Exploring mild enzymatic sustainable routes for the synthesis of bio-degradable aromatic-aliphatic oligoesters

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The application of *Candida antarctica* lipase B in enzyme-catalyzed synthesis of aromatic-aliphatic oligoesters is here reported. The aim of the present study is to systematically investigate the most favorable conditions for the enzyme catalyzed synthesis of aromatic-aliphatic oligomers using commercially available monomers. Reaction conditions and enzyme selectivity for polymeriza-tion of various commercially available monomers were considered using different inactivated/ activated aromatic monomers combined with linear polyols ranging from C_2 to C_{12}. The effect of various reaction solvents in enzymatic polymerization was assessed and toluene allowed to achieve the highest conversions for the reaction of dimethyl isophthalate with 1,4-butanediol and with 1,10-decanediol (88 and 87% monomer conversion respectively). M_w as high as 1512 Da was obtained from the reaction of dimethyl isophthalate with 1,10-decanediol. The obtained oligom-ers have potential applications as raw materials in personal and home care formulations, for the production of aliphatic-aromatic block co-polymers or can be further functionalized with various moieties for a subsequent photo- or radical polymerization.

Keywords: Cell activation · Cell death · Extracellular vesicles (EV) concentration · Industrial bioprocess · Microenvironment

Abbreviations: 1,2a, phthalic acid; **1,2e**, dimethyl phthalate; **1,3a**, isoph-thalic acid; **1,3e**, dimethyl isophthalate; **1,4a**, terephthalic acid; **1,4e**, dime-thyl terephthalate; **10p**, 1,10-decanediol; **12p**, 1,12-dodecanediol; **^{1}H-NMR**, proton nuclear magnetic resonance; **2p**, ethylene glycol; **4p**, 1,4-butanediol; **6p**, 1,6-hexanediol; **8p**, 1,8-octanediol; **CaLB**, *Candida antarctica* lipase B; **GPC**, gel permeation chromatography; **HiC**, *Humicola insolens* cutinase; **M_n**, number average molecular weight; **mQH$_2$O**, milliQ water; **M_w**, weight average molecular weight; **THF**, tetrahydrofurane

1 Introduction

Since the pioneer studies of Klibanov and Zaks, who first reported the activity of enzymes in organic media [1–3], the interest on enzymatic catalysis for industrial applica-tions had an exponential growth. Nowadays, enzymes are widely used in multi-ton-scale processes as deter-gent and feed additives, for starch conversion, in textile applications, and in many other applications related to polymer degradation, notably polysaccharide degrada-tion [4]. In contrast, for polymer synthesis, despite the huge potential, enzymes are still under-exploited. Yet, the plastics industry with the rising interest of the soci-ety towards environmentally-friendly processes/products needs an improved portfolio of 'green' techniques [5], especially for polyester-based materials, from which it is known that the manufacturing polycondensation process

involves high temperatures and vacuum and, therewith, is quite energy-consuming. Related to synthetic poly-mers, in the past, several enzyme-based approaches both for synthesis and for functionalization were reported. In particular, enzymes from the class of the hydrolases (e.g. lipases, cutinases and esterases) were found to be able to hydrolyze polyesters such as poly(lactic acid) [6, 7], poly(1,4-butylene adipate) [8] and similar co-polymers [9] as well as poly(ethylene terephthalate) [10–12]. Additionally, selected biocatalysts were also demonstrated to catalyze the opposite reaction in bulk or in presence of an organic solvent for the production of aliphatic polyesters [13], oligomers carrying side-chain functionalities [14, 15], and aromatic-aliphatic polyesters [16, 17]. Despite a large number of studies published on the synthesis of aliphatic polyesters, the biocatalyzed polycondensation of aromat-ic-aliphatic polyesters was at best of our knowledge by far less explored, even though this class of materials plays an important role as bulk polymer in the packaging industry and plastics industry in general.

In previous studies the lipase B from *Candida antarctica* (CaLB) [18] was found to be a well suited biocatalyst for such polycondensation reactions and was extensively reported for the synthesis of various aliphatic polyesters [19, 20]. Almost twenty years ago Park et al. reported on the synthesis of aliphatic-aromatic oligomers starting from trichloroethyl and trifluoroethyl aromatic esters [16]. The authors were successful in achieving polymerization products in a 300–800 g mol^{-1} M_W range. However, poly-condensation reactions using halogen-activated diesters produce reaction side products which need special han-dling for discarding. Five years later, Uyama et al. report-ed that also divinyl esters can be used at this purpose [17] but, unfortunately, the monomers turned out to be difficult to be produced/purified and have, consequently, not yet reached the market. Moreover, the long-term stabilities of the aromatic vinyl esters are limiting their use. Wu et al. reported on the synthesis of polyesters starting from sim-ple inactivated isophthalic acid [21] while Mezoul et al. reported a similar reaction starting from dimethyl esters of the same compounds, achieving the highest M_W reported ever for enzymatically-synthetized aromatic-aliphatic polyester (M_W of 55 000 g mol^{-1}) [22]. It is important to underline that recently also the CaLB-catalyzed polycon-densation of the renewable furandicarboxylic acid dime-thyl ester [23] and the use of aromatic polyols [24] were reported but neither was industrially implemented yet.

In the present work a systematic approach to elaborate the most favorable conditions for enzyme catalyzed syn-thesis of aromatic-aliphatic oligomers using commercially available monomers is presented. Six different aromatic monomers were combined with linear aliphatic polyols ranging from C_2 to C_{12} in order to investigate the selec-tivity of the enzyme together with the effect of solvents used. Consequently we believe this work contributes to the establishment of a strong mechanistic basis for the development of environmentally-friendly strategies for polyester oligomers production. This approach combines recyclable biocatalysts working in mild reaction condi-tions [25] with the possibility to incorporate functional moieties (known to be temperature-unstable) in the final reaction product [12, 13] together with a high enantio-and regioselectivity of the biocatalyst [25].

2 Materials and methods

2.1 Chemicals and reagents

Dimethyl terephthalate, 1,8-octanediol, ethylene glycol and 1,4-butanediol were purchased from Merck while 1,6-hexanediol and 1,12-dodecanediol were obtained from Tokyo Chemical Industry. All other chemicals and solvents were purchased from Sigma-Aldrich. All rea-gents and solvents were reagent grade and used without further purification if not otherwise specified.

2.2 Enzymatic preparations

Novozym® 435 is a commercial formulation (Sigma Aldrich) of lipase B from *C. antarctica* (CaLB), adsorbed on a macroporous methacrylic resin. The activity, assayed-based on hydrolysis of tributyrin, resulted to be 2400 U g_{dry}^{-1}. It has been demonstrated that most of the enzyme molecules of Novozym® 435 are localized in a shell of the bead with a thickness of ~100 μm [12]. The prepara-tion water content was determined to be below 0.3% w/w. The residual water content in the immobilized preparation was determined on aluminum plates. Therefore, a known amount of the biocatalyst was dried at 110°C for 8 h to constant weight. The water content is defined as the % of weight loss after drying.

2.3 Hydrolytic activity assay

The activity of enzyme preparations was assayed via tribu-tyrin assay as previously reported [15].

2.4 Synthetic activity assay

Quantification of the synthetic enzyme activity was per-formed based on the enzymatic synthesis of propyl laurate as previously described [6].

2.5 Enzymatic synthesis of aromatic-aliphatic polyesters

Aromatic dicarboxylic acids (or their esters) (2.0 mmol), linear polyols (2.0 mmol) and 10 mL of reaction solvents were mixed in a reaction vessel and stirred at 70°C until the monomers were completely solubilized. Lipase B from *C. antarctica* (CaLB) (10% w/w with respect to the total

amount of monomers) was then added to the reaction mixture and the reaction proceeded for 96 h in a Carou-sel 12 Plus Reaction Station (Radleys, United Kingdom) at 70°C and 1000 mbar under N_2 atmosphere. The used molar ratio of diester and polyol was 1.0:1.0. Samples were collected after 24, 48 and 72 h. After 96 h of incubation, the biocatalyst was removed via filtration in order to stop the polycondensation reaction. After solvent evaporation, the crude product was analyzed by ^{1}H-NMR and GPC without any further purification or precipitation. It was also ensured that no reaction occurred in the absence of an enzyme. All reactions were performed in duplicates.

2.6 ^{1}H-NMR

Nuclear magnetic resonance ^{1}H-NMR measurements were performed on a Bruker Avance II 400 spectrometer (reso-nance frequency of 400.13 MHz for ^{1}H) equipped with a 5 mm observe broadband probe head with z-gradients. $CDCl_3$ was used as NMR solvent if not otherwise specified.

2.7 Gel permeation chromatography (GPC)

GPC Samples were prepared and analyzed as previously described. [6] The molecular weights of the polymers were calculated using linear polystyrene calibration standards (250–70 000 Da). The injection volume was 40 µL.

3 Results

In the present work, enzyme catalyzed polycondensation of aromatic dicarboxylic acids with linear polyols with varying chain length was systematically investigated. In detail, terephthalic acid (1,4a), isophthalic acid (1,3a), phthalic acid (1,2a) or their corresponding esters dimethyl terephthalate (1,4e), dimethyl isophthalate (1,3e), dime-thyl phthalate (1,2e) were reacted with the linear poly-ols ethylene glycol (2p), 1,4-butanediol (4p), 1,6-hexan-ediol (6p), 1,8-octanediol (8p), 1,10-decanediol (10p) and 1,12-dodecanediol (12p) were investigated. All the reactions were catalyzed by lipase B from *C. antarctica* (CaLB) (Fig. 1) in its immobilized form known as Novozym® 435 (hydrolytic activity 2300 U g^{-1}) as biocatalyst. This enzymatic preparation was chosen since it was reported to be the most efficient biocatalyst when compared to various lipases derived from different organisms for polymeriza-tion of dicarboxylic acids divinyl esters with polyols [17] and of various aliphatic substrates [13, 16]. The tempera-ture (70°C) was chosen according to previous reports [21]. The selected temperature was reported to cause partial inactivation of the biocatalyst but only when long reaction times were applied [21]. Hence, this fact is important to be considered only when the recyclability of the biocatalyst is a major concern [14, 15].

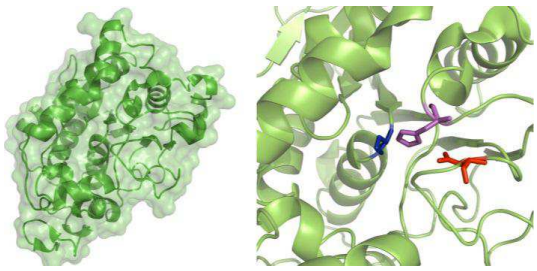

Figure 1. Structure of the biocatalyst Candida antarctica lipase B (CaLB) (left) and zoom-in of the catalytic triad (right). Red, aspartic acid (Asp187); Blue, Serine (Ser105); Magenta, Histidine (His224).

3.1 Biocatalyzed polycondensation of aromatic dicarboxylic acids with α,ω-alkylene glycols

In a first instance, we carried out the polycondensation between aromatic dicarboxylic acids with linear polyols. As reported in Table 1, among the aromatic dicarboxylic acids that were considered, only the polymerization of 1,3a with linear polyols ranging from C_4 to C_8 led to polycon-densation products. In particular, for the polymerization of 1,3a with 6p the highest monomers conversion of 15% was measured. No reaction products were observed from polymerization from 1,2a with 1,4a and in absence of the enzyme. These results are in agreement with those reported by Wu et al. [21]. However, it was not possible to calculate the molecular weight of the obtained poly(1,6-hexylene isophthalate) via GPC since only dimers and trimers were detected (M_n of 295 g mol^{-1}). The molecular weights of these reaction products identified via ^{1}H-NMR (Supporting information, Fig. S1) were considerably lower than those previously reported by Wu et al. An explanation for this fact could be the different reaction system (round bottom flask vs. Carousel 12 Plus Reaction Station) and the different solvent (diphenyl ether vs. toluene) used. In this study, we had specifically selected only those solvents allowing a direct work-up of the reaction products (filtra-tion with subsequent solvent removal) in order to avoid a multi-step extraction processes that in most of the cases requires halogenated solvents as reported for polymeriza-tions in diphenyl ether [26]. The reaction was stopped after 72 h of incubation due to solubility issues with the 1,2a and the 1,4a aromatic moieties, known to be poorly soluble in solvents commonly used for enzymatic synthesis such as heptane, toluene and tetrahydrofurane.

This first screening phase revealed that aromatic dicar-boxylic acids were not suitable for enzymatic polycon-densation reactions. The poor reactivity of these acids is connected to the high acidity of the aromatic dicarboxylic acids if compared to the linear ones described by Hollmann et al. [27] and could be due also to solubility limitations in the investigated media and/or lack of selectivity by the used biocatalyst. Wu et al. in their previous work had seri-

Table 1. Polycondensation of aromatic dicarboxylic acids with α,ω-alkylene polyols using CaLB as biocatalyst in toluene as reaction solvent. The reaction time was 72 h.

Substrates		Monomer conversion (%)[a)]
Dicarboxylic acid	Diol	
1,2a	2p, 4p, 6p, 8p, 10p, 12p	nd
1,3a	2p	nd
	4p	<10%
	6p	15%
	8p	<10%
	10p	nd
	12p	nd
1,4a	2p, 4p, 6p, 8p, 10p, 12p	nd

a) Calculated via ^{1}H-NMR. The aromatic ring of the dicarboxylic acid was assumed as constant. All reactions were performed in duplicates. nd, not detected

ous reproducibility issues for the reaction catalyzed by Novozym® 435 in diphenyl ether, with a variation of the obtained molecular weights of ±10 000 g mol^{-1} among the various batches [21]. The obtained monomers conversion rates were extremely low and no significant increase of the molecular weight of the produced oligomers was detected in these reported studies. Based on these findings, the utilization of aromatic dicarboxylic esters as monomers was investigated. It is important to note that no reaction products were observed when using THF as solvent for any of the tested aromatic dicarboxylic acids.

3.2 Biocatalyzed polycondensation of aromatic dicarboxylic esters with α,ω-alkylene glycols

Based on the fact that only isophthalic acid was converted in enzymatic polycondensation of aromatic diacids, we investigated the reactivity of their methyl esters. Indeed, using methyl esters, higher monomer conversions after 96 h of reaction were seen. However, only 1,3e and 1,4e gave polycondensation products (Table 2). It was recently reviewed how the reactions catalyzed by CaLB follow a ping-pong bi-bi mechanism, with the substrate that enters the active site of the enzyme and forms the first tetrahedral intermediate. The first product then leaves the active site with the formation of the acyl-enzyme. The second substrate enters the active site to generate the second tetrahedral intermediate then the final product leaves the active site and the enzyme is ready for another catalytic cycle. As in the case of serine proteases, a proton is transferred from serine, thus favoring the nucleophilic attack of the acyl carbon by the deprotonated alcohol. The enzyme catalyzes the reaction by stabilizing the nega-tively charged oxyanion by means of electrostatic interac-tions between Thr40 and Gln106 inside the so-called oxy-anion hole. The rate determining step of the reaction can be either the formation of the acyl-enzyme (acylation) or the deacylation steps. In transesterifications of secondary alcohols, the deacylation step is often rate limiting, especially when they are bulky. After the acylation of Ser105, the His224 residue receives a proton from the secondary alcohol. The latter acts as nucleophile by attacking the acylated-seryl ester with the eventual formation of the ester [28]. This is in agreement with results by Mezoul et al. [22], who first evidenced the lack of reactivity of the ortho position of methyl ester groups in enzymatic polymerizations. Here, the highest conversion ranging from 64 to 88% was obtained for the reaction of dimethyl isophthalate with the C_4–C_{10} polyols. Similar results were reported by Cruz-Izquierdo et al. [23] for enzymatic polymerization of 2,5-furanedicarboxylic acid dimethyl ester with linear polyols. In this study, enzymatic polymeriza-tion was also conducted using tetrahydrofurane (THF) as more polar solvent when compared to toluene. In this case, no polycondensation products were observed for 1,2e, 1,4e

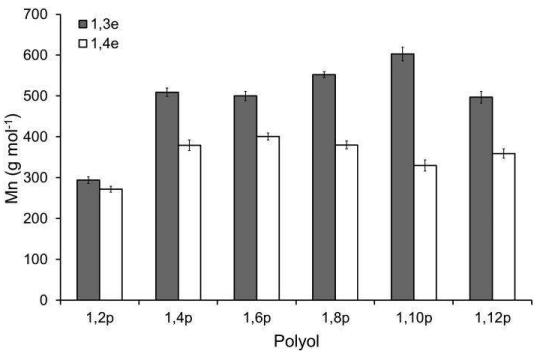

Figure 2. Number average molecular weight (*M*n) distribution for the reactions between dimethyl isophthalate (1,3e), dimethyl terephthalate (1,4e) and the C2–C12 linear polyols. Reaction conducted in toluene for 96 h using Novozym® 435 as biocatalyst. 1,2p, ethylene glycol; 1,4p, 1,4-butanediol; 1,6p, 1,6-hexanediol; 1,8p, 1,8-octanediol; 1,10p, 1,10-decanediol; 1,12p, 1,12-dodecanediol. All experiments were performed in duplicates.

Table 2. Polycondensation of aromatic dicarboxylic acids methyl diesters with α,ω-alkylene polyols using CaLB as biocatalyst in toluene as reaction solvent. The reaction time was 96 h.

Substrates		Monomer conversion (%)[a)]
Dimethyl ester	Diol	
1,2e	2p, 4p, 6p, 8p, 10p, 12p	nd
1,3e	2p	41%
	4p	88%
	6p	70%
	8p	64%
	10p	87%
	12p	58%
1,4e	2p	21%
	4p	43%
	6p	53%
	8p	35%
	10p	30%
	12p	24%

a) Calculated via ^{1}H-NMR by comparing the ratio between the methoxy groups and the aromatic ring of the dicarboxylic methyl ester (assumed as constant). All reactions were performed in duplicates.
nd, not detected

and the reaction conducted without biocatalyst while a limited conversion ranging from 11 to 20% was found for all the linear polyols (Supporting information, Table S1). An organic medium was used because it was not possible to conduct the polycondensation reactions in bulk due to the high melting points of the aromatic compounds at the used temperature (70°C).

The molecular weights and the polydispersity index of the reaction products (Fig. 2) indicate that despite good monomers conversion after 96 h of reaction, only a maximum M_n of 603 (corresponding to trimers and tetramers) was obtained for the reaction of 1,3e with 1,10p. These data are in agreement with previous reports [17, 22] but in disagreement with the studies of Wu et al. [21] who claimed a molar mass of 55 000 g mol^{-1} for the polymerization of isoph-thalic acid with 1,6-hexanediol while no molar masses and conversions were reported for the polymerization reaction of the same diacid with 1,4-butanediol.

For all used polyols, the obtained M_n was higher for the reactions where 1,3e was used as aromatic moiety (Fig. 2). This strongly agrees with the ^{1}H-NMR-calculated conversion rates that were always found to be higher for the 1,3 substituted monomer (Table 2).

4 Concluding remarks

In this work, immobilized lipase B from *C. antarctica* was found to be a suitable catalyst for polymerization of aromatic-aliphatic oligoesters in organic solvents. Polyesters were obtained from dimethyl terephthalate and dimethyl isophthalate with various dialcohols while no reactivity was found for terephthalic acid, phthalic acid and dime-thyl phthalate. The most suitable diols for the enzymatic synthesis were the C4–C10 polyols that led to monomer conversion of 64–88% for the polymerization with dimethyl isophthalate. The best solvent for this single-step extraction-free process resulted to be toluene. For future studies, the effect of other enzymes, reaction systems and biocatalyst recyclability [29, 30] on the reaction progression would be of high interest in order to achieve improved conversion rates in shorter reaction times and higher and reproducible product molecular weights. Moreover, molecular modeling studies are needed in order to understand the poor reactiv-ity of dimethyl terephthalate and the effect of the solvent on the reaction progression.

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Alessandro Pellis, Alice Guarneri and Martin Brandauer were involved in the experimental work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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