilized *Candida antarctica* lipase B (CALB, Novozym 435). The enzyme had an esterification activity of 0.06 $UA.mg^{-1}$ at the conditions described in the Experimental Section. The reactions were tested in four bioreactor operation modes, namely (i) batch, (ii) pulse fed-batch, (iii) fed-batch, and (iv) plug-flow. From now on the term *"fed-batch"* refers exclusively to the fed-batch bioreactor with continuous inflow. For all the bioreactors the reaction was performed at 65 ^{o}C (Table 2).

3.1   Batch Bioreactor

The biocatalytic polyester synthesis using CALB with the different biobased substrates was carried out as described in the Experimental Section. Two control reactions were set up. The first control included the substrates (diacid and diol) without CALB and without molecular sieves, while the second control contained substrate with molecular sieves and without CALB. In both control reactions we observed neither quantifiable substrate conversion nor product synthesis. The results for the biocatalytic synthesis of polyesters from the biobased building blocks in batch reaction are depicted in Figure 1 and Table 3.

The maximum conversions after 24 h for FDA in combination with BDO, isomannide in combination with succinic acid, and BDO in combination with succinic acid are 82.1 %, 93.8 %, and 85.6 %, respectively (Table 3). The initial rate of reaction are 1.08, 3.40 and 0.54 μmol.(mL.h)$^{-1}$ for FDA, isomannide, and BDO, respectively. The substrate consumption rate of reaction, calculated as the first derivative of the lines substrate concentrations vs. time, increases in the order isomannide - succinic acid >> FDA - BDO >> BDO - succinic acid during the time course analysis. Moreover, the volumetric productivities (mmol.(L.hour)$^{-1}$) for all the three reactions in batch operation were comparable (Table 3). According to the total of the results, batch bioreactor operation mode seems to be favourable for the conversion of isomannide in combination with succinic acid, showing not only high reaction conversions, but also high initial rate of reaction. Nevertheless, the productivity of isomannide-succinic acid was not that much different than the productivities of the other diacid-diols evaluated. The batch bioreactor was the best system in terms of conversions for the biocatalytic oligomerization of succinic acid in combination with isomannide.

3.2   Fed-Batch Bioreactor

Fed-batch bioreactor was used on the biocatalyzed synthesis of polyesters using immobilized CALB as described in the Experimental Section. The reaction was carried out in the presence of molecular sieves. At time zero the enzyme was added to the reaction medium free of substrate and the stock solution containing the substrates was pumped into the reactor. The average conversion of succinic acid and BDO between the hour 2 and 6 shows a rather constant value of 42.3% (±1.7). The maximum substrate conversion (49.9%) was reached after 24 h (Figure 2).

Table 3 Summary results of the different bioreactor operation modes.

Bioreactor operation mode	Substrate	Conversion (%)	Vol. Productivity (mmol.(l.h)$^{-1}$)	Initial reaction rate (mmol.(l.h)$^{-1}$)	CEOs (%)
Batch					
	BDO Succinic acid	85.6	0.36	0.54	52.4**
	Isomannide Succinic acid	93.8	0.39	3.4	41.6
	FDA BDO	82.1	0.34	1.08	36.9
Pulse fed-batch	BDO Succinic acid	99.6	0.62	0.98	71.1
	Isomannide Succinic acid	48.6	0.36	0.78	48.9
	FDA BDO	99.1	0.74	0.94	27.5
	Isomannide Succinic acid	14.2	0.06	-	21.9
	FDA BDO	66.2	0.28	-	27
Plug-flow * (Packed bed)	BDO Succinic acid	45.2	3.01	3.82	68.4
	Isomannide succinic acid	52.4	3.49	4.02	11.8
	FDA BDO	43.7	2.91	3.73	33.4

** By HPLC

Succinic acid in combination with isomannide shows a maximum conversion inferior to 40% and typically below 30%. The conversion at 24 h is 14.2% (Figure 3). The reaction of FDA in combination with BDO, however, shows a conversion higher than 57% and typically above 70%. The conversion decreases in time reaching a value of 66.2% at 24 h (Figure 4). Fed-batch bioreactor exhibits lower conversions than batch bioreactor at 24 h for all reactions performed (Table 3).
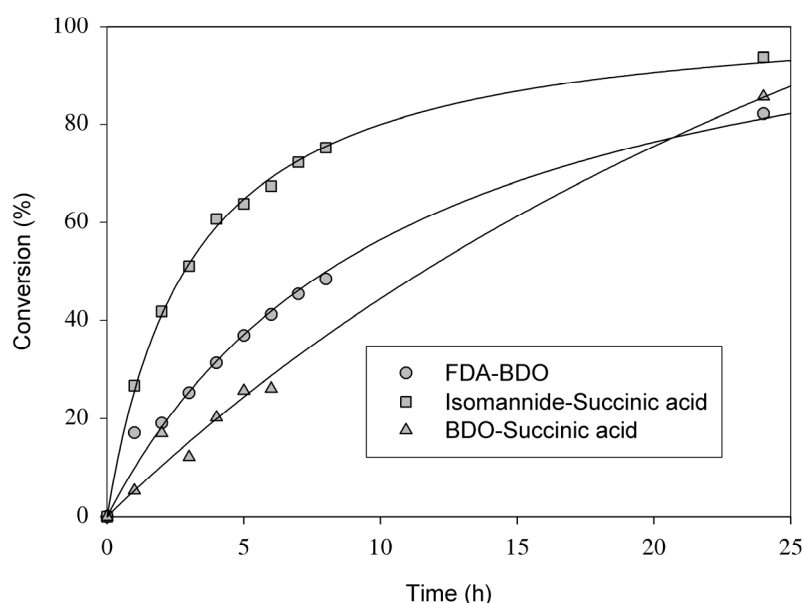

Figure 1 Time course analysis of FDA-BDO, isomannide-succinic acid, and BDO-succinic acid in batch bioreactor operation mode (substrate conversion).

The conversion of FDA in combination with BDO is higher than for the rest of diacid-diol pairs, which is contrary to what is found in the batch bioreactor. In addition, the volumetric productivities followed the order: FDA - BDO (0.28 mmol.(l.h)$^{-1}$) > BDO - succinic acid (0.21 mmol.(l.h)$^{-1}$) >> isomannide – succinic acid (0.06 mmol.(l.h)$^{-1}$, Table 3). These results compared with the batch operation mode demonstrate that fed-batch operation bioreactor mode in an CALB-catalyzed reaction it is not always associated to an increase in the total substrate conversion as has been shown elsewhere (Truppo et al. 2008).

3.3. Pulse Fed-Batch Process

The biocatalytic polyester synthesis in the presence of CALB with the different biobased substrate in a pulse fed-batch bioreactor was carried out during 24 h as described in the Experimental Section (Table 3). As it was mentioned before, the conversion is a result of the catalytic activity of the enzyme and it is due neither to the molecular sieves nor to the spontaneous reaction of the diacid-diol pair.
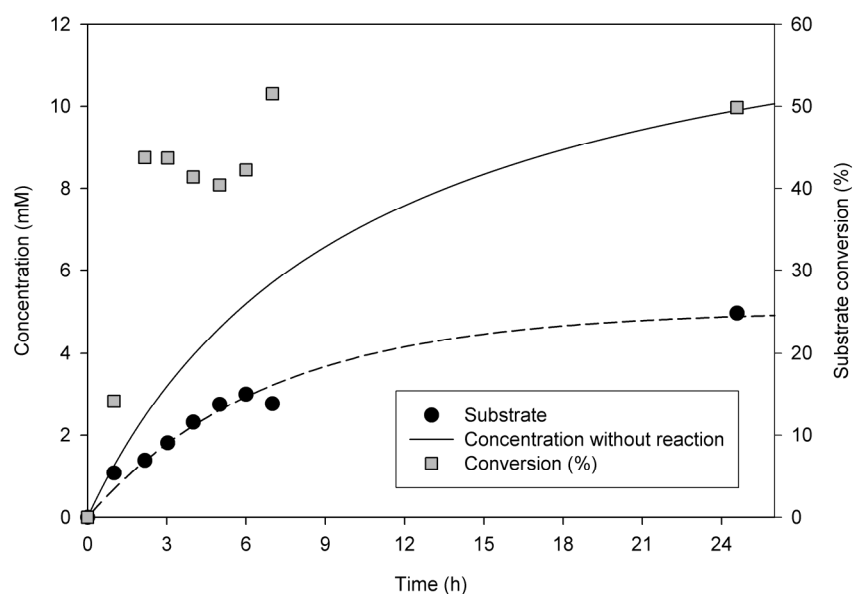

Figure 2 Time course analysis of succinic acid – BDO in fed-batch bioreactor operation mode. Substrate conversion and concentration.

The pulse fed-batch reaction was performed by drop-wise addition of 1 mL of stock solution every hour. The substrate conversions at time t were calculated taking into account the concentration (C_{theo}) into the bioreactor at time t when the reaction did not take place and the actual concentration (C_{Actual}) at time t. The results for the reaction of isomannide in combination with succinic acid (Figure 5) show that after 22 h 48.6% of the substrate was consumed. The slope of the line concentration against time is higher after the substrate addition at 22 h than at the beginning of the reaction. This is an indirect indication that the reaction proceeded slower at this time than at time zero. FDA in

combination with BDO, however, exhibits almost a total conversion after 22 h (99.1%, Figure 6).
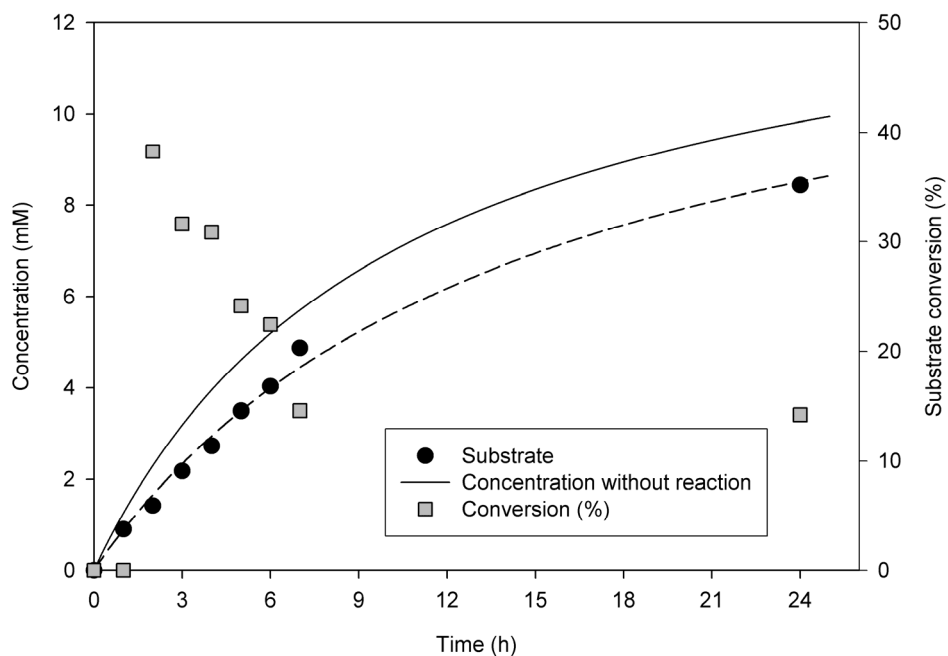

Figure 3 Time course analysis of isomannide - succinic acid in fed-batch bioreactor operation mode. Substrate conversion and concentration.
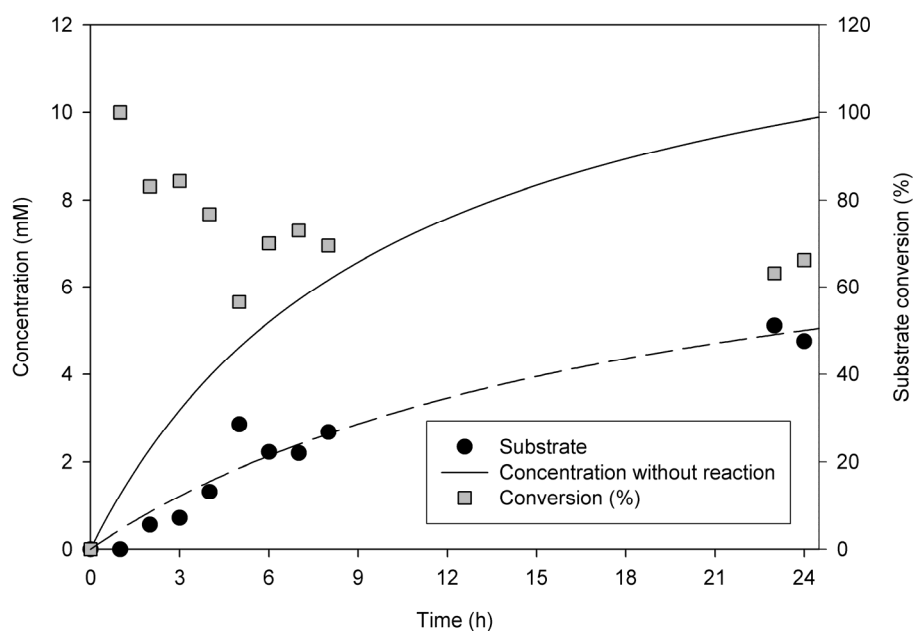

Figure 4 Time course analysis of FDA – BDO in fed-batch bioreactor operation mode. Substrate conversion and concentration.

As isomannide and succinic acid, the first derivative from the line concentration as function of time for FDA-BDO at the beginning of the reaction is higher than at 22 h (Figure 6). The decrease in the derivative value of FDA-BDO concentration after 22 h is an indirect indication of the reduction of the enzymatic reaction rate at this point.
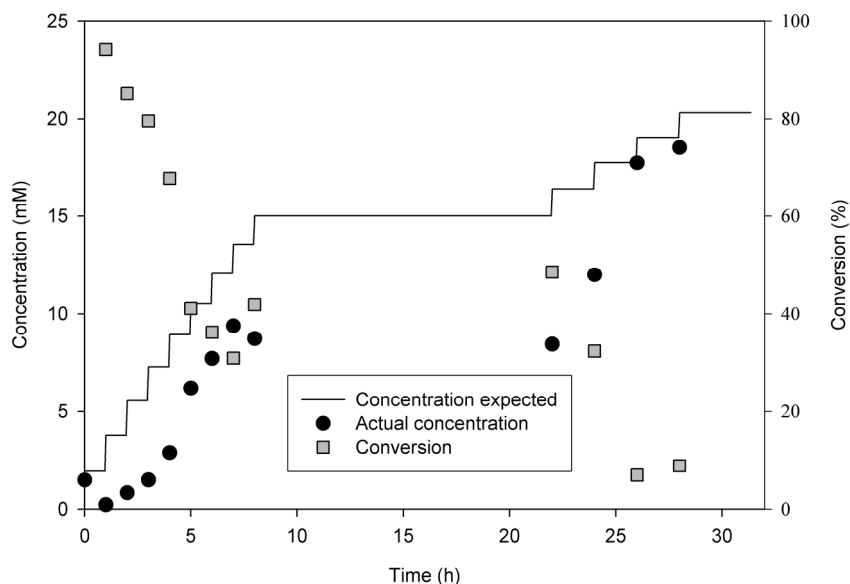

Figure 5 Pulse fed-batch operation mode for isomannide - succinic acid in pulse-batch bioreactor operation mode. Conversion and concentration.

The reaction carried out with BDO in combination with succinic acid shows that the first derivative of the function concentration against time at time zero was zero with a further increase until 8 h (Figure 7). The reaction proceeded overnight with complete consumption of the substrate after 24 h (99.6%). The pulse fed-batch bioreactor leads to a significant improvement of the substrate conversion compared with the batch reaction for the pairs FDA in combination with BDO and BDO in combination with succinic acid. The effect of this operation mode, however, was less for the reaction of isomannide in combination with succinic acid. The pulse fed-batch operation not only allows better conversion, but also the total amount of substrate that the system could handle was higher than for the batch operation.
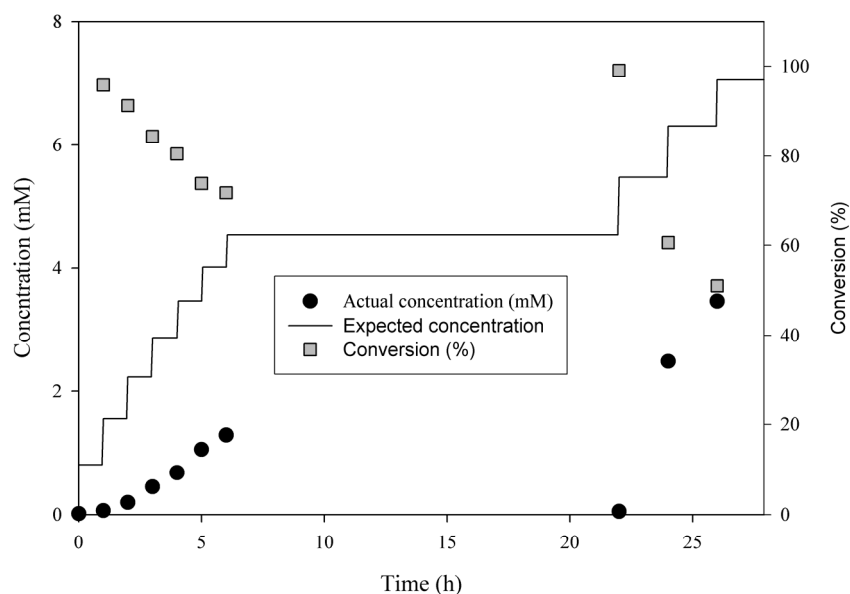
Figure 6 Pulse fed-batch operation mode for FDA – BDO in pulse-batch bioreactor operation mode. Conversion and concentration.

Moreover, the volumetric productivities for BDO-succinic acid (0.62 mmol.(l.h)$^{-1}$) and FDA-BDO (0.74 mmol.(l.h)$^{-1}$) were much higher than for all the other bioreactor operation mode already discussed, although was just comparable with batch bioreactor for isomannide-succinic acid (0.36 mmol.(l.h)$^{-1}$, Table 3).

3.4. Plug-Flow Bioreactor

The biocatalytic polyester synthesis in the presence of immobilized CALB with the different biobased substrates in a plug-flow bioreactor was carried out as described in the Experimental Section (Table 2). The breakthrough curve is shown in Figure 8. In general the behaviors for all the three diacid-diol pairs were similar. The inflection point for BDO in combination with succinic acid is 3.1 h. Similarly, the inflection point for succinic acid in combination with isomannide is 3.5 h and the inflection point for FDA in combination with BDO is 3.2 h. these values were calculated using the fitting of the experimental data shown in Figure 8 (Treybal 1987).
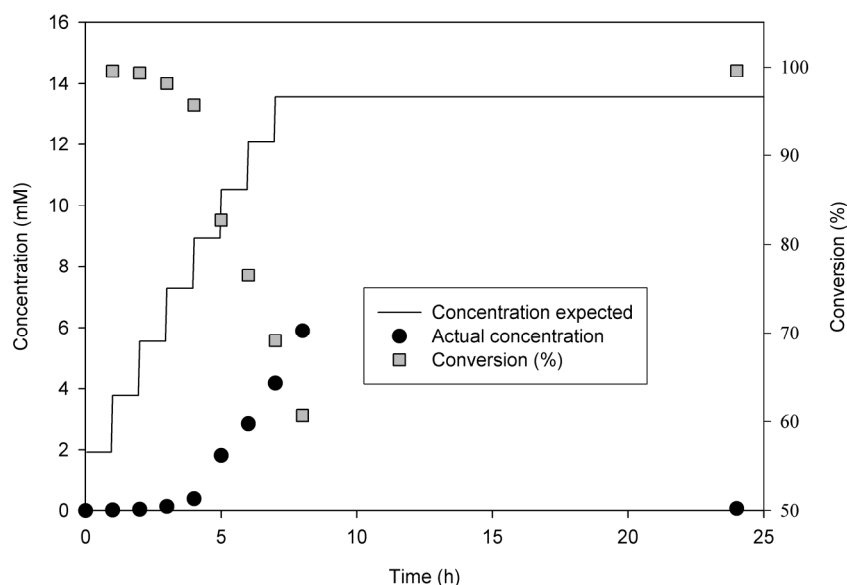
Figure 7 Pulse fed-batch operation mode for succinic acid − BDO in pulse-batch bioreactor operation mode. Conversion and concentration.

The conversions respect to the inflow substrate concentration at the inflection point are 45.2%, 52.4%, and 43.7% for BDO in combination with succinic acid, isomannide in combination with succinic acid, and FDA in combination with BDO, respectively. These values are similar to those obtained with the fed-batch, but in a shorter period of time (~ 3 h) and at higher inflow concentration (20 mM). Hence, the volumetric productivities were the highest among all the bioreactor operation mode tested, and followed the order: isomannide-succinic acid (3.49 mmol.(l.h)$^{-1}$) > succinic acid-BDO (3.01 mmol.(l.h)$^{-1}$) > BDO-FDA (2.91 mmol.(l.h)$^{-1}$). Therefore, plug-flow bioreactor offers an alternative for the enzymatic polyester synthesis of the biobased building blocks here studied with the advantage of shorter operation times, a continuous process and higher conversions. Interestingly, isomannide in combination with succinic acid exhibited the highest volumetric productivities and initial reaction rates among all the biobased building blocks pairs, when the used bioreactor operation mode had the initial substrate concentration at high values, namely batch and plug-flow bioreactors.
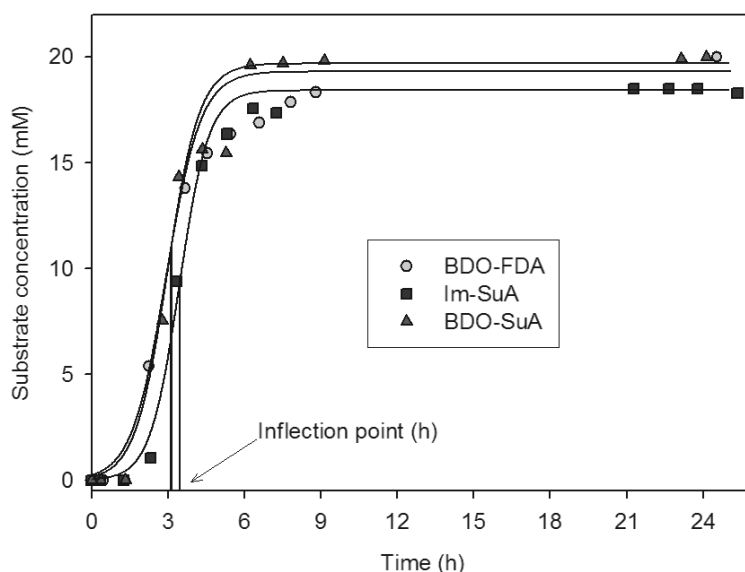
Figure 8 Breakthrough curve for BDO – FDA, isomannide - succinic acid, and BDO – succinic acid in plug-flow bioreactor operation mode.

Unfortunately due to the low solubility of both, succinic acid and isomannide in toluene / *tert*-butanol, a conclusive kinetic study could not be conducted, although our results suggest that initial substrate concentration influences the performance of the system. Despite that pulse fed-batch bioreactor is an operation mode scarcely explored in enzyme-catalyzed synthesis, it showed high potential in terms of substrate conversion and volumetric productivities compared with batch and simple fed-batch operation mode.

3.5. Product Characterization

The products obtained during the enzymatic polyesterification reaction were analyzed by MALDI-TOF. Product formation was analyzed at 24 h for batch, fed-batch, and pulse fed-batch bioreactor. The products for the plug-flow bioreactor were analyzed at the inflection point. MALDI-TOF analysis showed the formation of both cyclic (CEOs) and linear (LEOs) ester oligomers. For all the substrate combinations the formation of cyclic ester oligomers (CEOs) is promoted when pulse fed-batch bioreactor is used, while fed-

batch with continuous inflow displays lower formation of CEOs (Table 3). Succinic acid in combination with isomannide exhibits comparable CEOs formation when either pulse fed-batch (71.1%) or plug-flow bioreactor is used (68.4%), taking into account that these results compare the CEOs formation at the inflection point in the plug-flow bioreactor with the product formation at 24 h reaction in the pulse fed-batch. Moreover, isomannide in combination with succinic acid shows accumulation of CEOs when the operation mode is either batch (41.6%) or pulse fed-batch (48.9%). In the case of FDA in combination with BDO the different bioreactor operation modes do not give a substantial improvement in the CEOs formation. CEO_n states for the cyclic ester oligomer with n-times the combination diacid (A) and diol (B). Similarly the LEO AB_n states for the linear ester oligomer with n-times the combination diacid (A) and diol (B). The LEO AA_n and BB_n state for dicarbonyl and dihydroxyl terminal with n-times the combination diacid (A) and diol (B). Thus, AA_4 is the linear ester oligomer ABABABABA with a degree of polymerization (DP) of 9 according to the IUPAC definition of DP.

3.6.   Oligoesters produced from succinic acid in combination with 1,4-butanediol (BDO).

The CEOs synthesis from CALB-catalyzed reaction of BDO and succinic acid was promoted mainly in plug-flow and pulse fed-batch bioreactor. The degree of polymerization (DP) for the CEOs in plug-flow bioreactor is up to 10 (CEO_5, Figure 9D), while the LEOs do not present a DP larger than 9 (AA_4, Figure 9D). The most abundant CEO was CEO_3 that counted 44.6% of the total formed products. The product spectrum in pulse fed-batch, however, displayed oligomers with higher DP up to 12 (AB_6, CEO_6, Figure 9B) being the most abundant CEO the first in the series (CEO_1, 51.4%). Fed-batch with continuous inflow shows larger spectrum of compounds with a DP of 21 (AA_{10}, Figure 9C). The batch bioreactor exhibits higher DP of CEOs than LEOs (Table 3), being the most abundant compound AA_1 (Figure 9A).

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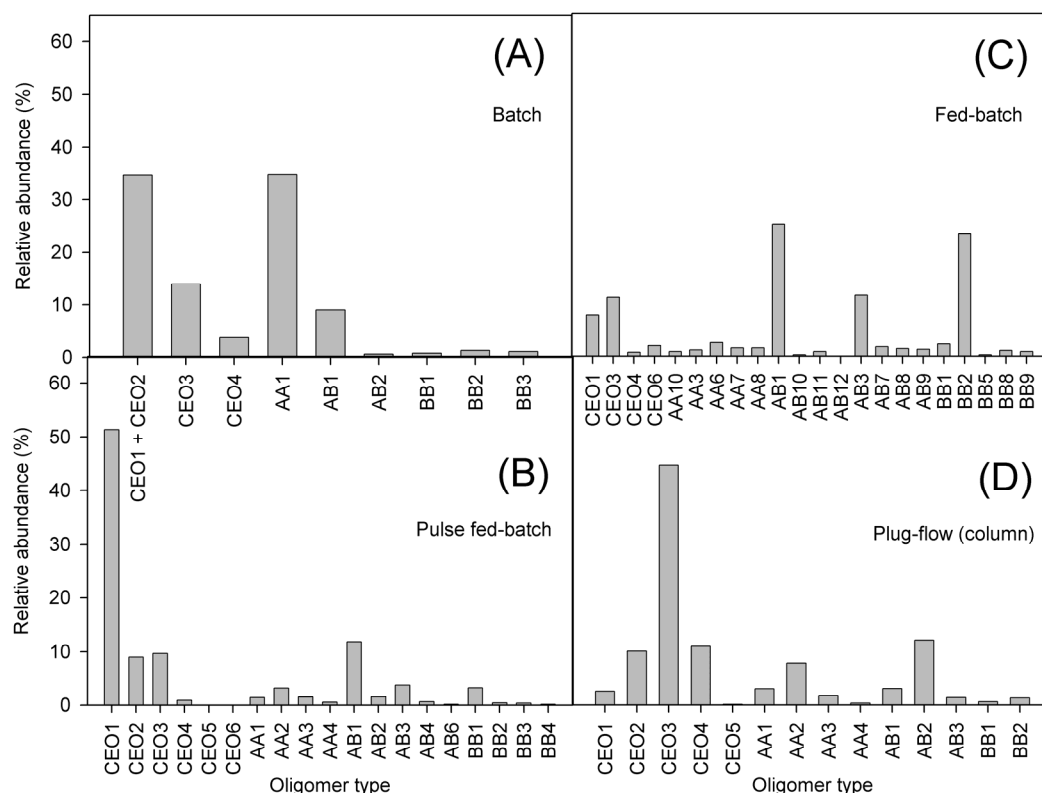
Figure 9 Products obtained in different bioreactor operation modes for succinic acid − BDO (see Table of symbols).

3.7. Oligoesters Produced from Isomannide in Combination with Succinic Acid.

The Batch bioreactor leads to the formation of short oligomers with a DP below 7 (CEO_3, AB_3, and BB_3; Figure 10A). Interestingly, pulse fed-batch leads to very similar results having the same sort of compounds and with comparable abundances (Figure 10B). Nevertheless, the abundance and spectrum of products in the case of plug-flow and fed-batch bioreactors is larger than under batch and pulse fed-batch operation. Fed-batch bioreactor shows ester oligomers with a DP up to 18 and 19 (BB_9, AB_9, CEO_9, Figure 10C) with almost an even occurrence for all the oligomers (AB_n, BB_n, AA_n, and $CEO_n \approx 25\% \pm 3$).

3.8. Oligoesters Produced from Furan-2,5-Dicarboxylic Acid (FDA) in Combination with 1,4-Butanediol (BDO).

Batch bioreactor shows formation of polyester with a DP up to 20 - 21 (AB_{10}, BB_{10}, and CEO_{10}; Figure 11A). CEOs counts for the 37 % of the total detected products.
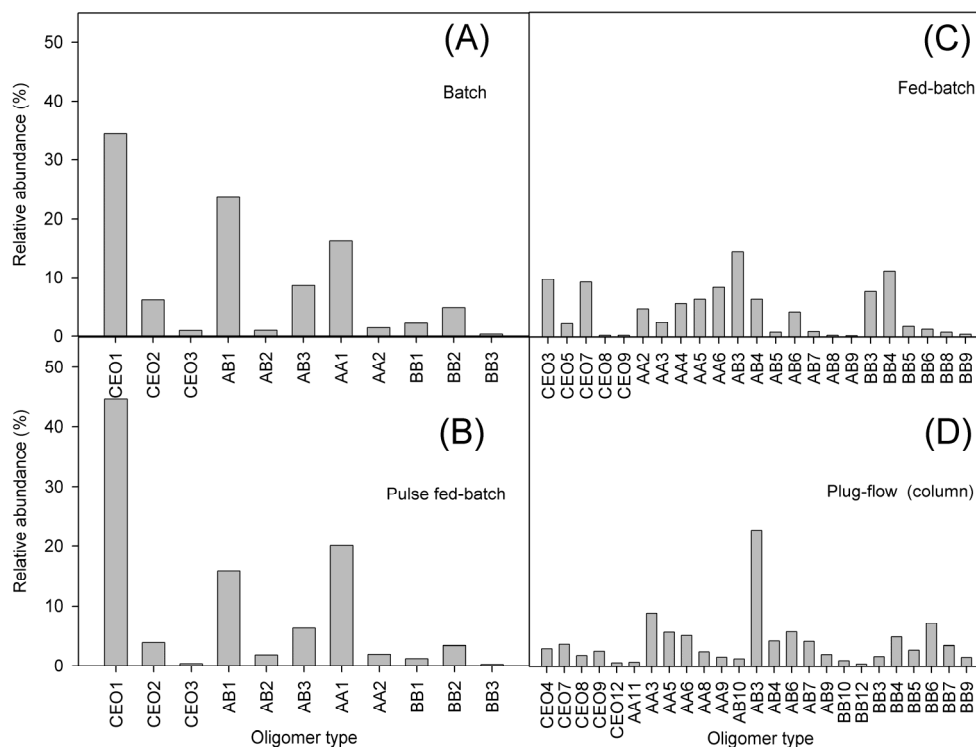
Figure 10 Products obtained in different bioreactor operation modes for succinic acid – isomannide (see Table of symbols).

Pulse fed-batch bioreactor shows still larger variety of polyesters than batch operation. CEOs with a maximum DP of 22 (CEO_{11}) were detected (Figure 11B). The abundance of CEOs (27.5%) is similar to the abundance of hydroxyl – carbonyl terminals (AB_n, 27.9%). Fed-batch bioreactor shows products with a lower DP than pulse fed-batch bioreactor (Figure 11C). AB_n is the most abundant oligomer and counts for the 38.1% of the total detected product, followed by CEO_n with 27.0%. Plug-flow bioreactor exhibits the larger DP among all the bioreactor operation modes (DP = 25 - 24, AA_{12}, AB_{12}, and CEO_{12}; Figure 11D). Abundance of CEOs was up to 33.4%, which is comparable value to that found

during batch operation (36.9%). The dicarbonyl terminal counts for 34.0% of the total detected product with preponderance of the first oligomers in the series (AA_2 - AA_6, Figure 11D).
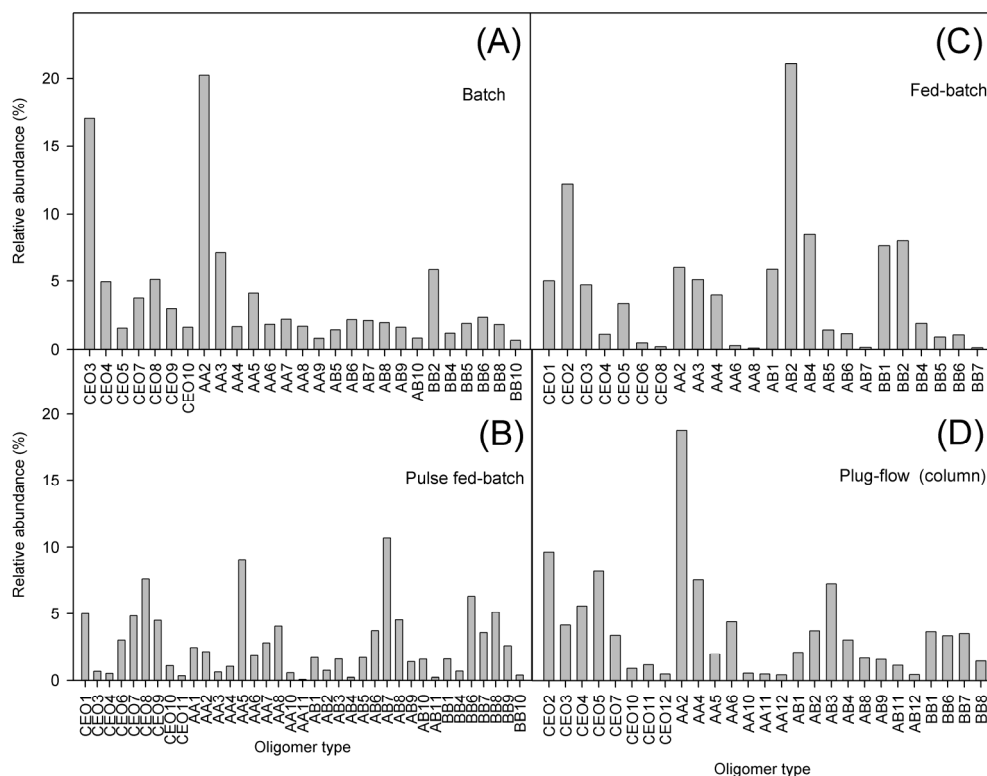
Figure 11 Products obtained in different bioreactor operation modes for FDA - BDO (see Table of symbols).

For most of the bioreactor operation modes the spectrum of products was wider for FDA – BDO than for BDO – succinic acid, and it was wider for BDO – succinic acid than for isomannide – succinic acid. Despite of the diversity of substrate here tested, the conversions were rather high and associated to a type of bioreactor. Hence, the results suggest the following facts: (i) the polyesters synthesis by CALB is not only a feature of the enzymatic system itself, but also it is strongly influenced by the bioreactor operation mode; (ii) the scarce abundance of CEOs for all the operation mode of succinic acid – isomannide, compared with the rest of the other building block combinations could be explained by sterical hindrance in the closure of formed open chain AB_n; (iii) bulky

substrates does not mean impossible reactions, since excellent substrate conversions and the formation of larger CEOs and LEOs occurred with FDA - BDO than for the other building block (iv) the choice of an optimum bioreactor operation mode depends of the system itself and it cannot be *a priori* decided.

1. CONCLUSIONS

The implementation of several strategies for the enzyme catalyzed synthesis of oligoesters from non-activated biobased building blocks was explored. The findings demonstrate that the occurrence of the different products (chemical species) is not only related with the intrinsic properties of the system, but also it is drastically determined by the way of bioreactor operation. The formation of CEOs from succinic acid and BDO was favoured under plug-flow and pulse fed-batch bioreactor. In addition, formation of CEOs from isomannide in combination with succinic acid occurred mainly under batch and pulse fed-batch operation conditions, although the total conversion at 24 h was superior in batch bioreactor than in the pulse fed-batch. Formation of CEOs from FDA in combination with BDO was not affected by the different bioreactor operation mode, but almost total substrate conversion at 24 h was reached in the pulse fed-batch bioreactor. In addition, the spectrum of products was wider for FDA – BDO than for BDO – succinic acid, and it was wider for BDO – succinic acid than for isomannide – succinic acid. In general the pulse fed-batch bioreactor had the most promising results in terms of substrate conversion and CEOs accumulation. This work paves not only the way for the biocatalytic synthesis of cyclic ester oligomers that have potential application the synthesis of novel biobased (co)polymers via ring opening polymerization (ROP), but also the possibility to explore bioreactors arrangements as a tool for gaining control on the biocatalyzed synthesis of CEOs. Hence, it is necessary to exploit the wealth of methods and techniques of bioreactor operation modes. For example conducting a thorough study of reusability of the enzyme, mass transfer into the system, influence of the particle size,

the characteristic times of the different subprocesses that happen into the bioreactor, namely: (i) inflow and mixing, (ii) mass transfer of the substrate from the bulk to the active-site of the enzyme, (iii) acylation – deacylation into the enzyme, and (iv) product release and mass transfer of the product toward the bulk, among others.

REFERENCES

Albertsson A-C, Varma IK. 2003. Recent Developments in Ring Opening Polymerization of Lactones for Biomedical Applications. Biomacromolecules 4(6):1466-1486.

Delhomme C, Weuster-Botz D, Kühn FE. 2009. Succinic acid from renewable resources as a C4 building-block chemical : a review of the catalytic possibilities in aqueous media. Green Chemestry 11:13-26.

Ding S, Tan T. 2006. L-lactic acid production by *Lactobacillus casei* fermentation using different fed-batch feeding strategies. Process Biochemistry 41(6):1451-1454.

Doran P. 1995. Bioprocess Engineering Principles Elsevier 439 p.

Gandini A. 2008. Polymers from Renewable Resources: A Challenge for the Future of Macromolecular Materials. Macromolecules 41(24):9491-9504.

Gandini A, Silvestre AJD, Neto CP, Sousa AF, Gomes M. 2009. The furan counterpart of poly(ethylene terephthalate): An alternative material based on renewable resources. Journal of Polymer Science Part A: Polymer Chemistry 47(1):295-298.

Haar R, Schols HA, van den Broek LAM, Saglam D, Frissen AE, Boeriu CG, Gruppen H. 2010. Molecular sieves provoke multiple substitutions in the enzymatic synthesis of fructose oligosaccharide-lauryl esters. Journal of Molecular Catalysis B: Enzymatic 62(2):183-189.

Habeych E, Barrios AFG, Restrepo-Boland A, Orduz S. 2010. Improved Production of *Bacillus thuringiensis* by Intermittent Fed-Batch Culture with Total Cell Retention. International Journal Of Chemical Reactor Engineering 8(A128):1-7.

Hilker I, Rabani G, Verzijl GKM, Palmans ARA, Heise A. 2006. Chiral Polyesters by Dynamic Kinetic Resolution Polymerization13. Angewandte Chemie International Edition 45(13):2130-2132.

Huang J, Schols HA, Klaver R, Jin Z, Voragen AGJ. 2007. Acetyl substitution patterns of amylose and amylopectin populations in cowpea starch modified with acetic anhydride and vinyl acetate. Carbohydrate Polymers 67(4):542-550.

Kiran KR, Hari Krishna S, Suresh Babu CV, Karanth NG, Divakar S. 2000. An esterification method for determination of lipase activity. Biotechnology Letters 22(19):1511-1514.

Kobayashi S, Ritter H, Kaplan D, Matsumura S. 2006. Enzymatic Synthesis of Polyesters via Ring-Opening Polymerization. Enzyme-Catalyzed Synthesis of Polymers: Springer Berlin / Heidelberg. p 95-132.

Kricheldorf HR. 1997. "Sugar diols" as Building Blocks of Polycondensates. Polymer Reviews 37(4):599-631.

Kricheldorf HR, Sabe C, Schwarz G, Krüger RP. 2003. Macrocycles 27: Cyclic aliphatic polyesters of isosorbide. Journal of Polymer Science Part A: Polymer Chemistry 41(21):3414-3424.

Lavalette A, Lalot T, Brigodiot M, Marechal E. 2002. Lipase-Catalyzed Synthesis of a Pure Macrocyclic Polyester from Dimethyl Terephthalate and Diethylene Glycol. Biomacromolecules 3(2):225-228.

Matsumura S. 2002. Enzyme-Catalyzed Synthesis and Chemical Recycling of Polyesters. Macromolecular Bioscience 2(3):105-126.

Matsumura S, Beppu H, Tsukada K, Toshima K. 1996. Enzyme-catalyzed ring-opening polymerization of β-propiolactone. Biotechnology Letters 18(9):1041-1046.

Matsumura S, Mabuchi K, Toshima K. 1997. Lipase-catalyzed ring-opening polymerization of lactide. Macromolecular Rapid Communications 18(6):477-482.

Mishra MK, Harini M, Kumaraguru T, Lakshmi Prasanna T, Fadnavis NW. 2011. A porous vessel bioreactor for gel entrapped biocatalysts: Kinetic resolution of trans-methyl (4-methoxyphenyl)glycidate by Lecitase® Ultra in gelatin organogel (Gelozyme). Journal of Molecular Catalysis B: Enzymatic 71(1-2):56-62.

Moore JA, Kelly JE. 1978. Polyesters Derived from Furan and Tetrahydrofuran Nuclei. Macromolecules 11(3):568-573.

Okajima S, Kondo R, Toshima K, Matsumura S. 2003. Lipase-Catalyzed Transformation of Poly(butylene adipate) and Poly(butylene succinate) into Repolymerizable Cyclic Oligomers. Biomacromolecules 4(6):1514-1519.

Schwarz G, Kricheldorf HR. 1996. New polymer synthesis. LXXXIII. Synthesis of chiral and cholesteric polyesters from silylated ``sugar diols''. Journal of Polymer Science Part A: Polymer Chemistry 34(4):603-611.

Sugihara S, Toshima K, Matsumura S. 2006. New Strategy for Enzymatic Synthesis of High-Molecular-Weight Poly(butylene succinate) via Cyclic Oligomers. Macromolecular Rapid Communications 27(3):203-207.

Treybal R. 1987. Mass transfer operations (Spanish version). New York: Mc Graw Hill. 863 p.

Truppo MD, Pollard DJ, Moore JC, Devine PN. 2008. Production of (S)-[gamma]-fluoroleucine ethyl ester by enzyme mediated dynamic kinetic resolution: Comparison of batch and fed batch stirred tank processes to a packed bed column reactor. Chemical Engineering Science 63(1):122-130.

Williams JB, Gusev AI, Hercules DM. 1997. Characterization of Polyesters by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. Macromolecules 30(13):3781-3787.

Xu X, Fomuso L, Akoh C. 2000a. Modification of menhaden oil by enzymatic acidolysis to produce structured lipids: Optimization by response surface design in a packed bed reactor. Journal of the American Oil Chemists' Society 77(2):171-176.

Xu X, Fomuso LB, Akoh CC. 2000b. Synthesis of Structured Triacylglycerols by Lipase-Catalyzed Acidolysis in a Packed Bed Bioreactor. Journal of Agricultural and Food Chemistry 48(1):3-10.

Yamamoto Y, Kaihara S, Toshima K, Matsumura S. 2009. High-Molecular-Weight Polycarbonates Synthesized by Enzymatic ROP of a Cyclic Carbonate as a Green Process. Macromolecular Bioscience 9(10):968-978.

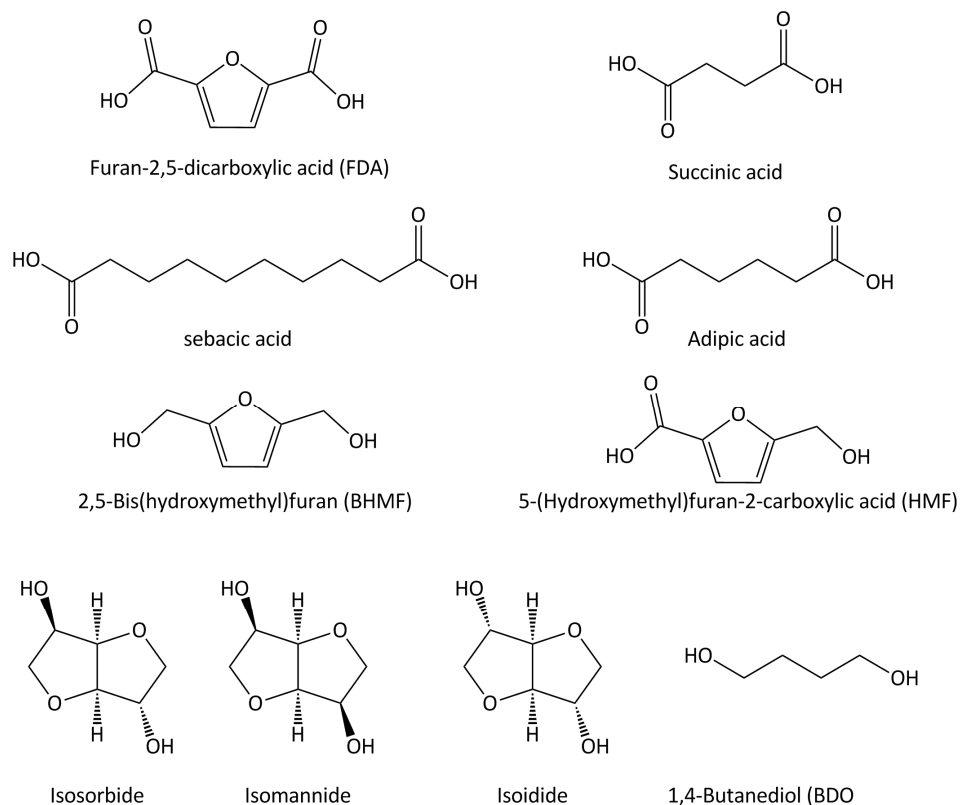Zhi-wei G, Charles J. S. 1988. Enzymatic synthesis of macrocyclic lactones. J. Am. Chem. Soc. 110:1999-2001.

General Discussion

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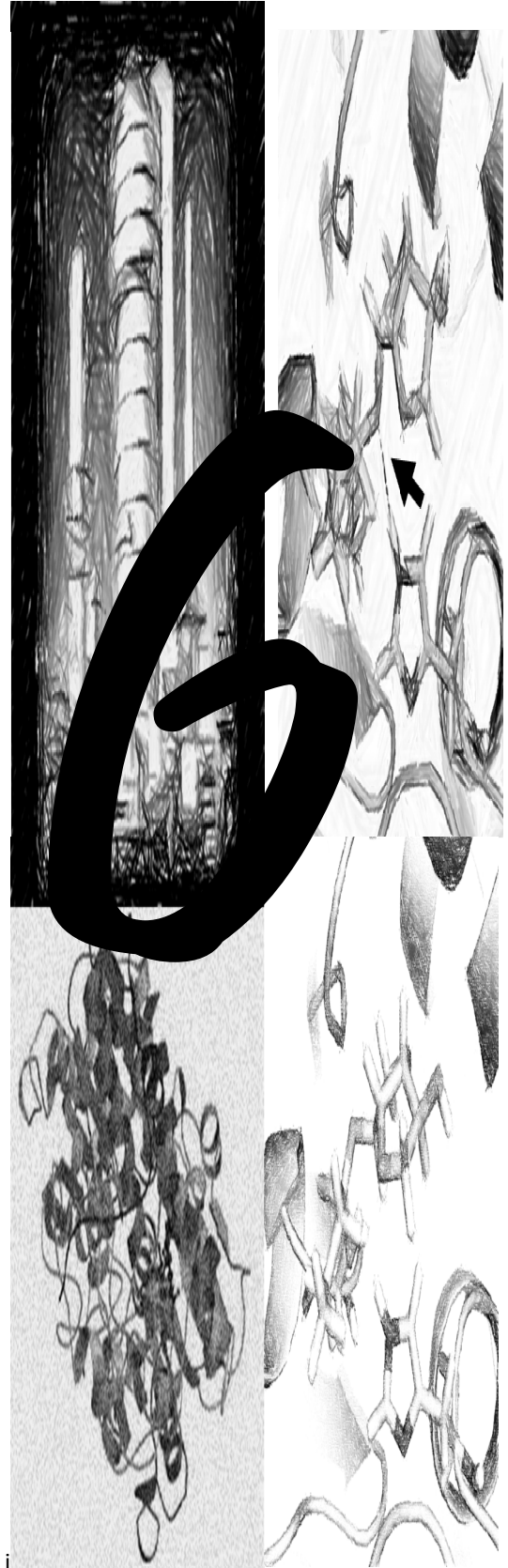
INTRODUCTION

The aim of this thesis was to evaluate the biocatalytic synthesis of cyclic ester oligomers derived from biobased monomers that could be used as building blocks for ring opening polymerization (ROP). The reactions were performed using a bulk commercial lipase: the lipase B from *Candida antarctica* (CALB) immobilized on a polymeric support (Novozym 435). Several substrates were taken into consideration: succinic acid, sebacic acid, adipic acid, furan-2,5-dicarboxylic acid (FDA), 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA), 2,5-bis (hydroxyl methyl)furan (BHMF), 1,4:3,6-dianhydro-D-glucitol (isosorbide), 1,4:3,6-dianhydro-D-mannitol (isomannide), 1,4:3,6-dianhydro-L-iditol (isoidide), and 1,4-butanediol (BDO) (Scheme 1). The building blocks were selected based on several criteria: (i) substrates that could lead to polymers already in use, but produced by chemical polycondensation, for which green, milder synthetic routes could be developed (i.e. succinic acid and 1,4-butandiol, for the polybutylene succinate) (ii) substrates that could produce new polyesters with potential high crystallinity or chirality (i.e. isomannide, isosorbide and the furan monomers) (iii) substrates are already/potentially produced from renewable resources (biobased building blocks) (iv) substrates that are thermolabile and therefore cannot be polymerized with the conventional chemical polycondensation (v) simple acid and alcohols (non-activated or "ready-to-use" substrates) (vi) flexible substrates (i.e. saturated aliphatic diacids and diols) in combination with more rigid molecules, like the saturated heterocylics from the group of dianhydrohexitols, namely isosorbide, isomannide and isoidide and the aromatic furan based monomers, and (vii) substrates that exhibit a stereoconfiguration. This work addresses the effect of the substrate configuration and the substrate size on the enzyme-based synthesis of polymers. Two aspects of the application of enzymes for the synthesis of cyclic ester oligomers from the selected building blocks have been addressed. First, the effect of the reaction conditions and substrate structure and size on the enzymatic chain elongation and cyclization. Second, the effect of the implementation of reactor

operation modes other than batch on the substrate conversion and yield of cyclic oligomers. This chapter discusses the results shown in previous chapters and compares with the current state of the art. We attempt to find more evidence of some of the results using tools such as simple calculations with molecular mechanics. Finally we conclude with some remarks and ideas for future research.

Furan-2,5-dicarboxylic acid (FDA)

Succinic acid

sebacic acid

Adipic acid

2,5-Bis(hydroxymethyl)furan (BHMF)

5-(Hydroxymethyl)furan-2-carboxylic acid (HMF)

Isosorbide   Isomannide   Isoidide   1,4-Butanediol (BDO

Scheme 1 biobased building blocks used in this work.

1. PRESENT WORK

2.1 Enzymatic Polymerization under Diluted Conditions Leads to Cyclic Ester Oligomers

Polyesters can be synthesized via either ring-opening polymerization (ROP) or polycondensation (PC). Since the work of Carothers (Carothers 1931) the most widely

applied polymerization synthesis route has been via PC. One of the drawbacks that chemists have had to tackle in PC has been the formation of short chain polyesters, namely LEOs and CEOs. Formation of both linear and cyclic oligomers is a shared feature of enzyme- and chemical-based systems. Kowalski et. al. explored the formation of cyclic and linear species using tin(II) octoate as catalyst in the polymerization of ε-caprolactone (Kowalski et al. 2000). Among the oligoesters formed, some of the CEOs included tin(II) in its structure, and the presence of a metal component in the product is a significant drawback. Therefore, it is interesting to find alternative for the synthesis of CEOs, which are the raw materials in ROP. The synthesis of CEOs according to the classical theory of Jacobson-Stockmayer can be classified in two large groups (Jacobson and Stockmayer 1950). : (i) formation of CEOs due to the ring-chain equilibrium that occurs in many polycondensations and ring-opening polymerizations (Scheme 2A) and (ii) end-to-end cyclization method that can be used for synthesizing polymers out of α, ω-bifunctional linear precursors (i.e. α, ω -hydroxy acid, Scheme 2B). The last one can be divided in two types: a bimolecular process where complementary terminal moieties (i. e. acid and alcohol) react to synthesize the CEO (bimolecular mechanism, Scheme 2B.i) and a process where the α, ω reactive groups of an α, ω-bifunctional linear molecule react to form the CEO (unimolecular mechanism, Scheme 2B.ii).

In this work, we have shown that in the enzymatic cyclization reaction, the cycles are formed on the active centre of the enzyme, via a unimolecular mechanism. Cyclic oligomers can be formed only from activated acyl-enzyme intermediates derived from α-hydroxy-ω-carboxy oligoesters (Enz-AB$_n$) by intramolecular nucelophilic attack of the α-hydroxy group on the acyl-serine bond on the active centre. So, the enzymatic cyclization occurs via a unimolecular mechanism as shown in route B.ii in Scheme 2, but mediated by the enzyme, and not by back-biting (intramolecular transesterification Scheme 2.A) or by a bimolecular mechanism. The reaction of 1,4-butanediol (BDO) in combination with succinic acid catalyzed by CALB led to the accumulation of CEOs in time, independent of the presence or absence of water (**Chapter 2**). Cyclic dimer, tetramer, hexamer and

octamer were formed and represented together 52% of the total products formed, the rest being linear oligoesters of similar chain length. The dimer CEO_1, was the most abundant cyclic oligomer. We proposed a mechanism that could illustrate the routes for the synthesis of CEOs from succinic acid and BDO, where the synthesis of the first oligomer AB_1 rules the progress of the reaction. We have showed that as long as monomers and short chain oligomers are present in the system, chain elongation mainly occurs by step-growth polymerization via the AB_1 synthon, while at later stages of the reaction, after complete consumption of the monomers, other pathways are involved. and larger oligoesters are responsible for chain growth. The following aspects can be emphasized: (i) at the beginning of the reaction the short LEOs are formed (i. e. AB_1, BB_1, etc), (ii) the addition of either acid (A) or alcohol (B) to AB_n leads to the formation of larger linear species (AA_n and BB_n), (iii) the LEOs species are competitive inhibitors together with the water formed as by-product during the reaction (Valivety et al. 1993), , (iv) only α-hydroxy- ω-carboxy LEOs (i.e. AB_n) can form cycles, (v) flexible (LEOs) can readily lead to the formation of either larger chains and/or CEOs, (vi) the reaction is controlled by the affinity of the enzyme for the different acylating species (AB_n, AA_n) present in the system, which is highest for A and AB_1, and lowest for AA_n and CEO_n. Recently, it was shown that the cyclic octamer (CEO_4) from succinic acid and 1,4-butanediol (Noguchi et al. 2006), adopts a compact structure stabilized by weak hydrogen bonds. Nevertheless, the octamer and all cyclic oligomers tested were good substrate for CALB for ring opening polymerization, suggesting that they can bind in the active center of the enzyme to initiate the reaction.

(A)

(B.i)

(B.ii)
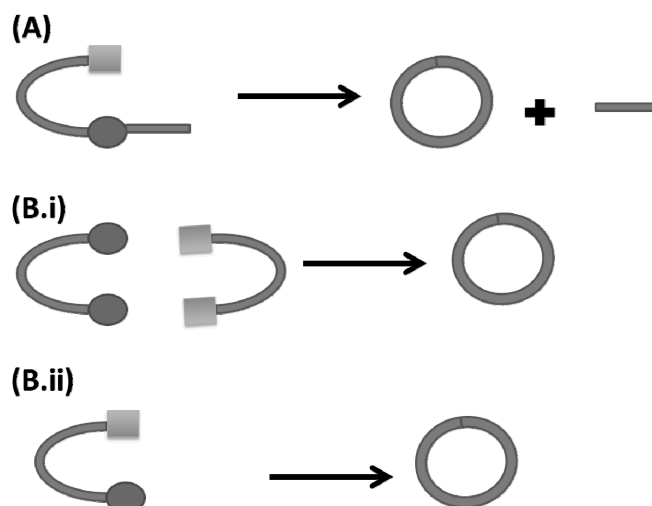
Scheme 2 Possible ways to carry out the formation of cyclic ester oligomers (CEOs), the square and the oval represents different moieties in the molecules, for example hydroxyl and carbonyl groups.

The model and the assumption proposed in **Chapter 2** for the formation of cyclic ester oligomers as major products in the enzymatic polymerization of succinic acid with 1,4-butanediol were validated by the results obtained for other monomers such as isomannide-succinic acid (**Chapter 3**) and furan based monomers (**Chapter 4**). For all reactions studied, cyclic ester oligomers were formed in the early stage of the reaction and were not degraded in time, as shown in Figure 1 below for the reaction between 2,5-furandicarboxylic acid (FDA) with 1,4-butanediol. Nevertheless, the size of the cycles was specific for each monomer combination (Table 1).
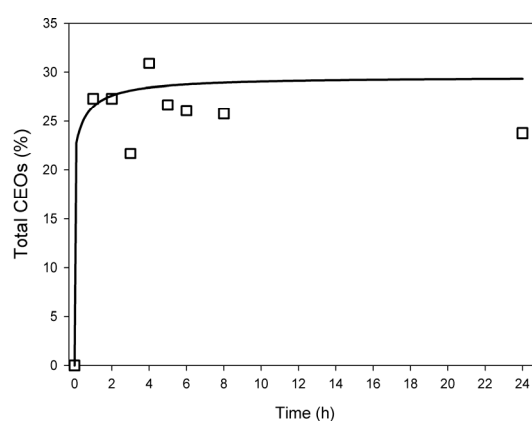

Figure 1 Total CEOs accumulation in time. Enzyme-catalyzed reaction of FDA in combination with succinic acid.

Table 1 Summary of some results from the biobased building blocks used in this thesis.

Substrate		Bioreactor type	CEO_n	Major CEO	LEO_n DP	CEOs	Substrate conversion (%)
Diol	Diacid						
BDO	Succinic acid	Batch	CEO_1 - CEO_4	CEO_1	1-3	52.4	85.6
		Fed-batch	CEO_1 - CEO_6	CEO_1	1-12	21.8	49.9
		Pulse fed-batch	CEO_1 - CEO_6	CEO_1	1-6	71.1	99.6
		Plug flow	CEO_1 - CEO_5	CEO_3	1-4	68.4	45.2
Isomannide	Succinic acid	Batch	CEO_1 - CEO_3	CEO_1	1-3	41.6	93.8
		Fed-batch	CEO_1 - CEO_9	CEO_3	1-9	21.9	14.2
		Pulse fed-batch	CEO_1 - CEO_3	CEO_1	1-3	48.9	48.6
		Plug flow	CEO_1 - CEO_{12}	CEO_4	1-12	11.8	52.4
BDO	FDA	Batch	CEO_3 - CEO_{10}	CEO_3	1-10	36.9	82.1
		Fed-batch	CEO_1 - CEO_8	CEO_1	1-8	27	66.2
		Pulse fed-batch	CEO_3 - CEO_{11}	CEO_1	1-11	27.5	99.1
		Plug flow	CEO_3 - CEO_{12}	CEO_2	1-12	33.4	43.7
BHMF	Succinic acid	Batch	CEO_1 - CEO_8	CEO_1	1-9	36.9	35.4
BHMF	Adipic acid	Batch	CEO_1 - CEO_4	CEO_1	1-4	11	43.3
BHMF	Sebacic acid	Batch	CEO_1 - CEO_2	CEO_1	1-4	15.3	39.1
HMFA	HMFA	Batch	DP6 - DP23	DP15	DP22	58.8	>99.9

The accumulation of CEOs in the medium using an enzyme-catalyzed system has been associated to phenyl-containing substrates, where the $\pi - \pi$ interactions of the aromatic ring, holds the structure of the cyclic (CEO_2) tetramer (<u>Hilker et al. 2008</u>; <u>Lavalette et al. 2002</u>). In this thesis, we demonstrate that the formation and accumulation of CEOs is not limited to phenyl-containing monomers, but applies to all monomers if they are substrates for lipase, as we have shown aliphatic chains, saturated heterocylic or aromatic furan based derivatives. We conclude that formation of cyclic ester oligomers is an intrinsic feature of the enzymatic polymerization.

2.2   Do Length and Flexibility Matter ?

We have shown in this thesis that, in the biocatalytic polymerization of biobased building blocks via AA/BB and AB condensation reactions, the ring-closure reaction occurs concurrently to the growth of linear polymer chains. Cyclic ester oligomers were obtained in systems containing only aliphatic, flexible monomers, like succinic acid and 1,4-butanediol, in reactions between flexible and more rigid monomers like (a) succinic acid with isomannide (rigid saturated heterocyclic), (b) C4, C6 and C10 dicarboxylic acids with 2,5-bishydroxymethyl furan (BHMF, aromatic ring) and (c) 1,4-butanediol with 2,5-furandicarboxylic acid (FDA, aromatic) and in the enzymatic homopolymerization of 5-hydroxymethyl-2-furoic acid (HMFA, aromatic). However, the total substrate conversion, the yield and the size of the cycles and the most abundant cycle formed was specific for each monomer combination. In the biocatalytic polymerization of BDO-succinic acid in batch mode, after 24 h reaction, cyclic oligomers ranging from dimer CEO_1 to octamer CEO_4 were obtained, at a substrate conversion of 86%. The cycles represented 52.4% of the total products formed, and dimer was the dominant cycle. Surprisingly, the isomannide-succinic acid systems yielded only the dimer CEO_1, hexamer CEO_3, and decamer CEO_5, while linear ester oligomers AB_n, BB_n and AA_n spanned the whole range from dimers to nonamers, for a total substrate conversion of 93.8%. Total cyclic

oligomers were 41.6% of the total products.

Significant differences were observed both in the conversion and the pattern of products obtained from the furan-based monomers. The substrate conversion was the highest for HMFA (99.9%) and decreased for 2,5-FDA (82%) and BHMF (35.4%, 43.1% and 39.1% in reaction with succinic acid, adipic acid and sebacic acid, respectively). The high reactivity of HMFA and 2,5-FDA is comparable with that observed in chemical reactions, and it is due to the electronic resonance effects between the aromatic ring and the π-electrons of the carboxyl substituents. However, BHMF shows lower reactivity than expected, possibly due to steric hindrance caused by the hydroxymethyl reactive group which is far from the planar furan core. The enzymatic homopolycondensation of HMFA produced CEOs (58.8%) and LEOs (41.2%) with DP from 6 to 23. The distribution of cyclic oligomers had an asymmetric bell-shape, with a peak at CEO_{15} and higher levels for DP < 15 (see Figure 3, Chapter 4). A trimer as the first member of the series of cyclic oligomers was also reported for the chemical homopolycondensation of HMFA mediated by chemical-based catalyst using 2-chloro-1-methylpyridinium iodide as condensing agent, pyridine as reaction medium, and at moderate temperatures (<60 $^{\circ}$C)(Hirai et al. 1984).

Enzymatic polymerization of FDA-BDO produced CEOs (37%) ranging from DP 6 to DP 20 and 63% LEOs of similar degree of polymerization. The hexamer (CEO_3) was the most abundant cycle. An interesting effect of the chain length of the acyl reagent on the cycle formation was observed for the reaction between BHMF and the aliphatic dicarboxylic acids with C4 (succinic acid), C6 (adipic acid) and C10 (sebacic acid). First, substrate conversion was higher for adipic acid than for succinic and sebacic acid. Similar effect was reported by McCabe and Taylor (McCabe and Taylor 2004) who found that the entropic component of the C6 acyl substrate was significantly less than that of C4 and C8 substrates. However, significant differences were obtained between the both the yield of CEOs formed (37% for C4, 11% for C6 and 15% for C10) and the size of CEOs formed (CEO_1 to CEO_8 for C4, CEO_1 to CEO_4 for C6 and CEO_1 to CEO_2 for C10). This clearly

indicates that other factors than the reactivity of monomers is important for the cycle formation, as we will discuss below. Considerable amount of work has been done to understand the stereospecificity of the alcohol side of the lipase binding site (Cygler et al. 1994), the preference of different lipases for different chain lengths and substitutuion of the acyl substrate according to the overall shape of the active site (Pleiss et al. 1998). It was shown that the preference of the acyl substrate is independent of the alcohol side (the oxyanion hole) of the active site (García-Alles and Gotor 1998). It is general rule that secondary alcohol are poor substrates as compared to primary alcohols. It is then remarkable that isomannide, a saturated heterocyclic with two secondary alcohol groups shows comparable reactivity with 1,4-butanediol in the enzymatic polymerization reaction.

DAH are rather rigid molecules compared with BDO, the V-shape configuration and the *cis*-fused rings form an angle of 120° between them. This configuration is favoured by the hydrogen atom attached to both carbons, 3 and 4 (Scheme 3), and oriented in opposite direction to the bending of the ring. This apparent rigidity found in DAH has been recently investigated using molecular mechanics and X-ray crystallography (Wu et al. 2011). The tridimensional structure of the 2,5-dicarboxylic derivative was described and one interesting finding was that the DAH skeleton is more flexible than it was assumed in the past. Although there is certain flexibility, the reactivity of the ring must be also affected by the orientation of the hydroxyl groups. In isomannide they are oriented inward the ring (*endo*-hydroxyl, Scheme 3). This orientation determines the high reactivity found in isomannide during enzyme-catalyzed reaction for the synthesis of oligoesters. In addition, the two reactive hydroxyl groups in isomannide are in the 1,4-positions of C4 chain, as in BDO. Significant differences have been observed between the amount and size of CEOs formed in the different reaction systems, as detailed above. Ring closure requires an intramolecular nucleophilic attack of the terminal hydroxyl group of the linear ester oligomer to the acyl-serine bond on the active site. Consequently, the oligomer chain should have some flexibility, to fold towards a V

conformation and access the acyl-serine bond through the oxoanion hole. Highest flexibility of the oligomer chain was expected for the oligomers constituted from flexible monomers like BDO and succinic acid, and this was confirmed by the high yield in CEOs (52% in the batch reaction and 71% in the pulse fed-batch operation). Surprisingly, the highest yield of CEOs in the batch system and the broadest range of cyclic oligomers was obtained by homopolymerization of HMFA, an aromatic, more rigid structure. However, the smallest CEO found was a hexamer. This suggest that the linear HMFA-homopolymers are highly flexible and fold in the active centre of the enzyme and that a minimum of 6 monomers are needed for ring closure. Figure 2 shows an HMFA tetramer docked in the active site of CALB, that adopted a folded, V-shape conformation. Similar behaviour can be assigned to the linear oligoesters derived from FDA and BDO. However, in the reaction between BHMF and aliphatic dicarboxylic acids both the yield and the size of CEOs decreases with the length of the aliphatic chain, being the highest for C4 (succinic acid). This does not imply that the flexibility of the oligomer chain decreases, but it may be due to a combination of the steric hindrance induced by the bulky hydroxymethyl substituent on the aromatic ring and the length of the aliphatic chain, preventing the correct binding into the alcohol side of the active centre. We can conclude that the length and the flexibility of the linear oligomer chain is the key factor to promote ring closure (the formation of cyclic oligoesters) and to control the size of the rings.
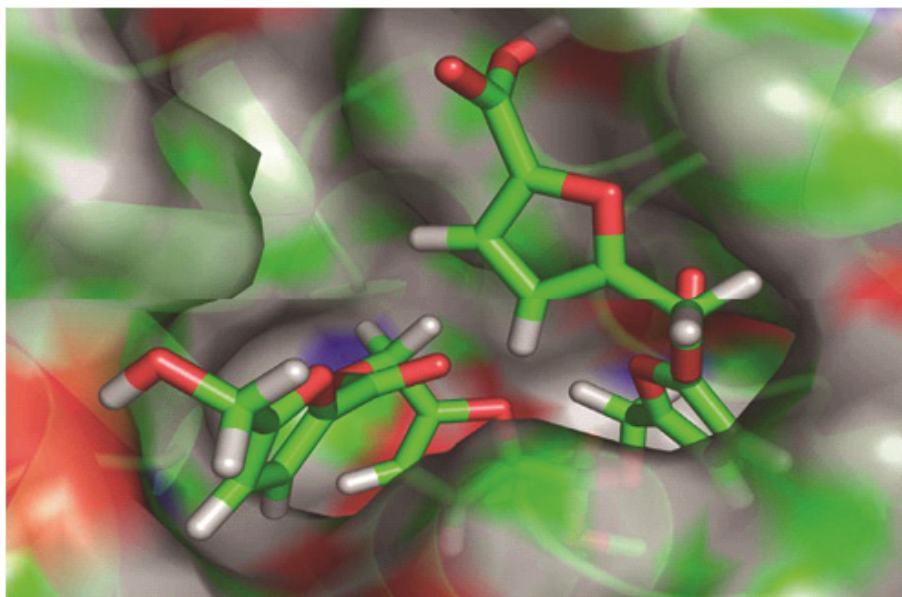
Figure 2. Docking of HMFA tetramer into the active center of CALB.

2.3 Three words about the enantiopreference of *Candida antarctica* lipase B (CALB)

The most attractive properties of enzyme-catalyzed reaction is the possibility to carry out them at low temperatures to obtain products in a very high chemo-, regio-, and enantioselective manner and also the flexibility to be combined enzymes with chemical catalyst (Hilker et al. 2006; Kobayashi 2010). Therefore, we addressed in **Chapter 3** the question if there is an enantiopreference of CALB with respect to dianhydrohexitol (DAH) isomers and if such enantiopreference follows the same pattern as in chemical catalyzed reactions. Preliminary experiments showed that the reactivity of the DAH isomers in the lipase catalyzed polymerization with succinic acid decreased from in the order isomannide > isosorbide > isoidide which is the opposite of the reactivity in chemically-catalyzed (poly)esterification. This can be explained only by the ennatiopreference of the alcohol side of the active site of the enzyme. This was confirmed by the substrate-imprinted docking. Productive docking solutions were found if the ester bond is formed with an *endo*-hydroxyl group, as found in isomannide and one of the isosorbide hydroxyl groups (C5, Scheme 3), 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, Scheme 3). An analysis of the substrates placement in the active site generated by docking shows that esterification at the *endo*-hydroxyl group leads to a reaction intermediate that fits into the CALB alcohol pocket and forms the hydrogen bonds required for stabilization of the transition state Scheme 3).
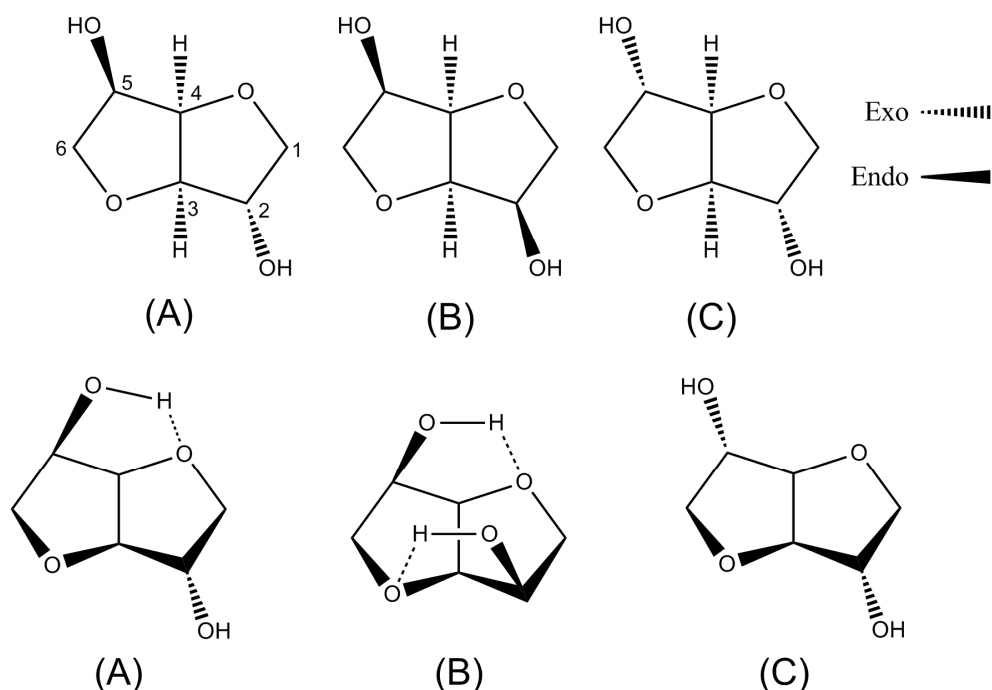
Scheme 3. 1,4:3,6-dianhydrohexitols (DAH). (A) 1,4:3,6-dianhydro-D-glucitol or isosorbide; (B) 1,4:3,6-dianhydro-D-mannitol or isomannide; and (C) 1,4:3,6-dianhydro-L-iditol or isoidide.

In the case of non-productive binding (the case of the *exo*-hydroxyl group), the reaction intermediate can fit into the alcohol binding pocket of CALB, but in a position that makes the hydrogen bonding unlikely (Figure 4, **Chapter 3**). Therefore, CAL-B preference for isomannide over isosorbide over isoidide is a direct result of the preference for the *endo*-hydroxyl groups. These results are in agreement with reported work on enzymatic isosorbide esterification. For example, the monosubstitution in the presence of CALB at 63 °C of isosorbide with ricinoleic acid resulted in 93% conversion of the *endo*-hydroxyl group ((R)-configurated) and less than 1% conversion of the *exo*-hydroxyl group ((S)-

configurated) (Boulifi et al. 2010). The preference of CAL-B for the R-enantiomers of the alcohol substrate has been well documented (Overmeyer et al. 1999; Reetz and Schimossek 1996) and has been used to produce chiral polyesters by dynamic kinetic resolution in a enzymatic- and chemical-catalyzed system from racemic 2,2-dimethyl-1,4-benzene dimethanol and dimethyl adipate (Hilker et al. 2006).

In order to understand more about the specificity found with CALB in combination with the three different DAH and the apparent contradiction respect to the results found in chemical catalyzed reactions, we did a simple calculation of molecular dynamics using ChemBio 3D Ultra 12.0. The algorithm used is based on the molecular mechanics model 2 (MM2). Among the parameters calculated, the 1,4-van der Waals energy represents the energy for the through-space interaction of atoms separated by two atoms, while the non-1,4 van der Waals term represents the energy for the through-space interaction between pairs of atoms that are separated by more than three atoms For example, in trans-2-butene, the Non-1,4 van der Waals energy term includes the energy for the interaction of a hydrogen atom bonded to C1 with a hydrogen atom bonded to C4 (Scheme 5). The 1,4 van der Waals energy term includes the energy for the interaction of a hydrogen atom bonded to C1 with a hydrogen atom bonded to C2 (Scheme 5). The value of the non-1,4 van der Waals energy was 215.3 (50.3), 190.5 (44.5), and 13.7 (3.2) $kJ.mol^{-1}$ ($kcal.mol^{-1}$) for isomannide, isosorbide, and isoidide, respectively (Scheme 3). According with the definition above shown, non-1,4 van der Waals energy quantifies indirectly the binding between the hydrogen bond attached to C5 and the oxygen present in the ether bond between C1 and C4 in isosorbide (Scheme 3). The non-1,4 van der Waals energy follows the order isomannide > isosorbide >> isoidide. This indicates that the hydrogen bond is stronger in the same way (isomannide > isosorbide >> isoidide), being the minimum for isoidide. The non-1,4 van der Waals energy for isoidide is almost 16 times smaller than for isomannide and that explain the high reactivity of isoidide and the low one for isomannide under chemical catalyzed conditions. The *endo*-oriented hydroxyl group (isomannide) is significantly less reactive than its *exo*-oriented

(isoidide) counterpart in chemical catalyzed polymerization (<u>Fenouillot et al. 2010</u>; <u>Noordover et al. 2006</u>). Hence, the reactivity found during CALB catalyzed reaction is a consequence of the interaction substrate-enzyme due to the sterical hindrance suffered by the *exo*-hydroxyl group found in isoidide and isosorbide as has been pointed out above and in **Chapter 3**.

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Cis-**butene**   *Trans*-**butene**
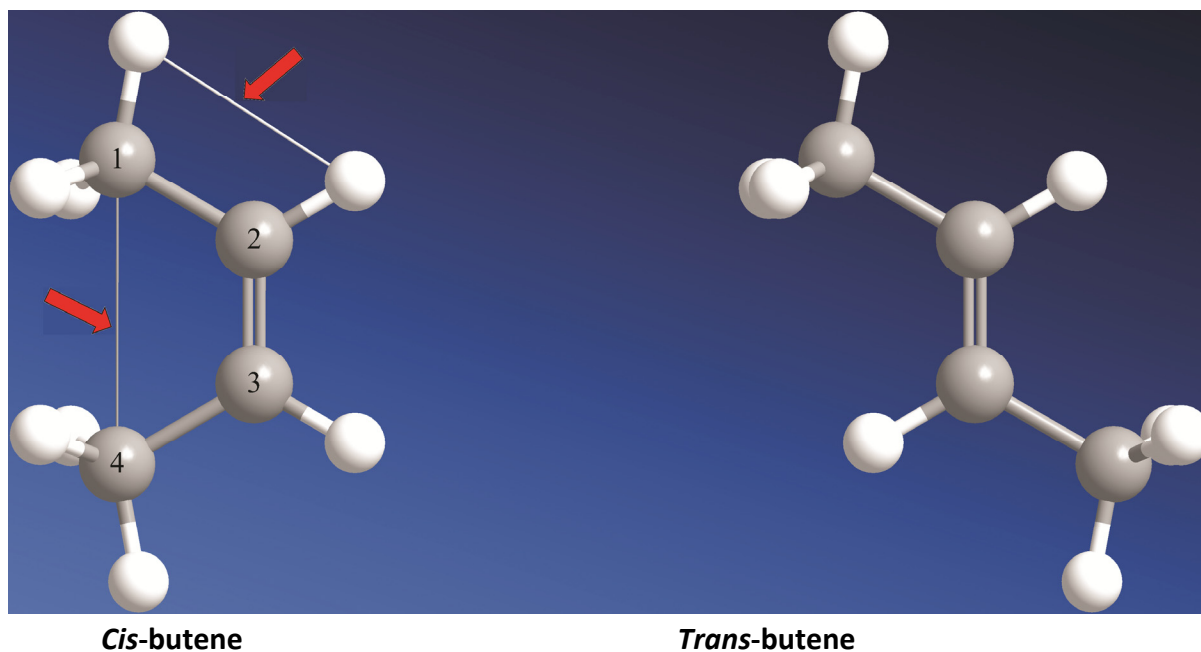
Scheme 5. schematic representation of the 1,4- *cis*- and *trans*- butane. The arrows represent the dummy bond involved on the estimation of the non-1,4 van der Waals energy and 1,4 van der Waals energy.

2.4   Bioreactors and Enzyme-Catalyzed Oligomerization

In Chapters 2, 3, and 4 of this thesis we have studied the enzymatic polymerization of several building blocks in a reactor operated in the batch mode. Batch operated stirred thank reactors are currently applied for industrial enzyme-catalyzed processes due to the their versatility, despite some drawbacks like the more complicated reuse and regeneration of the immobilized enzyme among others. In **Chapter 5** we have studied the effect of different reactor operation modes namely, plug-flow bioreactor, fed-batch

bioreactor, and pulse fed-batch on the substrate conversion, yield of cyclic oligoesters and size of the cycles formed for the following reactions: succinic acid in combination with 1,4-butanediol and isomannide, respectively and 2,5-FDA in combination with 1,4-butanediol. Figure 3 summarizes the findings exposed in Chapter 5 for the catalyzed reaction of BDO in combination with succinic acid in the presence of CALB. The highest substrate conversion (99.6%) was found during pulse fed-batch operation as well as the largest accumulation of CEOs (71.1%). The CEOs and LEOs produced had a DP up to 12 (CEO_6 and AB_6, Table 1). Fed-batch bioreactor operation mode, however, showed a substrate conversion of 49.9% and the lowest accumulation of CEOs (21.8%), even though it had the broader product distribution among the bioreactor operation mode studied with BDO-succinic acid. Hydroxyl-carbonyl terminal (AB_n) as well as diols (BB_n) were the most common type of products found into the fed-batch. Plug-flow bioreactor, however, showed the lowest substrate conversion (45.2%) despite of presenting a comparable abundance of CEOs (68.4%) than pulse fed-batch bioreactor (71.1%). Nonetheless, the highest productivity was reached in this operation mode (Figure 3). These results indicates that bioreactor operation mode has a strong influence on the CEOs production. In the particular case of BDO in combination with succinic acid plug-flow operation mode resulted in a better system for the synthesis of CEOs, although a system such as plug-flow bioreactor could be further improved with equivalent or better results than pulse fed-batch.
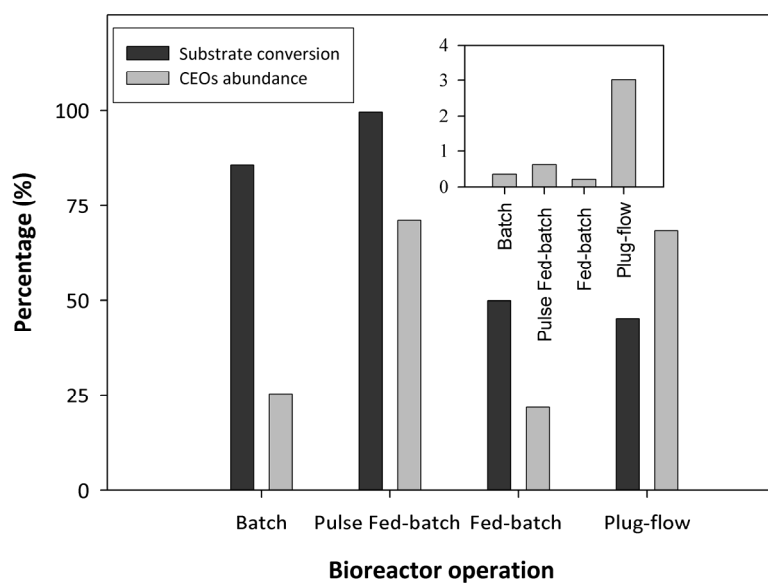
Figure 3 Succinic acid and 1,4-butanediol. Comparison of different bioreactor operation modes. Insert: bioreactor volumetric productivities $(mmol(l.h)^{-1})$ vs. type of bioreactor).

In the case of the enzymatic polymerization of isomannide in combination with succinic acid, the use of several bioreactor operation mode did not bring an evident improvement in the substrate conversion (Table 1 and Figure 4). The substrate conversion followed the order batch (93.8%) >> pulse fed-batch (48.6 %) ~ plug flow (52.4%) > fed-batch (14.2%). The CEOs accumulation was superior in pulse fed-batch (48.9%), followed by batch (41.6%), fed-batch (21.9%) and plug-flow bioreactor (11.8%). Although the CEOs amount produced in plug-flow bioreactor was the lowest, this operation mode exhibited the products with the highest molecular weight (DP = 24, CEO_{12} and 25, BB_{12}). This could be an indication that the fast reaction, 52.4% substrate converted in 3 h, leads easier to the elongation of the oligomers instead of cyclization. In other words, elongation of LEOs competes with cyclization of the linear chains. Moreover, plug-flow bioreactor exhibited the highest productivity among all the other bioreactors.

Figure 5 shows the results obtained for the reaction between FDA and BDO under the different operation modes. The substrate conversion was 82.1% after 24 h in batch bioreactor. The product mixture contained 36.9% CEOs, with a DP up to 20 (CEO_{10}). Batch operation mode together with plug-flow bioreactor at the break through point gave the highest concentration of CEOs (33.4%), although the substrate conversion at 24 h in the latter was only 43.7% (Figure 5). In contrast, pulse fed-batch bioreactor led to substrate conversion of 99.1% after 24 h, but with a CEOs formation of 27.5 % (Figure 5). This bioreactor operation mode presented a large spectrum of products with DP up to 23 (AA_{11}) for LEOs and 22 for CEOs (CEO_{11}). Fed-batch bioreactor exhibited a comparable accumulation of CEOs (27%, Figure 4) than pulse fed-batch bioreactor, although the variety of products was less (DP max. = 16, CEO_8 and 17 LEOs, AA_8) and substrate conversion was 66.2%. In this system the largest productivity was also for the plug-flow bioreactor. Some conclusions can be obtained from these observations: (i) similar to isomannide-succinic acid, the highest DP of CEOs was reached under plug-flow bioreactor, which suggests that high concentration of enzyme favours the cyclization of linear chains, (ii) likewise to succinic acid-BDO pulse fed-batch operation mode leads to

the largest substrate conversion, (iii) batch operation mode combine good substrates conversion with the largest CEOs accumulation, therefore this could be a system to be improved toward the increase of the CEOs formation from FDA - succinic acid keeping or increasing the current substrate conversion.

Figure 4 Succinic acid and isomannide. Comparison of different bioreactor operation modes. Insert: Bioreactor volumetric productivities (mmol(l.h)$^{-1}$) vs. type of bioreactor.

In general, an optimization of these results can be carried out. For example, lower inflow values and larger columns could bring a better accumulation of CEOs. These results proved that for biobased building blocks the bioreactor operation mode can bring substantial improvement in terms of synthesis of cyclic ester oligomers, productivities and DP.

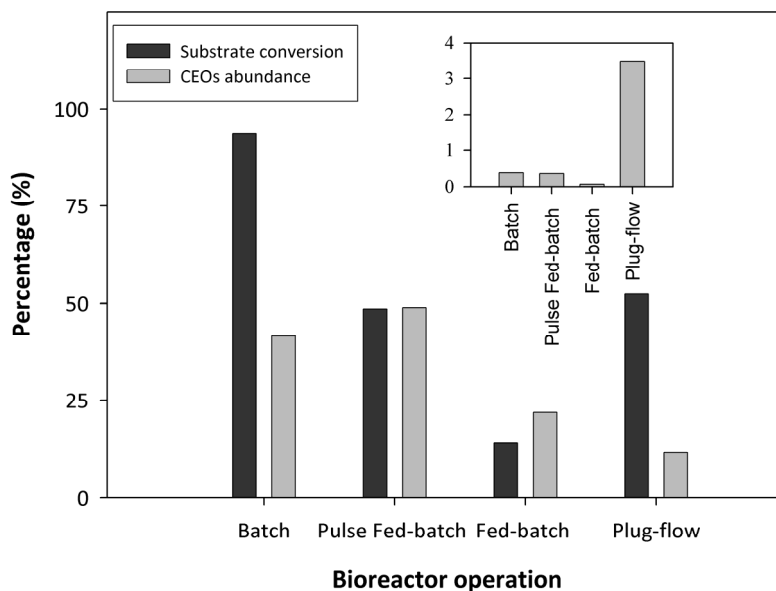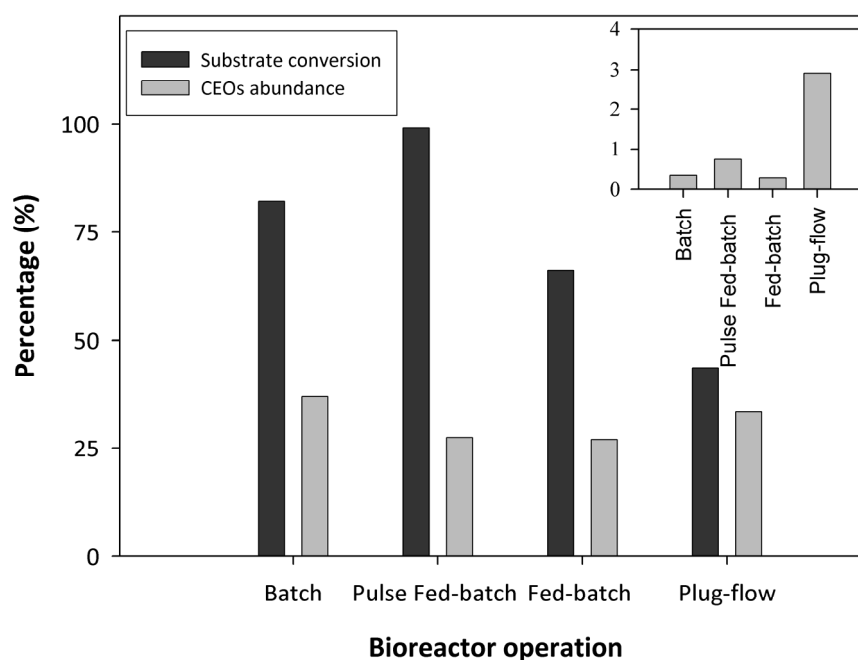**Figure 5** 1,4-butanediol and furan-2,5-dicarboxylic acid (FDA)Comparison of different bioreactor operation modes. (insert) Bioreactor volumetric productivities (mmol(l.h)$^{-1}$) vs. type of bioreactor

2.   FUTURE PROSPECTS

In this thesis, the biocatalytic synthesis of cyclic ester oligomers (CEOs) and linear ester oligomers (LEOs) derived from a range of biobased building blocks has been demonstrated. We have taken full advantage of the use of enzymes as catalyst. Reactions were carried out under mild conditions, thus making possible the polymerization of thermolabile monomers, like HMFA and BHMF. For the first time, we have showed that furan-based monomers and isomannide can be polymerized by lipase. We have exploited the ability of enzymes to work in anhydrous organic solvents and we have used a binary solvent mixture containing a hydrophobic solvent (*i.e.* toluene) and a hydrophilic one (*tert*-butanol) to increase the solubility of the non-activated dicarboxylic acids to promote the reaction. We have taken advantage of the enantio- and stereospecificity of the enzyme catalysis, and we have been able to polimerize isomannide, an aliphatic

heterocyclic secondary diol which was never mentioned as a substrate for the lipase before. Moreover, isomannide cannot be polymerized via chemical routes, and thus the enzymatic polymerization offers the chance to obtain a new class of biobased polyesters. We have shown that formation of cyclic ester oligomers by AA/BB and AB/AB polycondensation is an intrinsic attribute of the enzymatic polymerization, and that the abundance and the spectrum of CEOs formed is determined by the structure of the monomers and by the flexibility and length of the linear oligomer chain. For the first time, we have explored other reactor operation modes other than batch reactor for the enzymatic cyclization and polyesterification. We have shown that pulse fed-batch operation is more suitable for the formation of CEOs while plug-flow reactors are more suitable for the formation of longer linear oligoesters. There are, however, still many questions to be answered to make this concept viable. We propose few lines of research that we think could bring significant improvement of the technology and could bring a breakthrough for the industrial application of enzyme catalysis for the production of cyclic oligoesters and polyesters.

- The formation of CEOs can be influenced by application of several bioreactor operation modes and each operation mode here described has options to be further improved. As a suggestion, an experimental design could be helpful to reach better conversions, abundance of CEOs and polymerization degrees. For example taking pulse fed-batch bioreactor, the size of the pulse, the concentration of the substrate per pulse and the amount of enzyme present into the reactor could be variables to be taken into account at the time of an experimental design for any of the building blocks systems here tested. Similarly, plug-flow bioreactor can be improved changing for example, the column length, flow, load of enzyme and molecular sieves and porosity of the bed.

- A rigorous approach to tune the formation of CEOs combining the know-how described in this work is the regime analysis, which is based on the study of

characteristic times of the involved processes: (i) mixing time into the reactor, (ii) rate of reaction, (iii) residence time of the reactants (iv) rate of formation of the different products, etc. This approach will give an idea of which of the considered sub-processes is the limiting step to carry out the reaction, and therefore it is possible to make a more rational design of the process that improve the yield of the required product (i.e. CEOs).

- The success of enzymatic CEOs synthesis would be achieved not only using an empirical approach, but also by application of computational biochemistry and thermodynamical tools.

- In this study, we have used a commercial lipase as catalyst, and we have optimized reaction conditions to increase the yield of the reactions and we defined the basic molecular requirements for cyclic formation. An important step forward will be the integration of kinetic studies with *in silico* molecular modelling to understand in more detail the basis of the prevalent synthesis of cyclic vs. linear oligoesters to design CALB variants with increased activity for the synthesis of either cyclic oliogoesters or polyesters. Using protein engineering, optimal enzymes could be created for specific monomers and polymers.

- The research should be extended towards the enzymatic synthesis of polyesters based on the monomers described in this work to establish the full potential and the limitations of both the biobased monomers and the biocatalyst in terms of process efficiency and polymer properties. There would be several approaches to be explored, for example (a) enzymatic ring oligomerization with further ring-opening polymerization, (b) enzymatic oligomerization with further post-condensation, to produce (block)copolymers and (c) combination of ROP by chemical and enzymatic catalyst, among others.

REFERENCES

Boulifi NE, Aracil J, Martínez M. 2010. Lipase-catalyzed synthesis of isosorbide monoricinoleate: Process optimization by response surface methodology. Bioresource Technology In Press, Corrected Proof.

Carothers W. 1931. Polymeryzation. Chemical Reviews 8(3):353-426.

Cygler M, Grochulski P, Kazlauskas RJ, Schrag JD, Bouthillier F, Rubin B, Serreqi AN, Gupta AK. 1994. A Structural Basis for the Chiral Preferences of Lipases. Journal of the American Chemical Society 116(8):3180-3186.

Fenouillot F, Rousseau A, Colomines G, Saint-Loup R, Pascault JP. 2010. Polymers from renewable 1,4:3,6-dianhydrohexitols (isosorbide, isomannide and isoidide): A review. Progress in Polymer Science 35(5):578-622.

García-Alles LF, Gotor V. 1998. Lipase-catalyzed transesterification in organic media: Solvent effects on equilibrium and individual rate constants. Biotechnology and Bioengineering 59(6):684-694.

Hilker I, Rabani G, M. GK, Anja V, Palmans RA, Heise A. 2006. Chiral Polyesters by Dynamic Kinetic Resolution Polymerization. Angewandte Chemie International Edition 45(13):2130-2132.

Hilker I, Schaafsma AEJ, Peters RAH, Heise A, Nijenhuis AJ. 2008. Insight into lipase-catalyzed formation of macrocyclic oligoesters. European Polymer Journal 44(5):1441-1450.

Hirai H, Naito K, Hamasaki T, Goto M, Koinuma H. 1984. Syntheses of macrocyclic oligoesters from 5-hydroxymethyl-2-furancarboxylic acid. Die Makromolekulare Chemie 185(11):2347-2359.

Jacobson H, Stockmayer WH. 1950. Intramolecular Reaction in Polycondensations. I. The Theory of Linear Systems. The Journal

of Chemical Physics 18(12):1600-1606.

Kobayashi S. 2010. Lipase-catalyzed polyester synthesis – A green polymer chemistry. Proceedings of the Japan Academy, Series B 86(4):338-365.

Kowalski A, Duda A, Penczek S. 2000. Mechanism of Cyclic Ester Polymerization Initiated with Tin(II) Octoate. 2. Macromolecules Fitted with Tin(II) Alkoxide Species Observed Directly in MALDI−TOF Spectra. Macromolecules 33(3):689-695.

Lavalette A, Lalot T, Brigodiot M, Marechal E. 2002. Lipase-Catalyzed Synthesis of a Pure Macrocyclic Polyester from Dimethyl Terephthalate and Diethylene Glycol. Biomacromolecules 3(2):225-228.

McCabe RW, Taylor A. 2004. An investigation of the acyl-binding site of Candida antarctica lipase B. Enzyme and Microbial Technology 35(5):393-398.

Noguchi K, Kondo H, Ichikawa Y, Washiyama J, Okuyama K. 2006. 1,6,11,16-Tetra-oxacyclo-eicosane-2,5,12,15-tetra-one. Acta Crystallographica Section C 62(8):o528-o530.

Noordover BAJ, van Staalduinen VG, Duchateau R, Koning CE, van B, Mak M, Heise A, Frissen AE, van Haveren J. 2006. Co- and Terpolyesters Based on Isosorbide and Succinic Acid for Coating Applications: Synthesis and Characterization. Biomacromolecules 7(12):3406-3416.

Overmeyer A, Schrader-Lippelt S, Kasche V, Brunner G. 1999. Lipase-catalysed kinetic resolution of racemates at temperatures from 40 °C to 160 °C in supercritical CO2 Biotechnology Letters 21(1):65-69.

Pleiss J, Fischer M, Schmid RD. 1998. Anatomy of lipase binding sites: the scissile fatty acid binding site. Chemistry and Physics of Lipids

93(1-2):67-80.

Reetz MT, Schimossek K. 1996. Lipase-Catalyzed Dynamic Kinetic Resolution of Chiral Amines: Use of Palladium as the Racemization Catalyst. CHIMIA International Journal for Chemistry 50(12):668-669.

Valivety RH, Halling PJ, Macrae AR. 1993. Water as a competitive inhibitor of lipase-catalysed esterification in organic media. Biotechnology Letters 15(11):1133-1138.

Wu J, Eduard P, Thiyagarajan S, van Haveren J, van Es DS, Koning CE, Lutz M, Fonseca Guerra C. 2011. Isohexide Derivatives from Renewable Resources as Chiral Building Blocks. ChemSusChem 4(5):599-603.

SUMMARY

The 21 century faces new global challenges: food and water scarceness, more consciousness on sustainable development, the awareness of the extinction of the oil era, the global warming due to the carbon dioxide emissions, and the challenge of a globalized economy. The combination of these factors brings the humanity inexorable to seek new alternatives for food, energy, and technology. Such an evolution is also mirrored on the synthesis of new materials that accomplish certain characteristics, such as expected useful life, good performance on the applications, recyclability and degradability, etc. Among these materials polyesters are the most widely used. Historically the first attempt to synthesize polyester was in the 1930s by Carothers. Since the early work of Carothers, one of the most important difficulties has been the synthesis of polyesters with high molecular weight. The inconvenient found by Carothers persist until now for some new polyesters, for example the use of thermolabile substrates does not allow to synthesize polyesters with high molecular weight, good characteristics due to coloration of the final product, formation of unspecific bonds and additional operational problems (losses of raw material, use of harsh conditions, *in situ* removal of by-products, etc). Therefore, an alternative to overcome these problems is the use of ring-opening polymerization (ROP). This technique allows to work under less harsh conditions without the formation of by-products and even with using specific biocatalysts, and finally leading to polymers with larger molecular weight. Although ROP has several advantages over polycondensation, the unavailability of specific cyclic ester oligomers (CEOs) has prevented its massive application in the industry. Therefore, the

search of alternatives to synthesize CEOs is important. This work addresses this topic by the use of biobased building blocks in the presence of a efficient biocatalyst: *Candida antarctica* lipase B (CALB). The biobased building blocks were chosen taking into account flexible substrates such as 1,4-butanediol, adipic acid, sebacic acid, and succinic acid with more rigid ones such as 2,5-furandicarboxylic acid (FDA), 1,4:3,6-dianhydrohexitols (DAH, isomannide, isosorbide, and isoidide), 5-(hydroxymethyl)furan-2-carboxylic acid (HMFA), and 2,5-bis-(hydroxymethyl) furan (BHMF).

In **Chapter 2,** we present a proof of concept of the CALB-catalyzed cyclization of free acid and free alcohol in toluene based medium. Succinic acid with 1,4-butanediol in CALB-catalyzed reaction were used as reaction model. The substrates led to the stable formation of cyclic ester oligomers. Further, the use of pulse fed-batch bioreactor increases the synthesis of cyclic ester oligomers (CEOs). Not only an improvement in the total amount of CEOs is found, but also products with a higher molecular weight. In addition, a mechanistic model is presented to explain the formation of CEOs.

In (bio)chemistry the use of enantiomers plays an important role due to the characteristics that are conferred to the polymers produced out of them. Therefore, **Chapter 3** address the use of three outstanding sugar based enantiomers, 1,4:3,6-dianhydrohexitols (DAH) with succinic acid in CALB-catalyzed reaction. An experimental design was used to find better conditions that increase the total substrate conversion. Isomannide presents the largest conversion among the DAHs. Substrate-imprinted docking corroborates and explains the enantiopreference found by CALB. This finding is interesting compared with the reactivity found in chemical-catalyzed reaction of DAHs. These results pave the way to the synthesis of free DAH-based polymers that are difficult to synthesize by chemical means.

Among all biobased building blocks, furan derivatives are an alternative to replace widely used polymers such as polyethylene terephthalate (PET) due to the analogy between the

aromatic ring and the furan heterocyclic that confers similar characteristics to both types of polyesters. Therefore, in **Chapter 4** a diacid (2,5-furandicarboxylic acid, FDA), a diol (2,5-bis-(hydroxymethyl) furan, BHMF), and a hydroxyl acid (5-(hydroxymethyl)furan-2-carboxylic acid, HMFA) were polymerized in combination with BDO, sebacic acid, adipic acid, and succinic acid, and in the presence of CALB. The homopolymerization of HMFA reaches full conversion after 24 h and leads a broad variety of products (~60% CEOs). BHMF in combination with the alkyl diacids, however, reach more modest reaction yield and lower molecular weight products. Both the amount of cyclic ester oligomers (CEOs) and the degree of polymerization decrease with the increase of the aliphatic chain of the diacid: succinic acid > adipic acid > sebacic acid. In addition, FDA leads almost full conversion in 24 h and a variety of products, where CEOs were the 37% of the total.

Chapter 5 summarizes the most prominent substrate combinations found in previous chapters with different types of bioreactor operation modes as a tool to increase conversion and CEOs formation in CALB-catalyzed reaction. Batch bioreactor, fed-batch bioreactor, pulse fed-batch bioreactor, and plug-flow bioreactor operation mode are used. The chosen diacid /diol biobased building blocks were succinic acid -BDO, FDA - BDO, and BHMF - succinic acid. Almost full conversion of BDO-succinic acid, as well as of FDA-BDO is reached under pulse fed-batch operation, while isomannide-succinic acid exhibits the largest substrate conversion in batch bioreactor.

The general conclusion of this thesis is that CEOs from biobased building blocks can be synthesized by CALB-catalyzed reaction and the bioreactor operation leads to successful increase on CEOs synthesis. In addition, the spatial configuration of the substrates influences not only the reaction yield, but also the possibility to form CEOs in reactions catalyzed by CALB.

SAMENVATTING

De 21ste eeuw ziet zich geplaatst voor nieuwe wereldwijde uitdagingen: schaarste van voedsel en water, een toenemende bewustwording van de noodzaak tot duurzame ontwikkeling, het besef van het einde van het olietijdperk, het broeikaseffect als gevolg van koolstofdioxide emissies en verdergaande globalisering van de economie. De combinatie van deze factoren dwingt de mensheid te zoeken naar nieuwe alternatieven voor voedsel, energie en technologie. Een soortgelijke evolutie is ook waar te nemen in de materiaalkunde waar men zoekt naar syntheses van nieuwe materialen die voldoen aan bepaalde gewenste karakteristieken, zoals een lange levensduur, goede prestaties in de toepassingen, recyclebaarheid en/of gemakkelijke (biologische) afbreekbaarheid. Van al deze materialen worden polyesters het meest gebruikt. In de jaren dertig van de 19de eeuw heeft Carothers als eerste in de geschiedenis een succesvolle poging ondernomen om polyesters te synthetiseren. Een van de belangrijkste uitdagingen sinds het vroege werk van Carothers, blijft de synthese van polyesters met een hoog molecuulgewicht. De moeilijkheden die door Carothers werden ervaren, bestaan tot op de dag van vandaag voor polyesters. Het gebruik van thermisch labiele substraten laat bijvoorbeeld niet toe polyesters met een hoog molecuulgewicht te synthetiseren en geeft eindproducten met ongewenste karakteristieken, zoals verkleuring van eindproducten, vorming van niet-specifieke bindingen en verder operationele problemen (verlies van grondstof, gebruik van extreme condities, in situ verwijdering van bijproducten, etc.). Een mogelijkheid om deze problemen te overkomen is het gebruik van ring-opening polymerisatie (ROP). Deze

techniek staat toe te werken onder mildere condities zonder dat bijproducten worden gevormd. Zelfs gebruik van biokatalysatoren met een hoge specificiteit is mogelijk met als uiteindelijk resultaat de vorming van polymeren met een hoger molecuulgewicht. Ondanks dat ROP verschillende voordelen biedt ten opzichte van polycondensatie, zijn specifieke cyclische ester oligomeren (CEOs) nog altijd onvoldoende beschikbaar voor grootschalige toepassing in de industrie. De zoektocht naar alternatieven om CEOs te synthetiseren is daarom van belang. Dit onderwerp werd in het hier beschreven onderzoek aangepakt door gebruik te maken van bouwstenen, afkomstig van biomassa, in aanwezigheid van een efficiënte biokatalysator: *Candida antarctica* lipase B (CALB). De biobouwstenen werden gekozen uit flexibele substraten zoals 1,4-butaandiol, adipinezuur, sebacinezuur en barnsteenzuur, en meer rigide substraten als 2,5-furaandicarbonzuur (FDA), 1,4:3,6-dianhydrohextitolen (DAH, isomannide, isosorbide en isoidide), 5-(hydroxymethyl)furaan-2-carbonzuur (HMFA) en 2,5-bis-(hydroxymethyl)furaan (BHMF).

In Hoofdstuk 2 wordt een 'proof of concept' gepresenteerd van de CALB gekatalyseerde ringsluiting van vrij zuur en vrij alcohol in een op tolueen gebaseerd medium. Barnsteenzuur met 1,4-butaandiol werd als reactiemodel gebruikt in de CALB gekatalyseerde reactie. Deze substraten leidden tot de stabiele vorming van CEOs. Het gebruik van de 'pulse fed-batch bioreactor' verhoogde de synthese van CEOs. Niet alleen werd een verbetering van de totale hoeveelheid CEOs gevonden, maar ook producten met een hoger molecuulgewicht. Daarnaast wordt een mechanistisch model gepresenteerd om de vorming van CEOs te verklaren.

In de (bio)chemie is gebruik van enantiomeren belangrijk om de polymeren die hieruit worden gevormd van de gewenste karakteristieken te voorzien. In Hoofdstuk 3 wordt het gebruik beschreven van drie bijzondere, op suiker gebaseerde enantiomeren, te weten 1,4;3,6-dianhydrohexitolen (DAHs), met barnsteenzuur in de CALB gekatalyseerde reactie. Er werd gebruik gemaakt van een 'experimental design' om betere condities te

vinden en de conversie van substraat te vergroten. Van de DAHs liet isomannide de grootste conversie zien. 'Substrate-imprinted docking' bevestigt en verklaart de enantio-voorkeur van CALB. Deze resultaten openen de weg naar de synthese van vrije DAH-gebaseerde polymeren die moeilijk op chemische wijze zijn te synthetiseren.

Furaan derivaten zijn van de vele biobouwstenen een goed alternatief voor de vervanging van veel gebruikte polymeren zoals polyethyleen tereftalaat (PET). Dit als gevolg van de analogie tussen de aromaatring en de furaan heterocyclische ring, die vergelijkbare karakteristieken verlenen aan beide typen polyesters. Daarom werden in het werk beschreven in Hoofdstuk 4 een dizuur (2,5-furandicarbonzuur, FDA), een diol (2,5-bis-(hydroxymethyl)furan, BHMF) en een hydroxyzuur (5-(hydroxymethyl)furan-2-carbonzuur, HMFA) gepolymeriseerd in combinatie met BDO, sebacinezuur, adipinezuur en barnsteenzuur, in aanwezigheid van CALB. De homopolymerisatie van HMFA bereikte volledige conversie na 24 uur en leidde tot een breed scala aan producten (~60% CEOs). BHMF in combinatie met alkyl dizuren, bereikte daarentegen matige reactieopbrengsten en lagere moleculgewichten. Zowel de hoeveelheid cyclische ester oligomeren (CEOs), als de mate van polymerisatie, nam af met toenemende ketenlengte van het alifatische dizuur: barnsteenzuur > adipinezuur > sebacinezuur. FDA leidde bovendien tot volledige omzetting na 24 uur en een scala aan producten, waarvan 37% CEOs.

Hoofdstuk 5 geeft een samenvatting van de meest veelbelovende substraatcombinaties wat betreft verhoging van de omzetting en vorming van CEOs in de CALB gekatalyseerde omzettingen die in de voorgaande hoofdstukken worden beschreven en uitgevoerd zijn in verschillende typen bioreactoren, te weten batch, fed-batch, pulse fed-batch en plug-flow bioreactor. De gekozen dizuur/diol biobouwstenen waren barnsteenzuur - BDO, FDA - BDO en BHMF - barnsteenzuur. Nagenoeg volledige conversie van zowel BDO - barnsteenzuur als van FDA - BDO werd bereikt in een pulse fed-batch bioreactor, terwijl isomannide - barnsteenzuur de grootste substraat conversie liet zien in een batch bioreactor.

De samenvattende conclusie van deze thesis is, dat CEOs uit biobouwstenen kunnen worden gesynthetiseerd in CALB gekatalyseerde reacties en dat de door keuze van een geschikte bioreactor de CEOs opbrengst aanzienlijk kan worden verhoogd.

RESUMEN

La escasez de alimentos y agua, el incremento de la consciencia en el desarrollo sostenible, la certeza de la extinción de la era del petróleo, el calentamiento global debido al efecto invernadero por las emisiones de dióxido de carbono adicionalmente del desarrollo de una economía más globalizada son los nuevos retos en el siglo 21. La combinación de estos factores lleva a la raza humana a la inexorable búsqueda de nuevas alternativas en alimentos, energía y tecnología. Tal evolución se ve igualmente reflejada en la síntesis de nuevos materiales que presenten ciertas características tales como una vida útil garantizada del material, buen funcionamiento de tales materiales en las aplicaciones para las cuales son elaborados, reciclabilidad, degradabilidad, etc. Entre estos nuevos materiales los poliésteres se constituyen en los materiales mas ampliamente utilizados. Históricamente el primer intento de sintetizar poliéster fue llevado a cabo por Carothers en los años 30. Uno de los más importante problemas encontrados por Carothers fue la búsqueda de poliésteres de alto peso molecular y tal problema persiste hasta nuestros días con nuevos poliésteres. Problemas adicionales son, verbigracia, el uso de sustratos termolábiles que no permiten la síntesis de tales plásticos con alto peso molecular y con características aceptables debido a la formación de color que sobreviene por la oxidación parcial del sustrato o la formación de sub-productos como parte de reacciones indeseadas, además de problemas operacionales inherentes al proceso cómo pérdida del material inicial, uso de condiciones extremas, la remoción obligada de sub-productos de la reacción, entre otros. Por tal motivo, una alternativa para solucionar tales problemas es el uso de reacciones de polimerización por

apertura de ciclos (*ring-opening polymerization*, ROP). Ésta técnica permite el trabajo en condiciones de reacción moderada sin la formación de sub-productos mediante el uso de biocatalizadores específicos y como consecuencia se logran polímeros de mayor peso molecular que por medios convencionales (policondensación, PC). A pesar que ROP tienes numerosas ventajas sobre PC, la disponibilidad de oligo-ésteres cíclicos (CEOs) ha evitado la aplicación masiva de ROP a escala industrial. Por tal motivo, la búsqueda de alternativas para la síntesis de CEOs es un tema de vital importancia. De allí, el presente trabajo desarrolla éste tópico empleando sustratos sintetizados biológicamente en reacciones catalizadas por un eficiente biocatalizador: lipasa B de *Candida antarctica* (CALB). Los sustratos sintetizados biológicamente fueron escogidos teniendo en cuenta diferentes criterios como flexibilidad de las moléculas, enatioconfiguraciones, presencia de anillo aromático o no, entre otras, de allí se seleccionaron los siguientes sustratos: moléculas flexibles como 1,4-butanodiol, ácido adípico, ácido sebácico y ácido succínico en combinación con moléculas más rígidas como el ácido 2,5-furano dicarboxílico (FDA), 1,4:3,6-dianhidro hexitoles (DAH, isomanida, isosorbida, e isoidida), ácido 5-(hidroxi metil)-furano-2-carboxílico (HMFA), y 2,5-bis-(hidroximetil) furano (BHMF).

En el **Capítulo 2**, se demuestra experimentalmente el concepto de la reacción catalizada por CALB utilizando un par ácido y alcohol no modificados. Así la reacción entre ácido succínico y 1,4-butanodiol es utilizada como reacción modelo. La reacción llevada a cabo forma oligo-ésteres cíclicos en condiciones de reactor por lotes. Experimentos posteriores en reactor de lote alimentado por pulsos (*pulse fed-batch bioreactor*) demuestran ser una técnica que mejora la formación de los CEOs no solo en términos de preponderancia de este tipo de productos sino también con la formación de CEOs de mayor peso molecular. Éste capítulo concluye con el planteamiento de un modelo que explica la ruta que la reacción sigue para la formación de tales compuestos cíclicos.

En **el Capítulo 3** se estudia una de las características más interesantes de la (bio)química, la enantioselectividad de sustratos sintetizados biológicamente. Aquí se emplean tres

enantiómeros producidos a partir de azúcares (1,4:3,6-dianhidro hexitoles, DAH) en reacción con ácido succínico en presencia de CALB como biocatalizador. Un diseño de experimentos es utilizado con el fin de conseguir las mejores condiciones de reacción en términos de conversión de sustrato, dónde isomanida presenta la mejor conversión entre los DAHs utilizados. Posteriormente, el uso de un modelo del acoplamiento molecular del sustrato en el sitio activo de la enzima (*Substrate-imprinted docking*) corrobora los hallazgos experimentales y la enantiopreferencia mostrada por CALB, tal resultado es interesante comparado con la reactividad encontrada en reacciones catalizadas químicamente. Éstos hallazgos demuestran el principio de la síntesis de poliésteres que son difíciles de sintetizar químicamente.

Entre todos los sustratos sintetizados biológicamente, los derivados de furano son una alternativa para remplazar poliésteres ampliamente utilizados como el tereftalato de polietileno (PET) debido a la analogía entre el anillo de benceno y el heterociclo aromático del furano. De allí, en el **Capítulo 4** varios furano derivados son utilizados en combinación con algunos ácidos alquílicos, tales como: ácido 2,5-furano dicarboxílico (FDA), ácido 5-(hidroximetil) furano-2-carboxílico (HMFA), y 2,5-bis-(hidroximetil) furano (BHMF) en combinación con 1,4-butanodiol, ácido sebácico, ácido adípico y ácido succínico en presencia de CALB como biocatalizador. La homopolimerización de HMFA alcanza conversión total a las 24 horas con formación de una amplia gama de productos dónde los CEOs son aproximadamente el 60% de los productos detectados. Por el contrario, BHMF en combinación con los ácidos alquílicos logra un modesto porcentaje de conversión conjuntamente con productos de menor peso molecular. El rendimiento de la reacción al igual que el grado de polimerización de los productos formados decrementan con el incremento de la longitud de la cadena alquílica: ácido succínico > ácido adípico > ácido sebácico. Adicionalmente, la conversión de FDA es casi completa después de 24 horas de reacción, presentando una amplia variedad de productos, dónde los CEOs se constituyen en el 37% del total de productos detectados.

Finalmente en el **Capítulo 5** se utilizan los mejores resultados encontrados en los capítulos anteriores con los sustratos sintetizados biológicamente empleando diferentes tipos de operación de biorreactores como una herramienta para incrementar la conversión y el predominio de los CEOs en las reacciones catalizadas por CALB. Reactores operados por lotes, lotes alimentados, lotes alimentados por pulsos y en lecho empacado son empelados para tal fin. Los pares diácido / dialcohol de los sustratos sintetizados biológicamente fueron ácido succínico – BDO, FDA - BDO y BHMF – ácido succínico. El sustrato fue casi totalmente convertido cuando BDO – acido succínico y FDA – BDO fueron los sustratos bajo operación por lotes alimentados por pulsos, por el contrario, el par isomanida - ácido succínico mostró la mayor conversión de sustrato cuando el reactor fue operado por lotes.

Como conclusión general de esta tesis, la formación de CEOs a partir de sustratos sintetizados biológicamente en reacciones catalizadas por CALB es completamente posible y es fuertemente dependiente del tipo de sustrato escogido conjuntamente con el tipo de operación del reactor al igual que la configuración espacial del sustrato y su relación con CALB.

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Curriculum Vitae

David I. Habeych Narváez was born in Santa Marta (Colombia) on the 9th of December in 1975. In 1992 he completed his high-school studies at the *Colegio San Luis Beltrán* (Santa Marta) with distinction awarding a national scholarship for superior studies. Therefore, he attended the *Universidad Pontificia Bolivariana* (UPB, Medellín - Colombia). In 1998 he graduated with a degree in Chemical Engineering. He conducted his bachelor thesis in the area of biocatalysis in association with *Propal* (*Productora de Papeles S.A.*) and the Colombian Pulp and Paper Research Center. Further he pursued his Master studies on biotechnology (industrial bioprocesses) at the Universidad Nacional de Colombia (sede Medellín), graduating in 2003. His subject of the thesis was the evaluation of elicitors on plant cell cultures (calli) from *Catharanthus roseus* for the biosynthesis of indol alkaloids. In September 2004, he joined the post-master program on Bioprocess Design at Delft University of Technology (Delft, The Netherlands) receiving his diploma as Professional Doctorate in Engineering (PDEng). In September 2006 he worked in a bioprocess design project at DSM-Antiinfectives (Delft) during his second year of PDEng. In the same month he started his PhD research at Agrotechnology and Food Science group, subscribed to the Bioprocess Engineering department at Wageningen University, The Netherlands. The results of this research are described in this thesis. Between 1998 and 2001 David worked as docent and researcher at *Universidad Pontificia Bolivariana*, teaching general physics for engineers, biochemical engineering, heat transfer laboratory and plant cell culture, among others. Between 2001 and 2004 he worked at Universidad EAFIT as docent and researcher on bioseparations and biochemical engineering, among other courses. On the 1st of February of 2011, David started working as researcher at Twente University in the Thermochemical Conversion of Biomass (TCCB) group & at Wageningen University and Research Center at the Bioprocess Engineering group.

PUBLICATIONS

Habeych D., Eggink G., Boeriu C. Linear and Cyclic Ester Oligomers of Succinic Acid and 1,4-Butanediol: Biocatalytic Synthesis and Characterization. *Journal of Biocatalysis and Biotransformation*. 2011 (Aproved for publication)

Habeych David, Vanegas Diana, Juhl Benjamin, Pleiss Jürgen, Eggink Gerrit, Boeriu Carmen. Biocatalytic synthesis of polyesters from sugar based building blocks using immobilized *Candida antarctica* lipase B. *Journal of Molecular Catalysis B: Enzymatic*. V.71, 2011, P. 1-9.

Giraldo Catalina; Marín Luz Deisy; Habeych David. Sweeteners Purification from Stevia rebaudia Bertoni. *CENIC Ciencias Biológicas*, V.36, Especial Number, 2005. (In Spanish: *Obtención de Edulcorantes de Stevia Rebaudiana bertoni*).

Solarte Juan F.; Martinez Ana; Saez Alex; Habeych David. Culture Media Formulation from *Prosopis juliflora*. Revista EAFIT pag 9-17. Issue Jul-Sept. 2004. (In spanish: Evaluación de un medio de cultivo a partir del fruto de *Prosopis juliflora*)

Casas, Ana; Habeych, David; Velásquez, Jorge; Zapata, Jorge; Marín, Marta. Enzymatic Bleaching of Sugar Cane Bagasse Pulp: *Revista Ingeniería Química* (Spain). (2000). (In Spanish: *Industria Papelera-Preblanqueo enzimático de la pulpa de bagazo de caña de azúcar*)

Discipline specific activities

Courses
Biothermodynamic workshop   (The Netherlands, 2007)
Bioreactor Design and Operation (The Netherlands, 2008)
Thermodynamics in Biochemical Engineering (Germany, 2008)
Advanced course of biocatalysis (The Netherlands, 2006)

Conferences and meetings
Biofuel supply chain summit (Belgium, 2008)
12th Netherlands Biotechnology Congress
B-basic symposium (The Netherlands, 2007)
B-basic symposium (The Netherlands, 2008)
B-basic symposium (The Netherlands, 2009)
B-basic symposium (The Netherlands, 2010)
Biotrans (Hamburg, 2007)
Biocat (Switzerland, 2008)
Netherlands Process Technology Symposium (NPTS)
12th Netherlands Biotechnology Congress

General courses
EndNote workshop   (VLAG, 2006)
VLAG PhD week   (VLAG, 2007)
Academic writing   (CENTA, 2007)
Scientific writing   (CENTA, 2009)
Bioinformatics   (VLAG, 2007)
Techniques for writing and Presenting a scientific paper (VLAG, 2010)

Carreer perspective   (VLAG, 2010)

Optional courses and activities
PhD study tour Food & Bioprocess Engineering group (Japan, 2008)
Brainstorm-week Food and Bioprocess Engineering group (2008)
Brainstorm-week Food and Bioprocess Engineering group (2009)
Meeting Colloquia   (B-basic, 2007-2010)

Cover: tridimensional structure of *Candida antarctica* lipase B (CALB, front) and tridimensional structure of some of the substrates used in this research (back).
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